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[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007

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EDITORIAL

Roles of *Helicobacter pylori* BabA in gastroduodenal pathogenesis

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Abstract

Interactions between BabA and Lewis b (Le^b) related antigens are the best characterized adhesin-receptor interactions in *Helicobacter pylori* (*H pylori*). Several mechanisms for the regulation of BabA expression are predicted, including at both transcriptional and translational levels. The formation of chimeric proteins (*babA*/*B* or *babB*/*A* chimeras) seems to play an especially important role in translational regulation. Chimeric BabB/A protein had the potential to bind Le^b; however, protein production was subject to phase variation through slipped strand mispairing. The *babA* gene was cloned initially from strain CCUG17875, which contains a silent *babA1* gene and an expressed *babA2* gene. The sequence of these two genes differs only by the presence of a 10 bp deletion in the signal peptide sequence of *babA1* that eliminates its translational initiation codon. However, the *babA1* type deletion was found only in strain CCUG17875. A few studies evaluated BabA status by immunoblot and confirmed that BabA-positive status in Western strains was closely associated with severe clinical outcomes. BabA-positive status also was associated with the presence of other virulence factors (e.g. *cagA*-positive status and *vacA* s1 genotype). A small class of strains produced low levels of the BabA protein and lacked Le^b binding activity. These were more likely to be associated with increased mucosal inflammation and severe clinical outcomes than BabA-positive strains that exhibited Le^b binding activity. The underlying mechanism is unclear, and further studies will be necessary to investigate how the complex BabA-receptor network is functionally coordinated during the interaction of *H pylori* with the gastric mucosa.

Key words: *Helicobacter pylori*; BabA; Pathogenesis; Lewis antigens

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INTRODUCTION

The adherence of *Helicobacter pylori* (*H pylori*) to the gastric mucosa is widely assumed to play an important role in the initial colonization and long-term persistence in the human gastric mucosa. Analysis of the three completed *H pylori* genomes (strains 26695, J99, and HPAG1) has confirmed the presence of five major outer membrane protein (OMPs) families, which comprise approximately 4% of the *H pylori* genome. Among the families, members of the large Hop (*Helicobacter* outer membrane protein) family were the first characterized OMPs in *H pylori*. Several OMPs in the Hop family have been reported to act as adhesion molecules including the blood group antigen binding adhesin (BabA), sialic acid binding adhesin (SabA), adherence-associated lipoprotein (AlpA and AlpB), outer membrane inflammatory protein (OipA), and HopZ. Lewis b antigen (Le^b) and related fucosylated ABO blood group antigens are recognized by BabA[1], whereas sialyl-Lewis x and sialyl-Lewis a antigens (sLe^x and sLe^a) are recognized by SabA[2]. The corresponding receptors for AlpAB, OipA, and HopZ remain unknown. To date, BabA-Le^b is the best-characterized adhesin-receptor interaction in *H pylori*. In this review, I summarize recent data giving new insight into BabA and its role in pathogenesis.

IDENTIFICATION OF BABA

It is well known that Le^b is the dominant antigen in the

gastric mucosa of secretor-positive individuals[3], and the negative secretor status is associated with a Lewis a (Le^a)-dominant phenotype in the gastric mucosa (Figure 1). In 1993, two studies showed that *H pylori* can bind to fucosylated glycoconjugates containing Le^b structures on the surface of gastric epithelial cells within human biopsy specimens[4,5]. Studies using transgenic mice expressing the human Le^b epitope in gastric epithelial cells indicated that Le^b functions as a receptor for an *H pylori*-specific adhesin and mediates its attachment to the gastric pit and surface mucous cells[6]. Further studies using the same transgenic mice showed that *H pylori* was adherent to the surface of gastric epithelial cells, resulting in severe chronic gastric inflammation and atrophy; whereas *H pylori* was localized in the mucous layer in non-transgenic control mice[7].

In 1998, Ilver *et al* analyzed the blood group antigen-binding activity by measuring binding of *H pylori* to ^{125}I-labeled fucosylated blood group antigens[1]. Among 100 *H pylori* isolates examined, 66% bound the Le^b antigen; whereas 95% of the isolates did not bind the related Le^a, H-2, Le^x, or Le^Y antigens. The 78 K adhesin recognizing the Le^b antigen was detected on the bacterial cell outer membrane and was isolated by a combined ligand identification and purification technique and designated as blood group antigen-binding adhesin (BabA)[1]. Additional analyses revealed two sets of clones that encode two proteins with almost identical NH_2-terminal domains and completely identical COOH-terminal domains, but with divergent central domains. The corresponding genes were designated *babA* and *babB*; BabA but not BabB had Le^b antigen-binding activity. Therefore, the central domain in *babA* is believed to determine the specificity of receptor binding[1, 8-12]; however, the motifs of the *babA* gene that are involved in binding are still unknown.

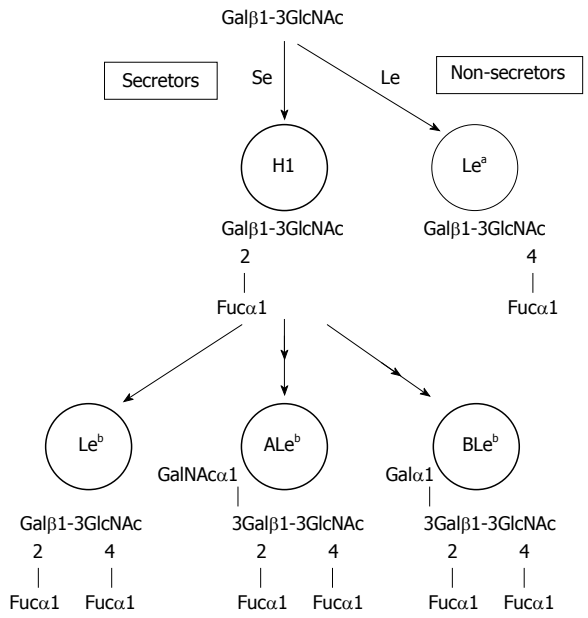

Figure 1 Biosynthetic pathways of Lewis antigens starting from type 1 lacto series core chains. Starting from type 1 core chains, an α1, 2-fucosyltransferase (Se) transfers fucose (Fuc) to the terminal galactose (Gal), resulting in the H-1 antigen (H1). H-1 antigen is a target for GalNAc- or Gal-transferases (in blood group A or B individuals) or remains unmodified (in blood group O individuals). These intermediates the are modified for the fucosylation step by an α1,3/4-fucosyltransferase (Le), resulting in the difucosylated histo-blood group antigens ALe^b, ALe^b and Le^b. Non-secretors are unable to produce an active Se product, and are only targets for the Le gene product Le^a.

FUNCTION OF BABA

BabA originally was defined as an adhesin binding to the Le^b antigen. The H-1 antigen is the carbohydrate structure that defines the blood group O phenotype in the ABO blood group system. Le^b, which is difucosylated, is formed by the addition of a branched fucose (Fuc) residue to H-1. The antigens that define blood group A and B phenotypes and corresponding antigens in the Lewis blood group system are formed by terminal N-acetylgalactosamine (GalNAc) or galactose (Gal) substitutions of H-1 and Le^b [A-1 and A-Lewis b (ALe^b), and B-1 and B-Lewis b (BLe^b) antigens, respectively; Figure 1].

Recently, Aspholm-Hurtig *et al* investigated the ability of BabA to bind Le^b, ALe^b and BLe^b[8]. Among 265 Le^b-binding *H pylori* strains from various geographic regions, more than 95% of *H pylori* strains are "generalists" (able to bind ALe^b and BLe^b in addition to Le^b); whereas a small subset of strains bind exclusively to ALe^b, and are called "specialist" strains. The authors proposed that the middle region of BabA was responsible for determining the different binding patterns; however, the specific motifs could not be identified[8]. Interestingly, "specialist" strains originated predominantly from South American individuals (where 60% of strains were classified as "specialist"), who are known to express almost entirely the blood group O phenotype. South American isolates in their study were from Peruvian and Venezuelan Amazon Amerindian populations and also from a Colombian mestizo (mixed Amerindian-European ancestry) population; probably most of these strains came mainly from European strains[13,14]. These data suggest that most specialist *babA* alleles may have arisen by mutation and/or recombination within the last 500 years. Therefore, the authors propose that such rapid evolution of BabA in response to host mucosal glycosylation patterns would enable the pathogen to adapt to their individual hosts while avoiding host immune responses, and contributes importantly to the extraordinary chronicity of human *H pylori* infection worldwide.

The mucins secreted by gastric mucous cells form a mucous gel layer covering the gastric mucosa. This gel layer is considered the first line of gastric mucosal defense against luminal noxious agents[15-17], and damage to the mucous gel is thought to precede gastric mucosal injury. The gastric surface mucous cells and gland mucous cells express the secretory mucins MUC5AC and MUC6/MUC5B, respectively[18,19]. The majority of *H pylori* reside in the gastric mucus overlying the epithelium. It is reported that *H pylori* could be co-localized with MUC5AC gastric mucin, but not with MUC6-producing cells in the glandular areas, suggesting

that adhesion is predominantly toward MUC5AC-specific ligands in gastric mucosa[20]. Subsequently, this binding phenotype could be correlated with the expression of an active BabA protein in *H pylori* and the presentation of the Le^b antigen in the gastric mucin MUC5AC[21,22]. However, since BabA-positive strains also attached to Le^b-negative MUC5AC of non-secretors, the involvement of additional epitopes and/or adhesins also must be involved[21]. In addition, binding of *H pylori* to MUC5B had been described[23] and a recent study confirmed that the binding was predominantly mediated by BabA and to a lesser degree by SabA adhesin[24].

LOCATION OF THE *BABA* GENE IN THE *H PYLORI* GENOME

H pylori 26695, J99 and HPAG1 each possess one *babA* allele (HP1243/JHP0833/HPAG1_0876) and one *babB* allele (HP0896/JHP1164/HPAG1_0320)[25-27]. Interestingly, the genomic locations of *babA* and *babB* genes are completely different among three strains (Figure 2). In strain J99, *babA* (JHP0833) is downstream of *hypD* (JHP0835) with a J99-specific gene (JHP0834) intervening, and *babB* (JHP1164) is downstream of *s18* (JHP1165). In strain 26 695, the locations of *babA* (HP1243) and *babB* (HP0317) are reversed. The chromosomal locations downstream of *hypD* and *s18* are referred to as locus A and locus B, respectively. In strain 26695, one gene encoding OMPs homologous to *babA* and *babB* (HP0317: denoted *babC*) with unknown function have been identified[27-29]. The location is referred to as locus C; interestingly, in strain HPAG1, the *babB* gene is located at locus C and *babC* gene at locus B. The *bab* genes initially were cloned from the strain CCUG17875, and this strain has two *babA* genes and one *babB* gene[1]. Gene inactivation experiments identified that only one gene (denoted *babA2*) had Le^b antigen-binding activity; whereas another gene (*babA1*) did not; *babA1* was located at locus B; however the locus of *babA2* was not determined[1].

The location of *babA* and *babB* in various clinical isolates of *H pylori* recently has been reported[29,30]. Hennig *et al*[30] analyzed a panel of 35 *H pylori* isolates and found that 24 (69%) contained *babA* sequences. In contrast, the *babB* gene was identified in 34 strains (97%). The *babA* gene was located at locus A for 19 strains (54%), at locus B for four strains (11%), and at locus C for three strains (9%). Four strains contain two copies of the *babA* gene, and the *babA* sequences found at two loci were identical in three strains and almost identical in one strain (i.e. three substitutions near the 5' ends of the genes in one strain), indicating that the multiple copies of *babA* presumably resulted from gene conversion (intragenomic nonreciprocal recombination) events. Importantly, two strains possessed the *babA* gene; however, the locus could not be identified, suggesting that there are additional unidentified chromosomal loci for *babA,* although *babA* may be found in one of three chromosomal loci in most cases.

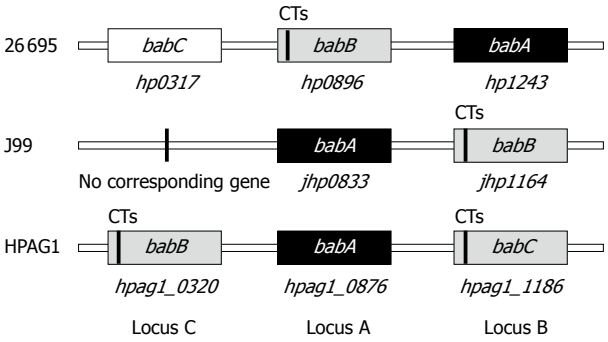

Figure 2 Genomic location of the *babA*, *babB*, and *babC* genes in strains J99, 26695, and HPAG1. CT: CT dinucleotide repeats.

Colbeck *et al*[29] analyzed a panel of 44 *H pylori* strains and found that 32 (73%) contained *babA* sequences. In contrast, the *babB* gene was identified in 41 (95%) isolates. The *babA* gene was located at locus A for 25 strains (57%) and at locus B for 18 strains (41%); locus C was not evaluated. Interestingly, although chromosomal DNA from low-passage-number, single-colony isolates was used, there was a mixed genotype in 30% (13/44) of the isolates, where the population of cells contained both *babA* and *babB* at the same locus. As a result, 11 strains were found to contain two copies of the *babA* gene including eight with mixed *babA* and *babB* at locus B.

Overall, from two detailed studies I conclude that the *babA* gene prefers to be located at locus A, some strains do not possess the *babA* gene, some strains possess multiple copies of the *babA* gene, and most strains possess the *babB* gene. The presence of *babB* might confer a stronger selective advantage than the presence of *babA*.

REGULATION OF BABA

Chimera formation between babA and babB

Several different mechanisms for regulation of BabA expression are predicted, including at the transcriptional and translational levels. As for translational regulation, the formation of chimeric proteins seems to play an important role. Chimera formation between *babA* and *babB* initially was reported by Pride and Blaser[11] who found that in two of 42 (5%) clinical isolates studied, the 5' regions of *babB* were replaced with the first 56 bp of *babA* (*babA/B* chimera). In addition, these authors showed that gene conversions frequently (10^{-3}) occur in *H pylori*, and the events are *recA*-dependent and DNase-resistant, indicating that they likely result from intragenomic recombination. *babA/B* chimeras also have been reported experimentally during *H pylori* infection in Rhesus monkeys[10].

In addition to *babA/B* chimeras, *babB/A* chimeras have been observed[9]. A *babA2* mutant from strain CCUG17875, defective in Le^b-binding, regained its activity by homologous recombination of a silent *babA1* gene into the *babB* locus, resulting in a chimeric *babB/A* gene. A silent wild-type *babA1* gene still was present.

The frequency of the *babA* mutant with Le^b-binding was approximately 10^{-5}. Detailed analyses of the chimeric *babB/babA* gene showed that the first 47 bp were *babB*-specific, the following 66 bp were shared between both *babA* and *babB*, and the remaining sequence was *babA*-specific. The second crossover event likely occurred within a region where the sequences of the *babA1* and the *babB* locus were identical. The chimeric BabB/A protein has the potential to bind Le^b; however, protein production was subject to phase variation through slipped-strand mispairing based on the number of Cysteine-Threonine (CT) dinucleotide repeats in the 5' region of the *babB* gene (switch "on" = functional and switch "off" = non-functional).

Initially, only five genes encoding the OMPs in *H pylori* (*oipA, sabA, sabB, babB* and *hopZ*) were reported to undergo phase variations in the 5' region such that not all strains produce functional proteins[25,27]. However, recent studies confirmed that phase variation is a method of regulating BabA production in some strains[10,29,30]. CT repeats were observed in 13 of 43 (30%) strains[29] and 4 of 22 (18%) strains[30]. Importantly, detailed analyses of the *babA* gene with CT repeats showed that the signal peptides are closely related to signal peptides of paralogous BabB proteins, whereas sequences further downstream were typical BabA sequences[30]. Taken together, these data suggest that the *babA* gene with the CT repeat might be the result of the translocation of *babA* into *babB* thereby generating a chimeric *babB/babA* gene. Interestingly, the *babC* gene in strain HPAG1 possessed CT repeats in the 5' coding region, whereas the *babC* gene in strain 26695 did not. These data suggest that *babB/C* chimera also might have occurred in some strains.

As described above, Colbeck *et al* found that there were cases with mixed *babA* and *babB* genes, especially at locus B[29]. The frequency of *babA* translocated at the *babB* locus was between 10^{-3} and 10^{-4}, which is in agreement with the frequency in strain CCUG17875[9]. Detailed investigation of 10 strains showed that the recombination event was identified from approximately 50 to 200 bp downstream of the ATG in five strains (all recombination occurred at locus B) and upstream of the ATG in the other five strains[29]. In the former case, the resulting gene forms the *babB/A* chimera, whereas complete recombination occurred in the latter case.

Overall, frequent translocation between *babA* and *babB* genes appears to be the main mechanism of regulating BabA expression. Therefore, *H pylori* uses both antigenic variation and phase variation to regulate *babA* expression.

Genomic mutations in the coding region of the babA gene

The *babA* gene initially was cloned from strain CCUG17875, which contains a silent *babA1* gene and an expressed *babA2* gene[1]. The sequence of these two genes differed only by the presence of a 10 bp deletion in the signal peptide sequence of *babA1* that eliminates its translational initiation codon. However, my group recently found that all 80 strains from a panel of Western and East Asian isolates contained an intact ATG start codon in the *babA* gene[31], and another group also reported the absence of the *babA1* type deletion[11,12,29,30,32]. Overall, the absence of a translation initiation codon, as described for *babA1* from CCUG17875, should be rare. Point mutations leading to stop codon, deletion and insertion in other parts of the *babA* gene also are not common; Hennig *et al* found one of 24 *babA*-positive strains (4%) contained a frameshift mutation that prevented expression of a full-length BabA protein (amino acid position at 55)[30].

Transcriptional regulation of BabA

Transcriptional regulation of BabA also has been reported. Backstrom *et al* found that only *babA2*, but not *babA1* was transcribed in strain CCUG17875[9]. Their analyses showed that *babA* transcription seemed to be regulated by the number of adenine [poly(A)] nucleotides within the -10 to -35 region of the *babA* promoter. The -10 and -35 region of the *babA2* sequences are highly homologous to the consensus for *E.coli* σ^{70} promoter sequences. This region was stable when the number of adenines was 10 (*babA2*) but would become non-functional when the number was 14 (*babA1*). The authors hypothesized that the poly(A) sequences between the -10 and the -35 sites could be prone to slippage mutations that allow changes in the level of transcription of downstream genes. However, other studies could not confirm that the -10 to -35 spacing played an important role in regulating *babA* expression[30,31]. Further studies will be necessary to fully interrogate the roles of transcriptional regulation of BabA.

Overall, there are several predicted mechanisms that may control BabA expression in some strains; however, there are many cases that remain unexplained. *H pylori* strains that do not produce BabA can be divided into five types, as shown in Table 1.

RELATIONSHIP BETWEEN BABA AND LE^B BINDING ACTIVITY

My group recently examined BabA protein and Le^b binding activity for 80 strains (40 from Japan and 40 from Colombia)[31]. BabA protein was measured by immunoblot analyses using anti-BabA antiserum (AK277), and Le^b binding activity was measured by binding of *H pylori* to ^{125}I-labeled fucosylated blood group antigens. *H pylori* strains were divided into two major groups: BabA-positive (76 strains) or BabA-negative (four strains). Semi-quantitative analyses of the BabA-positive strains allowed the BabA-positive strains to be classified into two distinct groups: those with high levels of BabA expression (68 strains) or those with low levels of BabA expression (eight strains). All of the 68 strains that exhibited Le^b binding activity produced high levels of BabA. The low and non-producer strains did not exhibit Le^b binding activity. Based on this finding, my group classified the strains into three distinct groups

Table 1 Five major types of *H pylori* strains that do not produce BabA

babA gene	Status
Negative	Include *babA/B* chimeras
Present	Regulated by slipped strand repairing and the status is "off" (probably equal to *babB/A* chimeras)
Present	Lack a translation initiation codon (single case of *babA1* in strain CCUG17875)
Present	Have a frameshift mutation(s) causing non-productive translation
Present	Without apparent mutations and without a hypothesis for the lack of expression

based on their expression levels of BabA: (1) BabA-high producers (BabA-H), which produce BabA protein at high enough levels to mediate Le^b binding, (2) BabA-low producers (BabA-L), which produce a small amount of BabA but not enough to mediate Le^b binding, and (3) BabA-negative strains, which do not produce any BabA protein.

BABA, LE^B BINDING ACTIVITY AND CLINICAL OUTCOMES

There currently are only a few studies that correlate the importance of BabA with clinical outcomes using immunoblot analyses[31,33,34]. My group recently performed large scale studies of 520 geographically diverse patients presenting with different clinical symptoms to evaluate BabA status by immunoblot analysis[31]. A total of 250 isolates from Western countries (150 strains from Colombia, 100 from the U.S.) and 270 isolates from East Asia (150 from Korea and 120 from Japan) were studied. All strains from East Asia expressed BabA protein. Twenty-four (9.8%) of Western strains were BabA-negative and were associated with milder gastric injury and lower *H pylori* density than BabA-positive status. BabA-negative status was inversely correlated with *cagA* status or *vacA* s1 genotype (i.e. only one (4.2%) and none (0%) of these BabA-negative strains were *cagA*- or *vacA* s1-positive, respectively). This is in agreement with previous studies that the *cagA* status was related to the presence of Le^b binding activity[1] and the presence of the *babA* gene[30].

Importantly, a small class of strains were BabA-positive but produced low levels of the BabA protein and lacked Le^b binding activity (BabA-L)[31]. Although these strains were functionally BabA-negative and were typically CagA-positive, they were more likely to be associated with duodenal ulcer, gastric cancer, and increased mucosal inflammation and atrophy than BabA-positive strains that exhibited *in vitro* Le^b binding activity (BabA-H strains) and BabA-negative strains. This finding suggests that either *in vitro* Le^b binding activity does not accurately reflect the severity of mucosal damage or that the clinical outcome or *in vitro* binding activity does not accurately reflect *in vivo* conditions. The underlying reason why strains with BabA-L status were more highly correlated with severe diseases than strains with BabA-H status is unknown, and it remains unclear whether expressing low levels of BabA have a direct role in the pathogenesis of gastroduodenal diseases. It is possible that BabA expression is influenced by the intragastric environment and that the phenotype of the BabA-L strains is an epiphenomenon rather than a cause of disease. It is possible that strong Le^b binding activity is associated with an inappropriate immune response resulting in severely inflamed mucosa. If so, the ability to change the BabA status from a high producer to low producer (i.e. Le^b binding to Le^b non-binding) would be advantageous for the organism, and a low producer might reflect an adaptation of *H pylori* that enhances survival in inflamed gastric mucosa. It also is possible that BabA expression down-regulates the proinflammatory effects of other putative virulence factors, such as the *cag* PAI and OipA.

DETECTION OF FUNCTIONAL *BABA* GENE

Most previous studies evaluating BabA (*babA*) status have used PCR techniques based on detection of the 10 bp deletion to distinguish between the *babA2* and *babA1* genes (Table 2)[35-53]. However, as described above, strains carrying the prototypical silent *babA1* gene are very rare, and in addition, the BabA protein levels often do not match the presence of the *babA* (*babA2*) gene[31]. Current terminology for *babA1* and *babA2* in the literature is confusing, and many researchers mistakenly understand that *H pylori* strains that do not produce BabA are either *babA* gene-negative or *babA1*-positive (= *babA* gene lacking a translation initiation codon). However, only one case with *babA1* has been reported, and BabA non-producing strains also usually possess non-functional silent *babA* gene sequences (i.e. 2, 4, and 5 in Table 2). Unfortunately, current PCR methods regard non-functional *babA* status as *babA2*-positive. In addition, a recent study confirmed that the PCR method used to detect *babA2* with only one primer pair previously designed yielded many false-negative results, probably due to sequence variation among strains[31].

Only a few studies have used a forward primer that is within the promoter region of the *babA* gene, a region that is identical to the sequence of *babA2* but different from that of *babA1* in strain CCUG17875[32,54,55]; however, recent analyses showed that the primers could also detect *babB* gene[31]. Overall, the information gained from currently used PCR-based methods must be interpreted with caution. In addition, I propose that researchers should not use current PCR-based methods in future studies.

Nonetheless, approximately half of the studies have suggested a correlation between *babA2*-positive *H pylori* in Western countries and increased risk of

Table 2 PCR-based genotyping for the *babA2* gene in *H pylori* positive cases *n* (%)

Study	Year	Population	Number studied	Prevalence of *babA2* gene						
				Total	Gastritis	PUD	Cancer	MALT	Duodenitis	Related to diseases
Western countries										
Gerhard *et al*	1999	Germany	114	82 (72)	18 (51)	23 (100)	21 (78)	20 (69)		Yes
Prinz *et al*[1,2]	2001	Germany	145	57 (39)	57 (39)					-
Rad *et al*[1]	2002	Germany	141	54 (38)	54 (38)					-
Zambon *et al*	2003	Italy	167	60 (36)	26 (28)	20 (49)			14 (42)	Yes
Oleastro *et al*	2003	Portugal	140	45 (32)	24 (23)	21 (58)				Yes
Podzorski *et al*	2003	USA	61	33 (36)	22 (36)					-
Oliveira *et al*	2003	Brazil	208	96 (46)	24 (32)	43 (54)	29 (56)			Yes
Rad *et al*[3]	2004	Germany	207	73 (35)	73 (35)					-
Lehours *et al*	2004	France	82	40 (49)	21 (54)			19 (44)		No
Gatti *et al*	2005	Brazil	89	42 (47)	37 (53)	3 (20)	1 (100)	1 (33)		No
Olfat *et al*	2005	Germany	92	41 (45)	19 (28)	22 (88)				Yes
		Sweden	74	33 (45)	21 (48)	12 (40)				No
		Portugal	91	31 (34)	12 (20)	19 (63)				Yes
		Finland	57	34 (60)	12 (46)	22 (71)				Borderline (P = 0.06)
Gatti *et al*	2006	Brazil	94	38 (40)	18 (41)	20 (40)				No
Asian countries										
Mizushima *et al*	2001	Japan	179	152 (85)	34 (81)	73 (85)	36 (90)	9 (82)		No
Yu *et al*	2002	China	104	83 (80)	83 (80)					-
Lai *et al*	2002	Taiwan	101	101 (100)	41 (100)	46 (100)	14 (100)			No
Han *et al*	2004	China	141	90 (64)	28 (65)	50 (65)	12 (57)			Yes (DU *vs* GU)
Zheng *et al*	2006	China	72	28 (39)	11 (39)	17 (40)				No
Lee *et al*	2006	Korea	135	83 (61)	64 (57)		19 (86)			Yes
Erzin *et al*	2006	Turkey	91	49 (54)	7 (23)	12 (43)	24 (73)			Yes

PUD: Peptic ulcer disease; MALT: Mucosal-associated lymphoid tissue; DU: Duodenal ulcer; GU: Gastric ulcer. [1]88% had German nationality and 12% were from other European countries; [2]Samples were examined from the antrum and the corpus, and the corpus data are presented (in the antrum, 55 were *babA2*-positive); [3]89% had German nationality and 11% were from other southern European countries.

developing significant clinical outcomes[38,44-46,52] and are in agreement with protein data as described above[31,33,34]. The prevalence of clinical isolates with a non-functional *babA2* gene without production of BabA protein may be low and negligible in some studies.

CONCLUSION

Several different mechanisms for regulation of BabA expression are predicted, including at both the transcriptional and translational levels. The formation of chimeric proteins seems to play an especially important role in translational regulation. The chimeric BabB/A protein has the potential to bind Le^b; however, the production was subject to phase variation through slipped-strand mispairing. Currently used PCR-based methods to evaluate BabA status do not take this mechanism of regulation into account, and information gained from currently used PCR-based methods must be interpreted with caution. I strongly recommend that researchers should not use PCR-based methods in their future studies. Recent studies evaluating BabA status by immunoblot confirmed that BabA-positive status in Western strains was closely associated with severe gastric injury, high *H pylori* density, and severe clinical outcomes. A small class of strains produced low levels of the BabA protein and lacked Le^b binding activity. Surprisingly, they were more likely to be associated with increased mucosal inflammation, atrophy, and severe clinical outcomes than BabA-positive strains that exhibit Le^b binding activity. The underlying reason is unclear, and further studies will be necessary to investigate how the complex BabA-receptor network is functionally coordinated during the interaction of *H pylori* with the gastric mucosa.

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EDITORIAL

Stem cells, a two-edged sword: Risks and potentials of regenerative medicine

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Abstract

The recent advancements in stem cell (SC) biology have led to the concept of regenerative medicine, which is based on the potential of SC for therapies aimed to facilitate the repair of degenerating or injured tissues. Nonetheless, prior to large scale clinical applications, critical aspects need to be further addressed, including the long-term safety, tolerability, and efficacy of SC-based treatments. Most problematic among the risks of SC-based therapies, in addition to the possible rejection or loss of function of the infused cells, is their potential neoplastic transformation. Indeed, SCs may be used to cure devastating diseases, but their specific properties of self-renewal and clonogenicity may render them prone to generate cancers. In this respect, 'Stemness' might be seen as a two-edged sword, its bright side being represented by normal SCs, its dark side by cancer SCs. A better understanding of SC biology will help fulfill the promise of regenerative medicine aimed at curing human pathologies and fighting cancer from its roots.

Key words: Stem cells; Regenerative medicine; Gastrointestinal diseases; Chronic liver diseases; Cell-based therapy

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A LESSON FROM THE PAST: STEM CELLS AND TUMORS

The recorded history of human cancer begins with an Egyptian papyrus, dating between 3000 and 2000 B.C., which describes breast tumors in humans. The nomenclature 'cancer' was first proposed by Hippocrates. The term derives from the Greek words carcinos and carcinoma, both literally meaning crab. Galen offered a possible explanation for this name based on similitude with either the morphology of cancer or with its tenacious and parasitic behavior. According to legend, Celsus attempted to classify pathologic masses into three categories: secundum naturam, associated with pregnancy; propter naturam, the tumefaction which develops following traumas and leads to tissue repair; and contra natura, synonymous with cancer[1,2]. After two millennia, this classification has found renewed perspective based on recent advancements in cell biology, with particular emphasis on the concepts of stemness and SCs.

In multicellular organisms, tissues are organized in a hierarchical manner, with SCs residing at the apex of the developmental pathway. SCs are defined as undifferentiated cells capable of self-renewal and differentiation into diverse mature progenies[3,4]. Therefore, SCs play a central role in tissue genesis, regeneration and homeostasis by providing new elements to increase tissue mass during pre- and post-natal growth, and replacing cell loss due to senescence or damage[5,6]. SCs are thought to alternate symmetric and asymmetric divisions, hence maintaining the property of self-renewal[7]. SCs possess a hierarchy of potentialities: from the totipotency of the zygote and its immediate progeny, to the pluripotency of embryonic SCs (ESCs), and the multi/unipotency of adult SCs (ASCs)[5,8,9]. ESCs are pluripotent cells derived from the inner cell mass of the blastocyst. ESCs can generate

any differentiated phenotype of the three primary germ layers by a process called determination[9,10]. At the end of embryogenesis, each tissue contains a heterogeneous population of cells at different stages of maturation, including relatively undifferentiated, self-renewing cells, termed adult SCs (ASCs). ASCs have a limited differentiation potential and are responsible for turnover and repair within the tissue of origin. ASCs have been identified in several organs, such as bone marrow (BM), gastrointestinal epithelium, skin, brain, muscle, and liver[8,11,12]. SCs colocalize with supporting cells in a physiologically limited and specialized niche, that varies in nature and location depending upon the tissue type[13,14]. The reciprocal interactions between SCs and their niche influence SC behavior: a complex network of developing signals regulates the balance between quiescence and the dividing state, leading ASCs toward self-renewal or differentiation[15,16]. According to the hierarchical model, long-term SCs (true SCs, extremely rare, with high differentiation potential and proliferative capacity) can give rise to short-term SCs (transit-amplifying or committed progenitors), which in turn are able to differentiate into mature elements providing tissue-specific functions[5,17]. Despite the paradigm of unidirectional cell determination, recent studies have shown that ASCs are endowed with an unexpected plasticity, as circulating adult progenitor cells can differentiate into mature cells of other tissue types[10,14,18]. A particularly high degree of plasticity is shown by bone marrow SCs (BM-SCs), which *in vivo* and *in vitro* studies have proved to be able to differentiate into a wide range of non-hematopoietic phenotypes[19-22]. It has also been demonstrated that BM-SCs normally circulate in the peripheral blood, and that the number of circulating SCs committed toward neuronal and hepatic differentiation increases following treatment with mobilizing agents[23]. This phenomenon has led to speculation about the existence of BM-derived pluripotent SCs, which could migrate from the peripheral blood into various tissues and contribute to normal turnover and repair following injury[5,15,24].

REGENERATIVE MEDICINE BASED ON STEM CELLS

The recent advancements in SC biology have led to the concept of regenerative medicine, which is based on SC potential for therapies aimed to facilitate the repair of degenerating or injured tissues[6]. SC-based therapies could be used to cure degenerative disorders associated with the loss of ASC functions, such as hematologic, cardiovascular, muscular and neurological diseases, gastrointestinal pathologies and chronic hepatopathies. SCs can be obtained from various sources, including embryos, fetal tissues, umbilical cord blood and adult organs. Once isolated, these cells may be forced to expand and differentiate into functional progenies suitable for cell replacement and tissue engineering[24]. ESCs, which have been isolated from humans and mice, can be maintained in an undifferentiated state indefinitely, though they seem to develop genetic abnormalities over long periods in culture[15,25]. ESCs and their derivatives might constitute an easily available source to obtain a large number of transplantable cells for regenerative treatments. Nevertheless, the possibility of immune rejection and teratoma/teratocarcinoma formation in the recipients represent major obstacles to the success and safety of ESC clinical applications[26]. A promising alternative source for SC-based treatments may be represented by cells established from fetal organs and placental tissues, which do not seem to form teratomas/teratocarcinomas in humans. In particular, several studies have indicated that umbilical cord blood SCs (CBSCs) are an easily accessible source of multipotent SCs, which may be readily available for transplantation, or for further expansion and manipulation prior to cellular therapies[8]. The plasticity and accessibility of CBSCs has provided the rationale for creation of CBSC unit banks, where these cells can be collected and stored for future use[24]. Finally, the manipulation and/or stimulation of ASCs seems to be the most promising tool for SC-based treatments, as it could improve the endogenous regenerative potential without risk of rejection and overcome the ethical and political issues related to embryonic and fetal SCs[6,8,24].

Focusing on ASC-based therapies in Gastroenterology, first attempts to translate regenerative medicine from theory to clinical practice have been made for various diseases, including celiac disease and inflammatory bowel disorders (IBD). In particular, following autologous BM transplantation, in a selected group of refractory celiac patients, significant histological improvement associated with impressive clinical progresses has been recorded[27,28]. Crohn's disease (CD) and ulcerative colitis (UC) are characterized by a status of chronic inflammation, mainly as a result of local immunological imbalance[29]. Several studies have suggested that either allogeneic or autologous BM-SC transplants may be effective in inducing CD and UC remission[30]. Various authors report their experience with IBD patients who underwent BM-SC transplantation for haematological malignancies and maintained a complete remission of their intestinal disease following transplantation[30]. The specific pathways and molecular mechanisms underlying the beneficial effects of HSC transplantation in IBD are still largely undefined. The immune system ablation followed by allogeneic transplantation of BM-SCs might provide a reset of the host immune system imbalance. Moreover, BM cells might contribute to tissue repair by facilitating neoangiogenesis and might also differentiate into epithelial cells and myofibroblasts[30,31]. The potential of BM-derived SCs in the treatment of IBD is currently being analyzed in clinical trials[30]. SCs might be used to cure other gastrointestinal pathologies, such as gastric ulcers, gastrointestinal motility disorders, and diabetes mellitus (DM)[32,33]. Regarding the latter, a major challenge in the treatment of DM is to provide patients with an insulin source that regulates glucose levels on a mandatory minute-to-minute basis[34,35]. In recent decades, new therapeutic strategies for the treatment of DM type I have been proposed, such as growth factor

administration, islet cell transplantation and also SC infusion to replace the dysfunctional beta-cells[35]. Different adult sources of extra-pancreatic SCs have been investigated, including CBSCs, whose efficacy for the treatment of DM has been shown in diabetic mice[36-39]. Another candidate for DM regenerative therapy is represented by BM-SCs. Numerous reports have showed that the infusion of BM-SCs can restore chemically-induced DM in mice[40,41]. Along with extra-pancreatic SC-based therapies, other researchers have focused their interest on endogenous pancreatic SCs (PSCs). The quest for an organ-bound PSC has received growing attention by the scientific community, because PSCs hold several advantages over extra-pancreatic sources, combining the ability for prolonged proliferation with an already established pancreatic commitment[35,42]. Finally, recent reports have demonstrated that extra-pancreatic, organ-bound SCs, such as liver SCs[43-46], human adipose tissue-derived mesenchymal SCs[47] and gastrointestinal SCs[48] can differentiate into islet cells. Unfortunately, there are still no functional studies that show biphasic insulin release upon glucose challenge by these cells.

In Hepatology, the most appealing application for SC-based therapies consists in the treatment of end-stage hepatic diseases. Chronic liver pathologies affect almost a fifth of the general population, often requiring an orthotopic liver transplantation (OLT)[49]. Given the donor organ shortage, various alternatives to OLT have been evaluated, including cell-based therapies which are currently under investigation all over the world. Cell-therapies in hepatology have numerous advantages when compared to OLT: the cells can be expanded *in vitro*, genetically manipulated, cryopreserved, obtained from the same patient and infused without major surgery. Possible cell-based treatments consist of hepatocyte transplantation and the development of bio-artificial liver systems (BALs). BALs have been mainly applied as supportive devices in patients excluded from or waiting for OLT and hepatocyte transplantation has limited overall success, related to the large amount of cells required to achieve acceptable function[50,51]. Therefore, SC-based therapies are emerging as new alternatives to OLT for end-stage liver pathologies. The most promising source for SC-based therapies is currently represented by BM-SCs and/or by mobilizing/proliferating agents, such as granulocyte-colony stimulating factor (G-CSF), which is able to both enhance the BM-SC mobilization into the peripheral blood and facilitate the endogenous liver SC activation[52,53]. BM-SCs seem to be physiologically involved in the processes of liver repair in humans[54,55]. The possible therapeutic potential of these cells has been investigated by intraportal autologous transplantation of BM-SC, which achieved some clinical improvement[56,57]. However, some authors reported negative results regarding BM-SC-therapies for end-stage liver disorders[58]. Other clinical approaches have been based upon the administration of G-CSF alone or in combination with the reinfusion of the mobilized BM-SCs. The feasibility, safety, and pattern of BM-SC mobilization following G-CSF treatment in patients affected by cirrhosis has been evaluated in a few clinical trials[59-64].

Overall, the use of ASCs for the treatment of gastrointestinal and hepatic disorders holds several advantages, such as easy accessibility, unlimited supply (given the possibility to expand the collected cells *in vitro*) and no risks of rejection or need for immunosuppressive therapies when autologous cells are employed. Nonetheless, some conceptual issues still limit the diffusion of such treatments into clinical practice. Firstly, on the basis of preclinical data, BM cells seem to facilitate gastrointestinal and hepatic regeneration mainly by a microenvironment modulation, which is likely to be transitory. In such a case, multiple treatments would presumably be required to achieve significant and lasting clinical results. Moreover, it has been observed that in some models of apparent transdifferentiation, SCs may actually be fusing with cells in the host tissue. Fusion phenomena between BM-SCs and other cells (Purkinjie cells, cardiomyocytes and hepatocytes) have been shown both *in vitro* and *in vivo*[15,24]. The implications of this discovery are notable: fusion and transdifferentiation are not synonymous, since transdifferentiation requires that a specific SC program be activated on the basis of extracellular signals, whereas in the case of fusion, the plasticity is trigged by endogenous factors upon mixing of the cytoplasm and joining of the nuclei. It must also be noted that the fused cells are aneuploid and potentially unstable[15]. Consequently, the possibility of cell fusion and the risk of malignant transformation of the transplanted cells, especially those pre-expanded *in vitro* before reinfusion, cannot be excluded and impose a need for careful evaluation and longer follow-up periods for assessing the safety and efficacy of these SC-based treatments[24].

STEM CELL ORIGIN OF CANCER AND CANCER STEM CELLS

In the nineteenth century, Virchow and Cohnheim proposed that some tumors, such as teratocarcinomas, exhibiting features of a whole range of different organs and therefore mimicking fetal development, could originate from embryonic rests[10,65-67]. Over 150 years later, the hypothesis of a SC origin of cancer lends itself to a modern-day interpretation of this theory: in a given tissue, somatic tumors could originate from the malignant transformation of a SC or its progeny during the determination process, a phenomenon called maturation arrest[10]. It is well accepted that carcinogenesis is a multistep process, involving accumulation of genetic mutations leading to the transformation of normal cells into tumorigenic cells. Every proliferating cell within a tissue may be targeted by carcinogenetic stimuli and undergo the process of transformation. Because of the specific characteristics of SCs, mutations within the SC compartment may result in cancer transformation[15]. Similarly, tumors might also arise from mutated progenitor cells which have regained the property of self-renewal, thereby dedifferentiating towards a SC phenotype[68-72]. Presumably, fewer mutagenic changes are required to transform a SC, in which the machinery to specify and

regulate self-renewal is already active, as compared to more committed progenitor cells, in which self-renewal must be activated ectopically[70]. Another potential source of tumorigenic cells may be represented by circulating pluripotent cells, originating from the BM and able to migrate into non-hematopoietic sites. The existence of such a population of SCs, whose properties are reminiscent of ESCs, has been suggested in humans and experimental animal models[73]. Once recruited, these cells may behave as normal SCs, and, therefore may accumulate mutations over time and initiate malignancies. Indeed, a recent report described a mouse model of gastric cancer induced by *H Pylori* infection, in which BM-derived cells were able to contribute to cancer development[74]. The hypothesis of a SC origin of tumors imposes caution when proposing SC-based therapies to treat human diseases. It is well known that ESCs may give rise to tumors, while cancers derived from ASC-therapies have never been reported. Nonetheless, the long-term safety of ASC infusion has not been adequately tested: preclinical studies and clinical trials with longer follow-up periods should be recommended prior to large-scale clinical applications of such cell-based therapies.

Along with the possible role of SCs in the cellular origin of tumors, mounting evidence suggests that cancer might be considered as a SC disease. Over the past 30 years, several studies have demonstrated that most cancers possess a hierarchic organization: the great majority of cancer cells cannot sustain the tumor mass, nor establish secondary lesions elsewhere in the body. Only a minority of cancer cells appear to be tumor-initiating and possess the metastatic phenotype. These cells have the property of self-renewal, can differentiate into any cell within the tumor population, and can migrate, establishing metastases. Given the similarities between normal SCs and tumor-initiating cells, the latter have been termed cancer SCs (CSCs)[75]. Studies on acute myelogenous leukemia (AML) firstly showed that only a small subset of cancer cells was capable of extensive proliferation both *in vitro* and *in vivo*. Two models have been proposed to explain this phenomenon: the stochastic theory and the cancer SC theory[76]. In the first model, processes of self-renewal versus differentiation occur randomly, so that every cancer cell has an equal probability of retaining self-renewal capacity. Conversely, the cancer SC theory postulates a hierarchical organization of functionally distinct cell subpopulations, at the apex of which resides a small population of tumor-initiating cells, responsible for cancer growth and progression. Such a hierarchical organization was first documented in hematological malignancies by Dick *et al*, who showed that only AML-initiating cells could induce AML when transplanted into SCID mice[77,78]. These results represented both the first direct demonstration of the existence of CSCs, and a proof of principle extendible to solid tumors. Currently, distinct populations of CSCs have been identified within the hematopoietic system[77,79], breast[80], brain[81], prostate[82,83], lung[84], skin, bone, kidney, ovary, head and neck cancers, and also gastrointestinal and liver tumors[85-90].

Tumor-initiating cells mimic SC properties to sustain the growth and spread of the tumor, while eluding the intrinsic and extrinsic controls that regulate homeostasis within SC populations. The unique properties of CSCs explain the failure of traditional chemotherapic strategies aimed at reduction of tumor mass by targeting proliferating cells: CSCs are usually quiescent and thus refractory to these treatments. The cancer SC hypothesis offers new insights for the development of therapeutic strategies in oncology, which will require a deep understanding of CSC molecular profile and biological behaviour[15,65]. Potential targets for CSC-based therapies in oncology might be found by comparing SC and CSC properties. i.e. it is well known that CSCs share molecular pathways involved in the maintenance of stemness (such as Wnt, Sonic Hedgehog, and Notch signalling) with SCs and that they are responsive to similar motomorphogens involved in both SC migration and cancer metastasis. The development of drugs antagonizing these signals may be helpful in inhibiting CSC proliferation and mobilization, therefore blocking cancer growth and metastasis[15,65]. Moreover, SCs and CSCs are able to secrete cytokines and angiopoietic factors which are critical for sustaining tumors, and that can be specifically targeted by anti-angiogenetic therapies[24]. However, an ideal CSC-based therapy would require targeting of CSCs, while sparing normal SCs. Indeed, despite similarities in terms of immunophenotype with their normal counterparts, some cell-surface markers and metabolic pathways must differs in CSCs compared with SCs, implying a biological uniqueness of CSCs. As a consequence, the identification of specific CSC-markers and pathways appears to be fundamental in order to develop novel therapeutic strategies in oncology. The quest for a surface marker which will enable isolation and further characterization of tumor-initiating cells within human cancers has already begun. Several studies have suggested that the CSC fraction within various tumors might be identified by the expression of CD133, a trans-membrane glycoprotein[91]. CD133 is expressed by progenitor cells belonging to neuronal, hematopoietic, epithelial and endothelial lineages and its expression has been reported in several tumor tissues, including melanomas, kidney, ovarian, colon and liver cancers[85-91]. In our opinion, CD133 might be useful to enrich the CSC fraction within some tumors, but it cannot be considered as a specific cancer SC-antigen. Indeed, CD133 is expressed by various normal SCs and also progenitor cells; moreover, upon a careful examination of the published studies, it seems that only a minority of CD133+ cancer cells is tumor-initiating[85-91].

STEMNESS AS A TWO-EDGED SWORD

A regenerative medicine based on SCs is no longer a future perspective, since SC research is already supporting an escalating industry, engaged in testing treatments for every sort of disease. Nonetheless, critical aspects need to be further addressed, including the long-term safety, tolerability, and efficacy of SC-based treatments, as well as their carcinogenic potential. Indeed, SCs represent the key to tissue genesis, regeneration and homeosta-

sis. However, for their specific characteristics, SCs may also represent a unique target for tumorigenic stimuli[16]. Stemness might be seen as a two-edged sword, its bright side being represented by normal SCs, its dark side by CSCs. This scenario leads to a reinterpretation of the previously mentioned Celsus' tumor classification, where ESCs represent the source of tumors secundum naturam; normal ASCs restore homeostasis following injuries, being responsible for tumors propter naturam; CSCs mimic normal ASCs in respect to self-renewal potential, but elude homeostatic regulation, resulting in tumors contra natura.

The CSC hypothesis imposes caution when proposing SC-based therapies, because infused SCs may degenerate into CSCs and give rise to neoplasms. This possibility should impose further preclinical studies prior to large-scale clinical applications of SC-based therapies. However, the CSC hypothesis also offers new insights for anti-cancer treatments, based upon the similarities and differences between SCs and CSCs. As a consequence, normal SC and CSC research must proceed side-by-side, because the identification of unique CSC targets requires a deep understanding of normal SC molecular profile and properties. The promise of regenerative medicine based on SCs imposes a better knowledge of SC and CSC biology, to help prevent and cure human pathologies and fight cancers from their roots.

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EDITORIAL

Role of cytokines in inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), represents a group of chronic disorders characterized by inflammation of the gastrointestinal tract, typically with a relapsing and remitting clinical course. Mucosal macrophages play an important role in the mucosal immune system, and an increase in the number of newly recruited monocytes and activated macrophages has been noted in the inflamed gut of patients with IBD. Activated macrophages are thought to be major contributors to the production of inflammatory cytokines in the gut, and imbalance of cytokines is contributing to the pathogenesis of IBD. The intestinal inflammation in IBD is controlled by a complex interplay of innate and adaptive immune mechanisms. Cytokines play a key role in IBD that determine T cell differentiation of Th1, Th2, T regulatory and newly described Th17 cells. Cytokines levels in time and space orchestrate the development, recurrence and exacerbation of the inflammatory process in IBD. Therefore, several cytokine therapies have been developed and tested for the treatment of IBD patients.

Key words: Cytokines; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Inflammation

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INTRODUCTION

Inflammatory bowel disease (IBD) comprises two forms, Ulcerative Colitis (UC) and Crohn's disease (CD). Currently, the pathogenesis of UC and CD is not completely understood, although the chronic relapsing inflammation is thought to be result from a dysregulated, aberrant immune response to intestinal flora in a context of genetic predisposition. In IBD, this loss of immune tolerance toward the enteric flora it is mediated by different molecules.

Cytokines are key signals in the intestinal immune system, and are known to participate in the disruption of the so-called normal state of controlled inflammation (physiological inflammation of the gut)[1]. Cytokines are small peptide proteins produced mainly by immune cells that facilitate communication between cells, stimulate the proliferation of antigen specific effector cells, and mediate the local and systemic inflammation in an autocrine, paracrine, and endocrine pathways[2]. In IBD, the innate immune response plays a critical role. Activated dendritic cells (DC) and macrophages secrete several cytokines that actively regulate the inflammatory response in UC and CD. Once secreted by these antigen presenting cells (APC), these cytokines trigger and differentiate many T cells activating the adaptive immune response. IBD has also a T cell dysregulation where clearance of overreactive and autoreactive cells is disturbed, in addition to an imbalance of Treg/Th1, Th2 and newly described Th17 cells populations in the activated state. The lack of appropriate regulation from T cells, or an over-production of effector T cells, participates in the development and exacerbation of IBD[3].

Altogether, APCs, Th1, Th2, T regulatory cells and

Table 1 Role of cytokines and cell lines involved in their production in patients with IBD

Cytokine	UC	CD	Cells involved in the production
TNF-α	Up-regulated	Up-regulated	Macrophages
TL1α	Unknown	Up-regulated	Th1
IL-1β	IL-1ra/IL-1 ratio	IL-1ra/IL-1 ratio	Macrophages
IL-6	Up-regulated	Up-regulated	Macrophages, DC, Th17 and others
IL-18	Not	Yes, not in all patients	Macrophages
TGF-β	Not clear, maybe defective signalling	Not clear, maybe defective signalling	Th0, Th3, Treg
IL-10	Not clear	Yes, up-regulated	Tr1 and Breg
IL-4	Not clear	Not clear	Th2, NK
IL-12	Up-regulated	Up-regulated	Macrophages, DC
IL-23	Yes	Yes	Macrophages, DC
IL-27	Not clear	Up-regulated	APCs
IL-17	Up-regulated	Up-regulated	Th17
IL-13	Up-regulated	Not	Th1, NK
IL-5	Up-regulated	Not	Th2, NK

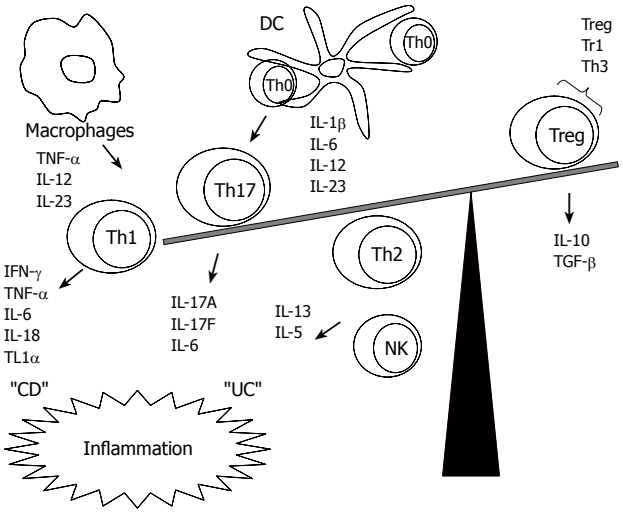

Figure 1 Cytokines imbalance between effector and T regulatory cells in IBD.

most recently characterized Th17 and their cytokine products play a complex role in IBD[4]. These cellular interactions are modulated by both traditionally studied cytokines (such as TNF-α, INF-γ, IL-1, IL-6, IL-4, IL-5, IL10, TGF-β) and others recently characterized (like IL-13, IL-12, IL-18, IL-23), considered to be either pro or anti-inflammatory, as shown in Table 1[5]. Although many common responses in IBD are mediated by cytokines, such as the regulation of the production of inflammatory mediators, reactive oxygen metabolites, nitric oxide, leukotriens, platelet-activating factor, and prostaglandins, activation of the nuclear factor κB (NF-κB) and inhibition of apoptosis, how cytokines determine the nature of the immune response in IBD may be quite different among IBD forms[6]. CD is associated with a Th1 T cell mediated response, characterized by enhanced production of IFN-γ and TNF-α. IL-12 and IL-23 govern the Th1 differentiation which in combination with IL-15, IL-18 and IL-21 will induce the stabilization of polarized Th1. On the other hand, in UC, the local immune response is less polarized, but it is characterized by CD1 reactive natural killer T cell production of IL-13 and Th2 cytokine production, as shown in Figure 1[7].

CLASSICAL PRO-INFLAMMATORY CYTOKINES

Lymphocytes and APCs orchestrate a lot of the inflammation in IBD, mainly the production of TNF-α, a 17-kD pleiotropic cytokine produced by innate immune cells as macrophages, monocytes, and also by differentiated T cells. TNF-α exerts its pro-inflammatory effects through increased production of IL-1β and IL-6, expression of adhesion molecules, proliferation of fibroblasts and procoagulant factors, as well as initiation of cytotoxic, apoptotic, acute-phase responses, and inhibition of apoptosis[8,9]. TNF-α expression in human macrophages was discovered in the colonic tissue and macrophages in both patients with CD and UC[10] and serum levels of TNF-α correlate with clinical and laboratory indices of intestinal disease activity[11]. Clinical studies have reported a dramatic improvement in CD patients treated with anti-TNF-α therapy such as infliximab, adalimumab and certolizumab pegol[12]. Reductions in the number of IFN-γ producing, lamina propria mononuclear cells (LPMC) in colonic biopsies results from anti-TNF-α treated patients[13].

The signalling of TNF-α starts with serum soluble TNF receptor I and Ⅱ (sTNF-R Ⅰ, Ⅱ) levels correlate with disease activity in IBD patients. More specifically, sTNF-RI is up-regulated in the serum of IBD patients compared to healthy controls and could be used as a marker for disease activity[14]. sTNF-RII levels are significantly more elevated in serum from active CD patients as compared to UC and could be used as an additional parameter to discriminate both diseases[12]. Recently TNF receptor type 1-dependent activation of innate responses was shown to reduce intestinal damage-associated mortality[15].

Related to the TNF-α, the TNF-like factor (TL1A) seems to stimulate IFN-γ secretion by binding to the death receptor 3 (DR3). A higher percentage of cells express the TL1A receptor DR3 in mucosal biopsies taken in CD and UC, and increased synthesis of IFN-γ has been observed to correlate with severity of disease in IBD patients[16]. This molecule links TNF related apoptosis in inflammatory intestinal epithelial lesions,

tumour-necrosis-factor related apoptosis inducing ligand (TRAIL) messenger RNA and protein were markedly up-regulated in IEC and lamina propria lymphocytes in animal model. Interferon-gamma and TNF-alpha potently induced TRAIL in IEC and TRAIL is highly up-regulated in IEC in inflammatory ileum and colon[9].

In addition to TNF-α, IL-1 seems to be important in the pathogenesis of IBD because of its immunological up-regulatory and pro-inflammatory activities. The IL-1 system consists of IL-1α and IL-1β, both of which are produced by various cell types through the initiation of cyclooxygenase type 2, phospholipase A, and inducible nitric oxide synthase (iNOS)[17]. The IL-1 system can be also highly regulated by IL-1 receptor antagonist (IL-1Ra), as supported by the findings of high plasma and tissue levels of IL-1Ra in patients with IBD, indicating that IL-1Ra may be part of the host mechanism for downregulation of inflammation[18]. The IL-1Ra/IL-1 ratio decreases with increasing IBD activity, while remaining constant in uninvolved CD and inflammatory control specimens: this may contribute to the pathogenesis of chronic gut inflammation[19]. Increased levels of IL-1 in IBD may be result of stimulation of colonic macrophages that can activate interleukin (IL)-1 converting enzyme (ICE) and hence release mature IL-1β into the colonic mucosa[20].

In contrast to other cytokines, IL-6 is a pleiotropic cytokine that exerts its proinflammatory effects largely by means of its soluble IL-6 receptor (sIL-6R). The combination of soluble IL-6 receptor (sIL-6R) and IL-6 stimulates cells that only express gp130 and not IL-6R, a process known as trans-signalling. IL-6 signalling through signal transducer and activator of transcription-3 (STAT3) has been extensively studied[21]. This system plays a central role in several immunologic reactions during the development of IBD, and circulating levels of IL-6 and sIL-6R correlate with many clinical features of CD and UC[22-24]. Blockade of IL-6 trans-signalling causes T-cell apoptosis, indicating that the IL-6-sIL-6R system mediates the resistance of T cells to apoptosis in CD[24]. Yamamoto *et al* (Kallen KJ[25]) introduced the anti-IL-6 receptor monoclonal antibody to a murine colitis model and found that the treatment with this antibody reduced IFN-γ, TNF-α, and IL-1β mRNA, and suppressed expression of several intracellular adhesion molecules in the colonic vascular endothelium. The anti-IL-6 receptor monoclonal antibody also abrogates murine colitis by effectively blocking the recruitment of leukocytes and increasing T-cell apoptosis[26]. Peripheral immune cells as well as colon epithelial and lamina propria cells with an active form of IL-6/STAT3 system may be responsible of the high correlation with the degree of mucosal inflammation[27]. The signaling of IL-6/STAT3 in the activation of mucosal T cells has been suggested as a major therapeutic target for the future[28]. STAT-3 itself induces the anti-apoptotic factors Bcl-2 and Bcl-xL, thus resulting in T-cell resistance against apoptosis. This circle of T-cell accumulation, mediated by apoptosis resistance, finally leading to chronic inflammation, can be blocked by anti-IL-6 receptor antibodies[29].

IL-18

IL-18 is produced by intestinal epithelial cells and was originally identified as an IFN-γ inducing factor, shares similarities with the IL-1 family in terms of its structure, processing, receptor, signal transduction pathway, and pro-inflammatory properties[30]. Recent studies have shown that the balance between this pleiotropic pro-inflammatory cytokine and its natural inhibitor, IL-18-binding protein (IL-18BP), may contribute to the pathogenesis of IBD[31]. A local increase of IL-18 expression has been demonstrated in chronic lesions of CD compared with uninvolved areas or normal controls[32], and an increase in IL-18 was also shown to be accompanied by marked increases in IL-18 receptor-positive immune cells as well as intense transcription of IL-18 induced by cytokines, such as IFN-γ, IL-1β, and TNF-α[33]. In a recent study has been reported that IL-18 up-regulation may be found only in a minority of patients with CD[34]. Furthermore, in the presence of IL-18, T cells from the inflamed CD tissue have been shown to produce less IL-10 than control tissue[35]. Although recombinant IL-18 alone induces significant proliferative responses in freshly isolated mucosal lymphocytes from CD patients[36,37], a synergy between IL-12 and IL-18 in activated macrophages may be a regulatory mechanism driving *lamina propria* lymphocytes toward a Th1 response in IBD[38]. It has been reported that cytokine IL-12 may act in synergy with IL-18 to promote the induction of IFN-γ, leading to severe gut inflammation in mice[39]. The development of Th1 CD4+ T cells in the intestinal mucosa is driven by IL-12, produced from activated macrophages, and IL-18, produced from activated macrophages and colonic epithelial cells. The synergistic effect is mainly caused by mechanisms involving the up-regulation of the IL-18 receptor by IL-12[40].

ANTI-INFLAMMATORY AND IMMUNOM ODULATORY CYTOKINES

IL-10

IL-10 is an anti-inflammatory cytokine that inhibits both antigen presentation and subsequent release of pro-inflammatory cytokines, thereby attenuating mucosal inflammation. The pivotal role played by IL-10 within the mucosal immune system has been extensively studied in the chronic ileo-colitis that develops in gene-targeted IL-10 knockout mice and by its therapeutic efficacy in several animal models of colitis[41]. An inactivation of IL-10 in mice results in an increased production of IL-12 and IFN-γ[42,43]. Inflamed tissues and granulomas of CD show low IL-10[44]. Melgar *et al*[45] reported a highly significant increase in IL-10 mRNA levels in T lymphocytes and in IL-10-positive cells in the colons of UC patients. Recently produc-

tion of IL-10 by regulatory T cells has been implicated as important issue in IBD[46]. Other regulatory cells that may participate in UC through the production of IL-10 are a regulatory B cells subtype called Bregs[47]. The importance of IL-10 production by B cells has been evidenced in IBD models and in humans[48,49], Mizoguchi *et al* showed that Bregs can be responsible for the suppression and/or recovery form acquired immune mediated inflammations by mechanisms that include IL-10 and TGF-β1 in IBD[47].

IL-4 and TGF-β

Overall, anti-inflammatory cytokines whose roles are less well characterized in IBD include IL-4 and TGF-β. IL-4 is a stimulatory molecule for B and T cells, and has known immunosuppressive effects in the intestine[50]. T-cell receptor alpha chain-deficient mice (TCR -/-) treated with anti-IL-4 monoclonal antibody showed a decrease in Th2-type mRNA cytokine production and an increase in expression of IFN-γ, suggesting that IL-4 plays a major role in inducing Th2-type CD4+ cells in the gut to shift towards a Th1 response[51]. Also another study showed that development of colitis in the TCR -/- mice depends on IL-4 rather than IFN-γ[52]. A study reported that the administration of IL-4 led to a significant reduction of the vascular endothelial growth factor (VEGF) production by peripheral blood mononuclear cells in active CD and UC patients[53].

Similarly, TGF-β is an inhibitory cytokine recognized as a key regulator of immunological homeostasis and inflammatory responses. Reduced TGF-β activity is considered to be responsible for the development of autoimmune disorders in several pathologic conditions including IBD[54]. Defective transforming growth factor TGF-β1 signaling due to high levels of Smad7 is a feature of IBD[55]. UC patients have exhibited increased production of TGF-β1 by LPMC as compared with both CD patients and controls, highlighting that although TGF-β acts on the systemic immune system to promote a potent immunosuppressive effect, locally TGF-β may demonstrate pro-inflammatory properties[56]. Evidence suggests that TGF-β can act in concert with epidermal, insulin-like, fibroblast growth factors, as well as VEGF to protect host tissue from luminal challenges and facilitate repair of mucosal injury in IBD[57,58]. As future therapy, the inhibition of Smad7 may reestablish TGF-β1 function and the suppression of colitis as proven in experimental models of colitis[59].

IL-12 and related cytokines

IL-12 and IL-23 belong to the IL-12 family of pro-inflammatory heterodimeric cytokines and comprises IL-12p40/IL-12p35 and IL-12p40/IL-23p19 subunits[60]. They are mainly produced by activated APCs and accessory cells such as DC and phagocytes[61]. The receptors for these cytokines are also heterodimeric IL-12 binds an IL-12Rβ-IL-12Rβ2 heterodimer, whereas IL-23 binds an IL-12Rβ1-IL-23R heterodimer[60]. The receptors for both IL-12 and IL-23 are mainly expressed on T cells, NK cells, and NKT cells. However, low levels of the receptor for IL-23 are also expressed on monocytes, macrophages, and DCs[61]. Both cytokines activate TYK2 and JAK2 as well as STAT1, STAT3, STAT4, and STAT5[60]. Although IL-12 activates STAT4 most efficiently, IL-23 preferentially activates STAT3[60]. Despite the similarities in receptor subunit and signaling, recent studies have shown that IL-12 and IL-23 drive divergent immunological pathways.

The expression of IL-12 is up-regulated in both active UC and CD biopsies and it correlates with activity index score[62]. Levels of IL12p40 and IL12Rβ2 are higher in early rather than in late CD suggesting that IL12-mediated modulation is strongly dependent on the stage of disease[63]. In particular, to drive adaptive immune responses, DCs (that sense the nature of the microorganisms in the intestine) are key producers of IL-12 in IBD[64].

In animal models, IL-23 showed to be essential for manifestation of chronic intestinal inflammation, whereas IL-12 is not. A critical target of IL-23 is a unique subset of tissue-homing memory T cells, which are specifically activated by IL-23 to produce the pro-inflammatory mediators IL-17 and IL-6[65].

Recently, another IL-12-related cytokine, IL-27, was described. IL-27 consists of EBI3, an IL-12p40-related protein, and p28, a newly discovered IL-12p35-related polypeptide. Mucosal expression of IL-23p19 and IL-27p28 transcripts correlate with the inflammatory activity in IBD both CD and UC. Particularly, IL-27p28 transcripts and EBI3 transcripts were significantly elevated only in active CD[66].

IL-17 and Th17 cells

Recently, a new T cell subset named "Th17", characterized by the production of IL-17, was identified as an important player in inflammatory responses[67]. Sequencing the human genome resulted in the discovery of an additional five members of the IL-17 family that were consecutively named IL-17B to IL-17F. IL-17A is exclusively produced by Th17 cells[68]. The production of IL-17 relies on STAT3 activation triggered by IL-23[69]. IL-17 in general induces the recruitment of immune cells to peripheral tissues, a response that requires NF-κB activation after IL-17 receptor engagement[70,71]. IL-17 also leads to the induction of many pro-inflammatory factors, including TNF-α, IL-6, and IL-1β, suggesting an important role for IL-17 in localizing and amplifying inflammation[72-74]. Furthermore, TNF-α and IL-6, which are both produced by Th17 cells, not only support Th17 cell development but also synergize with IL-17 to enhance the production of pro-inflammatory mediators[74]. Regulatory T cells CD4+CD25-Foxp3- could be a source of Th17 cells[75]. In human cells, IL-1, IL-6, and IL-23 promote human CD4+ to Th17 differentiation, but TGF-β1 is not needed like in mouse[76]. IL-17 as well as Th17 cells have both been found to be elevated in serum and intestinal tissue of IBD patients. IL-17 was not detected in inactive patients tissue as well as other colitis[77].

IL-13 and T cell response in UC

UC is characterized by a Th2 immune response in which IL-13, which is produced by specialized cells such as NK T-cells, was identified as an important effector cytokine[78]. In UC, IL-13 may impair epithelial barrier function by affecting epithelial apoptosis, tight junctions, and restitution velocity[79]. Both discoveries were made by determining the cytokine profile of LPMC isolated from tissue recovered from colonic resection from UC and CD patients. It was found that LPMC from UC patients secreted high amounts of Th2 cytokines IL-13 and IL-5[78,79]. This research group found that the IL-13 and IL-5 LPMC cells bear NK specific markers CD161 and recognize CD1d, indicating that they are NK T-cells[78]. These NK T-cells are considered "non-classical". The NK T-cells isolated from UC patients exhibited cytotoxicity towards an epithelial cell line (HT-29)[78]. This cell population possibly could be the cells causing epithelial cell cytotoxicity in UC described in the 1980s[80]. IL-13 signalling through the IL-13α2 receptor (IL-13Rα) in general is involved in induction of TGF-β1 production and fibrosis[81]. The signalling through IL-13Rα was important in the fibrosis caused by TGF-β1 in an animal model[82]. However, the extent to which this leads to the ultimate cascade of inflammation in UC remains to be determined.

NOVEL CYTOKINES INVOLVED IN IBD

Other cytokines like IL-21 and IL-22, which have been implicated in the pathophysiology of inflammatory and autoimmune diseases such as asthma, arthritis and lupus, play also an important role in IBD. IL-21 is a T cell derived cytokine member of the common gamma-chain-dependent cytokine family, which in general acts on intestinal epithelium helping to maintain the ongoing Th1 inflammation by inducing the production of IFN-γ[83,84]. IL-21 also has been shown to enhance the expansion of NK cells[85]. IL-21 is expressed by immune T and B cells and non-immune cells like fibroblasts, where it activates the metalloproteinase 1 production, and signalling through its receptor IL-21R it activates STAT-3 in T cells[86]. IL-21, like IL-6 and IL-23 is also involved in Th17 cell differentiation[87] and it is over-expressed in both CD and UC, with higher levels being found in CD[88].

IL-22 was originally described as an IL-9-induced gene and was named as IL-10-related T cell-derived inducible factor (IL-TIF)[89]. This cytokine shows 22% amino acid identity with IL-10 and belongs to a family of cytokines with limited homology to IL-10. IL-22 binds at the cell surface to a receptor complex composed of two chains belonging to the class II cytokine receptor family (CRF2): IL-22R1 and IL-10R2[90,91]. In the intestinal cells, particularly innate immune cells, the binding of IL-22 to its respective R1 chain induces a conformational change that enables IL-10R2 to interact with the newly formed ligand-receptor complexes. This in turn, activates a signal transduction cascade that results in rapid activation of several transcription factors, including STAT1/3 proteins[91]. The principal sources of IL-22 are natural killer and activated T and B cells. Th17 has proven a very important role in this matter[92]. IL-22 has proinflammatory functions in IEC and is upregulated in CD both in tissue and in serum[93,94]. Surprisingly, in a murine model of UC, Sugimoto *et al* demonstrated a novel protective role for IL-22, in which IL-22 attenuates in the intestine inflammation by inducing mucin membrane bound production by goblet cells[93,94]. Another recent paper showed that IL23R genotypes affect IL-22 serum concentrations, linking for the first time genetic CD susceptibility to Th17 cell function[94].

