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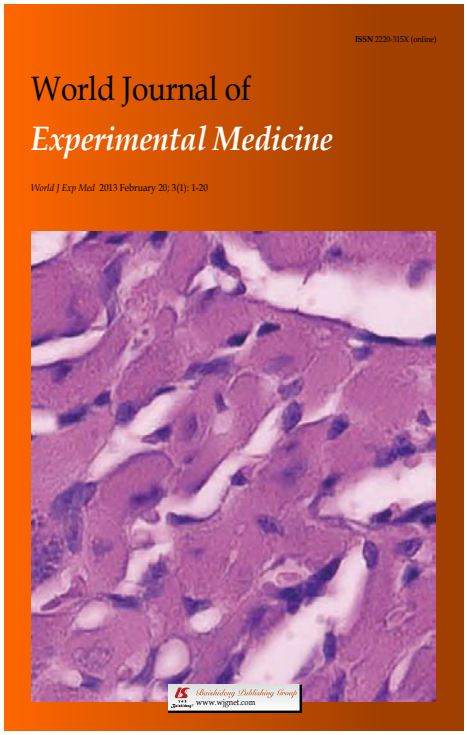

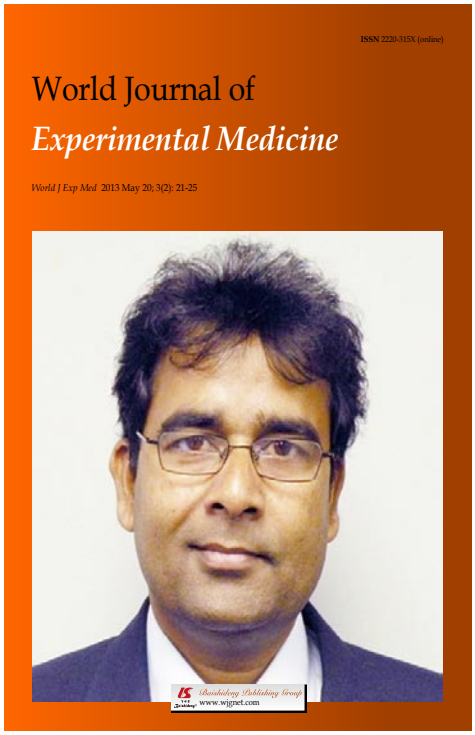

