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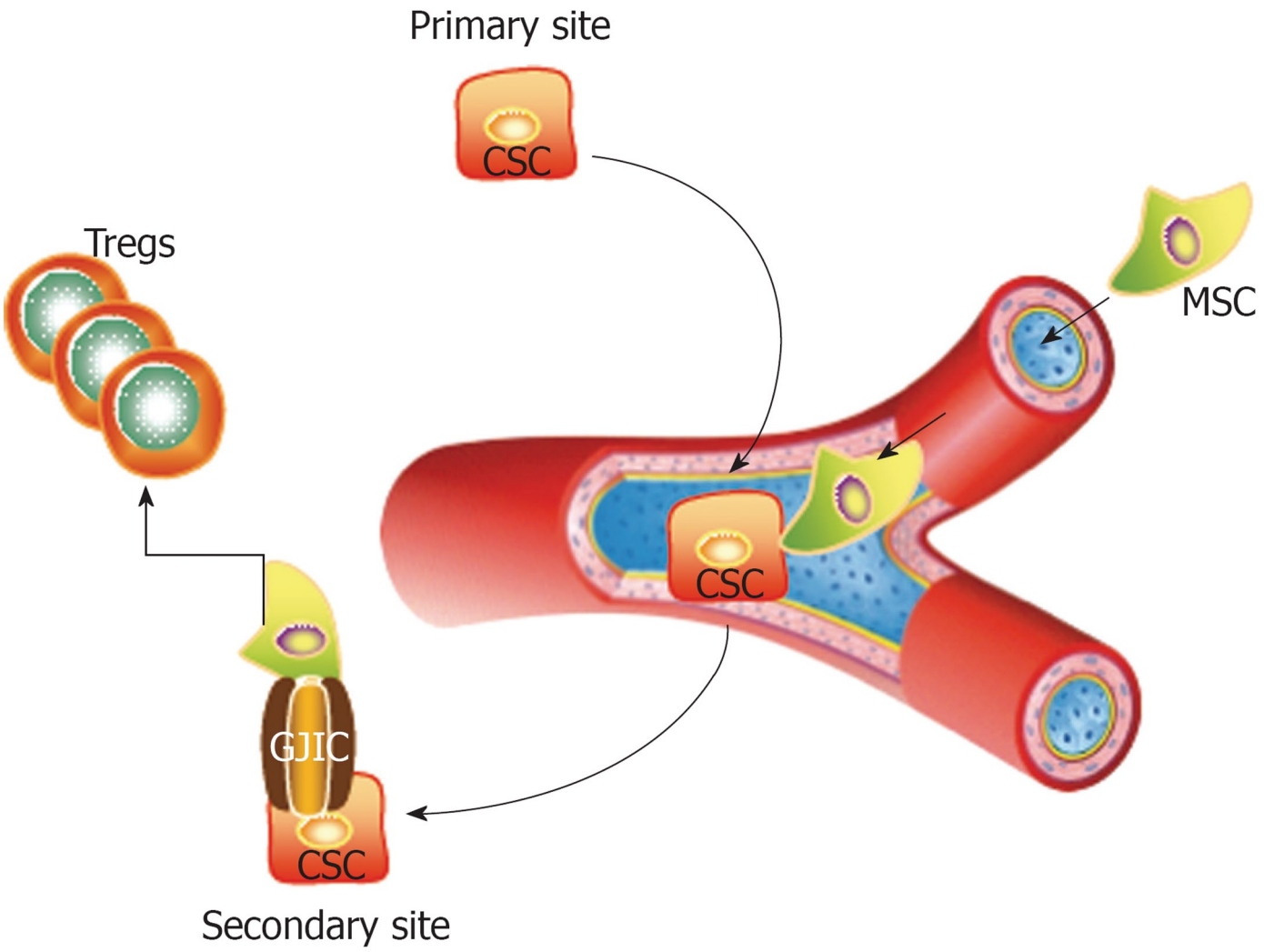

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EDITORIAL

Hepatitis C virus-host interactions: Etiopathogenesis and therapeutic strategies

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Abstract

Hepatitis C virus (HCV) is a significant health problem facing the world. This virus infects more than 170 million people worldwide and is considered the major cause of both acute and chronic hepatitis. Persons become infected mainly through parenteral exposure to infected material by blood transfusions or injections with nonsterile needles. Although the sexual behavior is considered as a high risk factor for HCV infection, the transmission of HCV infection through sexual means, is less frequently. Currently, the available treatment for patients with chronic HCV infection is interferon based therapies alone or in combination with ribavirin and protease inhibitors. Although a sustained virological response of patients to the applied therapy, a great portion of patients did not show any response. HCV infection is mostly associated with progressive liver diseases including fibrosis, cirrhosis and hepatocellular carcinoma. Although the focus of many patients and clinicians is sometimes limited to that problem, the natural history of HCV infection (HCV) is also associated with the development of several extrahepatic manifestations including dermatologic, rheumatologic, neurologic, and nephrologic complications, diabetes, arterial hypertension, autoantibodies and cryglobulins. Despite the notion that HCV-mediated extrahepatic manifestations are credible, the mechanism of their modulation is not fully described in detail. Therefore, the understanding of the molecular mechanisms of HCV-induced alteration of intracellular signal transduction pathways, during the course of HCV infection, may offer novel therapeutic targets for HCV-associated both hepatic and extrahepatic manifestations. This review will elaborate the etiopathogenesis of HCV-host interactions and summarize the current knowledge of HCV-associated diseases and their possible therapeutic strategies.

Key words: Hepatitis C virus; Hepatocellular carcinoma; Extrahepatic; Signalling; Therapy

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INTRODUCTION

Hepatitis C virus (HCV) infects more than 170 million people worldwide[1-3]. This virus is considered one of the major causes of both acute and chronic hepatitis. Persons become infected mainly through parenteral exposure to infected material by blood transfusions or injections with nonsterile needles. Although the sexual behavior is considered as a high risk factor for HCV infection, the transmission of HCV infection through sexual means, is less frequently[4,5]. Besides the cause of liver disease, the natural history of HCV infection (HCV) is also associated with the development of several extrahepatic manifestations[6,7]. Patients (40%-74%) infected with HCV might develop at least one extrahepatic manifestation during the course of the infection[6,7]. HCV-associated liver diseases range from chronic hepatitis to fibrosis, cirrhosis and hepatocellular carcinoma (HCC)[8]. Whereas, the extrahepatic manifestations include dermatologic, rheumatologic, neurologic, and nephrologic complications; and diabetes; arterial hypertension; autoantibodies and cryoglobulins[9,10].

Currently, the available treatment for patients with chronic HCV infection is interferon based therapies alone or in combination with ribavirin. Although a sustained virological response of patients to the applied therapy, a great portion of patients did not show any response[11]. Despite the mechanisms underlying the failure of interferon therapy are not well understood, several studies revealed that host response to interferon therapy is controlled by both viral and host factors[12]. Therefore, the current knowledge obtained from the functional analysis of both viral- and host factors, during the infection might help for the development of a novel therapeutic strategy in the future.

HCV GENOME AND ITS FUNCTIONAL ORGANIZATION

HCV belongs to the family Flaviviridae and is a single positive strand of RNA (about 9.6 kb) and contains a long open reading frame flanked by both 5' and 3' untranslated regions that are important for both translation and replication processes of the viral RNA genome[13,14]. Based on the sequence variation of HCV genome, six genotypes and more than 50 subtypes have been identified[15,16]. The RNA genome of the virus encodes for a single polyprotein that is mainly processed by cellular and viral proteases into at least 10 structural (Core, E1, E2/p7) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins (Figure 1).

Proteins that are derived from the amino-terminal of the viral polyprotein are called viral structural proteins, these include core and two envelope glycoproteins, E1 and E2. HCV core is a basic protein with variable molecular weights (17-21 kDa) and is characterized by its RNA-binding activity that is thought to be responsible for the comprising of the viral nucleocapsid[17-19]. However, the localization of HCV core protein in various subcellular compartments, including cytosol, lipid droplets, endoplasmic reticulum (ER), golgi apparatus, mitochondria, and nuclei suggests the contribution of HCV core protein in the modulation of different cellular processes[20].

The two HCV envelope glycoproteins E1 and E2 interact with cell surface molecules including CD81, claudin-1, scavenger receptor class B type I , and thereby facilitate the virus entry into the mammalian cells[21,22]. HCV p7 protein is a small transmembrane protein that is characterized by its functional activity as an ion channel protein[23]. In addition to the mentioned structural proteins, the non-structural protein NS2 is recognized to play a central role in polyprotein processing and virus assembly[24].

HCV non-structural proteins (NS3, NS4A, NS4B, NS5A, and NS5B) are essential for both viral RNA replication and polyprotein processing[25]. Besides its serine protease activity that is responsible for the cleavage of HCV polyprotein, and subsequently the generation of the amino termini of NS4A, NS4B, NS5A, and NS5B[26], NS3 serves as an RNA helicase and NTPase, and is considered an essential component of the RNA replicase complex[27,28]. NS4A, a small 54-amino-acid protein that forms a stable complex with the amino-terminal third of NS3, protease domain, and is required for a complete serine protease activity[29]. NS4B, an integral membrane protein that is mostly localized on the cytoplasmic side of the ER membrane and is implicated in assembly of the replicase complex on lipid rafts[30,31]. NS5A, a phosphoprotein that plays a role in viral resistance to interferon[32,33]. NS5A also plays a role in RNA replication, and virus assembly[34]. NS5B is the RNA-dependent RNA polymerase, and acts as the catalytic core of the macromolecular replicase complex essential for HCV RNA replication[25,35].

The functional analysis of HCV genome using cloned HCV gene expression in mammalian cells, the development of subgenomic or full-length replicon derived from HCV, and the generation of infectious HCV genotypes 1a and 2a in human hepatocyte derived cell lines have significantly contributed to the advancement of HCV research[36-39]. Recently, autophagy has gained importance as it plays an important role in HCV life cycle. Also, the role of HCV in the modulation of autophagy in hepatocytes has been reported[40-43]. HCV may induce accumulation of autophagosomes *via* the induction of ER stress and the unfolded protein response[40-43]. Similar to several viruses including poliovirus or coxsackie viruses, the induction of autophagosomes seems to play an important role in HCV replication[40-43]. Taken together, the knowledge obtained from the functional analysis of the molecular mechanisms of HCV-induced autophagy in hepatocytes may help for the development of a therapeutic strategy for treatment of HCV infection.

INTERFERENCE OF HCV PROTEINS WITH INTRACELLULAR SIGNAL TRANSDUCTION PROCESSES

The most studied transmembrane and intracellular signal

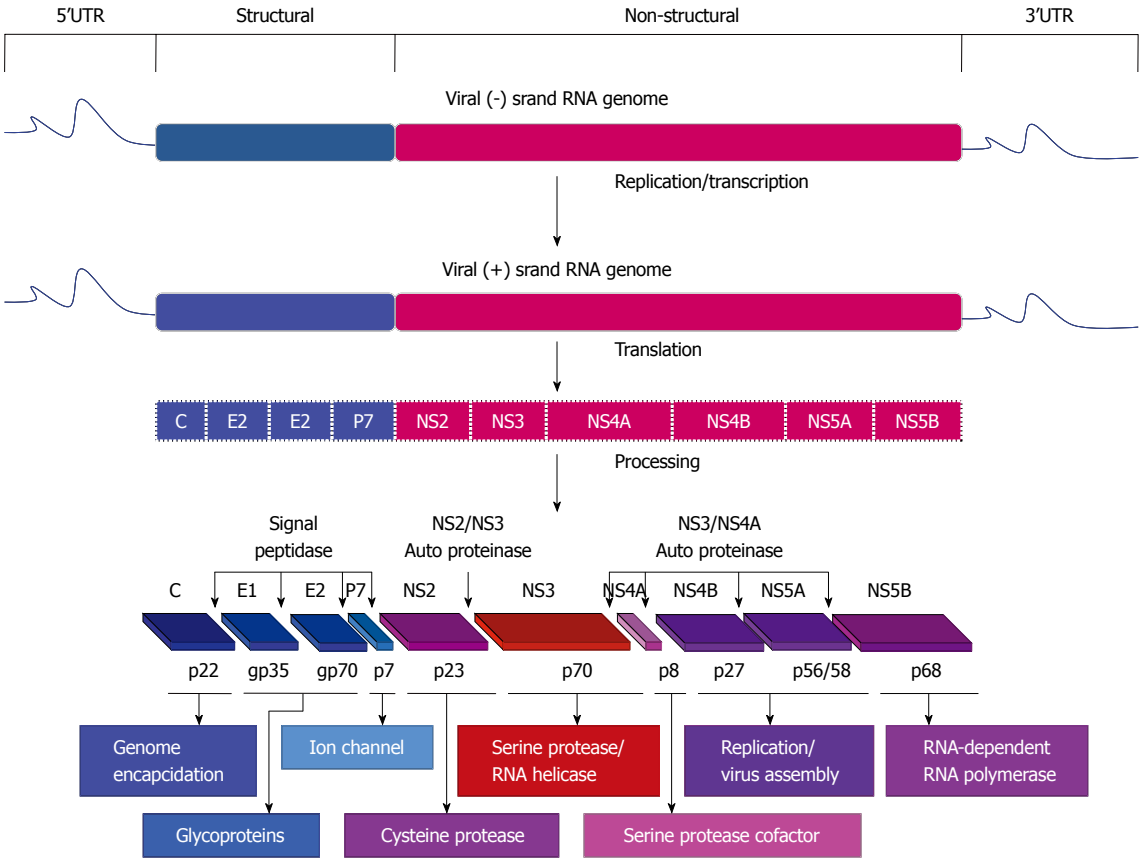

Figure 1 Hepatitis C virus genomic organization,replication,translation and generation of functional proteins. Proteins encoded by the hepatitis C virus (HCV) genome. HCV is formed by an enveloped particle harboring a plus-strand RNA of about 9.6 kb. The genome carries a long open-reading frame (ORF) encoding a polyprotein precursor of 3010 amino acids. Translation of the HCV ORF is directed *via* a 5' nontranslated region (NTR) functioning as an internal ribosome entry site; it permits the direct binding of ribosomes in close proximity to the start codon of the ORF. The HCV polyprotein is cleaved co- and post-translationally by cellular and viral proteases into ten different products, with the structural proteins core (C), envelop 1 (E1) and envelop 2 (E2) located in the N-terminal third, whereas, the non-structural (NS2, NS3, NS4A, NS4B, NS5A, NS5B) replicative proteins are located in the remainder. Putative functions of the cleavage products are shown.