POTENTIAL BIOLOGICAL THERAPIES DIRECTED TO CYTOKINES

Controlling the expression, production and activity of IL-23 as well as IL-17 is an approach that would allow the development of a novel treatment strategy with more anti-inflammatory efficacy and potentially with less suppressive effects on host defenses[95]. There are different biologic therapies directed to several cytokines tested in patients with IBD: fontolizumab (anti-interferon γ) is a humanized monoclonal antibody to interferon gamma. A small phase 2 study of fontolizumab at subcutaneous doses of 10 mg/kg in patients with moderate to severe CD demonstrated efficacy and safety[96]. A randomized clinical trial of 79 patients with CD receiving 1 mg or 3 mg of anti-IL-12 monoclonal antibody *versus* placebo demonstrated a response in 75% of CD patients compared with 25% in the placebo group[97]. Other antibodies have been generated against to T-cell subsets blockade including CD3+ cells (visilizumab) and CD25+ cells (daclizumab and basiliximab) for UC. Pilot studies have shown promising results in steroid-resistant UC patients[98]. IL-6 participates in a variety of critical functions, including T cell growth and differentiation, as well as B-cell proliferation. In a pilot study, where patients with active CD were treated with an antibody directed against the IL-6 receptor (Atlizumab), 80% responded at the full dose compared with 31% in the placebo group[99]. IL-11 is produced by cells of mesenchymal origin. A placebo controlled trial of subcutaneous IL-11 in patients with active CD did not demonstrate clear efficacy[100].

CONCLUSION

Cytokines are important in the pathogenesis of IBD and their manipulation has successfully reduced disease severity and maintained remission. Following the discovery of novel cytokines and the role they may play in gut mucosal immunity, as well as the emergence of new concepts and changing paradigms in IBD pathogenesis, the roles of several cytokines have been elucidated and tested in both preclinical animal models and clinical trials of patients with IBD. Complementary to this, proof of

concept for new cytokine targets is rapidly developing, with the possibility of future cytokine-based therapies that may offer greater specificity and decreased toxicity for the treatment of IBD. In addition, further applications of cytokine-based therapies in human clinical trials and preclinical animal studies are ongoing.

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CLINICAL PRACTICE GUIDELINE

Perspective on the practical indications of endoscopic submucosal dissection of gastrointestinal neoplasms

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Abstract

Endoscopic submucosal dissection (ESD) is a new endoluminal therapeutic technique involving the use of cutting devices to permit a larger resection of the tissue over the muscularis propria. The major advantages of the technique in comparison with polypectomy and endoscopic mucosal resection are controllable resection size and shape and *en bloc* resection of a large lesion or a lesion with ulcerative findings. This technique is applied for the endoscopic treatment of epithelial neoplasms in the gastrointestinal tract from the pharynx to the rectum. Furthermore, some carcinoids and submucosal tumors in the gastrointestinal tract are treated by ESD. To determine the indication, two aspects should be considered. The first is a little likelihood of lymph node metastasis and the second is the technical resectability. In this review, practical guidelines of ESD for the gastrointestinal neoplasms are discussed based on the evidence found in the literature.

Key words: Endoscopic submucosal dissection; Endoscopic mucosal resection; Gastrointestinal neoplasm; Treatment guideline; Lymph node metastasis

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GENERAL CONCEPT TO APPLY ENDOSCOPIC SUBMUCOSAL DISSECTION FOR GASTROINTESTINAL NEOPLASMS

Endoscopic submucosal dissection (ESD) is a new endoluminal therapeutic technique involving the use of cutting devices to permit a larger resection of the tissue over the muscularis propria in three steps: injecting fluid into the submucosa to elevate the lesion from the muscularis propria, precutting the surrounding mucosa of the lesion, and dissecting the connective tissue of the submucosa beneath the lesion. The major advantages of the technique in comparison with polypectomy and endoscopic mucosal resection (EMR) are these: the resected size and shape can be controlled; *en bloc* resection is possible even for a large lesion; and the lesions with ulcerative findings are also resectable[1,2]. Retrospective analyses of the comparison between ESD and EMR for the stomach epithelial neoplasms showed that ESD increased *en bloc* and histologically complete resection rates compared with EMR but was associated with longer average operation times and a higher incidence of intraoperative bleeding and perforation[3,4].

Two aspects are considered to determine the application of ESD for each lesion by each operator (Figure 1). The first is a little likelihood of lymph node metastasis and the second is the technical resectability. The former has been determined by the large numbers of surgically resected cases in each organ before establishment of ESD and the latter may be determined by the applied technique, the expertise of the operators, the location of the lesions or their characteristics. In terms of technical resectability, *en bloc* resection is more desirable than piecemeal resection for accurate assessment of the appropriateness of the therapy, because the depth of invasion and lymphovascular infiltration of cancer cells (that are considerable risk factors for nodal metastasis) are not accurately assessed by piecemeal resection. Almost all possible node-negative epithelial neoplasms can be resected *en bloc* by

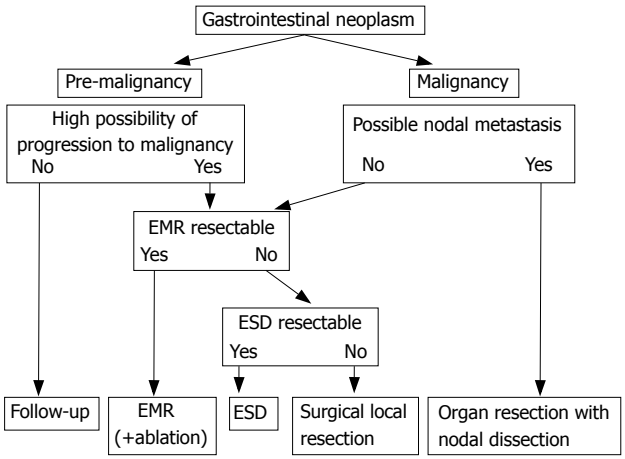

Figure 1 Algorism for endoscopic submucosal dissection of gastrointestinal neoplasms.

ESD, when they are treated by very experienced hands. This does not mean that all endoscopic resection should be performed as ESD. Polypectomy or endoscopic mucosal resection (EMR) are beneficial for patients with pedunculated neoplasms or small neoplasms because of the little invasiveness[5]. If the lesions are apparently pre-malignant neoplasms, piecemeal resection by using EMR may be permissible with the best balance of risks and benefits. Surgical organ resection with lymphadenectomy should be applied to those neoplasms with high probability of positive lymph nodes or failure in complete removal by ESD. Recurrent lesions can be also indicated for ESD, if they fulfill the criteria of no nodal metastasis, but indication should be carefully determined considering the risks of accompanying complications.

STOMACH EPITHELIAL NEOPLASMS

Aspects of nodal metastasis

Pre-malignant stomach epithelial neoplasms, gastric adenomas, have no nodal metastases. It is still controversial whether we should treat gastric adenomas endoscopically or follow them. A series with a small number of cases with a preoperative diagnosis of pre-malignant lesion revealed that 37% (16/43) of them were finally diagnosed as adenocarcinoma and a lesion > 1 cm was considered to pose a risk of malignancy[6]. Another study revealed that 6.8% (8/118) of cases were finally diagnosed as adenocarcinoma and high-grade dysplasia by endoscopic biopsy was considered to be an independent risk factor for malignancy[7]. Furthermore, preoperative diagnosis of depressed adenoma is considered to represent a higher risk of malignancy than protruding adenoma[8]. So, when the lesions have these characteristics, endoscopic treatments are recommended, similarly to intramucosal carcinomas. Although local recurrence should be taken into account, piecemeal resection by using EMR techniques to remove the apparent gastric adenomas is allowed.

In terms of malignant stomach epithelial neoplasms, the following types of early gastric cancers without lymphovascular infiltration of cancer cells may have little likelihood of nodal metastases: (1) intramucosal, differentiated adenocarcinoma without ulcer findings of any size; (2) intramucosal, differentiated adenocarcinoma with ulcer findings when the lesion is ⩽ 3 cm; (3) intramucosal, undifferentiated adenocarcinoma without ulceration when the lesion is ⩽ 2 cm; and (4) differentiated adenocarcinoma with minute submucosal penetration (500 micrometers below the muscularis mucosa; sm1) when the lesion is ⩽ 3 cm[9].

Technical aspects

When the endoscopists are well trained for ESD, the technical aspects may not restrict indications to perform ESD, based on the above criteria of no nodal metastasis. However, in our opinion, cases of ulcer findings with fusion of the muscle layer and the mucosal layer and cases of undifferentiated adenocarcinoma may be excluded from the indication or be carefully resected, at least until now. The former cases occur in cancers that previously had a deep ulcer extending into the proper muscle layer, where it is difficult to identify the gastric wall plane during submucosal dissection, which increases the possibility of perforation or incomplete resection by ESD[10]. In the latter cases, first, the margin is very unclear and the possibility of incomplete resection is fairly high, second, the clinical course after recurrence may be more miserable than that of differentiated-type, and third, the differentiation between ulcerative finding or biopsy-inducing fibrosis is sometimes difficult, even though small intramucosal undifferentiated adenocarcinoma with ulcer findings may be associated with nodal metastases[9].

ESOPHAGEAL SQUAMOUS EPITHELIAL NEOPLASMS

Aspects of nodal metastases

Low- and high-grade squamous intraepithelial neoplasms, including carcinoma *in situ* (m1), have no nodal metastases. It is still controversial whether one should treat these intraepithelial neoplasms endoscopically or just follow them. However, when the lesions are diagnosed as high-grade intraepithelial neoplasms, endoscopic treatment is recommended, to avoid future development of invasive carcinoma or to contain foci of invasive carcinoma[11,12]. Although local recurrence should be taken into account, piecemeal resection by using EMR techniques to remove the apparent intraepithelial neoplasms is allowed[13-15].

Esophageal squamous cell carcinomas invading the lamina propria (m2) pose little risk of nodal metastases. For those invading the muscularis mucosa (m3) and those with minute submucosal invasion (< 200 micrometers below the muscularis mucosa; sm1), the nodal metastases rate is 9.3% and 19.6%, respectively. The nodal metastases rate of m3 or sm1 cancers with 0-Ⅱ type, < 5 cm, well or moderately differentiated type, and no lymphovascular infiltration of cancer cells is 4.2%[16]. It has been reported that no nodal metastasis was found in patients with sm1, low

histologic grades, and no lymphovascular infiltration of cancer cells[17]. Therefore, for patients unwilling to undergo esophagectomy or chemoradiation and patients with comorbid diseases, ESD may be applied taking into consideration the risks of nodal metastases and treatment-related morbidity.

Technical aspects

When the endoscopists are well trained for ESD, the technical aspects by themselves may not restrict indications to perform ESD, except in special circumstances, such as lesions located in the diverticulum. When lesions spreading > 3/4 of circumference are resected as circular or semi-circular resection, post-operative stricture occurs to a high rate[18]. So, it is controversial to treat these lesions endoscopically. However, intensive balloon dilatations or tentative stent insertion may rescue from the stricture.

ESOPHAGEAL BARRETT NEOPLASMS

Aspects of nodal metastasies

Columnar intraepithelial neoplasms have no nodal metastases. Although local recurrence should be taken into account, piecemeal resection by using EMR techniques and additional ablation therapy to remove the apparent intraepithelial neoplasms is allowed[19-23].

There are no data about nodal metastases from the large numbers of surgically resected cases due to limited number of cases of esophageal columnar epithelial carcinomas at an early stage, although a small number of cases revealed no nodal metastasis for the intramucosal and sm1 cancer, where sm1 was determined by upper third of the submucosa[24]. There is no consensus whether one should apply to this kind of malignancy the same criteria that are applied to stomach epithelial neoplasms or esophageal squamous epithelial neoplasms as far as the depth of sm1 to be measured. International workshops of esophagogastric neoplasms adopted the cut-off line of 500 micrometers below the deeper muscularis mucosae, similarly to the stomach[25,26].

Technical aspects

Similarly to esophageal squamous epithelial neoplasms, the technical aspects by themselves may not restrict indications to carry out ESD, when the endoscopists are well trained for ESD. When lesions spreading > 3/4 of the circumference of the esophagus (a situation which commonly occurs in long segment Barrett epithelium) are resected (with circular or semi-circular resection), post-operative strictures occur at a high rate[19-23].

RECTAL EPITHELIAL NEOPLASMS

Aspects of nodal metastases

Pre-malignant rectal epithelial neoplasms, rectal adenomas, have no nodal metastases. From the standpoint of adenoma-carcinoma sequence, all adenomas, including diminutive polyps, are targets for endoscopic resection[27,28], although some investigators agree with endoscopic removal only if the size is > 5 mm[29]. *En bloc* resection is not always necessary for rectal adenoma or intramucosal carcinoma. However, higher rate of local recurrence was reported when multiple resections were performed[30-32]. Intramucosal carcinomas and those with slight submucosal invasion (< 1000 micrometers below the muscularis mucosa; sm1) without lymphovascular infiltration have little risk of nodal metastasis[33].

Tumor morphology and surface pit pattern are good endoscopic indicators for submucosal invasion. From this aspect, depressed lesions, laterally spreading tumors of non-granular type (LST-NG) and large protruding tumors are considered as good candidates for ESD because these lesions have a high risk of submucosal invasion, which may be difficult to diagnose preoperatively, and a thorough histopathological assessment of the resected specimen is essential. It is controversial whether one should perform ESD or piecemeal EMR for laterally spreading tumors of granular type (LST-G), because most lesions are intramucosal and the endoscopic prediction of invasiveness is highly feasible[34].

Technical aspects

Even for lesions that meet the criteria above, laparoscopic or open surgery may be selected in some institutions considering the location and size of the lesion. The lesions with submucosal fibrosis due to previous endoscopic treatment or biopsy are also resectable by ESD, even though the indication should be carefully weighed considering risks and benefits of ESD *vs* surgery[35,36]. The rectum is fixed to the retroperitoneum, therefore the endoscope is more easily manoeuvred than in other organs of the gastrointestinal tract. Furthermore, panperitonitis may be less likely than in the rest of the colorectum, even if the muscularis propria is teared, although penetration leads to air accumulation in the retroperitoneal space, which may then spread to a wider area[37,38].

COLONIC EPITHELIAL NEOPLASMS

Aspects of nodal metastases

The criteria for absence of nodal metastases are the same as those of rectal epithelial neoplasms (see above).

Technical aspects

There are several tortuous folds in the colon. Peristalsis and residual feces may sometimes disturb ESD procedure. So it is commonly believed that the technical difficulty of colon ESD exceeds those of the stomach, the esophagus, and the rectum, although there are many differences. In all cases, should one consider the substantial risks and expected benefits of ESD. However, promising results of ESD are reported from very experienced endoscopists at advanced institutions, similarly to those of the rectal epithelial neoplasms[39-43].

EPITHELIAL NEOPLASMS IN THE SMALL INTESTINE, INCLUDING DUODENUM

Aspects of nodal metastases

Pre-malignant epithelial neoplasms in the small intestine have no nodal metastases. Although local recurrence should be taken into account, piecemeal resection by using EMR techniques and ablation therapy to remove the apparent intraepithelial neoplasms is allowed[44]. There are no data about nodal metastases from the large numbers of surgically resected cases due to limited number of cases of epithelial carcinomas in the small intestine. There is no consensus whether one should apply the same criteria of stomach epithelial neoplasms or colorectal epithelial neoplasms to this malignancy.

Technical aspects

The small intestine, including the duodenum, is considered to be the most difficult organ where to perform ESD. The endoscope does not easily reach the target lesion and the organ is not fixed tightly except at the level of the duodenum, which results in fairly bad maneuverability. Peristalsis is the most active and the wall is the thinnest among the other gastrointestinal organs. Even is the resection is completed successfully, pancreatic juice and bile cause chemical damage to the mucosal wound, which may lead to prolonged bleeding and perforation. In our opinion, closure of the mucosal wound is recommended after ESD. When considering these issues, indication to perform ESD in the small intestine should be carefully assessed and limited. Due to the structural specificity of the papilla, ESD for ampullary neoplasms is not performed.

PHARYNGEAL EPITHELIAL NEOPLASMS

Aspects of nodal metastasis

Pre-malignant epithelial pharyngeal neoplasms have no nodal metastases. Although local recurrence should be taken into account, piecemeal resection by using EMR techniques and ablation therapy to remove the apparent intraepithelial neoplasms is permissible[45]. There are no data about nodal metastasis from the large numbers of surgically resected cases due to limited number of cases of pharyngeal epithelial carcinomas at an early stage. So, indication for invasive carcinoma is still controversial due to the lack of data. Owing to the structural differences, it is impossible to apply the criteria of esophageal squamous epithelial carcinomas for this malignancy.

Technical aspects

ESD is technically possible in this organ, and ESD may be the optimal endoscopic treatment not only because it enables an *en bloc* resection but also because it can prevent removal of excess mucosa of the pharynx, which is a very narrow and important organ related to swallowing and speech[46].

CARCINOID

Aspects of nodal metastasis

Carcinoids are classified based on organ site and cell of origin and occur most frequently in the gastrointestinal tract (67%) where they are most common in small intestine (25%), appendix (12%), and rectum (14%)[47]. Primary size > 2 cm, serosal penetration, and primary site in the small intestine are considered to be risk factors for metastases in the case of gastrointestinal carcinoids[48].

Nodal metastases are most commonly found with small intestine carcinoids (20%-45%), providing the rationale for an extended resection including the adjacent lymph node drainage area. Carcinoids of the appendix < 1 cm rarely metastasize, simply requiring appendectomy for treatment. Rectal carcinoids < 2 cm rarely metastasize, directing local excision, including endoscopic resection[49]. Another group revealed that colorectal carcinoids < 1 cm without lymphovascular infiltration could be curatively treated by local resection, but others would need radical nodal dissection[50]. Duodenal carcinoids < 2 cm may be excised locally because they rarely metastasize[51].

Multiple gastric carcinoids, usually no more than 1 cm, can be followed up by endoscopy and biopsy[52,53]. Sporadic gastric carcinoids should be treated by gastrectomy with lymphadenectomy, because some of those have nodal metastases even when they have a small size[54-56]. However, differentiation of types of gastric carcinoids is not always easy, so endoscopic resection, as a first step to obtain histology, may be acceptable for small gastric carcinoids < 1 cm to predict nodal metastases.

Technical aspects

Because almost all lesions for local resection are less than 1 cm in all the gastrointestinal organs, band ligation resection[57,58], cap-technique[59] or strip biopsy[60-62] result in good outcome. So the application of ESD for carcinoids may be limited. When the lesions are in intermediate size, such as 1-2 cm, or invade massively the submucosal layer, which may result in tumor-positive margin resection, ESD should be applied[36,63].

SUBMUCOSAL TUMOR

Aspects of metastases

Submucosal tumors (SMTs) are mesenchymal tumors, which may have very diverse origins. SMTs are classified and defined as benign or malignant based on a combination of size, histological, immunohistochemical, and ultrastructural criteria. The majority of them are classified into gastrointestinal stromal tumor (GIST), of muscular origin, of neurogenic origin, of vascular origin, and of adipose tissue origin. SMTs < 3 cm are generally considered benign tumors. SMTs > 3 cm with high mitotic counts are considered tumors at high-risk of malignancy. In case of GIST, the cutoff of the size between pre-malignancy and malignancy may be

2 cm. Sarcomas including malignant GIST generally do not metastasize to regional lymph nodes, but instead spread hematogenously to the liver or metastasize to the peritoneum[64]. Benign SMTs should generally only be treated if they are symptomatic. So the SMTs > 2 cm or 3 cm without evidence of metastasis may be candidates for local resection[65].

Technical aspects

From the rationale of ESD, the targets should originate from over the muscularis propria. The lesions originating from the inner layer of the muscularis propria may be resectable by careful resection over the outer layer of the muscularis propria, but the high probability of perforation and the artificial peritoneal dissemination by tear of the tumor capsule should be taken into consideration. When considering that the small size lesions located in the mucosal or submucosal layers are mostly benign, the indication of ESD for SMTs is quite limited, although some investigators reported promising results of ESD for SMTs[66,67].

FUTURE PERSPECTIVES

The perspectives on the current indication of ESD are described based on a review of data available in the literature until the end of 2007. Further investigations in both aspects, the assessment of nodal metastases and the technical innovations, may change widely the above perspectives in the future. Recently, a new application of ESD is being investigated in cooperation with laparoscopic surgeons for the treatment of possible node-positive gastric carcinoma and gastric GIST[68,69]. There is no doubt that these attempts will expand ESD into a new field, which will be added to the upcoming practical guidelines for ESD.

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"Melanosis" in the small and large intestine

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Abstract

Deposition of pigment in the intestinal mucosa is commonly observed by the endoscopist, especially within the colon, and particularly during investigations for constipation. Pigment may also be detected in the small intestine. Although labeled as melanosis, electron microscopy and X-ray analytical methods have provided evidence that this pigment is not melanin at all, but lipofuscin. Often, herbal remedies or anthracene containing laxatives are often historically implicated, and experimental studies in both humans and animal models have also confirmed the intimate relationship with these pharmacological or pseudo-pharmacological remedies. The appearance of melanosis coli during colonoscopy is largely due to pigment granule deposition in macrophages located in the colonic mucosa. The pigment intensity is not uniform, being more intense in the cecum and proximal colon compared to the distal colon. Possibly, this reflects higher luminal concentrations of an offending agent in the proximal compared to distal colon, differential absorption along the length of the colon, or finally, differences in macrophage distribution within the colon. Mucosal lymphoid aggregates normally display a distinct absence of pigment producing a "starry sky" appearance, especially in the rectosigmoid region. Interestingly, some focal, usually sessile, colonic mucosal neoplastic lesions, rather than submucosal lesions, may be better appreciated as pigment deposition may be absent or limited. If detected, removal and further histopathologic analysis of the polyp may be facilitated.

Key words: Melanosis; Lipofuscin; Melanosis coli; Constipation; Hemosiderin; Melanosis ilei; Melanosis duodeni; Anthraquinones

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INTRODUCTION

Prior studies using electron microscopy and X-ray analysis refined information regarding the composition of pigment granules in the intestinal tract. In particular, the distinction between lipofuscin, melanin and hemosiderin was more readily accomplished since separation of these pigment materials appeared to be possible based solely on their morphological characteristics[1]. X-ray analysis also permitted *in situ* analysis of tiny cellular inclusions in sectioned material with a high degree of sensitivity and sensitivity. As a result, a better understanding of pigments found in the intestinal tract has emerged.

PIGMENT GRANULES

Lipofuscin granules are residual bodies with undigested and/or oxidized lipids. These granules are thought to result from the processes of sequestration and enzymatic dissolution of cellular organelles within lysosomes. Electron microscopy is reported to show single membrane-bound bodies containing electron-dense lipid material along with electron-lucent or medium density neutral fat. Melanin is synthesized in the melanosome by oxidation of tyrosine to dopa and, eventually, melanin. Characteristic striated rod-like structures are observed under electron microscopy. Hemosiderin develops in residual bodies that result from macrophage phagocytosis of erythrocytes and/or their breakdown products. Lysosomal digestion results in electron-dense iron-containing particles. Each granule type is distinctive and has been well illustrated by others elsewhere[1].

MELANOSIS COLI

Melanosis coli is probably the most common pigmentation change seen in the intestinal tract mucosa

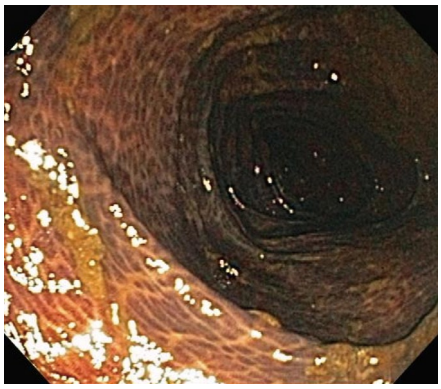

Figure 1 Typical alligator or snake-skin appearance of melanosis coli. Despite routine colon preparation, residual fecal debris is common, likely reflecting reduced colonic propulsive activity.

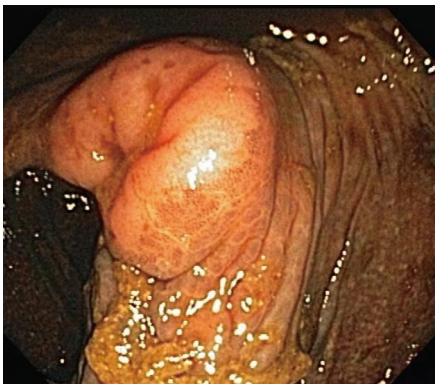

Figure 3 Ileal and cecal mucosa in melanosis coli. Melanosis is generally confined to the cecal mucosa although there is a very limited area of transition into ileal mucosa that is not pigmented.

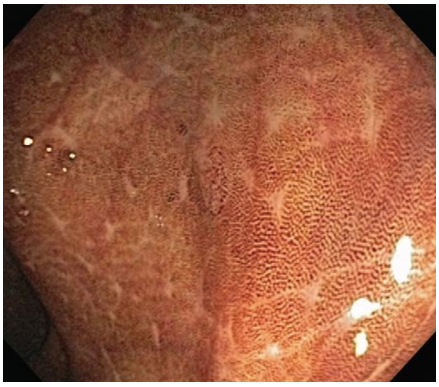

Figure 2 Rectosigmoid mucosa illustrating focal areas of patchy intense pigmentation as well as focal areas of absent pigment, the latter reflecting the presence of normal mucosal aggregates of lymphoid cells (so-called "starry sky" appearance of melanosis coli).

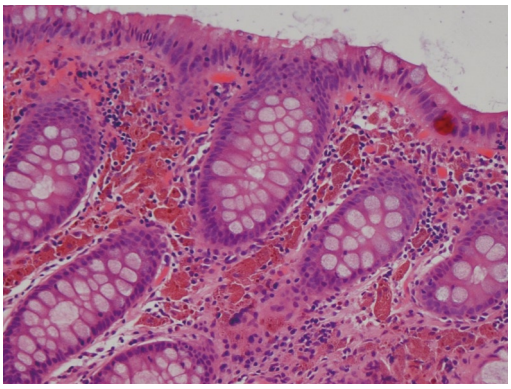

Figure 4 Photomicrograph showing typical pigment granule laden macrophages in the lamina propria.

during endoscopic evaluation and in biopsy materials submitted for histopathologic evaluation. It was, however, first described by Cruveilhier in 1829 before the emergence of endoscopic technologies. The abnormally brown or black pigmentation can even be visualized in the biopsy specimen in tissue forceps or on the filter paper prior to fixation immersion. Representative endoscopic appearances of melanosis coli are shown in Figures 1-3.

As shown, the pigment in melanosis coli is well localized within the colon as there is usually no pigment deposition in the more proximal small intestine, including the ileum. Occasionally, the pigment may extend through the entire colon, including the appendix and into the most distal ileal transition region with the cecum. It appears to be most intense and most readily detected in the cecum and ascending colon, but pigment changes are variable in their intensity, even within the same colon. In some areas of the colonic mucosa where lymphoid cell aggregates in the lamina propria are numerous, such as the rectum, the pigmented colonic mucosa shows a characteristic "starry sky" appearance, as these lymphoid cells do not accumulate intracellular pigment. As shown in Figure 4, a biopsy routinely treated with a standard hematoxylin-eosin stain shows lamina propria macrophages filled with brown colored pigment granules.

A variation in the intensity of the pigmentation through the length of the colon appreciated in some patients with melanosis coli at endoscopy may reflect differences in the luminal concentrations of possible offending agents (i.e., higher in proximal colon), such as laxatives or their byproducts. Alternatively, there may be differential regional rates of mucosal absorption within the colon. Finally, there may be colonic regional differences in the topographic distribution of macrophages within the colonic lamina propria. Studies on granule composition have demonstrated primarily lipofuscin, rather than the melanin pigmentation historically suggested by histochemical staining reactions. Indeed, it has been suggested by others that this entity might be more precisely labeled "pseudomelanosis coli" or colonic "lipofuscinosis". Recently, however, a lectin method for application to formalin-based and paraffin-embedded colon[2] was used to explore changes in biopsies from patients with melanosis coli associated with laxative use[3]. These studies revealed increased apoptotic bodies in the colonic epithelium with pigment accumulation in macrophages. The results based on application of these lectins were typical of lipofuscin as well as ceroids. In addition, however, an intense argentaffin reaction abolished by bleaching was present,

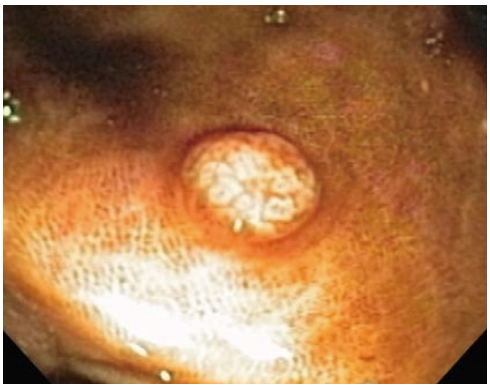

Figure 5 Easily visualized small sessile polypoid lesion in ascending colon with adjacent background pigmented mucosa typical of melanosis coli. Resected specimen confirmed absence of pigmented macrophages in the body of the resected adenoma.

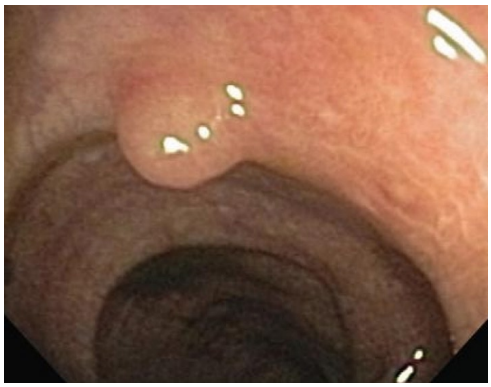

Figure 6 Small pigmented polypoid lesion similar to background pigmented colonic mucosa. Resected specimen revealed a submucosal leiomyoma along with pigmented macrophages in the overlying colonic mucosa due to melanosis coli.

most typical of a melanic substance. The apoptotic bodies in the colonic epithelium were thought to be due to laxative-induced cell death, not from natural programmed cell renewal. In addition, these apoptotic epithelial cells were believed to be the source of the pigment saccharides (as detected by lectins) while the melanic substance appeared to be derived directly from the anthraquinones[3].

Melanosis coli is most often detected during investigation for long-standing constipation, often in conjunction with a history of the chronic use of anthracene cathartics (including cascara, senna, aloes and rhubarb). Experimental studies in different mammalian species along with humans previously documented the appearance, disappearance and re-appearance of the pigment in colorectal mucosa with repeated cycles of laxative administration. Interestingly, as shown in Figure 5, pigment deposition also may spare some neoplastic colonic lesions, including both adenomas and carcinomas. Thus, biopsy or removal of these non-pigmented foci in melanosis coli has been recommended elsewhere to exclude the presence of neoplastic epithelial cells. Other lesions, including submucosal leiomyomas shown in Figure 6, however, may still have pigment deposits within mucosal macrophages overlying these focal submucosal lesions.

Anthraquinones appear to damage the colonic epithelial cells causing irreversible injury to some organelles. These cells are either shed into the colonic lumen, or the damaged organelles are sequestrated in autolysosomes in macrophages where digestion to residual lipofuscin bodies results. In some, lymph node involvement has also been observed. It is possible that this relatively selective colonic mucosal involvement may reflect the qualitative or quantitative differences in colonic microbial flora (as opposed to the small intestine). Alternatively, some other structural difference in colonic cells or their response to anthraquinone cathartics may be responsible for the colonic mucosal regionalization of the lipofuscin pigment deposition.

Treatment of this condition has not been established. Often, a recommendation is made to manage symptomatic constipation with fiber-containing foods or substances with mucilage, including psyllium, along with avoidance of anthraquinone cathartics.

MELANOSIS OF SMALL INTESTINE

Melanosis has been rarely recorded in the small intestine, at least, in the most readily visualized areas during routine endoscopic evaluation, including the duodenum or distal ileum. As noted earlier, pigment may extend for a very limited distance into the most distal ileum transitional mucosa in association with melanosis coli. Melanosis ilei alone and exclusive of pigmentation elsewhere, however, has also been noted, usually as an incidental observation during autopsy. In this setting, it has also been historically recorded to occur with carcinoma of the colon[4]. Pigment in the ileum is thought to arrive there through ingested materials found either in food or even inhaled dust that has been swallowed[5,6]. Although aluminum and silicon-containing compounds are common and may be detected as food additives or in medicines, hemosiderin pigment has also been detected in the ileum and hypothesized to result from intermittent bleeding within the upper gastrointestinal tract. Similarly, melanosis duodeni has also been related to occasional bleeding, usually from peptic ulceration[7-10].

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TOPIC HIGHLIGHT

Simon D Taylor-Robinson, MD, *Series Editor*

Hepatocellular carcinoma: Epidemiology, risk factors and pathogenesis

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Abstract

Hepatocellular carcinoma (HCC) is the commonest primary malignant cancer of the liver in the world. Given that the burden of chronic liver disease is expected to rise owing to increasing rates of alcoholism, hepatitis B and C prevalence and obesity-related fatty liver disease, it is expected that the incidence of HCC will also increase in the foreseeable future. This article summarizes the international epidemiology, the risk factors and the pathogenesis of HCC, including the roles of viral hepatitis, toxins, such as alcohol and aflatoxin, and insulin resistance.

Key words: Hepatocellular carcinoma; Epidemiology; Risk factors; Aetiology; Pathogenesis

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EPIDEMIOLOGY

Hepatocellular carcinoma (HCC) is the commonest primary cancer of the liver. Incidence is increasing and HCC has risen to become the 5th commonest malignancy worldwide and the third leading cause of cancer-related death, exceeded only by cancers of the lung and stomach[1]. The estimated incidence of new cases is about 500 000-1 000 000 per year, causing 600 000 deaths globally per year[2-6]. However, important differences have been noted between countries. Most cases of HCC occur in Asia[1] where several countries, particularly in East Asia, have a very high incidence (over 20 cases/100 000 population). For example, the incidence is 99 per 100 000 persons in Mongolia, 49 per 100 000 in Korea, 29 per 100 000 in Japan, and 35 per 100 000 in China[3]. Hong Kong and Thailand also have similarly high rates. Another region of concern is sub-Saharan Africa, particularly the western region of Africa, including Gambia, Guinea, and Mali, and also the Republic of Mozambique in south-east Africa. Areas with moderately high risk (11 cases/100 000-20 cases/100 000) include Italy, Spain and Latin American countries, and those at intermediate risk (5 cases/100 000-10 cases/100 000) include France, the United Kingdom, and the Federal Republic of Germany. A relatively low incidence (less than 5 cases/10 000) is found in the United States, Canada, and in Scandinavia. However, there are large areas of the world where the incidence is still unknown[3,7,8].

Table 1 Global frequency of new cases of hepatocellular carcinoma

Year (reference)	Total number	Males	Females
1990	437408	316300	121100
2000	564300	398364	165972
2002 (The World health report, 2003)	714600	504600	210000

Adapted from Parkin *et al*, 2001, 2005[2,5] and Bosch *et al*[8].

Although currently relatively low, the incidence of HCC is rising in developed western countries[9-12]. In the United States, there has been an increase of about 80% in the annual incidence of HCC during the past two decades. HCC rates increased from 1.4/100000 per year from 1976-1980 to 2.4/100000 per year from 1991-1995[3,10]. This increase has been most marked in men, with African-American men having higher incidence rates than US Caucasian men. This was explained by the emergence of hepatitis C during this same period, although the rise in immigration from HBV-endemic countries may also have played a role[9,10].

Other developed western countries have noted similar increasing trends. An increase in incidence of HCC has been reported in Italy, the United Kingdom, Canada, Japan, and Australia. The increase was reported among immigrants from parts of the world with high prevalence, such as sub-Saharan Africa and parts of Asia, being associated with a parallel increase in hospitalization and mortality for HCC[3,10]. In Egypt, between 1993 and 2002, there was an almost twofold increase in HCC amongst chronic liver patients[13].

However, it is not obvious when this rising trend, observed in many countries, will reach a peak. The Disease Control Center in Atlanta has estimated that deaths related to chronic hepatitis C in the United States will triple from the current rates of 8-10000 per year during the next decade. While most of these deaths will be due to liver failure and its complications, a considerable proportion can be expected to be due to HCC as well[9].

The most recent World Health report (World Health Organization[14], Table 1) indicated a total of 714600 new cases of HCC worldwide, with 71% among men (Figure 1). HCC is the 4th commonest cause of death due to cancer, after cancers of the respiratory system, stomach, and colon/rectum. Liver cancer ranked 3rd for male subjects and 5th for women. Geographically, there were 45000 liver cancer deaths in Africa, 37000 in the Americas, 15000 in the eastern Mediterranean, 67000 in Europe, 61000 in South-East Asia, and 394000 in the western Pacific region, including China and Japan. In the same year, 783000 persons died from cirrhosis, of which 501000 were men and 282000 were women[15].

The incidence of HCC increases with age, reaching its highest prevalence among those aged over 65 years[16,17]. Although HCC is rare before the age of 50 years in North America and Western Europe[18], a shift in incidence towards younger persons has been noted in the last two decades. HCC tends to occur in the background of cirrhosis of the liver. In western countries, this holds true in over 90% of cases, whereas in Asia and Africa the percentage of cases of HCC is higher in individuals with non-cirrhotic livers, compared to those with cirrhotic livers[3,19].

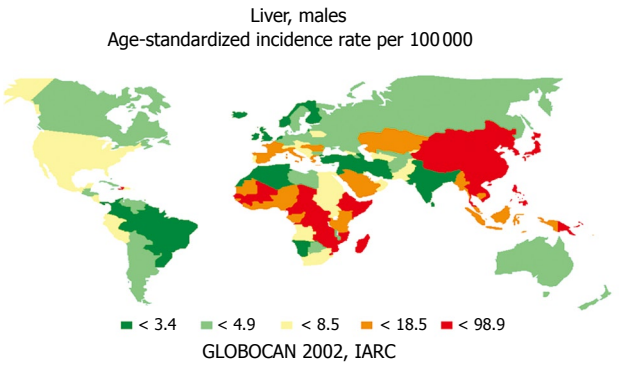

Figure 1 Age-standardized incidence rates of liver cancer in males per 100000 population (Adapted from GLOBOCAN 2002 with permission)[134].

RISK FACTORS

The major risk factor for the development of HCC is cirrhosis of the liver. However, about one quarter of HCC cases diagnosed in the United States do not have any known predisposing risk factors. The major known risk factors for HCC are viral (chronic hepatitis B and hepatitis C), toxic (alcohol and aflatoxins), metabolic (diabetes and non-alcoholic fatty liver disease, hereditary haemochromatosis) and immune-related (primary biliary cirrhosis and autoimmune hepatitis)[17]. Recently, the geographical variability in the incidence of HCC has been attributed to the changing distribution and the natural history of Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infection[20].

HCV

HCV is the most important risk factor for HCC in western European and North American countries, since epidemiological studies have shown up to 70% of patients with HCC have anti-HCV antibody in the serum[3,21-23]. Liver cancer has a higher prevalence in patients with HCV-associated cirrhosis than in non-viral aetiologies of chronic liver disease, while only a few cases of HCV-associated HCC have been reported in the non-cirrhotic liver, indicating that the virus possibly has a mutagenic effect[3,24,25].

The prevalence of HCV infection varies considerably by geographical region. African and Asian countries reported high HCV infection prevalence rates, while rates in North America, Europe and Australia have usually reported lower rates[26,27]. Egypt has the highest prevalence of HCV in the world[28-34] (predominantly genotype 4), which has been attributed to previous public health eradication schemes for schistosomiasis[28,34]. Even higher HCV infection rates, up to 60%, have been reported in older individuals, in rural areas such as the Nile delta, and in lower social classes[28,30,32,34].

The natural history of HCV infection has been investigated in several studies[3]. A Japanese study[35] reported a time lag of 13 years from infection by transfusion of

HCV infected blood to the development of chronic hepatitis. This time period was reported to be approximately 10 years in an American study and it took about 20 years for the same patients to develop cirrhosis of the liver[36]. Development of HCC took 28 years in the American subjects and 29 years in the Japanese cohort[35,36]. The annual risk of developing HCC in HCV-infected patients depends on the presence and severity of the underlying liver disease[3].

Up to 80% of HCV-infected individuals fail to eliminate the virus acutely and progress to chronic HCV infection[27,37-40]. Continuous inflammation and hepatocyte regeneration in the setting of chronic hepatitis and subsequent progression to cirrhosis is thought to lead to chromosomal damage and possibly to initiate hepatic carcinogenesis[27,41].

The rate of fibrotic progression following HCV infection is markedly variable, since the natural history of the disease typically extends over several decades[40,41]. The rate of fibrotic progression in HCV-infected patients is influenced by age at the time of infection, male sex, HCV genotype and alcohol consumption[42-50].

It is not clear whether any of these factors affect the onset of liver-related complications by mechanisms other than their effects on the rate of fibrotic progression. To determine which interactive variables were independent determinants of adverse clinical outcomes, Khan and colleagues examined the development of liver-related complications of chronic HCV in a large cohort of patients who were heterogeneous in age, country of birth, mode of HCV acquisition, HCV genotype, and histological and functional severity of liver disease. Patients were followed up for five years. These authors found that the major independent predictors of liver-related complications were sporadic transmission, advanced liver fibrosis at entry and low albumin[43].

HBV

The WHO has reported HBV to be second only to tobacco as a known human carcinogen[51]. Many studies on HCC risk following chronic HBV infection have been conducted in the East Asian countries, where most patients acquired HBV as newborn infants[52,53]. The incidence of HCC in HBV-related cirrhosis in this area of the world has been reported to be 2.7%[53]. The annual risk of HCC is 0.5% for asymptomatic HBsAg carriers and 0.8% for patients with chronic hepatitis B[53,54], while patients with HBV-cirrhosis have 1000 times higher risk of developing HCC, compared to HBsAg negative individuals[53,55]. Thus, it is likely that the probability of acquiring HCC increases with severity of underlying liver disease[53]. In Japan, the mean interval between HBV initial infection and the occurrence of HCC is 50 years. As most people are infected at birth, HBV-related cirrhosis usually develops earlier than in Western Europe or North America[35,53].

Few adequate studies have been performed in Europe or North America to address the issue of the incidence of HCC in individuals who are positive for HBsAg. Most of the studies in Western countries are based on small numbers of HBsAg positive patients and/or have not specifically analysed the group of HBsAg carriers. Additionally there is lack of uniformity in the timing of initiation of follow-up monitoring. In a cohort of 350 Western European patients with compensated cirrhosis, followed for about 6 years, the 5-year cumulative incidence of HCC was 6%[53,56,57]. A retrospective analysis of European patients with HBV-related cirrhosis found the 5-year incidence of HCC was 9%, irrespective of HBeAg or HBV DNA status at the time of diagnosis of cirrhosis[53,58].

HCC has been the first human cancer amenable to prevention using mass vaccination programmes. From a global perspective, the burden of chronic HBV infection is expected to decline because of the increasing utilisation of HBV immunization, since the early 1980s[20,59,60]. The Taiwanese mass vaccination program against HBV has considerably reduced the rate of HBsAg carrier in children and adolescents and consequently the incidence of childhood HCC[20,61,62]. The average annual incidence of HCC in children aged 6-14 years declined gradually (0.70 per 100 000 children in 1981-1986, 0.57 in 1986-1990 and 0.36 in 1990-1994). A significant decrease in HCC incidence in adults was also observed, 3-4 decades later[20,63].

HBV factors in HBV-related HCC

The mechanisms of carcinogenesis in HBV infection have been extensively studied, and a major factor is chronic necroinflammation with subsequent fibrosis and hepatocyte proliferation. However, HCC may occur in HBsAg carriers without cirrhosis. Both HBV and host hepatocytes may contribute to the final pathogenic outcomes, either individually or synergistically. Therefore, it is reasonable to consider that apart from host factors, viral factors are likely involved in HBV-related hepatocarcinogenesis[20].

Viral proteins in hepatocarcinogenesis

HBV may encode oncogenic viral proteins that may contribute to hepatocarcinogenesis[20]. For example, HBx is a well-known viral non-structural gene that has roles as a multifunctional regulator modulating gene transcription, as well as controlling cell responses to genotoxic stress, protein degradation, apoptosis, and several signalling pathways[20,64-67]. Although the specific mechanisms are still unknown, its critical role in liver malignant transformation has been demonstrated in studies of transgenic mice with HBx overexpression[20,68]. HBx protein has been shown to complex the tumor suppressor p53 protein and to suppress its function[53,69,70].

HBV genotype, basal core promoter (BCP) mutation and viral load in hepatocarcinogenesis

Several viral factors other than viral proteins as viral genotype, BCP mutations in the viral genome and viral load have been associated with hepatocarcinogenesis[20]. Eight HBV genotypes (A-H) have been described, based on genomic sequence divergence[20,71,72] These have distinct geographical and ethnic distributions: genotypes A and D prevail in Africa, Europe, and India; genotypes B and C in Asia; genotype E only in West Africa; and genotype

F in Central and South America[20,73]. It is reported that HBV genotype affects clinical outcome and treatment responses. For example, in Asia, genotype C is found to be commonly associated with more severe liver disease, cirrhosis and the development of HCC, compared to genotype B[20,65,68,74-78] whereas in Western Europe and North America, genotype D is more associated with severe liver disease and a higher incidence of HCC, than genotype A[20,79]. In addition to viral genotype, specific viral genomic mutations, particularly the BCP T1762/A1764 mutation, also correlate with HCC risk[20,80,81].

A prospective cohort study with 11 years of follow-up assessed the relationship between HBV viral load and mortality. Viral load was found to be associated with increased mortality from HCC and chronic liver disease in HBV-infected subjects. The relative risk (RR) for HCC mortality in patients with viral load $< 10^5$ copies/mL was 1.7 (95% CI, 0.5-5.7), whereas it was 11.2 (95% CI, 3.6-35.0) in patients with viral load $> 10^5$ copies/mL[74-76]. Viral load may thus be a useful prognostic tool in HBV infection.

HBV factors in young-onset HCC

Viral factors in association with the development of HBV-related HCC in young patients seem to be different from their old-aged counterparts[20,82]. Tsai and colleagues compared serum viral loads in young (less than 40 years of age) and older (over 40 years) patient groups in 183 HBV-related HCC patients and 202 HBV carriers. These authors found high serum HBV DNA levels were associated with the development of HCC in older patients, rather than those under 40 years[20,83]. Another study from Taiwan demonstrated that genotype B was significantly more common in patients with HCC, aged under 50 years, compared to age-matched inactive carriers (80% *vs* 52%, $P = 0.03$)[20,80]. This predominance was even more striking in younger patients with HCC, with 90% in those under 35 years. Most of these patients did not have cirrhosis. A further Taiwanese study reported that 26 children with HBV-related HCC were documented among 460 HBV carriers during 15 years follow up and genotype B was the major genotype (74%)[20,84]. These data suggest that genotype B-HBV may be associated with the development of HCC in young carriers without cirrhosis[20].

Viral factors in HCC in the absence of cirrhosis

Studies of HBV-related HCC in patients without cirrhosis have helped to explain the effect of viral factors in HCC development. Liu *et al* (2006) examined the role of BCP T1762/A1764 mutation, pre-core A1896 mutation and serum viral load in liver cancer, presenting in the absence of cirrhosis, by comparing 44 patients without cirrhosis, but with HBV-related HCC, to 42 individuals with cirrhosis and HBV-related HCC. These authors found that male gender, BCP T1762/A1764 mutation and viral load greater than 10^5 copies/mL were independently associated with the risk of HCC development in the absence of cirrhosis. They suggested that viral features predisposing to HCC might be similar between cirrhotic and non-cirrhotic groups[20,85].

Pre-S deletion in HCC

Recently, pre-S deletion of HBV has been found to be associated with the progression of liver disease and development of HCC in HBV carriers[20,86]. PreS deletion mutants hasten the storage of large envelope proteins in hepatocyte cytoplasm which can stimulate cellular promoters by inducing endoplasmic reticulum stress[53,87,88].

The interactions between pre-S deletion, PC mutation and BCP mutation of various stages of chronic HBV infection were investigated in 46 chronic HBV carriers and 106 age-matched carriers with different stages of liver diseases; 38 with chronic hepatitis, 18 with cirrhosis, and 50 individuals with HCC[87]. Logistic regression analysis demonstrated that pre-S deletion and BCP mutation were significantly associated with the development of progressive liver disease. Combinations of mutations, especially the pre-S deletion, rather than single mutation were correlated with a greater risk of progressive liver disease. Sequencing analysis showed that the deleted regions were more common in the 3' terminus of pre-S1 and the 5' terminus of pre-S2[20,86].

Combined hepatitis B and hepatitis C

Follow up studies have shown that patients with combined HCV and HBV infection have a higher risk of developing HCC than those with a HCV or HBV alone[3,53,89]. The cumulative risk of developing HCC was 10%, 21%, and 23%, respectively, after 5 years and 16%, 28% and 45%, respectively, after 10 years[3,90].

The HCC risk in subjects with both infections was investigated in a meta-analysis of 32 epidemiological studies between 1993 and 1997[53,91]. The OR for development of HCC in HBsAg positive, anti-HCV/HCV RNA negative subjects was 20.4; in HBsAg negative, anti-HCV/HCV RNA positive subjects, 23.6; and subjects positive for both markers, the OR was 135. These data suggest a more than additive effect of HBV and HCV coinfection on HCC risk. The two viruses may possibly act through common, as well as different, pathways in the carcinogenic process. Given that HBV acts as a cofactor in the development of HCV related cirrhosis and HCC, vaccination of patients with chronic hepatitis C against HBV has been recommended aiming to avoid further liver injury[53,92,93].

Coinfection of HBV and hepatitis D virus (HDV)

HDV coinfection with HBV is associated with increased liver damage. Verme and coworkers showed that HBsAg positive patients with HDV superinfection develop cirrhosis and HCC at an earlier stage (mean age 48 years), compared to HBsAg carriers without HDV infection (mean age 62 years)[53,94].

Coinfection with HIV

Chronic hepatitis C is more aggressive in HIV positive subjects, leading to cirrhosis and liver failure in a shorter time period[53,95]. Coinfection with HIV is a frequent occurrence because of shared routes of transmission. A recent study of HCC in HIV-HCV coinfected patients indicated rapid development of HCC in these patients[53,96].

Role of schistosomiasis

Schistosomiasis is a common parasitic infestation in some parts of the world. In Egypt, Schistosomiasis is a major public health problem and infection with *Schistosoma mansoni* constitutes the major cause of liver disease. From 1950s until 1980s, the Egyptian Ministry of Health (MOH) conducted a community-wide therapy campaign using parenteral tarter emetic to control the Schistosomiasis infestation. However, this unfortunately established a large reservoir of HCV infection in the country through needle re-usage at the time of treatment[97]. There is some epidemiological evidence that the presence of schistosomal infection may modify the course of hepatitis C genotype 4 co-infection and may lead to significantly more complications, such as portal hypertension at an earlier stage with accelerated progression to hepatitis C-associated fibrosis and thus quicker progression to HCC, than those patients who do not have a parasite burden[13,31-34].

Role of aflatoxin B1 (AFB1)

AFB1 is produced by a fungus of the genus, *Aspergillus* spp, in Asia and sub-Saharan Africa in which climatic factors and storage techniques favour the fungus to be a common contaminant of foods, such as grain, corn, peanuts and legumes. Areas with high exposure of AFB1 coincide with areas with a high prevalence of HCC. It has also been suggested that a high intake of AFB1 in HBV-infected patients is an added risk factor for HCC development[3,73,98,99]. It has been observed that areas with a high prevalence of HCC and high aflatoxin intake also correspond to areas with endemic HBV infection, and that patients at highest risk of developing HCC are those who are exposed to both HBV and AFB1[3,98].

Somatic mutations of the tumor suppressor p53 gene are the commonest genetic abnormality in human cancer and evidence supports a high level of p53 alterations in HCC. El Far and colleagues investigated p53 mutations in Egyptian patients with HCC and its relation to other prognostic factors, such as tumor grade, α-fetoprotein (AFP) and liver function tests to elucidate their implication in HCC pathogenesis. These authors found that p53 detection increased the frequency of HCC prediction from 79.5% to 86.3%. Moreover, significant positive correlation between p53 mutation and tumor size for tumor grade Ⅱ and Ⅲ was identified. Thus, serum concentration of p53 protein may be a potential non-invasive screening test for predicting risk of HCC[100].

It has been suggested that AFB1 can lead to HCC through inciting a specific mutation of codon 249 of the p53 tumor suppressor gene[101]. However, this mutation has also been found in patients who had previous contact with the HBV[3,78].

Pesticides

Pesticides exposure is one of the environmental factors hypothesized to increase the risk of HCC. Pesticides are considered to be possible epigenetic carcinogens through one or several mechanisms, such as spontaneous initiation of genetic changes, cytotoxicity with persistent cell proliferation, oxidative stress, inhibition of apoptosis, suppression of intracellular communication and construction of activated receptors[102,103].

A case-control study of HCC in HBV and/or HCV infected patients from Egypt suggested pesticides had an additive effect on the risk of HCC in rural males, amongst whom the use of carbamate and organophosphate compounds is commonplace[103].

Diabetes mellitus

A population-based study from the USA found diabetes to be an independent risk factor for HCC, regardless of chronic HCV or HBV infection, alcoholic liver disease, or non-specific cirrhosis. Diabetes was associated with a two- to threefold increase in HCC risk. About 60% of patients with HCC in this study were not diagnosed with chronic HCV-related or HBV-related hepatitis, alcoholic liver disease, or other known causes of chronic liver disease. Among these patients, 47% had diabetes, which was higher than those with other risk factors (41%). This suggests that diabetes may represent a considerable proportion of patients with idiopathic HCC[104].

An increased risk of HCC among patients with diabetes alone was also reported in a population based study using data obtained from the Denmark cancer registry[104,105]. Also, it is reported a threefold increased risk of liver cancer among patients hospitalized with diabetes, as well as and a fourfold risk in the presence of hepatitis, cirrhosis, and alcoholism in a Swedish study[104,106].

Diabetes, as part of the insulin resistance syndrome, has been implicated as a risk factor for non-alcoholic fatty liver disease (NAFLD), including in its most severe form, non-alcoholic steatohepatitis (NASH). NASH has been identified as a cause of both "cryptogenic cirrhosis" and HCC[104,107-112].

Diet

Many epidemiological studies have examined the relationship between diet and HCC risk[113]. The results are somewhat conflicting. Some studies have shown an inverse relationship between HCC and diets which are high in milk, wheat, vegetable, fish and fruit content. Other studies have shown no association.

With regard to egg consumption, two studies reported an inverse relation with HCC risk[114,115], while three others reported an increased risk[115-117]. Similarly, two studies[118,119] demonstrated that meat and animal protein consumption were associated with increased risk of HCC, although other studies[114-116] did not support this finding[113].

To verify if consumption of soya foods reduce the HCC risk, Sharp and his colleagues conducted a case-control study within a cohort of Japanese A-bomb survivors. They compared the pre-diagnosis intake of isoflavone-rich miso soup and tofu to HCC risk, adjusting for hepatitis B (HBV) and C (HCV) viral infections. They concluded that consumption of miso soup and other soya foods may reduce HCC risk and this is consistent with the results of epidemiological, animal and laboratory-based studies, as well as some clinical trials[120].

This is explained by the opposing effect of isoflavones on oestrogen and testosterone levels which reduce HCC risk, possibly by modifying the hormonal profile and reducing cell proliferation, associated with increased cancer risk. An alternative explanation may be that isoflavones provide an independent anti-tumour effect, such as suppression of angiogenesis, or stimulation of apoptosis[120].

A 41% reduction in HCC risk among coffee drinkers, compared to non-drinkers, has been observed in a meta-analysis study[121]. This favourable effect of coffee drinking was established both in studies from southern Europe[122-124], where coffee is widely consumed, and from Japan[125,126], where coffee intake is less frequent, and in subjects with chronic liver disease[121]. Some compounds in coffee, including diterpenes, cafestol, and kahweol, may act as blocking agents *via* modulation of multiple enzymes involved in carcinogenic detoxification as demonstrated in animal models and cell culture systems[119,127,128]. Moreover, coffee components modify the xenotoxic metabolism *via* induction of glutathione-S-transferase and inhibition of N-acetyltransferase[121,129]. Other components of coffee, including caffeine and antioxidant substances from coffee beans, have been related to favorable modifications in liver enzymes such as γ-glutamyltransferase and aminotransferase activities[119,130-133].

CONCLUSION

HCC is one of the commonest cancers worldwide. It is a major health problem and its incidence is increasing. The presence of cirrhosis is the major risk factor and worldwide this is largely due to chronic HCV and HBV infection. HCC carcinogenesis is likely to involve interplay of viral, environmental and host factors. The advent of mass-vaccination programmes for hepatitis B, particularly in East Asia is beginning to reduce prevalence rates for HCC in some countries, but for the most part, HCV-related HCC is increasing. Concerted strategies need to be developed for HCC surveillance in at risk populations.

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Essential oil of *Curcuma wenyujin* induces apoptosis in human hepatoma cells

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Abstract

AIM: To investigate the effects of the essential oil of *Curcuma wenyujin* (CWO) on growth inhibition and on the induction of apoptosis in human HepG2 cancer cells.

METHODS: The cytotoxic effect of drugs on HepG2 cells was measured by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. DNA fragmentation was visualized by agarose gel electrophoresis. Cell cycle and mitochondrial transmembrane potential (ΔΨm) were determined by flow cytometry (FCM). Cytochrome C immunostaining was evaluated by fluorescence microscopy. Caspase-3 enzymatic activity was assayed by the cleavage of Ac-DEVD-R110. Cleaved PARP and active caspase-3 protein levels were measured by FCM using BD™ CBA Human Apoptosis Kit.

RESULTS: Treatment with CWO inhibited the growth of HepG2 cells in a dose-dependent manner, and the IC50 of CWO was approximately 70 μg/mL. CWO was found to inhibit the growth of HepG2 cells by inducing a cell cycle arrest at S/G_2. DNA fragmentation was evidently observed at 70 μg/mL after 72 h of treatment. During the process, cytosolic HepG2 cytochrome C staining showed a markedly stronger green fluorescence than in control cells in a dose-dependent fashion, and CWO also caused mitochondrial transmembrane depolarization. Furthermore, the results clearly demonstrated that both, activity of caspase-3 enzyme and protein levels of cleaved PARP, significantly increased in a dose-dependent manner after treatment with CWO.

CONCLUSION: CWO exhibits an antiproliferative effect in HepG2 cells by inducing apoptosis. This growth inhibition is associated with cell cycle arrest, cytochrome C translocation, caspase 3 activation, Poly-ADP-ribose polymerase (PARP) degradation, and loss of mitochondrial membrane potential. This process involves a mitochondria-caspase dependent apoptosis pathway. As apoptosis is an important anti-cancer therapeutic target, these results suggest a potential of CWO as a chemotherapeutic agent.

Key words: Essential oil; *Curcuma wenyujin*; Apoptosis; HepG2; Caspase-3; Mitochondrial; Cytochrome C; Cleaved Poly-ADP-ribose polymerase

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer with more than 1 million fatalities occurring annually worldwide[1]. Most HCCs, unlike their normal counterparts, are quite resistant to death receptor-mediated apoptosis, because cell surface death receptors are cross-linked with either agonistic antibodies or soluble death ligand proteins[2,3]. HCCs also display high resistance to tumor necrosis factor-related

apoptosis-inducing ligand-mediated cell death[4,5], which, together with other apoptosis resistance mechanisms, suggests that alternative approaches are needed to control HCC growth and metastasis.

Ezhu, as a Chinese traditional medicine has been used for a long time. It belongs to the family of *Zingiberaceae*. This genus is composed of about 70 species of rhizomatous herbs which are distributed all over the world, and about 20 species could be identified in China. Actually, Chinese Pharmacopoeia indicated that the rhizomes of three species including *Curcuma phaeocaulis*, *C. kwangsiensis,* and *C. wenyujin* are used as *Ezhu*, which has been used for removing blood stasis, alleviating pain, and liver disease protection[6]. In order to control the quality of *Ezhu* and develop *Ezhu* as an effective therapeutic agent, we have developed quality control methods and conducted studies comparing the quantities of several chemical components of different types of *Ezhu*[7]. Nowadays in China, the essential oil of *Curcuma wenyujin* (CWO) has been used as injection to cure paediatric diseases such as acute upper respiratory infections, viral myocarditis, or acute pneumonia[8]. Besides, the essential oil of *Ezhu* has been used as a preparation to treat vaginitis[9]. Also, in some other countries, as in France, Japan, or India, the essential oil of *Ezhu* has been reported to possess antimicrobial[10], anti-bacterial, vasorelaxant[11], and anti-inflammatory activities[12]. In China, the essential oil of *Ezhu* has shown promising effects in the treatment of liver[13], gastric, lung, and cervical cancers. For instance, inhibitory effects of CWO on the growth of SMMC-7721 cells, cervical cells, L615 cells, and K562 cells have been reported[14]. In addition, we recently identified furanodiene, one of *Ezhu*'s ingredients, to activate p38 and to inhibit of ERK mitogen-activated protein kinase (MAPK) signaling in HepG2 cells. The result suggests *Ezhu* as a potential candidate for the treatment of liver diseases[15]. *Ezhu* has a long history on treating liver disease and protecting liver functions; however, there is no report about inhibitory effects of the essential oil of *Ezhu* on human HepG2 cell growth and the underlying mechanism of action. This study aimed determining the cytotoxicity of CWO, one species of *Ezhu,* in human hepatoma HepG2 cells and the underlying molecular mechanism of action.

MATERIALS AND METHODS

Materials

CWO was purchased from Zhejiang RuiAn Pharmaceutical Company (Lot No. 011001). CWO (0.1 mL) was diluted in 10 mL methanol. The solution thus obtained was filtered through a 0.45 μm Econofilter (Agilent Technologies, Palo Alto, CA, USA) and injected into Agilent Series 1100 (Agilent Technologies, USA) liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an autosampler, and a diode array detection (DAD) system, and analyzed under the conditions described in a previous report[7]. In brief, A Zorbax ODS column (250 mm × 4.6 mm I.D., 5 μm) with a Zorbax ODS C18 guard column (20 mm × 3.9 mm I.D., 5 μm) was used. Solvents that constituted the mobile phase were A (water) and B (acetonitrile). The elution conditions applied were: 0-15 min, linear gradient 30%-47% B; 15-30 min, isocratic 47% B; 30-40 min, linear gradient 47%-60% B; 40-50 min, linear gradient 60%-90% B; 50-60 min, linear gradient 90%-100% B; and finally, washing the column with 100% B for 10 min before reconditioning the column for 15 min with 30% B. The flow-rate was 1 mL/min and the injection volume was 10 μL. The column operated at 25℃. The analytes were monitored with DAD at 214 nm and 256 nm. In addition, methanol solutions, containing known concentrations of standards including curcumenone, curcumenol, neocurdione, curdione, isocurcumenol, furanodienone, curcumol, germacrone, curzerene, furanodiene, and β-elemene were prepared and subjected to the LC-DAD system for comparison. 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), JC-1 dye, caspase-3 assay kit, and 488 cytochrome C apoptosis detection kit were purchased from Molecular Probes. Human apoptosis kits were purchased from BD Bioscience.

Methods

Cell culture and drug treatment: The human hepatoma cell line HepG2 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Life Technologies Inc., Gaithersburg, MD), 100 μg/mL streptomycin, and 100 U/mL penicillin in 75 cm^2 tissue culture flasks in a humidified atmosphere at 37℃ with 5% CO_2. CWO was dissolved in 1 mL DMSO to make a 1 mmol/L stock solution and diluted to the concentrations as needed. The final volume of drug solution added to medium was 1%. Control samples contained 1% DMSO.

Growth inhibitory assay: Cells were seeded in 96-well microplates (1×10^5 cells/well in 100 μL medium). CWO was added to the cultures in serial concentrations and cultures were incubated for 48 h. Medium was discarded and 30 μL tetrazolium dye (MTT) solution (5 mg/mL in PBS) were added to each well. Plates were incubated for additional 4 h. DMSO (10 μL) was added to dissolve the formed formazan crystals. The plate was read in a microplate reader at 570 nm. MTT solution with DMSO (without cells and medium) acted as blank while the DMSO (1%)-treated cells served as control of 100% survival.

Agarose gel electrophoresis for analysis of DNA fragmentation: HepG2 cells were treated with the drug for 72 h while the DMSO (1%) containing medium treated cells served as control. Adherent cells (2×10^6/mL) were harvested, washed once with 400 μL PBS, and

taken up in 400 μL lysis buffer (containing 200 mmol/L Tris-HCl (pH 8.3), 100 mmol/L EDTA, and 1% SDS). Twenty μL of 10 mg/mL proteinase K were added for protein digestion, and the tubes were incubated in a 37℃ water bath overnight. The samples were allowed to cool down to room temperature before 300 μL saturated NaCl solution were added. After centrifugation for 15 min at 9000 r/min, supernatants were collected. DNA fibers were obtained by adding 1 mL cold absolute ethanol (EtOH) and a centrifugation for 20 min at 4℃ at 16000 r/min. DNA fibers were washed once with 500 μL of -20℃ 70% EtOH, and the DNA pellet was dried in a 70℃ oven. Fifteen μL TE buffer (10 mmol/L Tris-HCl pH 8.0, 1 mmol/L EDTA) containing 0.2 mg/mL RNase A were added. After an incubation at 37℃ for 90 min, 10 μL of each sample were loaded on a 1.5% TBE agarose gel to visualize DNA.

Cell cycle analysis: Cells were seeded in 6 well plates and treated with CWO at various concentrations for 48 and 72 h. DMSO (1%)-treated cells served as control. After treatment, media were discarded. The adherent cells were washed with PBS, and 300 μL trypsin were added for 5 min at room temperature to detach the cells. After centrifugation at 350 *g* at 4℃ for 5 min, the cell pellet was resuspended with 1 mL cold 70% EtOH at 4℃ for 12 h and centrifuged again for 5 min at 4℃ at 350 *g*. Finally, 1 mL propidium iodide (PI) staining solution (20 μg/mL PI, 8 μg/mL DNase free RNase) was added to the samples. The samples were analyzed by flow cytometry (FCM) (BD FACS Canto™). The results were analyzed by Mod Fit LT 3.0 software.

Measurement of mitochondrial transmembrane potential (ΔΨm): ΔΨm was assessed by JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide), Mitochondrial Potential Sensors (Molecular Probes, Leiden, Netherlands). Red fluorescence J-aggregate form of JC-1 indicates intact mitochondria, whereas green fluorescence shows monomeric form of JC-1 due to breakdown of the mitochondrial membrane potential. Cells were seeded in 6-well plates and then incubated with the desired concentrations of CWO for 48 h. The medium of each well was discarded, cells were treated with 1 mL medium containing 5 mg/mL JC-1 for 15 min at 37℃ and 5% CO_2 in the dark, washed twice in PBS, resuspended in 1 mL medium and measured by FCM.

Immunostaining of cytochrome C: Cytochrome C release was assessed by SelectFX Alexa Fluor 488 cytochrome C apoptosis detection kit (Molecular Probes, Leiden, Netherlands). Cells were seeded in 24-well plates, and treated with various amounts of CWO in an humidified atmosphere (37℃ in 5% CO_2) for 24 h while the DMSO (1%)-treated cells served as control. Media were discarded and the cells were washed with warm PBS, fixed with freshly prepared 4% formaldehyde in PBS for 15 min at 37℃, and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. The cells were washed, incubated in a blocking buffer (10% heat-inactivated normal goat serum (NGS) for 30 min at room temperature, and finally for 1 h with 1 μg/mL primary antibody (anti-cytochrome C, mouse IgG) at room temperature. Green fluorescence was observed by fluorescence microscopy.

Caspase-3 enzymatic activity assay: Caspase-3 enzymatic activity was determined by measuring the cleavage of Ac-DEVD-R110 according to the protocol with the caspase-3 assay kit supplied by Molecular Probes. Cells were treated with various amounts of CWO in an humidified atmosphere (37℃ in 5% CO_2) for 24 h while DMSO (1%)-treated cells served as control. Cells were harvested at a concentration of a minimum of 1×10^6/mL, pellets were collected, appropriate cell lysis buffer was added, and the samples were incubated on ice for 30 min. The samples were then centrifuged and supernatants were collected and transferred to microplates. Cell lysis buffer was used as a no-enzyme control to determine the background fluorescence of the substrate. At the same time, 1 μL of 1 mmol/L Ac-DEVD-CHO inhibitor was added to selected samples. One μL of DMSO was added to no-inhibitor samples to serve as control and incubated for 10 min simultaneously. Finally, 0.05 mmol/L Z-DEVD-R110 substrate was added and samples were incubated for 30 min prior to the fluorescence measurement.

Measurement of cleaved PARP and active caspase-3 protein levels: The BD™ CBA human apoptosis kit (BD, Franklin Lakes, USA) was applied to quantify the active caspase-3 and Poly-ADP-ribose polymerase (PARP) protein levels; cytometric Bead Array (CBA) employs a particle with a discrete fluorescence intensity to detect a soluble analyte. This kit provided two types of bead populations with distinct fluorescence intensities that have been coated with capture antibodies specific for cleaved caspase-3 and PARP. Cells were seeded in 6-well plates and incubated with various concentrations of CWO in an humidified atmosphere (37℃ with 5% CO_2) for 48 h. 1.0×10^6 cells per sample were counted, harvested, and washed with PBS. Fifty μL of cell lysis buffer was added to each sample for 30 min on ice and samples were vortexed at 10-min intervals. Pellet cellular debris was removed by centrifugation at 12500 r/min for 10 min. Protein concentrations were measured by 2-D Quant Kit (Amersham Biosciences, Piscataway, USA). Each sample was normalized in a final concentration of 0.2 μg/μL. Thirteen standard curves (standard ranging from 0 to 6000 U/mL) were obtained from one set of calibrators. For each sample and the standard mixture of lysate standard (caspase-3 and PARP beads), 50 mL of sample or standard of beads were added to the mixture of 50 mL of 2 mixed capture beads incubated for 1 h, and mixed 50 mL of PE detector bead for another 1 h. After that, samples were washed before data acquisition with a FCM. The results were analyzed by FCAP Array V1.0.

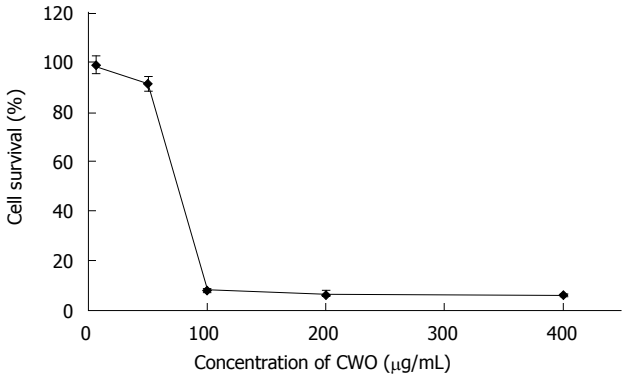


Figure 1 HepG2 cells were treated with the indicated concentrations of CWO for 48 h. Cell growth was determined by the MTT assay and was directly proportional to the absorbance at a wavelength of 570 nm. Data are expressed as mean ± SD from three independent experiments.