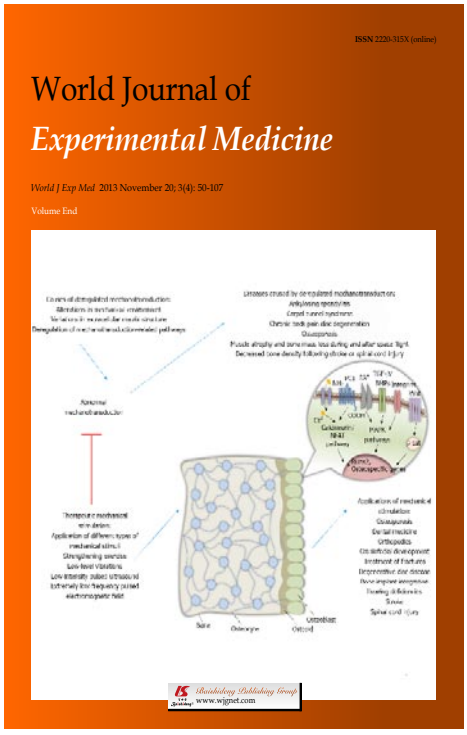

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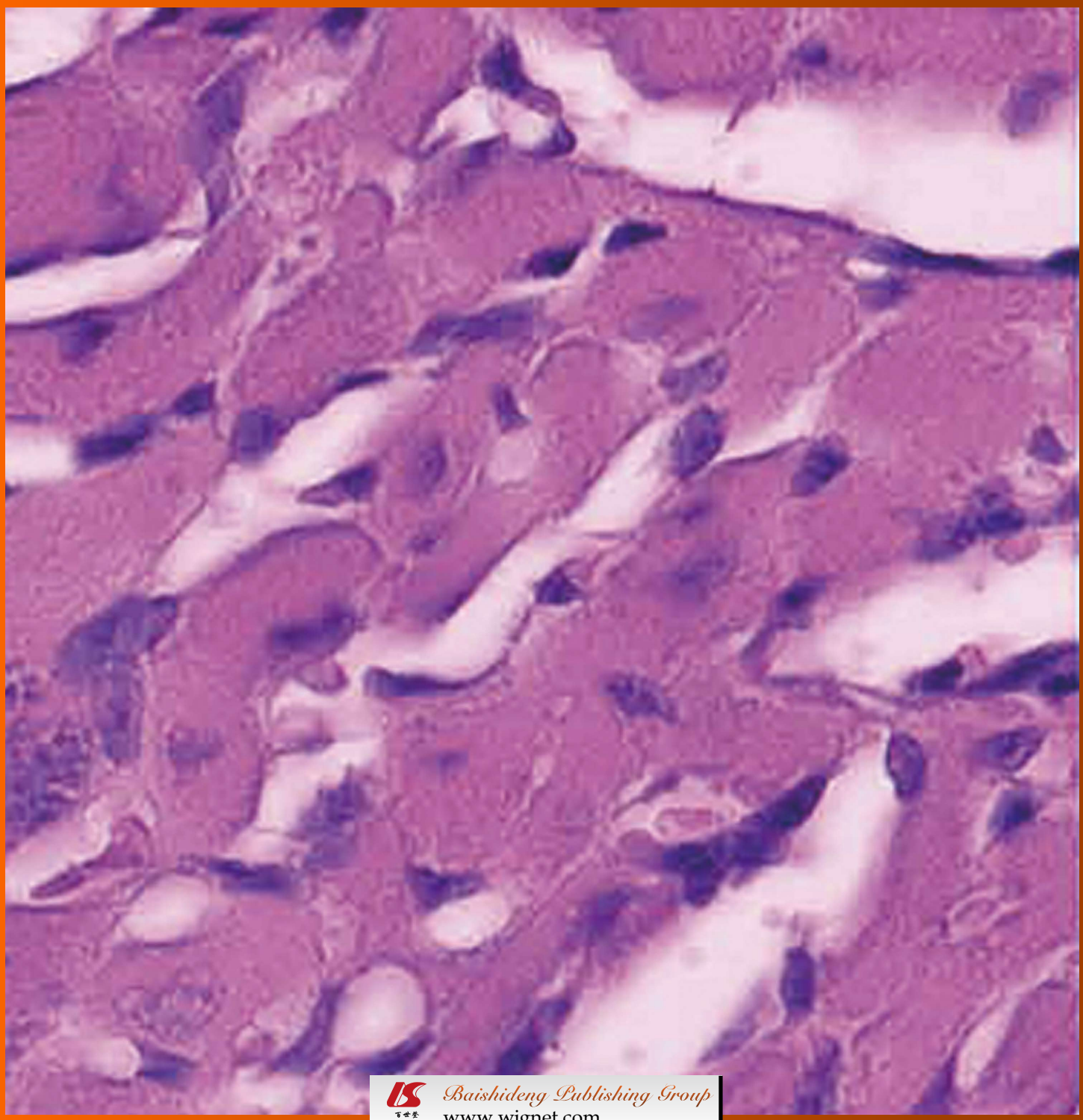