transduction pathways, in the liver, are the mitogen-activated protein kinases (MAPKs), the transforming growth factor (TGF)-β, and the Janus kinase (JAK), tumor necrosis factor (TNF)-α and sphingolipid (SP). However, the activation of these signaling pathways by either cytokines or growth factors leads to the regulation of specific cellular processes including proliferation, growth, differentiation, adhesion, migration, apoptosis, and both synthesis and degradation of the extracellular matrix[44-47].

As recognized, the replication cycle of HCV is an intracellular mechanism that requires the intracellular signal transduction processes of the host cell to ensure genome replication, transcription and translation[48,49]. A proposed model for the interference of HCV with cellular signal transduction processes is demonstrated in Figure 2.

MAPK signal transduction pathway

The legation of endothelial growth factor (EGF), hepatocyte growth factor (HGF) and TGF-α to their corresponding membrane receptors results mainly in the activation of the intrinsic tyrosine kinase that leads to ligand-receptor complex formation and subsequently autophosphorylation[50-52]. As a consequence, the formation of a transient complex from Ras proteins and GTP that subsequently mediates the activation of RAF and MAPKK kinases that, in turn, enhance the activation of MAPK by dual phosphorylation of threonine and tyrosine. Activated MAPK results in the phosphorylation of transcription factors such as cAMP response element-binding protein and Ets-related transcription factor 1 (ELK-1)[47,53-56]. Evolutionary, MAPK signal transduction pathway is considered as one of the oldest signal transduction pathways in eukaryotic cells. This kinase contains three different signal pathways: the extracellular regulated protein kinase (ERK, p42/44 MAPK), the stress activated protein kinase [stress activated protein kinases (SAPK), p38 MAPK, p38-RK or p38], and the c-Jun N-terminal kinase (JNK, p64/54 SAPK). All of these pathways are implicated in the regulation of cellular processes including cell growth, differentiation, maturation, proliferation and apoptosis[53-63]. In mammalian cells every single pathway is activated by two mitogen-activated protein kinase kinase (MKK), e.g., JNK is activated by MKK4 and MKK7, ERK is activated by MKK1 and MKK2, whereas p38 is activated by MKK3 and MKK6[54,55,64]. However, the dual role of MKK in the activation of JNK, ERK or p38 signal transduction pathways still remains to be investigated in detail. Although ERK has been shown to play a

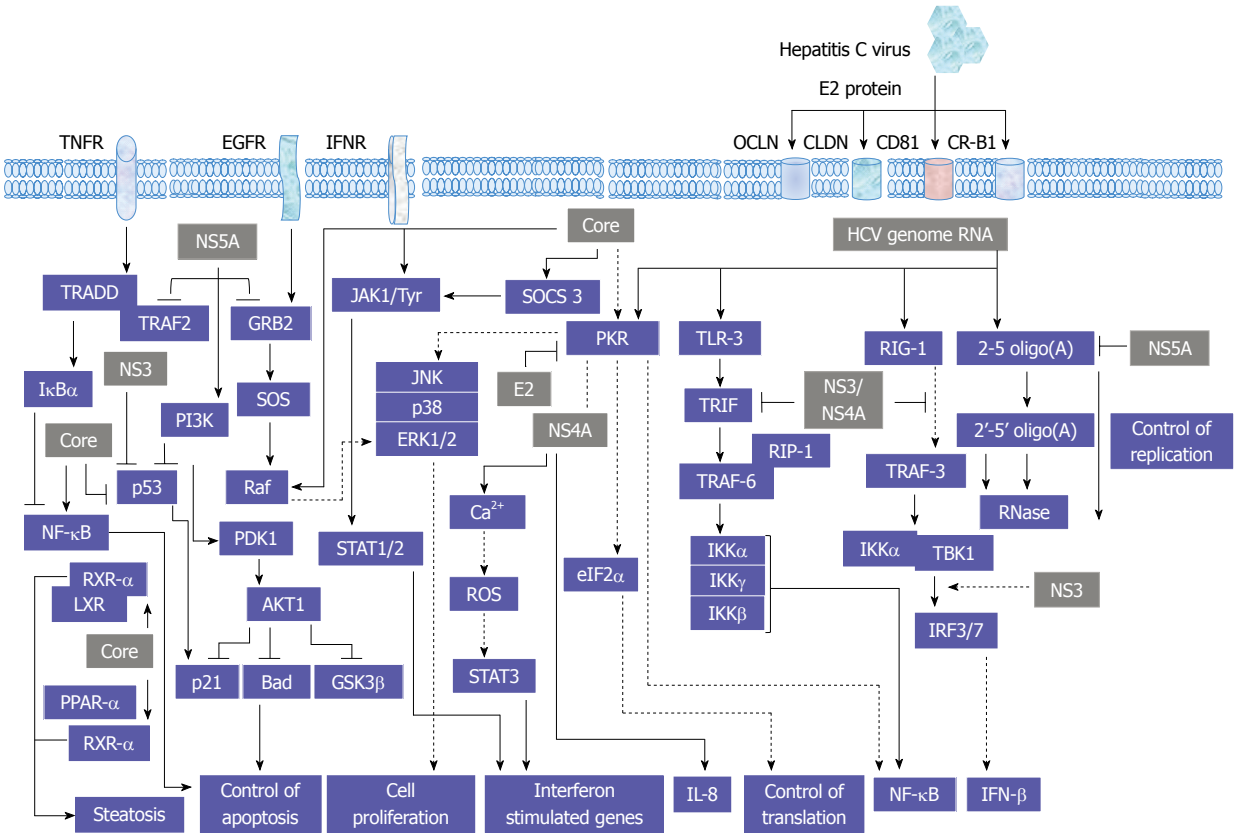

Figure 2 A proposed model for the consequences resulting from the interference of hepatitis C virus with signal transduction processes in host cells. HCV: Hepatitis C virus; TNFR: Tumor necrosis factor receptor; JAK: Janus kinase; EGFR: Endothelial growth factor receptor; IFN: Interferon; IFNR: IFN receptor; TRAF: Tumor necrosis factor associated factor; TRADD: TNFR-associated protein with death domain; JAK: Janus kinase; JNK: c-Jun N-terminal kinase; SOCS: Suppressor of cytokine signaling; PI3K: Phosphatidylinositol-3-kinase; ERK: Extracellular regulated protein kinase; RIP: Receptor-interacting protein; ROS: Reactive oxygen species; STAT: Signal transducers and activators of transcription; NF-κB: Nuclear factor κB; IL: Interleukin; PPAR: Peroxisome proliferator-activated receptor.

key role in the regeneration of liver cells[65,66], the role of p38, especially in hepatocytes regeneration, so far, is not clear[67,68]. Physiologically, the activity of JNK in the liver is minimal, however, its increase during liver regeneration or HCV infection may result from the direct effect of the elevated level of hepatic TNF-α[60,69]. Also, the promotion of cell proliferation in liver and non liver cells by HCV proteins is associated with the activation of MAP kinase signaling pathways JNK, p38 and ERK[47,54,55,70-72]. An overview of HCV-induced alteration of MAP kinase signaling pathways is summarized in Figure 3.

TGF-β signal transduction pathway

TGF-β is a cytokine family member that plays a key role in the regulation of different cellular events including growth, differentiation, adhesion, apoptosis, and synthesis and degradation of the extracellular matrix[47,73,74]. Although the elevation of TGF-β concentration is observed, during liver regeneration, no marked apoptosis was noted in hepatocytes[75]. However, the inhibition of TGF-β-mediated apoptosis in hepatocytes may be linked with parallel augmentation of Smads, and other antiapoptotic proteins such as Bcl-2 and Bcl-X in hepatocytes[76,77].

In the liver, TGF-β is responsible for hepatocytes regeneration, the development of fibrosis, and HCC, as well as for the proliferation and differentiation of epithelial cells[47,78]. Moreover, the elevation of TGF-β2 expression, in liver cells, during the course of HCV infection is associated with the development of neoangiogenesis[47,79]. Also, the higher the concentration of both TGF-β1 and TGF-β2 in the sera of patients with chronic liver diseases, such as chronic HCV infection, the more severe the liver failure; an evidence for the association between the level of these cytokines and the development of liver diseases including hepatic fibrosis, cirrhosis and HCC[47,80,81]. Simultaneously, in patients with chronic HCV, TGF-β1 serum concentration decreases and normalizes after successful antiviral therapy[82]. However, the inhibition of TGF-β pathway during the infection with HCV, hepatitis B virus (HBV), adenoviruses or human papilloma virus (HPV) has been reported[83]. Also, the mechanism whereby HCV protein NS5A inhibits the activity of TGF-β signal transduction pathway is reported[83]. The inhibition of TGF-β pathway by HCV protein NS5A results from its direct reaction with its specific receptor[84].

TGF-β signaling pathway appears to be most prominent at the interface between development and cancer both in liver and gut epithelial cells[85]. Since this signaling pathway is considered to play a pivotal role in the proliferation of embryogenic hepatocyte as well as in the formation of gastrointestinal cancers[86,87]. In addition, many studies have reported a reduction of TGF-β receptors in

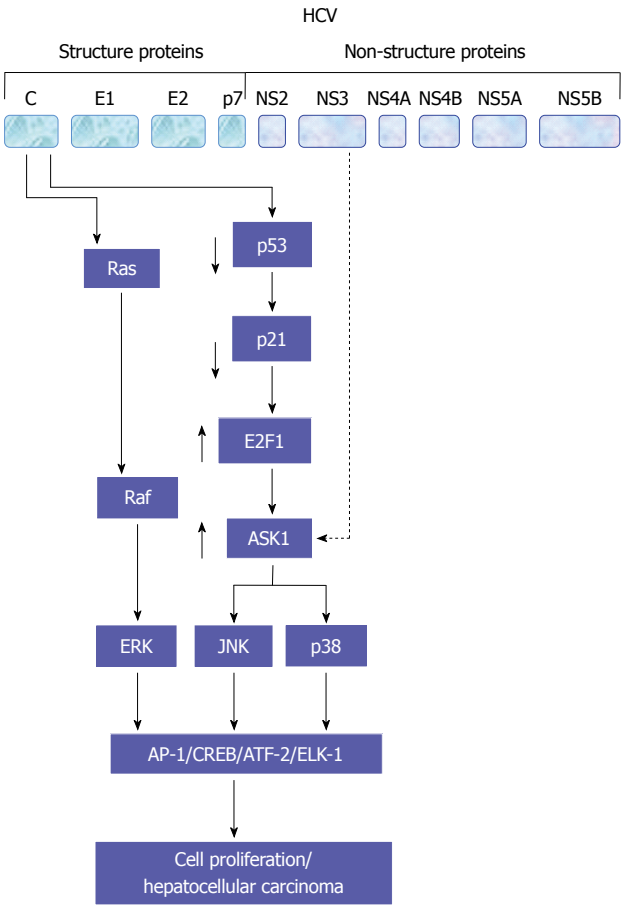

Figure 3 A schematic view for the molecular mechanisms,which are involved in the regulation of hepatitis C virus-induced associated cell proliferation. A central role for mitogen-activated protein kinase signaling pathways in the modulation of hepatitis C virus-associated hepatocellular carcinoma is also demonstrated. HCV: Hepatitis C virus; AP-1: Activator protein-1; JNK: c-Jun N-terminal kinase; CREB: cAMP response element-binding protein; ERK: Extracellular regulated protein kinase.

up to 70% of HCC[88,89]. Although Smad proteins have been shown to be impaired in different cancers, it appears to play a minor role in HCC[90,91]. Yet, TGF-β levels in serum and urine are increased in HCC patients[92,93]. However, High TGF-β levels have been noted during the course of advanced clinical stage of HCC[94,95]. This dual role of TGF-β signaling in HCC was explained by its effect on the tumor tissue microenvironment and on selective loss of the TGF-β-induced antiproliferative pathway[88]. However, the role of TGF-β signaling pathway in the development of both hepatic angiogenesis (Figure 4A) and HCC (Figure 4B) during the course of HCV infection is demonstrated in detail.