Statistical analysis

The data are expressed as mean ± SD from at least 3 independent experiments. Differences between groups were analyzed using a Student's *t*-tests.

RESULTS

CWO inhibits cell growth and induces DNA fragmentation in HepG2 cells

CWO treatment inhibited the growth of HepG2 cells in a dose-dependent manner, and the IC50 of CWO was approximately 70 μg/mL (Figure 1). We then examined whether CWO inhibited HepG cell growth through inducing cell death and apoptosis. HepG2 cell were treated with different concentrations of CWO for 72 h. Figure 2 shows that DNA fragmentation was evidently observed at a concentration of 70 μg/mL after 72 h of treatment.

CWO causes S/G_2 cell cycle arrest

The effect of different concentrations of CWO on cell-cycle progression was studied after 48 and 72 h of drug exposure. CWO treatment resulted in the accumulation of cells in S/G_2 phase with concomitant losses from G_0/G_1 phase (Figure 3). Since substantial proportions of cells were dead in groups treated with 50 μg/mL and 70 μg/mL CWO, only cultures treated with 35 μg/mL were used for the analysis as shown in Figure 4.

CWO causes mitochondrial transmembrane depolarization in HepG2 cells

Some chemotherapeutic drugs induced apoptosis *via* mitochondrial pathways by altering ΔΨm. To monitor the ΔΨm, we used JC-1 probe to determine the ΔΨm in cells that were treated with CWO for 48 h at different concentrations. Mitochondria with normal ΔΨm concentrate JC-1 into aggregates (red/orange fluorescence), while in depolarized mitochondria, JC-1 forms monomers (green fluorescence). As compared to non-treated HepG2 cells, green fluorescence increased while red/orange fluorescence decreased after CWO exposure. Figure 4 indicates an only minor shift from red/orange to green fluorescence in groups treated with 17.5 μg/mL and 35 μg/mL CWO, while 50 μg/mL and 70 μg/mL CWO led to significant changes in ΔΨm.

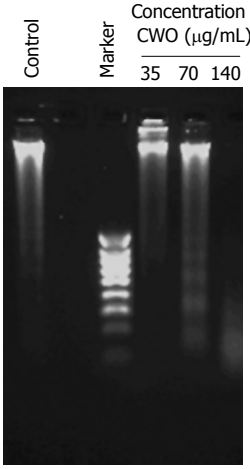


Figure 2 Agarose gel of electrophoresis of genomic DNA obtained from HepG2 cells treated with different concentrations of CWO for 72 h. DNA fragmentation with a ladder pattern is a characteristic for apoptosis.

CWO causes cytochrome C release from mitochondria into cytosol

Cytochrome C release from mitochondria to the cytosol is implicated in mitochondria dependent apoptosis[16]. Cytochrome C staining in the cytosol of HepG2 cells showed markedly stronger green fluorescence than in control cells in a dose-dependent fashion (Figure 5). CWO treated cells showed obvious punctuate green fluorescence staining or appeared to have green fluorescence accumulated in large aggregates compared to the control.

CWO activates caspase-3 enzymatic activity

Many studies previously have demonstrated that programmed cell death is associated with the activation of caspase as key elements involved in the sequence of events that lead to cell death[17]. Caspase-3 particularly, is essential for propagation of the apoptotic signal after exposure to many DNA-damaging agents and anticancer drugs. We examined caspase-3 activity after cells were treated with 17.5-70 μg/mL CWO for 24 h. The result clearly demonstrated that caspase-3 activity increased in a dose-dependent manner (Figure 6). Caspase-3 activities were 2 and 5.5 times higher in 35 μg/mL and 70 μg/mL CWO treated cultures, respectively, when compared to the control. When the cellular samples were incubated with specific Ac-DEVD-CHO inhibitors simultaneously, caspase-3 activity was blocked.

CWO increases cleaved PARP and active caspase-3 protein levels

Drug-induced cell death *via* apoptosis pathway, signaling can generally be divided into receptor- and mitochondrial-mediated pathways. These pathways converge at several downstream points including the mitochondria, caspase activation, and substrate cleavage[18]. Figure 7 showed that there was significant increase in protein levels of cleaved PARP and active caspase-3 in a dose-dependent manner as determined by BD™ CBA Human

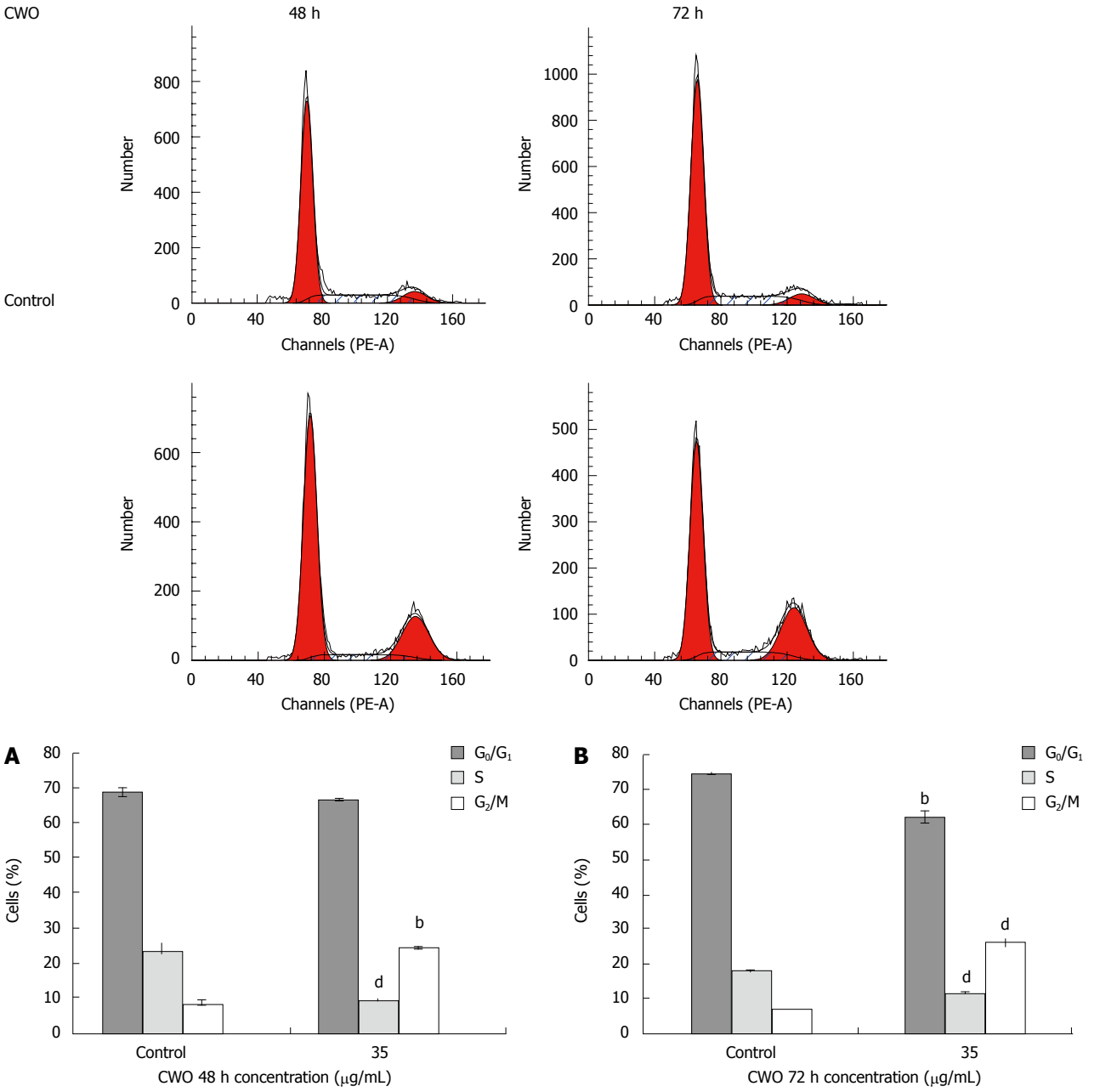


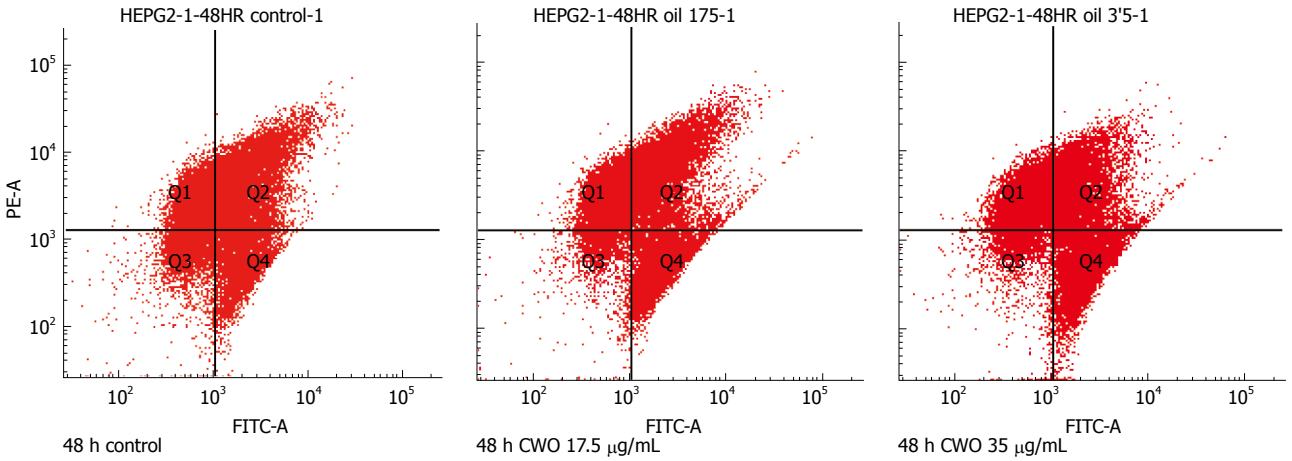
Figure 3 Effect of CWO on HepG2 cell cycle progression. Flow cytometric analysis of propidium iodide-stained HepG2 cells treated with 35 μg/mL CWO for 48 h (**A**) and 72 h (**B**). The results of HepG2 cells treated with CWO were analyzed by Mod Fit LT 3.0. Data expressed as mean ± SD from three independent experiments. $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* control.

Apoptosis Kit. HepG2 cells treated with 17.5, 35, and 70 μg/mL CWO were 6, 4, and 7 times higher in caspase-3 protein level than control group (Figure 7A). The level of cleavage of PARP had a 2-fold increase in HepG2 cells treated with 70 μg/mL CWO compared to the control (Figure 7B).

DISCUSSION

The essential oil of *Ezhu* and its ingredients have been widely used for treatment of malignant tumors in China[19] and have been identified to have hepatoprotective effects[20,21]. Previously, we have already identified an active ingredient furanodiene, a sesquiterpene compound, which have been isolated from CWO, one of species of *Ezhu*. Furanodiene has been found to induce apoptosis in HepG2 cells through activation of mitochondrial and caspase dependent pathway which involved activation of P38, and inactivation of ERK1/2 MAPK signaling cascades[15]. In the present study, we have found that CWO inhibited HepG2 cells growth with IC50 at approximately 70 μg/mL and it has been identified to inhibit HepG2 cell growth *via* inducing apoptosis as evidenced by activation of depolarization of ΔΨm, mitochondrial cytochrome C release, caspase-3, PARP cleavage, S/G_2 cell cycle arrest, and finally DNA fragmentation.

Active caspase-3 has been considered to be indicative of apoptosis[17] and another characteristic event of apoptosis is the proteolytic cleavage of PARP, a nuclear enzyme involved in DNA repair, DNA stability, and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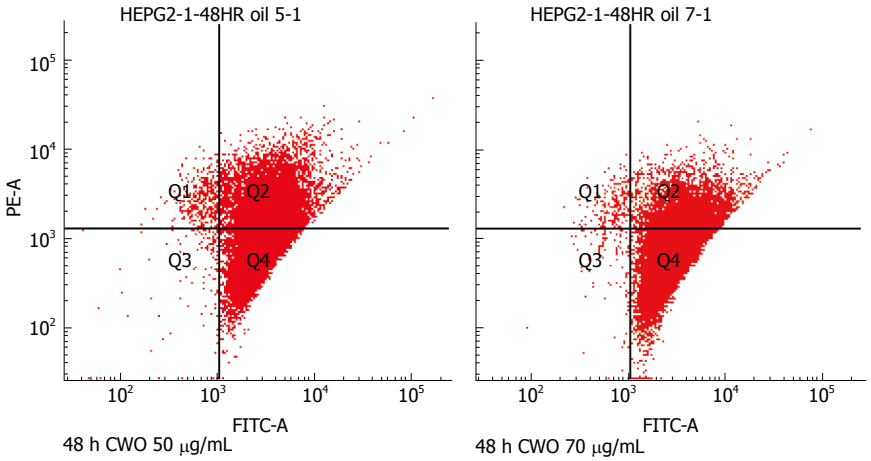
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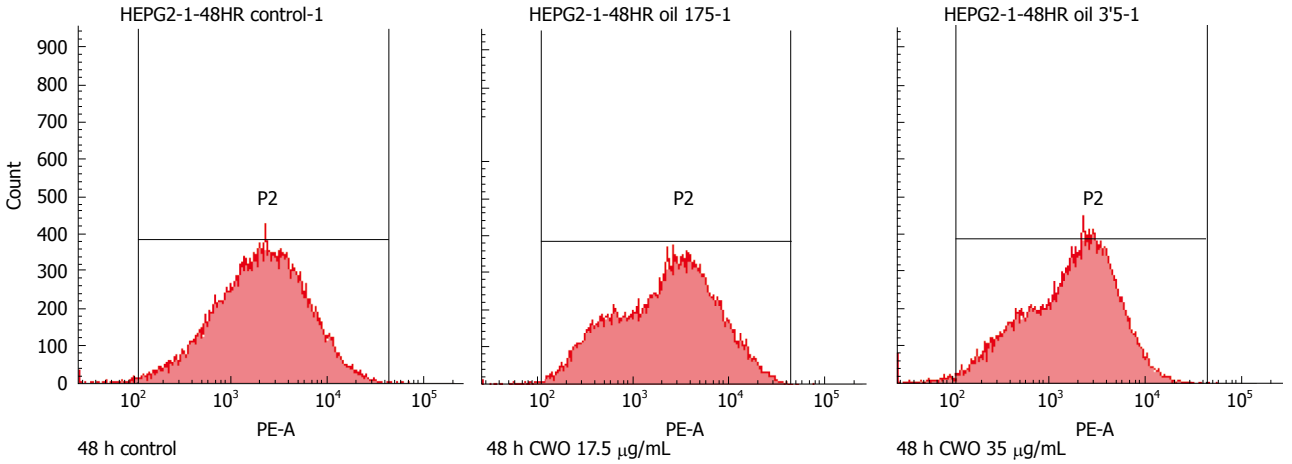
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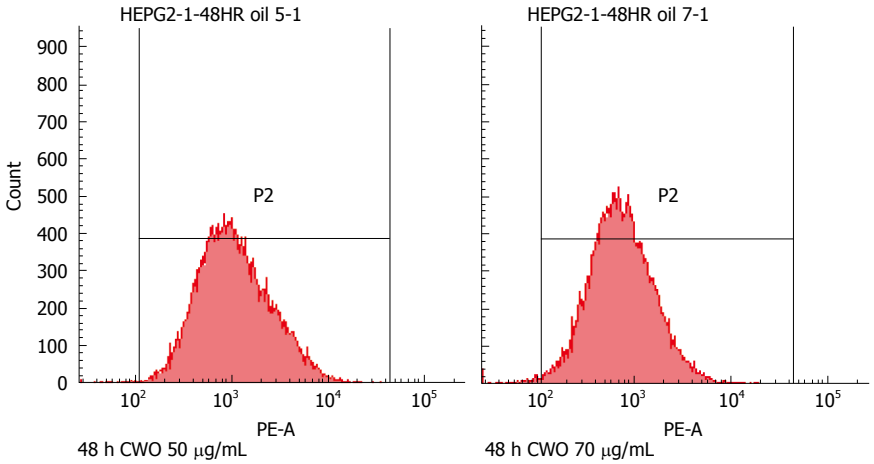
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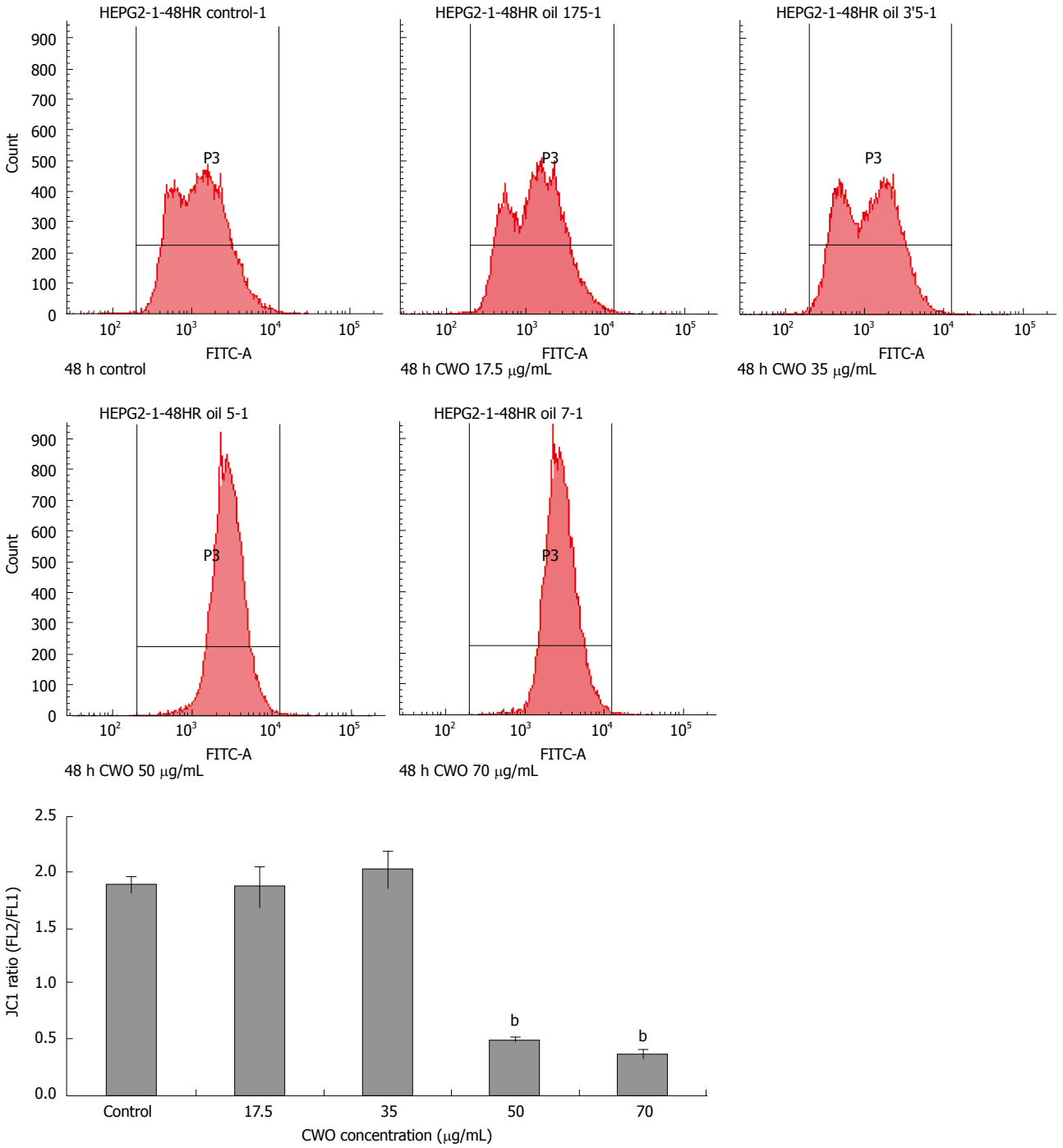

Figure 4 Analysis of change of ΔΨm in HepG2 cells. HepG2 treated with 17.5, 35 , 50, and 70 μg/mL for 48 h, were stained with JC-1 probe. The cells were analyzed by FCM. Red and green fluorescence were measured by FL2 and FL1 channel, respectively. Red fluorescence indicates intact mitochondrial potential while green fluorescence indicates breakdown of mitochondrial potential. The ratio of intensity of FL2 to FL1 indicates the change of ΔΨm. Data expressed as mean ± SD from four independent experiments. $^{b}P < 0.001$ *vs* control.

transcriptional regulation[22]. An experiment was performed to simultaneously and quantitatively evaluate CWO induced changes of active caspase-3 and cleaved PARP protein levels using CBA technology as demonstrated in Figure 7. As shown, active caspase-3 protein expressions were enhanced approximately 7-fold compared to the control. Meanwhile, we attempted to detect CWO-induced caspase-3 enzymatic activity increases using caspase-3 specific substrate Z-DEVE-R110, and have found that the enhancements disappeared when treated with Ac-DEVD-CHO inhibitor as shown in Figure 6. This showed the increases in caspase-3 enzymatic activities were specific to CWO treatment and suggested that CWO induces apoptosis *via* a caspase-3-dependent pathway.

CWO induced significant increase in caspase-3 enzymatic activity as well as the protein levels of active caspase-3 and cleaved PARP (Figure 7). These results suggested that CWO induced apoptosis *via* caspase pathway. Moreover, whether CWO induced apoptosis in HepG2 cell is mitochondria-dependent is unknown.

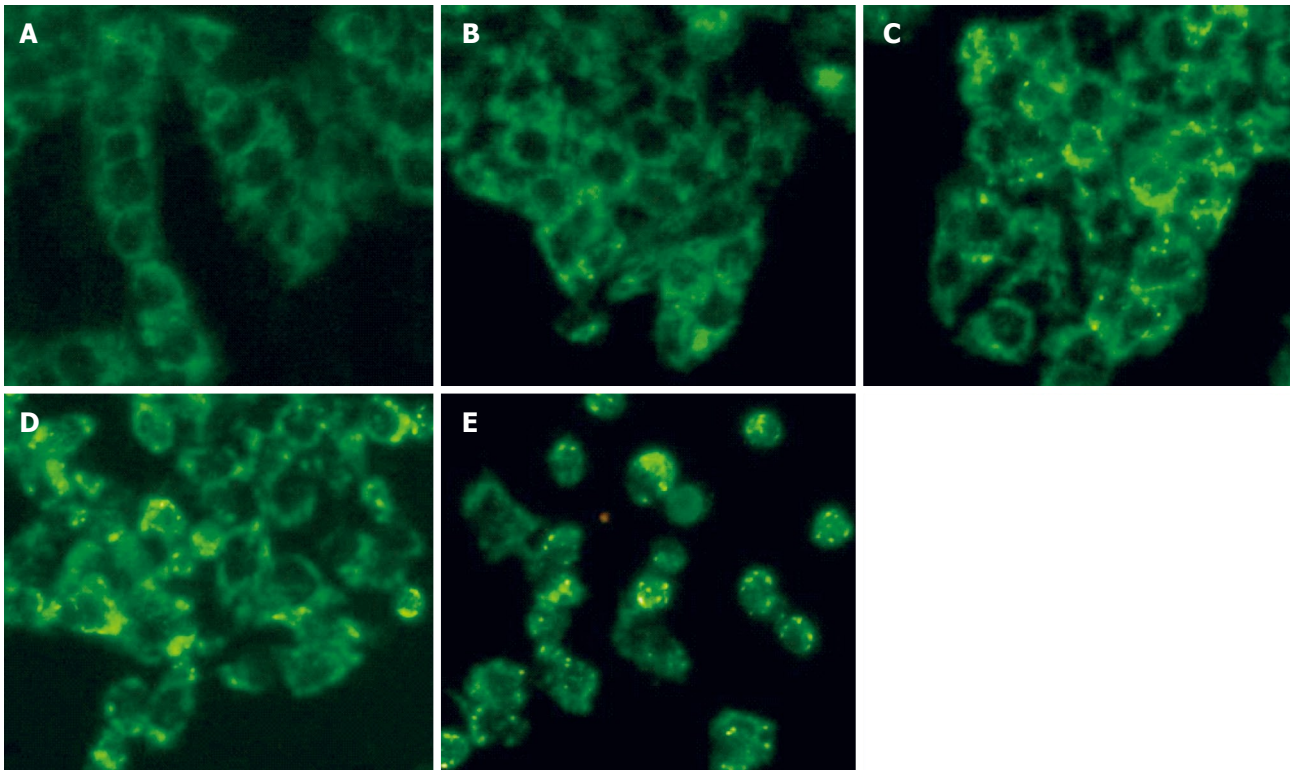

Figure 5 Cytochrome C release into the cytosol in CWO treated HepG2 cells for 12 h. Cytochrome C immunofluorescence was observed with fluorescent microscope. **A**: Control; **B**: 17.5 μg/mL; **C**: 35 μg/mL; **D**: 70 μg/mL; **E**: 120 μg/mL. Fine punctate/granular stainings for cytochrome C are observed. Cytochrome C release also increases the global cytosolic fluorescent signal. Similar results were obtained for 3 independent experiments (× 20).

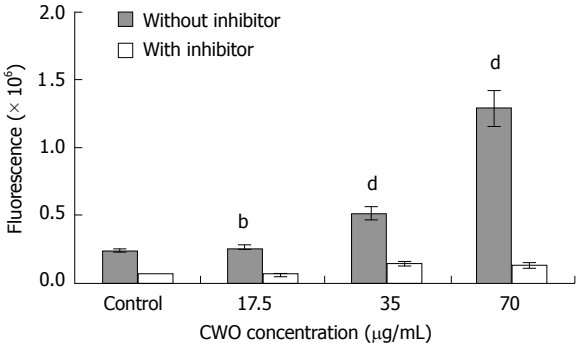

Figure 6 The fluorescence was increased in a dose-dependant manner after 24 h treatment with CWO. Caspase-3 cleaves substrate Ac-DEVD-R110 to emit green fluorescence. Higher fluorescent intensity indicates higher caspase-3 enzymatic activity. Ac-DEVE-CHO inhibitor can inhibit caspase-3 enzymatic activity. Data are expressed as mean ± SD from three independent experiments. $^{b}P < 0.001$, $^{d}P < 0.01$ *vs* control.

To address this question, the change of ΔΨm and mitochondrial cytochrome C release were determined. Figures 4 and 5 suggested that CWO-mediated apoptosis was accompanied with the ΔΨm as well as the release of mitochondrial cytochrome C into cytosol. These results demonstrated that a mitochondrial pathway was also involved in CWO-induced apoptosis.

It is now widely believed that p38 and JNK mediate apoptotic signals, while ERK promotes growth, differentiation, and proliferation. Nowadays, many studies have shown that p38 MAPK activation is necessary for cancer cell death initiated by a variety of anti-cancer agents[23]. Furthermore, different MAPK signaling pathways can be coordinately manipulated to enhance the efficacy of anticancer drug. Co-treatment of anticancer drugs with ERK inhibitors has been found to enhance anticancer effects. Anti-cancer drug paclitaxel (Taxol) induces tumor cell apoptosis through activating endogenous JNK in tumor cells[24]. When paclitaxel and ERK inhibitor were combined in cancer treatment, ERK inhibitor significantly enhances the JNK activation-mediated cytotoxic effect of paclitaxel. ERK inhibitor also found to enhance docetaxel-induced apoptosis of androgen-independent human prostate cancer cells[25].

CWO, possibly act as chemopreventive agents with respect to inhibition of the growth of human HepG2 cells through the induction of apoptosis. As apoptosis has become a new therapeutic target in cancer research, these results confirm the potential of CWO as an agent of chemotherapeutic and cytostatic activity in human HepG2 cells. Besides, in our previous study, furanodiene, isolated from CWO, has been identified induce HepG2 cell apoptosis through alternating MAPK signaling. Furanodiene obviously elevated phosphorylated form of P38 and reduced phosphorylated form of ERK1/2 in a dose-dependent manner, but a slightly and statistically insignificant change in phosphorylated form of JNK. Therefore, furanodiene induced-apoptosis in HepG2, involve activation of P38 and inhibition of ERK MAPK signaling. Whether CWO acts on these signaling pathways should be an interesting area for further study.

In short, we conclude that CWO induces apoptosis in HepG2 cells through activation of mitochondrial and caspase-3 pathway.

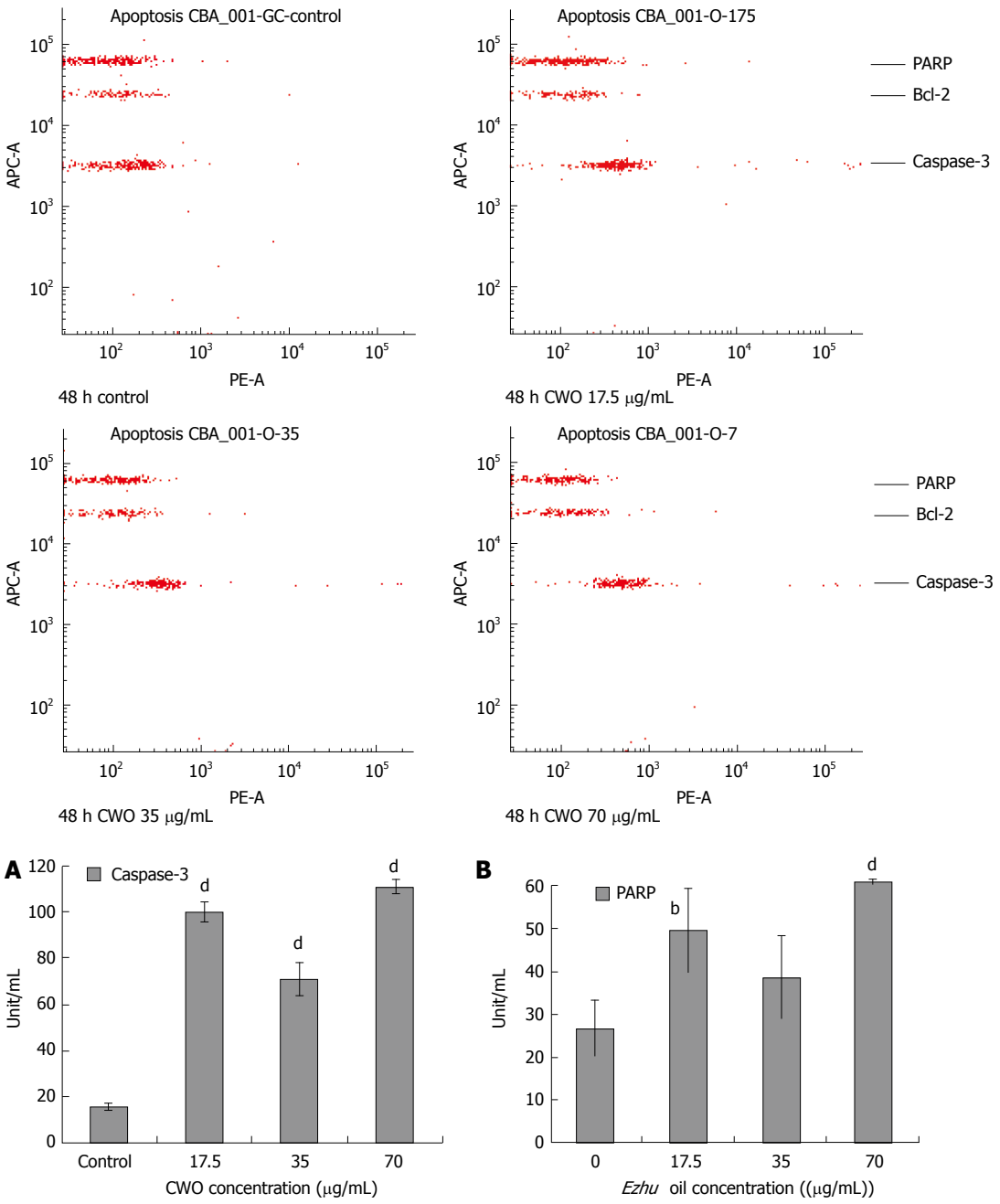

Figure 7 Protein expression levels of active caspase-3 and cleaved PARP in CWO-induced apoptosis. HepG2 cells were treated with medium alone (control) or different concentration of CWO for 48 h. Cells of each sample were counted to 1.0×10^6 and all the samples were normalized to final protein concentration in 0.2 μg/μL. It was detected with BD™ CBA Human Apoptosis Kit (BD, Franklin Lakes, USA) according to manufacturer instruction. The results were analyzed by FCAP Array V1.0. Active caspase-3 protein level in HepG2 was shown in (**A**) and the cleaved PARP protein level in HepG2 was shown in (**B**). The x-axis indicated the concentration of CWO while the y-axis indicated amount of proteins (unit per mL). Concentration of active caspase-3 and cleaved PARP in test samples were determined using the standard curve. Data expressed as mean ± SD from three independent experiments. $^bP < 0.01$, $^dP < 0.001$ *vs* control.

COMMENTS

Background

Ezhu has a long history on treating liver diseases and protecting liver functions. Chinese Pharmacopoeia indicated that the rhizomes of three species including *Curcuma phaeocaulis*, *C. kwangsiensis* and *C. wenyujin* are used as *Ezhu*. The essential oil of *Ezhu* has been reported to possess various biological roles such as antimicrobial, anti-inflammatory, and anti-tumor activity. Some sesquiterpene compounds isolated from essential oil of *Curcuma wenyujin* (CWO) has been identified to have hepatoprotective effects. The total effects of the complex interactions of different compounds in extracts isolated from CWO are not well characterized.

Research frontiers

Hepatocellular carcinoma (HCC) is the fifth most commonly diagnosed cancer with more than 1 million deaths reported annually worldwide. Many sesquiterpenes are identified to possess protective effects against carcinogenesis or tumor growth. For example, artemisinin, a sesquiterpene lactones, killed human oral cancer cells through apoptosis and may be useful as an alternative treatment for oral cancer. In order to develop effective means for prevention and treatment of HCC and related liver diseases, extensive research is being carried out to isolate and identify chemical extracts and pure compounds from Chinese medicine with hepatoprotective, anti-hepatoma, anti-multidrug resistant hepatoma, and anti-viral effects.

Innovations and breakthroughs

This is the first study to report the biological activity and mechanism of action of CWO on HCC cells.

Applications

CWO induce apoptosis in HepG2 cells through activation of mitochondrial and caspase-3-pathway and the result suggests the potential value of development of *Ezhu* on treatment of liver diseases and further study on anti-apoptotic effect of CWO may lead to identification of new lead compounds and novel drug targets for treatment of liver cancer and diseases.

Peer review

This is an interesting study showing that CWO inhibits growth and induces apoptosis in human HepG2 hepatoma cells. These findings are of interest.

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CLINICAL RESEARCH

Prevalence of microscopic colitis in patients with diarrhea of unknown etiology in Turkey

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Abstract

AIM: To investigate the prevalence and demography of microscopic colitis in patients with diarrhea of unknown etiology and normal colonoscopy in Turkey.

METHODS: Between March, 1998 to July, 2005, 129 patients with chronic non-bloody diarrhea of unexplained etiology who had undergone full colonoscopy with no obvious abnormalities were included in the study. Two biopsies were obtained from all colonic segments and terminal ileum for diagnosis of microscopic colitis. On histopathologic examination, criteria for lymphocytic colitis (intraepithelial lymphocyte $\geqslant$ 20 per 100 intercryptal epithelial cells, change in surface epithelium, mononuclear infiltration of the lamina propria) and collagenous colitis (subepithelial collagen band thickness $\geqslant$ 10 μm) were explored.

RESULTS: Lymphocytic colitis was diagnosed in 12 (9%) patients (Female/Male: 7/5, mean age: 45 year, range: 27-63) and collagenous colitis was diagnosed in only 3 (2.5%) patients (all female, mean age: 60 years, range: 54-65).

CONCLUSION: Biopsy of Turkish patients with the diagnosis of chronic non-bloody diarrhea of unexplained etiology and normal colonoscopic findings will reveal microscopic colitis in approximately 10% of the patients. Lymphocytic colitis is 4 times more frequent than collagenous colitis in these patients.

Key words: Diarrhea of unknown etiology; Microscopic colitis; Lymphocytic colitis; Collagenous colitis

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Erdem L, Yıldırım S, Akbayır N, Yılmaz B, Yenice N, Gültekin OS, Peker Ö. Prevalence of microscopic colitis in patients with diarrhea of unknown etiology in Turkey. *World J Gastroenterol* 2008; 14(27): 4319-4323 Available from: URL: http://www.wjgnet.com/1007-9327/14/4319.asp DOI: http://dx.doi.org/10.3748/wjg.14.4319

INTRODUCTION

Chronic diarrhea with no obvious reason is one of the challenges of gastroenterology. In 1980, Read *et al*[1] introduced microscopic colitis characterized by chronic diarrhea with normal endoscopic and radiologic findings, but with increased colonic mucosal inflammatory cells and epithelial lymphocytic infiltration on histologic examination. Later, Levison *et al*[2] emphasized that microscopic colitis covered all cases of colitis with normal colonoscopy, but abnormal histopathologic features and described lymphocytic colitis separately. Collagenous colitis, which is a closely related condition, was first described in 1976 as a separate subtype with additional histological finding of increased subepithelial collagen band thickness[3]. Thus, microscopic colitis is a condition with two subtypes having similar clinical, but different histological characteristics. In collagenous colitis subepithelial collagenous band thickness is important.

The prevalence of microscopic colitis has been difficult to estimate. The symptoms of microscopic

colitis have been frequently attributed to diarrhea-predominant irritable bowel syndrome, often for many years before diagnosis. Diagnostic awareness of these conditions by physicians in the geographic area of interest significantly effects the likelihood of diagnosis and, therefore, the prevalence.

Clinical and histological characteristics of microscopic colitis have been well established[4-8]. However, limited data is available regarding the prevalence, pathogenesis and progress of the disease and its treatment. The diagnosis is made only by histologic examination and most of these patients are treated and followed up erroneously as irritable bowel syndrome. Recently, several studies from Sweden and Iceland reported high prevalence of microscopic colitis[9-11]. In this prospective study we aimed to determine the prevalence of lymphocytic and collagenous colitis in Turkey in a subset of patients with chronic non-bloody diarrhea of unknown origin in which colonoscopy was not conclusive.

MATERIALS AND METHODS

Patients

Between March, 1998 and July, 2005, in three centers around Istanbul (two gastroenterology clinics and one private endoscopy laboratory), 129 consecutive patients with unexplained chronic (at least 3 mo duration), non-bloody diarrhea have undergone colonoscopy with visualization of terminal ileum and normal mucosal appearance noted. These patients were included in the study. Inclusion criteria are shown in Table 1. All patients underwent abdominal ultrasonography and/or computer tomography (CT). Patients who received radiotherapy, chemotherapy, or who had undergone operation related to bowel, stomach or gallbladder or patients with inflammatory bowel disease, chronic liver disease, renal disease or pancreatitis, and patients with the history of long term laxative and antibiotic use were excluded from the study. Stool consistency (liquid, semiliquid, soft), number of daily defecation, duration of diarrhea, and other gastrointestinal symptoms (abdominal pain, weight loss, *etc*) and previous medication were recorded.

Colonoscopy and histology

Patients were prepared for colonoscopy with 90 mL oral monobasic sodium phosphate and dibasic sodium phosphate. During colonoscopy two biopsies were taken from terminal ileum and all segments of the colon. Specimens were stained with HE and Masson's Trichrome or Van Gieson dyes.

Diagnostic criteria

Increased chronic inflammatory infiltration in the lamina propria, increased intraepithelial lymphocytes (IELs), degeneration of surface epithelium and increased mitosis in crypts were sought for the diagnosis of microscopic colitis. Over 20 IEL per 100 intercryptal epithelial cells (normal < 1-5/100) were deemed necessary for the diagnosis of lymphocytic colitis[7,8,12] (Table 2). For collagenous colitis subepithelial collagen band thickness was measured by ocular micrometer in Masson's Trichrome stained specimens. Thickness over 10 μm was required for the diagnosis[7,8,12] (Table 2).

Table 1 Inclusion criteria for study

Inclusion criteria
Diarrhea without blood (> 3 mo)
Normal stool microscopy
No growth in stool culture
Normal D-Xylose absorption test
Normal biochemical profile
Normal thyroid tests, normal serum gastrin
Negative antigliadin antibodies (IgA, IgG)
Negative *Clostridium difficile* toxins (A, B)
Negative HIV test
Normal urine 5-HİAA
Normal upper GI endoscopy
Normal abdominal US
Normal small bowel radiology
Normal duodenal biopsy
Normal colonoscopy including terminal ileum

Table 2 Histopathologic criteria for diagnosis of lymphocytic colitis and collagenous colitis

	Histopathologic criteria
Lymphocytic colitis	Chronic inflammatory infiltration in lamina propria
	Increased IELs
	Superficial epithelial degeneration and increased mitosis in crypts
	IELs/100 intercryptal epithelial cell > 20/100
Collagenous colitis	A diffusely distributed and thickened subepithelial collagen band > 10 μm
	Chronic inflammatory infiltration in lamina propria

RESULTS

During the mentioned period, colonoscopy was performed in a total of 9862 patients due to various reasons. One hundred and twenty-nine of those patients had chronic non-bloody diarrhea with no apparent cause even after laboratory and radiologic examination and full colonoscopy with terminal ileal visualization. These patients were included in the study. After colonoscopic biopsy of colonic segments in 114 patients, histopathologic examinations of colonic biopsies were normal. In all patients, biopsies from terminal ileum revealed normal epithelial features.

Fifteen patients (11.5%) had microscopic colitis (12 lymphocytic colitis and 3 collagenous colitis; 9%, 2.5%, respectively) (Figure 1A and B). Seven of the lymphocytic colitis patients were female, mean age was 45 ± 11.6 (27-63), mean duration of diarrhea was 22 mo (4-96) and mean number of daily defecation was 5 (3-9). Criteria of lymphocytic colitis were present in specimens obtained from all segments of the colon of these patients. Mean number of IEL per 100 intercryptal

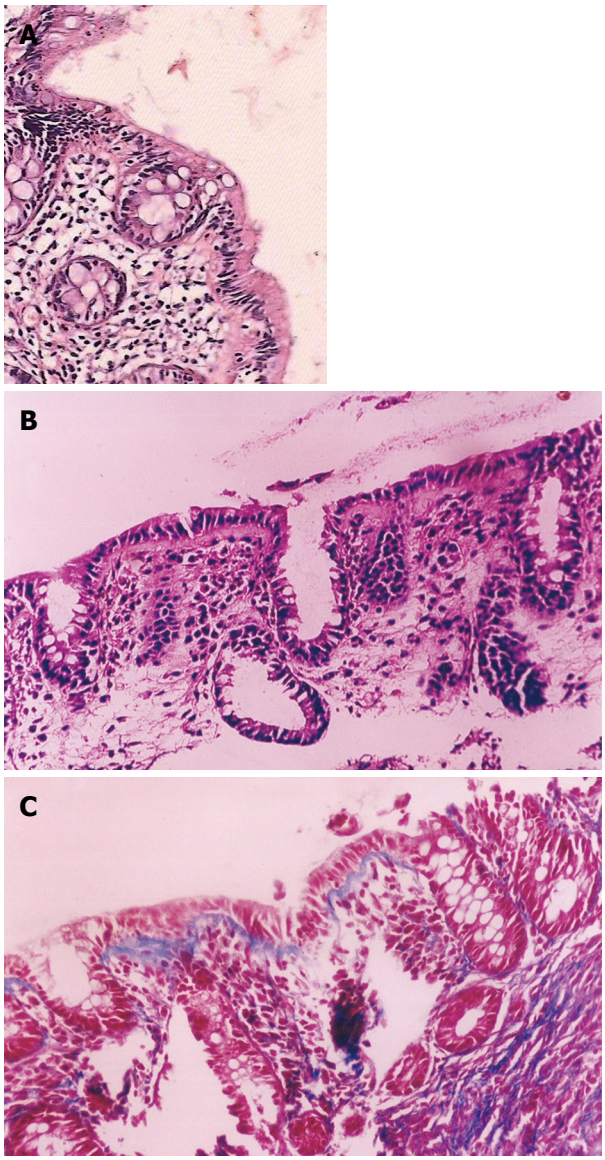

Figure 1 Pathologic view (× 200). **A**: Lymphocytic colitis. Note the increased number of chronic inflammatory cells in the lamina propria and within the surface epithelium (HE); **B**: Collagenous colitis. Note the subepithelial thick collagenous band (HE); **C**: Collagenous band thickness on Mason trichrome dye.

epithelial cells was 28.2 ± 6.8 (range 20-60) (Figure 1A). All patients with collagenous colitis were female (ages 65, 54 and 61 years) and their subepithelial collagenous band thickness was 31, 21 and 17.5 μm (Figure 1B and C). Mean durations of diarrhea were 34, 11 and 68 mo and mean daily stool frequencies were 5, 8 and 4 times, respectively.

DISCUSSION

Microscopic colitis, which is characterized by chronic watery diarrhea with normal radiological and endoscopic appearances, is diagnosed only by histopatologic examination. This condition which consists of two main subtypes (lymphocytic and collagenous colitis) is a relatively common cause of chronic watery diarrhea, often accompanied by abdominal pain and weight loss.

Studies from different countries reported microscopic colitis rates between 4%-13% in the cohort of population with non-bloody diarrhea of unknown origin[10-15]. In the current study, we found this rate to be 11.5% in Turkey.

Diagnosis of this condition is possible only with the awareness of health workers and careful assessment of the criteria. Therefore, the reported prevalence seems to change within years. In Sweden, microscopic colitis was reported in 4% of patients with non-bloody chronic diarrhea in 1993, but this rate was reported as 10% in 1998[9,10,13]. The prevalence of collagenous colitis in Sweden between 1984-1988 was $0.8/10^5$ inhabitants, but increased to $6.1/10^5$ inhabitants between 1996-1998[9,10,13,15]. Recently, higher prevalence values have been reported from Iceland where the mean annual prevalence of collagenous colitis was $5.2/10^5$ inhabitants and the mean annual incidence of lymphocytic colitis was $4.0/10^5$ inhabitants in the period 1995-1999[11]. According to various studies prevalence of collagenous colitis and lymphocytic colitis is 10-15.7/100 000 and 14.4/100 000, respectively[12-14,16].

In a study performed in Spain, lymphocytic colitis was found in 9.5% of patients who had undergone colonoscopy because of chronic diarrhea during a period of 5 years[14]. In this study, the prevalence of lymphocytic colitis was three times that of the prevalence of collagenous colitis, female/male ratio in lymphocytic and collagenous colitis was found 2.7/1 and 4.7/1, respectively. Female/male ratio were reported as 5/1 from Iceland and 2.1 from Sweden[10-15]. In reported series this ratio for collagenous colitis was found as 4/1-20/1[13-17]. In our study, female/male ratio for lymphocytic colitis was 1.4/1 and lymphocytic colitis was 4 times more than that of collagenous colitis.

Marshall *et al*[18] encountered 13 lymphocytic colitis and 1 collagenous colitis in their 111 chronic-diarrhea patients with unexplained etiology. In another study of 132 consecutive patients who had undergone colonoscopy for chronic diarrhea and abdominal pain, lymphocytic and collagenous colitis found in 21 (16%) and 7 (5%) of patients, respectively[19].

Mean ages of the patients with lymphocytic and collagenous colitis in other studies were between 51-59 years, and 64-68 years, respectively[13-17]. In our study, the mean age of the patients with lymphocytic colitis was 45 years (range 27-63). The mean age of our three collagenous colitis patients was 60 years.

In the studies of Lazenby *et al* and Baert *et al*, the mean IEL per 100 intercryptal epithelial cells was 34.7 and 29.4, respectively[5,8]. In the current study, the mean IEL per 100 intercryptal epithelial cells was 28.2. Normal subjects may have up to 1 to 5 IEL per 100 intercryptal epithelial cells. Some studies have reported that biopsy specimens from all segments of the colon revealed similar number of IEL and, therefore, biopsy obtained only from sigmoid colon would was enough for diagnosis[7,8,12]. In our study, the number of IEL was increased in all bowel segments.

Since subepithelial band thickness was less than 8 μm

in all our cases of lymphocytic colitis, the diagnosis of collagenous colitis and overlapping form was excluded. In normal subjects subepithelial collagen band thickness of 5-7 μm is considered as normal and band thickness of 7-80 μm is found in collagenous colitis[7,8,12]. In our 3 collagenous colitis patients the mean band thickness was 23 μm.

As yet, the etiology of lymphocytic colitis has not been well understood. Gastrointestinal infections, autoimmune diseases and various drugs (non-steroidal anti-inflammatory drugs, ranitidine, carbamazepine, simvastatin, ticlopidine, flutamide *etc*) were reported to be causative factors[12,16,17,20]. Some gastrointestinal rheumatologic disorders (celiac sprue, rheumatoid arthritis, uveitis, idiopathic pulmonary fibrosis, diabetes mellitus, pernicious anemia, autoimmune thyroiditis *etc*) and positivity of some autoantibodies, particularly antinuclear antibody (ANA) may be associated with both lymphocytic and collagenous colitis[14,21-24]. Giardiello *et al* found 4 ANA positive patients in their 12 lymphocytic colitis patients[24]. We found only one case of ANA positivity in our patients, but none of them were associated with any of the disorders or conditions mentioned above.

Patients with lymphocytic colitis were reported to be effectively treated with medications used in inflammatory bowel disease such as sulfasalazine and 5-ASA. If this regiment fails, bismuth subsalicylate, corticosteroids, azathioprine and cyclosporine may be given[12,15-17,25,26]. In the present study sulfasalazine or 5-ASA was used as first line treatment agents. Preliminary results show positive response in terms of symptom relief. Evaluation of long term outcome should wait completion of the study.

In conclusion, considering 11.5% of the patients with chronic diarrhea of unknown etiology and normal colonoscopy would have microscopic colitis, biopsy should be taken during colonoscopy in this subset of patients. Although the number of our cases was not enough to answer the question of how many biopsies should be taken and from which part of the colon, the fact that histopathological criteria were determined on all colonic regions in patients with lymphocytic colitis on whom biopsy was performed is promising in terms of diagnostic convenience. Lymphocytic colitis in Turkish patients was found to be 4 times more frequent than collagenous colitis.

COMMENTS

Background

Microscopic colitis is a chronic diarrheal disease with normal colonoscopic, but with abnormal histopathologic features. It is a disease with two subtypes of similar clinical but different histological features; lymphocytic colitis, which is characterized by pronounced colonic mucosal lymphocyte infiltration and collagenous colitis, which is characterized by increased subepithelial collagenous band thickness. In limited number of studies from various countries the rates of microscopic colitis in patients with chronic diarrhea have been reported between 4%-13%.

Research frontiers

Although the number of the cases was not enough to answer the question of how many biopsies should be taken and from which part of the colon, the fact that histopathological criteria were determined on all colonic regions in patients with lymphocytic colitis on whom biopsy was performed is promising in terms of diagnostic convenience.

Applications

Considering 11.5% of the patients with chronic diarrhea of unknown etiology and normal colonoscopy would have microscopic colitis, biopsy should be taken during colonoscopy in this subset of patients.

Terminology

Microscopic colitis is characterized by chronic watery diarrhea with normal radiological and endoscopic appearances. Lymphocytic colitis has similar characteristics with over 20 intraepithelial lymphocytes (IELs) per 100 intercryptal epithelial cells. Collagenous colitis has same characteristics with additional histological finding of increased subepithelial collagen band thickness.

Peer review

This is an epidemiologic study confirming findings reported from other countries about the frequency of lymphocytic and collagenous colitis and the importance of biopsies for the diagnosis. It's a nice paper, well written and well designed.

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BASIC RESEARCH

Effects of ethanol on insulin-like growth factor-Ⅰ system in primary cultured rat hepatocytes: Implications of JNK1/2 and alcoholdehydrogenase

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Abstract

AIM: To evaluate the effects of ethanol on the insulin-like growth factor-Ⅰ (IGF-Ⅰ) system involved in c-Jun N-terminal kinase (JNK1/2) and alcoholdehydrogenase (ADH) activity in primary cultured rat hepatocytes.

METHODS: Hepatocytes isolated from male Sprague-Dawley rats were incubated with various concentrations of ethanol for different durations of time. The cells were pretreated with SP600125 (10 μmol/L) and 4-MP (200 μmol/L), and then treated with ethanol (200 mmol/L). We then measured IGF-Ⅰ secretion, IGF-Ⅰ mRNA expression, cell viability and JNK1/2 activity by radioimmunoassay, RT-PCR, MTT assay and Western blot, respectively (n = 6).

RESULTS: Ethanol induced the activity of phospho (p)-JNK1/2, reaching a maximum at 60 min and then decreasing at 180 min. The effects of ethanol on the IGF-Ⅰ system were increased at 60 min (secretion: 7.11 ± 0.59 ng/mg protein *vs* 4.91 ± 0.51 ng/mg, mRNA expression: 150.2% ± 10.2% *vs* 101.5% ± 11.3%, P = 0.045) and then decreased at 180 min (secretion: 3.89 ± 0.25 ng/mg *vs* 5.4 ± 0.54 ng/mg protein; mRNA expression: 41.5% ± 10.4% *vs* 84.7% ± 12.1%, P = 0.04), however cell viability was decreased in a dose- and time-dependent manner. SP600125 blocked the ethanol-induced changes (at 60 min). Additionally, 4-methylpyrazole prevented the ethanol-induced decreases in the IGF-Ⅰ system, cell viability and p-JNK1/2 activity (at 180 min).

CONCLUSION: This study suggests that ethanol-induced p-JNK1/2 activation is associated with the IGF-Ⅰ system and cell viability in hepatocytes. Furthermore, alcohol dehydrogenase is involved in the relationship between ethanol-induced inactivation of p-JNK1/2 and the changes of the IGF-Ⅰ system and cell viability.

Key words: Insulin-like growth factor-Ⅰ; Insulin-like growth factor-Ⅰ receptor; C-Jun N-terminal kinase; Hepatocyte; Ethanol

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INTRODUCTION

Ethanol abuse exerts deleterious effects on the internal organs of the body, particularly the liver and brain, and alcohol-induced liver damage is one of the major causes of morbidity and mortality in alcoholics[1]. Ethanol alters hepatic carbohydrate and lipid metabolism as well as the synthesis of protein and DNA, which leads to hepatic dysfunction and cirrhosis[2]. Although the spectrum of ethanol toxicity is well known, the underlying pathophysiology of the signal transduction pathways has not been elucidated.

Ethanol alters cell functions *via* multiple signaling pathways, particularly those involving mitogen-activated protein kinases (MAPKs), which are involved in a variety of cellular responses including proliferation, differentiation, and apoptosis[3]. Several MAPK cascades have been identified, including those involving p42/44

and p38 MAPKs, and c-Jun N-terminal kinase (JNK1/2, also known as stress-activated protein kinase)[4]. JNK1/2 activity has been linked to the proliferation and apoptosis of hepatocytes[5].

Ethanol also induces prolonged activation of tumor necrosis factor (TNF)-stimulated JNK1/2 after hepatocytes are stimulated with various agonists, and prolonged activation of JNK1/2 and activator protein 1 (AP-1) is associated with the apoptosis and necrosis of hepatocytes that occurs in response to oxidative stress[4] and ischemia/reperfusion injury[6].

Insulin-like growth factor (IGF)- I is a peptide that plays an important role in regulating cell metabolism, growth, and differentiation[7]. The dose-dependent effects of ethanol on the IGFs system have been previously described in male rats[8]. The cellular action of IGF- I is mediated *via* the insulin-like growth factor- I receptor (IGF-IR), which exhibits tyrosine kinase activity[7]. IGF-IR is a key regulator of normal cellular processes, and plays a critical role in the development and progression of many types of cancer[9]. It has been reported that the renin-angiotensin system regulates the IGF- I system in hepatocytes[10], and it is known that retinoic acid inhibits growth-hormone-stimulated IGF- I production *via* protein kinase C (PKC)-δ in breast cancer cells. We recently found that the inhibitory effects of the ethanol-induced IGF- I system are related to p42/44 activity[11]. Although the relationships between ethanol-induced cellular action and apoptosis *via* MAPK including JNK1/2 activity have been reported previously, the secretion control mechanisms of the IGF- I system (IGF- I secretion, IGF- I mRNA expression, and IGF-IR activity) remain to be elucidated in primary cultured hepatocytes.

In the present study, we investigated the effects of ethanol on the IGF- I system, with particular attention to the JNK1/2 activity and alcoholdehydrogenase (ADH) in primary cultured rat hepatocytes.

MATERIALS AND METHODS

Materials

IGF- I antigen and IGF- I antibodies were purchased form GroPep (Adelaide, Australia), and the JNK1/2 inhibitor SP600125 was purchased from New England Biolabs (Beverly, MA, USA). An enhanced chemiluminescence (ECL) kit was purchased from Cell Signaling (Beverly, MA, USA). All routine culture media were obtained from Gibco-BRL (Grand Island, NY, USA). Aquasol, reflection X-ray film, and ^{125}I isotope were purchased from Dupont-NEN (Boston, MA, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from BioRad (Hercules, CA, USA). BSA (fraction V), glycine, SDS, acrylamide, glycerol, and Tween-20 were obtained from Sigma (St. Louis, MO, USA).

IGF- I radioimmunoassay

Recombinant human IGF- I was iodinated to a specific radioactivity of 150-300 Ci/g using the ^{125}I isotope following a modified version of the chloramine-T (Kodak, Grand Island, NY, USA) method. The specific activity of the iodinated IGF- I was typically 60-110 Ci/g protein. The iodination mixture was purified on a Sephadex G-50 column (150 cm) and pre-equilibrated with phosphate-buffered saline (0.1 mol/L, pH 7.4). The samples was then separated, after which the immunoreactive IGF- I was determined as previously described[11] with some modifications. All IGF- I data were expressed as nanograms of pure human IGF- I per milliliter, while assuming that equal cross-reactivity occurred between the rat and human IGF- I in the radioimmunoassay. Fifty microliters of rat polyclonal IGF- I antibody diluted to 1:1500 was added to 100 μL of each sample/standard and then incubated for 1 h at room temperature. Next, [^{125}I]-IGF- I was added at 20 000 cpm, and the samples and standards were then incubated for an additional 18 h at 4℃. Fifty microliters of horse serum (Sigma, St. Louis, MO, USA) was then added to the sample, which was then centrifuged at 3000 × *g* for 30 min. After discarding this supernatants, the radioactivities of the precipitates containing the bound [^{125}I]-IGF- I were counted with a gamma scintillation counter (Wallac, Finland). The intra- and interassay coefficients of variation for IGFs were 8% and 10%, respectively.

Isolation and culture of rat hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats weighing 200-300 g by a two-step perfusion procedure using 0.05% collagenase as described previously[12,13]. Cell viability, which was assessed by the exclusion of trypan blue, was 90% ± 5% (mean ± SD). Isolated hepatocytes were then plated onto collagen-coated plastic culture dishes (60 mm in diameter) at a density of 5 × 10^4 cells/cm^2 in Williams' medium E containing 10% FBS. The plates were then placed in a 5% CO_2 incubator for 3 h at 37℃, after which the medium was changed to FBS-free Williams' medium E. After an additional 30 min, ethanol, SP600125 and 4-methylpyrazole (4-MP) were added at various concentrations to the dishes, which were then immediately sealed with Parafilm. The cells were then incubated for 0-180 min at 37℃.

Cell lysis and quantification

After incubation, cells were rinsed twice with ice-cold phosphate-buffered saline, followed by the addition of lysis buffer comprising 20 mmol/L HEPES (pH 8.8), 136 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 10 mmol/L KCl, 2 mmol/L MgCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L dithiothreitol, 1 mmol/L benzamidine, 10 mmol/L β-glycerophosphate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 μg/mL pepstatin A. The cell lysates were then sonicated for 5 min using a Vibra Cell ultrasonic processor (Sonics and Materials, Danbury, USA). After centrifugation of the sonicated samples at 12 000 × *g* for 10 min at 4℃, the supernatants were

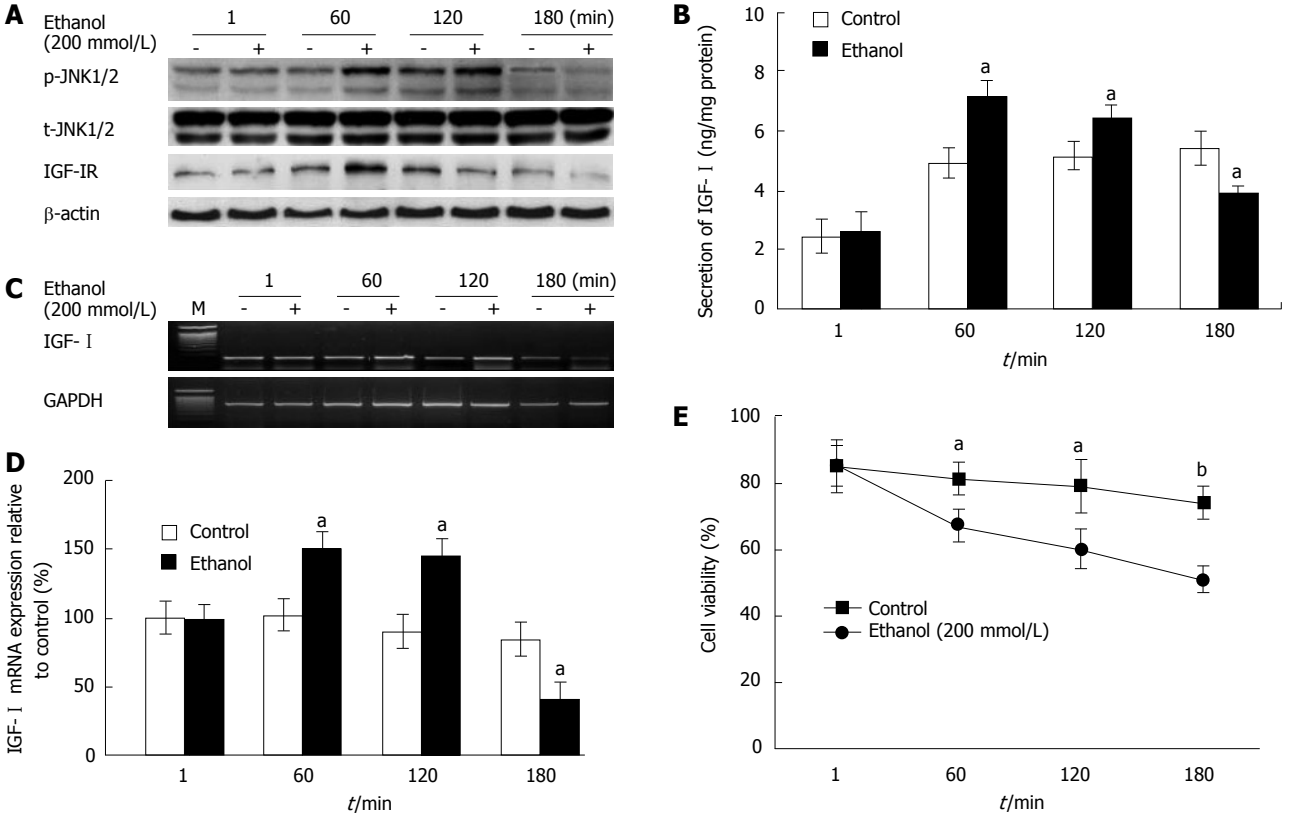

Figure 1 Time course of effects on the IGF-Ⅰ system, JNK1/2 activity, and cell viability induced by ethanol in primary cultured rat hepatocytes (mean ± SD). The cells were exposed to 200 mmol/L ethanol for 0, 60, 120 and 180 min. **A**: P-JNK1/2, t-JNK1/2, and IGF-IR activities; **B**: IGF-Ⅰ concentration; **C** and **D**: IGF-Ⅰ mRNA expression; **E**: Cell viability. β-actin (**A**) and GAPDH (**C**) were used as loading controls. The mRNA expression (as indicated by a band at 180 bp, C) was determined by densitometric analysis (**D**) of the amplification products. Data represent percentages relative to control. The cell viability (**D**) was determined by the MTT assay. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control ($n = 6$).

collected, and the protein concentrations were estimated using a bicinchoninic acid (BCA) protein assay kit (Pierce, Bonn, Germany).

Western blot

Cell lysates containing equal amounts of protein (20-30 μg) were fractionated by 10% SDS-polyacrylamide gel electrophoresis, after which the proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA) and then washed with 25 mmol/L Tris (pH 7.4) containing 137 mmol/L NaCl and 0.1% Tween-20. The membrane was then blocked with 25 mmol/L Tris (pH 7.4) containing 137 mmol/L NaCl and 0.1% Tween-20 containing 5% nonfat dry milk for 2 h at room temperature. The blots were then incubated with antibodies against p54/46 JNK1/2 and IGF-IR overnight at 4℃, after which they were incubated with antirabbit and antimouse horseradish peroxidase. After being washed, the blots were developed using an ECL kit and exposed to X-ray film to allow detection of the protein bands.

Cell viability

Standard MTT assay as described in literature was used with slight modification (2). MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma Co., MO. USA) was dissolved in isotonic phosphate buffer (IPB, pH 7.4) solution at 5 mg/mL and filtered to sterilize and remove insoluble residues. Hepatocytes were cultured in containing Williams' medium E containing 10% FBS and incubated for 4 h at 37℃ in serum free Williams' medium E. Cell survival was assayed by measuring the conversion of yellow, water-soluble tetrazolium MTT to blue, water-insoluble formazan. The absorbance was measured at 570 nm.

Statistical analysis

The statistical significance of differences between groups was determined using a Student's *t* test, with a probability value of $P < 0.05$ being considered to be indicative of statistical significance. All experiments were performed at least six times.

RESULTS

Time course of the effects of ethanol on the IGF-Ⅰ system, cell viability, and JNK1/2 activity

To evaluate the time course of the effects of exposure to ethanol on the IGF-Ⅰ system, cell viability, and JNK1/2 activity, primary cultured rat hepatocytes were exposed to 200 mmol/L ethanol for different times (1, 60, 120 and 180 min). The activity of p-JNK1/2 was observed at 60 and 120 min, and was decreased relative to control at 180 min by ethanol exposure (Figure 1A). However, the total (t)-JNK1/2 activity

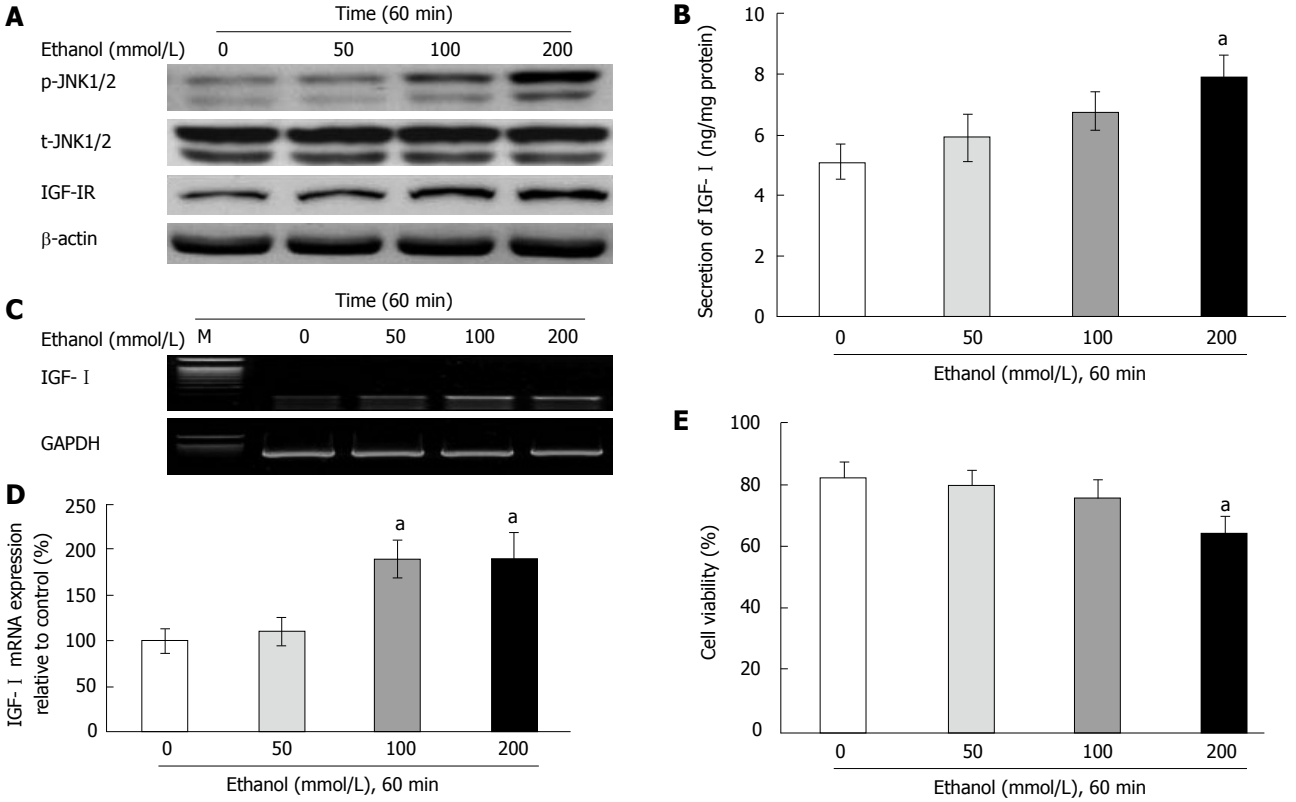

Figure 2 Effects of ethanol on the IGF- I system, JNK1/2 activity, and cell viability at different concentrations (0, 50, 100 and 200 mmol/L) in primary cultured hepatocytes (mean ± SD). The cells were exposed to ethanol at different concentrations for 60 min. **A**: P-JNK1/2, t-JNK1/2, and IGF-IR activities; **B**: IGF- I concentration; **C** and **D**: IGF- I mRNA expression; **E**: Cell viability. β-actin (**A**) and GAPDH (**C**) were used as loading controls. The mRNA expression (as indicated by a band at 180 bp, C) was determined by densitometric analysis (**D**) of the amplification products. Data represent percentages relative to control. The cell viability (**D**) was determined by the MTT assay. $^{a}P < 0.05$, *vs* control ($n = 6$).

was not affected. In addition, IGF-IR activity was also observed at 60 min, and it was decreased at 180 min (Figure 1A). The effects of ethanol on the secretion and mRNA expression of IGF- I were similar to changes in p-JNK1/2 activity, which increased at 60 (IGF- I secretion: 7.11 ± 0.59 ng/mg *vs* 4.91 ± 0.51 ng/mg protein; mRNA expression: 150.2% ± 10.2% *vs* 101.5% ± 11.3%, $P = 0.045$) and 120 min and then decreased at 180 min (IGF- I secretion: 3.89 ± 0.25 ng/mg *vs* 5.4 ± 0.54 ng/mg protein; mRNA expression: 41.5% ± 10.4% *vs* 84.7% ± 12.1%, $P = 0.04$; Figure 1B-D). The effects of ethanol on cell viability significantly decreased over time from 60 min onwards (at 60 min: 66.7% ± 5.12% *vs* 80.45% ± 5.21%, $P = 0.035$; Figure 1E).