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MINIREVIEW

Modulation of immune response in experimental Chagas disease

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Abstract

Trypanosoma cruzi (*T. cruzi*), the etiological agent of Chagas disease, affects nearly 18 million people in Latin America and 90 million are at risk of infection. The parasite presents two stages of medical importance in the host, the amastigote, intracellular replicating form, and the extracellular trypomastigote, the infective form. Thus infection by *T. cruzi* induces a complex immune response that involves effectors and regulatory mechanisms. That is why control of the infection requires a strong humoral and cellular immune response; hence, the outcome of host-parasite interaction in the early stages of infection is extremely important. A critical event during this period of the infection is innate immune response, in which the macrophage's role is vital. Thus, after being phagocytized, the parasite is able to develop intracellularly; however, during later periods, these cells induce its elimination by means of toxic metabolites. In turn, as the infection progresses, adaptive immune response mechanisms are triggered through the TH1 and TH2 responses. Finally, *T. cruzi*, like other protozoa such as *Leishmania* and *Toxoplasma*, have numerous evasive mechanisms to the immune response that make it possible to spread around the host. In our Laboratory we have developed a vaccination model in mice with *Trypanosoma rangeli*, nonpathogenic to humans, which modulates the immune response to infection by *T. cruzi*, thus protecting them. Vaccinated animals showed an important innate response (modulation of NO and other metabolites, cytokines, activation of macrophages), a strong adaptive cellular response and significant increase in specific antibodies. The modulation caused early elimination of the parasites, low parasitaemia, the absence of histological lesions and high survival rates. Even though progress has been made in the knowledge of some of these mechanisms, new studies must be conducted which could target further prophylactic and therapeutic trials against *T. cruzi* infection.

Key words: *Trypanosoma cruzi*; Chagas disease; Innate and adaptive immune response

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INTRODUCTION

Trypanosoma cruzi (*T. cruzi*), the etiological agent of Chagas disease, affects nearly 2 500 000 people in Argentina and 18 million in Latin America. The parasite presents two stages of medical importance in the host, the amastigote, intracellular replicating form, and the extracellular trypomastigote, the infective form. That is why control of the infection requires a strong humoral and cellular immune response; hence, the outcome of host-parasite interaction in the early stages of infection is extremely important. In humans the disease presents different clinical and immunological periods: the acute period, characterized by the presence of trypomastigotes in the bloodstream, associated with immunosuppressive phenomena[1], which is asymptomatic in 95% of cases[2] and remits spontaneously, to enter in a second indeterminate phase, after 3

or 4 mo, which can last the rest of the host's life with no clinical signs. It is characterized by low parasitaemia and positive serology and, in later years, approximately 30% of infected people develop some degree of cardiac or digestive pathology in the chronic period of infection. This is attributed to direct action of the parasite, or to autoimmune reactions induced by *T. cruzi*. Gironès *et al*[3] critically reviewed the evidence in favour of and against autoimmunity through molecular mimicry as responsible for Chagas disease pathology from clinical, pathological and immunological perspectives. Also in this sense, Bonney *et al*[4] observed that vaccination with heat-killed *T. cruzi* induces the development of autoimmunity *via* molecular mimicry and other mechanisms and potentially fatal cardiomyopathy. Their results show that exposure to *T. cruzi* antigen alone is sufficient to induce autoimmunity and cardiac damage, yet additional immune factors, including a dominant TH1/TH17 immune response, are likely required to induce cardiac inflammation.

Immune response to *T. cruzi* is highly complex and involves many components, both effectors and regulators. The unspecific immunosupression that occurs during the first stage of the infection and *T. cruzi*'s capacity to adapt and evade this response allow it to invade cells and spread, which means that the parasite may remain indefinitely in the host's tissues because it is not completely eliminated[1].

The process in which trypomastigotes enter the host's cells involves several stages: initial parasite-cell contact, trypomastigote adhesion, early induction of immune response, which causes modifications to the membrane proteins. The parasite has been claimed to enter the host's cell using a variety of mechanisms: (1) it enters professional phagocytic cells by phagocytosis; (2) the cellular membrane emits pseudopodia, modifications are produced in the actin filaments, and protein tyrosine kinases such as PI-3 are activated; this process culminates with the formation of a parasitophorous vacuole and soon after lysosomes and endosomes are recruited[5]; (3) it enters non phagocytic cells by means of endocytosis but there is no emission of pseudopodia in the host's cell; and (4) another mechanism involves direct penetration by the parasite in the cell by means of membrane invagination, with an important intake of energy[6].

After entering, the parasite lodges in the cytoplasmic vacuolar compartment where a gradual differentiation process occurs from trypomastigote to amastigote[7,8], and the latter divide by means of binary fission, to then become trypomastigotes once again; they leave the cell to spread *via* lymph and blood, and infect other cells in which they once again go through the replication cycle. *T. cruzi* primarily infects cells belonging to the reticuloendothelial system, nerve and muscle tissue, including cardiac fibres[9].