TNF-α signal transduction pathway

As recognized, macrophages, monocytes, mast cells and NK cells are the main source of TNF-α production that is considered to be one of the major mediators of the antiviral inflammatory response, which results in the enhancement of lymphocytes proliferation and differentiation, production of acute phase proteins and cell apoptosis[60,96,97].

The two essential TNF-α membrane receptors are the TNF-R1 and TNF-R2[98]. TNF-R1 is an extracellular transmembrane receptor that consists of extracellular, transmembrane and intracellular [death domain (DD)] domains. TNFR1 is considered to play a key role in liver, and is expressed in hepatocytes as well as in kupffer cells and hepatic sinusoidal endothelial cells[99-101].

Activated TNF-R1 binds, *via* the DD, to an adaptor protein TNFR-associated protein with DD, which afterwards activates Fas associated DD proteins, TNF associated factor 2 and receptor-interacting protein. All of these proteins influence different signal transduction pathways, which are involved in the regulation of apoptosis[102], and anti-apoptotic effects of TNF-α as the activation of nuclear factor κB (NF-κB) factor, as well as JNK and ERK from MAPK signal transduction pathway[103].

Although the main target cells of HCV infection are the hepatocytes, the infection of B lymphocytes by HCV virus is documented[104]. Therefore, the participation of both innate and adoptive immune system at the etiopathogenesis of HCV during the course of HCV infection is expected.

The induction of TNF-α, during the infection with chronic HCV, results mainly in the activation of NF-κB pathway that subsequently stimulates transcription of genes encode for cytokines, acute phase proteins, immunoglobulins (Ig) and adhesion factors[60,103,105]. The ligation of TNF-α to TNF-R1, depending on the activated cellular proteins, leads to either cell proliferation or apoptosis[106].

TNF-α plays a diverse role in HCV infection. The activation of TNF-α has a pivotal role in the inflammatory process of chronic hepatitis C, and TNF-α levels correlate with the degree of inflammation[107,108].

HCV viral proteins including core, NS3 and NS4B protein have been reported to be involved in the modulation of cell proliferation[54,55,60,109,110] and production of proinflammatory cytokines TNF-α through NF-κB (Hassan *et al*[47,54,60], 2007), activator protein-1 (AP-1) and serum response element (SRE)[55]. AP-1 is a complex of homo- or heterodimers encoded by c-jun and c-fos family genes[55]. However, the ability of AP-1 to stimulate proliferation seems to be growth factors[47,111], oncogenes and inflammatory peptides- dependent mechanism[112]. SRE regulates the promoters of immediate early genes such as c-fos and PIP92. MAPK cascade activation phosphorylates Elk-1 factor binding with SRE and serum response factor[113]. Thus, the created complexes affect transcription of genes taking part in cell proliferation. A schematic view demonstrates HCV-mediated pathways leading to TNF-α production is outlined in Figure 5.

JAK signal transduction pathway

JAK signal transduction pathway can be activated by different cytokines and growth factors. This intracellular pathway operates in hepatocytes[114,115] as well as in immune[116], hematopoietic[117,118] and neural system cells[119]. After extracellular ligand-receptor interaction, receptor multimerization and the activation of JAK1, JAK2, JAK3 and tyrosine kinase 2 (Tyk2) is observed[120]. The receptor-

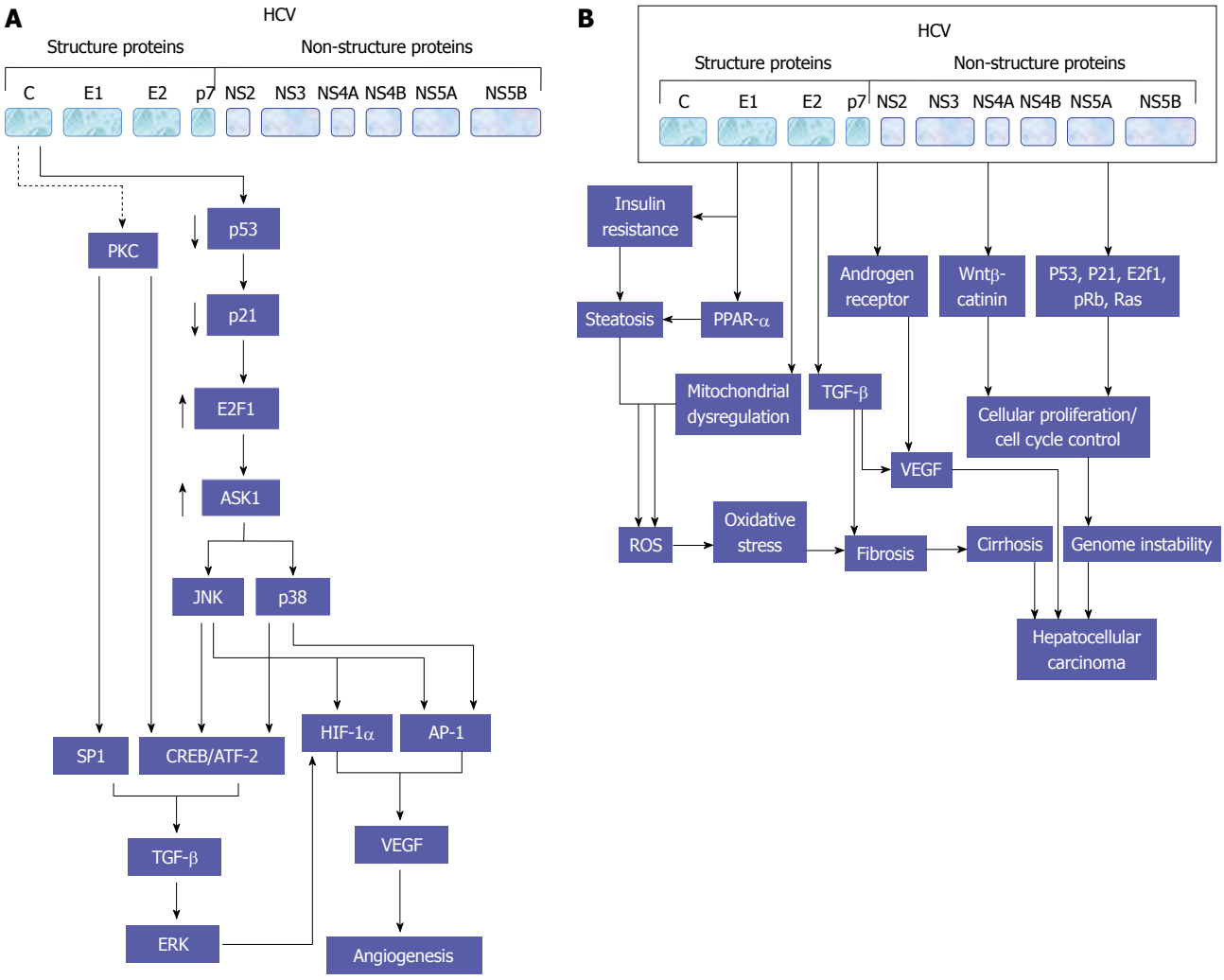

Figure 4 A proposed model for hepatitis C virus - mediated effects in liver cells and the modulatory role of transforming growth factor β in the regulation of both angiogenesis (A) and hepatocellular carcinoma (B). PKC: Protein kinase C; HCV: Hepatitis C virus; AP-1: Activator protein-1; CREB: cAMP response element-binding protein; JNK: c-Jun N-terminal kinase; ERK: Extracellular regulated protein kinase; TGF: Transforming growth factor; VEGF: Vascular endothelial growth factor; ROS: Reactive oxygen species; PPAR: Peroxisome proliferator-activated receptor.

kinase complex phosphorylates cytoplasmic SH-2-containing transcription factors: signal transducers and activators of transcription (STAT)1-6. STATs are specifically inhibited by protein inhibitors of activated STAT and by suppressor of cytokine signaling (SOCS) through negative feedback control[121]. SOCS proteins include SOCS 1, 2, 3 and cytokine-induced Src homology 2 protein, which bind to JAK kinase inhibiting its enzymatic activity[122]. STATs perform different, often opposing functions in the liver. STAT1 is mainly activated by interferon (IFN) type I (IFN-α/β) and IFN type Ⅱ (IFN-γ). Its essential function in liver is the participation in antiviral immune defense, as well as in the development of inflammation and apoptosis. IFN-α/β and IFN-γ are ligands for STAT2, whose major function is antiviral defense. Membrane the IFN-α/β receptor (IFNAR) is a complex of two subunits: IFNAR1 and IFNAR2. IFNAR2 presents three diverse forms: full-length IFNAR2c is responsible for signal transduction and transcription process, whereas short form IFNAR2b and soluble form IFNAR2a inhibit these processes[123]. The complex IFN-α/β - IFNAR activates JAK1 and Tyk2 kinases. IFN-γ takes effect by IFN-γ receptor (IFNGR): IFNGR1 and IFNGR2. STAT3 function is especially regulated by interleukin (IL)-6 and its family members such as cardiotrophin-1, oncostatin M, IL-11, leukemia inhibitory factor or ciliary neurotrophic factor, by IL-10, IL-22, EGF and HCV proteins. STAT3 participates in the acute phase response, stimulates hepatocytes regeneration and regulates lipid and carbohydrate metabolism in the liver[124]. Moreover STAT3 is one of the main anti-HCV defense elements that act by increasing the IFN-α antiviral effect and by its direct cytoprotective and anti-inflammatory influence on hepatocytes[125]. IL-6 and its related cytokines bind gp130 receptor protein, which plays a key role in liver regeneration.

Furthermore, the activation of gp130 is independent of the activation of other kinases, such as MAKP[71]. The ligand-gp130 complex activates JAK1, JAK2 and Tyk2 and subsequently leads to the activation of STAT1-3. However, the modulation of JAK1, JAK2 and Tyk2-

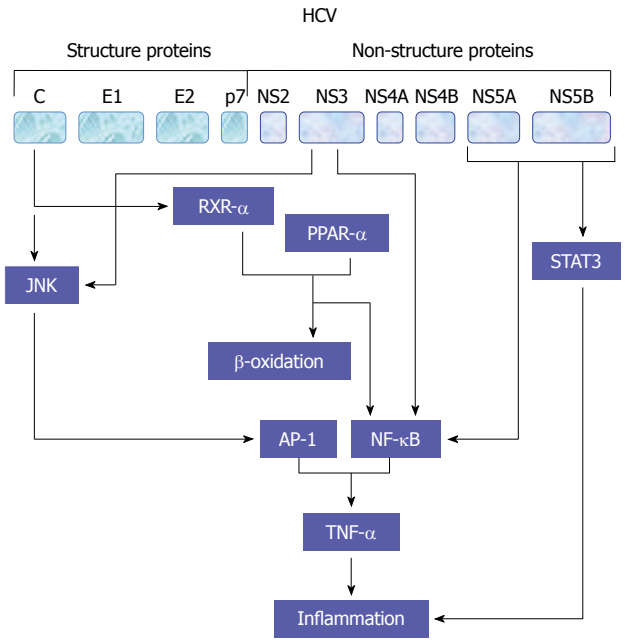

Figure 5 A suggested model for hepatitis C virus -associated inflammation and the role of tumor necrosis factor α in the regulation of this mechanism. HCV: Hepatitis C virus; JNK: c-Jun N-terminal kinase; AP-1: Activator protein-1; NF-κB: Nuclear factor κB; PPAR: Peroxisome proliferator-activated receptor; TNF: Tumor necrosis factor; STAT: Signal transducers and activators of transcription.

mediated activation of STAT1-3 factors by HCV infection or by HCV structural and non-structural proteins has been demonstrated. Thus, the inhibition of the transmembrane and intracellular signal transduction pathways could be a new therapeutic target in chronic HCV treatment. HCV structural proteins Core, E2 and non-structural protein NS5A were reported to reduce the number of IFN-α receptors (IFN-α R1 and IFN-αR2c) and subsequently inhibit IFN-α-induced activation of STAT1-3[126-128]. As a result, viral replication, as well as inflammation and fibrosis in the liver, is augmented and has a negative effect on IFN-α treatment response among patients with severe liver damage. However, HCV does not affect IFN-γ function, and in consequence, STAT1 activation[129]. Moreover, the production of IFN-γ by NK cells during HCV infection is associated with the inhibition of hepatocytes regeneration[124]. Also, STAT4 has been shown to be activated by IL-12 and to play a critical role in hepatocytes damage during hepatic ischemia/reperfusion injury[130]. Whereas, STAT5 that is mainly activated by growth factors and involved in the regulation of the expression of genes encoding cytochrome P450, HGF and insulin growth factor 1, which are essential for hepatocytes metabolism, growth and differentiation[131,132]. STAT6 that is mainly regulated by IL-4, IL-12 and IL-13 and contributes in Th2 lymphocytes response during viral hepatitis and reduces hepatocytes damage during hepatic ischemia/reperfusion injury[133]. An overview demonstrating the interference of HCV with JAK/STAT, cytokines and IFN-associated pathways is outlined in Figure 6.