Dose-dependent effects of ethanol on the IGF- I system, cell viability, and JNK1/2 activity

To investigate the dose-response effects of ethanol on the IGF- I system and JNK1/2 activity, the cells were exposed to ethanol at different concentrations (0, 50, 100 and 200 mmol/L) for 60 min. The effects of ethanol on the p-JNK1/2 activity increased in a dose-dependent manner (Figure 2A). In addition, the activity of p-JNK1/2 relative to control was maximal in response to exposure to 200 mmol/L ethanol, whereas the t-JNK1/2 activity was not affected (Figure 2A). Furthermore, the changes in IGF- I secretion, mRNA expression, and IGF-IR activity were increased by ethanol in a dose-dependent manner (Figure 2A-C). The treatment with 200 mmol/L ethanol showed significantly decrease of IGF- I secretion, mRNA expression and IGF-IR activity when compared with the control (IGF- I secretion: 7.89 ± 0.71 ng/mg *vs* 5.09 ± 0.56 ng/mg protein, mRNA expression: 191.5% ± 27.4% *vs* 100% ± 13.1%, $P = 0.032$; Figure 2A-D). These results were similar to those of the ethanol-induced p-JNK1/2 activity. However, cell viability was significantly decreased by exposure to 200 mmol/L ethanol (65.2% ± 4.9% *vs* 83.2% ± 4.2%, $P = 0.024$; Figure 2E).

Relationships between ethanol-induced activation of JNK1/2, the IGF- I system, and cell viability

The JNK1/2 inhibitor SP600125 was used to determine if ethanol-induced activation of p-JNK1/2 (at 60 min) was related to the IGF- I system and cell viability. The ethanol-induced activations of p-JNK1/2 and IGF-IR were blocked by treatment with 10^{-5} mol/L SP600125 (Figure 3A). In addition, the temporary increases in secretion (8.02 ± 0.67 ng/mg protein) and mRNA expression (208.8% ± 23.4%) of IGF- I induced by ethanol were also blocked by SP600125 (IGF- I secretion: 3.78 ± 0.42 ng/mg protein, mRNA expression: 113.87% ± 27.5%, $P = 0.024$; Figure 3B-D), whereas t-JNK1/2 activity was not affected (Figure 3A). The ethanol-induced decrease in cell viability (64.2% ± 5.5%) was recovered by SP600125 (77.6% ± 4.1%, $P =$

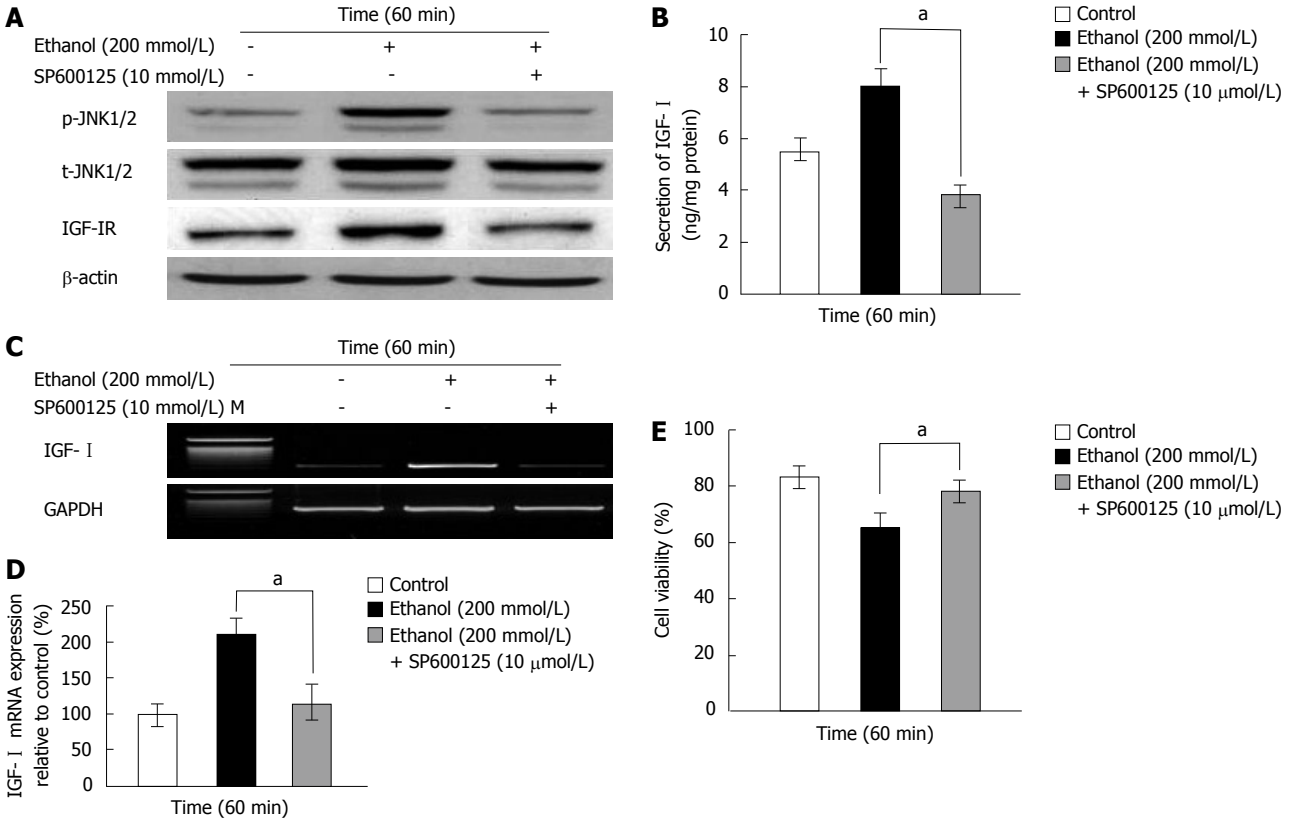

Figure 3 Effects of JNK1/2 inhibitor on the IGF- I system, cell viability, and activity of JNK1/2 induced by ethanol at 60 min in primary cultured rat hepatocytes (mean ± SD). The cells were pretreated with 10 μmol/L SP600125 30 min before being exposed to 200 mmol/L ethanol for 60 min. **A**: p-JNK1/2, t-JNK1/2, and IGF-IR activities; **B**: IGF- I concentration; **C** and **D**: IGF- I mRNA expression; **E**: Cell viability. β-actin (**A**) and GAPDH (**C**) were used as loading controls. The mRNA expression (as indicated by a band at 180 bp, C) was determined by densitometric analysis (**D**) of the amplification products. Data represent percentages relative to control. The cell viability (**D**) was determined by the MTT assay. $^{a}P < 0.05$ *vs* control ($n = 6$).

0.045; Figure 3E). These results together demonstrate that the transient changes in the ethanol-induced IGF- I system and the decreased cell viability were related to p-JNK1/2 activity.

Relationships between ADH and decreased changes in the IGF- I system by ethanol-induced JNK1/2 activity

To determine the effects of ADH (alcohol dehydrogenase) on the ethanol-induced inactivation of p-JNK1/2 in the IGF- I system and decreased cell viability at 180 min, cells were exposed to 200 mmol/L ethanol after being pretreated with the ADH inhibitor 4-MP (200 μmol/L). The ethanol-induced inactivation of p-JNK1/2 and IGF-IR was recovered by 10^{-5} mol/L 4-MP, whereas the t-JNK1/2 activity was not affected (Figure 4A). The ethanol-induced decreases in the secretion (4.54 ± 0.52 ng/mg protein) and mRNA expression (24.5% ± 17.1%) of IGF- I and cell viability (50.3% ± 5.5%) were also recovered by pretreatment with 4-MP (IGF- I secretion: 10.3 ± 0.79, mRNA expression: 109.4% ± 21.8%, cell viability: 77.2 ± 7.2, $P = 0.035$; Figure 4B-E). These results together indicate that the decreases in ethanol-induced p-JNK1/2 activity, IGF- I system and cell viability were related to the ADH.

DISCUSSION

Ethanol exerts toxic effects on almost all organs, particularly liver and brain. The liver is a major metabolic organ in which most IGF- I is produced and secreted, and its mediators are responsible for alcohol-induced liver injury[14].

In the present study, we showed that ethanol transiently increased p-JNK1/2 activity at 60 min and then decreased it at 180 min. It has been reported that ethanol activates MAPKs[1,2], and acute exposure of primary cultured rat hepatocytes to ethanol for 60 min increases the activities of p42/44 MAPK and p-JNK1/2, with both activities gradually decreasing thereafter[15]. Exposure to ethanol has been shown to cause prolonged activation of p-JNK1/2; however, this response was attenuated in hepatocytes obtained from rats chronically exposed to ethanol for 6 wk[16]. Our result is similar with those of previous studies mentioned above, in which exposure time to ethanol causes activation and inactivation of p-JNK1/2 activation in rat hepatocytes.

The activation of p-JNK1/2 normally occurs in response to growth stimuli and is involved in cell proliferation[17]. This is also related to apoptosis and antiapoptosis, and is activated by cytokines or stress stimuli such as osmotic shock, UV light, and heat[9,18]. IGF- I system has been reported to protect against a variety of chemical cellular injuries that induce apoptosis[19]. We previously showed that ethanol decreased the synthesis and secretion of IGF- I and the activity of IGF-IR, an effect that is related to cell

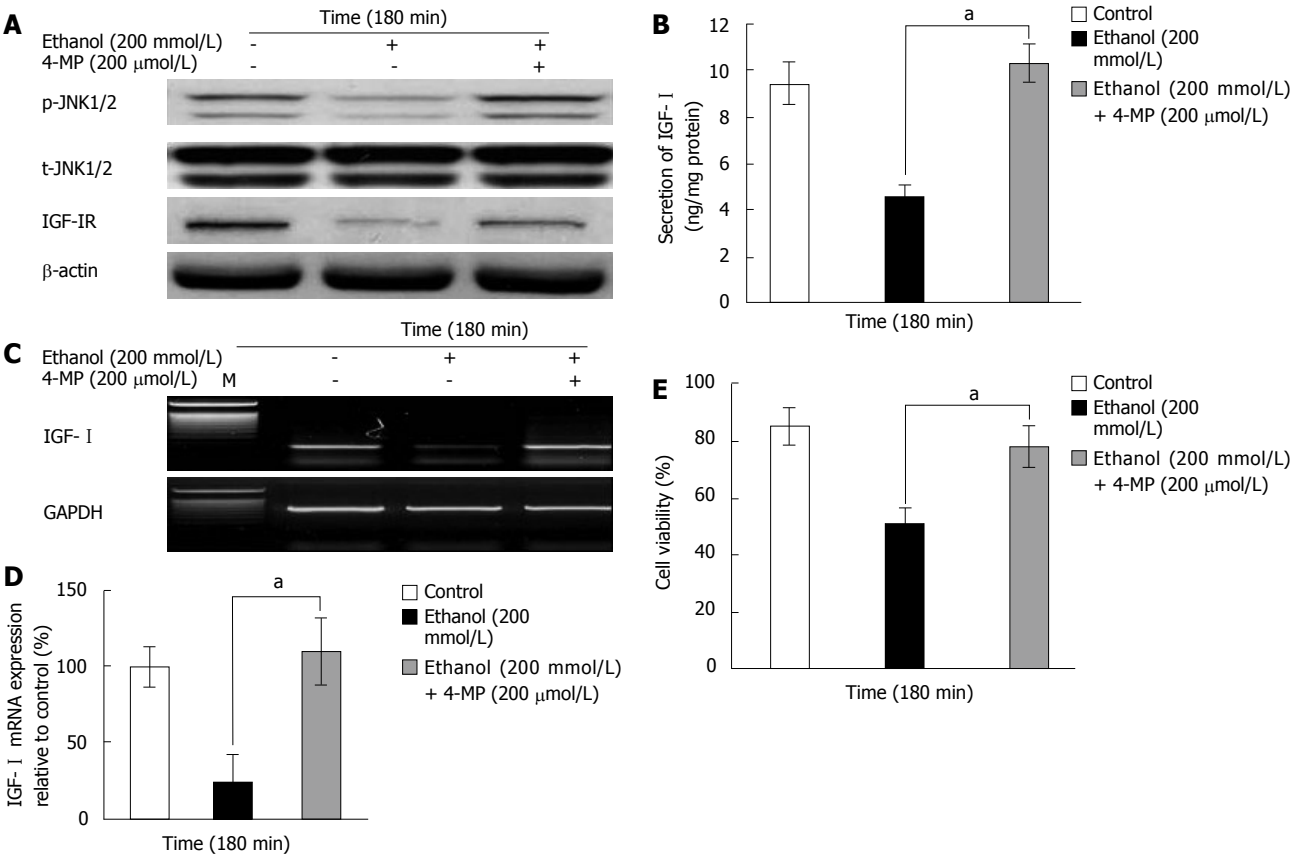

Figure 4 Effects of ADH inhibitor on the IGF- I system, cell viability, and JNK1/2 activity induced by ethanol at 180 min in primary cultured rat hepatocytes (mean ± SD). The cells were pretreated with 200 μmol/L 4-MP 30 min before being exposed to 200 mmol/L ethanol for 180 min. **A**: p-JNK1/2, t-JNK1/2, and IGF-IR activities; **B**: IGF- I concentration; **C** and **D**: IGF- I mRNA expression; **E**: Cell viability. β-actin (**A**) and GAPDH (**C**) were used as loading controls. The mRNA expression (as indicated by a band at 180 bp, C) was determined by densitometric analysis (**D**) of the amplification products. Data represent percentages relative to control. The cell viability (**D**) was determined by the MTT assay. $^{a}P < 0.05$ *vs* control ($n = 6$).

proliferation and differentiation[11]. In the present study, ethanol-induced transient activation of p-JNK1/2 increased in the IGF- I system, but this decreased when p-JNK1/2 was inactivated. Furthermore, IGF-IR activity also regulates ethanol-induced secretion and synthesis of IGF- I . These results are consistent with our previous study that ethanol-induced p42/44 activity was related to the secretion and synthesis of IGF- I in primary cultured rat hepatocytes[11]. In our previous study, although there were different doses (5%-20%), chronic ethanol treatment caused dose-dependent decreases in the secretion and synthesis of IGF- I in liver and blood *in vivo*[8].

We also used a JNK1/2 inhibitor to verify whether the ethanol-induced transient activation of p-JNK1/2 may alter the IGF-I system. The results reported here confirmed that the effects of ethanol on p-JNK1/2 activation, the IGF- I system, and cell viability were recovered by an inhibitor of the JNK1/2 activity. We suggest that acute exposure to ethanol affects not only p42/44 MAPK but also p-JNK1/2 activities, which in turn alter the IGF- I system. It has been reported that ethanol-induced transient activation of p-JNK1/2 indicates pro-apoptosis, while a prolonged activation induces anti-apoptosis in hepatocytes[20]. Hepatocytes express two JNK genes (JNK1 and JNK2) and bile acids cause activation of both JNK1 and JNK2, but JNK1 activation causes apoptosis whereas JNK2 activation protects against apoptosis[21]. It has been reported that ethanol causes more pronounced activation of JNK 1 compared to JNK 2, suggesting a role for this preferential activation of JNK 1 in ethanol-induced apoptosis of hepatocytes[15].

Interestingly, we found that cell viability is always decreased by ethanol. However, there was a transient activation of p-JNK1/2 and the subsequent inactivation of p-JNK1/2 in parallel with changes of the IGF- I system. These results suggest that transient activation of p-JNK1/2 with increment of the IGF- I system lead to pro-apoptotic events and transient resistance of hepatocytes. Also, the ethanol-induced inactivation of p-JNK upon decrease of the IGF- I system indicate that the cells have already passed the threshold for proliferation or survival against the ethanol-induced toxicity.

The ADH is an enzyme involved in ethanol metabolism that appears to provide the link between the effects of ethanol-induced p-JNK1/2 activity on the IGF- I system and cell viability at 180 min but not at 60 min (data not shown). It has been reported that the level of 4-MP decreased by approximately 90% in rat hepatocytes following exposure to ADH and ethanol[22].

Ethanol rapidly activates p-JNK1/2, which is associated with the response of the endoplasmic reticulum to stress, which in turn causes inhibition of ADH[23]. Acute exposure to 200 mmol/L ethanol may also activate p-JNK1/2 *via* acetaldehyde-dependent[15] and acetaldehyde-independent[24] pathways in rat hepatocytes. It was reported that acetaldehyde produced by ethanol oxidation activates p42/44 MAPK and p-JNK1/2 in rat hepatocytes[1,2,15]. We previously reported that ethanol-induced changes of the IGF- I system are related to ADH activity[11]. These results suggest that the decrease in p-JNK1/2 activity induced by exposure to ethanol for 180 min regulates the decrease of IGF- I system and cell viability, with these effects being related to ADH. However, the effects of the ethanol-induced transient activation of p-JNK1/2 on the increment of IGF- I system were not due to ADH.

In conclusion, this study suggest that ethanol-induced p-JNK1/2 activation is related to changes in the IGF- I system and cell viability in hepatocytes. Furthermore, ethanol-induced inactivation of p-JNK1/2 is involved in the IGF- I system and cell viability *via* ADH. These findings might be helpful to understand the pathogenesis of liver damage induced by ethanol, and may lead to a rational therapeutic intervention against ethanol toxicity.

COMMENTS

Background

Ethanol-induced liver damage is unavoidable upon exposure to alcohol. Moreover, enhanced c-Jun N-terminal kinase (JNK1/2) and alcohol dehydrogenase (ADH) activity have been linked to the ethanol induced hepatotoxicity. Insulin-like growth factor- I (IGF- I) system has been reported to protect against a variety of chemical cellular injuries that induce apoptosis; however it has not been well defined whether the IGF- I system is associated with the ethanol-induced JNK and ADH activity.

Research frontiers

It was reported that acetaldehyde produced by ethanol oxidation activates p42/44 MAPK and p-JNK1/2 in rat hepatocytes. We previously reported that ethanol-induced changes of the IGF- I system are related to p42/44 activity. To investigate the importance of IGF- I system *via* JNK and ADH, we performed this study using specific inhibitors.

Innovations and breakthroughs

We found that there was increase and then decrease in the IGF- I secretion and mRNA expression during ethanol treatment. The activity of JNK was also temporary increased and then decreased by ethanol. However, cell viability was monotonically decreased. Both JNK and ADH inhibitors blocked ethanol-induced changes of IGF- I system and cell viability.

Applications

The present study evaluated the changes of the IGF- I system indicating that the potential value of IGF- I system for patients with ethanol-induced liver damage. Moreover, this study demonstrates that ethanol-induced IGF- I system is involved in the activities of JNK1/2 and ADH.

Terminology

IGF- I system has been reported to protect against a variety of chemical cellular injuries and promote proliferation of hepatocytes. The liver is a major metabolic organ in which most IGF- I is produced and secreted, and its mediators are responsible for alcohol-induced liver injury.

Peer review

This manuscript describes the activation of JNK1/2 activity by ethanol treatment of rat hepatocytes and subsequent changes in IGF expression and secretion as well as changes in proliferation. These effects could be inhibited by the specific inhibitors of JNK1/2 as well as an inhibitor of ADH. This manuscript is well-written, clear and concise with a thorough results section.

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BASIC RESEARCH

Protective effect of prednisolone on ischemia-induced liver injury in rats

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Abstract

AIM: To investigate the effects of prednisolone on cell membrane bleb formation, calpain μ activation and talin degradation during hepatic ischemia-reperfusion injury in rats.

METHODS: The hilar area of the left lateral and median lobes of rat liver (68%) was clamped for 60 min and followed by 120 min reperfusion. Prednisolone was administered at 1.0, 3.0, or 10 mg/kg at 30 min before ischemia. In addition to biochemical and microscopic analyses, activation of calpain μ was determined using specific antibodies against the intermediate (activated) form of calpain μ. Degradation of talin was also studied by Western blotting.

RESULTS: In the control and prednisolone (1.0 mg/kg) groups, serum aspartate transaminase (AST) and alanine transaminase (ALT) level were elevated, and cell membrane bleb formation was observed after 120 min of reperfusion. Moreover, calpain μ activation and talin degradation were detected. Infusion of prednisolone at 3.0 or 10 mg/kg significantly suppressed serum AST and ALT, and prevented cell membrane bleb formation. At 10 mg/kg, prednisolone markedly suppressed calpain μ activation and talin degradation.

CONCLUSION: Prednisolone can suppress ischemia-reperfusion injury of the rat liver. Its cytoprotective effect is closely associated with the suppression of calpain μ activation and talin degradation.

INTRODUCTION

Hepatic ischemia-reperfusion injury is a serious complication but unavoidable problem in liver surgery including liver transplantation and hepatic resection[1]. The most important consequence of this pathological process is multiple organ failure with a high mortality rate. Therefore, there is a considerable interest in the prevention of hepatic ischemia-reperfusion injury. Steroid therapy suppresses liver injury by a variety of mechanisms, including increased tissue blood flow and suppression of oxygen free radicals, arachidonic acid derivatives, lysosomal proteases (cathepsins) and cytokine production[2-5]. However, the exact intracellular mechanisms of steroid action on hepatic ischemia-reperfusion injury remains unknown.

Exposure of hepatocytes to hypoxia or oxidative stress is thought to result in a rise in intracellular $Ca2^{+}$ concentrations ($[Ca^{2+}]_i$), initiating cell membrane bleb formation, an early event leading to cell death[6,7]. Although the molecular mechanisms of bleb formation are unknown at present, Ca^{2+}-dependent disruption of the cytoskeleton is considered to play an important role in the blebbing of plasma membrane[8,9]. Calpain μ, a Ca^{2+}-sensitive form of Ca^{2+}-activated neutral protease (EC 3, 4, 22, 17), has been shown to degrade various cytoskeletal proteins such as talin, α-actinin and

filamin[10-14].

Steroids are the most potent anti-inflammatory and immunosuppressive agents[15]. They inhibit the synthesis of almost all known cytokines and cell surface molecules required for immune function, and suppress the activity of nuclear factor kappa B (NF-κB)[16]. This inhibition is mediated by the induction of IκBα inhibitory protein, which traps activated NF-κB in inactive cytoplasmic complexes. On the other hand, inflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α, are involved in the pathophysiology of hepatic ischemia-reperfusion injury[17]. The liver also plays a central role in the metabolism of these acute reactant cytokines. In the liver, these mediators are produced in large amounts by Kupffer cells or endothelial cells[18], and are released rapidly under various insults like hypoxia[17].

In the present study, we investigated the cytoprotective mechanisms of prednisolone using an experimental model of ischemia-reperfusion injury of rat liver, with special emphasis on the activation of calpain μ and degradation of talin.

MATERIALS AND METHODS

Animals

Thirty-seven adult male Wistar (Aburabi Laboratory, Shiga, Japan) rats weighing 220-260 g were used in the study. All procedures were carried out in accordance with the guidelines set forth in the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. Rats were anesthetized by an interperitoneal injection of pentobarbital sodium (50 mg/kg body weight), and underwent laparotomy. Before vascular clamping, heparin sodium (50 units) was intravenously injected to prevent blood coagulation.

Prednisolone

A highly potent antagonist of prednisolone was obtained from Shionogi Pharmaceutical Co., Japan. Its molecular formula is $C_{25}H_{31}NaO_8$ and molecular weight is 482.51 (Figure 1). Animals were divided into two groups: rats treated with 0.9% normal saline solution were assigned as the control group (n = 10), and those treated with intravenous injection of prednisolone (1.0, 3.0, or 10 mg/kg) as the prednisolone group (n = 27) (1.0, 3.0 and 10 mg/kg) (Figure 2).

Partial liver ischemia

An intravenous catheter was placed in the tail vein, through which prednisolonewas infused at 30 min before vascular clamping. Partial (68%) hepatic ischemia was induced by clamping the branches of the portal vein, and hepatic artery feeding the left lateral and median lobes, the branches of the right lateral (24%) and caudate (8%) lobes were not clamped[13,14]. In this condition, intestinal congestion or other complications did not occur throughout the experiment (Figure 3).

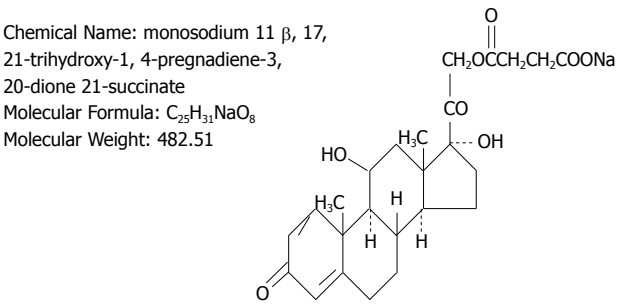

Figure 1 Structure of prednisolone.

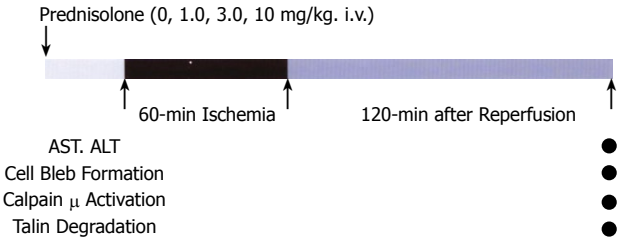

Figure 2 Experimental model.

Measurement of serum aspartate transaminase (AST) and alanine transaminase (ALT)

Blood samples were collected at 120 min after declamping, and serum samples were stored at -80℃ until biochemical analysis. Serum AST and ALT concentrations were determined by a Spot Chem kit (Spotchem Co., Kyoto, Japan).

Cell membrane bleb formation

In a separate set of experiments similar to those described above, tissue samples were obtained for histological examination after *in situ* perfusion and fixation with 0.1% glutaraldehyde and 4% paraformaldehyde, which were infused via the portal vein. Biopsy specimens were also processed for routine histopathologic examination[13,14].

Antibodies against intermediate (activated) form of calpain μ

The preparation and characteristics of an antibody that specifically recognizes the N-terminal peptide of intermediate (activated) (NH_2-AQVQKQC-COOH) form of calpain μ (78 kDa) have been described previously[13,14].

Western blot analysis

Liver tissues were immediately frozen by liquid nitrogen and stored at -80℃ for 3 d. For Western blot analysis, samples were homogenized in an ice-water bath using a radioimmune protein assay buffer [1.0% Nonidet P-40 (Iwai Kagaku Co Ltd, Tokyo, Japan); 0.1% deoxycholic acid; 150 mmol/L sodium chloride; 50 mmol/L Tris hydrochloride; 1.0 mmol/L phenylmethylsul-fonyl fluoride, pH 7.5] containing 5 mmol/L ethylene glycol-bis (b-aminoethyl ether)-N, N, N' N'-tetra acetic acid (EGTA) and 5 μmol/L leupeptin. After centrifugation

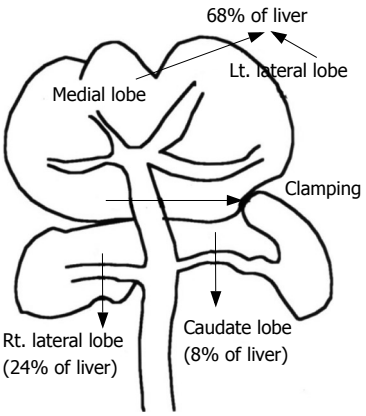

Figure 3 Partial liver ischemia by clamping hilar area of left lateral and medial lobes without causing intestinal congestion. Lt.: Left; Rt.: Right.

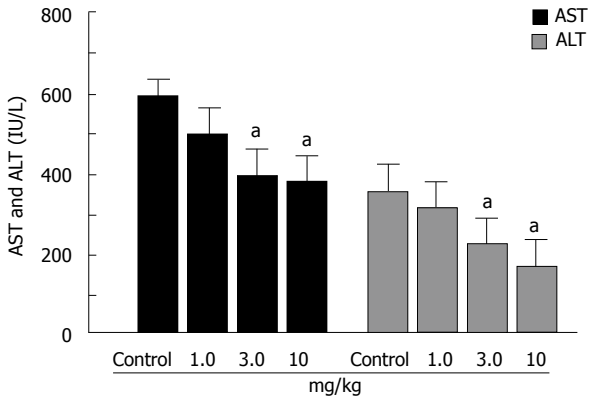

Figure 4 Effects of prednisolone on the AST and ALT levels in animals with 60 min of partial hepatic ischemia. $^{a}P < 0.05$.

at 3000 r/min for 20 min at 4℃, the supernatant (25 μg of protein) was subjected to Western blot analysis using an antibody against the intermediate form of calpain μ or an anti-talin antibody (Sigma Chemical Co., St. Louis, MO). The amount of these polypeptides was determined by a densitometric analysis.

Statistical analysis

Data were expressed as means ± SD. Differences in transaminase levels, calpain μ activation and talin degradation among various groups were tested for statistical significance using the Student's *t* test and Dunnett's multiple comparison test. A *P* value of less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

Serum AST and ALT

Serum AST and ALT concentrations were increased at 120 min after declamping in the control group. Prednisolone reduced the concentration of both transaminases in a dose-dependent manner. Differences between control and prednisolone levels were significant at prednisolone dose of 3.0 and 10 mg/kg (Figure 4).

Histological findings

To further investigate the cytoprotective effect of prednisolone, liver tissues were examined histologically (Figure 5). In the control group, at 120 min after declamping, the cell structure of hepatocytes was not clear due to membrane bleb formation. Furthermore, numerous membrane microparticles were present in the sinusoidal space. In contrast, membrane blebbing was rarely seen and the cell structure was well preserved in prednisolone-treated (3.0 and 10 mg/kg) rats.

Activation of calpain μ

Using a specific antibody to the intermediate (78 kDa) form of calpain μ, Western blotting was performed to examine the relationship between calpain μ activation and cell injury of the liver (Figure 6). The activated form of calpain μ appeared in all groups at 120 min after vascular declamping. However, prednisolone inhibited calpain μ activation in a dose-dependent manner. The difference in calpain μ activation was significant between control and high dose prednisolone (10 mg/kg) rats.

Degradation of talin

Proteolysis of talin, a favorable intracellular substrate of calpain μ was also investigated by Western blotting (Figure 7). Talin was markedly degraded in control and low-dose prednisolone (1.0 mg/kg) rats 120 min after vascular declamping. However, at 3.0 and 10 mg/kg, prednisolone significantly suppressed talin degradation, compared to the control group.

DISCUSSION

The major findings of our study were that prednisolone inhibited calpain μ activation in ischemia-reperfusion injury of the rat liver and that the degree of calpain μ activation closely correlated to the morphological changes in hepatocytes, i.e., cell membrane bleb formation. Furthermore, we also showed that prednisolone reduced the level of talin degradation in ischemic liver tissues. These changes were associated with improved overall liver function as reflected by lowering of serum AST and ALT concentrations, relative to the control.

Calpain μ is a major Ca^{2+}-dependent cytosolic protease so far described[13,14] and is activated following increased $[Ca^{2+}]_i$ in hepatocyte injury. Since cell membrane bleb formation is an irreversible phenomenon leading to cell necrosis, the role of calpain activation in cell membrane blebbing through the proteolysis of cytoskeletal proteins[15] is considered to be particularly important. Furthermore, we have previously reported that talin and α-actinin were degraded simultaneously with calpain μ activation in oxidative stress-induced hepatocyte injury, and that all these events were suppressed by a specific calpain inhibitor, calpeptin[10-14]. Thus, the beneficial effects of prednisolone on hepatic ischemia-reperfusion injury may be at least due to inhibition of calpain μ activation.

Recent studies have demonstrated that hepatocyte

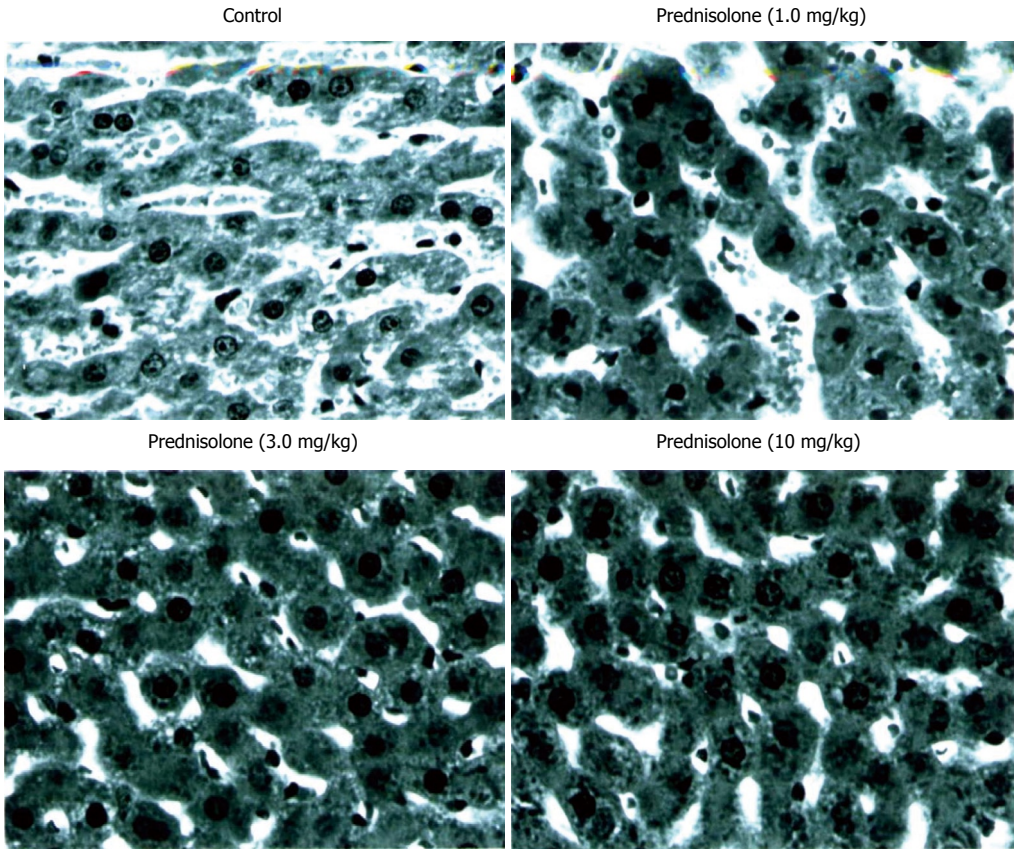

Figure 5 Effects of prednisolone on histopathological findings in animals with 60 min partial hepatic ischemia. T (HE, × 400).

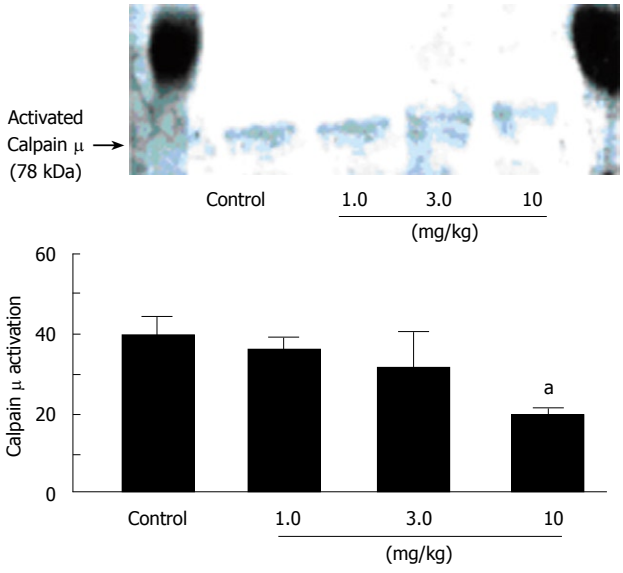

Figure 6 Effects of prednisolone on calpain μ activation in animals with 60 min of partial hepatic ischemia. $^aP < 0.05$.

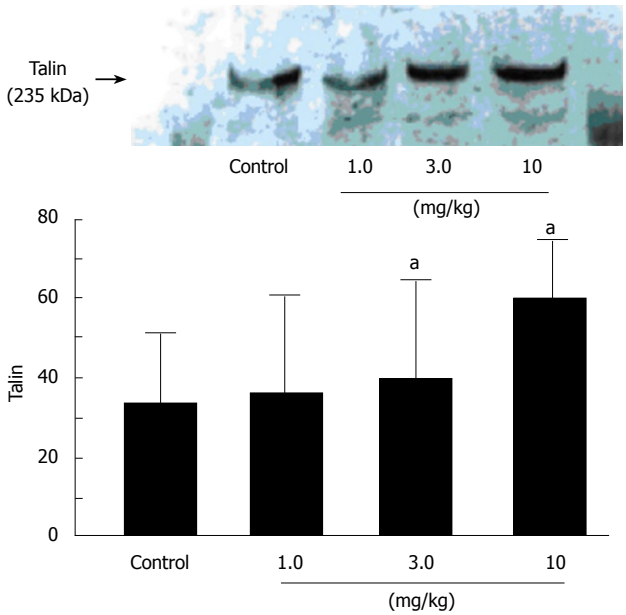

Figure 7 Effects of prednisolone on the degradation of talin in animals with 60 min of partial hepatic ischemia. $^aP < 0.05$.

injury is initiated by a rise in $[Ca^{2+}]_i$, which results from Ca^{2+} release from the internal storage sites and Ca^{2+} influx[7,11,12], and is closely correlated with the magnitude of ischemic insult. On the other hand, the pathogenesis of hepatic ischemia-reperfusion injury is complex and multifactorial. Various factors such as the reactive oxygen species[19], platelet-activating factor[20], thromboxane A_2[21], leukotriene B_4[22] and endothelin-1[23] have been identified. In addition, inflammatory cytokines like interleukin-1β and tumor necrosis factor-α also play an important role in hepatic ischemia-reperfusion injury[17]. All these substances induce a rise in $[Ca^{2+}]_i$. These findings indicate that in addition to prostaglandin E1 and prostacyclin[13] which directly suppress the rise in $[Ca^{2+}]_i$, other agents like corticosteroids may inhibit hepatic ischemia-reperfusion injury by suppressing the production of these extracellular mediators. In fact, corticosteroids have been shown to inhibit the generation of oxygen free radicals[24], and reduce

hepatic ischemia-reperfusion injury[25]. In the present study, we demonstrated that at a dose of 10 mg/kg, prednisolone inhibited degradation of talin, as well as calpain μ activation in the ischemic liver. Since the increase in $[Ca^{2+}]_i$ induces calpain μ activation, inhibition of calpain μ activation by prednisolone suggests a possible suppression of increased $[Ca^{2+}]_i$ in hepatocytes. Therefore, it is possible that prednisolone inhibits hepatic ischemia-reperfusion injury by suppressing the generation of extracellular mediators with subsequent abrogation of the rise in $[Ca^{2+}]_i$.

Consistent with the beneficial effects on extra- and intracellular mediators, these findings suggest that preoperative administration of glucocorticoids may prevent hepatic ischemia-reperfusion injury and subsequent systemic reactions. However, the inhibitory effect of prednisolone on the calpain μ activation was not completely similar to the results of the serum transaminase levels, and membrane bleb formation. From the clinical point of view, a complete inhibition of hepatic ischemia-reperfusion injury may be an ultimate goal in liver surgery. To accomplish this, treatment with a combination of several agents that inhibit various steps of hepatic ischemia-reperfusion injury is probably necessary since all agents reported so far produce only a partial inhibitory effect. The multiplicity approach for hepatic ischemia-reperfusion injury (e.g. prostaglandin E1 and prednisolone) is now under investigation.

In conclusion, we demonstrated in the present study that prednisolone suppressed ischemia-reperfusion injury of the rat liver. Its cytoprotective effect was partial, but was closely associated with inhibition of calpain μ activation and suppression of talin degradation. The effects of a combination of two or more agents of different inhibitory mechanisms should be examined in the future to reduce the complications encountered during hepatic surgery.

COMMENTS

Background

Hepatic ischemia-reperfusion injury is a serious complication but unavoidable problem in liver surgery including liver transplantation and hepatic resection. The most important consequence of this pathological process is multiple organ failure with a high mortality rate. Steroid therapy suppresses liver injury by a variety of mechanisms, including increased tissue blood flow and suppression of oxygen free radicals, arachidonic acid derivatives, lysosomal proteases (cathepsins) and cytokine production. In this study, authors investigated the cytoprotective mechanisms of prednisolone using an experimental model of ischemia-reperfusion injury of rat liver, with special emphasis on the activation of calpain μ and degradation of talin.

Research frontiers

Steroids are the most potent anti-inflammatory and immunosuppressive agents. They inhibit the synthesis of almost all known cytokines and cell surface molecules required for immune function, and suppress the activity of nuclear factor kappa B (NF-κB). This inhibition is mediated by the induction of IκBα inhibitory protein, which traps activated NF-κB in inactive cytoplasmic complexes. On the other hand, inflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α, are involved in the pathophysiology of hepatic ischemia-reperfusion injury. The liver also plays a central role in the metabolism of these acute reactant cytokines. In the liver, these mediators are produced in large amounts by Kupffer cells or endothelial cells, and are released rapidly under various insults like hypoxia.

Innovations and breakthroughs

Recent studies have demonstrated that hepatocyte injury is initiated by a rise in $[Ca^{2+}]_i$, which results from Ca^{2+} release from the internal storage sites and Ca^{2+} influx, and is closely correlated with the magnitude of ischemic insult. On the other hand, the pathogenesis of hepatic ischemia-reperfusion injury is complex and multifactorial. All these reactive oxygen species induce a rise in $[Ca^{2+}]_i$. This studyfound that at a dose of 10 mg/kg, prednisolone inhibited degradation of talin, as well as calpain μ activation in the ischemic liver.

Applications

In this research, authors found calpain μ is an important enzyme related to hepatic ischemia-reperfusion injury. The mechanism of calpain μ is first reported in living organism in this research.

Peer review

The study deals with protective effect of prednisolone antagonist on ischemia-induced liver injury. The authors have shown that the compound used offered protection for the liver. This is a well written and interesting study.

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RAPID COMMUNICATION

Univariate and multivariate analysis of risk factors for severe *clostridium difficile*-associated diarrhoea: Importance of co-morbidity and serum C-reactive protein

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Abstract

AIM: To investigate risk factors for severe *clostridium difficile* associated diarrhoea (CDAD) in hospitalized patients.

METHODS: We analysed risk factors for severe CDAD (associated with systemic signs of hypovolemia) in 124 hospitalized patients by retrospective chart review.

RESULTS: Severe CDAD was present in 27 patients (22%). Statistical analysis showed a significant association with a higher 30-d mortality (33% *vs* 4%, $P < 0.001$) and a higher proportion of longer hospital stay exceeding 14 d (74% *vs* 52%, $P = 0.048$). Charlson co-morbidity score (OR 1.29 for 1 point increment, $P < 0.05$) and serum C-reactive protein at diagnosis (OR 1.15 for 10 mg/L increment, $P < 0.001$) were independent predictors of severe CDAD.

CONCLUSION: Patients with a severe level of co-morbidity and high serum C-reactive protein levels at the time of diagnosis should receive particular attention.

Key words: *Clostridium difficile*; Nosocomial diarrhoea; Co-morbidity; C-reactive protein; 30-day mortality

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INTRODUCTION

Clostridium difficile associated diarrhoea (CDAD) is the most common cause of healthcare-associated diarrhoea and results in a wide spectrum of disease severity ranging from asymptomatic carriage to life-threatening enterocolitis and death[1-5]. Recently, a new epidemic strain producing higher levels of toxin has emerged in Canada and the US[5-8] as well as in some European countries which results in CDAD with higher morbidity and mortality[9-13]. Many studies have investigated risk factors for infection with *Clostridium difficile* (*C. difficile*) and subsequent development of CDAD. Thus, advanced age, severe comorbidity[14], hospitalisation[15], antibiotic exposure, immunosuppressive therapy[16,17] and treatment with motility influencing or acid-suppressive drugs have all been reported as risk factors for CDAD[18-21]. In contrast, less is known about risk factors associated with a severe course of CDAD in hospitalized patients.

MATERIALS AND METHODS

We conducted a retrospective analysis of CDAD in hospitalized patients to identify possible risk factors for a severe clinical course. Our institution is a community hospital treating approximately 19 000 in-patients per year. Using a computer-based search, we identified 186 positive stool tests for *C. difficile* toxin B from 142 patients who fulfilled the case definition for CDAD between October 2003 and August 2006. After chart review 18 cases were excluded: 5 patients had multiple admissions and only the first admission was included, 5 patients were younger than 18 years and in 8 patients

Table 1 Patient characteristics

Patient characteristics	Data
Age[1] (yr)	76 (18-93)
Sex	
Female	71 (57%)
Male	53 (43%)
Nursing home residency	19 (15%)
Charlson´s comorbidity score	4 (0-10)
GI procedures including PEG and surgery	13 (10%)
Previous medication:	
Antibiotic therapy within 6 wk prior to onset CDAD	101 (81%)
Acid-suppressive therapy	66 (53%)
Immunosuppressive therapy	25 (20%)
Opioid use	57 (46%)
Laxative use	30 (24%)
Clinical features of CDAD	
Hospital-acquired CDAD	101 (81%)
Interval onset of diarrhoea to CDAD therapy ⩾ 7 d	45 (37%)
Body temperature ⩾ 38℃	56 (45%)
Severe CDAD	27 (22%)
Laboratory at diagnosis:	
White blood cell count (G/L)	14.1 (4.6-81.3)
CRP (mg/L)	118 (2-413)
Creatinine (mg/L)	11.5 (3.1-110.5)
Sodium (mmol/L)	136 (114-145)
Potassium (mmol/L)	3.52 (2.43-5.07)
Continuation of initial antibiotic therapy despite CDAD	71 (57%)
Antibiotic therapy for CDAD	113 (91%)
Length of hospital stay > 14 d	70 (56%)
30-d mortality	13 (10%)

[1]Data are given as median (range) or number (percentage).

Table 2 Univariate analysis of risk factors for severe CDAD

Variable	Non-severe CDAD (n = 97)	Severe CDAD (n = 27)	P
Male sex	41/42	12/44	
Nursing home residency	12/12	7/26	
Hospital-acquired CDAD	76/78	25/93	
Immunosuppressive therapy	15/15	10/37	< 0.05
Previous antibiotic therapy	78/80	23/85	
Acid-suppressive therapy	47/48	19/70	
Therapy with opioids	40/41	17/63	
Laxative use	19/20	11/41	< 0.05
GI procedures including PEG and surgery	13/13	0/0	
Continuation of initial antibiotic therapy	52/54	19/70	
Antibiotic treatment for CDAD	88/91	25/93	
Body temperature ⩾ 38℃	38/39	18/67	< 0.05
Therapy ⩾ 7 d after onset diarrhoea	33/34	12/44	
Length of hospital stay > 14 d	50/52	20/74	< 0.05
30-d mortality	4/4	9/33	< 0.001
Age (yr)	74 ± 12	77 ± 12	
Charlson´s score (points)	3.4 ± 2.2	5 ± 2.6	< 0.001
White blood cell count (G/L)	15.3 ± 9.9	21.6 ± 10.4	< 0.01
C-reactive protein (mg/L)	109 ± 79	223 ± 92	< 0.001
Creatinine (mg/L)	14 ± 13	24 ± 17	< 0.01
Sodium (mmol/L)	135 ± 5	133 ± 7	
Potassium (mmol/L)	3.6 ± 0.5	3.4 ± 0.5	

data were incomplete, leaving 124 patients for further analysis. We recorded patient age, sex, nursing home residency, comorbidity to calculate the Charlson comorbidity score[22,23] previous and concomitant medication (systemic antibiotic treatment within 6 wk preceding diagnosis, continuation of the initial antibiotic therapy after diagnosis of CDAD, use of opioids or laxatives), predisposing medical or surgical procedures (endoscopy, percutaneous gastrostomy, nasogastric tubes, chemotherapy or radiotherapy) as well as vital signs (heart rate, blood pressure and body temperature) and laboratory parameters (white blood cells, C-reactive protein, sodium, potassium, creatinine) at the time of diagnosis. In addition, we recorded the length of hospital stay, the period until beginning therapy for CDAD after the onset of diarrhoea, whether a specific antibiotic therapy for CDAD was instituted or not and the 30-d mortality after initial diagnosis of CDAD.

Patients with more than three loose stools per day on more than two consecutive days with a positive stool test for *C. difficile* toxin were diagnosed as CDAD[16,24]. Hospital-acquired CDAD was assumed if the onset of diarrhoea was > 72 h after hospital admission or if there had been a hospital admission for CDAD within the previous 6 wk. Severe CDAD was defined as profuse diarrhoea associated with a positive shock index (heart rate bpm/systolic blood pressure mmHg >1.5) at initial diagnosis[10]. All other patients were classified as non-severe CDAD.

Comparisons between the two groups of severe and non-severe CDAD were performed by Student t test for normally distributed data, proportions were analysed by χ^2 or F test as appropriate. A two-sided error level of $P < 0.05$ was considered statistically significant. Variables significantly associated with severe CDAD in univariate analysis together with risk factors reported in the literature were entered into a multivariate analysis. Statistical analysis was computed with SPSS version 14.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Demographics and results of initial evaluation

Patient characteristics are summarised in Table 1. Many patients had a comorbidity resulting in a median Charlson comorbidity score of 4. The majority of patients had hospital-acquired CDAD, 27 patients (22%) had severe CDAD, the overall 30-d mortality was 10% (13/124); all patients who died were >70 years.

Analysis of possible risk factors

Univariate analysis for comparison of patients with non-severe (n = 97) and severe CDAD (n = 27) revealed that immunosuppressive therapy, laxative use, body temperature ⩾ 38℃, length of hospital stay > 14 d, 30-d mortality, Charlson comorbidity score, white blood cell count, serum levels of C-reactive protein and creatinine were all significantly associated with severe CDAD (Table 2).

Table 3 Multivariate analysis of possible risk factors for severe CDAD

	P	OR; 95% CI
Variable (unit)		
Charlson´s score (points; 1-point increments)	< 0.05	1.39; 1.06-1.83
Body temperature ⩾ 38℃		1.15; 0.35-3.83
Immunosuppressive therapy		1.84; 0.45-7.49
Acid-suppressive therapy		1.28; 0.39-4.21
Opioid use		2.50; 0.80-7.84
Laxative use		2.66; 0.79-8.97
C-reactive protein (mg/L; 10 mg/L increments)	< 0.01	11.2; 10.3-12.1
White blood cell count (G/L; 1 G/L increments)		1.01; 0.96-1.06
Creatinine level (mg/L; 10 mg/L increments)		12.5; 9.2-16.8
Reduced Model		
Charlson´s score (points; 1-point increments)	< 0.05	1.29; 1.02-1.61
C-reactive protein (mg/L; 10 mg/L increments)	< 0.001	1.15; 1.08-1.22

A borderline statistically significant association was found for comedication with acid-suppressive therapy or opioids. By contrast, severe CDAD was not associated with nursing home residency, presence of hospital-acquired CDAD, continuation of the initial antibiotic therapy after diagnosis or increasing age. Multiple logistic regression analysis confirmed a significant association of severe CDAD and Charlson comorbidity score (OR 1.29 for 1 point increment, $P < 0.05$) and levels of serum C-reactive protein (OR 1.15 for 10 mg/L increment, $P < 0.001$; Tables 2 and 3).

DISCUSSION

The major findings in this retrospective analysis were a 22% rate of severe CDAD significantly associated with relatively high 30-d mortality (33% *vs* 4%, $P < 0.001$) and a higher proportion of a hospital stay exceeding 14 d (74% *vs* 52%, $P < 0.05$). In addition, comorbidity assessed by the Charlson comorbidity score ($P < 0.05$) and serum C-reactive protein at the time of diagnosis ($P < 0.001$) were identified as independent risk factors for severe CDAD in multivariate analysis.

The rate of severe CDAD and associated 30-d mortality in this study are relatively high. Infection with the recently emerging strain BI/NAP1 associated with severe courses of CDAD[2,8-11] is an unlikely explanation, since this strain had not been documented in Germany at the time of our retrospective analysis[25]. Therefore the most likely explanation are advanced age (median 76 years) and high comorbidity (median Charlson score of 4) of our cohort. The observed association of disease severity with comorbidity assessed by the Charlson comorbidity score is in line with reports on an association of severe CDAD with cognitive impairment[16], number of chronically affected organ systems[26], cardiac disease, malignancy, chronic obstructive pulmonary disease, pre-existing renal failure and other severe disease[27,28]. Our data support the hypothesis that comorbidity is an important risk factor for severe CDAD and the Charlson comorbidity score, which includes most of these conditions, might be a useful tool to identify patients at particular risk for severe CDAD. We also identified serum levels of C-reactive protein as independently associated with severe CDAD. In fact, serum C-reactive protein was a far better predictor of severe CDAD than white blood cell count, which has been described by others[28,29]. Thus, at the median Charlson comorbidity score of our cohort (4 points) a C-reactive protein level of 250 mg/L at diagnosis predicted a higher than 50% probability for severe CDAD. Perhaps more sensitive markers of inflammation such as procalcitonin might be even more useful in the evaluation of disease severity.

Other known risk factors for CDAD[2,18,30] might also be relevant for severe CDAD. In line with these data we found comedication with laxatives, opioids and acid-suppressive therapy associated with severe CDAD in univariate analysis although these risk factors could not be confirmed in multivariate analysis. In contrast, a variety of other putative risk factors for severe CDAD could not be confirmed. Thus, we did not detect an association of severe CDAD with increased age[27,30], which is probably due to the already advanced median age of our cohort. Moreover, prolonged antibiotic use *per se*, continuation of the antibiotic therapy after the diagnosis of CDAD, gastrointestinal procedures or surgery, which have all been reported as risk factors for *C. difficile* colonisation and CDAD[2,17] were not associated with severe disease in this study.

In conclusion, comorbidity and serum levels of serum C-reactive protein were identified as predictors of severe CDAD. Patients with strong comorbidity and high serum C-reactive protein levels at the time of diagnosis should be treated with particular attention.

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COMMENTS

Background

Clostridium difficile associated diarrhoea (CDAD) is the most common cause of healthcare-associated diarrhoea. It results in a wide spectrum of disease severity ranging from asymptomatic carriage to life-threatening enterocolitis and death with associated health care costs.

Research frontiers

A variety of studies has investigated risk factors for the development of CDAD. Thus, advanced age, severe comorbidity, hospitalisation, antibiotic exposure, immunosuppressive therapy as well as treatment with motility influencing or acid-suppressive drugs were identified as risk factors for CDAD. However, little is known about risk factors for associated with a severe course of CDAD in hospitalized patients.

Innovations and breakthroughs

The major findings reported are a 22% rate of severe CDAD which was significantly associated with relatively high 30-d mortality and a higher proportion of a hospital stay exceeding 14 d. Moreover, comorbidity assessed by the Charlson comorbidity score and levels of serum C-reactive protein at the time of diagnosis were identified as independent risk factors for severe CDAD

in multivariate analysis.

Applications

The major findings of this study should help to identify hospitalized patients with a particular risk for a severe course of CDAD. An early identification of patients at risk would allow a more timely intervention probably improving both morbidity and mortality.

Peer review

The paper describes important risk factors for a severe course of CDAD in hospitalized patients which have a potential for everyday clinical practice. It's an interesting paper.

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RAPID COMMUNICATION

Efficacy of 6-mercaptopurine treatment after azathioprine hypersensitivity in inflammatory bowel disease

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Abstract

AIM: To investigate the efficacy of 6-mercaptopurine (6-MP) in cases of azathioprine (AZA) hypersensitivity in patients with inflammatory bowel disease.

METHODS: Twenty nine previously confirmed Crohn's disease (CD) (n = 14) and ulcerative colitis (UC) (n = 15) patients with a known previous (AZA) hypersensitivity reaction were studied prospectively. The 6-MP doses were gradually increased from 0.5 up to 1.0-1.5 mg/kg per day. Clinical activity indices (CDAI/CAI), laboratory variables and daily doses of oral 5-ASA, corticosteroids, and 6-MP were assessed before and in the first, sixth and twelfth months of treatment.

RESULTS: In 9 patients, 6-MP was withdrawn in the first 2 wk due to an early hypersensitivity reaction. Medication was ineffective within 6 mo in 6 CD patients, and myelotoxic reaction was observed in two. Data were evaluated at the end of the sixth month in 12 (8 UC, 4 CD) patients, and after the first year in 9 (6 UC, 3 CD) patients. CDAI decreased transiently at the end of the sixth month, but no significant changes were observed in the CDAI or the CAI values at the end of the year. Leukocyte counts (P = 0.01), CRP (P = 0.02), and serum iron (P = 0.05) values indicated decreased inflammatory reactions, especially in the UC patients at the end of the year, making the possibility to taper oral steroid doses.

CONCLUSION: About one-third of the previously AZA-intolerant patients showed adverse effects on taking 6MP. In our series, 20 patients tolerated 6MP, but it was ineffective in 8 CD cases, and valuable mainly in ulcerative colitis patients.

Key words: Inflammatory bowel disease; Azathioprine; 6-mercaptopurine; Side effects; Efficacy

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INTRODUCTION

5-aminosalicylate is usually ineffective in the maintenance treatment of steroid induced remission in idiopathic inflammatory bowel (IBD) diseases, i.e. ulcerative colitis (UC) and Crohn's disease (CD)[1]. In the vast majority of cases immunosuppressive treatment is necessary to maintain the remission. Azathioprine (AZA) has been advised in the treatment of UC and CD since the middle of 1960's[2]. It is worth starting if the patient is corticosteroid resistant (the effective dose does not lead to remission) or dependent (discontinuation of the corticosteroid causes relapse)[3]. Azathioprine and its first metabolite 6-mercaptopurine are effective immunomodulators, but contrary to the corticosteroids, purine analogues have a late onset of action[4]. Their maximum effect can only be expected after 3-6 mo[5]. Azathioprine is offered as first choice, but it can cause early hypersensitivity reaction (fever), or gastrointestinal side effects (nausea, vomiting, and diarrhoea) in the first two weeks in 5%-10% of patients. In these cases, 6-mercaptopurine (6-MP) may be effective without side effects[6]. However, there are few data about the clinical efficacy of changing the AZA to 6-MP therapy in cases having hypersensitivity reactions after the first AZA medication.

MATERIALS AND METHODS

Between 2002 and 2005, 29 IBD patients (15 women and 14 men) were treated with 6-mercaptopurine due to azathioprine hypersensitivity. The drug Purinethol, 50 mg (Laboratoire GlaxoSmithKline) was approved by the National Institute of Pharmacy. The mean age of the

Table 1 The number of IBD patients in the three groups, according to extents, fistulas and surgeries

	Disease	No. of patients	A	B	C	D	E	F
Group1	UC	7	1	2	4	-	-	-
Group1	CD	2	-	2	-	-	-	-
Group2	CD	8	3	2	2	1	8	16
Group3	UC	8	1	-	7			1
Group3	CD	4	-	3	1	-	4	4
		29	4	9	14	1	12	21

UC-A: Distal; UC-B: Left sided; UC-C: Pancolitis; CD-A: Small bowel only; CD-B: Colon only; CD-C: Small bowel + colon, CD-D: Upper GI + small bowel + colon; CD-E: Number of fistulas; CD-F: Number of surgeries.

patient's was 40.1 years (range 19-66 years), and mean time to 6-MP treatment from the IBD diagnosis was 5.4 years (range 0.1-16.4 years). Fifteen patients had UC and 14 patients had CD. Table 1 contains the number of IBD patients in the 3 groups, according to extents, fistulas and surgeries. 6-MP test dose (50 mg/d for 7-10 d) was administered first. The therapy had to be discontinued in the first two weeks because of the same or similar hypersensitivity reactions as taking azathioprine during the initial doses in 9/29 patients (7/9 UC, 2/9 CD). Among the 9 patients, 4 had hypersensitivity reactions, including fever, and 5 were intolerant due to GI side-effects. During the very short interval (7-10 d) between the AZA start and the appearance of adverse events, we did not observe side-effects, such as leucopenia, abnormal LFTs or pancreatitis. Medication had to be suspended in 8/20 patients during the first 6 mo because it was ineffective. Decreasing the CDAI score not more than 70, and at least 3 score values in the CAI, was considered to be a treatment failure. All of them were CD patients; their treatment was continued by methotrexate and/or infliximab, if required.

Twelve patients tolerated 6-mercaptopurine without side-effects for more than six months with clinical efficiency. Four of 12 had Crohn's disease, 8/12 had UC. During the study 9 patients (6 UC, 3 CD) were treated for more than a year. The initial dose of 6-MP was 50 mg/d and it was increased, if possible, up to 1.5 mg/kg per day. The clinical activity, 6-MP and corticosteroid (prednisolone, methylprednisolone, separately) as well as the laboratory variables of acute inflammatory process were recorded. The medication was initiated at the first visit, and follow-up visits after the first, third, and sixth, twelfth months after the initial therapy. Crohn's Disease Activity Index (CDAI) and Clinical Activity Index of Ulcerative Colitis (CAI) were scored and calculated at each visit. According to the calculated values, the actual activity was grouped as inactive (1), mild (2), moderate (3) or severe (4). The following blood chemistry variables were determined, erythrocyte sedimentation rate, hematocrit, white cell and platelet counts, blood iron level, CRP, and fibrinogen.

STATA V8 program was used for statistical analysis. The repeated measurements of ANOVA and correlation analysis were used. Associations of variables between activity groups were analyzed by one way method of ANOVA.

RESULTS

The baseline means ± SD of CDAI/CAI, CRP, ESR, WBC, platelets, and the changes over time demonstrated decreased inflammatory reactions (Table 2). Relationships between time (at six months and one year), activity, and laboratory variables are presented in Table 3. Results of the analysis corresponded to 12 patients at 6 mo (8 UC, 4 CD), and 9 mo (6 UC, 3 CD) at the end of the first year (repeated measurement of ANOVA). CDAI decreased significantly only at the end of the sixth month in 4 of the 12 patients in whom 6-MP treatment was successful, but a similar decrease could not be demonstrated in the CAI of UC. At the end of the year, such an alteration could not be detected in any of the activity indices. CRP, referring to the severity of the inflammation, decreased and the iron level increased significantly at the end of the year (Table 3). Correlation between the calculated activity index numeric values (CDAI, CAI) and laboratory variables (correlation analysis) is presented in Table 4. The laboratory variables associated with the numeric values of the activity revealed a decrease in the inflammatory reaction in UC, but not in CD, due to the small number of CD cases. Besides the amelioration of the inflammatory reaction, the direct suppressive effect on the bone marrow may also be associated with reductions in the numbers of leukocytes and platelets. Treatment had to be discontinued temporarily in 1 case because of leukopenia at the end of the year. In another case, a significant bone marrow depression developed, necessitating surgical intervention (ileal-pouch anal anastomosis - IPAA) after drug cessation. Hepatotoxic side-effects and pancreatitis were not observed during the year. Table 5 shows the correlation between the activity groups - inactive (1), mild (2), moderate (3), severe (4) - and the values of laboratory variables in UC and CD patients (one way ANOVA). The laboratory variables correlated substantially better with the group classification than with the activity index numeric values.

The average doses of 6-MP and corticosteroids administered at the time of the visits is presented as mg/kg (Figure 1). Prednisolone treatment could be omitted and the dose of methylprednisolone could be tapered to one third at the end of the year. At the end of the first year 5/9 patients (3 UC, and 2 CD) became steroid free.

DISCUSSION

Systemic corticosteroid treatment is often needed in relapses of idiopathic inflammatory bowel diseases (UC, CD)[7]. The dose depends on the activity of the disease with severe cases treated intravenously[8]. If the patient responds to the intravenous regime, treatment should be switched to oral administration, and oral doses should be tapered gradually and finally terminated. If

Table 2 Changes of CDAI/CAI, CRP, ESR, WBC, PLT variables in the third group (mean ± SD)

Disease	Months	CDAI	CAI	CRP	ESR	WBC	PLT
UC	0	-	7.8 ± 6.3	16 ± 10	30.4 ± 16.2	9.3 ± 3.3	384 ± 175
UC	3	-	6.9 ± 6	8.3 ± 3.5	28.3 ± 16.5	10.3 ± 4.5	336 ± 119
UC	6	-	6.1 ± 6.7	11 ± 19	31.9 ± 32.7	6.8 ± 2	348 ± 197
UC	12	-	1.1 ± 1	4.6 ± 3.5	27 ± 17.2	6.1 ± 2.6	283 ± 87
CD	0	146 ± 128	-	39.8 ± 26.7	41.3 ± 11.6	9.9 ± 2.7	431 ± 136
CD	3	145 ± 124	-	23.3 ± 10.7	29.2 ± 14.1	7 ± 3	332 ± 66
CD	6	167 ± 68	-	21 ± 15	17.6 ± 28.6	9.9 ± 4.2	317 ± 89
CD	12	46 ± 56	-	6 ± 4	18 ± 14.7	6.2 ± 1.3	247 ± 80

Table 3 Significant changes after six and twelve months of 6-MP treatment

Variable	6 mo	12 mo
CDAI	$P = 0.0144$ ($n = 4$)	NS $n = 3$
CAI	NS $n = 8$	NS $n = 6$
Leukocyte	NS	$P = 0.0057$
CRP	NS	$P = 0.0206$
Serum iron	NS	$P = 0.0459$

NS: Not significant.

Table 4 Correlation of activity index numbers with the laboratory variables

Variable	CAI/UC	CDAI/CD
Thrombocyte	$P < 0.001$	NS
Serum iron	$P = 0.006$	NS
CRP	$P = 0.008$	NS
Haematocrit	$P = 0.022$	NS
Leukocyte	$P = 0.071$ (BS)	NS
ESR 1st h	$P = 0.077$ (BS)	NS

NS: Not significant; BS: Borderline significance.

Table 5 Correlation between activity groups and laboratory variables

Variable	1-4 UC (*P*)	1-4 CD (*P*)
Fibrinogen	0.0046	NS
Thrombocyte	0.0060	0.0350
CRP	0.0061	NS
Leukocyte	0.0144	0.071 (BS)
Haematocrit	0.0270	NS
Serum iron	0.0808 (BS)	NS

BS: Borderline significance.

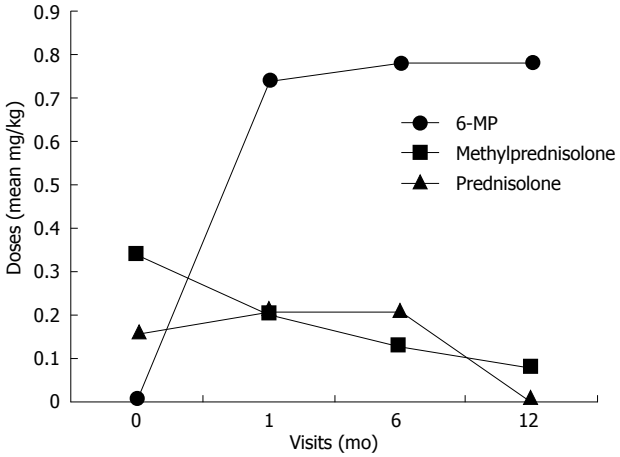

Figure 1 Changes of the 6-MP and oral steroid mean doses.

the patient proves to be steroid resistant, or dependent, immunosuppressive treatment is suggested[9]. Azathioprine is recommended first, where it is the most frequently used immunosuppressive drug for IBD[10]. An oral dose of 1.5-2.5 mg/kg per day is usually effective, but requires monitoring[11]. Therapeutic effects and side-effects of AZA show great variability among the patients due to the various concentrations of the therapeutic and toxic metabolites[12]. The application is hampered in 9%-25% of the patients due to its toxic effects. Hypersensitivity reactions (fever), or gastrointestinal side effects (nausea, diarrhea), can occur during the first weeks of the treatment[13]. According to McGovern *et al*, imidazole that is cleaved of the AZA molecule can be responsible for the development of this process[14]. AZA is a pro-drug that is converted to 6-MP through a non-enzymatic step. Further metabolism of 6-MP depends on three competing enzyme pathways. Hepatotoxic 6-methylmercaptopurine (6-MMP) is produced by thiopurine methyltransferase (TPMT), a key enzyme of toxic and therapeutic metabolites. Measurement of its activity helps determine individual doses[14,15]. Catabolism, *via* xanthine oxidase (XO), forms inactive 6-thiouric acid which is eliminated through the urine. Metabolism, *via* hypoxantin-guanin phosphoribosyl transferase (HPRT), leads to the formation of cytoactive 6-thioguanine nucleotide (6-TGN) that binds to the DNA and RNA, and is the active molecule responsible for the late side-effects in a dose-dependent manner. The proportion of these three enzymes determines the effective 6-TGN level. In case of low TPMT activity (due to enzyme polymorphisms) metabolism shifts to the production of 6-TGN, where high concentrations are associated with efficacy, but above a certain level (> 450 pmol/108 erythrocyte), 6-TGN has a myelotoxic effects[16]. Bone marrow depression that is a late toxic reaction usually occurs in the first three months[17]. Other mechanisms participate in the development of this process as well since this side-effect is noticed with normal and high TPMT activity. During the occurrence of immunosuppressor activity in IBD, a decrease in the number of lamina propria plasma cells and altered function of the lymphocytes and killer cells is detected. According to previous studies that need further

confirmation, the therapeutic effect of AZA is explained by its apoptosis inducing property that is independent from 6-TGN. Tiede *et al* showed that azathioprine and its 6-thioGTP metabolite alters apoptosis of T cells[18]. The activity of certain genes in the cells is repressed by the metabolite which induces apoptosis in a mitochondrial manner.