In order to progress with regard to knowledge of the immune response set off by *T. cruzi* infection and to analyze whether it is possible to modulate this complex response, several experimental models have been developed. A model for vaccinating mice with *Trypanosoma rangeli* (*T. rangeli*), a parasite closely related to *T. cruzi*, but nonpathogenic to humans[10-12], has been designed in our laboratory[13]. *T. rangeli* shares areas of geographical distribution, epidemiological characteristics, and antigenic and immunogenic components with *T. cruzi*. Specific diagnosis becomes difficult by means of classical serological methodologies because it induces a response of crossed antibodies. Moreover, both parasites cannot be morphologically differentiated[14-16]. The antigenic similitude between *T. rangeli* and *T. cruzi* has been shown by means of different methods by numerous research groups[17-20].

T. rangeli presents an enzyme, sialidase, with neuraminidase activity which is fundamentally expressed in the epimastigote stage and, unlike *T. cruzi*, does not present transialidase. Recent studies show that the sialidase system is very complex and can take on different expressions in different strains of the parasite, owing to genetic mutations[21]. In addition, it induces a complex modulation of the immunological mechanisms of the infected vector (*Rhodnius* genus) causing a reduction in the production of soluble mediators such as nitric oxide, oxygen free radicals, and the inhibition of phagocytosis as well as humoral response, among others, which favours the development of the parasite and results in the death of the vector[11].

The strategy of vaccinating with a parasite that is nonpathogenic to humans is based on the fact that, in the event of the future development of a vaccine for human use, and accepting the role played by autoimmune mechanisms in the pathology of Chagas disease, the possible induction of auto-aggression due to vaccination must be avoided[3,4].

In our experimental model, two groups of mice were used, one vaccinated with *T. rangeli* (at least $n = 6$ in each experiment) and then challenged by *T. cruzi*, and another group of control animals ($n = 6$), which were only infected with *T. cruzi*. A fixed number of 1500 virulent parasites were used to infect and the starting time of the infection was determined.

We observed that previously vaccinated mice showed very low parasitaemia, high survival rates and an absence of histological and autoimmune lesions, while mice that were only infected showed high parasitaemia, high mortality and severe histopathological alterations in the heart, skeletal muscle, spleen and liver[13,22,23]. For histological studies, mice from each group: vaccinated with *T. rangeli* and afterward challenged with *T. cruzi* (V) ($n = 6$) and non-vaccinated but infected with *T. cruzi* (I) ($n = 6$), were killed with ether anesthesia. Hearth, spleen, liver and skeletal muscles from the quadriceps were immediately removed from each mouse, fixed in buffered, 10% formalin (pH 7.0), and embedded in paraffin wax. One-half of each organ was cut into 5-μm-thick sections, and they were stained with haematoxylin-eosin. At least 20 areas from each section were checked for parasites and histopathology under a 40-x objective in a blind study.

The Figures 1 and 2 show a representative experi-

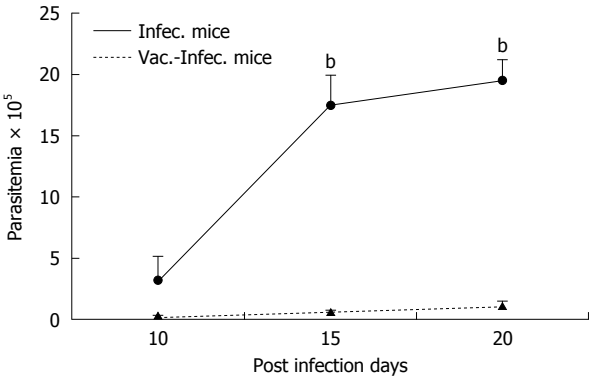

Figure 1 Parasitemia levels (geometric mean ± SE) in *Trypanosoma cruzi* infected mice (I) and in mice previously vaccinated with *Trypanosoma cruzi* and challenged with *Trypanosoma cruzi* (V-I). The differences in parasitemia levels were evaluated by *t*-test: $^{b}P < 0.001$.

ments. Similar results were obtained with two strains of *T. rangeli* from different origins, isolated in Colombia and Brazil, which revealed that the capacity to protect mice against lethal infection by *T. cruzi* is a characteristic common to different strains of *T. rangeli*. This result represents a clear advantage for the future preparation of possible vaccines for animal or human use[24].