SP signal transduction pathway

Although SPs are considered to be the major components of eukaryotic plasma membranes and mediators of cell-to-cell interactions, their role as second messengers in transmembrane and intracellular signal transduction is documented[134]. The main function of the SP signal transduction pathway is the modulation of specific cell reactions including proliferation, growth arrest, differentiation, apoptosis and calcium homeostasis (Boya *et al*[135], 2005). SP pathways can be activated by many pro-apoptotic and promitotic factors[136-138].

Ceramide is the most intensively studied second messenger of SP signal transduction pathway. Ceramide can mediate its antiproliferative effect by the activation of JNK, SAPK, cathepsin D, methionine adenosyl transferase 1A and caspase 3, leading to the destruction of the cytoskeleton, nuclear and plasma membranes[139]. In addition to its antiproliferative effects, ceramide has the ability to trigger mitochondrial dysfunction by the enhancement of reactive oxygen species (ROS) accumulation and cytochrome c release[140]. However, the antiapoptotic function of ceramide depends on its ability to decreases the intracellular level of anti-apoptotic proteins of the Bcl-2 family as well as the activity of anti-apoptotic enzymes such as Ca^{2+}-kinases including protein kinase C (PKC), PKCα and PKCβα/Akt[141]. Besides its role in the regulation of both cell survival and death, ceramide can also inhibit autophagocytosis by a mechanism based on the enhancement of apoptotic pathway[135,142]. As known, autophagocytosis is an intracellular process that relies on degradation of damaged, dead or used cell structures to prolong cell life[142].

Sphingosine (SFO) that is synthesized mainly from the hydrolysis of Ceramide by ceramidases is a member of the second messengers of SP signal transduction pathway. SFO plays a key role in promotion of apoptosis by the enhancement of ROS production in mitochondria and activation of caspase 3, 7 and 8[137]. Also, the inhibition of AKT by SFO leads to the augmentation of the cellular effects of both cytochrome c and caspase-3[137].

Besides its negative effect on DNA synthesis, methylation and replication, SFO reduces the activity of protein kinases including, PKC, calmodulin-dependent protein kinase and insulin receptor kinase[137], and thereby leads to disturbances of nuclear proteins phosphorylation including RNA polymerase, topoisomerase Ⅱ, histones and matrix proteins[143].

Some studies underline the proliferative character of SFO suggesting that low cellular concentrations of SFO leads to stimulation of cell proliferation and DNA synthesis, whereas the high concentrations is associated with the induction of apoptosis. SFO-1-phosphate (S1P) that mainly synthesized from SFO, has been reported to have an anti-apoptotic potential[135]. An increase in the intracellular level of S1P can activate cell proliferation and its passing from G1 phase to S phase, augment the general number of cells resting in S phase, shorten the time needed for cell division, enhance survival rate of

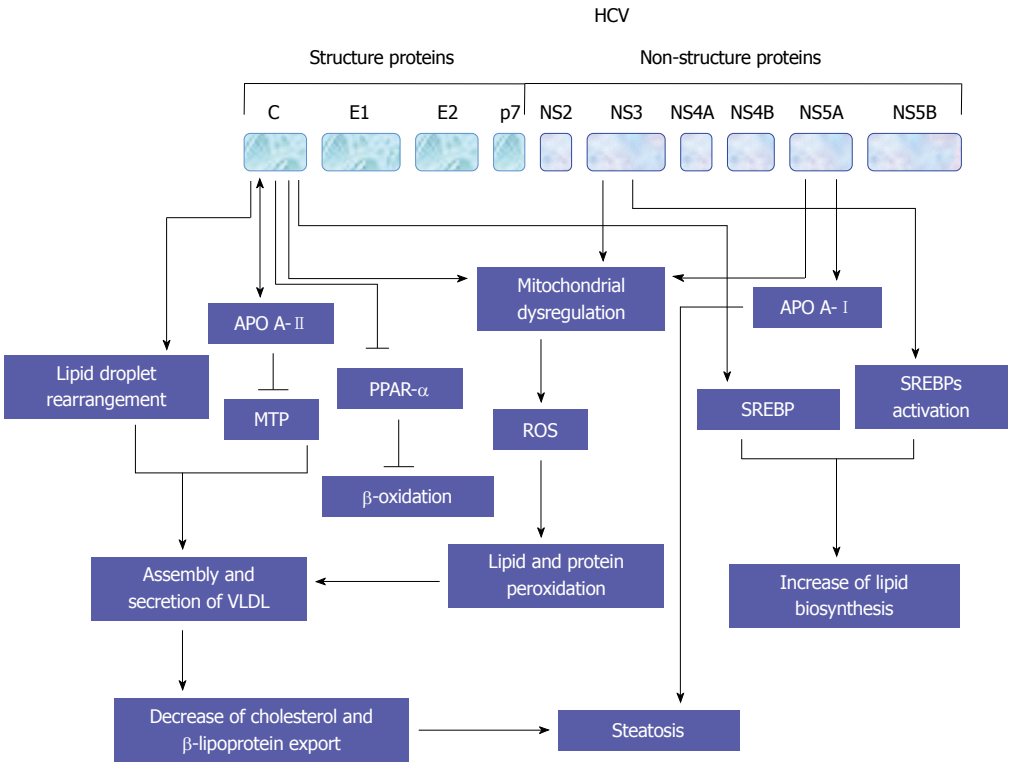

Figure 6 Proposed model for the molecular mechanisms, which are involved in the regulation of hepatitis C virus-associated steatosis. HCV: Hepatitis C virus; ROS: Reactive oxygen species; PPAR: Peroxisome proliferator-activated receptor.

cells subjected to pro-apoptotic factors, mobilize calcium ions from intracellular compartments, influence cytoskeletal architecture and the processes of cell migration and adhesion[144]. S1P modulates cell functions in two different ways: as an intracellular messenger and as a ligand of G protein-coupled receptors, known as endothelial differentiation genes (Edg) - Edg-1, -3, -5, -6 and -8[144]. Cer can be phosphorylated by ceramide kinase to ceramide-1-phosphate (C1P), which can be dephosphorylated back to ceramide by C1P phosphatase[145]. Similarly to S1P, C1P promotes cell proliferation[145]. Recently, some studies have shown that the inhibition of SP metabolism can be a new therapeutic target for HCV infection[146].

HCV-ASSOCIATED STEATOSIS

Fatty liver (liver steatosis) is recognized as a histological phenotype of HCV infection that is occurring in all patients independent from the genotype[147,148]. Although the development of steatosis seems to be a direct consequence of viral protein expression, the molecular mechanism of its occurring appears to be genotype-specific[149].

The promotion of lipid homeostasis by HCV is mediated by the increasing of lipogenesis *via* a process including the activation of ER membrane bound transcription factors (SREBPs), reducing oxidation and lipid export[149]. As recognized, the main function of SREBPs is the regulation of the transcription of genes that encode for the enzymes which are essential for the biosynthesis of both cholesterol and fatty acid[150]. However, the suppression of both HCV replication and release in response to the inhibition of SREBP[151], or by the fatty acid synthase, an enzyme that is primarily involved in the biosynthesis of fatty acids[151,152], suggest that the host lipid metabolic pathways is considered a potential target for the treatment of HCV infection. A schematic view suggests HCV-mediated pathways leading to the development of steatosis are outlined in Figure 7.

HCV-RELATED CRYOGLOBULINEMIC

Cryoglobulinemia is defined as the presence of circulating Ig that precipitate at temperatures below 37 ℃ and redissolve on rewarming[153]. Such an *in vitro* phenomenon is detectable in a wide number of chronic infectious and immunological disorders, as well as in some hematological malignancies[153-155].

Cryoglobulinemia is usually classified into serological subsets namely type I or monoclonal cryoimmunoglobulinemia that is composed by single monoclonal Ig, mixed cryoglobulinemia (MC) that contains a mixture of polyclonal IgG and monoclonal (type Ⅱ) or polyclonal (type Ⅲ) IgM rheumatoid factor (RF)[156]. Type I cryoglobulinemia is frequently associated with hematological disorder including multiple myeloma, immunocytoma or Waldenstrom's macroglobulinaemia, and is mostly asymptomatic except in the case of hyperviscosity syndrome[157-159]. Whereas, MC is characterized by a typical triad purpura, weakness, arthralgias as well as by multisystem organ involvement including chronic hepati-

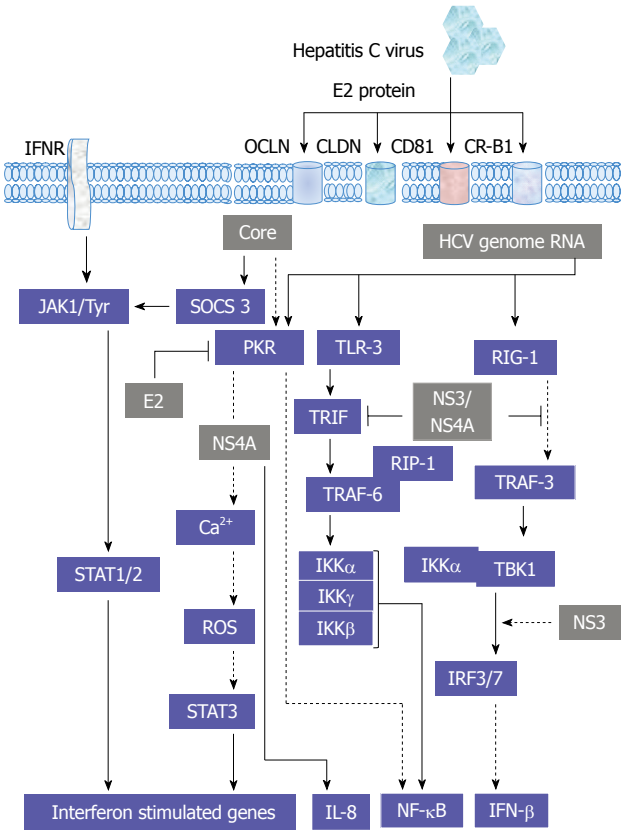

Figure 7 A schematic overview for the interference of hepatitis C virus with cytokines-associated Janus kinase/signal transducers and activators of transcription and cytokines-associated pathways. HCV: Hepatitis C virus; JAK: Janus kinase; EGFR: Endothelial growth factor receptor; IFN: Interferon; IFNR: IFN receptor; TRAF: Tumor necrosis factor associated factor; JAK: Janus kinase; SOCS: Suppressor of cytokine signaling; RIP: Receptor-interacting protein; ROS: Reactive oxygen species; STAT: Signal transducers and activators of transcription; NF-κB: Nuclear factor κB; IL: Interleukin; PPAR: Peroxisome proliferator-activated receptor.

tis, membranoproliferative glomerulonephritis, peripheral neuropathy, skin ulcers, widespread vasculitis, and less frequently lymphatic and hepatic malignancies[154-156,160].

The pathological feature of MC is a leucocytoclastic vasculitis, including both small and medium sized vessels, which are responsible for cutaneous and visceral organ involvement[154-156,160,161]. However, based on clinico-serological and pathological alterations, the terms MC and cryoglobulinemic vasculitis (CV) are referred to the same clinical syndrome[160-162].

CV is considered to be a relatively rare disorder; its prevalence among different countries show a great geographical heterogeneity[155,156,160].

Currently, there are no available classification/diagnostic criteria for CV. However, in the clinical practice, the main diagnostic parameters include serum mixed cryoglobulins with RF activity, low C4, orthostatic skin purpura, and leukocytoclastic vasculitis of small/medium-sized blood vessels secondary to the deposition of circulating immune-complexes and complement[154-156,160,161].

The causative role of hepatotropic viruses in the development of CV had been hypothesized previously[163-165], when a role of HBV in another systemic vasculitis - the polyarteritis nodosa had been demonstrated[166]. However, soon after the identification of CV in patients with HBV infection[167], the role of HCV in the development of CV has been considered[168,169], the majority HCV patients was characterized by the presence of CV[170], an evidence for the association between the chronic infection with HCV and the development of CV.

Currently, the role of HCV infection in the modulation of CV has been consequently established in several studies[160,169-172]. Therefore, a direct role for HCV in the formation of immune-complex-mediated vasculitis is considered. Besides its role as a main triggering factor of CV, HCV infection is thought to play an important role in the underlying lymphoproliferative disorder[160,169-172]. However, the detection of both active and latent HCV viral replication in the peripheral lymphocytes of patients with HCV infection and/or CV suggesting further a dual feature for HCV as both hepato- and lymphotropic virus[169,171]. Also, the affinity of lymphoid tissue to the infection with HCV supports further the occurring of both autoimmune and lymphoproliferative disorders in patients with chronic HCV infection[160,170-174].