In the view of the clinical efficacy, results achieved by 6-MP treatment can be compared with azathioprine. Considering bioequivalence, 6-MP shows the same efficacy as half (0.75-1.5 mg/kg per day) of the daily dose of AZA (1.5-2.5 mg/kg per day)[19]. There are few data about the clinical use of 6-MP[20] and only some investigated treatment results of 6-MP in AZA-intolerant patients suffering from IBD. Boulton-Jones *et al* reported a cohort of 19 patients who had failed AZA therapy. Ten had CD and 9 UC[21]. The AZA dose prior to discontinuation ranged from 50 to 150 mg. Eleven of 19 patients were able to tolerate 6-MP in a dose ranging from 50 to 100 mg (median 100 mg). Two of 8 patients developed fever; other adverse events were vomiting, leucopenia, skin rash, headache, and abdominal pain. The treatment could be continued in 6 patients from the 11 in the study performed by Bowen *et al* and were successful only in 4 cases[22]. McGovern *et al* achieved steroid independent remission in 15 of 29 patients[23]. Similar results were achieved by Doménech *et al*[24].

In our CD patients, favourable effects were attained only in 4/14 cases, at the end of the first 6 mo with 6-MP (average: 0.75 mg/kg per day). Eight CD patients were withdrawn due to ineffective therapy within the same time. Later, at the end of the first year, the benefit of the therapy was lost in all patients. In these cases, higher doses (more than 1.5 mg/kg per day) might increase the response and perhaps the number of adverse events[25]. Decreased inflammatory reactions were more significant in UC. The efficacy of the drug was indicated by the improvement of biological parameters associated with inflammation, which permitted a gradual reduction in the steroid. It is worth mentioning that clinical activity indexes (CDAI and CAI) were less sensitive than the laboratory variables at presenting decreased inflammation[26]. The correlation between activity groups and the laboratory variables showed substantially better statistical association. Considering the efficacy in case of our patients without 6-MP intolerance, it should be mentioned that more significant improvement could be achieved by using higher doses (mainly in CD). Measurement of the 6-TGN and 6-MMP metabolites and TPMT activity is recommended for the safe dosage of thiopurine analogues, and to avoid the development of late toxic effects, although none of these parameters ensure total safety[27]. In patients with early AZA intolerance treated with 6-MP thereafter, our results showed wide individual variation of 6-MP doses and responses. Patients on effective doses may develop adverse reactions at any time; therefore, blood counts and liver function tests have to be checked in every second week at the beginning, then every month, and later on in every third month. Because of the potential for life-threatening side-effects, the drug is recommended in cooperating patients only[28].

AZA and 6-MP have been accepted as maintenance therapies in IBD, but controversy exists regarding optimal dosing and benefits of therapeutic drug monitoring of metabolites[29]. The AZA baseline dose (1.7 mg/kg per day) proved to be effective in maintaining remission in a French cohort of CD patients, which was lower than the dose used in clinical trials (2.5 mg/kg per day). Nielsen *et al* in their review in the year 2001, suggested a 0.25 ± 0.5 mg/kg daily initial (AZA equivalent) dose for the 6-MP, increasing to 1.0 ± 1.5 mg/kg daily[30]. Su and Lichtenstein, three years later, advocated "the optimal dose for the treatment of active CD is generally considered 2.5 mg/kg per day for azathioprine and 1.5 mg/kg per day for 6-MP"[17]. In our AZA intolerant patients, the initial dose was 50 mg/d with a stepwise increase after the third and sixth month with 25 or 50 mg/d up to 1.5 mg/kg per day according to the tolerability of the patients. The low dose and the step up policy may play a role in the poor therapeutic response, especially in very active Crohn disease patients in the second group.

It is worth starting with 6-MP therapy in patients with hypersensitivity reactions to AZA. One third of the patients will be intolerant, and another third will not gain any benefit, especially those who had serious active CD. However, patients with mild or moderate UC/CD might have the advantage of being able to tolerate the daily dose of more than 1 mg/kg.

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RAPID COMMUNICATION

Epstein-Barr virus is associated with gastric carcinoma: The question is what is the significance?

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Abstract

AIM: To examine the possible role of the Epstein-Barr Virus (EBV) in the development of gastric adenocarcinoma (GC). It is unclear whether EBV is involved in GC development or is a consequence of gastric inflammation secondary to immunosuppressive treatments.

METHODS: A systematic review was carried out of all published observational studies on the temporal association between EBV and GC, with a view to determine a causal relationship.

RESULTS: The present study showed that the worldwide crude prevalence of EBV in gastric adenocarcinoma was 8.29%. The prevalence varied from 7.08% for intestinal type and 9.82% for diffuse type of GC. It was observed that Western and Central Asian countries had a significantly higher frequency of EBV positive cases compared to South-Eastern countries. America had the highest EBV-GC prevalence whereas Europe had the lowest.

CONCLUSION: The present review has demonstrated a high prevalence of EBV in gastric adenocarcinoma. However, studies designed to assess a temporal relationship and histological association using sensitive techniques should be carried out to establish the role of EBV in GC carcinogenesis.

Key words: Epstein-Barr virus infection; Gastric carcinoma; Systematic review

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INTRODUCTION

Gastric adenocarcinoma (GC) is the second most common cause of cancer-related mortality and a major public health problem worldwide[1]. GC has a distinct geographical distribution with the highest incidence rates in Asia and South America and the lowest incidence in western countries[1-3].

The Epstein-Barr virus (EBV) is a ubiquitous virus, with carcinogenic properties, which has been linked to the development of several malignancies including nasopharyngeal carcinoma and Burkitt's lymphoma[4]. It has been estimated that over 90% of the population worldwide has been exposed to EBV, although, not all infected individuals develop EBV-related disease[4]. After infection, EBV remains in a latent state in B-cell lymphocytes, at a rate of 1 in 10^6 circulating cells. The reason why it is difficult to identify EBV is perhaps due to the fact that expression of only a small number of viral proteins allows the maintenance and control of cell proliferation[5,6].

EBV is a common co-infection in several diseases and some authors have suggested that it may represent a late event in the GC carcinogenesis, after *H pylori* infection[7]. However, there is lack of specific information implicating EBV in the pathogenesis of GC. The worldwide occurrence of EBV positive GC is estimated at >50 000 cases/year, although, it remains unclear whether the presence of EBV is the cause or a consequence of GC[8].

Therefore, we carried out a systematic analysis of all observational studies on EBV and GC with a view to assess a possible temporal and statistical association.

MATERIAL AND METHODS

Articles search

All studies included in the present review (n = 494) were selected from the Medline database using the following subject headings: (1) EBV or Epstein-Barr or "EBV-associated membrane antigen, Epstein-Barr virus" [Substance Name] or "Epstein-Barr Virus Infections" [MeSH] or "Epstein-Barr viral capsid antigen" [Substance Name] or "Epstein-Barr virus early antigen" [Substance Name]; (2) virus OR ("Viruses" [MeSH] or "Virus Activation" [MeSH] or "DNA Tumor Viruses" [MeSH] or "Tumor Virus Infections" [MeSH]; (3) gastric or stomach or ("Stomach" [MeSH] or "Stomach Diseases" [MeSH]; (4) cancer or neoplasms OR "Neoplasms" [MeSH]. Furthermore, we also searched the reference list of all published articles.

Inclusion/exclusion criteria

All original published studies in English, French, Spanish and Portuguese on EBV infection and gastric carcinoma were included in the analysis (n = 494). Studies were excluded for the following reasons (Figure 1): studies referring to other types of cancer (n = 140); use of languages such as Russian, Chinese, Japanese or Italian (n = 30); articles other than those containing research data, such as reviews, letters to the editor and case-reports (n = 155); *in vitro* and *in vivo* experiments (n = 81); and studies referring to histological types other than gastric adenocarcinoma (n = 44).

Data extraction and statistical analysis

The studies included in the present analysis (n = 103) were reviewed by all the authors, in order to obtain the necessary information, such as: design of study (case-control or retrospective), source of tissue sample (formalin embedded tissues, fresh tissues or peripheral blood samples), study population, geographical distribution, histological type of the lesion and EBV diagnostic methodology (*in situ* hybridization, Polymerase Chain Reaction, immunohistochemistry and PCR followed by Enzyme Linked Immunoassay). Pooled frequencies were estimated by country, region and continent and were adjusted for the type of cancer, sample source and EBV diagnostic methodology.

RESULTS

General observations

Our study included a total of 103 published articles which analysed the frequency of EBV in GC in 21 different countries: 9 from Asia (China, India, Japan, Taiwan, Korea, Papua New Guinea, Pakistan, Kazakhstan and Turkey), 7 from Europe (UK, France, Germany, Italy, Netherlands, Poland and Russia) and 5 from America (USA, Mexico, Brazil, Chile and Colombia).

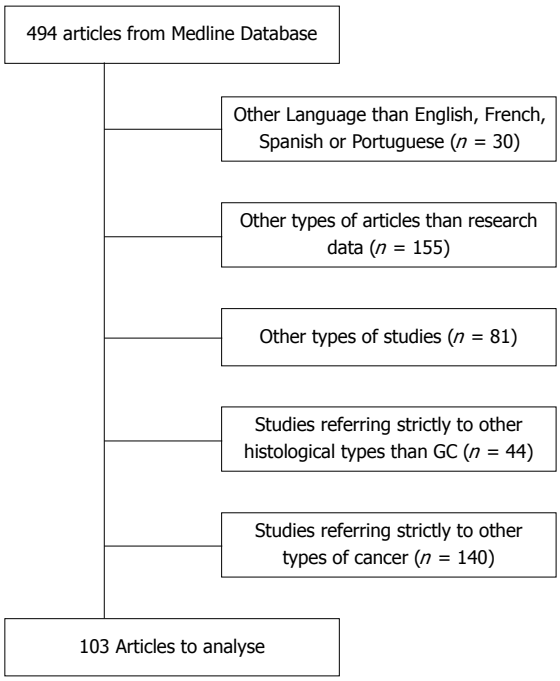

Figure 1 Application of Inclusion/exclusion criteria.

Study design

Our study material consisted of 96 retrospective studies, 6 prospective studies and one case-control study.

Sample source

The study samples comprised of the following: 92 studies were conducted on formalin embedded tissues, 10 on fresh tissues and one on peripheral blood samples.

EBV diagnostic methodology

The most common methodology used for the diagnosis of EBV was *In Situ* Hybridization (ISH) for EBER1 (n = 88), followed by Polymerase Chain Reaction (PCR) for the BamHI-W region of EBV (n = 6), PCR for EBNA1 (n = 3), immuno-histochemistry for LMP1 (n = 2), PCR followed by Enzyme Immunoassay (PCR-EIA) for BamHI-W, and ISH for BamHI-W in one study each. In two studies, it was not possible to determine the methodology used for EBV detection.

Statistical analysis

A total of 114 studies, comprising of 33471 cases were evaluated. The great majority of studies were conducted in Asian countries (n = 77) with a total of 29 076 individuals, followed by Europe (n = 18) with 2296 individuals and America (n = 19) with 2099 individuals (Table 1). With respect to the diffuse type of GC, a total of 5846 cases were analyzed, of which 4862 individuals were from Asian countries, 173 from Europe and 811 from America (Table 1). By contrast, intestinal type of GC was reported in 7786 cases, including 6367 individuals from Asia, 338 from Europe and 1082 from

Table 1 Distribution of Epstein-Barr virus-association with gastric adenocarcinoma

	Gastric adenocarcinoma					Gastric adenocarcinoma intestinal type					Gastric adenocarcinoma diffuse type				
	n	Total	EBV +	Crude frequency (%)	Adjusted frequency (%)	*n*	Total	EBV +	Crude frequency (%)	Adjusted frequency (%)	*n*	Total	EBV +	Crude frequency (%)	Adjusted frequency (%)
EUROPE	18	2296	201	8.75	7.96	4	338	27	7.99	2.94	4	173	9	5.2	7.83
East Europe	4	293	43	14.7	26.3	1	13	0	0	---	1	19	4	21.1	---
Poland	3	87	25	28.7	40	1	13	0	0	---	1	19	4	21.1	---
Russia	1	206	18	8.74	---	---	---	---	---	---	---	---	---	---	---
Central Europe	13	1938	154	7.95	7.58	3	325	27	8.31	2.94	3	154	5	3.25	4.55
United Kingdom	4	682	12	1.76	1.7	---	---	---	---	---	---	---	---	---	---
France	1	59	5	8.47	---	1	21	0	0	---	1	22	1	4.55	---
Germany	1	35	3	8.57	---	1	17	1	5.88	---	1	18	2	11.1	---
Netherlands	7	1162	134	11.5	7.58	1	287	16	9.06	---	1	114	2	1.75	---
South Europe	1	65	4	6.15	---	---	---	---	---	---		---	---	---	---
Italy															
ASIA	77	29076	2324	7.99	9.65	21	6367	430	6.75	5.82	21	4862	441	9.07	11.2
South-East Asia	72	28760	2253	7.83	9.56	19	6290	414	6.58	5.82	18	4665	398	8.53	6.89
Taiwan	3	486	77	15.8	20	2	129	23	17.8	19	2	103	24	23.3	21.2
Papua New Guinea	1	150	2	1.33	---	1	72	0	0	---	1	78	2	1.33	---
Korea	13	4784	300	6.27	5.7	2	233	4	1.72	1.71	2	319	22	6.9	6.89
Japan	45	21956	1750	7.97	11.6	11	5480	353	6.44	5.82	11	4011	346	8.63	11.2
China	10	1384	124	8.96	7.74	3	376	34	9.04	10.7	2	154	4	3.25	1.43
South Asia	2	112	21	18.8	17.6	---	---	---	---	---	1	70	8	11.4	---
India	1	60	20	33.3	---						1	70	8	11.4	---
Pakistan	1	52	1	1.92	---						---	---	---	---	---
Central-western Asia	3	204	51	25	10.8	2	77	16	20.8	26.9	2	127	35	27.6	37.7
Kazakhstan	2	139	14	10.1	9.89	1	48	1	2.08	---	1	91	13	14.3	---
Turkey	1	65	37	56.9	---	1	29	15	51.7	---	1	36	22	61.1	---
AMERICA	19	2099	249	11.9	11.3	14	2082	95	4.56	8.3	13	811	124	15.3	13.3
South America	8	927	124	13.4	12.2	7	503	47	9.34	8.77	6	381	75	19.7	18.8
Colombia	2	294	31	10.5	9.92	1	91	11	10.5	---	1	86	12	14	---
Chile	1	278	53	20.2	---	2	178	23	12.9	14.5	3	193	52	26.9	29.6
Brazil	4	355	40	11.3	11.3	4	234	13	5.56	4.35	2	102	11	10.8	6.55
Central America	4	630	55	8.73	9.86	3	241	8	3.32	2.84	3	319	38	11.9	12.8
Mexico															
North America	7	542	70	12.9	10.2	4	338	40	11.8	10.8	4	111	11	9.91	8.63
United States															
GLOBAL ANALYSIS	114	33471	2775	8.29	9.67	39	7786	552	7.08	5.88	38	5846	574	9.82	12.1

Distribution of EBV association with Gastric Adenocarcinoma.

America (Table 1).

The largest sample size for gastric adenocarcinoma was obtained from South-east Asia (28 760 cases), followed by Central Europe (1938 cases) and South America (927 cases). With respect to the intestinal and diffuse types of GC, the largest sample was from South-east Asia (6290 and 4665 cases respectively), followed by South America (503 and 381 studies respectively) and Central Europe (325 and 154 studies, respectively).

Table 1 shows the crude and adjusted frequency of positive EBV results in different countries considering the world as a discrimination factor. The worldwide crude EBV positive prevalence was 8.29% for gastric adenocarcinoma, with 7.08% for intestinal type and 9.82% for diffuse type. The corresponding adjusted prevalence was estimated as 9.67%, 5.88% and 12.1%, respectively. The adjustment of EBV positive prevalence resulted in minor changes from the crude estimates. America registered the highest adjusted EBV positive prevalence for both gastric adenocarcinomas and the diffuse type of GC (11.3% and 15.3%, respectively). On the other hand, Europe had the lowest adjusted EBV positive prevalence for gastric adenocarcinoma, and intestinal and diffuse types of GC (7.96%, 2.94%, and 7.83%, respectively).

DISCUSSION

As a Group I carcinogenic agent, EBV has been intensively studied over the past 40 years[9]. It has been demonstrated consistently that EBV has an association with nearly 100% of gastric lymphoepithelioma-like carcinomas[10-12], which are believed to have similar pathogenic mechanism as nasopharyngeal carcinoma[7].

Despite the presence of several hundred published studies demonstrating an association between EBV and GC, the pathogenic role of EBV in gastric carcinogenesis remains to be established[7]. Nevertheless, there is strong evidence to directly implicate EBV in GC development: presence of EBV in all cancer cells detected by in situ hybridization of EBER1, but not in the surrounding epithelial cells[13]; monoclonality of the viruses in neoplastic cells as judged by the analysis of single terminal repeats of EBV DNA[14]; and elevation of immunoglobulin A and B antibodies against viral

capsid antigen several months before the clinical presentation of the disease[15].

The pathogenic mechanism of EBV-GC is not well understood. EBV is transmitted through the saliva, and primary infection occurs at the oropharyngeal mucosa, with the involvement of the Waldeyer's ring followed by infection of naive B-cells as they circulate near the infected cells[16]. After infection, EBV establishes a persistent state in the B-cells, and is maintained *ad eternum*. From time to time, the infected B-cells become activated as a result of immune suppression of the host, and the infected cells are able to enter other tissues[16]. Since the main receptor (CD21/CR2, the receptor for the C3d component of complement) for EBV is normally absent from gastric epithelial cells[17,18], EBV infection has been suggested to be accomplished by the binding of EBV particles with IgA antibodies which are then engaged by gastric epithelial cells[7,19]. Once inside the gastric cells, EBV activates its latency state by expressing latency proteins which induce cell proliferation and viral maintenance. Recent studies have shown that in gastric carcinoma, EBV expresses a different pattern of proteins than those seen in Burkitt's lymphoma and nasopharyngeal carcinoma, suggesting a different oncogenic mechanism in gastric carcinoma[20,21].

The development of gastric carcinoma is a multistep event, progressing from normal to preneoplastic lesions to highly malignant tumours[20]. Despite strong evidence implicating *H pylori* as the main etiological factor for GC development, several other environmental and genetic co-factors have been reported[22]. At present, it is generally accepted that EBV infects gastric cells depending on a permissive environment and a genetically susceptibility host, upon an inflammatory state of gastric epithelium, which is in agreement with the findings that EBV-GC are monoclonal tumours[22]. Moreover, there are clinico-pathological features that are seen consistently in EBV-GC, such as: higher prevalence in males, with no impact on stromal invasion or survival[11,12,23]; tumour histology varying from moderately differentiated tubular to poorly differentiated solid type[17,24-29]; and a predisposition for the upper two-thirds of the stomach.

By performing a systematic review of all published studies worldwide, we have attempted to analyze the role of EBV in GC development, by estimating and comparing the worldwide occurrence of EBV-GC and by elucidating their temporal relationship and statistical association.

Our study included 103 published reports from 21 different countries distributed over 3 continents (Europe, Asia and America). Gastric adenocarcinoma is the second most common cause of cancer-related mortality worldwide and is the 14th overall cause of death[2]. Worldwide, there are widespread differences in gastric cancer rates, with the highest rates seen in Japan, China and South America, and much lower rates in Western Europe and the United States[2,22]. As expected, the great majority of studies were conducted in Asian countries, with 80 general reports on EBV and gastric adenocarcinoma, 20 on diffuse type and 21 on intestinal type of GC. The crude frequency of EBV positive cases in Asian countries was 8.07%, 9.13% and 6.73% for GC, diffuse type and intestinal type, respectively. Moreover, western and central Asian countries had significantly higher frequency of EBV positive cases than south-eastern countries.

Despite these studies, the exact association between EBV and GC remains to be established. Moreover, recent data reinforces the importance of the differences in the association of EBV with gastric carcinoma among different ethnic communities[30,31], with respect to temporal relationship, histological association and the application of sensitive methodologies for EBV diagnosis in GC. Nevertheless, the present review emphasizes the need for further studies designed to elucidate EBV-GC carcinogenesis model.

COMMENTS

Background

Gastric adenocarcinoma is a major public health problem worldwide. Epstein-Barr Virus (EBV) infects over 90% of population worldwide and has been linked as a late event in the gastric carcinogenesis process, after *H pylori* infection.

Research frontiers

Despite evidence to suggest the existence of biological plausibility, there is lack of information about the role of EBV in gastric cancer, either as a risk factor for the development of cancer or as a predictive marker for major outcomes after medical therapy.

Innovations and breakthroughs

A systematic review was conducted on 103 studies from 21 different countries representing 33471 cases of cancer. Most of the studies (n = 96) were retrospective and used formalin embedded tissues (n = 92) and used In Situ Hybridization (ISH) for EBER1. The adjusted prevalence of EBV was 9.67%, 5.88% and 12.1%, for gastric adenocarcinoma, intestinal type and diffuse type, respectively. America registered the highest adjusted EBV prevalence (11.3% and 15.3%, respectively) for both gastric adenocarcinomas and diffuse type. By contrast, Europe had the lowest adjusted EBV prevalence (7.96%, 2.94%, and 7.83%, respectively), for gastric adenocarcinoma, intestinal and diffuse type.

Applications

Differences in EBV prevalence may provide further insight into research on gastric carcinogenesis and the natural history of this disease.

Peer review

The present study is relevant as it emphasizes the lack of prospective studies and the absence of methodologies that can discriminate past from current viral infection. The focus of research and clinical application may become relevant in future studies.

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RAPID COMMUNICATION

Postoperative change of anti-Thomsen-Friedenreich and Tn IgG level: The follow-up study of gastrointestinal cancer patients

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Abstract

AIM: To study the influence of tumor removal on the serum level of IgG antibodies to tumor-associated Thomsen-Friedenreich (TF), Tn carbohydrate epitopes and xenogeneic αGal, and to elucidate on the change of the level during the follow-up as well as its association with the stage and morphology of the tumor and the values of blood parameters in gastrointestinal cancer.

METHODS: Sixty patients with gastric cancer and 34 patients with colorectal cancer in stages Ⅰ-Ⅳ without distant metastases were subjected to follow-up. The level of antibodies in serum was determined by the enzyme-linked immunosorbent assay (ELISA) using synthetic polyacrylamide (PAA) glycoconjugates. Biochemical and haematological analyses were performed using automated equipment.

RESULTS: In gastrointestinal cancer, the TF antibody level was found to have elevated significantly after the removal of G3 tumors as compared with the preoperative level (u = 278.5, P < 0.05). After surgery, the TF and Tn antibody level was elevated in the majority of gastric cancer patients (sign test, 20 *vs* 8, P < 0.05, and 21 *vs* 8, P < 0.05, respectively). In gastrointestinal cancer, the elevated postoperative level of TF, Tn and αGal antibodies was noted in most patients with G3 tumors (sign test, 22 *vs* 5, P < 0.01; 19 *vs* 6, P < 0.05; 24 *vs* 8, P < 0.01, respectively), but the elevation was not significant in patients with G1 + G2 resected tumors. The postoperative follow-up showed that the percentage of patients with G3 resected tumors of the digestive tract, who had a mean level of anti-TF IgG above the cut-off value (1.53), was significantly higher than that of patients with G1 + G2 resected tumors (χ^2 = 3.89, all patients; χ^2 = 5.34, patients without regional lymph node metastases; P < 0.05). The percentage of patients with a tumor in stage I, whose mean anti-TF IgG level remained above the cut-off value (1.26), was significantly higher than that of patients with the cancer in stages Ⅲ-Ⅳ (χ^2 = 4.71, gastric cancer; χ^2 = 4.11, gastrointestinal cancer; P < 0.05). The correlation was observed to exist between the level of anti-TF IgG and the count of lymphocytes (r = 0.517, P < 0.01), as well as between the level of anti-Tn IgG and that of serum CA 19-9 (r = 0.481, P < 0.05). No positive delayed-type hypersensitivity reaction in skin test challenges with TF-PAA in any of the fifteen patients, including those with a high level of anti-TF IgG, was observed.

CONCLUSION: The surgical operation raises the level of anti-carbohydrate IgG in most patients, especially in those with the G3 tumor of the gastrointestinal tract. The follow-up demonstrates that after surgery the low preoperative level of TF antibodies may be considerably increased in patients with the carcinoma in its early stage but remains low in its terminal stages. The stage- and morphology-dependent immunosuppression affects the TF-antibody response and may be one of the reasons for unresponsiveness to the immunization with TF-antigens.

Key words: IgG antibodies; Thomsen-Friedenreich; Tn; αGal; Gastrointestinal cancer; Immunosuppression; CA 19-9

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INTRODUCTION

Cancer immune surveillance is considered to be important in the anti-tumor protection of the host. However, immunity not only protects the host from cancer but also may promote tumor growth, creating an immuno-resistant cancer cell phenotype ("cancer immunoediting"). In the advanced stages of cancer, tumor escapes the immune control under the immunosuppressive conditions[1]. The removal of the tumor appears to disturb the immunoediting process and may reverse immunosuppression. The suppressed antigen-specific antibody responses in tumor-bearing mice may be reversed after the surgical removal of the primary tumor even in the existence of a disseminated metastatic disease[2].

Mucin-type tumor-associated carbohydrate antigens (TACA), the Thomsen-Friedenreich (TF) antigen and its precursor, Tn, are frequently expressed in malignant tumor cells. They deserve to be studied as targets for active specific immunotherapy[3]. Human blood serum contains natural TF- and Tn antibodies whose subpopulations may bind the corresponding antigens on human tumor cell lines[3-5]. It is not yet clear which role antibodies play in the natural anti-cancer defense system. The level of TF- and Tn antibodies was significantly decreased in the serum of primary (not operated) patients with cancer, including patients with the disease in its early stage[6-9]. Furthermore, the low level of anti-TF IgG was associated with a lower differentiated carcinoma and advanced gastric cancer that suggests an implication for antibodies in the progression and pathology of the tumor[10]. The high level of anti-TF IgG in the serum of primary patients with gastric cancer is closely associated with survival[11]. The dynamic changes of the level of TF- and Tn-antibodies in the serum of patients with cancer and its association with survival have been insufficiently studied. Investigations have mainly focused on clinical trials of antigen-specific immunotherapy[12,13]. The authors have undertaken a long-term follow-up of cancer patients to determine changes in the postoperative level of TF- and Tn antibodies, as well as to elucidate the association of this level with the progression of cancer, and survival. For comparison, the level of xenoreactive antibodies to the αGal epitope was determined. In humans, the αGal epitope is absent because the α1-3 galactosyltransferase gene was inactivated in evolution, but approximately 1% of immunoglobulins have an anti-αGal specificity[14].

In this work, the influence of the surgical removal of the tumor on the level of antibodies and its change during the follow-up was investigated. The association of the level of antibodies with the stage and morphology of the tumor, as well as values of blood parameters was studied. Also, the delayed-type hypersensitivity reaction to TF-PAA conjugates in skin testing was examined.

MATERIALS AND METHODS

Subjects

The investigation was carried out in accordance with the ICH GCP Standards and approved by Tallinn Medical Research Ethics Committee. Informed consent was obtained from subjects under study. The follow-up study was undertaken of patients with a verified diagnosis of gastric (n = 60) and colorectal cancer (n = 34) of stages I -IV by using the pTNM system[15]. Patients with distant metastases of the cancer or those who received chemo- and X-ray therapy were not subjected to study. The median age of the patients was 60 years (the age ranging from 30 to 75 years). The venous blood samples were taken before and after surgery at intervals from three to sixteen months, with a further follow-up during two to twelve years. The extended D2 gastrectomy with lymphadenectomy or, additionally, with the splenectomy in gastric cancer, as well as the resection of local lesions of colorectal cancer was performed. In advanced cancer regional lymph node metastases were also removed. In some patients concomitant diseases were documented. Breast cancer was diagnosed in three, benign diseases, in five, anaemia and diabetes mellitus, in two cases. The other sporadic manifestations were Parkinson's disease, carcinoma of the uterus and chronic hepatitis.

Glycoconjugates

Synthetic polyacrylamide (PAA) glycoconjugates with a single reiterative epitope were used in comparative immunoassays[16]. The homogeneity of PAA-conjugates enables a precise detection of epitope-specific antibodies. The following PAA-conjugates were used: the TF disaccharide, Galβ1-3GalNAcα; Tββ, Galβ1-3GalNAcβ; Tn, GalNAcα; αGal or a B-blood group disaccharide, Galα1-3Galβ; SiaLe^a (CA 19-9 tetrasaccharide), Neu5Acα2-3Galβ1-3 (Fucα1-4) GlcNAcβ. Tris-PAA, tris (hydroxymethyl) aminomethane-PAA, was used as a negative control because of its low background and good reproducibility in immunoassay[8]. The TF-PAA as a substituted PAA containing 0.1 mol of TF *per* 1 mol of PAA was used because of its elevated binding to human IgG antibodies. The rest of the polyacrylethanolamide-conjugates had 0.2 mol of a saccharide *per* 1 mol of PAA. All PAA-conjugates were received from Lectinity, Russia.

The determination of the level of epitope-specific IgG antibodies in sera by enzyme-linked immunosorbent assay (ELISA)

The method has been described elsewhere[8]. The dilution of serum was 1:50-1:200. The antibody levels were calculated as a ratio $A_{test}/A_{control}$ where A_{test} is the absorbance with PAA-glycoconjugate and $A_{control}$, with Tris-PAA. The variation coefficient was 3%. To diminish

Table 1 The effect of gastrectomy on the antibody level in patients with gastric cancer

Detection of antibodies	Mean ± SD	Increase	Median of difference[1]	Difference[2]		*P* (sign test)
				+	-	
TF (*n* = 28)				20	8	
Preoperative level	1.95 ± 1.86					
Postoperative level	2.66 ± 2.61	36.4%	0.28			< 0.05
Tn (*n* = 29)				21	8	
Preoperative level	2.64 ± 2.24					
Postoperative level	3.36 ± 2.84	27.3%	0.38			< 0.05

[1]Values of the postoperative (the first analysis after surgery) minus preoperative antibody level; [2]The number of patients having the positive or negative difference.

the variation of the test, the serum samples taken before and after surgery were analyzed using the same plate.

Clinical analysis of blood samples

Biochemical and hematological analyses were performed at the Oncological Centre of the North-Estonian Regional Hospital. The following automatic equipment was used: a Hitachi 912 and Elecsys 2010, Roche Diagnostics; a Sysmex XE-2100, Sysmex Corporation.

Blood samples were taken before and after surgical operation during the planned visits to the physician for health control. The antibody levels were correlated with those of the C-reactive protein (CRP), tumor markers (CA19-9, CEA), the alanine aminotransferase, glucose, haemoglobin, circulating red blood cells (count), leukocytes (count), neutrophils (%, count), monocytes (%), lymphocytes (%, count), platelets (count) and eosinophils (%). The concentration of CRP was determined by a turbidimetric method and that of tumor markers, by an electrochemiluminescence immunoassay.

Skin test

Antigens: TF-PAA, *Mr* 30 and 1000 ku; Tββ-PAA, *Mr* 1000 ku. The antigens (50-100 μg) were injected intradermally and the delayed-type hypersensitivity reaction was monitored twice: through 24 and 48 h. The reaction was considered positive if erythema > 5 mm was developed.

Statistical analysis

The Mann-Whitney (*U*-test), the sign test with a null hypothesis (median = 0) for a paired-sample comparison, the Chi-squared test and the regression analysis were used in the study. The differences were considered significant when $P < 0.05$. The graphs were plotted by means of a SigmaPlot 2000 program and Statgraphics Plus 5.1.

RESULTS

The influence of the removal of the tumor on antibody levels

To investigate the effect of the surgical removal of the tumor on antibody levels, the serum samples taken before and after surgery were analyzed. The subtracted values of the postoperative minus preoperative level of antibodies varied and showed an abnormal distribution, however, were mostly positive. The median of differences was also positive (Tables 1 and 2). A sign test for a paired-sample comparison showed the postoperative level of TF- and Tn antibodies to have significantly increased in patients with gastric cancer. Patients with gastric cancer, whose postoperative level of TF- and Tn antibodies was higher than the preoperative one, predominated significantly over those having a lower postoperative level (Table 1, $P < 0.05$). In gastrointestinal cancer, the TF antibody level was found to have elevated significantly after the removal of G3 tumors as compared with preoperative level (median 1.42 and 1.23, respectively, $u = 278.5$, $P < 0.05$). The elevation of the level of anti-Tn and αGal IgG after surgery was not significant in U-test owing to the variation in levels. However, the paired-sample sign test demonstrated a significant (more than 75%) predomination of patients having a postoperative elevation of TF, Tn or αGal antibody level over those having a lower postoperative level after resection of G3 tumors. These differences were not significant in patients with G1 and G2 resected tumors (Table 2). The level of all three antibodies was increased after surgery in 12% (mainly patients having G3 tumors) and reduced in 4% of patients with gastrointestinal cancer. The change of antibody levels was not associated with the transfusion of erythrocytes after surgery.

The postoperative follow-up

The clustered distribution of the combined pre- and postoperative level of antibodies was observed. The clusters were separated with the following cut-off values: TF 1.26, 1.53; Tn 1.88, 2.38; αGal 2.18; 2.80. Patients whose level of antibodies was higher than the first cut-off value were considered responders. The follow-up showed that the percentage of patients with G3 resected tumors of the digestive tract, whose individual mean level of anti-TF IgG remained above the cut-off value, exceeded significantly the percentage of patients with G1 + G2 resected tumors (Table 3). In the case of the other antibodies these differences were not observed.

In gastric or gastrointestinal cancer, the percentage of anti-TF IgG responders was significantly higher in stage Ⅰ than in stages Ⅲ-Ⅳ (Table 4). In gastric cancer without metastases (N0 in stages Ⅰ-Ⅱ *vs* N1 + N2) the percentage of anti-TF IgG responders tended to increase. The proportion of Tn- or αGal-responders was not increased in stage Ⅰ *vs* stages Ⅲ-Ⅳ of the disease.

It was established that despite the elevation of anti-TF IgG level after surgery of patients with advanced cancer, its level during the follow-up varied only slightly, remaining mostly low (Figure 1).

The relation of antibody levels to the values of blood parameters

Linear regression analysis showed an existing correlation

Table 2 Postoperative changes of the level of antibodies in gastrointestinal tumor of different grades

Grade[1]	Detection of antibodies	Mean ± SD	Increase	Median of difference	Difference		*P* (sign test)
					+	-	
G1 + G2	TF, preoperative	2.03 ± 2.04					
(*n* = 24)	postoperative	2.43 ± 2.86	19.7%	0.05	15	9	
G3	TF, preoperative	1.84 ± 1.71					
(*n* = 27)	postoperative	3.03 ± 3.20	64.7%	0.29	22	5	< 0.01
G1 + G2	Tn, preoperative	2.46 ± 1.80					
(*n* = 30)	postoperative	2.99 ± 2.45	21.5%	0.12	18	12	
G3	Tn, preoperative	2.45 ± 2.18					
(*n* = 25)	postoperative	3.15 ± 2.92	28.6%	0.42	19	6	< 0.05
G1 + G2	αGal, preoperative	4.10 ± 2.55					
(*n* = 32)	postoperative	4.62 ± 3.20	12.7%	0.17	20	12	
G3	αGal, preoperative	5.09 ± 3.34					
(*n* = 32)	postoperative	6.61 ± 3.70	29.9%	1.20	24	8	< 0.01

[1]G1+G2-well-and moderately-differentiated carcinomas.

Table 3 The percentage of patients with the mean level of anti-TF IgG above the cut-off value (1.53) by histological grading: The postoperative follow-up study

Cancer	G1 + G2		G3		χ^2	*P*
	n	> cut-off	*n*	> cut-off		
Gastric, all	22	9.1%	38	26.3%	2.58	0.108
Gastric, without metastases (N0)	16	6.2%	22	31.8%	3.64	0.056
Gastrointestinal, all	47	8.5%	47	23.4%	3.89	0.049
Gastrointestinal (N0)	35	5.7%	26	26.9%	5.34	0.021

Table 4 The percentage of patients with the mean level of anti-TF IgG above the cut-off value of 1.26 (responders) in relation to cancer progression: The postoperative follow-up study

Cancer	*n*	> cut-off	χ^2	*P*
Gastric, N0	38	39.5%	2.92	0.088
Gastric, N1 + N2	22	18.9%		
Gastric, stage Ⅰ	24	41.7%	4.71	0.03
Gastric, stages Ⅲ-Ⅳ	18	11.1%		
Gastrointestinal, stage Ⅰ	29	37.9%	4.11	0.043
Gastrointestinal, stages Ⅲ-Ⅳ	28	14.3%		

between the level of anti-TF IgG and the count of lymphocytes (Figure 2). As well, the correlation between the level of anti-Tn IgG and the serum level of tumor marker CA 19-9 was established: $r = 0.481$, $P = 0.011$, $n = 27$ (Tn-responders); $r = 0.495$, $P = 0.002$, $n = 36$ (all patients). No correlation between the level of antibodies and the values of other biochemical or hematological parameters was established.

Skin test

No positive delayed-type hypersensitivity reaction in skin test challenges with TF-PAA in any of the fifteen patients, including those with a high level of anti-TF IgG, was observed. In some cases the weak erythema did not exceed 3 mm. Neither side effects nor complications were recorded.

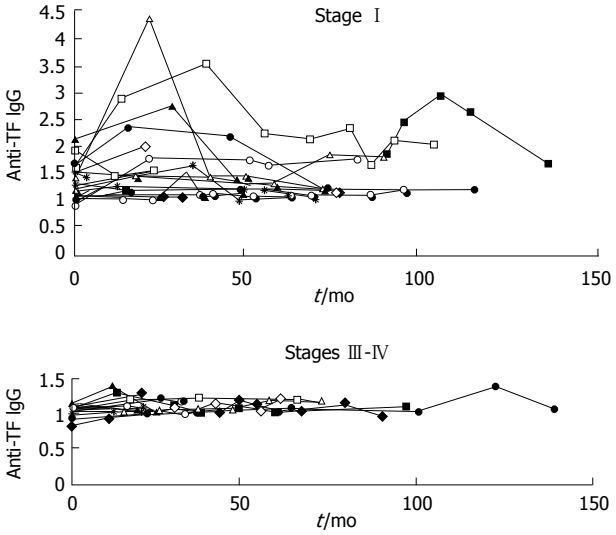

Figure 1 Changes of the level of anti-TF IgG in gastric cancer in stages Ⅰ and Ⅲ-Ⅳ during follow-up.

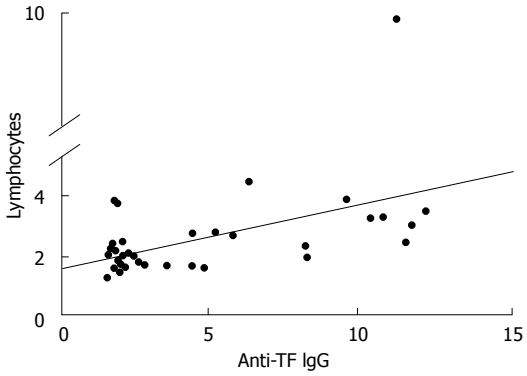

Figure 2 The correlation between the level of anti-TF IgG and the count of lymphocytes (10^9/L) in gastrointestinal cancer patients, whose anti-TF IgG level exceeded a cut-off value of 1.53. $r = 0.517$, $P = 0.001$, $n = 37$, $y = 0.215x + 1.380$.

DISCUSSION

The investigation of the level of serum IgG antibodies to tumor-associated carbohydrate epitopes (TF, Tn) and xenogeneic for human αGal epitope has been carried out earlier. The preoperative level of TF and Tn antibodies

was significantly lower in patients with gastrointestinal cancer, including stage I, than in blood donors, whereas the difference in the level of αGal IgG between cancer patients and blood donors was not significant[8]. The preoperative level of anti-TF IgG antibodies in patients with gastric carcinoma having regional lymph node metastases was found to be significantly lower than that in patients without metastases[10].

In gastrointestinal cancer the level of TF antibodies in responders with regional metastases was increased after surgery but the follow-up showed the percentage of TF-responders to be significantly higher among patients with cancer in stage Ⅰ (Table 4). This is due to a minor and short-term postoperative elevation of the antibody level in the serum of patients with lymph node metastases in stages Ⅲ-Ⅳ of the disease. The level of anti-TF IgG in the serum of five patients with gastrointestinal cancer in stages Ⅱ-Ⅳ, which was high prior to surgery rose even more after surgery and remained high for a long time (not shown). However, in most patients the TF IgG immune response in the terminal stages of the disease was suppressed whereas in stage Ⅰ both the stimulation and suppression of the immune response took place (Figure 1). Since with cancer progression no significant differences were observed in the level of Tn and αGal antibodies, the immunosuppression associated with advanced cancer concerns TF antibodies mainly. In this study, the level of αGal antibodies was determined because their low level may be indicative of humoral immunodeficiency disorders[17]. However, the immunosuppression associated with advanced gastrointestinal cancer appeared not to affect the level of αGal antibodies. In gastric cancer the higher level of anti-αGal IgG was observed in patients with a larger tumor[10]. In patients with pancreatic cancer, who often suffer from severe immunosuppression[18], a high level of anti-αGal IgG was observed as well.

The positive correlation between the level of TF antibodies and the count of lymphocytes in TF-responders (n = 57, r = 0.400, P = 0.002) appeared to reflect the adaptive immune response and provided a further explanation for the involvement of anti-TF IgG in cancer-associated immunosuppression. In patients whose level of anti-TF IgG was higher than the second cut-off value (1.53, Figure 2) this correlation was more pronounced. However, during the follow-up of some patients no such correlation was observed. This is indicative of the existence of a complex relationship between both the parameters which depend on pathological conditions. A decreased preoperative count of lymphocytes and postoperative surgery-related lymphocytopenia occurred in patients with gastrointestinal cancer. Besides, the T helper deficiency was more frequently observed in patients with regional nodal metastases[18].

The above findings are supportive of the idea that patients with a disease of an early stage and its minimal residue after surgery are more responsive to active immunotherapy. The pre- and postoperative level of TF-antibodies, the pattern of TF-expression in tumor, and the individual profile of immune response to the immunization with TF-antigen should be taken into account when selecting the contingent for immunotherapy.

The postoperative elevation of the level of carbohydrate-specific antibodies, viz. Forssman antibodies, has been documented earlier[19,20]. In our study, the postoperative elevation of antibody levels was found to be related to the low-differentiated carcinoma. Thus, the levels of all carbohydrate-specific antibodies, including those investigated earlier[21], were elevated in most patients after the surgical removal of G3 tumors, while in patients with G1 and G2 resected tumors, the differences in antibody levels before and after surgery were insignificant (Table 2).

In the preoperative examination of patients with gastrointestinal cancer, significant differences were established in the level of anti-TF IgG between them, namely, its lower level was associated with a lower-differentiated carcinoma in stages Ⅰ-Ⅱ[10]. These lower levels were increased significantly after surgery as shown in the present study. Moreover, the significant preponderance of patients with an elevated postoperative level of anti-TF IgG in the case of G3 resected tumors over those with G1 + G2 resected tumors was observed during the follow-up.

In general, the study shows that in most patients with the low-differentiated carcinoma the lower preoperative level of TF antibodies increases after surgery and has a tendency to remain elevated in the early stages of the cancer.

Taken together, the above results may be interpreted as follows: (1) A specific suppressive influence of the tumor on the production of TF antibodies is associated with the stage and grade of the tumor; the surgical removal of the primary tumor (especially G3 tumors) with lymphadenectomy may reverse the suppression of TF antibodies in the early stage of the disease; (2) The mainly low-differentiated carcinoma has an unspecific suppressive influence on the production of anti-carbohydrate antibodies. This influence may also be reversed by the removal of the tumor.

The specificity of human anticarbohydrate antibodies and their natural targets have been poorly studied. Natural anti-carbohydrate immunoglobulins are mostly antibodies of IgM-class. The high level of anti-TF and Tn IgG observed in some patients with cancer may be a sign of an acquired immune response which is indicative of the switching of antibody to the IgG-class. The anti-TF and anti-Tn IgG were affinity-purified from the serum of patients by using synthetic TF- and Tn-PAA sorbents[22,23]. TF antibodies demonstrated a high activity of binding to mucins isolated from human malignant tumors, but only in 15% of tumor extracts, whereas the high activity of Tn antibodies was not observed. The analysis of the specificity of purified anti-TF IgG, the mutual and complete inhibition of serum antibodies by TFα and TFβ conjugates, and a good correlation between the levels of anti-TFα and anti-TFβ IgG in sera manifest that human anti-TF IgG is specific to both TFα and β anomers, with preference to the latter[23]. In this respect, antibodies resemble human monoclonal antibodies, which are able to bind different carcinoma cell lines and immunostained

mucin-related tumor tissues[24].

The level of anti-Tn IgG is correlated with that of CA 19-9 in the serum. This seems to be unusual because the CA 19-9 antigen (SiaLe^a) differs structurally from Tn and contains no GalNAc residues. The correlation is not due to the cross-reactivity of antigens because the affinity-purified anti-Tn IgG did not react with SiaLe^a-PAA in the ELISA. This correlation may be explained by a similar relationship between both independent parameters and pathological conditions. Cancer progression or disorders of excretion (cholestasis and other disorders) may provoke the elevation of the level of CA 19-9[25] and Tn antibodies, respectively. Since the tumor marker CA 19-9 is a prognostic factor in colorectal cancer[26], the correlation between parameters may be indicative of the possible prognostic significance of antibodies as well.

Synthetic TF and Tn glycoconjugates deserve to be studied from a viewpoint of development of anti-cancer vaccines[12,13]. Synthetic PAA-glycoconjugates may be promising preparations because they have been described well and can be modified by epitope density or supplemented with the amplifier of immune response and synthesized in necessary quantities. The results of the skin test performed by the authors show the TF-PAA to be a safe and non-toxic glycoconjugate. The lack of the delayed-type hypersensitivity reaction indicates that the TF antibody response took place through the T-cell independent mechanism, which is typical of carbohydrate antigens.

The high preoperative level of anti-TF as well as anti-MUC1 IgG was closely associated with the survival of patients with gastric cancer[11]. However, the possible protective mechanism of TF antibodies in cancer has yet remained unclear. The TF antigen seems to play a crucial role in the adhesion of cancer cells to the endothelium through the interaction of galectin-3[27,28]. We suppose that even if TF antibodies are not cytotoxic for TF-expressed tumor cells, they may exhibit an anti-adhesive effect by blocking the TF- galectin-3 mediated metastatic spread. Whether anti-TF and anti-Tn IgG are prognostic factors and how their level in the follow-up is associated with survival will be shown by further investigations.

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COMMENTS

Background

Tumor-associated carbohydrate antigens (TACA), the Thomsen-Friedenreich (TF) antigen and Tn are frequently expressed in malignant tumors. Human blood serum contains TF- and Tn antibodies but it is not yet clear which role antibodies play in the natural anti-cancer defense system. The TF antigen seems to play a crucial role in the metastatic mechanism due to adhesion of cancer cells to the endothelium. The high level of TF antibodies in the serum may be favourable if antibodies could block the TF- mediated metastatic spread. The level of TF- and Tn antibodies is significantly decreased in the serum of primary (not operated) patients with cancer. The high level of anti-TF IgG in the serum of primary patients with gastric cancer is closely associated with survival.

Research frontiers

The relationship of the immune system with the tumor is far from a clear understanding. Cancer immune surveillance is considered to be important in the anti-tumor protection of the host. The growing tumor escapes the immune control under the immunosuppressive conditions. The surgical removal of the tumor may reverse the immunosuppression.

Innovations and breakthroughs

The dynamic changes of the level of TF- and Tn-antibodies in the serum of patients with cancer have been insufficiently studied. Investigations have mainly focused on short-term clinical trials of antigen-specific immunotherapy. Authors have undertaken a long-term follow-up of cancer patients to determine changes in the postoperative level of TF- and Tn antibodies, as well as to elucidate the association of this level with the progression of cancer, and survival. The association of the level of antibodies with the stage and morphology of the tumor, as well as values of blood parameters was studied. Also, the delayed-type hypersensitivity reaction to TF- polyacrylamide (PAA) conjugates in skin testing was examined.

Applications

The patients with a disease of an early stage may be more responsive to TF-specific active immunotherapy. Synthetic TF and Tn glycoconjugates deserve to be studied from a viewpoint of development of anti-cancer vaccines and in diagnostic aims.

Terminology

The epitope is an antigenic determinant, viz. saccharide. The PAA glycoconjugate is a polymeric molecule with covalently bound saccharide. The anomer (α or β) is a spatial structure of saccharides.

Peer review

This is interesting and provocative work. The authors present most of their results in terms of changes in antibody levels. It would be important for them to state what the basal level of these antibodies is, and to give us some idea of what percentage change is represented.

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RAPID COMMUNICATION

Procedure-related musculoskeletal symptoms in gastrointestinal endoscopists in Korea

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Abstract

AIM: To determine the prevalence and risk factors of work-related musculoskeletal disorders in gastrointestinal endoscopists in Korea.

METHODS: A survey of musculoskeletal symptoms, using a self-administered questionnaire, was conducted on 55 endoscopists practicing in general hospitals or health promotion centers.

RESULTS: Forty-nine (89.1%) endoscopists reported musculoskeletal pain on at least one anatomic location and 37 (67.3%) endoscopists complained of pain at rest. Twenty-six (47.3%) endoscopists had severe musculoskeletal pain defined as a visual analogue score greater than 5.5. Factors related to the development of severe pain were (1) standing position during upper endoscopy, (2) specific posture/habit during endoscopic procedures, and (3) multiple symptomatic areas. Finger pain was more common in beginners, whereas shoulder pain was more common in experienced endoscopists. Sixteen percent of symptomatic endoscopists have modified their practice or reduced the number of endoscopic examinations. Only a few symptomatic endoscopists had sought professional consultation with related specialists.

CONCLUSION: The prevalence of musculoskeletal pain in endoscopists is very high. The location of pain was different between beginners and experienced endoscopists. Measures for the prevention and adequate management of endoscopy-related musculoskeletal symptoms are necessary.

Key words: Endoscopy; Endoscopist; Musculoskeletal symptom

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INTRODUCTION

Muscle and joint pains are common complaints in individuals whose job requires repetitive isometric maneuvers or awkward body positions[1]. Musculoskeletal pains have been reported in individuals with different occupations such as bus drivers, unskilled laborers,

musicians, physical therapists, and computer keyboard operators[1-7]. Recently, ergonomic mechanisms related to the development of work-related musculoskeletal disorders (MSD) have drawn substantial interest[8-10].

Work-related musculoskeletal symptoms are common in certain medical specialists such as laparoscopic surgeons and dentists[11,12]. However, there are very few studies on MSD in gastrointestinal endoscopists. The incidence of musculoskeletal injuries has been variously reported from as low as 13% for neck pain[13] to as high as 57% for back pain[14]. To the best of our knowledge, a detailed study in endoscopists' on the severity of musculoskeletal symptoms, symptom-related risk factors, and doctor's response to their own symptoms has not been performed in eastern countries. The purpose of the present study was to assess the prevalence, severity, risk factors, and clinical impact of work-related MSD in gastrointestinal endoscopists in Korea.

MATERIALS AND METHODS

From June 2006 to September 2006, 55 endoscopists practicing in 4 general hospitals and 2 health promotion centers were included in the present study. Endoscopists expressed their willingness to participate and completed a self-reported questionnaire. The questionnaire was largely structured; although some questions were kept open. Data were collected on the age, gender, duration of endoscopy practice, underlying musculoskeletal disease, and postures and habits during endoscopy. Workload parameters included total duration of endoscopy practice, weekly working hours and monthly number of endoscopic procedures. The areas of musculoskeletal pain were marked on a figure of the human body, and the severity of pain at each site was expressed using a 100 mm visual analogue scale (VAS), a standard measurement tool in pain research. The presence of severe pain was defined as a VAS value greater than 55 mm[15,16]. The participants were also asked as to whether their symptoms affected their ability to perform endoscopic procedures, how they managed their symptoms, and whether they had endoscopy-related symptoms other than MSD.

Continuous data were expressed as mean ± standard deviation (SD) or median with range. Categorical data analysis was conducted using the chi-square test. Continuous data were analyzed using the independent *t* test. All *P* values were 2-tailed and *P* values less than 0.05 were considered statistically significant.

RESULTS

General characteristics of the endoscopists

Fifty-five endoscopists (male 37, female 18) participated in the study. The median age was 39 years (range, 28-47 years), and the median duration of practicing endoscopy was 39 mo (range, 1-228 mo). The average procedure time per week was 19.5 ± 7.7 h. The average number of endoscopies performed per month was 270.2 ± 153.2.

Eighty-three percent of the endoscopists reported possible endoscopy-related non-musculoskeletal symptoms, such as decreased visual acuity (63.6%), chronic fatigue (60%), depressive mood (18.2%), dizziness (14.5%), headache (12.7%), and skin allergy (1.8%).

Prevalence of musculoskeletal pain among the endoscopists

Forty-nine (89.1%) endoscopists reported musculoskeletal pain on at least one anatomic location. The average number of symptomatic areas was 3.9 ± 2.8. Forty endoscopists (72.7%) had pain at more than one anatomic location. Thirty-seven endoscopists (67.3%) had pain at rest. The VAS value of the most painful area was 5.4 ± 2.2. The musculoskeletal pain developed at 27.5 ± 37.9 mo (range, 1-156 mo) after starting endoscopy.

The location and incidence of musculoskeletal pain during endoscopic procedures or at rest are shown in Figure 1A. The most commonly reported painful area during endoscopic procedures was right shoulder, followed by left shoulder and left finger. There was little difference in the overall distribution of the painful areas at rest. However, neck pain and upper back pain were relatively frequent.

The most painful site during endoscopic procedures was the left finger (16.4%), followed by left shoulder and right wrist (14.5%), left wrist (9.1%) and right shoulder (7.3%). The left and right shoulder (12.7%) were the most painful areas at rest, followed by neck (9.1%), right upper back and lower back (5.5%).

Risk factors associated with severe musculoskeletal pain

Twenty-six endoscopists (47.3%) had severe musculoskeletal pain with a VAS value greater than 55 mm[15,16]. Three factors were statistically related to the development of severe musculoskeletal pain: (1) standing position during upper endoscopic procedures, (2) specific posture or habit during endoscopic procedures, and (3) multiple symptomatic areas (Table 1). The proportion of endoscopists with severe musculoskeletal pain was slightly higher in female than in male endoscopists (61.1% and 40.5%, respectively; $P = 0.152$).

Comparison between beginners and experienced endoscopists

Endoscopists were divided into two groups (beginner versus experienced) by the total duration of practicing endoscopy (39 mo, Table 2). In the beginner group, the weekly procedure time was longer and the number of endoscopic examinations was greater. However, there was no significant difference in the prevalence of musculoskeletal pain, number of symptomatic areas, and VAS value of the most painful area between the two groups.

By contrast, the location of pain was different between the two groups (Figure 1B and C). During a

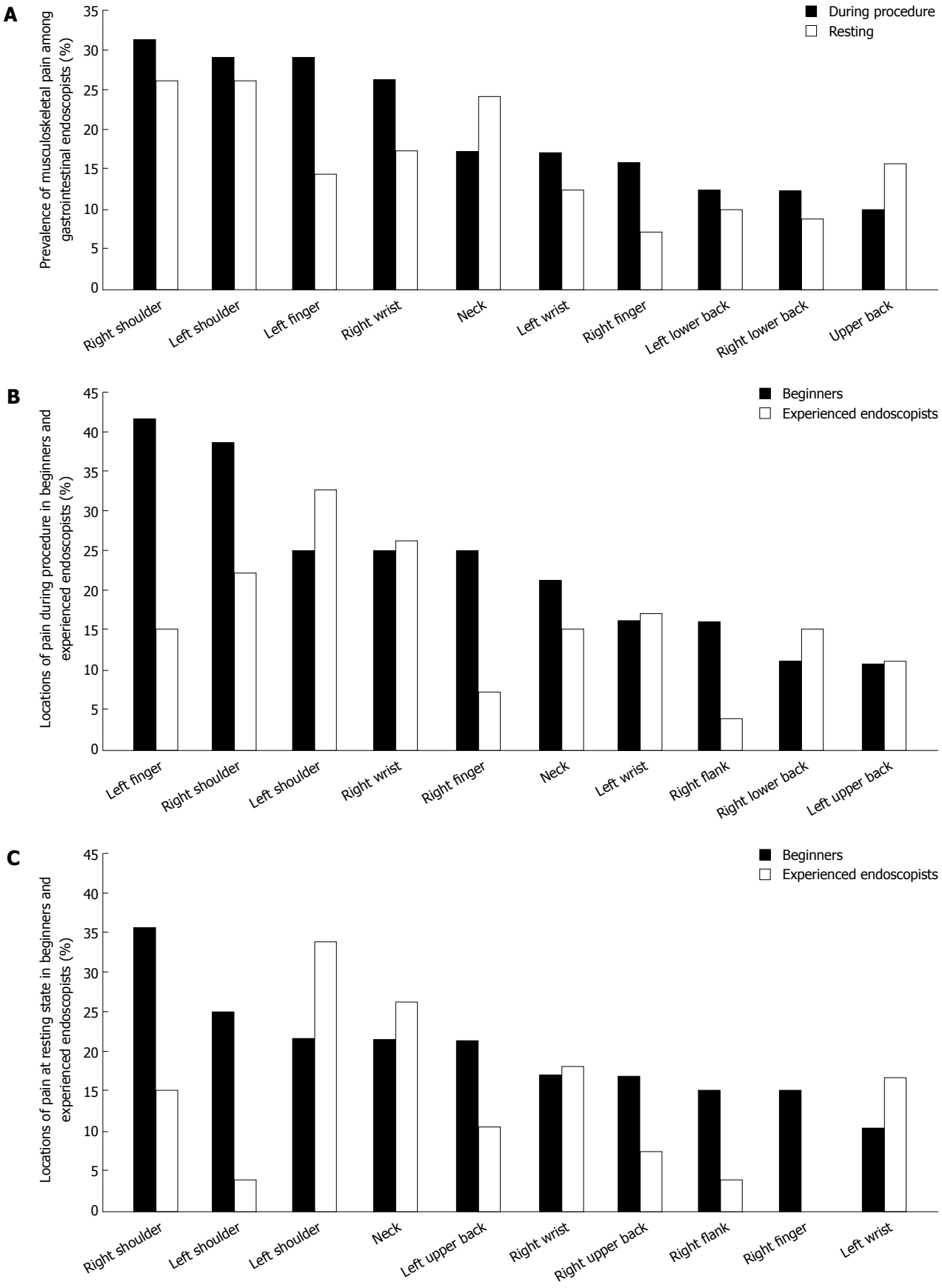

Figure 1 **A**: Prevalence of musculoskeletal pain in gastrointestinal endoscopists in Korea. Right shoulder pain was the most common symptom both during endoscopic procedures and at rest; **B**: Prevalence of musculoskeletal pain in beginners and experienced endoscopists during endoscopic procedures ($^{a}P < 0.05$); **C**: Prevalence of musculoskeletal pain in beginners and experienced endoscopists at rest.

procedure, the left finger was the most commonly reported painful area in beginners (42.9%, 12/28), whereas the

Table 1 Comparison of demographic and procedure-related variables between endoscopists with or without severe (VAS > 5.5) musculoskeletal pain

	With severe musculoskeletal pain (n = 26)	Without severe musculoskeletal pain (n = 29)	P
Age (median, range, yr)	34 (29-47)	36 (28-45)	
Sex			
Male	15	22	
Female	11	7	
Career			
Full time faculty	16	15	
Trainee	10	14	
Duration of practicing endoscopy (mean, range, mo)	40.5 (1-204)	39 (1-228)	
Posture in upper endoscopy			0.027
Mainly standing	25	21	
Mainly sitting	1	8	
Posture in colonoscopy			
Mainly standing	11	12	
Mainly sitting	15	17	
Specific posture or habit during endoscopy (%)	11 (68.8)	5 (31.3)	0.041
Number of endoscopies per month (mean ± SD)	280.5 ± 159.3	260.1 ± 149.7	
Time of endoscopic procedure per week (mean ± SD, h)	20.5 ± 8.1	17.9 ± 7.2	
Number of painful areas (mean ± SD)	4.8 ± 3.2	2.3 ± 2.1	0.002

Table 2 Comparison of musculoskeletal pain and endoscopic workload between beginners and experienced endoscopists

	Beginners (n = 28)	Experienced endoscopists (n = 27)	P
Pain over at least one anatomic location (%)	26 (92.8)	23 (85.2)	
Severe pain with VAS value > 5.5 (%)	15 (53.6)	13 (48.1)	
Multiple painful areas (%)	22 (78.6)	18 (66.7)	
Number of painful areas (mean ± SD)	3.9 ± 3.0	3.1 ± 2.9	
VAS value of the most painful area (mean ± SD)	4.9 ± 2.3	4.7 ± 3.1	
Duration of endoscopic procedure per week (mean ± SD, h)	23.7 ± 7.6	14.9 ± 5.2	0.001
Number of endoscopies per month (mean ± SD)	322.4 ± 159.9	216.1 ± 127.4	0.009

VAS: Visual analogue scale.

left shoulder was the most commonly reported painful area by experienced endoscopists (33.3%, 9/27) (Figure 1B). The distribution of the most painful area during endoscopic procedures was also different in the two groups. The most painful area was the left finger (21.4%, 6/28) in beginners, followed by right wrist (10.7%, 3/28), left shoulder (7.1%, 2/28) and left wrist (7.1%, 2/28). However, the most painful locations in experienced endoscopists were the left shoulder (22.2%, 6/27), followed by right wrist (18.5%, 5/27), left finger (11.1%, 3/27) and left wrist (11.1%, 3/27). The distribution of pain and the most painful areas at rest were very similar in the two groups (data not shown).

Response of endoscopists to the musculoskeletal pain

Among endoscopists with musculoskeletal pain, 16.3% (8/49) reported that they had modified their practice or reduced the number of the endoscopic procedures. A majority (81.6%) of endoscopists with musculoskeletal pain managed their symptoms by themselves using stretching (67.3%), exercising (8.2%) and rest (6.1%). Fourteen endoscopists (28.6%) reported the use of medications such as nonsteroidal anti-inflammatory drugs or topical analgesic patches.

Only 14.3% (7/49) of symptomatic endoscopists had sought advice from specialists on musculoskeletal disorders or had undergone a specific diagnostic work-up. Three endoscopists were diagnosed to have a sprain (n = 1) or a cervical intervertebral disk herniation (n = 2). In the two endoscopists with herniated disc, one required a 4-wk sick leave until the symptoms improved and the other had modified the practice and reduced the number of endoscopic procedures.

DISCUSSION

Work-related MSDs or overuse syndrome are a group of diseases resulting from repetitive action at the work place[17]. Collagen failure and connective tissue damage results in inflammation, pain, and further weakening of the tissues. Such a vicious cycle can lead to permanent injury and disability if the tissues are not allowed to heal properly[13]. Work-related injury is an important cause of missed workdays and impaired performance at work.

We observed that MSD is very prevalent in gastrointestinal endoscopists in Korea. A majority of endoscopists experienced pain at multiple anatomic areas (72.7%) and two-third (67.3%) complained of the pain at rest. The prevalence of MSD observed in the present study is higher than that noted in previous reports by other groups[13,14]. One possible explanation is that the endoscopic workload of the doctors in the present study

was very high. In this regard, a previous study reported that the endoscopic volume measured in terms of hours per week, number per week, or percentage of working time was strongly associated with the development of musculoskeletal injuries among endoscopists[13]. Workload-associated factors such as the number or duration of work were also related to the prevalence of MSD in other occupations[1,3,18].

In the present study, 26 (47.3%) endoscopists complained of severe musculoskeletal pain, defined as a VAS value greater than 5.5 (Table 1). Endoscopists with severe pain were more likely to have multiple painful areas than those without severe pain. Contrary to our expectations, there was no significant difference in the endoscopic workload between them. This may be due to the small number of participants of the present study. However, ergonomic factors like bad posture during the procedure may be important in the development of endoscopy-related musculoskeletal symptoms. With respect to this hypothesis, we observed that ergonomic factors, such as specific posture/habit during the procedure and standing position in upper gastrointestinal endoscopy, correlated significantly with the development of severe musculoskeletal pain.

In the present study, the experience level of endoscopists was not related to the prevalence of musculoskeletal pain, the number of symptomatic areas, and the severity of the most painful regions. However, there were differences in the distribution of the symptomatic areas (Figure 1B and C). For example, finger pain was more common in beginners, whereas shoulder pain was more common in experienced endoscopists. This was especially true when the endoscopist was performing a procedure. The exact reason of this difference is unclear, but muscles and/or joints frequently used during an endoscopic procedure may differ with the experience of the endoscopist. In this respect, it should be noted that beginners tend to depend more on the movement of the knob during endoscopy.

The best approach to MSD must be preventive. As noted in a previous report[13], endoscopists in the present study tended to neglect or tried to alleviate their symptoms by themselves without resorting to professional help. However, we found that a small proportion (16.3%) of symptomatic endoscopists modified the procedure patterns or reduced the number of endoscopic procedures. The overall efficacy of an endoscopy unit may be negatively influenced by these factors. Measures for the prevention of work-related MSD may improve the productivity of healthcare institutions. The importance of ergonomics in work-related MSDs has been studied in a certain fields[19,20]. Endoscopists should also take advantage of such studies.

A major limitation of our study was that it was difficult to determine whether a particular symptom was related to endoscopy or not. This was related to our study design using a self-administered questionnaire without an objective assessment of the symptoms. However, we presumed that a great proportion of the symptoms in the present study were endoscopy-related. Another limitation was that the participants were working in either general hospitals or health promotion centers, where the volume of endoscopic procedures exceeds the average endoscopic workload. About one-half of endoscopies, in general, were performed under sedation, the proportion of upper to lower endoscopy was approximately three to two, and that of therapeutic endoscopies was almost twenty percent, although this aspect was not investigated in the present survey. Finally, there was no follow-up data in the present study. Large-scaled follow-up studies in various clinical settings are needed.

In conclusion, the prevalence of musculoskeletal symptoms in endoscopists is very high, and the majority of symptomatic endoscopists do not seek professional consultation. The pattern of musculoskeletal pain among beginners and experienced endoscopists was different, suggesting multiple ergonomic mechanisms for symptom development. Measures for the prevention and adequate management of endoscopy-related musculoskeletal symptoms are necessary.

COMMENTS

Background

Work related musculoskeletal disorder (MSD) is a common problem in individuals whose job requires repetitive isometric maneuvers or awkward body positions. However, the prevalence of MSD among endoscopists is not well known. The present study was designed to investigate the prevalence and risk factors of work-related MSD in gastrointestinal endoscopist in Korea.

Research frontiers

We investigated the incidence, severity, location and clinical impact of work-related MSD during endoscopic procedures and at rest in gastrointestinal endoscopists. The present study is the first detailed study designed to investigate MSD in gastrointestinal endoscopists in eastern countries.

Innovations and breakthroughs

We used a self reported questionnaire in 55 endoscopists practicing in 4 general hospitals and 2 health promotion centers. The severity of MSD was assessed by the visual analogue scale, a standard measurement tool in pain research.

Applications

In the present study, the prevalence of musculoskeletal symptoms among endoscopists was very high, and the majority of symptomatic endoscopists did not seek professional consultation. The pattern of musculoskeletal pain in beginners and in experienced endoscopists was different, suggesting multiple ergonomic mechanisms for symptom development. We hope the present study would lead to interest in work-related MSD and ergonomics in gastrointestinal endoscopists.

Peer review

The present study has shown that the development of severe pain in gastrointestinal endoscopists is related to ergonomic factors, such as specific posture/habit and the standing position during endoscopic procedures. The study suggests that endoscopists need to focus on teaching a beginner about proper posture and manipulation techniques in order to prevent musculoskeletal symptoms.

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RAPID COMMUNICATION

Resting energy expenditure and glucose, protein and fat oxidation in severe chronic virus hepatitis B patients

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Abstract

AIM: To study and determine the resting energy expenditure (REE) and oxidation rates of glucose, fat and protein in severe chronic hepatitis B patients.

METHODS: A total of 100 patients with liver diseases were categorized into three groups: 16 in the acute hepatitis group, 56 in the severe chronic hepatitis group, and 28 in the cirrhosis group. The REE and the oxidation rates of glucose, fat and protein were assessed by indirect heat measurement using the CCM-D nutritive metabolic investigation system.

RESULTS: The REE of the severe chronic hepatitis group (20.7 ± 6.1 kcal/d per kg) was significantly lower than that of the acute hepatitis group (P = 0.014). The respiratory quotient (RQ) of the severe chronic hepatitis group (0.84 ± 0.06) was significantly lower than that of the acute hepatitis and cirrhosis groups (P = 0.001). The glucose oxidation rate of the severe hepatitis group (39.2%) was significantly lower than that of the acute hepatitis group and the cirrhosis group (P < 0.05), while the fat oxidation rate (39.8%) in the severe hepatitis group was markedly higher than that of the other two groups (P < 0.05). With improvement of liver function, the glucose oxidation rate increased from 41.7% to 60.1%, while the fat oxidation rate decreased from 26.3% to 7.6%.

CONCLUSION: The glucose oxidation rate is significantly decreased, and a high proportion of energy is provided by fat in severe chronic hepatitis. These results warrant a large clinical trail to assess the optimal nutritive support therapy for patients with severe liver disease.