On the other hand, it was demonstrated[25] that, in the acute period of experimentally infected mice, *T. cruzi* induces a response that presents different patterns in each different immune system compartment, splenomegaly, lymphoid subcutaneous tissue expansion, persistent polyclonal activation of lymphocyte T and B, and at the same time, thymus and mesenteric node atrophy.

A critical event during early stages of the infection is the innate immune response, in which the macrophage's role is vital. Thus, after being phagocytized, the parasite is able to develop intracellularly; however, during later periods, these same cells induce its elimination by means of toxic metabolites. In turn, as the infection progresses, adaptive immune response mechanisms are triggered through the TH1 (cellular) and TH2 (humoral) responses.

INNATE IMMUNE RESPONSE

Soluble mediators and cells

Early in the infection, *T. cruzi* induces an intense inflammatory response, which plays a crucial role in the disease's pathogenesis. In experimental models, some of the immunological events that take place during the first few hours after infection are known. Indeed, it has been observed that *T. cruzi* antigens induce activation of the natural killer (NK) cells prior to expansion of T lymphocytes[26]. During this stage the macrophages induce a cascade of cytokines: initially they produce interleukin (IL)-12, which acts on NK cells to induce the production of interferon (IFN)γ, which in turn increases the production of IL-12, tumor necrosis factor (TNF)α and NO in the macrophage, thus contributing to the elimination of the parasite[27]. At the same time, both types of cells synthesize regulatory cytokines such as IL-10 and IL-4 to reduce the harmful effects associated with excess stimulation of the immune system[28]. In very early stages of the infection, components of *T. cruzi*, including its DNA and membrane glycoconjugates, trigger the innate response through their interaction with Toll like Receptors: TLR2, TLR4 and TLR9 in macrophages and dendritic cells. After activation, both cells secrete cytokines and chemokines, and increase the expression of their co-stimulatory molecules, inducing endocytosis and intracellular death. As mentioned above, an adequate production of proinflammatory cytokines such as IFNγ, TNFα, IL-1, IL-12, IL-6 and IL-18 is essential for controlling infection by intracellular parasites[29]. Meanwhile, it has been observed that IL-17 might regulate the recruitment of inflammatory cells and the differentiation of TH1 in heart tissue[30]. Therefore, in order to resolve the *T. cruzi* infection, a balance is necessary between the immune response mediated by TH1 and by TH2[31]. TH1 cells are responsible for the production of inflammatory cytokines, while TH2 cells have an anti-inflammatory function and are involved in the antibody mediated response. IL-12 and IL-18 produced by dendritic cells and macrophages promote the development of TH1 cells that produce IFNγ, while IL-4 induce the expansion of TH2 cells and of high amounts of IL-10. As a result of this process, regulation of the cellular response occurs due to a reduction in the activation of dendritic cells and in the macrophage's microbicidal activity. In addition, IL-4 takes part in inducing transforming growth factor (TGF)β which regulates the activity of the antigen presenting cells and enhance the susceptibility of infection by *T. cruzi*[32].

In this sense, we observed in our experimental model[31,33] (n = 6 for each group and each experiment performed) that vaccinated animals had a significant increase of IL-12, down regulation of the proinflammatory cytokines, IL-6, IFNγ, TNFα, and increase of soluble TNF receptors sTNFIR and sTNFIIR, which inhibit the deletereous activity of TNFα, in accord with Camargo *et al*[27]. Also Chandrasekar *et al*[34] detected proinflammatory cytokine production (IL-6, TNF-α, IL-1β) in the myocardium of *T. cruzi* infected mice, which suggests the probable involvement of the production of these molecules *in situ* in the injury of the myocardial function. The diminished production of proinflamatory cytokines in the immunized group of our model, associated with higher survival rates, suggests that both, IL-6 and TNF-α, are probably involved in the fatal outcome of the infected mice.