Although the HCV infection presents homogenous distribution worldwide, the geographical heterogeneity in the context of HCV-associated immune disorders is common[174]. Thus, the involvement of particular HCV genotypes, environmental and/or host genetic factors may play a central role in the development of HCV-associated CV.

Although the strong affinity of the HCV envelop protein E2 to CD81 and subsequently the modulation of immunological disorders[175], the HCV, based on its biological features and several laboratory studies, seems to be insufficient to drive the different autoimmune-lymphoproliferative disorders in infected patients[173,174,176]. CD81 is a cell surface protein that expressed in both hepatocytes and B-lymphocytes[177], and is recognized to play an important role by the entry of HCV particles[178], in addition to the modulation of HCV-induced autoimmunity[179]. Therefore, the elevation of HCV-associated autoimmune diseases including CV during the course of infection[180,181], may be a consequence of the interaction between HCV-E2 and CD81 leading the increase of the frequency of VDJ rearrangement in antigen-reactive B-cell[169]. Thus, the expansion of B-lymphocyte may be the main actor that is responsible for the observed autoantibody production during the course of HCV infection[174,182-184]. Also, another mechanisms including the molecular mimicry such as HCV antigens or host autoantigens may be involved in the activation of B lymphocyte and thereby increase the production of autoantibodies[160].

In addition to its immunopathogeneicity, HCV is reported to exert oncogenic potential that is mainly involved in the development of HCC[47,54,60,109,185], and in the lymphomagenesis and, possibly, in other malignancies including thyroid cancer[184-186]. However, the modulation of TNF signaling of the host cells by HCV NS5A pro-

tein and the inhibition of JAK-STAT pathway by HCV core following the treatment with either IL-6 or IFN-γ stimuli[126,186,187], suggesting an important role for HCV in the dysregulation of the immune system.

The outcome and the severity of CV among patients are largely variable, also the behavior of the disease is mostly unpredictable and patient show usually a relatively benign clinical course. Thus, based on its complicated etiopathogenesis the treatment of CV syndrome must deal with the total clinical picture of the conflicting conditions including: HCV infection, autoimmune, and lymphoproliferative alterations[160]. Thus, according to the pathogenetic process leading to HCV infection and subsequently to the appearance of CV, the treatment of the disease may be applied at three different levels by means of etiologic, pathogenetic, and/ or symptomatic therapies.

DERMATOLOGIC MANIFESTATIONS OF HCV

Dermatologic manifestations of HCV are classified, based on the disease to be proven, are either a suspected etiology or causation. The causal manifestation of the dermatologic diseases results from direct infection of HCV in the skin, lymphocytes, dendric antigen-presenting cells, and blood vessels[188]. Whereas, the etiological manifestation of dermatological diseases results indirectly when the disruption of another organ infected or affected by HCV is associated with skin manifestations, but not specific or typical of skin responses in relation to HCV-infected or affected organ[189,190]. The causal manifestation is directly mediated by HCV infection as evidenced by the detection of HCV-RNA particles in epidermal cells[191] as well as by the induction of epiphenomena, that results mainly from the disruption of immune responses, in the skin of HCV-infected patients[192,193]. Also, the leukocytoclastic vasculitis that is due to cryoglobulinemia is considered a good evidence for a specific skin manifestation that results in a great part from the production of Ig, that is associated with rheumatoid characteristics causing an immune complex-mediated vasculitis and thereby presents a good example for etiological skin manifestation[194,195]. Such skin responses result from a wide range of causes, for example the release of thyroid hormone in early HCV-linked autoimmune thyroiditis[196,197]. Also, chronic active hepatitis that mostly leads to fibrotic liver disease in patients with chronic hepatitis C infection can lead to the development of cutaneous vascular changes such as palmar erythema or spider nevus[197,198]. Moreover, arteriovenous hemangioma, a benign acquired cutaneous vascular lesion, has been reported, in patients with chronic active hepatitis associated with HCV infection[199].

Another category of dermatologic manifestations in HCV infections includes porphyria cutanea tarda (PCT), an example for HCV-related disease in which causation is either unexplained or undeniable[200].

HCV-ASSOCIATED LICHEN PLANUS

Oral lichen planus (LP) is a chronic inflammatory condition that affects the oral mucous membranes with a variety of clinical presentations, including reticular papular, plaque-like, atrophic, and ulcerative lesions[201]. Oral LP affects about 0.1% to 4% of the population, it is a middle-aged disease that is more common among women[202]. The occurring of LP is induced by a wide range of factors including both bacterial and viral infections that thought to trigger the regulation of cell-mediated mechanisms leading the formation of oral LP lesions[203,204]. The association of LP with chronic liver disease is reported[205], and seems to be geographical dependent disease[206]. However, the risk of chronic liver disorders in LP patients appears to be f age, sex, alcohol consumption and even hepatitis B infection (HBV)-independent[207]. Nevertheless, most patients with LP and chronic liver disease are not HBV[208,209] or hepatitis G virus-infected[210-213]. Although LP is rarely associated with various hepatic conditions such as Wilson's disease, haemochromatosis, primary sclerosing choloangitis, and α-1-antitrypsin deficiency[214,215], the association of LP with primary biliary, and HCV infection is reported in several studies[209,216-224]. Thus, HCV-associated hepatic disease may precede LP onset or may be diagnosed together with it[225].

The geographic heterogeneity in the prevalence of HCV infection is reported in patients with other HCV-related extrahepatic conditions, such as serum autoantibodies, PCT or lymphoma[225], suggesting a genetic differences among the studied populations. Indeed, HCV-related oral LP seems to be associated mainly with the HLA-DR6 allele (Nagao *et al*[226], 1996) and this could partially explain the particularity of the geographic heterogeneity of HCV infection- associated LP. However, the pathogenetic link between LP and HCV is not fully understood, since the molecular mimicry between the virus and host epitopes is unexpected, and the viral factors including the genotype or the viral load[226-228]. Although the histological features of lesional tissue from HCV-positive or HCV-negative patients showed no substantial differences[229,230], the presence of HCV in oral LP lesional tissue becomes object of several investigations[222,230-233]. However, the presence of replicative intermediate HCV-RNA in LP specimens provide a strong evidence for the association between HCV infection and the development of LP in HCV infected patients[222,230-233]. Although, the compartmentalization of HCV in the oral mucosa, the infection with HCV does not seem to cause direct damage to epithelial cells in oral LP[234].

HCV AND TYPE 2 DIABETES

The epidemiological link between HCV and type 2 diabetes mellitus (T2DM) is common and widely reported[235-238], and the infection with HCV increase the risk of T2DM development[239]. Although the processes of T2DM development in patients with HCV infection is not fully described, the molecular mechanisms, which are

involved in the regulation of HCV-induced insulin resistance is studied in details[240-243].

Chronic infection with HCV is mostly associated with insulin resistance that subsequently leads to the development of the metabolic disease T2DM. Thus, apart from the well-investigated complications of diabetes, the appearance of insulin resistance in patients with chronic HCV infection leads mostly to the enhancement of fibrosis, and cirrhosis, and in turn to the development of HCC[244]. Besides the liver complications, patients with insulin resistance show a poor response to antiviral therapy[245].

As known, insulin is an anabolic hormone that is secreted by pancreatic β-cells. This enzyme is essential for the maintenance of glucose homeostasis[246,247].

The pathway of insulin signaling pathway is involved mainly in the regulation of different cellular processes including the activation of insulin receptor (IR), IR substrates (IRS), phosphatidylinositol-3-kinase, Akt and PKC isoforms ζ and λ[248,249]. The activation of Akt promotes storage of excess glucose as glycogen by phosphorylating glycogen synthase kinase and subsequently suppresses gluconeogenesis by inhibition of phosphoenol-pyruvate carboxykinase and glucose-6 phosphatase. Whereas, the activation of Akt is involved in the translocation of the glucose transporter GLUT4 to the plasma membrane, and subsequently the enhancement of glucose uptake[248].

The direct interference of HCV with the insulin signaling cascade is experimentally documented in several studies[250]. Also, in patients with chronic hepatitis C, direct interactions between HCV and insulin signaling components occur and may result in insulin resistance, which in turn, may progress to T2D in at-risk individuals. In the transgenic mouse model[149], the core-encoding region of HCV is sufficient to induce IR. This effect was reversed by treatment with anti-TNF-antibodies, which suggested an increased level of serine phosphorylation of IRS-1 as induced by TNF-α. Thus, the core protein may induce IR indirectly *via* stimulation of the secretion of TNF-α. However, *in vitro* models suggest a direct interaction of the core protein with the insulin signaling pathway. An increased proteasome degradation of the IRS-1 and -2 *via* the activation of the SOCS-3[251]. Also, a genotype-specific mechanisms, in which down-regulation of peroxisome proliferator-activated receptor γ and up-regulation of SOCS-7 was observed in cells transfected with the core protein of genotype 3[252]. A schematic view suggests HCV-mediated pathways leading to insulin resistance during the course of HCV infection is shown in Figure 8.

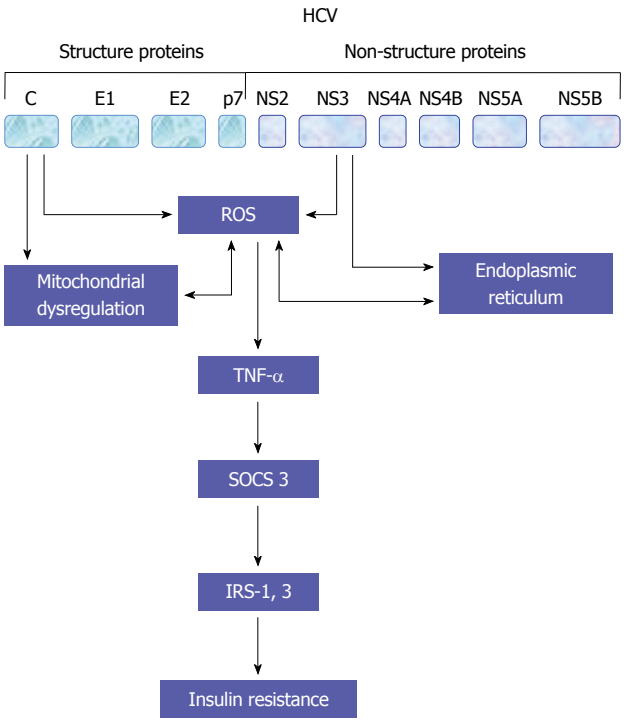

Figure 8 A proposed view for molecular mechanisms, which are involved in the modulation of hepatitis C virus-mediated insulin resistance during the course of hepatitis C virus infection. TNF: Tumor necrosis factor; HCV: Hepatitis C virus; SOCS: Suppressor of cytokine signaling; ROS: Reactive oxygen species; STAT: Signal transducers and activators of transcription; IRS: Insulin receptor substrates.

CONCLUSION

The replication cycle of HCV is host-dependent processes that require the intracellular signal transduction pathways of target cells to govern the nuclear factors, which are essential for the promotion of both transcriptional and translational mechanisms of the viral genome. Although the role of extracellular processes in the modulation of etiopathogenesis of HCV infection is not completely described, the role of intracellular signal transduction processes in the modulation of HCV-host interactions is established and seem to be the main actor in the regulation of HCV-associated both liver diseases and extrahepatic manifestations. The alteration of the physiological status of the intracellular signal transduction processes in response to their interaction with HCV viral proteins is thought to be responsible for the cause of the severe complications of chronic HCV infection including liver disease (e.g., hepatitis, fibrosis, cirrhosis and HCC) and extrahepatic manifestations (e.g., dermatologic, rheumatologic, neurologic, and nephrologic completions; and diabetes; arterial hypertension; autoantibodies and cryglobulins).

Thus, the knowledge obtained from the functional analysis of the intracellular signal transduction processes using an HCV artificial cellular systems may help to identify a new therapeutic target for the treatment of chronic HCV infection and diseases.

Alterations in cellular proteins and their regulation during HCV infection are clearly involved in the development and progression of HCV-associated both hepatic and extrahepatic diseases.

Both host lipid metabolic and very low-density lipoproteins (VLDL) pathways play a central role in the regulation of different viral processes including replication, assembly, secretion and entry. Thus, the association of HCV with VLDL is thought to be a virus strategy

to evade host immune defense by masking the putative antigenic moieties from immune recognition. The understanding of the mechanistic details underlying the interactions between viral and host lipid metabolic pathways will help to identify potential host cell factors that may be required for HCV and the infectious processes and thereby gives the opportunity to design a potential therapeutic approach in order to eradicate HCV infection, and to decrease lipid metabolism-associated extrahepatic manifestation during the course of HCV.

The infection with HCV is more likely to favor IR in response to the accumulation of the viral proteins including core, NS3 and NS5A. However, the induction of IR by HCV infection is not merely because of glucose imbalances rather it involves upregulation of the gluconeogenic and lipogenic genes that promote glucose intolerance and progresses towards IR, a step towards HCC.