Key words: Chronic severe viral hepatitis; Energy metabolism; Respiratory quotient; Malnutrition

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INTRODUCTION

The liver plays a pivotal role in controlling carbohydrate metabolism by maintaining glucose concentrations in the normal range, as well as in protein and fat metabolism. This is achieved by a tightly regulated system of enzymes and kinases that regulate nutrient breakdown and synthesis in hepatocytes. Many studies have shown that cirrhotic patients have nutrient and energy metabolism imbalance, which lead to malnutrition and seriously affect their prognosis[1,2]. On multivariate regression analysis, the Child-Pugh's score is a good independent predictor of malnutrition. With respect to energy metabolism, there is no consensus on energy expenditure, but there is a consensus that respiratory quotients are lower in liver cirrhosis patients than in healthy subjects. Therefore, resting energy expenditure (REE), calorie intake, and energy balance should be routinely assessed in cirrhotic patients in order to identify hypermetabolic and hypometabolic patients. The nutritional and metabolic parameters in these patients are indispensable for design-

ing and prescribing personalized nutritional strategies for the treatment of muscle malnutrition, which can thus improve their morbidity and mortality rates[3]. Protein-energy malnutrition is frequently observed in liver cirrhosis patients; disorders of protein metabolism and energy metabolism are closely correlated with protein-energy malnutrition. It has been shown that, in protein metabolism disorders, the synthesis and degradation rates of albumin decreased and the serum half-life of albumin became longer[4]. These changes are closely correlated with the prognosis of cirrhosis patients. Therefore, it is important for clinicians to identify and treat metabolic disorders in liver cirrhosis patients. Currently, it is thought that supplementation with branched-chain amino acids (BCAAs) is useful for improving protein metabolism disorders, and that a late evening snack (LES) improves the catabolic state of advanced liver cirrhosis patients. Long-term oral supplementation with a BCAA mixture was found to be better than an ordinary diet[5,6]. Recently, it was shown that the ingestion of a 200 kcal rice LES can improve nutritional metabolism in cirrhosis patients. Furthermore, a short course of recombinant human growth hormone (rhGH) and insulin-like growth factor- I (IGF- I) raised albumin levels and tended to improve energy metabolism in liver cirrhosis patients. The exogenous administration of IGF- I has hepatoprotective and antifibrogenic actions in experimental liver cirrhosis[7-11]. Therefore, proper nutritional support is very important in promoting the recovery of liver disease patients. However, the characteristics of the energy metabolism of severe chronic hepatitis patients are not clear. Thus, the aim of the present study was to explore the characteristics of the energy metabolism of severe chronic hepatitis patients so as to provide data that could be used for optimal nutritional support.

MATERIALS AND METHODS

Subjects

One hundred patients with liver diseases were enrolled from July 2006 to September 2007. They were categorized into 3 groups according to their disease recovery stage: acute hepatitis group (n = 16; hepatitis A, n = 10; hepatitis E, n = 6), severe chronic hepatitis B-related hepatitis group (n = 56), and hepatitis B-related cirrhosis group (n = 28, Table 1). Of the severe chronic hepatitis B-related hepatitis patients, 14 patients (10 males, 4 females), whose status changed from the acute severe stage to the recovery stage within 8 wk, were randomly selected for assessment twice. The patients' average age was 42.5 years (range, 36-62 years). The patients all met the diagnostic criteria of the "Symposium on Viral Hepatitis and Liver Diseases in 2000"[12]. Briefly, the severe chronic hepatitis was defined based on the following inclusion criteria: (1) a history of chronic hepatitis or liver cirrhosis; (2) severe asthenia and a serum total bilirubin more than 171 μmol/L; and (3) prothrombin time activity (PTA) less than 60%. All enrolled patients had not received nutritional support and rational regime treatment, anti-viral agents and steroid. The study was explained to the patients and/or their relatives, and written informed consent was obtained. The study was approved by the Ethics Committee of the Beijing You'an Hospital of Capital Medical University.

REE and glucose, protein and fat oxidation rates

The REE and the carbohydrate, protein and fat oxidation rates were determined by indirect heat measurement. The REE was determined using the CCM-D nutrition metabolism investigation system (Medgraphics Company, United States). The average O_2 amount consumed and the CO_2 amount produced per minute by the subjects were used to calculate the actual REE using the Weir formula; subsequently, the respiratory quotient, the ratio of the average O_2 amount consumed to the CO_2 amount produced per minute by the subjects, was calculated. Twenty-four-hour urine samples were collected from all subjects for the determination of the urea nitrogen level using the HITACHI7170 automatic biochemistry analyzer (Japan). The protein oxidation rate, non-protein-respiratory quotient (npRQ), the carbohydrate (CHO) and the fat oxidation rates were calculated from the collected data using a computer program. The predicted REE value was calculated using the Harris-Benedict formula based on the subject's height, weight and age.

The subjects were required to fast for at least 8 h prior to testing. In order to avoid muscle activation; they stayed in bed in the morning for at least 30 min. The room temperature was kept between 24℃ and 26℃, with a humidity of 45%-60%. The volume and gas were calibrated for the CCM-D nutrition metabolism investigation system; and the subject's data on height, weight, gender and age were put into the system. The value of energy metabolism may be related to body weight. Therefore, the REE/kg of all patients were also evaluated.

Liver function assessment

Serum samples were collected from all subjects, and the following liver function indices were determined by the HITACHI7170 automatic biochemistry analyzer (Japan): alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TBIL), albumin (ALB), proalbumin (PA), cholinesterase (CHE), and cholesterol (CH).

Statistical analysis

The data is presented as mean ± SD. The means were compared between groups using variance analysis. Data from severe virus hepatitis patients in the severe stage and the recovery stage were compared using the paired t test. SPSS 10.0 statistical software was used for all analyses. $P < 0.05$ was considered statistically significant.

RESULTS

Energy metabolism characteristics

The REE of the severe chronic hepatitis group and the

Table 1 Clinical characteristics of the patients

Group	*n*	Age (yr)	Male/Female	ALT (U/L)	AST (U/L)	T-Bil (mg/dL)	ALB (g/L)	PA (g/L)	CHE (g/L)	CH (g/L)
1	16	34 ± 16	10/6	154.3 ± 184.8	68.3 ± 58.4	4.4 ± 6.4	36.6 ± 4.4	142.0 ± 66.0	6050.9 ± 2243.6	164.9 ± 49.4
2	56	46 ± 13	49/9	189.2 ± 374.9	156.8 ± 159.4	20.4 ± 9.8	29.8 ± 4.6	52.5 ± 27.5	2723.6 ± 1422.2	52.3 ± 36.2
3	28	53 ± 10	24/4	68.3 ± 118.9	80.2 ± 80.6	5.8 ± 7.2	29.5 ± 7.2	62.2 ± 34	2628.6 ± 1416.3	114.9 ± 42.7

Group 1: Acute hepatitis group; Group 2: Chronic severe hepatitis group; Group 3: Cirrhosis group. PTA: Prothrombin activity. PTA = (control PT-8.7)/(patients PT-8.7) × 100%.

Table 2 REE and oxidation rates in different groups of patients with liver disease (mean ± SD)

Group	REE (kcal/d)	REE/kg (kcal/d)	RQ	npRQ	CHO (g/d per kg)	Fat (g/d per kg)	Protein (g/d per kg)
1	1586.7 ± 783.0	25.8 ± 11.3	0.90 ± 0.05	0.92 ± 0.21	3.8 ± 2.1	0.5 ± 0.5	1.1 ± 0.6
2	1388.5 ± 334.5	20.7 ± 6.1	0.84 ± 0.06[a]	0.88 ± 0.18	1.8 ± 1.0[a]	0.9 ± 0.7	0.9 ± 0.6
3	1317.9 ± 266.3	19.8 ± 3.6	0.88 ± 0.08	0.91 ± 0.09	2.7 ± 1.4	0.9 ± 2.0	1.0 ± 0.6
P value	0.126	0.014	0.001	0.441	0	0.721	0.525

Group 1: Acute hepatitis group; Group 2: Chronic severe hepatitis group; Group 3: Cirrhosis group. [a]$P < 0.05$, 2 *vs* 1 and 3 *vs* 1.

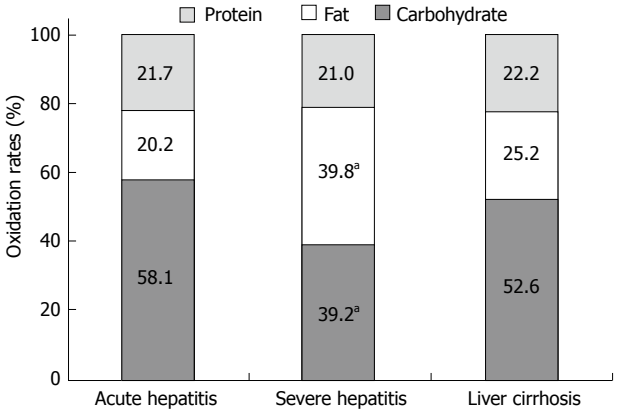

Figure 1 Oxidation rates of protein, carbohydrate and fat in the severe hepatitis group, the acute hepatitis group, and the cirrhosis group. [a]$P < 0.05$, the acute hepatitis group *vs* the cirrhosis group. There is no significant difference between groups of severe hepatitis and liver cirrhosis.

acute hepatitis group was not significantly different from that of the cirrhosis group. However, the REE per kg (REE/kg) of the acute hepatitis group was significantly higher than that of the chronic severe hepatitis group and the cirrhosis group ($P = 0.014$), but the difference in the REE/kg between the chronic severe hepatitis group and the cirrhosis group was not significant. The respiratory quotient of the severe chronic hepatitis group (0.84 ± 0.06) was significantly lower than that of the acute hepatitis group and the cirrhosis group ($P = 0.001$). The respiratory quotients of the chronic hepatitis group (0.90 ± 0.05) and the cirrhosis group (0.88 ± 0.08) were not significantly different (Table 2).

The proportion of energy supplied by the three major substrates (carbohydrate, fat and protein) differed among the groups. Protein oxidation rates were not significantly different among the groups; they ranged from 21.0% to 22.2%. The carbohydrate oxidation rate of the severe hepatitis group (39.2%) was significantly lower than that of the acute hepatitis group and the cirrhosis group ($P = 0.048$). The fat oxidation rate of the severe hepatitis group (39.8%) was significantly higher than that of the acute hepatitis group and the cirrhosis group ($P = 0.01$). The carbohydrate and fat oxidation rates of the acute hepatitis group and the cirrhosis group were not significantly different ($P = 0.472$). Energy supplied by carbohydrate oxidation accounted for 50% or more of the total energy supplies (Figure 1). The actual consumptions per kg weight per day of protein and fat did not differ significantly among the three groups. However, the carbohydrate consumption per kg weight per day was highest in the acute hepatitis group and lowest in the chronic severe hepatitis group; there were significant differences among the groups in carbohydrate consumption ($P < 0.0001$, Table 2).

Energy metabolism differences between severe hepatitis patients at severe and recovery stages

The energy metabolism of the 14 patients with severe chronic hepatitis whose disease improved from the severe stage to the recovery stage was assessed twice. Four of these patients had been given growth hormone (4.5 IU/d for 2 wk). Significant improvement in the biochemical indices was seen in the patients at the recovery stage: ALT decreased from 561.2 ± 818.5 U/L to ALT 35.7 ± 9.37 U/L, T-Bil decreased from 17.4 ± 5.5 mg/dL to 5.3 ± 2.7 mg/dL, and PTA increased from 38.2% ± 29% to 72.7% ± 35.9%. After the improvement of liver function, the REE was not significantly changed compared to that obtained prior to the improvement; there was no significant change in the REE per kg weight. However, the carbohydrate oxidation rate increased from 41.7% to 60.1%, while the fat oxidation rate decreased from 26.3% to 7.6%. There was a non-significant trend for the respiratory quotient (RQ) value to increase ($P = 0.105$, Table 3).

DISCUSSION

The liver plays a unique role in carbohydrate metabolism by maintaining glucose concentration levels in the normal

Table 3 Dynamic determination of REE and oxidation rates in severe chronic hepatitis patients (n = 14, mean ± SD)

Patient	REE (kcal/d)	REE/kg (kcal/d per kg)	RQ	CHO (%)	Fat (%)	Protein (%)
Severe stage	1366.1 ± 140.7	19.1 ± 3.3	0.86 ± 0.05	41.7 ± 18.3	26.3 ± 27.2	32.1 ± 24.8
Recovery stage	1306.7 ± 497.8	17.9 ± 6.2	0.91 ± 0.06	60.1 ± 11.7	7.6 ± 38.6	32.3 ± 30.9
P value	0.761	0.638	0.105	0.075	0.232	0.990

range. This is achieved by a tightly regulated system of enzymes and kinases that regulate glucose breakdown and synthesis in hepatocytes. This process is under the control of glucoregulatory mediators, of which insulin plays a key role. Therefore, the liver is the major organ of substrate and energy metabolism. It has recently been noted that the energy metabolism of patients with end-stage liver diseases, such as cirrhosis, was altered compared to normal controls, they showed evidence of malnutrition, a high metabolism, a lower RQ value, and a relatively higher fat oxidation rate[2,4,6,11]. Long-term oral supplementation with a BCAA mixture is better than ordinary food taken as a late evening snack for improving the serum albumin level and the energy metabolism of cirrhosis patients[5,10].

Severe chronic hepatitis is a severe liver disease in which extended liver tissue necrosis caused by chronic viral hepatitis or hepatitis cirrhosis may lead to liver failure. Nutritional support is an important component of comprehensive therapy. However, the characteristics of the energy metabolism of severe chronic hepatitis patients have never been previously reported. The present study found that the energy metabolism of patients with severe chronic hepatitis had several unique characteristics.

First, the REE of severe chronic hepatitis patients was not significantly different from that of acute hepatitis and cirrhosis patients, who did not have increased energy metabolism. The REE per kg weight was similar in the severe chronic hepatitis group to that in the cirrhosis group, and both were lower than that in the acute hepatitis group. This may be due to the fact that the REE/kg value in the severe chronic hepatitis patients was lower than that in normal controls. This is different from the high energy metabolism condition found in cirrhosis patients, which is widely acknowledged. Tajika[2] found that energy metabolism was normal in 58% patients, and only 12% of patients had low energy metabolism. This can be related to the high energy metabolism of acute hepatitis patients. Plauth[6] showed that chronic hepatitis C patients had a high energy metabolism related to their viral load; and their high energy metabolism resolved with anti-viral treatment. Therefore, it is likely that high energy metabolism is related to the presence of acute hepatitis.

Second, severe the chronic hepatitis group had a notably higher fat oxidation rate than the acute hepatitis group and the cirrhosis group, and a lower glucose oxidation rate than the acute hepatitis group and the cirrhosis group. There was no difference in the protein oxidation rate among the three groups.

Third, the REE before and after liver function improvement in the severe hepatitis patients was compared. The REE of these patients was not changed, but they had a lower fat oxidation rate and a higher carbohydrate oxidation rate; however, these differences were not statistically significant, perhaps as a result of the small sample size. Recently, Plauth[6] showed that TIPS could improve energy metabolism and malnutrition in cirrhosis patients. Growth hormone treatment can improve the liver function and energy metabolism of severe hepatitis patients. From these results, it would appear that severe chronic hepatitis patients cannot utilize carbohydrate. With a notable decrease in the glucose oxidation rate, they can only obtain energy by increased fat utilization. Glucose utilization returns to normal when the patients recover. The inability to use glucose may be due to insulin resistance in chronic liver disease patients[8]. An increase in the RQ value can be used as a marker of recovery in these patients. Tajika[2] also found that, in cirrhosis patients, the non-protein respiratory quotient (npRQ) was an independent risk factor for survival; and patients with a lower npRQ had a worse prognosis.

In the present study, substrate and energy metabolism of cirrhosis patients was not significantly different from that of acute hepatitis patients. This may be related to patient selection. The cirrhotic patients selected were in Child grade A or B, resulting in no significant difference between the cirrhosis patient group and the acute hepatitis group. Multivariate regression analysis confirmed that the Child-Pugh's score is a better independent predictor of malnutrition than the other variables. However, the REE, TEE, calorie intake and energy balance need to be routinely assessed in cirrhotic patients in order to identify hypermetabolic and hypometabolic patients, which account for approximately 30% of patients. In these patients, the nutritional and metabolic parameters are indispensable for designing and prescribing personalized nutritional strategies for the treatment of the patients' muscle malnutrition, thus improving their morbidity and mortality rates.

In conclusion, in the present study, severe chronic hepatitis patients had a lower resting energy metabolism per kg weight than acute hepatitis patients, but similar to cirrhosis patients. Severe chronic hepatitis patients had a significantly higher fat oxidation rate and a significantly lower glucose oxidation rate than acute hepatitis and cirrhosis patients. As the severe chronic hepatitis patients' condition improved, the glucose oxidation rate increased. An increase in the RQ value can be used as a marker of recovery in these patients. The REE and the oxidation rates of various substrates determined by indirect heat measurement can be used to determine the optimal nutritive support therapy for severe liver disease patients.

COMMENTS

Background

The liver plays a pivotal role in glucose, fat, protein and energy metabolism.

Many studies have shown that patients with liver cirrhosis have nutrient and energy metabolism imbalance, which lead to malnutrition and seriously affect their prognosis. However, the characteristics of the glucose, fat, protein and energy metabolism in patients with severe chronic hepatitis are not clear.

Research frontiers

It has recently been noted that the energy metabolism of patients with end-stage liver diseases such as cirrhosis was altered compared to normal controls. They showed evidence of malnutrition, a high metabolism, a lower respiratory quotient (RQ) value, and a relatively higher fat oxidation rate. Long-term oral supplementation with a branched-chain amino acids (BCAA) mixture is better than ordinary food taken as a late evening snack for improving the serum albumin level and the energy metabolism of cirrhotic patients. Severe chronic hepatitis, which extended hepatic cell necrosis caused by chronic viral hepatitis B, leads to liver failure. Nutritional support is an important component of comprehensive therapy. However, the characteristics of the glucose, fat, protein and energy metabolism in patients with severe chronic hepatitis remain unclear.

Innovations and breakthroughs

The characteristics of the energy metabolism of severe chronic hepatitis patients have not been previously reported. The authors studied the disturbed homeostasis of energy, carbohydrate, fat and protein metabolism in severe chronic viral hepatitis B patients, and found that these patients had increased fat oxidation and reduced carbohydrate metabolism, which intended to improve when the liver function became normal. An increase in the RQ value can be used as a marker of recovery in these patients.

Applications

The measurement of resting energy expenditure (REE) and the oxidation rates of various substrates can be used to determine the optimal nutritive support therapy for severe liver disease patients. This research is expected to warrant a large clinical trail to assess the optimal nutritive support therapy for severe liver disease patients.

Peer review

The authors have estimated REE and RQ of patients with severe chronic hepatitis B. And most of energy consumed by these patients is provided by fat, not carbohydrate. The study is interesting and valuable.

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RAPID COMMUNICATION

Orthotopic liver transplantation as a rescue operation for recurrent hepatocellular carcinoma after partial hepatectomy

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Abstract

AIM: To compare post-orthotopic liver transplantation (OLT) survival between patients with recurrent hepatocellular carcinoma (HCC) after partial hepatectomy and those who received *de novo* OLT for HCC and to assess the risk factors associated with post-OLT mortality.

METHODS: From July 2003 to August 2005, 77 consecutive HCC patients underwent OLT, including 15 patients with recurrent HCC after partial hepatectomy for tumor resection (the rescue OLT group) and 62 patients with *de novo* OLT for HCC (the *de novo* OLT group). Thirty-three demographic, clinical, histological, laboratory, intra-operative and post-operative variables were analyzed. Survival was calculated by the Kaplan-Meier method. Univariable and multivariable analyses were also performed.

RESULTS: The median age of the patients was 49.0 years. The median follow-up was 20 mo. Three patients (20.0%) in the rescue OLT group and 15 patients (24.2%) in the *de novo* OLT group died during the follow-up period (P = 0.73). The 30-day mortality of OLT was 6.7% for the rescue OLT group *vs* 1.6% for the *de novo* OLT group (P = 0.27). Cox proportional hazards model showed that pre-OLT hyperbilirubinemia, the requirement of post-OLT transfusion, the size of the tumor, and family history of HCC were significantly associated with a higher hazard for mortality.

CONCLUSION: There are no significant differences in survival/mortality rates between OLT as *de novo* therapy and OLT as a rescue therapy for patients with hcc. Pre-OLT hyperbilirubinemia, post-OLT requirement of transfusion, large tumor size and family history of HCC are associated with a poor survival outcome.

Key words: Orthotopic liver transplantation; Liver cancer; Resection; Recurrence; Survival

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. An estimated number of 372000 new cases of HCC are diagnosed each year, constituting 4.6% of all new cancers (6.3% in men, 2.7% in women)[1,2]. Endemic areas include the Far East and Africa. The number of patients with HCC in China comprises approximately 40%-50% of all HCC patients of the world and HCC is the 2nd leading cause of death among cancer mortalities in the country[3]. While the etiology and pathogenesis of HCC are not entirely clear, the major contributing factors for the high prevalence of HCC in the country include chronic hepatitis B infection and dietary contamination of aflatoxin[4].

Partial hepatectomy with tumor resection and orthotopic liver transplantation (OLT) are the two commonly used surgical modalities for HCC treatment. Partial hepatectomy with tumor resection may be performed as a definitive or bridging therapy. Post-operative recurrence of HCC is common in patients undergoing hepatectomy, affecting long-term outcome. For patients with recurrent HCC after partial hepatectomy, rescue treatment options include percutaneous ethanol injection (PEI), trans-catheter

arterial chemoembolization (TACE), and surgical resection of recurrent tumor[5,6]. In addition, OLT appears to be a valid rescue option for these patients[7]. Poon *et al*[7] reported that OLT as a salvage operation is feasible and effective in patients with transplantable small HCC (solitary ≤ 5 cm, or 2 or 3 tumors ≤ 3 cm) who initially had primary resection and had preserved liver function. However, OLT has not been routinely performed in patients with recurrent advanced HCC[8]. In our clinical practice, we found that these patients with recurrent HCC, even at an advanced stage, might still benefit from OLT as a rescue operation. We hypothesized that OLT as a rescue operation for patients with recurrent HCC after initial resection surgery may have a similar outcome to these patients with HCC who had *de novo* OLT. The aims of the study were to compare post-OLT survival rates between patients who have recurrent HCC and those who have no history of resection surgery, and to evaluate the risk factors associated with postoperative mortalities.

MATERIALS AND METHODS

Patients

This is an historical cohort study involving 77 consecutive patients who underwent OLT for HCC from July 2003 to August 2005 in Eastern Hepatobiliary Surgery Hospital, Shanghai, China, a tertiary referral center specializing in surgical treatment of HCC and a variety of other liver disorders. The data were extracted from a prospectively maintained database for OLT which was approved by the Institutional Ethics Committee. The 77 eligible patients were divided into two groups: the study group with OLT performed as a rescue operation for recurrent HCC after initial partial hepatectomy for tumor resection (the rescue OLT group, $n = 15$) and the control group with OLT performed as the 1st line *de novo* therapy for HCC (the *de novo* OLT group, $n = 62$).

Inclusion and exclusion criteria

The inclusion criteria were patients aged > 18 years and those with HCC who underwent OLT. The exclusion criteria were patients who had OLT for etiologies other than HCC and patients who had OLT combined with transplantation of other organ(s).

Criteria for diagnosis and selection

The diagnosis of HCC was based on a combined assessment of clinical presentations, history of HBV infection and liver cirrhosis, imaging data (ultrasound, CT scan, and/or MRI), preoperative laboratory evaluation (alpha-fetoprotein), and pre- and/or postoperative histopathology. Patients considered to be candidates for OLT met the following criteria: (1) primary or recurrent HCC met Shanghai Criteria for OLT[9], i.e., tumor size ≤ 9 cm, number of tumors ≤ 3, the absence of macrovascular tumor embolism, and the absence of extrahepatic metastasis; (2) the ability to take anti-rejection medications after OLT; and (3) the absence of significant comorbidities. Written informed consent was obtained from all patients before the surgery.

Demographic and clinical variables

A panel of demographic and clinical variables were evaluated, including age, sex, excessive alcohol use, tobacco use, family history of HCC, HBV and/or hepatitis C infection, liver cirrhosis, diabetes, cardiopulmonary diseases, renal insufficiency, the time from the tumor detection to OLT, donor source, preoperative blood biochemistry, pre-OLT Child-Pugh scores[10], post-OLT pTNM tumor staging (UICC, International Union Against Cancer, 1953), intraoperative variables, postoperative histopathology and postoperative course.

Clinical outcomes

The primary outcomes were estimated based on the 30-d postoperative mortality, overall survival, and tumor-free survival, and the secondary outcomes were risk factors associated with the mortality.

Statistical analysis

Descriptive statistics were computed for all factors. These include medians and percentiles for continuous factors and frequencies for categorical factors. Time of follow-up is defined as either months from OLT to death, or months from OLT to last follow-up visit. A Kaplan-Meier plot was used for graphical representation of survival probabilities by recurrence of the liver cancer. Univariable and multivariable Cox proportional hazards models were used to estimate the hazard rates for several factors of interest. A stepwise selection method was used to choose the final multivariable model using a 0.50 and 0.25 significance criterions for entering and remaining in the model, respectively. A significance level of 0.05 was considered for all analyses. SAS version 9.1 software (SAS Institute, Cary, NC) and R 2.0.1 software (The R Foundation for Statistical Computing) were used to perform all analyses.

RESULTS

Procedure

In a median waiting period of 2 mo (ranging from 4 d to 3 mo), all 77 patients underwent cadaver OLT. Two patients (13.3%) in the rescue OLT group and 11 patients (17.7%) in the *de novo* OLT group underwent TACE or PEI therapy before OLT operation. As a part of our routine clinical protocol, end-to-end anastomoses of the inferior vena cava, portal veins, hepatic arteries, and bile ducts of the donors and recipients were performed. At anhepatic phase, 4000 units of hepatitis B immunoglobulin (HBIG) were injected intramuscularly. At the completion of the operation, methylprednisolone 500 mg was infused intravenously. As a part of the post-OLT protocol, 74 patients (96.1%) received one to three 6-d courses of intravenous 5-fluorouracil 500 mg on post-operative day 1 and day 4, mitomycin 2 mg

Table 1 Demographic and clinical data

Factors	Rescue OLT group (n = 15)	De novo OLT group (n = 62)
Age (yr)[1]	50.0 (46.0-55.0)	49.0 (44.0-55.0)
Male, No. (%)	14 (93.3)	51 (82.3)
Excessive alcohol use, No. (%)	5 (33.3)	13 (21.0)
Tobacco use, No. (%)	7 (46.7)	52 (83.9)
Family history of liver cancer, No. (%)	3 (20.0)	8 (12.9)
Hepatitis B infection, No. (%)	15 (100.0)	57 (91.9)
Diabetes, No. (%)	2 (13.3)	7 (11.3)
Liver cirrhosis, No. (%)	15 (100.0)	60 (96.8)
Months from tumor detection to OLT[1]	1.0 (1.0, 3.0)	2.0 (1.0, 3.0)
Child-Pugh Score, No. (%)		
A	7 (46.7)	39 (62.9)
B	6 (40.0)	23 (37.1)
C	2 (13.3)	0 (0.0)
UICC pTNM tumor staging[1]	3.0 (3.0, 3.0)	3.0 (3.0, 3.0)
Total bilirubin (μmol/L)[1]	22.8 (15.0, 54.6)	32.1 (23.0, 55.4)
Direct bilirubin (μmol/L)[1]	10.4 (5.8, 35.3)	14.4 (9.8, 29.5)
Albumin (g/L)[1]	35.3 (32.1, 42.1)	34.5 (32.0, 37.6)
Prealbumin (g/L)[1]	12.7 (9.3, 15.3)	10.3 (6.9, 14.5)
Alanine aminotransferase (U/L)[1]	90.1 (37.7, 183.9)	48.9 (37.8, 91.7)
Asparate aminotransferase (U/L)[1]	85.4 (36.5, 150.0)	65.5 (47.1, 99.4)
Prothrombin time (s)[1]	14.5 (13.3, 17.9)	15.2 (13.4, 17.8)
Activated partial thromboplastin time (s)[1]	34.7 (31.8, 38.0)	34.8 (30.7, 41.3)
OLT operating room time (h)[1]	8.0 (7.2, 8.1)	6.8 (5.7, 7.7)
OLT anhepatic time (min)[1]	65.0 (60.0, 81.0)	62.0 (56.0, 74.0)
OLT intra-op bleeding (L)[1]	2.0 (1.5, 2.3)	1.5 (1.2, 2.0)
OLT intra-op transfusion (L)[1]	1.6 (1.0, 2.0)	0.8 (0.4, 1.4)
OLT post-op transfusion (L)[1]	1.2 (0.8, 2.0)	0.4 (0.0, 1.4)
Pre-OLT α-fetoprotein (U/L)[1]	7.5 (4.9, 27.1)	362.6 (20.4, 3480.0)
Post-OLT α-fetoprotein (U/L)[1]	5.3 (4.1, 10.9)	15.7 (5.4, 120.4)
Change in α-fetoprotein after OLT[1]	4.2 (0.8, 21.8)	337.9 (13.3, 3023.5)
Postoperative histopathology[1]		
Size of largest tumor in diameter (cm)[1]	3.0 (1.5, 6.0)	3.6 (2.5, 6.0)
Number of tumor[1]	1.0 (1.0, 4.0)	1.0 (1.0, 2.0)
Histology differentiation of tumor, No. (%)		
Moderate	8 (53.3)	9 (14.5)
Poor	7 (46.7)	53 (85.5)
Tumor vascular invasion, No. (%)		
None	5 (33.3)	26 (41.9)
Microvascular	8 (53.3)	33 (53.2)
Macrovascular	2 (13.3)	3 (4.8)
Tumor in right lobe, No. (%)	13 (86.7)	53 (85.5)
Post-OLT treatment, No. (%)		
None	1 (6.7)	2 (3.2)
Chemotherapy	14 (93.3)	60 (96.8)
Time of follow-up (mo)[1]	18.0 (12.0, 24.0)	21.0 (14.0, 32.0)

[1]Statistics presented are medians (Q25, Q75).

on post-operative day 2 and day 5, and carboplatin 100 mg on postoperative day 3 and day 6. Post-operative anti-rejection regimens included tacrolimus, methylprednisolone, and mycophenolate mofetil. The blood levels of tacrolimus were maintained at 10-15 ng/mL for 3 mo after OLT and 5-10 ng/mL thereafter. Intravenous methylprednisolone was started on postoperative day 1, at 50 mg per 6 h, and tapered to 20 mg per day. On discharge from the hospital, intravenous methylprednisolone was switched to oral prednisone 15 mg per day with tapering. Prednisone was discontinued at 3 mo post-operatively.

Table 1 summarizes descriptive statistics for all 77 patients in the rescue OLT (n = 15) and *de novo* OLT (n = 62) groups.

Clinical data on the rescue OLT group

Table 2 presents detailed information about the tumor, at the time of tumor resection in the study group. All patients in the rescue OLT group underwent radical tumor resection because of the lack of a donor liver, even though the patients met Shanghai Criteria for OLT. The patients had partial hepatectomy with a tumor-free margin of $\geqslant$ 1.5 cm which was confirmed by postoperative histopathologic evaluation. The majority of patients underwent postoperative adjunctive therapy, including TACE in 12 (80.0%) patients and PEI in 2 (13.3%) patients.

Survival data

Within a median follow-up of 20 mo (interquartile

Table 2 Initial clinical data of HCC at time of tumor resection in the study group (n = 15)

Factors	*n*
Years since tumor detection[1]	4.0 (3.0, 5.0)
Mean diameter of tumor (cm)[1]	4.5 (3.0, 8.0)
Tumor-free interval after resection (mo)[1]	26.0 (6.0, 38.0)
Microsatellite lesions (%)	
0	11 (73.3)
1	4 (26.7)
Number of post-resection trans-catheter Arterial chemoembolization	
0	3 (20)
1	4 (26.7)
2	5 (33.3)
3	2 (13.3)
6	1 (6.7)
Number of post-resection percutaneous ethanol injection	
0	13 (86.7)
3	1 (6.7)
16	1 (6.7)
Tumor in the left lobe (%)	12 (80)
Unifocal tumor (%)	15 (100)
Vascular invasion (%)	
None	10 (66.7)
Microvascular	3 (20)
Macrovascular	2 (13.3)
Non-encapsulated tumor (%)	9 (60)

[1]Statistics presented are median (Q25, Q75).

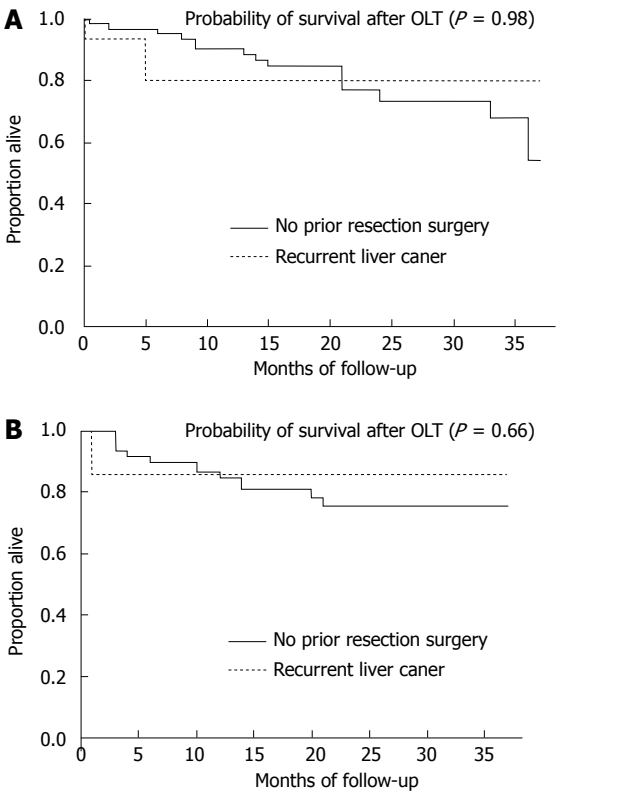

Figure 1 Probability of overall survival and tumor-free survival after ortotopic liver transplantation- Kaplan-Meier curves. **A**: Interaction between time and groups was verified as the two curves cross each other suggest non-proportionality of the hazards but this is not found statistically significant (P = 0.98); **B**: Interaction between time and groups was verified as the two curves cross each other suggest non-proportionality of the hazards, but not statistically significant (P = 0.66).

range: 14-29 mo), 18 (23.4%) patients died: 3 (20%) in the rescue OLT group and 15 (24.2%) in the *de novo* OLT group. One patient from each group died in 30 d of OLT (6.7% *vs* 1.6%) because of disseminated intravascular coagulation and pneumonia, respectively. One patient in the control group died of graft-versus-host disease 2 mo after OLT. The main cause of the death was recurrent tumor and metastasis, and 15 patients died more than 30 d after OLT.

Risk factors associated with survival outcome

Table 3 presents univariable hazard rates for the association between several factors of interest and overall survival rate. Younger age, pre-operative hyperbilirubinemia, large tumor size, family history of liver cancer, and tumor microembolism were found to significantly influence the hazard for post-OLT mortality. Figure 1 presents the Kaplan-Meier curves of overall survival and tumor-free survival by the two groups, respectively. Interaction between time and the study groups was verified as the two curves cross each other suggested non-proportionality of the hazards; but this was not found statistically significant.

Table 4 shows the results of the multivariable Cox proportional hazards model. Total bilirubin, the requirement for post-operative transfusion, the size of the largest tumor, family history of HCC, and microembolisms remained in the final model. Recurrence of HCC was kept in the final model because of clinical importance, even though it was not found significantly associated with survival rate. The hazard of dying after OLT increases by 1% for every 1 unit increase in total bilirubin. Also, for every 1 L increase in the amount of post-OLT blood transfusion, the hazard of dying increases by 40%. In addition, the hazard of dying increases by 30% for every 1 cm increase in the size of the largest tumor. Finally, subjects with a family history of HCC have 7.7 times of the hazards of dying that of those without a family history of HCC.

DISCUSSION

Partial hepatectomy and OLT are effective treatment modalities for HCC patients with underlying cirrhosis[11-13]. The surgical outcome of the two approaches appeared to be comparable, with each of the treatment modalities having its advantages and disadvantages. For example, operative mortality was lower for partial hepatectomy which may be preferably applied to HCC patients with a well compensated cirrhotic liver, while OLT can be performed selectively in patients with tumor recurrence and/or decompensated liver. Although the decision of choice on partial hepatectomy and OLT can be difficult to make[14-16], it appears that the latter treatment modality may have a survival advantage[17,18]. The main barrier for the routine application of OLT in the patient population has been the availability of a donor organ. On the other hand, partial hepatectomy can be offered as a sole surgical treatment or as a bridging procedure for OLT. However,

Table 3 Univariable Cox proportional hazards models

	Factor	Hazard ratio (95% CI)	*P*
Demographic	Age	0.92 (0.88-0.97)	< 0.001
	Gender (male *vs* female)	1.6 (0.36-6.9)	0.54
	Tobacco (yes *vs* no)	1.7 (0.48-5.8)	0.42
	Excess alcohol use (no *vs* yes)	1.1 (0.37-3.4)	0.83
	Family history liver cancer (yes *vs* no)	4.4 (1.6-11.9)	0.004
	Diabetes (yes *vs* no)	1.4 (0.42-5.0)	0.57
	Interval from tumor detection to OLT in mo	0.99 (0.91-1.07)	0.74
Disease groups	Recurrent liver cancer (yes *vs* no)	1.02 (0.29-3.5)	0.98
Preoperative laboratory tests	Total bilirubin	1.01 (1.004-1.01)	< 0.001
	Direct bilirubin	1.01 (1.01-1.01)	< 0.001
	Albumin	0.99 (0.90-1.10)	0.91
	Pre-albumin	0.91 (0.82-1.02)	0.11
	Alanine aminotransferase	1.00 (1.00-1.01)	0.57
	Asparate aminotransferase	1.00 (1.00-1.01)	0.15
	Prothrombin time	1.04 (0.92-1.2)	0.51
	Activated partial thromboplastin time	1.01 (0.96-1.08)	0.62
Tumor histopathology	Size of largest tumor (cm)	1.1 (1.03-1.2)	0.009
	Tumor staging	1.6 (0.43-6.2)	0.47
	Tumor range	1.3 (0.90-1.9)	0.16
	Tumor location (left *vs* right)	1.2 (0.33-4.0)	0.82
	Microembolism (micro *vs* none)	2.1 (0.67-6.7)	0.2
	Microembolism (macro *vs* none)	10.5 (2.3-48.3)	0.003
Preoperative staging	Child-Pugh Score	1.5 (0.67-3.4)	0.31
Intraoperative factors	OLT operative room time (h)	1.1 (0.80-1.6)	0.48
	OLT anhepatic time (min)	0.97 (0.92-1.01)	0.11
	OLT intra-op bleeding (L)	0.47 (0.20-1.1)	0.097
	OLT intra-op transfusion (L)	0.73 (0.43-1.2)	0.24
	OLT post-op transfusion (L)	1.1 (0.90-1.4)	0.33

Table 4 Multivariable Cox proportional hazards model

Factors	Hazard ratio (95% CI)	*P*
Recurrent liver cancer (yes *vs* no)	1.7 (0.38-7.2)	0.5
Total bilirubin	1.01 (1.006-1.02)	< 0.0001
OLT post-op transfusion (L)	1.4 (1.1-1.8)	0.008
Size of largest tumor (cm)	1.3 (1.1-1.4)	0.0004
Family history liver cancer (yes *vs* no)	7.7 (2.2-26.9)	0.001
Microembolism (yes *vs* no)	3.4 (0.85-13.5)	0.08

partial hepatectomy can be associated with a short-term risk for postoperative hepatic failure with a 5%-10% mortality and a 30%-50% morbidity[19,20] and with a long-term risk for tumor recurrence, affecting 80% of the patients at 5 years[21-24]. Since the recurrence of the tumor after partial hepatectomy is common, rescue medical, radiographic, and surgical therapies are often needed.

There are scanty data on the clinical outcome of OLT as a rescue operation for patients with recurrent HCC after the initial tumor resection. We evaluated 77 consecutive patients with OLT, of whom 15 patients had rescue OLT for recurrent HCC. All the 15 patients had concomitant liver cirrhosis. These patients all had postoperative single or multiple sessions of TACE and/or PEI. We found no difference in the overall and tumor-free survivals between the rescue OLT and *de novo* OLT groups, suggesting that OLT may be a valid option for patients with recurrent HCC after the tumor resection.

In addition to its application in advanced stage HCC, OLT can also be a valid treatment option for patients with early stage tumors if partial hepatectomy is not amenable[25], even though the survival advantage of OLT over partial hepatectomy in these patients has not been confirmed[26,27]. When compared with partial hepatectomy, OLT for resectable HCC may offer a survival benefit in a subset of patients as long as the donor organ is available within 6-10 mo[16].

OLT has increasingly been performed for HCC where reported 1- and 2-year cumulative survival rates were 90.0% and 65.6%, and the disease-free survival rates were 77.5% and 62.5%, respectively[28]. The 3-year survival reached 77%-80%[29,30]. Poon *et al*[7] reported that a 5-year overall survival and tumor-free survival of OLT for patients with recurrent small HCC (diameter $\leqslant$ 3 cm) after initial tumor resection were 48% and 0%, respectively. The 5-year survival rate of 422 HCC patients with OLT was 44.4%, and histologic grade of HCC and tumor size (> 5 cm) were found associated with tumor-free survival[31]. Multiple factors may have contributed to the improvement in survival in HCC patients after OLT, including appropriate selection of candidate patients, the application of surgical techniques minimizing the risk for intraoperative tumor spread, and postoperative adjunct medical therapy.

The most commonly used criteria for transplantation for HCC patients are the Milan criteria: solitary tumor $\leqslant$ 5 cm in diameter or 2 or 3 tumor nodules with the largest diameter $\leqslant$ 3 cm and the absence of macroscopic vascular invasion or extrahepatic metastasis[11]. In the current study, we used the Shanghai Criteria[9], i.e., tumor size $\leqslant$ 9 cm, number of tumors $\leqslant$ 3, the absence of macrovascular tumor embolism(s), and the absence of

extrahepatic metastasis. The main difference between the Milan Criteria and Shanghai Criteria was the tumor size with cut-off 5 cm *vs* 9 cm.

A variety of factors were reported to be associated with a poor surgical outcome after OLT, including large tumor size[13,28,31,32], the presence of vascular invasion[13], the presence of portal vein thrombosis[28], and poor histologic differentiation[31]. In the current study, the risk factor associated with a poor survival were pre-OLT hyperbilirubinemia, the requirement of post-OLT blood transfusion, large tumor size, family history of liver cancer, and the presence of tumor microembolism. The recurrence of HCC after the tumor resection as an indication of OLT was not found associated with a poor survival outcome after OLT. Therefore, OLT appears to be a valid option as rescue operation for recurrent HCC after tumor resection.

Post-operative corticosteroid use may be associated with a high risk for tumor recurrence in patients with OLT. Mazzaferro *et al*[11,33] reported that discontinuation of corticosteroid use for 3-6 mo in HCC patients with OLT had a lower risk for tumor recurrence and post-operative long-term corticosteroid use had a 4-fold increase in tumor recurrence after OLT. In addition, post-operative corticosteroid use appears to pose a higher risk for post-operative infection and metabolic side effects. It appears that post-operative immunosuppression with omission of cortisteroid use may be safe[34]. In our study protocol, all patients had corticosteroid tapering and discontinued the agent at one month after OLT.

There are some limitations to the study. First, this is not a randomized trial with a small sample size in the rescue OLT group (n =15), which would have been subjected to a type Ⅱ error. Second, longer follow-up would be needed. Finally, the study was conducted in a tertiary care center specializing in surgical treatment of HCC and other liver disorders and there might have been a selection bias.

In conclusion, there are no significant differences in survival/mortality rates between OLT as a *de novo* therapy and OLT as a rescue therapy for patients with HCC. Pre-OLT hyperbilirubinemia, post-OLT requirement of transfusion, large tumor size, and family history of HCC are associated with a poor survival outcome.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Liver transplantation (LT) appears to be a valid rescue option for these patients. However, LT has not been routinely performed in patients with recurrent advanced HCC. Meanwhile, the risk factors of orthotopic liver transplantation (OLT) for HCC patients are unclear. So we hypothesized that OLT as a rescue operation for patients with recurrent HCC after initial resection surgery may have a similar outcome to these patients with HCC who had *de novo* OLT. The aims of the study were to compare post-OLT survival rates between patients who have recurrent HCC and those who have no history of resection surgery; and to evaluate risk factors associated with postoperative mortalities.

Research frontiers

Liver transplantation is one of the hotspots in researches on liver tumors. But few studies have tried to find the risk factors of OLT for HCC using the statistical method.

Innovations and breakthroughs

No significant differences were found in survival/mortality rates between OLT as a *de novo* therapy and OLT as a rescue therapy for patients with HCC in this study. And Pre-OLT hyperbilirubinemia, post-OLT requirement of transfusion, large tumor size, and family history of HCC were found associated with a poor survival outcome.

Applications

This study shows that there were no significant differences in survival/mortality rates between OLT as a *de novo* therapy and OLT as a rescue therapy for patients with HCC. And pre-OLT hyperbilirubinemia, post-OLT requirement of transfusion, large tumor size, and family history of HCC were associated with a poor survival outcome. The main limit in this research are the biology and genetics of tumors.

Peer review

The authors described excellent results of the OLT for HCC patients using statistical methods. This article identified that recurrent HCC was not a risk factor of OLT. Meanwhile, pre-OLT hyperbilirubinemia, post-OLT requirement of transfusion, large tumor size, and family history of HCC play important roles in the prognosis of OLT. The findings are potentially important for planning of further studies.

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RAPID COMMUNICATION

Effect of antidepressants on body weight, ethology and tumor growth of human pancreatic carcinoma xenografts in nude mice

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Abstract

AIM: To investigate the effects of mirtazapine and fluoxetine, representatives of the noradrenergic and specific serotonergic antidepressant (NaSSA) and selective serotonin reuptake inhibitor (SSRI) antidepressant respectively, on body weight, ingestive behavior, locomotor activity and tumor growth of human pancreatic carcinoma xenografts in nude mice.

METHODS: A subcutaneous xenograft model of human pancreatic cancer cell line SW1990 was established in nude mice. The tumor-bearing mice were randomly divided into mirtazapine group [10 mg/(kg·d)], fluoxetine group [10 mg/(kg·d)] and control group (an equivalent normal saline solution) (7 mice in each group). Doses of all drugs were administered orally, once a day for 42 d. Tumor volume and body weight were measured biweekly. Food intake was recorded once a week. Locomotor activity was detected weekly using an open field test (OFT).

RESULTS: Compared to the fluoxetine, mirtazapine significantly increased food intake from d 14 to 42 and attenuated the rate of weight loss from d 28 to 42 (t = 4.38, P < 0.05). Compared to the control group, food intake was significantly suppressed from d 21 to 42 and weight loss was promoted from d 35 to 42 in the fluoxetine group (t = 2.52, P < 0.05). There was a significant difference in body weight of the mice after removal of tumors among the three groups. The body weight of mice was the heaviest (13.66 ± 1.55 g) in the mirtazapine group and the lightest (11.39 ± 1.45 g) in the fluoxetine group ($F_{(2,12)}$ = 11.43, P < 0.01). The behavioral test on d 7 showed that the horizontal and vertical activities were significantly increased in the mirtazapine group compared with the fluoxetine and control groups ($F_{(2,18)}$ = 10.89, P < 0.01). These effects disappeared in the mirtazapine and fluoxetine groups during 2-6 wk. The grooming activity was higher in the mirtazapine group than in the fluoxetine group (10.1 ± 2.1 *vs* 7.1 ± 1.9) (t = 2.40, P < 0.05) in the second week. There was no significant difference in tumor volume and tumor weight of the three groups.

CONCLUSION: Mirtazapine and fluoxetine have no effect on the growth of pancreatic tumor. However, mirtazapine can significantly increase food intake and improve nutrition compared with fluoxetine in a pancreatic cancer mouse model.

Key words: Pancreatic carcinoma; Mirtazapine; Fluoxetine; Body weight; Nude mice; Locomotor activity; Ethology

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Jia L, Shang YY, Li YY. Effect of antidepressants on body weight, ethology and tumor growth of human pancreatic carcinoma xenografts in nude mice. *World J Gastroenterol* 2008; 14(27): 4377-4382 Available from: URL: http://www.wjgnet.com/1007-9327/14/4377.asp DOI: http://dx.doi.org/10.3748/wjg.14.4377

INTRODUCTION

Pancreatic adenocarcinoma is the fourth leading cause of cancer-related death in the United States[1]. However, its incidence has increased steadily in China in recent years. At the time of diagnosis, 80% of patients present with either locally advanced or metastatic disease. In recent years, although gene therapy and biological targeting therapy can significantly inhibit the growth of pancreatic cancer in animal experiments, there is no satisfactory

therapy for pancreatic cancer patients to extend their median survival time and improve their quality of life. The overall five-year survival rate is less than 5%[2].

The stress associated with the diagnosis and treatment of pancreatic cancer can cause significant psychiatric morbidity. It was reported that pancreatic cancer patients have the highest rate of major depression compared with other cancer patients[3]. Depression occurs in 47%-71% of patients with pancreatic cancer[4,5]. Unfortunately, depression adversely affects many clinical oncology outcomes. It can prolong hospital stay, augment the complication of therapy, decrease the ability to care oneself, reduce the compliance with medical treatment, lead to a poorer quality of life, and even shorten survival time[6-8]. Antidepressant medications not only improve depressive symptoms of patients with cancer but also reverse these adverse impacts[9].

Clearly, antidepressant treatment constitutes one of the new strategies of cancer adjuvant therapy. However, data on treatment of depression with antidepressants in cancer patients are relatively scarce. The effect of different agents on distressing symptoms of cancer patients is still a subject for discussion. At present, selective serotonin reuptake inhibitor (SSRI) antidepressants are recommended as the first-line therapy for major depressive patients. Furthermore, mirtazapine is a new noradrenergic and specific serotonergic antidepressant (NaSSA), which stimulates 5-HT_1 receptors, but blocks serotonin 5-HT_2 and 5-HT_3 receptors and histamine H_1 receptors[10], which may be associated with increasing appetite and weight gain. Recent studies have shown that serotonin has been extensively implicated in the regulation of ingestive behavior[11,12].

Therefore, we performed experiments using fluoxetine and mirtazapine as representatives of SSRI and NaSSA, respectively. The aim of the study was to study the effects of oral mirtazapine and fluoxetine on body weight, food intake, locomotion and tumor growth in a subcutaneous pancreatic tumor model.

MATERIALS AND METHODS

Drugs and reagents

Mirtazapine was kindly provided by Organon, Oss, the Netherlands. Fluoxetine was purchased from Eli Lilly & Co (Indianapolis, IN). RPMI1640 and fetal bovine serum were purchased from Gibco (Grand Island, NY).

Experimental animals

BALB/c nu/nu male and female mice (5 wk old, weighing 17-20 g) of SPF class were purchased from the Experimental Animal Center, Guangzhou University of Chinese Medicine. The mice were housed under pathogen-free conditions in the Animal Center of Sun Yat-Sen University (4-5 mice per cage at 22 ± 1℃ room temperature) with free access to water and standard rat chow, in a 12 h light-dark cycle.

Pancreatic cancer cell line and culture conditions

Human pancreatic cancer cell line SW1990 was a kind gift from the Second Affiliated Hospital of Sun Yat-Sen University. The cell line was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Monolayer cultures were maintained on a culture flask and incubated in a mixture of 50 mL/L CO_2 and 950 mL/L oxygen at 37℃. Trypsinization was terminated with a medium containing 10% FBS and the cells were washed once with a serum-free medium and resuspended in Hank's balanced salt solution. Only single-cell suspensions displaying greater than 90% viability were used for injection.

Establishment of subcutaneous xenograft model

To produce SW1990 donor tumors, 3×10^6 cells per animal in a total volume of 0.2 mL were inoculated subcutaneously into the right flank of a nude mouse. Tumor size was measured *via* callipering. When the subcutaneous solid tumor reached approximately 1 cm in diameter and was aseptically removed from the donor animals. Macroscopically necrotic tissues and the remaining healthy tumor tissues were cut with scissors and minced into approximately 1 mm^3 pieces in Hanks' balanced salt solution containing 100 units/mL penicillin and 100 μg/mL streptomycin. A small incision was then made through the right dorsal flank and a tumor tissue piece was implanted subcutaneously beneath the dorsal flank skin of a nude mouse. We established a subcutaneous pancreatic cancer model as previously described[13] with certain modifications.

Experimental design

After tumor transplantation, the mice were randomly assigned into three groups (7 mice each group). Treatment was initiated one day after tumor transplantation as the first day experiment. The first group received an equivalent normal saline solution as control. The second and third groups received 10 mg/(kg·d) mirtazapine and 10 mg/(kg·d) fluoxetine, respectively[14], once a day for 42 d. Oral application was chosen as it is the standard application of antidepressants. For the study, the treated mice were closely monitored for any side effects and sacrificed on d 43. The transplanted tumor sizes were measured with a caliper, twice a week, and the tumor volume was calculated using the formula[15]: $V = W^2 \times L/2$, where W is the width and L is the length of the tumor. Body was weighed biweekly and food intake was expressed as daily consumption in gram per animal weekly.

Open field test (OFT)

OFT is a widely used test to evaluate the emotion and locomotor activity in rodents. As a test of spontaneous (unconditioned) behavior, it allows the animal to exhibit a wide range of behaviors and is therefore highly suitable for the study of complex phenomena such as anxiety or depression[16]. The open field apparatus used is a rectangular chamber (35 cm × 35 cm × 20 cm) made of plexigal, which was built from black walls and white floor. The floor of the open field was divided into 25 identical squares by 4 × 4 black lines[16]). A video camera

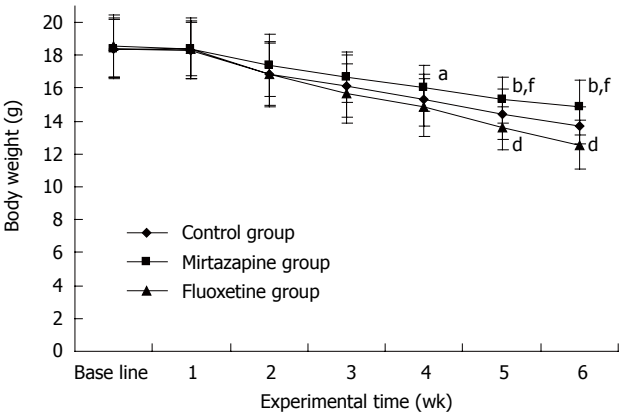

Figure 1 Effects of antidepressants on body weight of nude mice. Data are represented as mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* fluoxetine group, $^{d}P < 0.01$ *vs* control group, $^{f}P < 0.01$ *vs* control group.

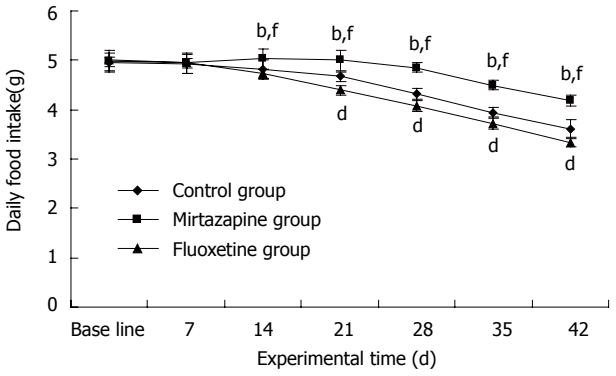

Figure 2 Effects of antidepressants on food intake of mice. Data are represented as mean ± SD. $^{b}P < 0.01$ *vs* fluoxetine group, $^{d}P < 0.01$ *vs* control group, $^{f}P < 0.01$ *vs* control group.

was placed 1.5 m above the apparatus. After each trial, the apparatus was cleaned with water containing 0.1% acetic acid. The behavioral parameters registered during the first 5 min exposure to the open field apparatus were horizontal activity (the number of squares an animal entered), rearing known as vertical activity (the number of times an animal was standing on its hind legs with forelegs in the air or against the wall), grooming activity (the number of paws or tongues used to clean or scratch the body), which could reflect a stable individual trait "nonspecific excitability level". The OFT was performed weekly between 13:00 and 15:30. Any abrupt loud noise could markedly inhibit locomotion and even induce prolonged immobility of the mice. Therefore, the testing room was comprised merely of the background noise.

Statistical analysis

Statistical analysis was performed with the SPSS 13.0 for Windows. Data were expressed as mean ± SD. Pancreatic tumor weight, tumor volume and behavioral parameters were compared using one-way ANOVA. Significant differences in body weight and food intake were determined by two-way ANOVA and the Student-Newman-Keuls test for multiple comparisons between groups. $P < 0.05$ was considered statistically significant based on a two-tailed test.

RESULTS

Effects of long-term antidepressant treatment on body weight

The change in body weight during the treatment is shown in Figure 1. The mice had a progressive weight loss. The body weight of mice in the three groups was very close in the first week. In the first 3 wk of treatment, the body weight of mice in the mirtazapine group was greater than that of mice in the other groups. However, no significant difference was observed. In wk 4, the body weight of mice was significantly greater in the mirtazapine group (16.00 ± 1.41 g) than in the fluoxetine group (14.86 ± 1.77 g) ($F_{(2,12)} = 4.2$, $P < 0.05$). The effect of mirtazapine lasted until the end of experiment. Nevertheless, the body weight was significantly decreased in the fluoxetine group in week 5-6 compared with the control group ($P < 0.01$, Figure 1). The body weight of mice after removal of the tumor was also significantly increased in the mirtazapine group (13.66 ± 1.55 g) but decreased in the fluoxetine group (11.39 ± 1.45 g) compared with the control group (12.56 ± 1.29 g) ($F_{(2, 12)} = 11.43$, $P < 0.01$).

Effects of antidepressants on mice ingestive behaviour

Daily food intake of the tumor-bearing mice was gradually reduced over the whole treatment period (Figure 2). At initiation of the study, no difference was observed in ingestive behavior of mice in different groups. On day 14, food consumption of mice was significantly increased in the mirtazapine group (5.03 ± 0.16 g) compared with the fluoxetine (4.73 ± 0.11 g) and control groups (4.79 ± 0.16 g) ($F_{(2,12)} = 23.31$, $P < 0.01$). Mirtazapine exerted its effect to the end of experiment. However, fluoxetine treatment significantly decreased food consumption of mice compared with the control group from day 21 to 42 ($P < 0.01$, Figure 2).

Locomotor behavior in open-field apparatus

Mirtazapine and fluoxetine significantly increased the locomotor activity of mice in the OFT. In the first week of behavioral test, the horizontal activity and vertical activity were significantly increased in the mirtazapine group (117.3 ± 16.4, 95.3 ± 13.6) compared with the fluoxetine group (95.3 ± 13.6, 13.0 ± 4.2) and control group (80.6 ± 18.0, 7.9 ± 3.4) ($F_{(2,18)} = 10.89$, $F_{(2,18)} = 97.09$, $P < 0.01$, Figure 3A and B). There was no difference in grooming activity among the three groups. However, the grooming activity was significantly higher in the mirtazapine group (10.1 ± 2.1) than in the fluoxetine group (7.1 ± 1.9) ($F_{(2,18)} = 4.90$, $P < 0.01$, Figure 3C) in the second week. Meanwhile, the horizontal and vertical activities were significantly increased in the mirtazapine and fluoxetine groups compared with the control group ($P < 0.01$, Figure 3A and B). Nevertheless, these parameters obtained from the mice treated with mirtazapine did not differ from those treated with fluoxetine during week 3-6.

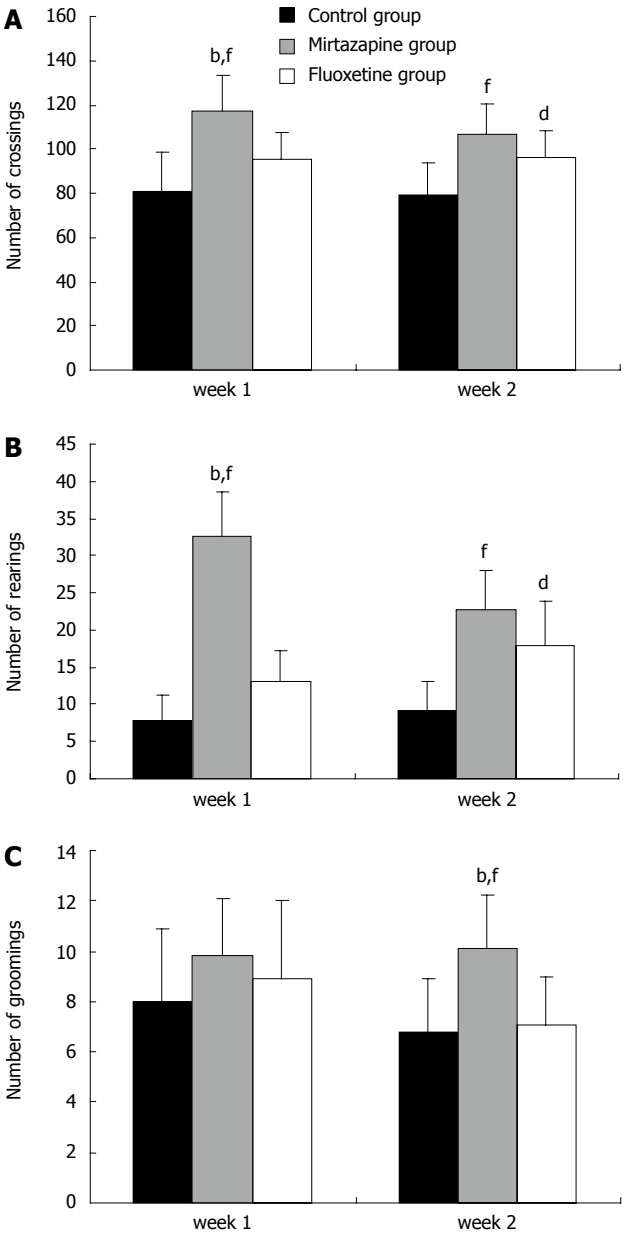

Figure 3 Horizontal (**A**), vertical (**B**) and grooming (**C**) activity in pancreatic tumor-bearing mice in the OFT. Data are represented as mean ± SD. $^{b}P < 0.01$ *vs* fluoxetine group, $^{d}P < 0.01$ *vs* control group, $^{f}P < 0.01$ *vs* control group.

Effects of antidepressants on growth of pancreatic cancer in vivo

As shown in Figure 4, the tumor xenograft grew very rapidly with the prolongation of experiment. Nevertheless, no significant difference was observed in tumor volume of each group at any time point during the whole experiment. After 6-wk treatment, the animals were killed when the tumors were removed and weighed. However, no significant difference in tumor weight was detected in the mirtazapine group (1.18 ± 0.20 g), fluoxetine group (1.20 ± 0.28 g) and control group (1.23 ± 0.34 g) ($F_{(2,18)} = 0.06$, $P > 0.05$).

DISCUSSION

The results of the present study show that daily oral mirtazapine (10 mg/kg) could significantly increase

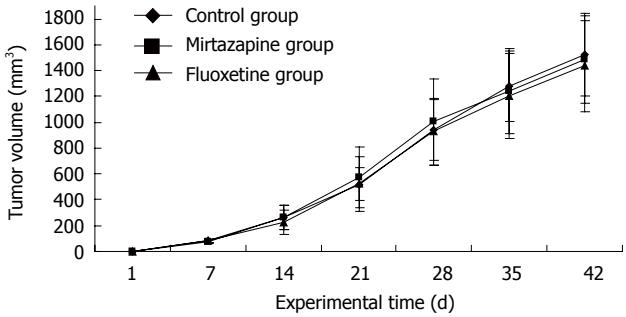

Figure 4 Effect of antidepressants on the growth of pancreatic cancer xenografts. Data are represented as mean ± SD.

food consumption and attenuated the rate of weight loss. However, treatment with fluoxetine (10 mg/kg) significantly suppressed food intake and promoted weight loss. Mirtazapine and fluoxetine showed their effects on the regulation of food intake and body weight to the end of experiment, suggesting that there is an extensive implication between serotonin and food intake. One explanation for the effects may be that the plasma half life of the two drugs is very long. It was reported that pharmacological agents that increase the levels of 5-HT in the central nervous system (CNS) suppress food intake, whereas drugs that antagonize the actions of 5-HT increase food intake[17]. Mirtazapine is a potent antagonist at postsynaptic $5HT_2$ and $5HT_3$ receptors which may potentially increase the appetite and body weight[18-20]. However, fluoxetine augments serotonergic activity by selectively inhibiting the reuptake of neurotransmitter, and reduces food intake and body weight in both animals[21, 22] and human beings[23], and is thus used in the treatment of obesity[24].

We examined the behavioral effects of mirtazapine and fluoxetine in the OFT. The horizontal activity fully reflected the animal activity, rearing the degree of curiosity to the novel surroundings, and grooming the level of alert against the novel environment. Mirtazapine and fluoxetine significantly increased the locomotor activity of pancreatic tumor-bearing mice compared with the control group. In the initial behavioral test, the horizontal activity was significantly increased in the mirtazapine group compared with the fluoxetine and control groups. Rearings were also significantly increased in the mirtazapine group compared with the fluoxetine and control groups (Figure 3A and B). Grooming activities increased earlier in the mirtazapine group than in the fluoxetine group. The results of the present study are consistent with the reported data[25,26], showing that antidepressants increase locomotor activity in a depressed model of normal rats. Nevertheless, mirtazapine adapted faster to the new environment and elevated earlier the alert of tumor-bearing mice against the novel environment. These findings indicate that mirtazapine is better than fluoxetine to tolerate the stress associated with the diagnosis and treatment of pancreatic cancer.

To our knowledge, the effect of mirtazapine and fluoxetine on the growth of pancreatic cancer in nude mice has not been reported. In the present study, the

tumor volume at any time points and tumor weight were not significantly different in the three groups. Interestingly, a previous study demonstrated that fluoxetine is neither a complete carcinogen nor a tumor promoter[27], which is in agreement with our results obtained from pancreatic tumor-bearing mice. Moreover, Abdul *et al*[28] reported that the growth of subcutaneous PC-3 xenografts in athymic nude mice is significantly inhibited by antidepressants. Mirtazapine and fluoxetine also did not exhibit any toxicity throughout the whole treatment, suggesting that mirtazapine and fluoxetine can be safely used in the treatment of depression in pancreatic cancer patients.

Pancreatic cancer patients have not only distressing symptoms such as appetite loss, nausea, vomitting, weight loss, sleep disturbances and pain, but also psychiatric comorbidities such as adjustment disorder, depression frequently accompanying the disease process. It was reported that patients with pancreatic cancer have a weight loss of 83%-87% and approximately 30% of the patients have a weight loss of over 10%[29].

In summary, mirtazapine as an adjuvant therapy is beneficial to the pancreatic cancer patients with depression[30]. Mirtazapine as the first-line therapy for depressed patients with advanced pancreatic cancer has a bright future. Nevertheless, further investigation and evaluation of mirtazapine are needed before it is widely used in clinical practice.

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COMMENTS

Background

The treatment of pancreatic cancer remains a great challenge. The majority of patients with pancreatic cancer develop major depression. Depression adversely affects many clinical outcomes. Antidepressant treatment has been accepted as one of the new strategies in cancer adjuvant therapy. However, systemic studies on the treatment of depression in patients with cancer have not been well documented. The effect of different antidepressants on distressing symptoms of cancer patients is a subject for further evaluation.

Research frontiers

At present, fluoxetine is one of the selective serotonin reuptake inhibitor (SSRI) antidepressants which are recommended as the first-line therapy for depression. Mirtazapine belongs to a new family of noradrenergic and specific serotonergic antidepressants (NaSSA) used in the treatment of major depression.

Innovations and breakthroughs

On the basis of previous data, this was the first study examining the effects of mirtazapine and fluoxetine on the growth of pancreatic cancer in nude mice. The results of the present study show that mirtazapine could significantly increase food intake and attenuate the rate of weight loss in experimental mice. However, fluoxetine could significantly suppress food intake and promote weight loss in tumor-bearing mice.

Applications

To summarize the actual application values, mirtazapine neither inhibits nor promotes pancreatic tumor growth according to the findings from this study. The results support the hypothesis that mirtazapine as an adjuvant therapy is superior to fluoxetine for pancreatic cancer patients with depression.

Peer review

The title accurately reflects the major contents of the article. On the basis of previous researches, this paper is an original research article on the effect of mirtazapine and fluoxetine on the growth of human pancreatic carcinoma in nude mice. The findings are of great interest and provide a foundation for their application in clinical practice. The conclusions are reliable and valuable.

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RAPID COMMUNICATION

Prognostic impact of metastatic lymph node ratio in advanced gastric cancer from cardia and fundus

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Abstract

AIM: To investigate the prognostic impact of the metastatic lymph node ratio (MLR) in advanced gastric cancer from the cardia and fundus.

METHODS: Two hundred and thirty-six patients with gastric cancer from the cardia and fundus who underwent D2 curative resection were analyzed retrospectively. The correlations between MLR and the total lymph nodes, positive nodes and the total lymph nodes were analyzed respectively. The influence of MLR on the survival time of patients was determined with univariate Kaplan-Meier survival analysis and multivariate Cox proportional hazard model analysis. And the multiple linear regression was used to identify the relation between MLR and the 5-year survival rate of the patients.

RESULTS: The MLR did not correlate with the total lymph nodes resected (r = -0.093, P = 0.057). The 5-year overall survival rate of the whole cohort was 37.5%. Kaplan-Meier survival analysis identified that the following eight factors influenced the survival time of the patients postoperatively: gender (χ^2 = 4.26, P = 0.0389), tumor size (χ^2 = 18.48, P < 0.001), Borrmann type (χ^2 = 7.41, P = 0.0065), histological grade (χ^2 = 5.07, P = 0.0243), pT category (χ^2 = 49.42, P < 0.001), pN category (χ^2 = 87.7, P < 0.001), total number of retrieved lymph nodes (χ^2 = 8.22, P = 0.0042) and MLR (χ^2 = 34.3, P < 0.001). Cox proportional hazard model showed that tumor size (χ^2 = 7.985, P = 0.018), pT category (χ^2 = 30.82, P < 0.001) and MLR (χ^2 = 69.39, P < 0.001) independently influenced the prognosis. A linear correlation between MLR and the 5-year survival was statistically significant based on the multiple linear regression (β = -0.63, P < 0.001). Hypothetically, the 5-year survival would surpass 50% when MLR was lower than 10%.