The high IFN-γ production during acute *T. cruzi* infection has been also widely demonstrated[35,36]. This finding is generally associated with protective effects since IFN-γ enhances trypanocidal activity of the macrophages *via* a nitrogen oxide mediated mechanism[37,38]. In this sense, we have observed a high production of IFN-γ in both vaccinated and control experimental groups. This finding is in agreement with Reed *et al*[39], who detected high IFN-γ levels in both susceptible and resistant mice. Moreover, in our mentioned works it was observed that in immu-

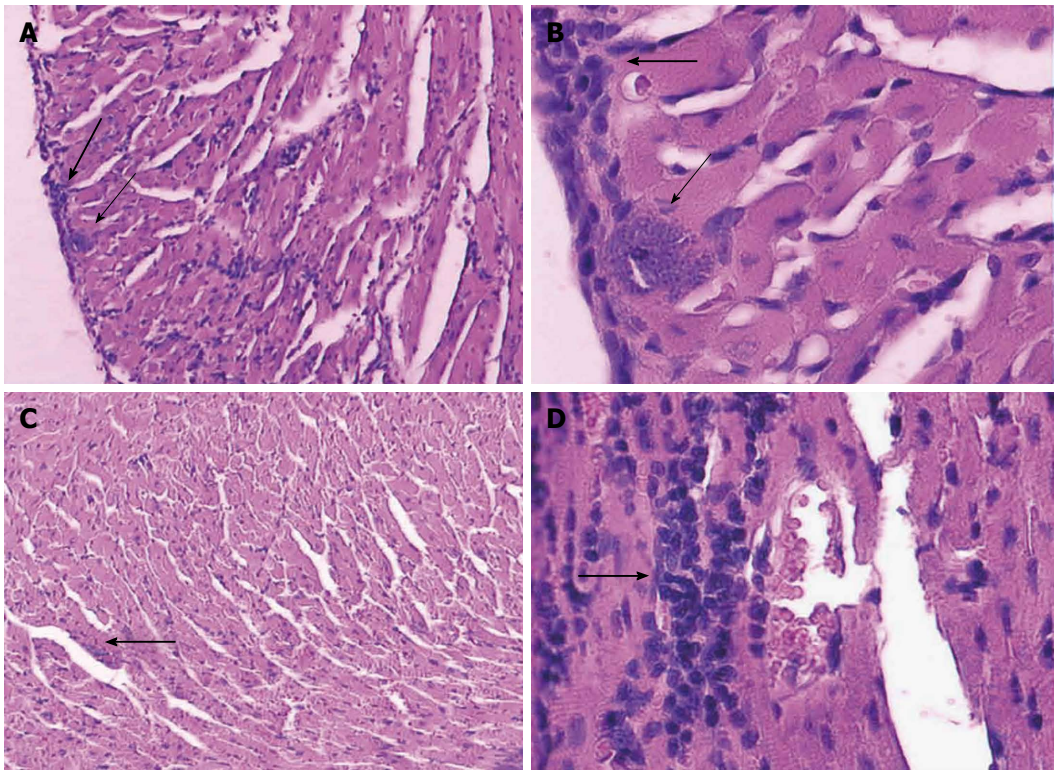

Figure 2 Histological studies: all sections were stained with hematoxylin and eosin. Histological sections of heart. A, B: Control groups show nests of amastigotes (thin arrows) and mononuclear cell infiltration (thick arrow); C, D: Representative sections from mice vaccinated with *Trypanosoma cruzi*. Infected mice show focal mononuclear cell infiltrates (thick arrows). No amastigote nest was observed (A, C: 100 ×; B, D: 400 ×).

nized animals, the ratio IFN-γ/IL-10 was greater than in the non immunized-infected mice. Taken together, these results suggest that, in vaccinated - infected animals, the action of protective IFN-γ could be more effective, without the antagonist action of IL-10. On the other hand, in all the experiments performed, the serum concentration of IL-10 correlated with parasitemia levels. These results are consistent with those of Reed *et al*[39], who detected a lower IL-10 production in resistant mice compared to the susceptible ones. Furthermore, Abrahamsohn *et al*[40] observed a lower number of parasites and higher IFN-γ production in IL-10 KO mice than in the wild type ones.