As recognized, the development of HCC is mostly associated with activation of different signaling pathways including Ras/Raf/MAP kinase, cyclin/cyclin-dependent kinase and wnt-1 pathways. The Constitutive expression of HCV viral proteins including core and NS3 results in a high basal activity of MAP kinase pathway and thereby potentiates hepatocyte transformation as well as the regulation of HGF, senescence and differentiation.

Also, HCV core protein can modulate the expression of cyclin-dependent inhibitor p21, which is considered the major target of p53 and triggers mainly the activities of cyclin/cyclin-dependent kinase complexes, which is implicated in cell-cycle control and tumor formation.

Moreover, the transcriptional upregulation of both Wnt-1 and its downstream target WISP-2 by HCV core protein suggested a possible role for Wnt-1 pathways in the modulation of HCV core and NS5A proteins in the development of HCC.

The functional analysis of JAK signal transduction pathway in the context of HCV-host interactions opened a new research option for a better understanding the mechanisms of HCV resistance to IFN-α therapy. JAK pathway is known to be the principal signaling pathway for IFN-α. Therefore, the inhibition of this pathway by viral proteins or by the reduction IFN-α receptors in response to the accumulation of both HCV structural (C and E2) and non-structural protein (NS5A) proteins may contribute to the resistant mechanisms of HCV to IFN therapy. Besides the negative effect on the virological response to IFN-α treatment, the reduction of IFN-α receptors plays a central role in the suppression of IFN-α-mediated activation of STAT1-3 and subsequently augments viral replication, inflammation and fibrosis during the course of HCV infection.

Therefore, the better understanding of the molecular mechanisms, which are involved in the regulation of virus-host cell interactions may help to develop a new therapeutic strategies. These therapeutic strategies may help to decrease or even to inhibit HCV-associated both hepatic and extrahepatic diseases, and make IFN-α therapy more effective in HCV-infected patients.

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OBSERVATION

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Would cancer stem cells affect the future investment in stem cell therapy?

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Abstract

The common goal within the overwhelming interests in stem cell research is to safely translate the science to patients. Although there are various methods by which this goal can be reached, this editorial emphasizes the safety of mesenchymal stem cell (MSC) transplant and possible confounds by the growing information on cancer stem cells (CSCs). There are several ongoing clinical trials with MSCs and their interactions with CSCs need to be examined. The rapid knowledge on MSCs and CSCs has now collided with regards to the safe treatment of MSCs. The information discussed on MSCs can be extrapolated to other stem cells with similar phenotype and functions such as placenta stem cells. MSCs are attractive for cell therapy, mainly due to reduced ethical concerns, ease in expansion and reduced ability to be transformed. Also, MSCs can exert both immune suppressor and tissue regeneration simultaneously. It is expected that any clinical trial with MSCs will take precaution to ensure that the cells are not transformed. However, going forward, the different centers should be aware that MSCs might undergo oncogenic events, especially as undifferentiated cells or early differentiated cells. Another major concern for MSC therapy is their ability to promote tumor growth and perhaps, to protect CSCs by altered immune responses. These issues are discussed in light of a large number of undiagnosed cancers.

Key words: Mesenchymal stem cells; Cancer stem cells; Cytokines; Regulatory T-cells; Breast cancer; Major histocompatibility antigen

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INTRODUCTION

There are ongoing experimental studies and clinical trials to use stem cells, including mesenchymal stem cells (MSCs), to deliver drugs to tumors[1-7]. There are several advantages of using MSCs but mostly, their ability to be delivered across allogeneic barrier[8]. MSCs can be available as "off-the-shelf" source for immediate delivery and this is mostly due to the cells' immune suppressor functions[9]. However, a subset of MSCs expresses the major histocompatibility antigen, which can mediate the cells' ability to function as antigen presenting cells, and also to cross-present antigens[10-12]. These immune functions of

MSCs could also compromise their safety. However, this editorial is focused on the potential confound of cancer stem cells (CSCs) and will therefore not discuss possible confounds of MSCs in the event of switch to immune enhancer role. Similarly, although the literature indicates that MSCs, unlike embryonic stem cells, are less likely to form tumors, this topic will not be focused in this editorial, although it is an important issue for safety of MSCs as these stem cells move towards the clinic.

CSCs

The definition of CSC is not different from any other stem cells. Thus, it is expected that a CSC will be present at low frequency, self-renew and initiate tumors[13]. The reports indicate that CSCs could be radioresistant[14]. Unlike the detailed hierarchy for hematopoietic stem cells (HSCs), similar steps in lineage commitment have not been elucidated for other stem cells, including CSCs. This gap in the field of stem cell is however not a surprise since the field is considered as relatively new, as compared to the HSCs, which has been studied for decades. To explain the complexity going forward, to develop a detailed hierarchy of stem cells, we can take a look at the HSCs after decades of research. Additional information is evolving on these stem cells, indicating that the mapping of lineages continues to be a subject in progress[15]. Thus, therapies will be developed in the realm of unresolved science.

Tumor initiating cells have been traditionally referred as dormant cancer cells, which are considered as those responsible for cancer resurgence[13,16,17]. There are arguments if CSCs differ from tumor initiating cells[18]. Since a stem cell should be able to repopulate a system and self-renew, it could be argued that a tumor initiating cell, which can repopulate a system, will not be a stem cell unless the cell can self-renew. Regardless, as the field of CSCs begin to develop into a hierarchy, the field of stem cell therapy will benefit because decisions could be made on which cancer cells will be affected by other stem cells, such as MSCs[19,20]. This point is important because MSCs, which are currently in clinical trials[21-23], can support and also protect cancer growth[19,20,24].

The major problem that is envisioned for stem cell therapy is linked to undiagnosed cancers. This population represents about 30% of middle aged subjects[25]. As would be expected, stem cell treatment would be likely indicated for a similar cohort of individuals who would be considered as middle-age. The delivery of stem cells to older and middle-aged individuals is particularly important since MSCs could support tumor growth as well as to protect the tumor cells from immune clearance[19,20]. Going forward, to ensure the safety of MSC treatments, it will be paramount to encourage scientific studies to understand how the delivery of stem cells might affect the undiagnosed cancers. Such studies will require indepth experimental analyses.

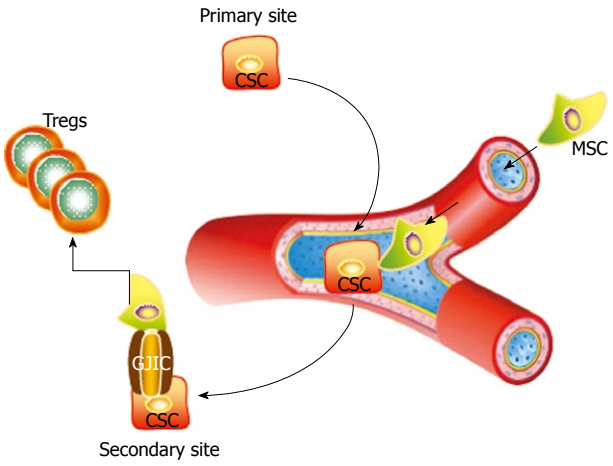

Figure 1 The cartoon shows cancer stem cells at the primary site where the tumor was developed and the cell moving to a secondary site through the blood circulation. In a scenario where mesenchymal stem cells (MSCs) are given to the subject, the cancer stem cell (CSC) can form gap junctional intercellular communication (GJIC) with the MSCs and/or can suppress the immune system through mechanism such as expansion of regulatory T-cells (Tregs). GJIC can facilitate the exchange of small microRNA between the cancer stem cell and MSCs.

MSC-CSC INTERACTION

A difficult question to be answered is to predict whether stem cell therapy should be designed with strategies to retain the dormant cancer cells in their quiescent phase or to also be prepared to treat the cancer if the stem cell treatment induced tumor growth and metastasis. Answers to this question are not simple because MSCs have varied effects on tumors, including the ability to maintain a quiescent phenotype or to support tumor growth[19,20,24]. There is also an ethical issue if patients with a history of cancer should be disqualified for a treatment in which he or she could benefit.

MSCs are suggested and also in ongoing clinical trial to directly deliver drugs to target tumors[26]. The main disadvantage of using MSCs for this purpose is their ability to support cancer growth and progression[24,27]. While the use of MSCs for targeted therapy is an excellent method, the approach could affect the CSCs, through direct interaction and also through their immune suppressive effect[19,24]. It is expected that the drugs will target the rapidly dividing cancer cells. However, if the drugs cannot target the stem cell subset, the MSCs could protect these cells to encourage dormancy.

Perhaps MSCs could be protective by inducing and expanding immune suppressor regulatory T-cells and/or to deliver small RNA either through the formation of gap junctions or through exosomes[28,29]. There are reports to support the presence of small RNA within exosomes released from tumor cells[30,31]. In general, stem cells are not metabolically active cells and are therefore expected to be "rich" sources of RNA that could be delivered to the tumor cells. CSCs could benefit from the close location of MSCs to establish gap junctional inter-

cellular communication, which can facilitate the passage of small microRNA that benefits a dormant phase of the cancer cell[29,32].

CONCLUSION

This editorial highlights the issue of CSCs as a serious consideration when MSCs and other similar cells are used in therapy. Shown in Figure 1 is a CSC from the primary site migrating through the circulation to a secondary site. If the CSC contact a MSC, this can lead to the formation of gap junctional intercellular communication for the exchange of small microRNA[29] and/or can prevent immune target by inducing the expansion of regulatory T-cells.

Dormancy of cancer has been described, although it is yet to be determined if the CSCs are responsible for a dormant phase. Regardless, robust research is required to understand how stem cells such as MSCs affect pre-existing CSCs and their progenies. There is intense research to develop methods to prevent the transition of dormant cancer cells into rapidly growing cells. However, these strategies are proposing to retain the dormant cells in the individuals. Perhaps this is a good strategy since 30% of individuals have undiagnosed occult cancer. On the other hand, this could be a problem if transplanted MSCs could support the transition of the dormant cancer cells into aggressively dividing cells. It is therefore a serious consideration to carefully review the issues on CSCs and MSCs for safe treatment.

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ORIGINAL ARTICLE

Standardizing a simpler, more sensitive and accurate tail bleeding assay in mice

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Abstract

AIM: To optimize the experimental protocols for a simple, sensitive and accurate bleeding assay.

METHODS: Bleeding assay was performed in mice by tail tip amputation, immersing the tail in saline at 37 ℃, continuously monitoring bleeding patterns and measuring bleeding volume from changes in the body weight. Sensitivity and extent of variation of bleeding time and bleeding volume were compared in mice treated with the P2Y receptor inhibitor prasugrel at various doses or in mice deficient of FcRγ, a signaling protein of the glycoprotein VI receptor.

RESULTS: We described details of the bleeding assay with the aim of standardizing this commonly used assay. The bleeding assay detailed here was simple to operate and permitted continuous monitoring of bleeding pattern and detection of re-bleeding. We also reported a simple and accurate way of quantifying bleeding volume from changes in the body weight, which correlated well with chemical assay of hemoglobin levels (r^2 = 0.990, P < 0.0001). We determined by tail bleeding assay the dose-effect relation of the anti-platelet drug prasugrel from 0.015 to 5 mg/kg. Our results showed that the correlation of bleeding time and volume was unsatisfactory and that compared with the bleeding time, bleeding volume was more sensitive in detecting a partial inhibition of platelet's haemostatic activity (P < 0.01). Similarly, in mice with genetic disruption of FcRγ as a signaling molecule of P-selectin glycoprotein ligand-1 leading to platelet dysfunction, both increased bleeding volume and repeated bleeding pattern defined the phenotype of the knockout mice better than that of a prolonged bleeding time.

CONCLUSION: Determination of bleeding pattern and bleeding volume, in addition to bleeding time, improved the sensitivity and accuracy of this assay, particularly when platelet function is partially inhibited.

Key words: Mouse or mice; Tail bleeding assay; Prasugrel; Platelets; Hemostasis; FcRγ

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INTRODUCTION

Bleeding assay is widely used as an *in vivo* assessment of

haemostatic action of platelets in rodents, particularly in genetically modified mice or following treatment with anti-platelet drugs[1]. However, the protocol of the bleeding assay varies considerably among laboratories[2]. This assay involves a longitudinal incision or transverse amputation of the tip of the tail. Bleeding time is largely determined by the interaction between platelets and damaged vessel wall leading to hemostatic plug formation. The bleeding monitoring is usually conducted at room temperature either by blotting with filter paper every few seconds until bleeding ceases[3-6] or dipping the tail into tubes in a preset time-frame to determine the duration of bleeding[7,8]. Bleeding time is usually defined as the time of the first cessation of bleeding although recurrence of bleeding is known to occur[9], and accumulated bleeding time is also commonly used[9-11]. It is also common to immerse the tail in isotonic solution at 37 ℃[9,12-17].