CONCLUSION: The MLR is an independent prognostic factor for patients with advanced gastric cancer from the cardia and fundus. The decrease of MLR due to adequate number of total resected lymph nodes can improve the survival.

Key words: Stomach neoplasms; Lymph node metastasis; Metastatic lymph node ratio; Lymphadenectomy; Prognosis

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INTRODUCTION

At present, patients with advanced gastric cancer from the cardia and fundus still have a poor prognosis despite some therapeutic modalities. Lymph node metastasis is considered one of the most important prognostic factors[1-3]. And lymphadenectomy is fundamentally critical in radical surgery. Its standardization highly depends on the accuracy of prognosis evaluation according to the classification of lymph node metastasis. The current American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC) TNM system (1997) has established the classification

system based on the number of metastatic lymph nodes. D2 curative resection, which includes gastrectomy and D2 lymphadenectomy, required dissection of all the Group 1 and Group 2 nodes classified by anatomical location. However, with the development of D2 lymphadenectomy, larger lymph nodes dissected may enable to fine larger metastatic lymph nodes, which induces a migration in the staging system. The ratio of the number of metastatic lymph nodes over the total number of resected lymph nodes is introduced to prognosis evaluation. It was reported that metastatic lymph node ratio (MLR) can minimize the stage migration effect caused by increasing total dissected lymph nodes, also can help refine the current TNM stage system[4,5]. Though many studies on the prognostic significance of MLR in gastric cancer have been carried out, relevant researches on advanced gastric cancer from the cardia and fundus are still rare. Therefore, the aim of this retrospective study was to discuss the clinical impact of MLR in patients with gastric cancer from the cardia and fundus, and provide further evidence for rational lymphadenectomy.

MATERIALS AND METHODS

Materials

Two hundred and thirty-six cases, diagnosed as primary gastric cancer from the cardia and fundus were treated with curative resection at the Department of Oncology, Affiliated Union Hospital of Fujian Medical University, Fuzhou China, between January 1996 and June 2002. The surgical procedure was defined as curative when no grossly visible tumor tissue (metastasis or lymph node involvement) remained after the resection and the resection margins were histologically normal. There were 197 male (83.5%) and 39 female (16.5%) patients aged from 30 to 79 years with a mean of 58.8 ± 9.8 years. All patients received a D2 or more extended dissection of all the Group 1 and Group 2 lymph nodes or more according to the Japanese Classification of Gastric Carcinoma (JCGC)[6]. Among the 236 patients, total gastrectomy (TG) was performed in 190 patients, and proximal subtotal gastrectomy (PSG) in 46. Lymph nodes were meticulously dissected from the en bloc specimens, and the classification of the dissected lymph nodes was determined by specialized surgeons who reviewed the excised specimens after surgery based on the JCGC. Clinical and histopathologic data of each patient were collected and recorded in a specifically designed data collection form. The histopathologic spectrum included papillary adenocarcinomas (47/236, 20%), tubular adenocarcinomas (101/236, 43%), mucinous adenocarcinomas (29/236, 12%), poorly differentiated adenocarcinomas (36/236, 15%), undifferentiated carcinomas (8/236, 3%) and others (15/236, 6%) according to the World Health Organization classification system. Based on the 5th Edition of UICC TNM system[7], T category is defined as follows: T2: tumor invades muscularis propria or submucosa; T3: tumor penetrates serosa without invasion of adjacent structure; T4: tumor invades adjacent structures. N category is defined as follows: N0: no regional lymph node metastasis; N1: metastasis in 1 to 6 regional lymph nodes; N2: metastasis in 7 to 15 regional lymph nodes; N3: metastasis in more than 15 regional lymph nodes. Among our patients, there were 25 at stage pT2, 118 at pT3 and 93 at pT4, respectively, while there were 42 pN0, 97 pN1, 68 pN2 and 29 pN3 respectively. Finally, 48 cases (20%) were categorized as stage Ⅱ, 128 (54%) as stage Ⅲ and 60 (25%) as stage Ⅳ. All the patients received postoperative chemotherapy, using 5-FU as the dominant agent. No patientreceived postoperative radiotherapy. The follow-up was carried out by specialized investigators, who were trained about the follow-up system for clinical observation. The median follow-up for the entire cohort was 44 mo (range: 1-136 mo). A total of 222 cases were followed up with a rate of 94.0%.

Methods

All calculations were performed using the SPSS 11.5 statistical package. Correlation analysis was made to find the relationship between MLR and the total lymph nodes, positive nodes and the total lymph nodes. Cumulative survival was determined *via* the Kaplan-Meier method, with univariate comparisons between groups through the log-rank test. Covariates that remained significant through the univariate analysis were selected for multivariate analysis. Cox regression was used for multivariate analysis, with a forward stepwise elimination model. A multiple linear regression model to correlate MLR with 5-year survival was obtained based on Kaplan-Meier 5-year survival estimates for each MLR interval, using midpoint of MLR interval as the independent variate. Significance of differences was assumed at P values of less than 0.05.

RESULTS

MLR of different pT/pN subcategories

From the 236 cases, a total of 5615 lymph nodes were picked up and histologically examined, with 1610 positive and 4005 negative. The median total LN number was 23 (range 7-74, mean 23.8 ± 8.8 per patient), the median number of positive LNs was 5 (range 0-44, mean 6.8 ± 6.8 per patient), and that of negative LNs was 16 (range 0-48, mean 16.9 ± 9.3 per patient). The MLRs were 12.0%, 26.3% and 36.2% in cases with pT2, pT3and pT4, (χ^2 = 138.9, P < 0.001), and 16.8%, 42.7% and 68.9% in cases with pN1, pN2, and pN3, respectively (χ^2 = 820.7, P < 0.001). Figure 1 shows a trend of MLR according to different pT/pN subcategories. The MLR ascended as the invasion deepened, or the number of metastatic nodes increased.

Correlation analysis between MLR and total lymph nodes, positive lymph nodes and total lymph nodes

The MLR did not correlate with the total lymph nodes dissected (r = -0.093, P = 0.057), whereas positive lymph nodes did (r = 0.173, P = 0.008). Figures 2 and 3 show the scatters of these two groups. The results revealed that, in the same extent of lymphadenectomy, MLR

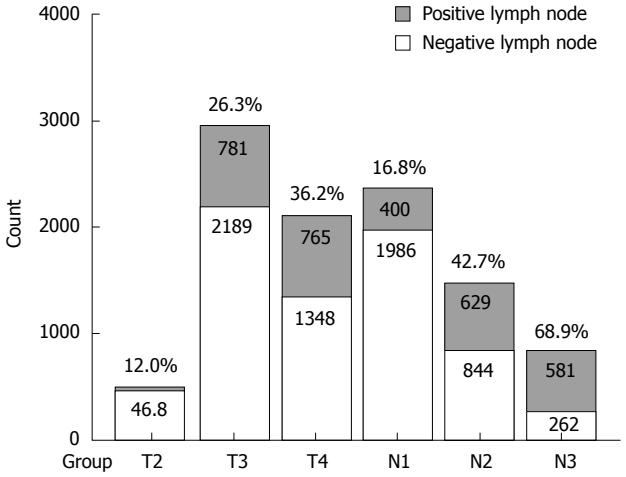

Figure 1 MLR of different pT/pN subcategories.

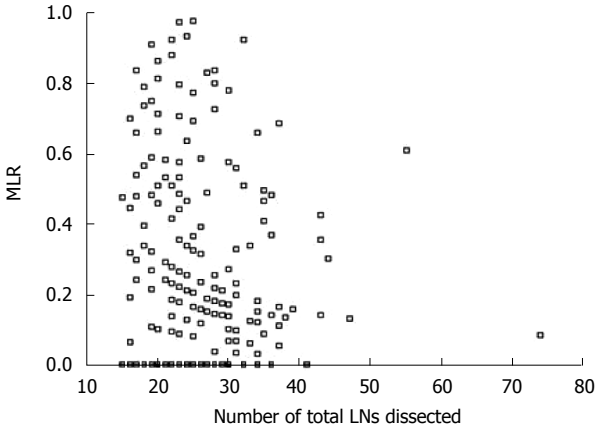

Figure 2 Scatter of MLR and total LNs dissected.

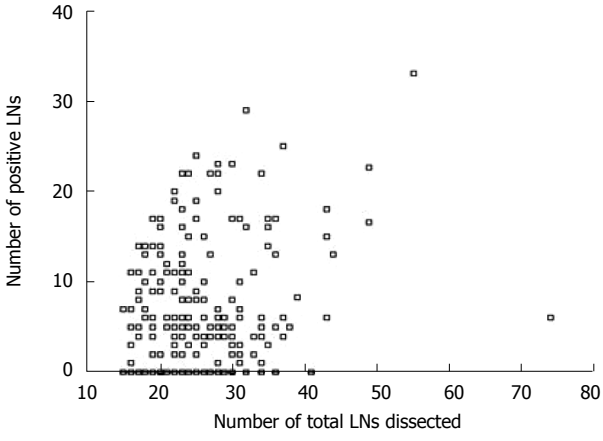

Figure 3 Scatter of positive LNs and total LNs dissected.

would not increase with the number of total lymph nodes, but the number of positive lymph nodes would.

Univariate survival analysis

The 5-year overall survival rate of the entire cohort was 37.5%. The clinicopathological variables tested in the univariate analysis are shown in Table 1. Factors influencing the 5-year survival rate were patient gender

Table 1 Univariate survival analysis of MLR and clinicopathological characteristics in 236 patients undergoing curative surgery

Characteristics	*n*	Median survival time (mo)	5-yr survival (%)	χ^2	*P*
Gender				4.26	0.0389
Male	197	45	36.3		
Female	39	51	43.6		
Age (yr)				1.18	0.2777
< 60	103	45	39.0		
⩾ 60	133	46	36.6		
Tumor size (cm)				18.48	0.0000
< 3	88	60	45.5		
3-6	97	47	39.7		
> 6	51	32	18.1		
Borrmann's type[1]				7.41	0.0065
Borrmann Ⅰ, Ⅱ	172	50	41.7		
Borrmann Ⅲ, Ⅳ	64	41	26.5		
Histological type				5.07	0.0243
Differentiated	213	47	38.9		
Undifferentiated	23	35	19.9		
pT category				49.42	0.0000
pT2	25	72	84.0		
pT3	118	54	40.2		
pT4	93	29	20.8		
pN category				87.7	0.0000
pN0	42	65	73.2		
pN1	97	55	42.4		
pN2	68	27	19.9		
pN3	29	17	0.0		
Num of dissected LNs				8.22	0.0042
< 15	36	27	22.4		
⩾ 15	200	49	39.1		
MLR				34.3	0.0000
< 10%	59	63	60.5		
~ 20%	58	46	34.1		
~ 30%	33	44	33.5		
> 30%	86	28	21.0		
Type of gastrectomy				2.98	0.0844
TG	190	46	39.3		
PSG	46	45	31.3		

[1]Borrmann's type: Macroscopic appearances of primary tumor, classified as Type Ⅰ: polypoid tumors; Type Ⅱ: ulcerated carcinomas with demarcated and raised margins; Type Ⅲ: ulcerated carcinomas without definite limits, infiltrating into the surrounding wall; Type Ⅳ: diffusely infiltrating carcinomas.

($P = 0.0389$), tumor size ($P < 0.001$), Borrmann type ($P = 0.0065$), histological grade ($P = 0.0243$), pT category ($P < 0.001$), pN category ($P < 0.001$), total number of dissected lymph nodes ($P = 0.0042$) and MLR ($P < 0.001$). Patient age ($P = 0.2777$) and type of gastrectomy ($P = 0.0844$) had no significant influence on the survival.

Multivariate survival analysis

Multiple survival analysis was calculated by the Cox's proportional hazard regression model. In order to confirm the influence of MLR, the prognostic factors considered at univariate analysis were analyzed first by stepwise regression, including gender, tumor size, Borrmann type, histological grade, pT category, pN category and total number of dissected lymph nodes except MLR. As a result, there were four independent, statistically significant prognostic parameters: tumor

Table 2 Multiple stepwise regression analysis with Cox proportional hazards model

Characteristics	β	Wald	*P*	RR	95% CI for RR	
					Low	High
MLR excluded						
Tumor size		6.665	0.039			
3-6 cm *vs* < 3 cm	0.222	1.938	0.164	1.249	0.913	1.707
> 6 cm *vs* < 3 cm	0.501	6.636	0.010	1.650	1.127	2.414
pT category		27.747	0.002			
pT3 *vs* pT2	0.761	9.184	0.000	2.140	1.308	3.499
pT4 *vs* pT2	1.286	24.369	0.000	3.617	2.171	6.026
pN category		65.066	0.000			
pN1 *vs* pN0	0.618	9.792	0.002	1.855	1.260	2.733
pN2 *vs* pN0	1.224	30.727	0.000	3.402	2.206	5.244
pN3 *vs* pN0	2.156	57.470	0.000	8.639	4.947	15.090
Num of total LNs	-0.682	10.684	0.001	0.506	0.336	0.761
MLR included						
Tumor size		7.985	0.018			
3-6 cm *vs* < 3 cm	0.307	3.863	0.049	1.359	1.001	1.845
> 6 cm *vs* < 3 cm	0.512	7.353	0.007	1.668	1.152	2.415
pT category		30.821	0.000			
pT3 *vs* pT2	0.772	9.940	0.002	2.165	1.339	3.500
pT4 *vs* pT2	1.343	26.759	0.000	3.832	2.303	6.375
MLR	2.569	69.390	0.000	13.06	7.134	23.900

Table 3 Estimation based on multiple linear regression model (stepwise method)

Parameters	β	*t*	*P*	95% CI for β
Tumor size	-0.11	-1.97	0.051	
MLR	-0.63	-10.4	0.000	-0.71 to -0.51
Intercept (constant)	0.56	19.0	0.000	0.48 to 0.59

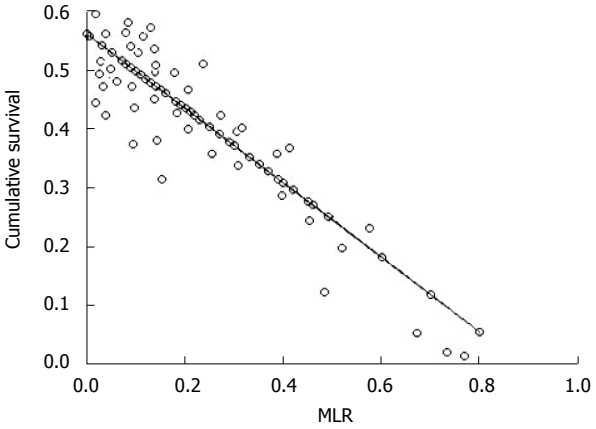

Figure 4 Regression line of MLR impact on the 5-year survival rate.

size (P = 0.039), pT category (P = 0.002), pN category (P < 0.001) and total number of dissected lymph nodes (P = 0.001). When MLR was included in the Cox's regression, the overall fit of the Cox model increased (likelihood ratio test with and without MLR: 397 and 305, respectively). Tumor size (P = 0.018), pT category (P < 0.001) and MLR (P < 0.001) were remained to be independent prognostic factors, with MLR being the most significantly independent factor. The risk ratios and their 95% confident interval are listed in Table 2.

MLR impact on overall survival

Linear trend test found that tumor size and MLR both had a linear correlation to the 5-year overall survival rate, and the correlation coefficients (r) were -0.157 (P = 0.019) and -0.655 (P < 0.0001), respectively. Obviously, MLR showed a much stronger correlation than tumor size. Despite tumor size and overall survival related, only MLR can reach statistically significant differences according to the multiple linear regressions (P < 0.0001). As shown in Table 3, the regression equation of the assumed statistically linearity was y = -0.63x + 0.56. Figure 4 presents a regression line of this calculated MLR effect on the 5-year survival. The hypothetical baseline 5-year survival (based on the y-intercept, i.e. MLR 0%) was 56%. For every 10% added to MLR, the calculated 5-year survival rate decreased by 6.3%. Hypothetically, the 5-year survival would surpass 50% when MLR was lower than 10%.

DISCUSSION

Over the last few decades, the rising trends in incidence rates for upper third gastric cancer have been reported by many investigators around the world[8,9]. Cancer of the gastric cardia and fundus is commonly found in late or the advanced stage at the initial diagnosis[10,11]. It was warranted to link the poor prognosis to lymph node metastases for cancer of this anatomical location reported with a higher frequency of perigastric lymph nodes and higher proportion of overall lymph node metastasis[12,13]. A prospective study conducted by de Manzoni *et al*[14] showed that 56.9% of patients with types Ⅱ and Ⅲ cardia cancer had nodal spread. Di *et al*[15] set up a research on lymph node involvement in gastric cancer for different sites, showing an involvement in 80.4% of cases for upper third cancers. In the present study, 82.2% of cases were found to have lymph node metastasis. And the overall MLR was 28.7%. These data confirmed the cited reports.

Lymph node metastasis is one of the most important prognostic factors for gastric cancer after curative resection. Methods of metastatic lymph nodes evaluation are still under investigation and being continuously improved, including some immunohistochemical methods, in order to predict the prognosis and guide the therapy. But few special proteins expressed in gastric cardia cancer, compared with non-cardia cancer. For example, with regard to the mucin phenotype, MUC1 and MUC5AC expression was less frequent in cardia carcinomas than in non-cardia carcinomas[16]. Therefore, lymph node status mainly depends on routine pathological examinations, previously based on the anatomical station of metastatic lymph nodes, and is classified by the number of metastatic regional lymph nodes[17-19]. The problem of lymph node classification based on number of metastatic ones is the stage migration, induced by larger lymph nodes dissected. Researches on prognostic impact of MLR have been done in patients with colon cancer, pancreatic cancer, breast cancer and other carcinomas[20-23] to find its advantage

in predicting survival outcome after curative resection. MLR was introduced as a more reliable prognostic factor for gastric cancer[24,25]. In our study, a greater MLR was associated with poorer survival by univariate analysis. Multivariate analysis further identified that the MLR was a most important independent prognostic factor among the other factors evaluated, including pN category. This phenomenon was in agreement with those reported by some other investigators. It may be a superior indicator for lymph node classification system. Relevant data were reported about the grouping of patients with gastric cancer with lymph node metastasis ratios. By imitating the pN category of UICC/AJCC, most researchers selected three or four different groups. Different N ratio cutoffs have been proposed[26-29], such as 0%, 1% to 9%, 10% to 25%, > 25%; 0%, < 25%, < 50%, > 50% and so on. Many authors did not describe a specific method for the selection of the reported cutoffs. Hyung *et al*[30] discovered that the cutoff values were 10% for T3N1M0 and 25% for T3N2M0 by analysis of the prognosis according to MLR. In the present study, we found the 5-year survival would surpass 50% when MLR was lower than 0.10.

Our study also showed that MLR can reflect the number of LNs examined and the quality of LN dissection. Curative resection was the determining factor to improve the 5-year survival in this type of tumor[31]. Although D2 radical resection for patients with cancer of gastric cardia and fundus is widely accepted[32], how many lymph nodes should be removed to accurately predict clinical outcome has not been determined. Barbour *et al*[33] suggested patients with Siewert types Ⅱ and Ⅲ adenocarcinoma of the GEJ should undergo adequate lymphadenectomy to permit examination of ⩾ 15 lymph nodes allowing the accurate identification of prognostic variables. Removal of ⩾ 15 lymph nodes is associated with more accurate survival estimates for patients with advanced disease. Gee *et al*[34] stated that preferably 20-25 lymph nodes were necessary for determining prognosis and treatment for tumors of the gastroesophageal junction. In the present study, MLR did not correlate with the total lymph nodes while the number of metastatic LNs did. This finding indicated that the extent of lymphadenectomy was adequate when MLR value did not fluctuate with the resected number of lymph nodes. Obviously, it requires a certain amount of lymph nodes to be dissected. McKee *et al*[35] pointed out, MLR may be confounded by the small number of nodes examined from each patient, it should not be used for prognostic information in patients with fewer than 15 nodes examined. The median total LN number was 23 (mean 23.8 ± 8.8 per patient) in this study, so we suggested D2 lymphadenectomy in order to ensure adequate dissection.

In conclusion, MLR has advantages in providing a more precise prognostic evaluation. We should pay attention to the clinical impact of MLR on prognosis of gastric cancer located in cardia and fundus when performing a D2 radical resection. It is warranted to make efforts to reduce MLR, preferably lower than 10%, in order to achieve better therapeutic efficiency.

COMMENTS

Background

The incidence rates of gastric cancer located in cardia and fundus have increased in recent years. Though the staging system of gastric cancer refines step by step, staging techniques never stop updating. So far, few studies have investigated the relative contribution of metastatic lymph node (LN) ratio to the prognosis evaluation with advanced gastric cancer from cardia and fundus.

Research frontiers

Some researches have shown that metastatic lymph node ratio (MLR) is an excellent predictor for survival outcome in patients with colon cancer, pancreatic cancer, breast cancer and other carcinomas. Some related studies on gastric cancer also found the potential of the MLR on prognostic evaluation, but without a consensus on stratification cutoffs, especially lack of data for advanced gastric cancer located in cardia and fundus.

Innovations and breakthroughs

The authors retrospectively reviewed 236 patients with gastric cancer from cardia and fundus who were treated with D2 radical resection in a hospital in Fujian between 1996 and 2002, to investigate the validity of metastatic LN ratio as a prognostic factor. The study not only divided MLR into some different grades for survival analysis, but also set up a regression to discover the relation between MLR and survival.

Applications

The authors suggest that metastatic LN ratio can provide more dependable and accurate information on the extent of LN metastasis and lymphadenectomy for advanced gastric cancer located in cardia and fundus. Moreover, it is an important prognostic factor and can provide further evidence for rational lymphadenectomy.

Peer review

This is an interesting and well-done study investigating the prognostic role of MLR in cancer of gastric cardia and fundus. This is a well-written and significant paper.

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CASE REPORT

Evidence for colorectal sarcomatoid carcinoma arising from tubulovillous adenoma

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Abstract

Sarcomatoid carcinomas of the colorectum are rare tumors that display both malignant epithelial and stromal components. Clinically, they are aggressive tumors with early metastasis. Due to their infrequent occurrence, the pathogenesis is poorly understood. We report a case of a 52-year-old woman who presented with a rectal mass and intermittent hematochezia. Superficial biopsies during colonoscopy revealed a tubulovillous adenoma with high-grade dysplasia. Endoscopic ultrasonography confirmed an invasive nature of the mass, and deeper biopsies revealed the presence of neoplasm with mixed histological components. The surgically-excised specimen demonstrated the presence of poorly differentiated spindle cells underneath the tubulovillous adenoma and an intermediate stage of invasive adenocarcinoma. Based on the histological appearance and immunohistochemical studies, a diagnosis of sarcomatoid carcinoma was made. Only nine cases of sarcomatoid carcinomas of the colorectum have been reported to date. As a result, the terminology and pathogenesis of sarcomatoid carcinoma remain speculative. To the best of our knowledge, this is the first report of co-existence of sarcomatoid carcinoma and invasive adenocarcinoma with tubulovillous adenoma; all stages represented within the same tumor. This observation supports the "monoclonal theory" of pathogenesis with an adenoma-sarcoma progression with or without an intermediate stage of carcinoma.

Key words: Sarcomatoid carcinoma; Tubulovillous adenoma; Adenocarcinoma; Rectum; Cytokeratin

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INTRODUCTION

Sarcomatoid carcinoma is a rare malignant tumor characterized by a combination of epithelial and mesenchymal elements. Over the past 100 years, sarcomatoid carcinomas have been increasingly recognized at different anatomic locations including the head and neck, respiratory tract, and female reproductive organs[1-3]. Within the gastrointestinal tract, the oropharynx and esophagus are the most commonly affected areas[1,4-7]. To our knowledge, sarcomatoid carcinomas rarely occur in the colon, with only nine cases reported in the English literature[8-12]. A possible reason is likely due to their marked similarity to malignant mesenchymal tumors, such as gastrointestinal stromal tumors, malignant fibrous histiocytoma, and leiomyosarcoma[12-16]. As a result, the natural history, pathogenesis, and treatment of these unusual tumors are poorly understood. In general, sarcomatoid carcinoma of the colon has been described as an aggressive neoplasm with an associated poor prognosis.

First described by Virchow in 1864, a variety of terms

have been used to describe sarcomatoid carcinomas. They include carcinosarcoma, pseudosarcomatous carcinoma, carcinoma with mesenchymal stroma, and spindle cell carcinoma[17]. These varied terminologies for sarcomatoid carcinomas reflect the uncertainty of its histogenesis and classification. Several theories have been proposed to explain the histopathogenesis of sarcomatoid carcinoma; however, these theories remain speculative.

We report an unusual case of a mixed rectal tumor containing a superficial tubulovillous adenoma with deeper areas of high-grade malignant spindle cells and an invasive adenocarcinoma; all stages represented within the same tumor. This could support an adenoma-to-sarcomatoid progression either directly or indirectly via an intermediate stage of adenocarcinoma. In addition, we discuss the basis of pathologic diagnosis, proposed theories regarding its histopathogenesis and review the clinical features of this heterogenous tumor.

CASE REPORT

A 52-year-old white female presented to the emergency department with a prolapsed rectal mass and intermittent rectal bleeding over the past 10 years. Until presentation, she had attributed her symptoms to hemorrhoidal disease and had performed digital reduction of the prolapsed 'mass' from time to time. Increased bleeding and frequency of prolapse made her seek medical attention. Her past medical history was unremarkable; however, there was a family history of colon cancer in her father. Physical examination revealed a large, firm, ulcerated mass that prolapsed from her rectum. Serum levels of carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9 were not elevated. Colonoscopy confirmed the presence of a large sessile exophytic rectal growth with a velvety, multi-lobulated surface, just proximal to the dentate line (Figure 1A and B). Several biopsies were performed using a jumbo forceps, which revealed a tubulovillous adenoma with high-grade dysplasia. However, the possibility of an adjacent invading tumor beneath the tubulovillous adenoma could not be ruled out given the lack of depth from the biopsy. An extensive staging workup including a CT scan of the chest and abdomen showed no signs of distant metastasis. To further evaluate the tumor for any presence and extent of local invasion, a rectal endoscopic ultrasound (EUS) was performed. At EUS, the invasive nature of the mass became apparent as the tumor invaded into the lamina propria and into the muscularis mucosa (Figure 1C, star). Additionally, an 8-mm hypoechoic well-defined malignant-appearing lymph node was found adjacent to the mass, suggestive of lymphatic metastasis (Figure 1D, arrow). During this procedure, a 1-cm × 1-cm piece of the tumor was snared off to obtain deeper sections of the mass for further histopathological diagnosis, and which demonstrated a neoplasm with mixed histological components consisting of tubulovillous adenoma (Figure 2A and B) and poorly differentiated spindle cells (Figure 2C to E). Subsequently, the patient underwent an abdominoperineal resection of this mass. *Ex-vivo* pathologic analysis of the surgically removed rectum showed a superficial layer of tubulovillous adenoma with high-grade dysplasia arising just proximal to the dentate line and extending 6.2 cm proximally (Figure 3A and B). Its deeper sections featured islands of intermediate stage invasive adenocarcinoma with poorly organized glandular structures (Figure 3C) within a background of poorly differentiated sheets of spindle cells. Immunohistochemical studies showed strong positivity for cytokeratin in both epithelial and stromal components of the tumor (Figure 3D). Histological analysis of all 41 lymph nodes showed no evidence of metastasis including the 8- mm hypoechoic lymph node seen on EUS. Based on the histological appearance and immunochemical studies, a diagnosis of sarcomatoid carcinoma was made. The disease was clinically staged according to the American Joint Committee on Cancer as a Stage I (T1N0M0) colorectal cancer. The patient's postoperative course was uneventful and she remains free of tumor recurrence or metastasis after an 8-mo follow up.

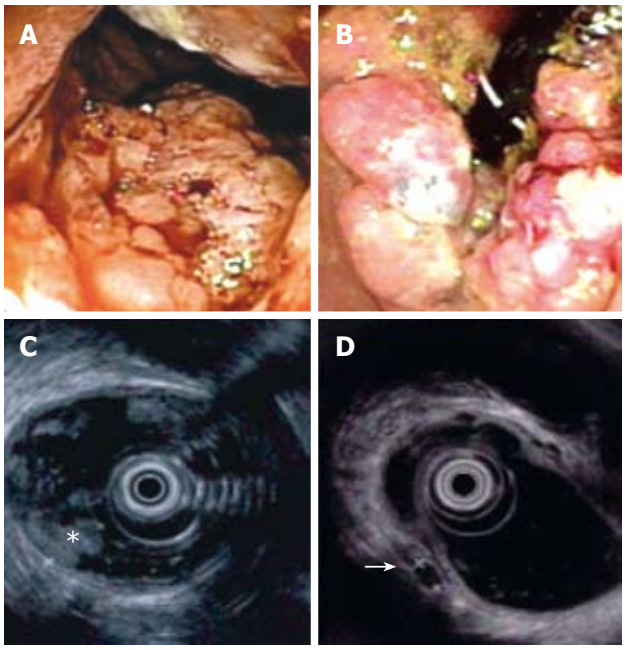

Figure 1 Colonoscopy demonstrates. **A**: Large sessile polypoid growth with velvety surface and superficial ulceration in the rectum (forward view); **B**: Multilobulated, smooth surfaced exophytic nature of the tumor upon retroflexion. Sonographic images at rectal EUS showing; **C**: An infiltrative mass with invasion of the muscularis mucosa (star); **D**: An 8-mm hypoechoic lymph node suspicious for lymphatic metastasis (arrow).

Case tissue

Tissue from the rectal tumor was fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 4 μm in thickness, floated onto positively charged slides, and dried overnight at 70℃. From each block, 5 micron thick sections were cut and stained with haematoxylin and eosin (HE). For immunohistochemical analysis, the avidin-biotin complex method was used with the following antibodies: pancytokeratin (Figure 3D), vimentin (Figure 3E), smooth muscle actin (SMA), S100, Bcl-2, CD34, smooth muscle myosin (SMMS), p53 (Figure 4A), CD117 (Figure 4B), desmin, and PDGFR-alpha

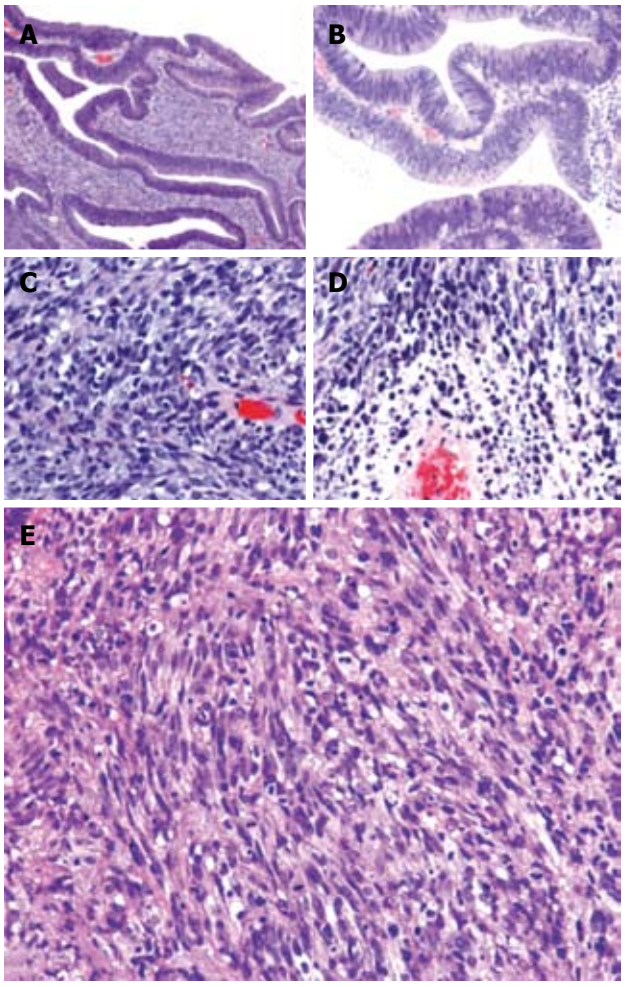

Figure 2 Histology of the rectal biopsy using HE stains. **A**: Tubulovillous adenoma and underlying spindle cell tumor. The aggressive spindle cell lesion infiltrates directly underneath the adenoma (x 10); **B**: A higher magnification view of the adenomatous component (x 20). High-grade spindle cell lesion (x 40) showing: **C**: Cigar shaped nuclei, nuclear pleomorphism, a high mitotic rate; **D**: Tumor necrosis; **E**: Smooth muscle-like spindle sheets of cells in the sarcomatoid component (x 40).

(Figure 4C). The immunohistochemical profile is listed in Table 1. Appropriate positive and negative control tissues were incubated in parallel with the case slides to confirm the specificity of each antibody.

Gross findings

The surgical specimen consisted of a sigmoid and rectal segment measuring about 36.5 cm in length with a luminal diameter varying from about 4.8 cm at the proximal end to 8.0 cm at the distal end (Figure 3A and B). Located at the distal end of the specimen was an exophytic, circumferential, and fungating mass measuring about 5.5 cm in length along the gastrointestinal tract, rising approximately 1.3 cm from the luminal surface, with a greatest diameter of 6.2 cm. Just distal to the fungating mass was a squamous anal mucosa measuring about 0.8 cm in length. The remainder of the colonic mucosa was covered by small papules, which were slightly whiter than the gray mucosal background and measured about 0.1-0.3 cm in greatest dimension. These papules covered about 80% of the total surface of the colon and were most prominent in the

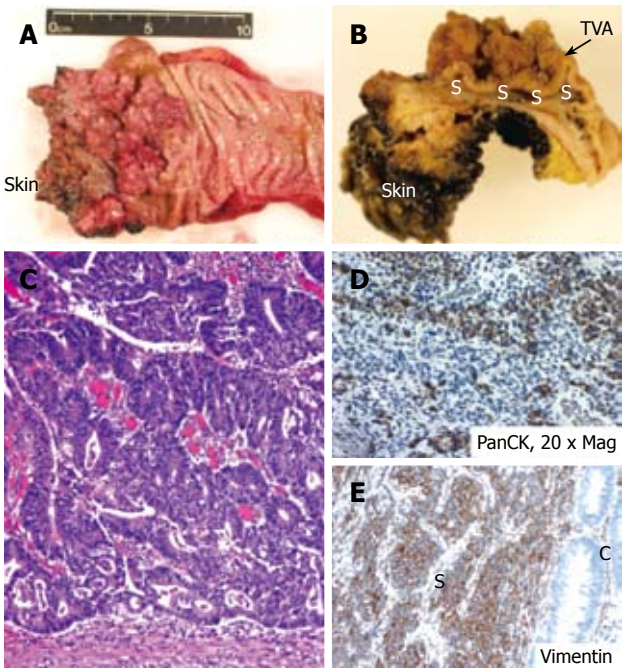

Figure 3 Gross appearance of the surgically-removed rectosigmoid mass (**A** and **B**). **A**: The luminal side view demonstrates the proximity to the dentate line; anal skin is labeled for orientation; **B**: Upon sectioning the sample through the sagittal plane, the lateral view identifies the sarcomatoid smooth component (marked as S) underneath the velvety tubulovillous adenoma component (marked as TVA); **C**: Invasive adenocarcinoma with high nucleus/cytoplasm ratio within the deeper sections; **D**: Pancytokeratin was diffusely positive in both epithelial and mesenchymal components (x 20); **E**: Vimentin showed expression in the sarcomatous (S) component only, with no staining in the carcinoma (C) portion.

Table 1 Antibodies used for histological evaluation of the tumor, and a summary of the results

Antibody	Concentration	Company	Result
Smooth muscle actin (SMA)	1:2	Dako	Negative
S100	1:1000	Zymed	Negative
Bcl-2	1:60	Dako	Negative
CD34	1:30	Becton Dickinson	Negative
PDGFA-α	1:120	Santa Cruz Biotechnology	Non-contributory
Smooth muscle myosin (SMMS)	1:100	Dako	Negative
CD117	1:150	Dako	Negative
Desmin	1:100	Dako	Negative
Vimentin	1:100	Dako	Negative
p53	1:100	Dako	Positive
Pancytokeratin	1:100	Zymed AE1/AE3 Becton Dickinson Cam 5.2	Patchy positive

distal area near the fungating mass. No ulcers or strictures were noted. Forty-one lymph nodes were isolated from the adipose tissue surrounding the bowel wall and were negative for any evidence of metastasis.

Histological findings

The initial superficial biopsies from the large rectal mass showed a tubulovillous adenoma with high-grade dysplasia; however, the possibility of adjacent invasive tumor could not be ruled out given the lack of depth from the

Table 2 Summary of demographics and outcome of cases reported in the English literature with the diagnosis of colorectal sarcomatoid carcinoma

Case	Author, yr	Age (yr)	Sex	Site	Distant metastasis	Outcome
1	Weidner, 1986	73	M	Sigmoid	On follow up	4 yr, DOD
2	Chetty, 1993	72	F	Cecum	On initial visit	3 mo, DOD
3	Roncaroli, 1995	71	F	Rectum	On follow up	6 mo, DOD
4	Isimbaldi, 1996	86	F	Ascending colon	None	2 yr, disease free
5	Shoji, 1998	78	M	Descending colon	None	16 mo, disease free
6	Takeyoshi, 2000	82	M	Rectum	On follow up	6 mo, DOD
7	Kim, 2001	41	F	Sigmoid	On follow up	4 mo, DOD
8	Di Vizio, 2001	56	F	Descending colon	On follow up	21 mo, DOD
9	Kim, 2005	71	M	Ascending colon	On initial visit	Unspecified
10	Present case	52	F	Rectum	None	8 mo, disease free

DOD: Died of disease.

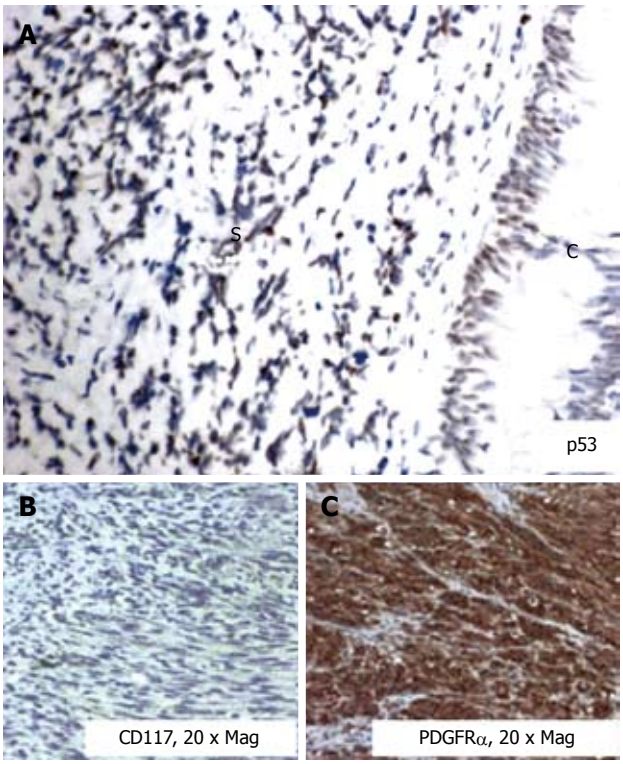

Figure 4 Immunohistochemical characteristics of the resected tumor showed. **A**: p53 was expressed in both carcinomatous (C) and sarcomatous (S) components within the tumor, with a relative increase in the latter; **B**: Staining for CD117 was negative; **C**: PDGFR stained diffusely positive in both the components (x 20).

biopsies. Subsequently, a larger piece of tumor tissue, obtained during a EUS procedure, revealed a neoplasm with mixed histological components consisting of high-grade spindle cells and a tubulovillous adenoma (Figure 2). The high-grade spindle cell tumor showed cigar shaped nuclei, nuclear pleomorphism, a high mitotic rate, a high nucleus/cytoplasm ratio, and central necrosis (Figure 2C-E). The spindled cells appear to infiltrate from the deep aspect of the biopsy into the mucosa and surround the adenomatous glands in an aggressive fashion. The tubulovillous adenoma, similar to the previous biopsy, contained abundant dysplastic epithelial glands with very few mucin.

The surgical specimen of the sigmoid colon and rectum revealed an invasive well-differentiated adenocarcinoma in a background of a large tubulovillous adenoma (Figure 3C). In addition, there was a focal sarcomatous component consisting of high-grade spindle cells. The carcinoma was composed of neoplastic epithelial cells, arranged in nests forming glandular structures with increased nuclear/cytoplasmic ratio and prominent nucleoli. The invasive carcinoma was confined to the submucosa (pT1) without gross or microscopic evidence of muscular invasion. Surgical margins were free of tumor and there was no evidence of metastasis in all 41 lymph nodes. According to the TNM classification, the pathological stage was pT1N0.

Immunohistochemistry

Immunohistochemical analysis revealed focal, but strong staining for pancytokeratin (Figure 3D), focal staining for vimentin (Figure 3E), smooth muscle actin, and patchy cell clusters of desmin in the spindle cell component of our specimen. Although staining for p53 revealed immunoreactivity in both the epithelial and sarcomatous components, it was relatively increased in the sarcomatous component (Figure 4A). In addition, the sarcomatous component was weakly positive for CD34 and Bcl-2, but negative for CD117 (Figure 4B). The epithelial component was diffusely immunoreactive to pancytokeratin and negative to smooth muscle actin, desmin, CD34, CD117, and Bcl-2. Since there is a subset of gastrointestinal stromal tumors that are negative for CD117 and positive for PDGFR-α, we performed an immunohistochemical staining for PDGFR-α, which stained diffusely positive in both the spindle and epithelial cells of the overlying tubulovillous adenoma (Figure 4C). Taken together, the strong cytokeratin staining and the focal positivity for smooth muscle markers, the tumor adjacent to the tubulovillous adenoma was diagnosed as sarcomatoid carcinoma rather than a carcinosarcoma or CD117-negative GIST.

DISCUSSION

Sarcomatoid carcinoma of the colon is a rare clinical and pathological entity. To the best of our knowledge, only nine cases of colonic sarcomatoid carcinoma have been reported in the English literature (Table 2), the present case being the tenth. Histologically, sarcomatoid carci-

nomas of the colon contain both epithelial and mesenchymal components. The epithelial component of these tumors mainly consists of high-grade adenocarcinoma, while the accompanying sarcomatous component often demonstrates a spindled appearance with varying degrees of mesenchymal-like differentiation.

The nomenclature of these bi-differentiated tumors has been a matter of debate despite the advent of immunohistochemistry and electron microscopy. In the past, the term pseudosarcoma was used to describe the condition in which the epithelial component was malignant and spindle cells were benign[17-19]. Later, Matsusaka *et al* showed that pseudosarcoma and sarcomatoid carcinoma were histologically and clinically the same conditions[20]. Moreover, several reports have used carcinosarcoma interchangeably to describe sarcomatoid carcinoma, further confusing the diagnosis and classification of these tumors[13,16,21]. Rosai J explained that when the sarcomatous component is mainly composed of spindle cells but still identifiable as epithelial ones morphologically or immunohistochemically, the diagnosis should be called sarcomatoid carcinoma[22]. However, when sarcomatous components reveal typical specialized differentiation such as obvious striation of rhabdomyosarcoma or osteoid produced by malignant neoplastic cells, the diagnosis should be called carcinosarcoma[22]. For cases without any histologically-identifiable differentiation, immunostaining for cytokeratin or other epithelial markers may help in determining whether the neoplasm matches the qualifications for sarcomatoid carcinoma or carcinosarcoma. The common denominator of all sarcomatoid carcinomas is the immunoreactivity of epithelial markers such as cytokeratin for both the sarcomatous and epithelial components. However, if sarcomatous elements do not express epithelial markers, the term carcinosarcoma is the preferred diagnosis[17,23]. In our case, both the sarcomatous and epithelial components were immunoreactive to pancytokeratin, confirming our diagnosis of sarcomatoid carcinoma.

The histogenesis of sarcomatoid carcinoma remains unclear and controversial despite more than a century of conjecture. Proposed explanations of the bi-differentiated appearance include the multiclonal hypothesis on one hand, and on the other hand the "collision theory", suggesting that the two tumor components are derived from separate and distinct malignant cell clones[5,24]. Other investigators suggest a clonal origin (monoclonal hypothesis) of the tumor since common characteristics have been seen between the different cellular populations as well as an observed transitional population[25,26]. For example, the presence of cytokeratin in spindle cells within sarcomatoid carcinomas of various anatomical locations supports the epithelial origin of these cells[17,19,27,28]. The observed characteristics in the monoclonal hypothesis could either be due to a malignant transformation of a pluripotent stem cell capable of epithelial and mesenchymal differentiation or the sarcomatous element arising from a metaplastic transformation of the carcinomatous element. Some postulate that this sarcomatous transition from carcinomatous cells could be related to retrovirus infection[29]. Sarcomatoid carcinomas demonstrated very rarely to be "collision tumors" with sharply defined sarcomatous and carcinomatous components without any shared or transitional features. There is, however, strong molecular evidence that supports the monoclonal origin of most sarcomatoid carcinomas[30]. Several genetic studies involving loss of heterozygosity (LOH) with microsatellite markers and pattern of X chromosome inactivation have demonstrated a common origin of the admixed components[31,32]. In addition, Delahunt *et al* described progressive accumulation of p53 proteins in the phenotypic conversion of carcinoma into sarcomatoid phenotype, thus indicating an increasing clonal dominance of dedifferentiated tumor cells carrying p53 mutations[33].

In our case, the surgically removed rectal tumor demonstrated both tubulovillous adenoma and adenocarcinoma adjacent to each other with sarcomatous elements right beneath the tubulovillous adenoma. This histological finding strongly suggests that the two components originated from a multipotent epithelial cell, and that the sarcomatous components originated with differentiation from adenoma to sarcomatoid phenotype during tumor progression through an intermediate stage of adenocarcinoma. To further support a monoclonal origin and a possible tumor progression sequence in our case, immunohistochemistry for p53 protein showed increasing accumulation of p53 from tubulovillous adenoma to adenocarcinoma and finally to the sarcomatous area (Figure 4A). The adenoma-to-carcarcinoma sequence is well known; however little is known whether the sarcomatous phenotype is part of this tumor progression sequence. To our knowledge, this is the first case that demonstrates a clear histological adenoma-adenocarcinoma-sarcomatoid phenotype sequence progression all in one image.

Clinical features of all ten cases (including our present report) showed a mean age of 68 years (range 41-86) and a slight predilection for females (Table 2). Sarcomatoid carcinomas can be located anywhere in the large bowel, but a preference for the distal colon (including the descending colon, sigmoid colon, and rectum) is seen in most cases. Lymph nodes and distant sites of metastasis disclose a predominance of the malignant epithelial component. However, only one case has shown metastasis from the sarcomatous element, indicating the aggressive nature of the epithelial component[13]. Despite radical surgery, chemotherapy, or radiotherapy, prognosis for sarcomatoid carcinoma of the colon remains poor. Seven cases revealed distant metastasis either at initial or follow-up visit, and six of the ten cases died within five years of diagnosis. Due to the rarity of cases, no specific conclusions can be made regarding prognostic factors.

Treatment for sarcomatoid carcinomas of the colon should follow similar guidelines for colonic adenocarcinomas, as no specific treatment guidelines are available for the management of this tumor. In addition, extensive follow-up should be warranted given the poor prognosis associated with this rare clinical entity. Nine of the ten reported cases (including ours) of colonic sarcomatoid carcinomas have undergone resection of the primary tumor; however, only two of the ten cases have survived over the

past two years despite adjuvant therapy. In a recent 8-mo follow-up, our patient is still alive with no evidence of metastasis after surgical resection of the primary tumor.

In summary, sarcomatoid carcinoma of the colon is a highly aggressive neoplasm that leads to a poor patient outcome despite clinical intervention. Endoscopic biopsy specimens containing lesions with spindle cell morphology should raise the differential diagnosis of sarcomatoid carcinoma with immunostaining for cytokeratin to help confirm the diagnosis. The histogenesis of sarcomatoid carcinoma is still unclear; however, the morphological appearance and immunohistochemical analysis of our case strongly suggests an adenoma-carcinoma-sarcomatoid phenotype sequence progression as the likely pathogenesis of this rare tumor. Clinical management should follow the diagnostic and therapeutic guidelines for colorectal adenocarcinomas given the paucity of cases. Further studies and collection of cases are needed to establish proper therapeutic interventions.

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CASE REPORT

AIDS-associated plasmablastic lymphoma presenting as a poorly differentiated esophageal tumor: A diagnostic dilemma

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Abstract

Plasmablastic lymphoma (PBL) is a rare form of diffuse large B-cell lymphoma characterized by weak/absent expression of conventional B-cell markers and strong expression of plasma cell markers. It is strongly associated with human immunodeficiency virus (HIV) and Epstein Barr virus infection, and shows an unusual tropism to the oral cavity. Herein we describe a patient with AIDS who presented with weight loss and dysphagia owing to a large gastroesophageal mass. His radiographic and endoscopic findings and long history of cigarette consumption suggested carcinoma. Biopsy demonstrated a poorly differentiated tumor stained negatively to routine lymphoid markers including CD20. However, gene rearrangement studies confirmed a B-cell process and a more detailed immunohistochemical analysis revealed the cells stained positively for CD138 (plasma cell antigen). These findings were diagnostic of PBL. Our report reviews the wide differential diagnosis of PBL and underscores the importance of a broad array of viral and molecular studies needed to establish this diagnosis.

INTRODUCTION

An increasingly large number of HIV-infected patients are developing HIV-associated, but not AIDS-defining neoplasms including esophageal cancer, head and neck malignancies, lung cancer, liver and anal neoplasms and renal cell carcinoma[1]. The pathogenesis of these tumors is complex and is in part related to alcohol and cigarette consumption, the consequences of chronic inflammation including hepatitis C virus (HCV)-associated cirrhotic liver disease and the presence of oncogenic viruses such as human papilloma virus, Epstein Barr virus (EBV) and hepatitis B virus (HBV). Many of these patients are receiving highly active anti-retroviral therapy (HAART) and are no longer destined to die from AIDS-related complications.

Plasmablastic lymphomas (PBLs) were originally described in HIV-infected patients as an aggressive variant of diffuse large B-cell lymphoma (DLBCL), with a peculiar tropism for the oral cavity[2]. PBLs are composed of rapidly growing, large neoplastic cells displaying some degree of plasma cell differentiation. Phenotypically, PBLs display an unusual immunohistochemical profile characterized by weak or absent expression of conventional B-cell markers

coupled with strong expression of plasma cell markers. Recent reports have identified this neoplasm in extra oral sites in both HIV seropositive and seronegative individuals.

Herein, we describe the clinical course of a patient with AIDS who presented with a constrictive and ulcerating esophageal mass which was thought initially to be a poorly differentiated carcinoma, but after a more detailed immunohistochemical evaluation, proved to be PBL. We emphasize the unusual clinical features of this rare form of non-Hodgkin's lymphoma (NHL) and the diagnostic challenges associated with its identification.

CASE REPORT

A 40-year-old Caucasian male with a 25-pack-year history of cigarette consumption but no alcohol or illicit drug use, sought medical attention. He had lost 20 lbs over a period of 2 mo, and complained of loss of appetite and progressive odynophagia (solids > liquids). He also experienced low-grade fevers and drenching night sweat that was not ameliorated by acetaminophen. His past medical history was significant for HIV and chronic active HBV co-infection, diagnosed a year earlier when, while homeless, he presented with muscle weakness and altered mental status. His initial CD4+ count was < 50 cells/μL and his HIV viral load was > 100 000 copies/mL. Neurological evaluation led to a diagnosis of AIDS-associated encephalopathy and myelopathy. He was transferred to a skilled nursing facility where he received rehabilitatory care along with once daily HAART consisting of a fixed dose coformulation of tenofovir 300 mg and emtricitabine 200 mg, ritonavir 100 mg and atazanavir 300 mg. His condition gradually improved and 6 mo later his CD4+ count increased to 180 cells/μL and his HIV viral load fell to < 75 copies/mL.

On physical examination he appeared disheveled and emaciated with dry mucous membranes, proximal muscle wasting but no hairy leukoplakia or oral candidiasis. There was no lymphadenopathy or hepatosplenomegaly, and his myelopathy-associated spastic gait and lower extremity hyperreflexia were stable. Laboratory tests included: white blood count 7600 cells/mm$^{[3]}$; hemoglobin 12.1 mg/dL; platelet count 303 × 10^3/μL; total protein 7.2 mg/dL; albumin 4 mg/dL; lactate dehydrogenase 496 IU/L (normal range, 125-243 IU/L); normal electrolyte and hepatic transaminase levels; positive HBV surface antigen and HBV e antigen; HBV DNA 9360 copies/mL and negative Hepatitis A virus and HCV serologies. His CD4+ count had dipped to 103 cells/μL, but his HIV viral load remained non-detectable. A chest roentgenogram demonstrated a retrocardiac soft tissue density. Chest and abdominal computed tomogram (CT) further showed the density to be a 4.9 cm × 5.1 cm concentric distal esophageal mass associated with extensive gastric wall thickening (Figure 1). The dominant mass corresponded to an area of intensely increased metabolic activity (SUV = 40.3) and was associated with right iliac adenopathy

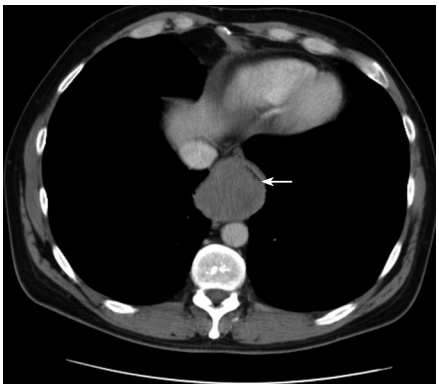

Figure 1 CT scan of chest highlighting the large distal esophageal mass. Note the impressive constriction of the esophageal lumen (arrow).

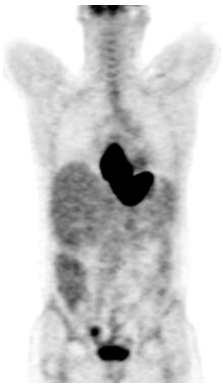

Figure 2 Whole body PET scan demonstrates intensely increased metabolic activity corresponding to the large esophagogastric mass. There is also focal increased activity in a right iliac node.

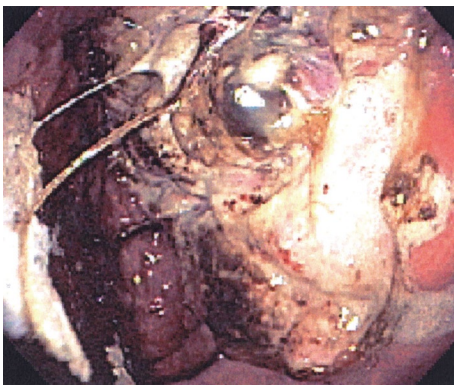

Figure 3 Upper Endoscopic evaluation shows esophageal mass with ulcerative features which extended into the gastric fundus.

(SUV = 40.6) on a whole body F-18 Fluorodeoxyglucose positron emission tomography (PET) scan (Figure 2).

Upper endoscopic evaluation revealed that the mass constricted 40% of the esophageal lumen and extended into the proximal stomach where a large ulcerative lesion was identified (Figure 3). Biopsies of the tumor showed a poorly differentiated, malignant neoplasm composed of irregular sheets of cells, which in many areas were largely necrotic. Cells were cytologically atypical with somewhat eccentric vesicular nuclei and prominent nucleoli (Figure 4A). On initial review of the biopsy, a poorly differentiated carcinoma of gastroesophageal origin was favored. However, immunohistochemical evaluation showed that the tumor cells stained negatively

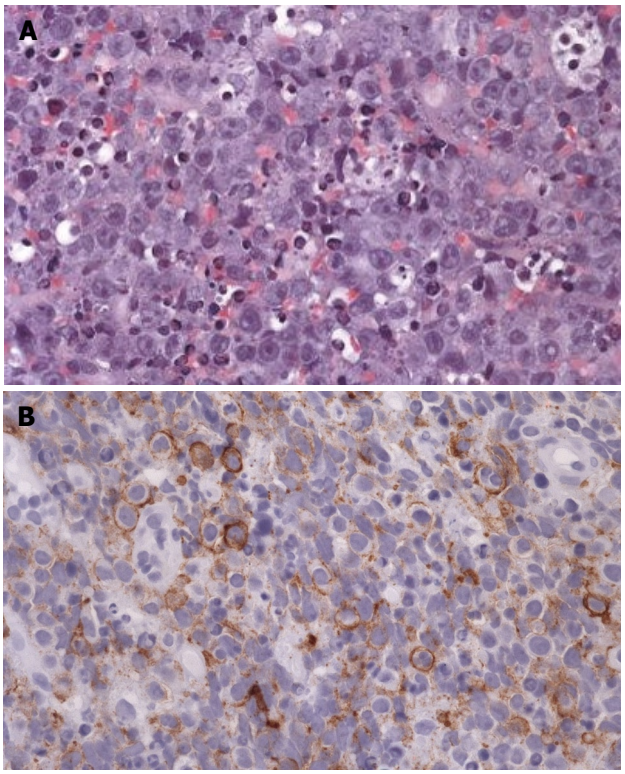

Figure 4 **A**: HE image shows a poorly differentiated, malignant neoplasm composed of irregular sheets of cells. Cells are cytologically atypical with somewhat eccentric vesicular nuclei and prominent nucleoli (x 40); **B**: Immunohistochemistry shows patchy but strong staining of tumor cells for CD138 (x 40).

for epithelial markers (cytokeratin), lymphoid markers (CD20, CD3, CD30 and PAX-5) and melanocytic markers (HMB-45, melan-A and S100). Based on these inconclusive results, additional immunohistochemical stains for markers of lymphoid and plasmacytoid differentiation was performed. The tumor cells were stained strongly positive for CD-45 (leukocyte common antigen) and CD-138 (plasma cell marker) and weakly positive for CD-79a (pan B-cell marker) (Figure 4B). Tumor cells were also positive for the B-cell transcription factors Bob-1 (focal) and Oct-2 (focal weak). In situ hybridization studies were positive for EBV but negative for human herpes virus type-8 (HHV-8, Table 1). A clonal rearrangement of the immunoglobulin heavy chain gene was identified on polymerase chain reaction analysis.

Following a diagnosis of PBL, the patient was treated with combined chemotherapy consisting of liposomal doxorubicin, cyclophosphamide and etoposide (LACE regimen) in addition to HAART[3]. He tolerated the therapy well, with mild nausea with each treatment cycle, alopecia and a single episode of culture negative neutropenic fever. A follow-up CT scan taken after the second treatment cycle showed dramatic improvement with just mild residual wall thickening of the distal esophagus. After completing a total of six cycles of chemotherapy, his dysphagia and weight loss resolved and a CT-PET scan showed complete resolution of the abnormal activity in esophagus and right iliac region.

Table 1 Immunohistochemical findings of esophagogastric mass

Type	Antigen	Immunoreactivity
Epithelial markers	Cytokeratin 35BH11	Negative
	EMA	Diffusely positive
	BER-EP4	Negative
Lymphoid markers	CD-45	Positive
	CD-3	Negative
	CD20	Negative
	CD79a	Equivocally positive in some cells
	PAX-5	Negative
	CD30	Negative
	CD138	Positive (patchy, strong)
B-cell transcription factors	Bob-1	Positive
	Oct-2	Positive
Melanocyte markers	Melan-A	Negative
	HMB-45	Negative
	S100	Negative
Viral markers	HHV-8	Negative
	EBV (*in situ* hybridization)	Positive

EMA: Epithelial membrane antigen; PAX-5: Paired box gene-5; HHV-8: Human Herpes Virus type-8; EBV: Epstein Barr virus.

His post-chemotherapy upper endoscopic evaluation did not reveal persistent NHL and 6 mo later, he remains in remission.

DISCUSSION

In 1997, Delecluse and colleagues were the first to describe in HIV-infected patients, the occurrence of a high-grade malignant lymphoma subtype named PBL which exclusively involved the oral cavity[2]. These tumors possessed a unique immunohistochemical phenotype characterized by their failure to express common lymphoid markers while stained positively for plasma cell markers[4]. Over the past decade, the clinical spectrum of this NHL has expanded to include extra oral involvement in patients with and without HIV infection. Unusual sites of PBL involvement have included the skin, nasal and paranasal sinuses, long bones, lungs, stomach, anorectum, omentum, testes, spermatic cord, bone marrow, sacrococcygeal cysts and central nervous system[5-10].

PBL accounts for 2.6 % of all AIDS-related NHLs, and rarely represents the sentinel manifestation of AIDS[11-13]. In HIV-negative individuals, PBL is often associated with iatrogenic immunosuppression such as seen with organ transplantation[14]. It has also been reported in association with Azathioprine and Infliximab therapy for management of inflammatory bowel disease[15,16]. PBL has been diagnosed in children as young as age 7, but the majority of the reported cases involve middle-aged adults.

Patients with PBL can be divided into three distinct categories[17-20]. The first and the more common PBL variant is localized to the oral mucosa, although the tumor may also involve nodal or extranodal sites. Histologically, this variant is characterized by a

Table 2 Differential diagnosis of PBL

Tumor subtype	Carcinoma	PBL	Common DLBCL	BL	Plasmacytoma
Tumor cell size	Large	Large	Large	Intermediate	Intermediate
CD20	-	- to ±	+	+	-
CD45	-	Variable	+	+	-
CD138	-	+	-	-	+
VS38c	-	+	- to ±	-	+
MUM1	-	+	-	-	+
EBV	-	+	-	+	-
HHV-8	-	Variable	-	-	-

PBL: Plasmablastic lymphoma; DLBCL: Diffuse large B cell lymphoma; BL: Burkitt's lymphoma; CD: Cluster of differentiation; MUM-1: Multiple myeloma oncogene-1-protein; EBV: Epstein Barr virus; HHV-8, human Herpes Virus type-8; +: Expression of the antigen in the majority of cells; -: Absence of antigen expression; ±: Weak antigen expression.

monomorphic population of immunoblasts with no or minimal plasmacytic differentiation. The second PBL category is distinguished by its plasmacytic differentiation and extra oral presentation. The tumor cells are composed predominantly of immunoblasts, plasmablasts and mature plasma cells. The third variant of PBL has also been reported in association with HHV-8 and multicentric Castleman's disease. Patients typically present with lymphadenopathy and splenomegaly, often with plasmablasts circulating in the peripheral blood.

Despite morphologically resembling B-cell immunoblasts, PBL is associated with plasma cell immunophenotype, with loss of B cell markers (CD20) and surface immunoglobulin, and acquisition of plasma cell surface markers [VS38c, CD38 (syndecan-1), MUM-1, CD-138]. The plasmablasts are variably immunoreactive for CD45 and CD-79a[6,11,15]. They usually lack Bcl-6, the germinal center-associated B-cell antigen and PAX-5/BSAP, a nuclear factor that is present from the precursor B-cell stage and in all mature B cells but lost in terminal differentiation to plasma cells[11,17]. Newer B-lineage markers like the transcription factors OCT.2 and BOB.1 may be helpful to confirm the B-cell origin of these tumors[20].

EBV infection is strongly associated with PBL. *In situ* hybridization for EBV Encoded RNA in tumor specimens has reportedly ranged from 60% to 100%, suggesting that EBV plays an important role in PBL pathogenesis[9,15,17,21]. In contrast, HHV-8 is not consistently associated with PBL, although rare cases have identified HHV-8 in conjunction with HIV infection, PBL and multicentric Castleman's disease[9,15,17,22,23]. Patients with chronic HBV infection are more likely to develop NHLs, but specific cases of PBL have not been documented in this population[24].

The differential diagnosis of PBL may overlap with a variety of other clinicopathologic entities (Table 2). When PBL presents as an oral lesion, it may be confused with periodontal disease like odontogenic cellulitis, KS or melanoma[12]. Carcinomas can be distinguished from PBLs based on presence of immunoreactivity for epithelial markers such as cytokeratin. Primary effusion lymphoma, as opposed to PBL, usually presents with serous effusions without detectable tumor masses, and is strongly associated with both HHV-8 and HIV infection. The presence of serum monoclonal proteins, and/or bone involvement with radiographically evident lytic lesions, favors the diagnosis of plasma cell myeloma rather than PBL[25].

In the pre-HAART era, HIV-infected patients with PBL were destined to die from their disease shortly after their NHL diagnosis. In the initial report by Delecluse and colleagues, 9 of 11 patients with long-term follow-up had a median survival of 6 mo[2]. In the HAART era, patient prognosis appears better and prolonged survival is the goal of treatment. Among six patients treated with anthracycline-based multiagent chemotherapy in conjunction with HAART, five were alive and disease free with a median follow-up of 22 mo[15]. The importance of an intact immune system in preventing or controlling PBL is underscored by reports of tumor regression with HAART alone and in the absence of chemotherapy[26-28]. Ironically, the early phase of immune reconstitution may be a fertile ground for NHL development. Our patient's diagnosis of lymphoma within 1 year of HAART initiation, and in the context of a rapidly improving CD4+ cell count and non-detectable HIV viral load may be another example[29].

Finally, our patient's complaint of dysphagia in the setting of a large esophagogastric mass, together with his long-standing history of smoking was disconcerting for carcinoma. Though the history of fever, night sweat and an elevated LDH raised the possibility of a lymphoma, the finding of poorly differentiated tumor cells stained negatively for routine lymphoid markers of B and T-cell differentiation did not support this diagnosis. But the subsequent demonstration of the plasma cell antigens, which are not typically part of the routine immunohistochemical panel, in conjunction with a broader array of viral and molecular studies helped us to establish the diagnosis of PBL.

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CASE REPORT

Severe chest pain in a pediatric ulcerative colitis patient after 5-aminosalicylic acid therapy

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Abstract

Severe reactions to mesalamine products are rarely seen in pediatric patients. We report a case of a 12-year-old boy who had a severe cardiac reaction to a mesalamine product Asacol. Past medical history is significant for ulcerative colitis (UC) diagnosed at 9 years of age. Colonoscopy one week prior to admission revealed pancolitis. He was treated with Asacol 800 mg three times per day and prednisone 20 mg/d. He was subsequently admitted to the hospital for an exacerbation of his UC and started on intravenous solumedrol. He had improvement of his abdominal pain and diarrhea. The patient complained of new onset of chest pain upon initiating Asacol therapy. Electrocardiogram (ECG) revealed non-specific ST-T wave changes with T-wave inversion in the lateral leads. Echocardiogram (ECHO) revealed low-normal to mildly depressed left ventricular systolic function. The left main coronary artery and left anterior descending artery were mildly prominent measuring 5 mm and 4.7 mm, respectively. His chest pain completely resolved within 24-36 h of discontinuing Asacol. A repeat echocardiogram performed two days later revealed normal left ventricular function with normal coronary arteries (< 3.5 mm). Onset of chest pain after Asacol and immediate improvement of chest pain, as well as improvement of echocardiogram and ECG findings after discontinuing Asacol suggests that our patient suffered from a rare drug-hypersensitivity reaction to Asacol.

Key words: Mesalamine; 5-aminosalicylic acid; Ulcerative colitis; Pericarditis; Drug hypersensitivity reaction

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INTRODUCTION

Mesalamine is a well-known treatment for ulcerative colitis. Drug reactions to mesalamine are uncommon, and most often include skin rash and hypereosinophilia[1]. Severe reactions to mesalamine products are rarely seen in pediatric patients. Cardiac complications have been reported as a rare extraintestinal manifestation of inflammatory bowel disease (IBD) and mainly manifest as pericarditis[2-4]. However, cardiac complications may be associated as a very rare drug reaction to 5-aminosalicylic acid (5-ASA) products[1,5,6]. The difficulty lies in distinguishing between these two etiologies. We herein report a case of a 12-year-old boy with ulcerative colitis who had a severe cardiovascular reaction to a mesalamine product, Asacol.

CASE REPORT

This is a 12-year-old boy with a past medical history significant for ulcerative colitis (UC) diagnosed at 9 years of age, along with psoriasis and arthritis. He was initially placed on steroids and Pentasa when he was first diagnosed. The Pentasa was discontinued after he developed a rash. It was difficult to distinguish his psoriasis from a potential drug reaction as a cause of the rash. He was subsequently weaned from his steroids after approximately 2 mo.

He presented to the hospital three years after his initial diagnosis with a two-month history of exacerbation of his UC. His symptoms continued to progress. He had 6-8 bloody stools per day 2 wk prior to hospital admission. Colonoscopy 6 wk into the flare revealed pancolitis. He was started on Asacol 800 mg three times per day and prednisone 20 mg once a day for his UC flare. The patient was subsequently admitted for worsening abdominal pain, bloody diarrhea as well as fever, fatigue, myalgia, and extremity pain. He also complained of new onset of severe chest pain progressively worsening since

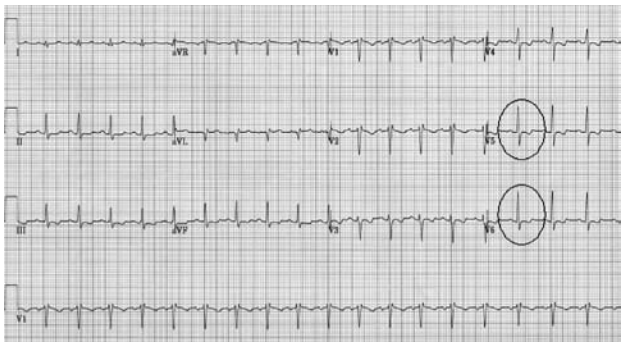

Figure 1 Non-specific ST-T wave changes with T-wave inversion in the lateral leads (circles).

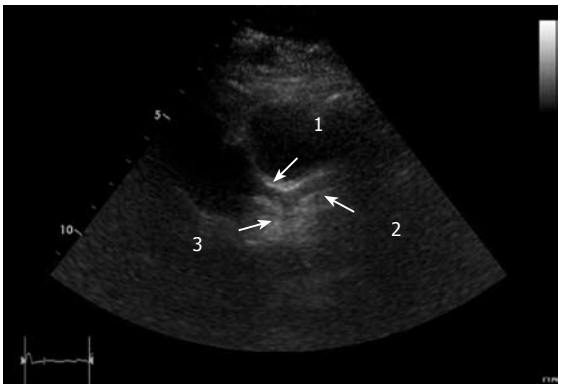

Figure 2 Echocardiogram-parasternal short-axis view showing mildly dilated left main coronary artery (1), left anterior descending artery (2), left circumflex coronary artery (3).