The Figure 3 shows the levels of IL-6, IL-10, IFN-γ and TNF-α in a representative experiment, in different groups of mice. From the results obtained in mice treated only with saponina adjuvant and after infected, the effect of the adjuvant alone became evident. In this group of animals a delay in the increase of parasitemia was detected, but the systemic production of IL-6 and TNF-α and the mortality rate were very similar to those in non vaccinated-infected group. This could be due to the unspecific action of the adjuvant on the immune system, which is not sufficient to help control the infection. Additionally, the results of these experiments suggest that, in this experimental model, the levels of IL-6 and TNF-α seem to be earlier markers of fatal outcome than the parasite load[33].

Likewise, the vaccinated group had very low levels of IL-10, which allowed IFNγ to maintain its protective activity, activating macrophages, essential for the elimination of parasites, unlike the control group, which showed high levels if IL-10, which blocks activation of the macrophages and their microbicidal function[31,33]. It was not possible to detect IL-4 or IL-5 in either group with the methodology used. Taken together, these results show that vaccination with *T. rangeli* made it possible to induce a profile of the cytokines different from that of the non immunized-infected mice, with a delicate balance between TH1 and TH2, suitable for overcome the infection. This modulation of the synthesis and liberation of cytokines and soluble receptors was also observed during the acute period of natural human infection[41].

On the other hand, during the process of invading the host cell, *T. cruzi* interacts with different receptors of the macrophage to induce its own phagocytosis. Different molecules of the family of Toll type receptors recognize different molecular patterns associated with bacteria, viruses, fungi and protozoa. As a result, innate immune response mechanisms and the development of the subsequent adaptive response are triggered[42].

With regard to NO, it is considered to be the most important early soluble mediator produced by immune system cells. Macrophages recognize antigen microorganisms through their different receptors (Toll-like, NLRs and RIG-like) and trigger the production of inflammatory mediators inducing the activity of the inducible Nitric Oxide synthase enzyme. This enzyme is produced by the antigen presenting cells and may inhibit the expression