Whilst the current protocols permit a valid *in vivo* bleeding assay, significant limitations exist. Notably, the within-group variation is usually rather large[3-5,12,14-21], making this assay less sensitive in identifying between-group differences. Further, the amount of bleeding was not determined in a majority of studies apparently due to the notion that bleeding time itself is sufficient. However, we recently observed in mice treated with clopidogrel at different doses that while bleeding time was similar, bleeding volume differed significantly between groups, indicating that bleeding time alone was insufficient in differentiating the dose-dependent effect of anti-platelet interventions. Thus, it is necessary to improve and standardize the bleeding assay method[2]. For this purpose, we described here our experimental details of the assay for assessment of (1) dose-dependent effect of an anti-platelet drug prasugrel and (2) phenotype of FcRγ knockout (FcRγ$^{-/-}$) mice with controversy reports on bleeding time[5,20]. In comparison with bleeding time measurement only, our method does show improved accuracy and sensitivity and yet simple to operate without requiring special equipment.

MATERIALS AND METHODS

Animals and drug treatment

Male C57Bl/6 mice at 12-17 wk of age were used. We also studied bleeding time and volume in FcRγ$^{-/-}$ mice (in C57Bl/6 background) a GPVI receptor signaling molecule. Previous studies showed that FcRγ$^{-/-}$ mice either had unchanged or prolonged bleeding time[4,18]. FcRγ$^{-/-}$ mice were kindly provided by Prof. Shaun Jackson (Australian Blood Disease Centre). All experimental procedures were approved by a local animal ethics committee and were in accordance with the Australian code of practice for the care and use of animals for scientific purposes as described by the National Health and Medical Research Council of Australia.

Using the bleeding assay, the dose-dependent effect of the purinergic receptor antagonist prasugrel was studied. Prasugrel tablets of 5 mg each (Eli Lilly, United States) were crushed to fine power, suspended in 10% methyl cellulous and given orally by daily gavage (0.2 mL/mouse) for 3 d. Six doses of prasugrel were tested at 0.015, 0.05, 0.15, 0.5, 1.5 and 5 mg/kg per day, respectively. This range of dosages was determined according to previous studies at a high dose of 5 mg/kg in mice[10,22,23] and clinically relevant doses (5-10 mg/d per person) of ≤ 0.15 mg/kg[24]. Bleeding assay was conducted on day-3 at 5-6 h after the last dose. Control mice received no treatment. For positive control, thrombocytopenia was induced in one group of mice (n = 6) by treatment with CD41 antibody (BD Biosciences) injection daily at 0.5 mg/kg (ip) for 3 doses[10]. This regime reduces circulating platelets by 90%[25].

Tail bleeding assay

Animals were anesthetized with a mixture of ketamine, xylazine and atropine (at 100, 10 and 1.2 mg/kg, respectively) and body weight (accurate to mg) was obtained. Animals were placed in prone position. A distal 10-mm segment of the tail was amputated with a scalpel. The tail was immediately immersed in a 50-mL Falcon tube containing isotonic saline pre-warmed in a water bath to 37 ℃ (Figure 1A). The position of the tail was vertical with the tip positioned about 2 cm below the body horizon. Each animal was monitored for 20 min even if bleeding ceased, in order to detect any re-bleeding. Bleeding time was determined using a stop clock. If bleeding on/off cycles occurred, the sum of bleeding times within the 20-min period was used. The experiment was terminated at the end of 20 min to avoid lethality during the experiment as required by the local animal ethics committee. Body weight, including the tail tip, was measured again, and the volume of blood loss during the experimental period was estimated from the reduction in body weight. At the end of experiment, animals were killed by anesthesia overdose.

Hemoglobin assay

To validate the accuracy of measurement of bleeding volume by changes in the body weight, in a separate batch of mice, blood cells were separated by centrifuge at 4000 r/min for 5 min at room temperature. The supernatant was removed and erythrocytes were re-suspended in 2 mL of lysis buffer (BD Pharm Lyse). After 10 min incubation in the lysis buffer, tubes were centrifuged at 10 000 r/min for 5 min. Concentrations of hemoglobin were measured spectrophotometrically using a Micro plate spectrophotometer at 550 nm (BioRad). OD readings for hemoglobin were plotted against respective changes in body weight (accurate to in mg).

Statistical analysis

Results are presented as mean ± SE or otherwise specified. Correlation analysis was performed using the least-square method. Between-group comparison was made by analysis of variance followed by the Newman-Keuls multiple-comparison test or unpaired t test. $P < 0.05$ was considered statistically significant.

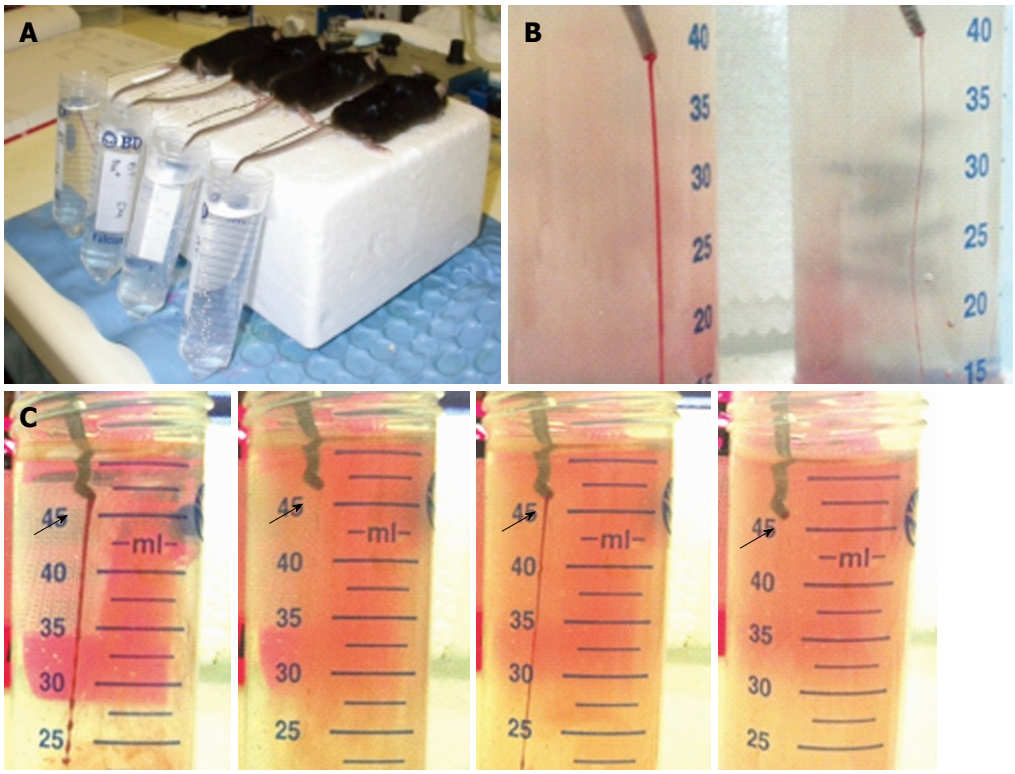

Figure 1 Setting-up of tail bleeding assay in mice. A: Bleeding assay was performed by amputation of the tail tip which was immediately merged in saline (37℃); B: Photo showing difference in the thickness of blood streams in animals receiving prasugrel at 5 (left) or 0.5 (right) mg/kg per day; C: Starting/stopping bleeding cycles (arrows) observed in a FcRγ$^{-/-}$ mouse.

RESULTS

Body weights ranged from 23 to 29 g and the averaged group means were similar among the groups (24 to 26 g). In our experimental setting, untreated mice had an average bleeding time of 2 min and bleeding volume of 0.05 mL. None of the control and untreated mice showed re-bleeding. Platelet depletion, induced by treatment with CD41 antibody, markedly prolonged bleeding time up to 20 min in all mice with an average bleeding volume of 1.07 mL. Difference in the severity of bleeding was easily detected based on the thickness of tail bleeding stream (Figure 1B). Re-bleeding was found in 40% of mice receiving prasugrel at doses ≤ 0.5 mg/kg and in 6/8 FcRγ$^{-/-}$ mice, and was readily visible (Figure 1C). This usually occurred with progressive thinning of the bleeding stream till complete stop for a period (usually a few minutes), followed by abrupt restart of bleeding.

An excellent correlation between changes in body weight and corresponding OD readings by spectrophotometry for hemoglobin concentrations (r^2 = 0.990, P < 0.0001, Figure 2), validating the accuracy of evaluation of bleeding volume from changes in body weight in our experimental setting.

All animals treated with prasugrel at higher doses (i.e., 1.5 or 5 mg/kg per day) had a bleeding time over 20 min and bleeding volume of 0.7 to 1.8 mL each (1.06 ± 0.22 mL, mean ± SD). In animals receiving prasugrel at medium doses (i.e., 0.5 or 0.15 mg/kg), while bleeding time was over 20 min in all animals except one in 0.15 mg/kg group (Figure 3A), bleeding volume was significantly lower compared with either the 5 mg/kg prasugrel group or PD group (Figure 3B). This was in contrast to a lack of difference in bleeding time at intermediate dosages of prasugrel (0.15 or 0.5 mg/kg, Figure 3A and B).

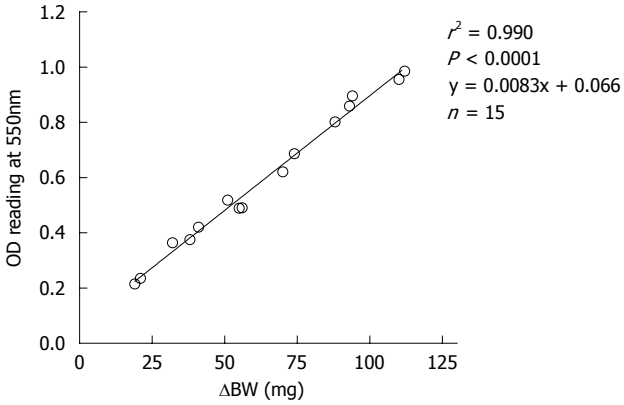

Figure 2 Correlation between changes in the body weight and OD readings for hemoglobin concentrations. Blood was collected in saline and blood cells were separated by centrifugation and erythrocytes re-suspended in lysis buffer. Concentrations of hemoglobin were measured spectrophotometrically.

Combined data from prasugrel experiment showed a significant correlation between bleeding time and bleeding volume (r^2 = 0.613, P < 0.01, y = 0.046x - 0.056). However, there was a wide range of bleeding volumes in animals with bleeding time exceeding 20 min (Figure 3C), suggesting a dissociation of bleeding time and bleeding volume

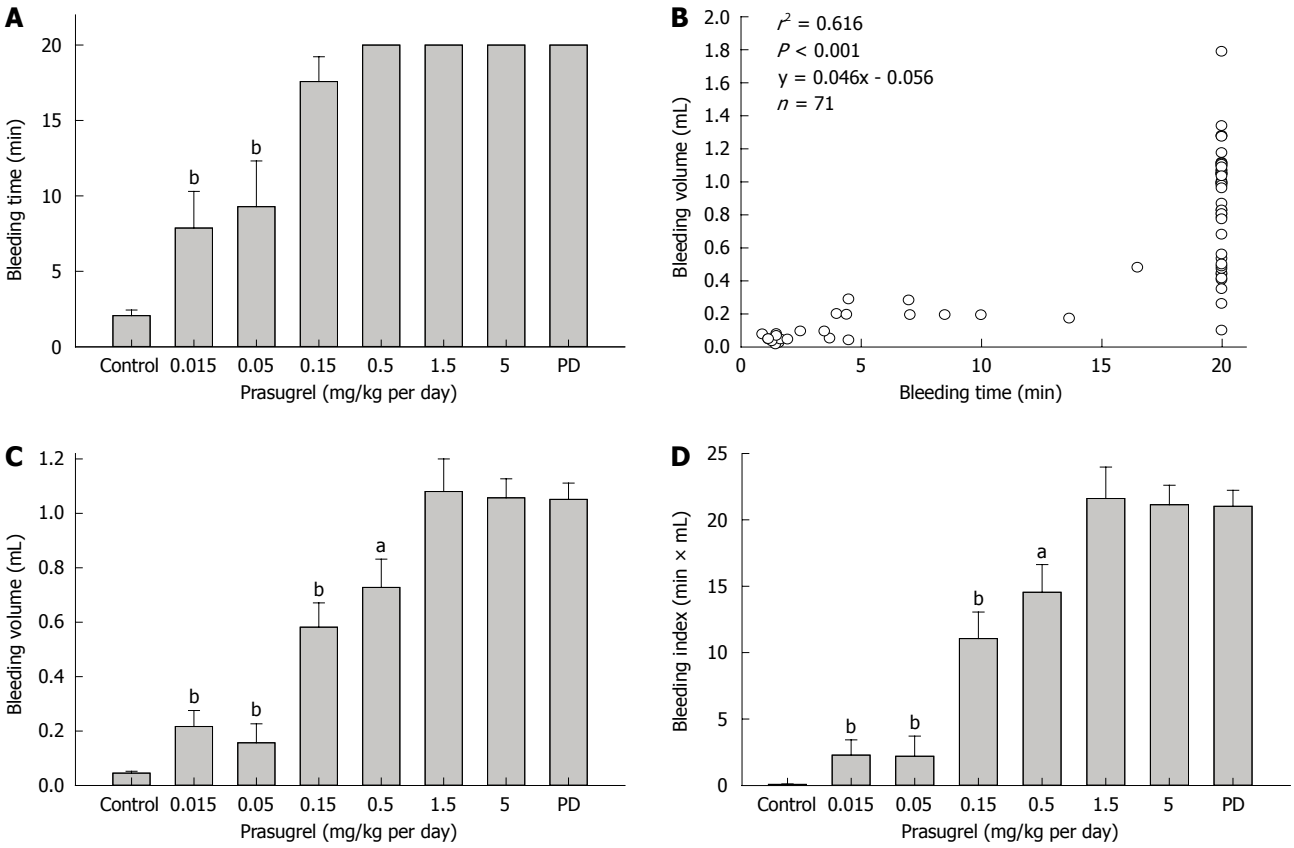

Figure 3 Dose-dependent reduction by the anti-platelet drug prasugrel on bleeding parameters. Bleeding time failed to detect dose-effect relation only at two lowest doses (0.015 and 0.05 mg/kg per day, A), whereas bleeding volume was significantly lower *vs* 5 mg/kg per day starting from 0.15 mg/kg per day (B). Panel C shows the correlation between bleeding time and volume. Note the wide variation of bleeding volume in animals with bleeding time exceeding 20 min (C). The bleeding index was derived as the product of time (min) and volume (mL, D). Parameters of all treated groups were significantly higher than the untreated control. Platelet depletion (PD) group served as a positive control. $^{a}P < 0.02$, $^{b}P < 0.001$ *vs* 5 mg/kg prasugrel group. A total of 8 groups of mice (n = 6-13) were studied.