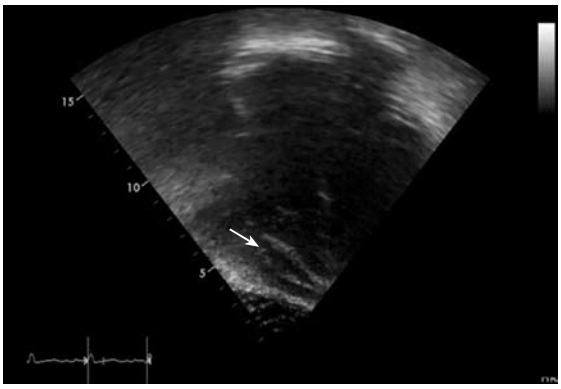

Figure 3 Echocardiogram-4-chamber view showing pericardial effusion (arrow).

starting Asacol therapy. The chest pain was not related to exercise and not associated with palpitations, shortness of breath, syncope, pallor, cyanosis or altered by changes in posture or breathing.

On admission, his vitals included a temperature of 38℃, pulse rate of 126 beats per minute, respiratory rate of 18 breaths per minute and a blood pressure of 107/65 mmHg. He was started on intravenous solumedrol 8 mg every 6 h with subsequent improvement of his abdominal pain and diarrhea. Chest pain, however, persisted and the chest pain worsened shortly after ingesting Asacol. Electrocardiogram (ECG) was performed and revealed non-specific ST-T wave changes with T-wave inversion in the lateral leads (Figure 1). Troponin T, CK-MB, Brain Natruritic Peptide, and CRP levels were ordered to evaluate for possible pericarditis or a myocardial abnormality. The results of this lab work were normal. Echocardiogram (ECHO) revealed low-normal to mildly depressed left ventricular systolic function. Calculated shortening fraction was 26% and left ventricular size was within normal limits. The left main coronary artery and left anterior descending artery were mildly prominent measuring 5 mm and 4.7 mm, respectively (Figure 2). The ECHO also revealed trivial circumferential pericardial effusion that was hemodynamically insignificant (Figure 3). A spiral CT scan ordered to evaluate for possible pulmonary embolism in the setting of an elevated d-dimer was unremarkable except for a small amount of pericardial fluid.

The patient's chest pain completely resolved within 24 h after discontinuation of Asacol. A repeat echocardiogram performed two days later revealed normal left ventricular function with a shortening fraction of 33%, normal coronary arteries (< 3.5 mm) and a trivial pericardial effusion. The patient was subsequently discharged from the hospital on prednisone and methotrexate. His UC remained in remission upon follow-up by his pediatric gastroenterologist 2 mo after hospital discharge. An ECHO and ECG were also repeated in the follow-up 2 mo after hospital discharge, revealing continued resolution of coronary artery dilation (Figure 4). The ECG was normal without the S-T wave abnormality noted 2 mo earlier.

DISCUSSION

Mesalamine has been found to be a beneficial medication in the treatment of patients with ulcerative colitis. The mechanism of action of mesalamine is unknown; however, it is thought to exert its action topically as opposed to systemically. Mucosal production of arachidonic acid metabolites, through both the cyclo-oxygenase and the lipoxygenase pathways, is thought to be increased in patients with inflammatory bowel disease. Mesalamine acts by a variety of mechanisms which include blocking cyclo-oxygenase thereby inhibiting prostaglandin synthesis, reducing antioxidant and pro-inflammatory cytokine synthesis, reducing lymphocyte metabolism and reducing expression of adhesion molecules. All these work to decrease inflammation in the colon[7,8].

Chest pain along with electrocardiographic and echocardiogram findings in a pediatric patient on a 5-ASA product should alert the physician to the possibility of drug reaction. We have presented a pediatric patient with ulcerative colitis who developed cardiac signs and symptoms associated with pericarditis soon after starting a 5-ASA product. Determining the etiology of pericarditis in this setting can be complex as this presentation has been reported as a rare extraintestinal manifestation of inflammatory bowel disease[2-4]. Chest pain may be associated with other more common gastrointestinal conditions such as gastroesophageal reflux disease (GERD). Chest pain in IBD may also be caused by respiratory infection and pleural inflammation which were ruled out in our patient. Patients with IBD have a higher propensity to develop pulmonary embolism (PE) and patients often

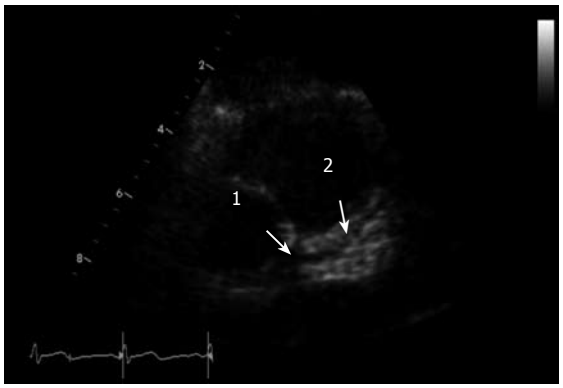

Figure 4 Echocardiogram-parasternal short-axis view showing normal left main coronary artery (1), normal left anterior descending artery (2).

present with acute onset of chest pain[4]. The normal spiral chest CT ruled out PE in our patient.

In our case, there was a temporal relationship between the onset of the chest pain and the administration of Asacol. The characteristics of the chest pain were not typical of pericarditis but the ST-T wave changes and the pericardial effusion which resolved after discontinuation of the Asacol were suggestive of the diagnosis. Normal troponin and CKMB levels do not support the diagnosis of myocarditis, although there was a mild but clear change in ventricular systolic function which also resolved after the Asacol was stopped. It is unclear why the coronary arteries were mildly prominent at the time of the diagnosis, which would suggest a vasculitis process such as Kawasaki disease.

This case illustrates the importance of eliciting a thorough medical history and being aware of the timing when new medications are started. It is imperative that any new onset of chest pain, especially in this setting, should be evaluated via cardiac enzymes, EKG and echocardiogram to quickly diagnose any complication caused by either the inflammatory bowel disease itself or a rare adverse drug reaction.

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CASE REPORT

Management of rectal foreign bodies: Description of a new technique and clinical practice guidelines

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Abstract

A number of techniques have been described to remove rectal foreign bodies. In this report, a novel endoscopic technique using a pneumatic dilatation balloon normally used in achalasia patients is presented. In addition, a systematic review of the literature was performed for non-operative methods to remove foreign bodies from the rectum. These results are summarised, presented as a practical at-a-glance overview and a flow chart is offered to guide the clinician in treatment decisions. The design of the flow chart was based on the aims to treat the patient preferably on an outpatient basis with minimally invasive techniques and if possible under conscious sedation rather than general anaesthesia.

Key words: Foreign body; Rectum; Rectal; Removal; Review

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INTRODUCTION

Intentional or unintentional insertion of rectal foreign bodies is not uncommon and often poses a serious challenge on the clinician. Objects can be inserted for diagnostic or therapeutic purposes, or self-treatment of anorectal disease, by criminal assault and accident or, most commonly, for sexual purposes. Most patients with rectal foreign bodies present to the emergency room usually after efforts to remove the object at home. Many endoscopic and surgical techniques to remove rectal foreign bodies have been described in the literature and the reported variety in foreign bodies is as large as the number of techniques used to remove them[1-46]. The descriptions in the available literature are anecdotic and consist largely of case reports or case series[1-46].

In this report, a novel endoscopic technique to remove rectal foreign bodies using a pneumatic dilatation balloon normally used in achalasia patients is presented. In addition, a systematic review of the literature was performed for non-operative methods to remove foreign bodies from the rectum. These results are summarized and a practical flow chart is presented to guide the clinician in his or her treatment decisions.

CASE REPORT

A 19-year-old man presented at the emergency department, 12 h after insertion of a high pressure container with tanning spray into his rectum. A plain abdominal radiograph (Figure 1) showed the container in the rectosigmoid region. There were no signs of perforation. A flexible sigmoidoscopy was performed under conscious sedation. The object was located just above the rectosigmoid junction. The container could not be extracted by bimanual manipulation. An attempt to remove the object with conventional endoscopic instruments, such as polypectomy snares, was unsuccessful.

The sigmoidoscope could be passed alongside the foreign body to its proximal end. A guide wire was left behind with the sigmoidoscope removed. Subsequently, a 40 mm pneumatic dilatation balloon (Rigiflex®, Boston Scientific), normally used in achalasia patients, was inserted over the guide wire and inflated just above the container (Figure 2). For safety purposes, the sigmoidoscope was reintroduced alongside the catheter of the balloon to allow endoscopic visual control of

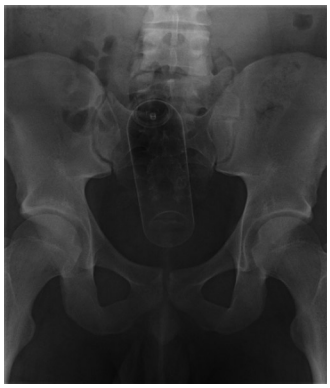

Figure 1 Plain abdominal radiograph showing the foreign body impacted in the rectosigmoid.

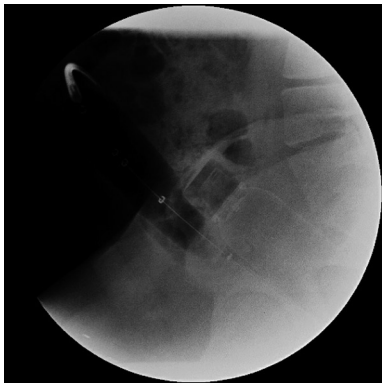

Figure 2 Lateral view of abdominal radiograph depicting the foreign body with the achalasia balloon inflated just above the container.

the distal end of the container in the rectum. Gentle traction was exerted on the balloon catheter, and the container was successfully removed under fluoroscopic and endoscopic control (Figure 3).

DISCUSSION

A large number of surgical and non-surgical techniques have been described to remove rectal foreign bodies[1-46]. Our case illustrates that for removal of foreign bodies retained in the rectosigmoid, extraction with a pneumatic dilatation balloon, inflated above the foreign body, may be an elegant and safe alternative when conventional techniques fail. Our technique has not been described before as revealed by a systematic review of the literature. We performed a systematic PubMed search from 1966 to present, using the search terms 'rectal', 'rectum', 'colorectal', 'foreign', 'bodies' and 'endoscopic'. Only reports in English were included. The results of the systematic search of the literature, specified for the type of foreign body, are summarized in Table 1[1-36]. Table 1 also summarizes endoscopic techniques and non-endoscopic techniques for removing foreign bodies. In addition to the reports presented in the Table 1, several case series have been published without detailed information on the techniques used to remove various foreign bodies[18,22,25,37-46].

An algorithm was provided to guide the clinician in his or her treatment decisions, partly based on the methods presented in the Table 1 (Figure 4). We included only those methods most commonly used and excluded rare treatment variants.

The first step in the evaluation is that one should always be aware of the possibility of a large bowel perforation and perform radiological investigations. Plain abdominal radiography or water soluble contrast enemas may be helpful. An abdominal X-ray will also provide information on the localization of the foreign body, whether it is below or above the rectosigmoid junction. If perforation of the bowel has occurred, immediate laparotomy is warranted. If there are no signs of perforation, several management approaches can be tried. Our aim was to treat the patient on an outpatient basis with minimally invasive techniques and preferably under conscious sedation instead of general anaesthesia.

First, digital removal of the object should be attempted, if necessary with the patient at different positions. If this approach fails, one can try bimanual manipulation. The next step is the insertion of an endoscope with subsequent attempts to grasp the foreign body with regular endoscopy accessories like polypectomy snares. When this fails, it may be helpful to

Figure 3 The removed container.

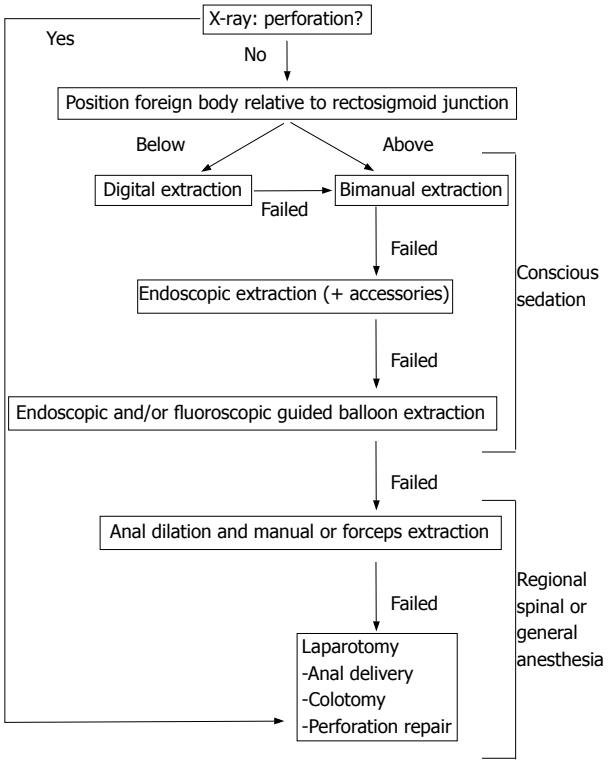

Figure 4 Algorithm for the removal of a colorectal foreign body.

Table 1 Overview of reports on endoscopic and non-endoscopic removal of rectal foreign bodies

Type foreign body	Technique	Anaesthesia	Author[Ref.]
Ballpoint pen	Polypectomy snare[1]	-	Richter[1]
Water filled balloon	Puncture and forceps[1]	-	Wolf[2]
Chicken bone	Polypectomy snare[1]	-	Tarnasky[3]
Toothpick	Polypectomy snare[1]	-	Over[4]
Apple	Defragmentation by APC[1]	None	Glaser[5]
Glass bottle	Biopsy forceps[1]	General	Huang[6]
Vibrator	Polypectomy snare[1]	None	Huang[6]
Glass test tube	Inflated Sengstaken tube[1]	-	Hughes[7]
Test tube	Polypectomy snare[1]	-	Kantarian[8]
Enema tip	Polypectomy snare[1]	-	Kantarian[8]
Vibrator	Polypectomy snare, biopsy forceps[1]	-	Kantarian[8]
Pencil	Polypectomy snare[1]	-	Vemula[9]
Iron bar	2-channel colonoscope and wires[1]	-	Ahmed[10]
Bottle neck	Inflated Foley cathether[1]	General	Humes[11]
Spray container	Achalasia balloon[1]	None	Present report
Spongy toy ball	Obstetric vacuum extractor	General	Feigelson[12]
Vibrator	Obstetrical forceps, anal dilation	Local	Haft[13]
Vibrator	Uterine vulsellum	Local	Levin[14]
Aftershave bottle	Rubber-shod bone olding clamp	Spinal	Siroospour[15]
Chicken bone	Digitally	None	Davies[16]
Aerosol-can Cap	Tenaculum forceps, anal dilatation	General	Aquino[17]
Vase	Filling with plaster	General	Couch[18]
Glass jar	Extraction with plaster rolls	Spinal	Graves[19]
Glass jar	Endotracheal tube, anal dilation	Local	Garber[20]
Apple	Bimanual manipulation	Local	Sharma[21]
Glass jar	Inflated Foley catheter	General	Yaman[22]
Glass bottle	Obstetric vacuum cup	General	MacKinnon[23]
Glass bulb	3 inflated Foley catheters	-	Diwan[24]
Thermometer	Biopsy forceps	General	Huang[6]
Vibrator	Transanal Kocher clamps	Local	Huang[6]
Bowling bottle	Obstetric forceps	General	Huang[6]
Perfume bottle	Manually	Spinal	Busch[25]
Piece of wood	Manually	General	Jansen[26]
Toothbrush case	Inflated Fogarty catheter	-	Wigle[27]
Oven mitt	Forceps after anal dilation	General	Losanoff[28]
Sink waste pipe	Obstetric forceps	General	Peet[29]
Metallic boule	Electromagnet	General	Coulson[30]
Carrot	Myomectomy screw	-	Vashist[31]
Glass	Obstetric vacuum extractor	Spinal	Johnson[32]
Rubber ball	Manual extraction, anal dilation	General	Nivatvongs[33]
Wooden rod	Bimanually, anal dilation	Spinal	Nivatvongs[33]
Bottle	Manually after anal dilation	General	Gopal[34]
Dildo	Myomectomy screw	-	Clark[35]
Light bulb	Abdominal compression	Spinal	Konishi[36]

-: No description; APC: Argon-plasma coagulation; [1]Endoscopic removal of rectal foreign bodies.

use devices that can be inflated in the rectosigmoid, such as a Foley catheter or an achalasia balloon. Such a device prevents a vacuum that might develop upon extraction of the foreign body and may also be directly used to remove the object.

If these interventions fail, we refer the patients to the operating theatre. Full relaxation of the anal sphincter muscles can be achieved by local, spinal or general anaesthesia. Sometimes, bimanual manipulation of the relaxed abdominal wall under spinal or general anaesthesia may evade surgery. Patients should be consented for a laparotomy prior to general anaesthesia should the manual or endoscopic removal fail.

Finally, when conservative measures fail, laparoscopic or laparotomic approaches are indicated. After removal, sigmoidoscopy is generally recommended to rule out perforations. In the largest series of patients with rectal foreign bodies described thus far (n = 93), it was found that objects retained for more than 2 days, those larger than 10 cm and those located proximal to the rectum increase the likelihood of surgery[37].

In conclusion, many techniques are available for the extraction of rectal foreign bodies. If possible, patients should be treated with minimally invasive techniques and preferably on an outpatient basis under conscious sedation.

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CASE REPORT

Gastric infiltration of diffuse large B-cell lymphoma: Endoscopic diagnosis and improvement of lesions after chemotherapy

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Abstract

Diffuse large B-cell lymphoma (DLBCL) is the most common histologic subtype of the non-Hodgkin's lymphoma (NHL) accounting for about 40% of all NHLs. This is a case report about the endoscopic appearance of a DLBCL with infiltration to the stomach in a 39-year-old female. She had a 6-mo history of lumbar and left upper quadrant pain with intermittent episodes of melena. A computer tomography (CT) scan showed mural thickening of the gastric antrum. Endoscopic examination revealed multiple gastric ulcers. Definite diagnosis could be made by endoscopic biopsies and the patient had a good response to chemotherapy. This response correlated well with a further endoscopic follow-up. A follow-up endoscopic examination could be considered to evaluate a good response to chemotherapy in DLBCL patients with secondary gastric dissemination.

Key words: Diffuse large B-cell lymphoma; Non-Hodgkin's lymphoma; Gastric infiltration

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INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common histologic subtype of non-Hodgkin's lymphoma (NHL) accounting for about 40% of all NHLs[1,2]. Tumors may be localized or confined to one side of the diaphragm in 20% to 40% of cases (Stage Ⅰ or Ⅱ). Stage Ⅳ or disseminated disease is observed in approximately 40% of patients and is usually characterized by extranodal extramedullary infiltration[3,4]. Sites of extranodal involvement in DLBCL can include the stomach/gastrointestinal system among others[5-7]. In this report, we describe a patient with a stage Ⅳ DLBCL infiltrating the stomach diagnosed at endoscopic examination and an excellent response after 6 cycles of chemotherapy.

CASE REPORT

A 39-year-old female was referred for an upper endoscopy because of melena, weight loss and a retroperitoneal mass. She had a 6-mo history of lumbar and left upper quadrant pain. Two months before admission she presented with nausea, early satiety, and intermittent episodes of melena and malaise. At admission she also reported a 5 kg weight loss and nocturnal diaphoresis. Physical examination revealed bilateral supraclavicular lymphadenopathy, hepatosplenomegaly and a palpable tender epigastric mass. A computer tomography (CT) scan showed cervical and mediastinal lymphadenopathy, bilateral pleural effusion, two pulmonary nodules in the upper right lobe, hepatosplenomegaly and a retroperitoneal

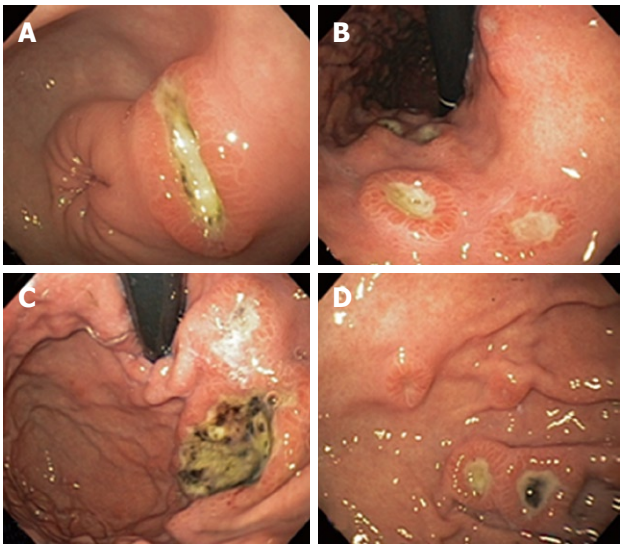

Figure 1 Endoscopic appearance of DLBCL infiltration of gastric antrum (**A**), lesser curvature (**B**), fundus (**C**), and body (**D**).

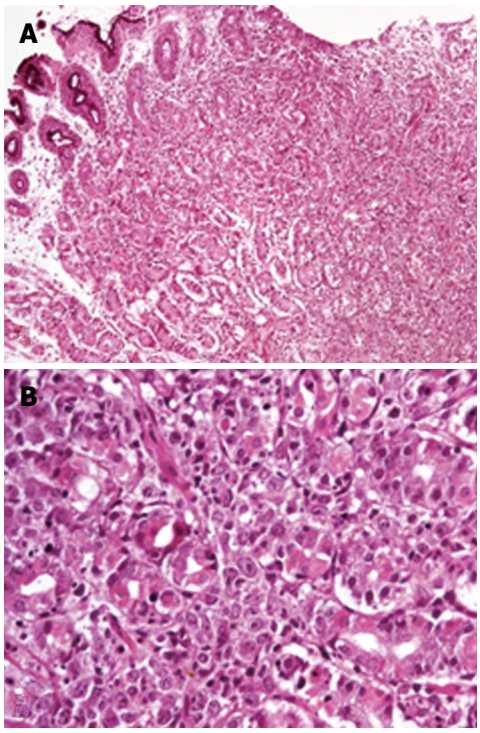

Figure 2 Gastric mucosa biopsy showing diffuse infiltration of lamina propria with distortion of the glandular architecture (**A**) and diffuse infiltration by large lymphoid cells (centroblast-type) that surround and destroy the gastric glands (**B**).

mass with mural thickening of the gastric antrum. A cervical lymph node biopsy was obtained and the patient underwent an upper endoscopic examination. At endoscopy, we observed multiple gastric ulcers without active bleeding. The ulcers had elevated margins, ranging from 5 to 15 mm in diameter and their base was covered by fibrin and/or necrotic material. The margins of the ulcers showed a characteristic erythematous, congestive, mosaic-like pattern suggestive of infiltration (Figure 1). Multiple biopsies were taken from the ulcer margins. Histopathologic analysis revealed a centroblastic DLBCL

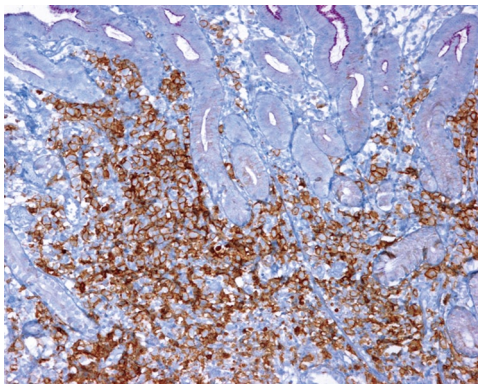

Figure 3 Gastric biopsy showing infiltration by atypical lymphoid cells in the gastric mucosa with intense positivity for CD20 at immunohistochemical analysis.

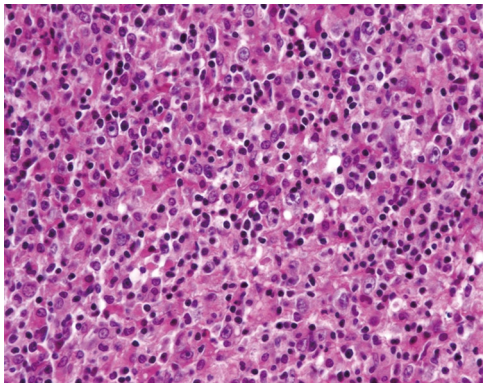

Figure 4 Lymph node biopsy showing neoplastic lymphocytes with fine nuclear chromatin, some of which have 2 or 3 peripheral nucleoli (centroblastic type) and others have prominent central nucleoli (immunoblastic type).

positive for CD20 at immunohistochemical analysis (Figures 2 and 3). The histologic results from the lymph node biopsy confirmed the diagnosis (Figure 4). The patient received rituximab, cyclophosphamide, adriamycin, vincristine and prednisone (R-CHOP) chemotherapy and omeprazole (20 mg *po* twice a day). Her clinical symptoms improved dramatically after 2 cycles of chemotherapy. After 3 cycles of chemotherapy she underwent a follow-up upper endoscopy (6 wk after the first endoscopy) that showed the presence of scarring tissue in majority of the ulcer sites and improvement at the sites where the ulcers with a bigger size were located. A final upper endoscopy performed after 6 cycles of chemotherapy (12 wk after the first endoscopy), showed almost complete resolution of the lesions (Figure 5). She denied early satiety or pain and had no signs of gastrointestinal hemorrhage.

DISCUSSION

Disseminated DLBCL (stage Ⅳ) is seen in approximately 40% of patients and the gastrointestinal tract is the most common site of extranodal NHL accounting for 20% to 60% of newly diagnosed cases[8-10]. Disseminated nodal disease requires systemic chemotherapy as opposed to localized primary gastric NHL that is potentially curable

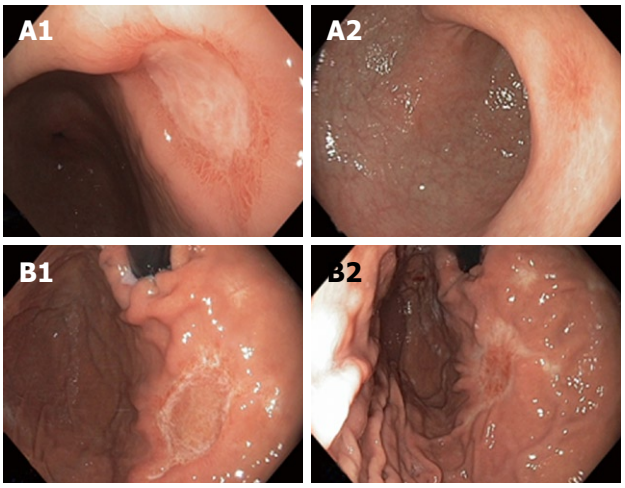

Figure 5 Endoscopic appearances of gastric antrum (**A1**, **A2**) and fundus (**B1**, **B2**) after 3 and 6 cycles of chemotherapy respectively.

with local radical treatment[11]. Thereby, it is crucial to make an early and reliable identification of the disease to initiate a correct therapy. The characteristics of the lesions at endoscopic examination can differ between primary and secondary gastric NHL. Kolve *et al*[12] described the endoscopic differences between 176 patients with primary NHL and 29 with secondary NHL, the lesions had macroscopical polypoid or exulcerative infiltrating changes. Primary low-grade gastric NHL was mainly characterized by diffuse infiltration and unifocal growth pattern with bulky disease in 80% of the cases with high-grade malignancy, whereas the lesions with secondary involvement showed a multifocal growth pattern in 66% of cases with bulky disease in 35%[12]. Our case showed a multifocal pattern of ulcerative lesions with elevated margins. Infiltrative disease was suspected on the basis of her clinical presentation, the number of ulcers and the erythematous mosaic-like pattern at margins of the lesions. Biopsies taken at the margin sites were diagnostic. This description of gastric infiltration by this specific type of neoplasia could help the endoscopist to suspect or identify this entity since appearance of the ulcers at endoscopic examination was very characteristic. The reason to perform the second endoscopic examination was to correlate the endoscopic findings with the improvement in the clinical picture and the radiological follow-up. Obviously, omeprazole treatment could also improve gastric ulcers, but we consider that in such a short period of time and considering the nature of the disease, it is highly improbable that without chemotherapy, we would have observed the same results. This shows that a follow-up endoscopic examination could also be taken into consideration to evaluate a good response to chemotherapy in DLBCL patients with secondary gastric dissemination.

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CASE REPORT

Perforation of the duodenum by an ingested toothbrush

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Abstract

We report a rare case of duodenal perforation caused by an ingested 12-cm long toothbrush handle. A 22-year-old female presented with intermittent epigastric pain for 6 d after swallowing a broken toothbrush. The swallowed toothbrush could not be removed from the second portion of the duodenum by endoscopy. Laparotomy revealed a perforation in the anterior wall of the duodenal bulb. The toothbrush was removed *via* the perforation which was debrided and closed. There were no postoperative complications.

Key words: Duodenum; Endoscopy; Laparotomy; Perforation; Toothbrush

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INTRODUCTION

Ingestion of various types of foreign bodies, such as toothpicks, fish or meat bones, screws, coins, metal clips, teeth, dental prosthesis, and spoon handles, has been reported[1-3]. Most ingested foreign bodies pass through the gastrointestinal tract spontaneously without causing untoward effects. However, sometimes these foreign bodies cause obstruction or perforation of the gastrointestinal tract necessitating surgical intervention[2-5]. Here, we report a rare case of duodenal perforation resulting from an ingested toothbrush.

CASE REPORT

A 22-year-old female presented with intermittent epigastric pain for 6 d. Eight days prior to hospitalization, she experienced nausea and foreign body sensation in the throat. She attempted to induce vomiting by irritating the pharynx with the distal end of a toothbrush handle. The toothbrush broke at the junction of the handle and the brush head. Unfortunately, she accidentally swallowed the handle of the broken toothbrush, which was 12 cm long. She had no passage of tarry noted after swallowing the foreign body. However, she experienced epigastralgia 2 d after ingesting the toothbrush. Endoscopy was performed twice at another hospital. However, the swallowed toothbrush could not be removed. The patient denied any history of excessive alcohol consumption or illicit drug use. Her medical history was otherwise unremarkable.

Upon admission, the patient was afebrile, and her vital signs were normal. Mild tenderness but no rebound pain was noted in the upper abdomen. Laboratory values were as follows: 10.0 g/dL hemoglobin, 30.5% hematocrit, 7100/mm^3 white blood cells (WBC), 30 U/L aspartate aminotransferase (AST), 32 U/L alanine aminotransferase (ALT), and 72 U/L alkaline phosphatase. Chest and plain abdominal X-ray examinations were unremarkable. Endoscopic examination revealed a broken toothbrush handle in the second portion of the duodenum (Figure 1A). The blunt end of the toothbrush faced distally and the broken end, proximally against the duodenal wall (Figure 1B). There was an ulcer on the anterior wall of the duodenal bulb. Endoscopic removal of the toothbrush was reattempted but failed.

Surgery was performed on the second day of hospitalization. Laparotomy revealed a perforation in the anterior wall of the first portion of the duodenum. The perforation had necrotic edges and was sealed off by the infundibulum of the gallbladder. The toothbrush was removed through this perforation which was debrided and then closed primarily with interrupted silk sutures rein-

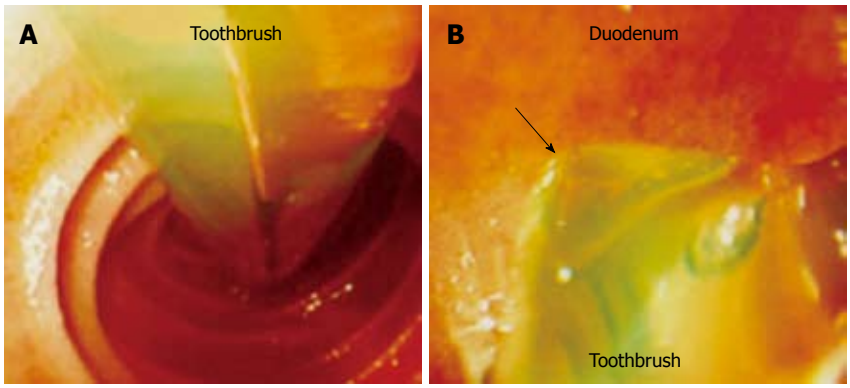

Figure 1 A toothbrush handle found in the second portion of the duodenum (**A**) and the pointed broken end (arrow) of the toothbrush handle impinged on the wall of the duodenal bulb (**B**).

forced with an omental patch. The patient's postoperative course was uneventful. The patient resumed oral intake on the fourth postoperative day and was discharged from the hospital 7 d after surgery.

DISCUSSION

Several factors are associated with the ingestion of foreign bodies. Children usually swallow foreign bodies because of carelessness. In adults, poor vision, mental disease, drug addiction, wearing of dentures, and rapid eating have been implicated as the etiologic factors of foreign body ingestion[2-5]. In addition, excessive alcohol intake and extremely cold fluids may dull the sensitivity of the palatal surface and increase the risk of swallowing foreign bodies[6]. The majority of ingested foreign bodies pass uneventfully through the gastrointestinal tract[2-5]. However, in some patients, the ingested foreign body may cause impaction, perforation, or obstruction. Perforation of the gastrointestinal tract may be associated with a considerably high mortality and morbidity. Gastrointestinal tract perforation may cause peritonitis, abscess formation, inflammatory mass formation, obstruction, fistulae, and hemorrhage[2,6]. In addition, foreign body perforation of the gastrointestinal tract may involve adjacent structures such as the kidneys, psoas muscle, and inferior vena cava[7,8]. Rare cases of foreign body migration to the pleura, heart, kidneys, or liver have been reported[9-12]. Most deaths in patients with foreign body perforation of the gastrointestinal tract are due to fulminant sepsis[2,13,14]. Therefore, efforts should be made to remove the ingested foreign bodies if they cannot pass through the gastrointestinal tract spontaneously.

In this case, the broken toothbrush handle was entrapped in the duodenal sweep and resulted in perforation of the duodenum. Intestinal injury resulting from an ingested foreign body tends to occur in areas of acute angulation but it may occur in all segments of the gastrointestinal tract[6]. The retroperitoneal, relatively immobile, and rigid nature of the duodenum as well as its deep transverse rugae and sharp angulations make it a common site for the entrapment of long and sharp-ended objects. Furthermore, the properties of foreign bodies determine the degree of complications caused by them. Thin and sharp foreign bodies such as chicken and fish bones, toothpicks, and straightened paperclips carry a higher risk of perforation. Long, slender, sharp-ended items have difficulty in traversing the tortuous gastrointestinal tract[2,3]. In a review of 31 cases of toothbrush ingestion, no episodes of spontaneous passage were reported[15]. Complications related to pressure necrosis, including gastritis, mucosal tears, and perforations, occurred in several of these cases.

Although a conservative approach toward foreign body ingestion is justified, early endoscopic removal of the ingested foreign body from the stomach is recommended[2,4,15]. Ertan *et al*[16] reported the first case of successful removal of a swallowed toothbrush. Other authors found the endoscopic approach unsuccessful due to the size and shape of the ingested toothbrush[17,18]. Esophageal perforation during the endoscopic extraction of a toothbrush has been reported[6]. In addition, objects longer than 6-10 cm have difficulty in passing the duodenal sweep[19]. Therefore, in cases of unsuccessful removal of gastric foreign bodies that are longer than 6.0 cm, surgical removal of them should be considered. Wishner and Rogers[18] reported a case of successful laparoscopic removal of a toothbrush from the stomach. Laparotomy in this case is justified because it is difficult to remove a toothbrush from the duodenum *via* a laparoscopic approach.

In conclusion, an ingested toothbrush cannot pass spontaneously through the gastrointestinal tract. Early removal of the ingested toothbrush is advised to avoid impaction of the toothbrush at the duodenum and to minimize morbidity. Endoscopic removal should be performed by a skilled endoscopist. If this is not possible or unsuccessful, a surgical approach is recommended.

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CASE REPORT

Flare-up of ulcerative colitis after systemic corticosteroids: A strong case for Strongyloides

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Abstract

Super-imposed infection with intestinal organisms can mimic a flare-up of underlying disease in patients with inflammatory bowel disease (IBD). We report a case of patient with long standing ulcerative colitis (UC), who presented with abdominal pain, diarrhea and low-grade fever after receiving systemic corticosteroids for an unrelated disorder. Despite a negative stool examination, a peripheral eosinophilia reappeared upon tapering down of a corticosteroid dose. Subsequently, duodenal biopsies showed evidence for Strongyloides, presumably acquired 20 years ago when the patient was residing in Brazil. The patient fully recovered following anti-helmintic therapy. This case underscores the importance of considering Strongyloides in the work-up of flaring-up IBD patients, even if a history of residing or traveling to endemic areas is in the distant past.

Key words: Ulcerative colitis; Infection; Parasites; Eosinophilia; Strongyloides

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INTRODUCTION

Although exacerbation of ulcerative colitis (UC) usually poses a little diagnostic dilemma, physicians should remain cognizant to the possibility of an alternative cause for patient symptoms.

CASE REPORT

A 54-year-old practicing physician, native of Brazil who immigrated to Israel 20 years ago, was hospitalized in the Neurology Department with severe cluster headache attack, which responded to oral dexamethasone at 16 mg/d. History was also notable for left-sided UC controlled by 5-aminosalycilates (5-ASA) over the past 11 years. In the previous 2 years, the patient was lost to follow-up but reported he was in clinical remission. Two weeks after his hospital discharge from the Neurology Department, he presented again with non-bloody diarrhea, abdominal pain and muscle pain.

Upon examination a fever of 37.7℃ was noted, and right abdominal tenderness was appreciated. The rest of the physical examination was unremarkable. Complete blood count showed that his white blood cell count was 8.4 K/mcl, with a normal differential count. Hemoglobin level was 14.6 g/dL. Chemistry results were all normal except for an albumin of 2.6 g/dL. Urinalysis showed leukocyturia and urine culture-yielded *E.coli*. Abdominal plain film showed small fluid levels.

Although the patient's abdominal pain and diarrhea suggested a possible UC flare-up, the lack of rectal bleeding, the right-sided abdominal tenderness and the muscular complaints raised the suspicion of a super-imposed infectious process in addition to a urinary tract

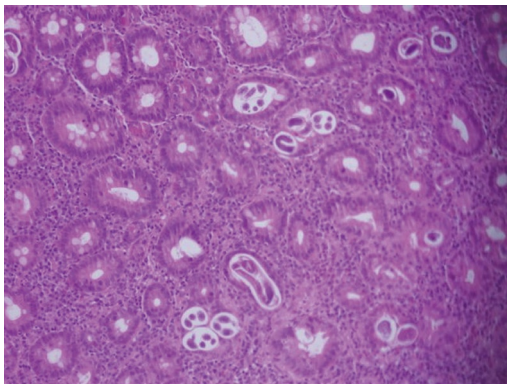

Figure 1 Histology of the duodenum showing chronically and actively inflamed mucosa with rhabditoid developing larvae, developing eggs and adult Strongyloides.

infection. Steroid myopathy causing muscles' pain was also considered. Prednisone was tapered to 5 mg over 4 d and ofloxacin was started, but the patients' symptoms persisted and a fever up to 38℃ was noted. Stool culture, examination for ova & parasites and *C. difficile* toxin assay were all negative. Following steroid tapering, an elevation of eosinophil count to 15.5% of white blood cells was noted, with an absolute count of 980/mcl. Re-inspection of the blood count obtained in the Neurology Department before initiation of dexamethasone revealed peripheral eosinophilia (1240/mcl), which went unnoticed. The patient underwent gastroduodenoscopy, which showed a hyperemic edematous duodenal wall. Colonoscopy showed edematous mucosa and punctate submucosal hemorrhages extending along the colon from the descending colon to the cecum. The rectum appeared relatively spared.

Histology from the colon revealed chronic eosinophilic inflammation and formation of eosinophilic abscesses. Duodenal histology revealed Strongyloides larva (Figure 1). CMV PCR in blood and tissue CMV immunohistochemistry were negative.

Ivermectin at a dose of 12 mg once daily was initiated with prompt resolution of all symptoms. During the 4-year follow-up, the patient was well, but experienced several mild flare-ups of UC, controlled with 5-ASA therapy. Repeat colonoscopies showed left sided colitis, and histology was compatible with UC. Two subsequent gastoduodenoscopies with biopsies did not disclose evidence of strongyloides

DISCUSSION

Strongyloides stercoralis is endemic in tropic and subtropic areas of the world, but has also been reported in residents of certain regions of the US and in coal miners in non-endemic areas[1,2]. Most cases present with pulmonary and/or upper gastrointestinal symptoms[3]. However, Strongyloides-associated colitis can occur as part of hyperinfestation following altered host immune status[4,5]. In this situation, larvae traveling down from the duodenum penetrate the colon wall rather than continue to be excreted in the stool[4]. The ensuing eosinophilic-predominant colitis may mimic new onset of UC[2,4]. Alternatively, it can emerge after corticosteroid treatment of a patient with well established UC, and can be mistaken for a refractory exacerbation[1,3,6].

The diagnosis of Strongyloides is often problematic. Stool examination is negative in 50%-70%[7,8] of patients. Eosinophilia was present in 84% of patients in one series[7], but in only 32% of corticosteroids-treated patients in another series[9]. Our patient had eosinophilia upon his prior admission to the Neurology Department that went unnoticed. Serology testing for Strongyloides-specific antibodies is also helpful, although not widely available. Upper endoscopy usually reveals hyperemic edematous duodenal mucosa with white villi[10] and colonoscopy may show mucosal edema, erosions and ulcerations[11], but none of these findings is specific. Duodenal aspirates and/or biopsy can assist the diagnosis, but are dependent upon worm burden and amenable to sample error[7]. Moreover, even when larvae are present in the intestinal or colonic wall, the diagnosis can be over-looked by the inexperienced or unsuspecting pathologist[1,12], or may be mistaken for eosinophilic gastroenteritis[13].

Strongyloides can persist in the host for his or her entire life-time[14]. Indeed, in our patient, infection was probably acquired in Brazil, twenty years prior to clinical presentation. Thus, albeit rare, the potential fatal consequences of untreated strongyloidiasis, make it imperative to consider this diagnosis in flaring inflammatory bowel disease (IBD) patients with even a distant history of residence or travel in endemic areas, or in patients failing to respond to standard therapy[15]. Eosinophilia should be excluded before initiation of immunosuppression, given the hazards of over-looking a dormant parasitic infection.

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CASE REPORT

Rapid re-emergence of YMDD mutation of hepatitis B virus with hepatic decompensation after lamivudine retreatment

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Abstract

Lamivudine has a high rate of antiviral resistance. Sequential treatment of anti-hepatitis B virus (HBV) is commonly used for lamivudine resistance. We report 4 cases of patients with rapid redetection of HBV mutants during the lamivudine retreatment. The four patients received lamivudine as an initial treatment of HBV and adefovir and lamivudine as a rescue therapy consecutively. HBV-DNA level, YMDD mutations and adefovir -resistant mutations (RFMP) were tested every 3 mo during the sequential treatment. All the patients showed YMDD mutations during the initial lamivudine therapy. After adefovir therapy for lamivudine resistance, they showed viral breakthrough. Adefovir was switched to lamivudine, however, it did not induce viral suppression at all, rather increased HBV-DNA with rapid reemergence of the YMDD mutations. All the patients had ALT flares, and hepatic decompensation occurred in two patients. After switching to adefovir combined with entecavir or lamivudine for a rescue therapy, the patients had reduction in HBV-DNA and ALT improvement. These cases demonstrated that lamivudine retreatment of patients with preexposed lamivudine resistance leads to rapid reemergence of YMDD mutation with significant viral rebounds and subsequent hepatic decompensation. Sequential administration of lamivudine in patients with a prior history of YMDD mutation should be abandoned.

Key words: Hepatitis B; Lamivudine; Adefovir dipivoxil; Mutations

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INTRODUCTION

Lamivudine has been commonly used in the treatment of chronic hepatitis B as a first-line antiviral agent. Long-term lamivudine therapy can usually suppress hepatitis B virus (HBV) replication, however, prolonged monotherapy leads to the emergence of lamivudine-resistant HBV mutants[1-3]. The emergence of rtM204I/V (YMDD) mutation of HBV polymerase gene is associated with rebounds in serum HBV DNA and flares of transaminase level[4].

Adefovir dipivoxil is an effective rescue therapy for lamivudine-resistant HBV[5,6]. However, adefovir resistance occurs more frequently in second-line treatment of lamivudine-resistant patients than in naïve patients[7]. It is well known that sequential monotherapy with antiviral agents induces the sequential occurrence of viral mutations[8]. We here report the rapid redetection of YMDD mutants and hepatic decompensation in patients with prior lamivudine resistance during lamivudine retreatment.

CASE REPORTS

Patient 1 (Figure 1) was a 46-year-old man with HBeAg-positive chronic hepatitis B. Lamivudine was started initially and good virological and biochemical responses were observed in the patient. However, HBV-DNA increased and rtM204I mutation was detected by restriction fragment length polymorphism (RFMP) assay[9] after 2 years of lamivudine therapy.

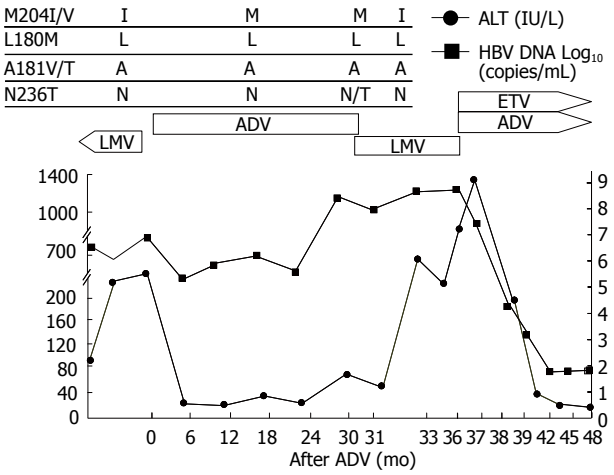

Figure 1 Viral responses and clinical courses in a 46-year-old man with chronic hepatitis (patient 1). The underline represents sequential evolution of lamivudine- or adefovir- resistant genotypic mutations in HBV polymerase gene. LMV: Lamivudine; ADV: Adefovir; ETV: Entecavir; ALT: Alanine aminotransferase.

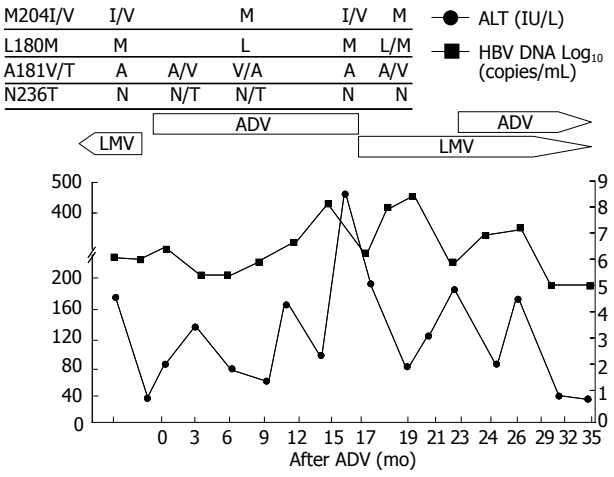

Figure 2 Viral responses and clinical courses in a 49-year-old man with compensated cirrhosis (patient 2). The underline represents sequential evolution of lamivudine- or adefovir- resistant genotypic mutations in HBV polymerase gene. LMV: Lamivudine; ADV: Adefovir; ALT: Alanine aminotransferase.

After lamivudine was switched to adefovir, HBV-DNA dropped by 2 logs with ALT normalization. Viral breakthrough (defined as a $\geqslant$ 1 $\log_{10}$ increase in HBV DNA from nadir after initial viral response) and rtN236T mutation developed after 30 mo of adefovir therapy. Lamivudine was restarted with discontinuation of adefovir. After lamivudine was restarted, the rtM204I mutation reemerged and rtN236T adefovir-resistant mutants disappeared 1 mo after the treatment. He showed persistently high levels of HBV-DNA, ALT and total bilirubin. Lamivudine was changed to adefovir and entecavir combination treatment as a rescue therapy. After the combination treatment, HBV-DNA and ALT dropped significantly.

Patient 2 (Figure 2), a 49-year-old man with cirrhosis, showed the similar response to lamivudine retreatment as shown in patient 1. Viral breakthrough with YMDD mutation developed after 15 mo of the initial lamivudine treatment. Lamivudine was switched to adefovir, however, there was no decrease in HBV-DNA level but ALT elevation. After reintroduction of lamivudine for adefovir resistance, ALT level decreased without a significant drop in HBV-DNA level initially. Adefovir-resistant mutant was replaced by wild type, however, reemergence of the rtM204I/V mutation followed by HBV-DNA rebound and ALT fluctuation were observed. Adefovir was added to the ongoing lamivudine therapy.

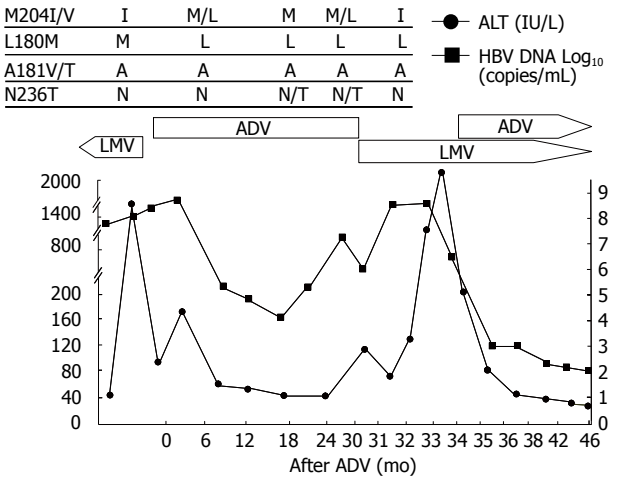

Figure 3 Viral responses and clinical courses in a 50-year-old woman with decompensated cirrhosis (patient 3). The underline represents sequential evolution of lamivudine- or adefovir- resistant genotypic mutations in HBV polymerase gene. LMV: Lamivudine; ADV: Adefovir; ALT: Alanine aminotransferase.

Patient 3 (Figure 3), a 50-year-old woman, had cirrhosis and received lamivudine as an initial treatment. After 16 mo of lamivudine therapy, she had elevated HBV-DNA followed by hepatic decompensation. Mutation profile showed rtM204I and rtL180M at the time of viral breakthrough. Lamivudine was switched to adefovir, and HBV-DNA decreased and her liver function was restored. Viral breakthrough occurred after 27 mo of adefovir treatment and rtN236T mutation was detected at this time. Lamivudine monotherapy was reintroduced consecutively. Rapid increase in HBV-DNA with the rtM204I mutants 2 mo after the lamivudine retreatment and ALT flare with jaundice and ascites occurred subsequently. The rtN236T mutation changed to wild type after the LMV treatment. Adefovir was added to lamivudine as a rescue therapy, and a rapid drop in HBV-DNA and ALT level was observed. The bilirubin decreased from the peak level of 18.7 to 1.6 mg/dL 6 mo after the combination treatment.

Patient 4 (Figure 4), a 50-year-old man with cirrhosis, received lamivudine for 14 mo. He had viral breakthrough with mild elevation of ALT, therefore lamivudine was changed to adefovir. However, he had no viral response though he showed a normal ALT level during the adefovir treatment. Adefovir was switched to lamivudine due to the high viral load. The ALT level was stable despite no decrease in HBV-DNA. After 2 mo

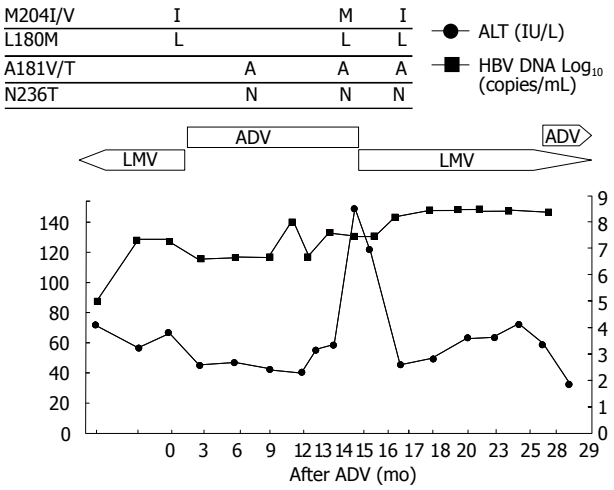

Figure 4 Viral responses and clinical courses in 50-year-old man with compensated cirrhosis (patient 4). The underline represents sequential evolution of lamivudine- or adeforvir- resistant genotypic mutations in HBV polymerase gene. LMV: Lamivudine; ADV: Adefovir; ALT: Alanine aminotransferase.

of lamivudine retreatment, HBV-DNA rebound with ALT flare developed. HBV mutation rtM204I was reappeared at this time. Adefovir was added to the ongoing lamivudine treatment.

All the patients were positive for HBeAg and had no seroconversion of HBeAg during the sequential antiviral treatment except for patient 3. Patient 3 had HBeAg loss after the lamivudine/adefovir combination therapy. No death occurred, however, two of them showed hepatic decompensation following the reemergence of YMDD mutations and viral breakthrough.

DISCUSSION

These cases showed that reintroduction of lamivudine could induce rapid reemergence of lamivudine-resistant mutations in chronic hepatitis B patients with prior lamivudine resistance. Lamivudine is well tolerated and significantly reduces HBV-DNA level[10]. However, lamivudine resistance associated with mutations in the polymerase gene, particularly in rtM204I/V known as YMDD mutant, occurs at a rate of 14%-30% annually[3,4]. Development of lamivudine resistance is associated with high baseline HBV-DNA level, duration of lamivudine treatment, precore variant, insufficient serum HBV-DNA suppression and elevated serum ALT level during treatment[11-13]. All the cases had positive HBeAg and high viral load at the time of lamivudine resistance. The high viral replication status may predispose to develop antiviral resistant mutations and subsequent viral breakthrough.

Adefovir is an effective rescue therapy for lamivudine-resistant HBV and significantly improves biochemical, virological, and histological features of lamivudine-resistant patients[5,6]. Compared with lamivudine, adefovir is associated with a low rate of antiviral resistance[14]. However, adefovir resistance occurs more common in patients with preexisting lamivudine resistance[7]. Among our cases, rtN236T mutation was detected in two patients at the time of viral breakthrough after adefovir rescue therapy. The other two showed no virologic response, and rtA181V was found in one patient.

YMDD mutations, mainly rtM204I in our cases, disappeared after the adefovir treatment, suggesting that cessation of antiviral therapy leads to disappearance of drug-resistant mutations. However, it was reported that lamivudine-resistant HBV mutants reappear rapidly after reintroduction of lamivudine[15]. Once antiviral-resistant HBV mutants have been selected, the mutants are archived even if treatment is stopped. In our cases, YMDD mutation reemerged within 3 mo after reintroduction of lamivudine, suggesting that re-treatment to which the virus has been previously resistant has a limited efficacy even after wild-type YMDD has restored. It could not be excluded that the adefovir-resistant mutations may affect the rapid emergence of lamivudine-resistant mutation.

The emergence of lamivudine resistance can result in viral breakthrough, flares of hepatitis and worsening of the initial histological improvement[1,2]. In patients with cirrhosis, severe exacerbation of hepatitis may result in hepatic failure[16]. It was reported that patients with YMDD mutations experience a higher rate of hepatitis flares than those without YMDD mutations[17], which is possibly because the YMDD locus takes part in cellular immune response in some cases.

Serum HBV DNA level was higher than baseline in the cases after emergence of the YMDD mutant before exacerbation occurred. Two patients experienced a very rapid deterioration of hepatic function accompanying the reemerging YMDD mutants only a few months after lamivudine reintroduction. These findings clearly demonstrate that sequential monotherapy, particularly retreatment with the antiviral agent to which the HBV has been previously resistant, should be avoided.

Combination therapy with adefovir and lamivudine or other antiviral agents could be a better option to prevent the emergence of antiviral-resistant mutations in patients with lamivudine- or adefovir -resistance. Combination therapy is not likely to improve virologic response, but rather decrease the rate of viral resistance[18,19]. Unfortunately, combination therapy could not be commonly used in Korea so far because of the high cost of antiviral agents. Other antiviral agents, such as tenofovir or pegylated interferon, could be promising agents for multi-drug resistant HBV[20,21]. All the patients in this study received combination therapy with adefovir and lamivudine or entecavir as a rescue therapy for lamivudine resistance. After the combination treatment, the HBV DNA and ALT level dropped and hepatic function was restored. It was reported that entecavir has a potent antiviral efficacy against naïve HBV and lamivudine-refractory HBV[22]. Case 1 received adefovir and entecavir as the rescue therapy. These two agents have antiviral activity on wild type or YMDD mutants without cross resistance. The other three patients received adefovir and lamivudine combination treatment. Case 3 showed a rapid reduction in HBV DNA level and HBeAg seroconversion soon after the combination

therapy. Long-term data are not available from cases 2 and 4. However, they showed a significant decrease in HBV DNA level and improvement in hepatic function after combination therapy.

Taken together, lamivudine retreatment may induce rapid reemergence of the YMDD mutation and significant viral rebound and subsequent hepatic flare in patients with preexposed YMDD mutants. Sequential lamivudine treatment of those with a prior history of YMDD mutation should be abandoned.

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LETTERS TO THE EDITOR

Nutritional therapy for active Crohn's disease

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Abstract

Nutritional therapy for active Crohn's disease (CD) is an underutilized form of treatment in adult patients, though its use is common in the paediatric population. There is evidence that nutritional therapy can effectively induce remission of CD in adult patients. Enteral nutrition therapy is safe and generally well tolerated. Meta-analysis data suggest that corticosteroids are superior to nutritional treatment for induction of remission in active CD. However, the potential side effects of such pharmacotherapy must be taken into consideration. This review examines the evidence for the efficacy of elemental and polymeric diets, and the use of total parenteral nutrition in active CD.

Key words: Crohn's disease; Nutrition; Dietary; Treatment; Steroids

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TO THE EDITOR

Nutritional therapies studied for the induction of remission in active Crohn's disease (CD) include enteral nutrition (EN), and total parenteral nutrition (TPN). EN by means of a polymeric diet can be given *via* the nasogastric or per oral route. Compliance tends to be greater with polymeric nutrition support than with an elemental diet, as the feed is considered more palatable. Polymeric diets provide nitrogen in the form of whole protein, and carbohydrates as hydrolysates of starch. Fat is most often provided as medium chain fatty acids. Fiber is commonly added to polymeric feeds though there is little evidence to suggest that it has a substantial positive or negative effect in hospitalized patients[1]. Elemental diets contain nutrients in simple forms (such as amino acids, simple carbohydrates, fats, vitamins and minerals) requiring little or no digestion to take place prior to absorption.

The theory behind the mechanism of action of an elemental diet is multi-factorial. Malnutrition can have effects on immune function and wound healing, as well as psychological and cognitive effects. Improvements in wound healing by ensuring a good nutritional status would theoretically lead to enhanced mucosal healing. Increased gut permeability has been implicated in the pathophysiology of CD. This is thought to relate to abnormalities in the tight junctions between enterocytes allowing an increase in luminal antigen uptake-potentially a factor contributing to increased inflammatory activity[2]. Treatment with an elemental diet has been shown to decrease intestinal permeability[3]. The incidence of CD dramatically increased in the twentieth century[4]. This coincides with many changes in our daily lives, including changes in our dietary intake. As elemental diets involve the ingestion of specific substances, it may be that pro-inflammatory antigens are avoided. The normal commensal bacterial population of the gut may play a role in the development of inflammation in CD, though the mechanism is unclear. In experimental animal models, inflammation does not develop in mice reared in a sterile environment[5]. An early study suggested the amount of bacteria per gram of faeces was reduced in patients on an elemental diet, though there is no consensus on this issue[6]. The constituents of an elemental diet are primarily absorbed in the proximal small bowel-proximal to the most commonly affected sites of inflammation in CD. The reduction in the workload of digestion and absorption, and a reduction in peristalsis and digestive tract secretions may also play a role[7]. In general, elemental diets contain a low proportion of fat compared to polymeric or normal diets. A recent Cochrane review concluded that there is a non-significant trend towards greater efficacy with

very low fat and long-chain triglyceride elemental diets compared to standard elemental diet regimes[8].

High quality randomised controlled trials looking at the use of nutritional therapy for the management of acute CD are difficult to perform. There have been very few studies where the remission rate with nutritional therapy is as low as that seen in the placebo arm of drug trials in active CD. So, if we accept that nutritional treatments have an effect, then the question arises as to the magnitude of this effect. According to some studies, remission rates may be as high as 84% with the use of an elemental diet[9]. The Cochrane review (2007) of enteral feeding in active CD provides us with very useful meta-analysis data[8]. As found in previous meta-analyses, the Cochrane review concluded that steroids have superior efficacy to enteral nutrition in inducing remission[8]. The exact role of enteral nutrition (EN) in adults to treat active CD is therefore undefined.

TRIALS COMPARING ELEMENTAL DIET TO CORTICOSTEROIDS

A number of studies have been conducted looking into the efficacy of elemental diets in CD patients. Riordan *et al*[9] studied 136 patients with active CD. An elemental diet was introduced and all other CD treatments discontinued. The intention was to give an elemental diet for 2 wk, though 31% of the patients did not tolerate the diet for more than 1 wk. Of the 78 remaining patients, 84% achieved disease remission after a 14-d treatment course. The group was then split into 38 patients receiving a tapered course of prednisolone and advice on healthy eating, and 40 patients receiving placebo instead of steroid-this 'diet' group was asked to introduce one new food each day and exclude foods that worsened symptoms. There was a median remission of 3.8 mo in the steroid group compared to 7.5 mo in the diet group.

Gorard *et al*[10] compared 22 patients given an elemental diet (4 wk treatment) to 20 patients receiving prednisolone (0.75 mg/kg daily for 2 wk followed by reducing doses). All participants were CD patients requiring hospitalisation for an acute flare of the disease. Nine of the twenty-two patients (41%) in the diet arm of the trial withdrew because of intolerance. Disease activity was measured using a simple disease activity index. The reduction in disease activity was similar between the diet (score of 4.8 reducing to 1.7) and prednisolone (score of 5.3 reducing to 1.9) groups. In addition to this, similar reductions in C-reactive protein, and increases in serum albumin concentration were found. The probability ratio of remaining patients in remission was, however, much lower in the diet group. At 6 mo, this probability was 0.67 after steroid compared to 0.28 after elemental diet.

TRIALS COMPARING POLYMERIC DIET TO CORTICOSTEROIDS

Many trials involving polymeric and enteral diets in CD have been conducted in children, due to the perceived need to avoid corticosteroids, to alleviate the additional risk of growth failure. Day *et al*[11] looked at 27 children with active CD (15 new diagnoses, 12 with known disease). They gave a polymeric feed as the exclusive source of nutrition for 6-8 wk either per oral or *via* a NG tube. No other medical therapy was used at that time. Twenty-four of the twenty-seven children completed the 6-8 wk course, while the other three did not tolerate enteral feeding. At the end of the treatment period, 80% of the newly diagnosed patients and 58% of the known-CD patients had entered remission. The CD remained inactive in all of the newly diagnosed patients with entered remission, over the mean 15.2-mo follow-up period.

Borrelli *et al*[12] conducted a trial comparing polymeric diet to corticosteroids in 37 children with active treatment in naïve CD patients. The study period was 10 wk, and after this time 15 of the 19 children (79%) receiving a polymeric diet had remission compared to 12 of the 18 patients in the corticosteroid group (67%). The differences were not statistically significant. An additional interesting aspect of this trial was that mucosal healing was assessed by endoscopy with histology at weeks 0 and 10. The proportion of children with mucosal healing was significantly higher in the polymeric diet group (74%) than in the corticosteroid group (33%).

The use of a polymeric diet in adult patients with CD has also been studied. Gonzalez-Huix *et al*[13] conducted a randomised controlled trial comparing adults with acute CD receiving 1 mg/kg per day prednisolone (n = 17) followed by a reducing course, to those on a polymeric diet and no medication (n = 15). The polymeric feed was given *via* a fine-bore NG tube and no other nutrition allowed. The polymeric diet patients went back to a normal diet after clinical remission was achieved. Of the seventeen patients in the steroid group, fifteen entered remission after a mean time of 2 wk. One patient had an intestinal perforation requiring surgery, and the others entered remission after being started on a polymeric diet. Of the 15 patients in the polymeric diet group, 12 entered remission after a mean time of 2.4 wk. Of the 3 treatment failures, one improved when steroids were given, and the other two were said to have failed as they did not enter remission after 4 wk on the polymeric feed. Patients from both arms of the trial were started on oral 5-ASA preparations prior to discharge. The cumulative probability of relapse during the follow-up period was higher after steroid treatment than after polymeric diet though this did not reach a statistical significance.

There is some evidence to suggest that the amount and type of fat in polymeric feeds may have an impact on its efficacy in CD patients. Gassull *et al*[14] hypothesised that a polymeric diet rich in monounsaturated fatty acids (MUFA) would be more effective in inducing remission in active CD patients than an identical diet but with polyunsaturated fatty acids (PUFA)-precursors of some inflammatory cytokines. They randomised 62 patients with active CD to either one of these diets for no longer

than 4 wk, or to 1 mg/kg per day prednisolone. The steroid group reacted as expected from previous studies with a 79% remission rate. However, the diet group did not fare as well. Only 20 % in the MUFA group entered remission, while 52% in the PUFA group achieved this target. These results were quite the opposite of those expected. Leiper *et al*[15] conducted a randomised trial in 54 patients with active CD. They received a polymeric diet with either high or low long-chain triglyceride (LCT) content. A staggering 39% of patients withdrew from the trial within 3 wk because of an inability to tolerate the diet, which was offered by either the oral or nasogastric route. Of those completing the trial, the response rate was 46% for the low LCT group and 45% for the high LCT group, respectively, thereby demonstrating no significant difference in efficacy with differing fat composition.

COCHRANE COLLABORATION REVIEW OF ENTERAL NUTRITION THERAPY FOR THE INDUCTION OF REMISSION IN ACTIVE CD

There have been four meta-analyses looking at the use of enteral nutrition therapy in comparison to steroids to induce remission in active CD patients. Overall, each of these meta-analyses showed steroids to be more effective than enteral nutrition strategies. However, when two large trials were excluded because of the concomitant use of other medicines in the steroid arm, both enteral nutrition and steroids were seen to have an equal efficacy. A recent review from the Cochrane Collaboration studied the results from trials comparing different types of enteral nutrition (EN) to each other, and trials comparing the use of EN to steroids[8]. When looking at differences between diet formulations used to treat patients with acute CD, they performed a meta-analysis which included data from 188 adult patients treated by elemental diet and 146 patients given a polymeric diet. No significant difference was found in the results achieved between elemental and polymeric diets.

Sub-group analysis showed no difference between formulae with high fat *versus* low fat content. Differences in the amount of fat in the form of high *versus* low long-chain triglyceride were also shown not to be significant. Meta-analysis of trials comparing enteral feeding to corticosteroids compared data from 192 enteral nutrition patients *versus* 160 patients treated with steroid, which revealed a pooled odds ratio of 0.33 favouring steroid treatment.

TOTAL PARENTERAL NUTRITION AS A TREATMENT FOR ACTIVE CD

Controlled trials of total parenteral nutrition (TPN) in CD patients are few and far between. Greenberg *et al*[16] conducted a trial in 51 patients with active CD. They were randomised to either TPN and nil by mouth (n = 17), partial parenteral nutrition (PPN) and supplementary nutrition with a liquid feed of a defined formula *via* a NG tube (n = 19), or PPN and supplementary normal food (n = 15). Remission occurred in 71% of patients on TPN, in 58% of patients on the PPN/defined formula diet, and in 60% of patients on PPN/normal diet. Of those achieving remission, the chance of successfully remaining in remission at one year was 42%, 55%, and 56%, respectively. The differences were found not to be significant. The total bowel rest achieved through TPN was therefore not thought to be of importance.

DISCUSSION

There is a disappointing lack of quality studies on the use of TPN in active CD patients. It is difficult to find a place for TPN as a treatment for active CD. The efficacy of TPN does not seem to be greater than that suggested by other trials of EN or steroids. TPN is known to be associated with an increased risk of adverse events, such as sepsis, although perhaps it has a place in patients intolerant to both EN and steroids. The value of TPN in malnourished patients with intestinal failure due to CD is beyond doubt.

There would seem no logical reason to choose EN over steroids for the vast majority of our patients. The European Society for Parenteral and Enteral Nutrition published guidelines in 2006 on the use of enteral nutrition in gastroenterology[17]. They suggested that a role could be found for EN in active CD in the following circumstances: steroid intolerance, patient refusal of steroids, EN in combination with steroids in undernourished individuals, and in patients with an inflammatory stenosis of the small intestine.

EN plays a greater role in children with active CD. In this group of patients, EN has been shown to have an efficacy equal to steroids. Therefore, it would seem perfectly reasonable to prescribe EN instead of steroids in the hope of avoiding steroid side-effects, including deleterious effects upon growth and development of children. The use of corticosteroids increases the risk of permanent growth failure in children, and 20%-30% will become adults with an abnormally short stature whether or not they are exposed to prolonged courses of steroids[18].

The nutritional status in those with acute CD is important. Differences are seen between patients in an active phase of disease and those in remission. Weight loss is found in up to 75% of patients hospitalized with an exacerbation of CD, with a negative nitrogen balance present in more than 50%, whereas the majority of patients in remission are of normal nutritional status[17]. The role of nutritional therapy in the maintenance of a good nutritional status in CD patients is important, especially as the condition itself will predispose to malnutrition.

When EN is to be used, the type of formula does not make any difference to the efficacy. Polymeric diets are less expensive and more palatable than elemental

diets, and therefore it would seem reasonable to suggest that there is no place for the elemental diet.

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Falk Workshop: Perspectives in Liver
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www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
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February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
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June 4-7, Helsinki, Finland
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June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
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June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

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June 25-28, Barcelona, Spain
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June 26-28, Bratislava, Slovakia
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July 9-12, Paris, France
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September 18-19, Mainz, Germany
Falk Symposium 166:
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September 20-21, Mainz, Germany
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Digestive Disease Week 2009

November 21-25, London, UK
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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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