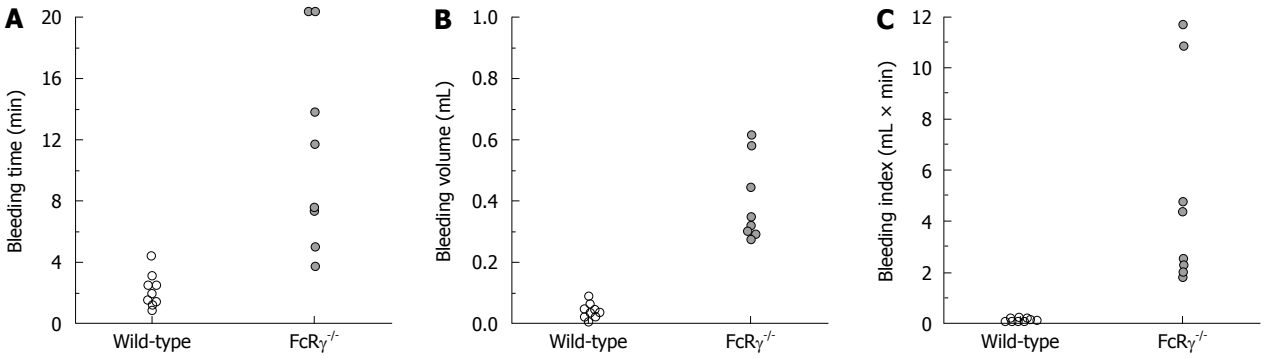

Figure 4 Tail bleeding time and volume measured in wild-type and FcRγ$^{-/-}$ mice. Bleeding assay was performed by tail tip amputation and then immersing the tail in saline at 37℃. FcRγ$^{-/-}$ mice showed a significant increase in both bleeding time and volume ($P < 0.01$ *vs* wild-type mice) and development of recurrent bleeding in 6/8 of animals studied. Note that the better separation of FcRγ$^{-/-}$ and control mice by bleeding volume or index than that by bleeding time.

during the period studied. Thus, there was virtually lack of good association between bleeding volume and time. Considering the significance of both bleeding time and volume in the bleeding assay, we calculated from this set of data "bleeding index" as the product of time (in min) and volume (in mL, Figure 3D). This index was similar to that of bleeding volume in detecting dose-dependent inhibition of hemostasis by prasugrel. However, the variation of this index is greater than that of bleeding volume.

Under our modified experimental conditions and use of the tail amputation method, two FcRγ$^{-/-}$ mice had continuous bleeding up to 20 min and the rest of 6 mice showed recurrent bleeding episodes of 3 to 6 times within the 20 min period (Figure 4A). Bleeding volume in FcRγ$^{-/-}$ mice was significantly greater than that of wild-type controls (Figure 4B). This phenotype of FcRγ$^{-/-}$ mice was identified better by bleeding volume than by bleeding time.

DISCUSSION

The bleeding assay is a widely used test to explore hemostatic function of platelets. However, the details of the assay method have never been standardized. In the current study, we performed the assay by transecting the tip of the tail, immersing the tail in 37 ℃ saline, continuous monitoring of bleeding pattern, and determining bleeding volume from changes in body weight. Our results suggest that the details applied in the assay yielded a simple and sensitive determination of a partially inhibited hemostatic function of platelets. We emphasize that rather than describing novel techniques, by providing our experimental details we aimed to contribute to standardizing this *in vivo* assay.

The reported methods of estimating bleeding volume involve collection of blood into a fixed volume of saline and measurement of concentrations of hemoglobin spectrophotometrically or quantification of red blood cell density[9,11,17,18]. We found that loss of the body weight at the end of the assay was a valid approach to determining bleeding volume. During the 20-min experimental period, there was no loss of body weight due to excreting urine or feces. While increase in weights of saline containing tubes should also reflect the amount of blood loss, we found inconsistency of changes in weights of Falcon tubes containing 50 mL saline and pre-soaked in water bath, apparently due to evaporation of surface moisture, water loss due to trace amount left with the cap or attached to mouse tail. By measuring bleeding time and volume, we tested the effect of prasugrel over a wide range of dosages. The bleeding time was able to show a dose-dependent inhibition of platelet activity only at high doses. A significant but partial inhibition by prasugrel at medium doses (e.g., 0.5 and 0.15 mg/kg per day) was detected by a reduced bleeding volume without concomitant change in bleeding time. Further, those animals with bleeding time over 20 min showed a considerable variation in the bleeding volume over the assay period, indicating the limitation of bleeding time as a hemostatic index. By comparison with the bleeding time alone, our results show that either bleeding volume or bleeding index is more sensitive in detecting prasugrel dose-related inhibition of platelet function. This view is in keeping with a recent study showing a significant increase in the bleeding volume in mice deficient of Rab27b while bleeding time was found unreliable[26].

Johansen *et al*[11] described an automated system that included a vision camera and specially designed software for monitoring bleeding time. Volume of blood loss was derived from measurement of haemoglobin concentration[11]. Such a set-up represents an accurate bleeding assay for detection of multiple cycles of bleeding as well as blood loss, which is desirable to laboratory practice. In our setting, we visually and easily observed recurrence of bleeding in mice with either genetic (FcRγ$^{-/-}$) or pharmacological (prasugrel) intervention leading to partial inhibition of platelet activity. Re-bleeding pattern is mostly likely due to some residual capability in these animals in formation of platelet thrombi to stop bleeding. However, a partial inhibition of platelets renders an unstable structure and poor adhesion to vessel wall of thrombi that can be washed away under arterial blood flow, similar to that described by Folts as a cyclic variation of blood flow of a stenotic artery with endothelial damage[27].

The location and the way of inducing tail vascular injury are also critical for the outcomes of the assay. Whereas Sarratt *et al*[20] reported a prolonged but variable bleeding time in FcRγ$^{-/-}$ mice with tail amputation, Mangin *et al*[5] observed no change in the bleeding time in FcRγ$^{-/-}$ mice using a longitudinal cutting method. Our finding of a prolonged bleeding time in FcRγ$^{-/-}$ mice using the amputation method was consistent with a previous report[20]. Further, we documented a significant increase in the bleeding volume in FcRγ$^{-/-}$ mice. Our findings in FcRγ$^{-/-}$ mice clearly indicate a defect in GPVI signaling and hemostatic action of platelets, as one would have expected[24,28,29]. One potential variable of the bleeding assay comes from different ambient environment by either exposing the tip of the tail to room temperature and air (causing dryness) or immersing the tail in 37 ℃ saline. Dejana *et al*[12] determined the tail blood flow by radioisotope labeled microsphere method and found a 3-fold higher tail flow under 37 ℃ *vs* that under the room temperature. However, bleeding time was actually shorter at 37 ℃ than that at 23 ℃[12]. Thus, to prevent peripheral hypothermia and resultant vasoconstriction, a regional temperature of 37 ℃ and moisture are the optimal environment for hemostatic testing relative to that of room temperature and dryness. Other factors potentially affecting the assay might include body temperature, type of anesthetics used and hemodynamic conditions of animals. However, we observed in a previous study that there was no difference in bleeding time between mice with and without myocardial infarction[10].

Whereas tail bleeding is a useful and commonly used *in vivo* assay without requirement for specialized equipment, there is a need to standardize the details of the assay. We recommend the following: First, tail amputation appears better than longitudinal tail incision. Second, instead of exposure to the air and room temperature, it is recommended to submerge the amputated tail in saline at 37 ℃. In addition to keeping constant temperature and moisture at the cut surface, this also allows for continuous monitoring of bleeding stream without disturbance of the wound. Third, visual monitoring of bleeding should continue even after bleeding ceases for likely re-bleeding, a phenomenon that is common when hemostatic action of platelets is partially inhibited[29-31]. Fourth, the volume of bleeding can be easily determined by change in body weight at the end of the assay. Whilst "bleeding index" derived from our two sets of data does not appear to provide further information than that by the bleeding volume, this index takes into consideration of both bleeding time and volume.

In conclusion, the details of bleeding assay described

here are easy to perform and allow for detection of recurrent bleeding and bleeding volume, both parameters being useful in assessment of the haemostatic action of platelets.

COMMENTS

Background

Determination of haemeostatic activity of platelets *in vivo* is important for phenotypic determination of genetically altered mice or assessment of anti-platelet drugs. Tail bleeding assay has been widely adopted for this purpose with the bleeding time as the most commonly used parameter. This index, however, suffers from limitations of a large individual variation making this assay an insensitive one based on many reports. Furthermore there is considerable inconsistency among laboratories in performing the assay rendering data comparison difficult.

Research frontiers

There is an urgent need of standardizing and optimizing the current method of bleeding assay. In the current study, effort was given to establish a simple protocol for this assay and to compare bleeding time and bleeding volume as separate measures.

Innovations and breakthroughs

This paper provides detailed description of the tail bleeding assay by continuously monitoring bleeding pattern and measurement of bleeding volume. The results from drug dose-effect determination and from a genetically modified mouse strain all showed improvement in sensitivity and reduction in individual variation using the method described and by determination of the bleeding volume.

Applications

The detailed description of the assay method in this paper would be helpful towards standardizing and optimizing the tail bleeding assay. *In vivo* determination of the anti-platelet agent prasugrel using this assay provides useful reference information for future studies testing this class of agents in mice. The authors provide explanation for contradictory reports by different groups on bleeding assay of FcRγ knockout mice.

Terminology

The genetically modified mouse strains have been widely used for research on platelet activity. Mice are also commonly used for assessment of anti-platelet drugs. The only *in vivo* measure of platelet activity is tail bleeding assay. The study would contribute to the optimization and standardization of this assay.

Peer review

This study addresses an area of interest with obvious importance to the hematologists. The manuscript presents an interesting assay of platelet haemostatic activity in mice. It is clearly written.

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Fungal Pathogens: From Basic Biology to Drug
Santa Fe, NM, United States

January 20-20, 2012
Exploiting Bacteriophages for Bioscience, Biotechnology and Medicine
London, United Kingdom

January 22-27, 2012
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Ventura, CA, United States

February 7-12, 2012
Gene Silencing by Small RNAs
Vancouver, British Columbia, Canada

March 4 -10, 2012
Malaria Experimental Genetics
Hinxton, Cambridge, United Kingdom

March 12-13, 2012
2nd Annual International Conference on BioInformatics and Computational Biology Special Track: Stem Cell Research
Singapore

March 12-13, 2012
BICB 2012: 2nd Annual International Conference on BioInformatics and Computational Biology (updated)
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March 31-April 3, 2012
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Electron transfer at the microbe-mineral interface
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6th Broadening Microbiology Horizons in Biomedical Science Meeting
Stratford-Upon-Avon, United Kingdom

May 6-12, 2012
4th ASM Conference on Prokaryotic Cell Biology and Development
Montreal, Canada

May 7-19, 2012
Bioinformatics and comparative genomes analyses
Napoli, Italy

May 8 - 10, 2012
Exploring Human Host-Microbiome Interactions in Health and Disease
Cambridge, United Kingdom

May 30-31, 2012
European Lab Automation
Hamburg, Germany

June 3-8, 2012
Anaerobes in Health and Disease; How to Isolate, Identify and Look for Resistance in a Cost-Effective Way
Szeged, Hungary

June 16-21, 2012
Gene transcription in yeast
Girona, Spain

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15th International Conference on Experimental Mechanics
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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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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/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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