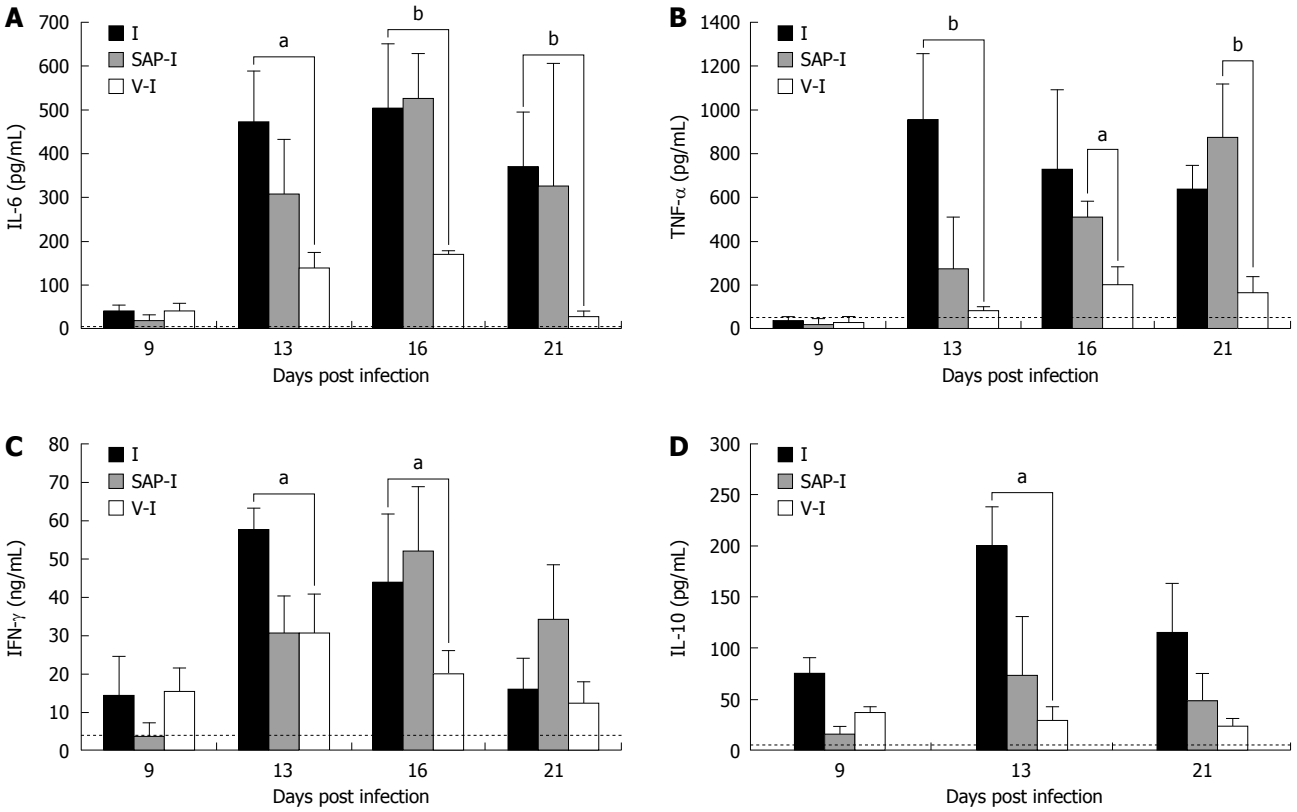

Figure 3 Kinetic of circulating cytokine levels in mice infected with *Trypanosoma cruzi* (A), in mice treated with saponin adjuvant and infected and in mice vaccinated with *Trypanosoma cruzi* and after infected (A-D). mean ± SE cytokine are represented. The area below the pointed line indicates the 95% confidence limit of each cytokine observed in plasma from uninfected mice. A: Interleukin (IL)-6; B: Tumor necrosis factor (TNF)-α; C: Interferon (IFN)-γ; D: IL-10. The differences in cytokine levels among I and SAP-I groups with respect to V-I group were evaluated by *t*-test: $^{a}P < 0.05$, $^{b}P < 0.01$.

of class II histocompatibility molecules. However, when effector cells are activated by inflammatory stimuli, important amounts of NO are synthesized, causing modifications in the cellular microenvironment[43].

At high concentrations, NO inhibits the synthesis of IL-12 and apoptosis, contributing to regulating the TH1/TH2 balance since TH1 cells are more susceptible to this process than TH2 cells[44]. In addition, this favours the proliferation of regulatory T cells during the acute experimental infection and inhibits the expression of molecules involved in adhesion and migration of cells. NO exerts its cytotoxic function on *T. cruzi*, affecting growth factors, for example by nitrosylation of the haem group, reducing the availability of iron. It is also the most important mediator in the destruction of intracellular amastigotes[45]; however, it has been shown that an excess of NO has harmful effects on the host's tissues[45,46]. In this sense, the results obtained in our studies are in agreement with these authors. In fact vaccinated animals revealed a modulation of NO levels, and the subsequent absence of lesions in the host, unlike the control group, which showed significantly increased levels of this metabolite[31].

Meanwhile, with respect to the cells, macrophages play an indispensable role in the primary response to pathogens but also take part in the resolution process of the inflammation and homeostasis of tissues. The function of macrophages is polarized towards an inflammatory or a regulatory profile, depending on the microenvironment they are in[47]. This cell population can be activated by classical way (type 1) dependent on IFNγ and TNFα, or by an alternative way (type 2) stimulated by IL-4 and IL-13[48]. Classically activated ones are currently grouped in M1, alternatively activated ones in M2a, those that polarize towards a TH2 response in M2b, and those whose stimulation is mediated by glucocorticoids and TGFβ in M2c. Therefore the different types of macrophages are involved in different response to pathogens, tumours and autoimmune diseases, showing markers exclusively associated with the function they play[49]. There is an important consumption of oxygen during the process of phagocytosis. The respiratory burst caused by macrophages and neutrophils is regarded as a powerful microbicidal mechanism. Oxygen free radicals are toxic to pathogens and prevent colonization by microorganisms in tissues. However, most of M1 macrophages' microbicidal activity is put down to NO and its derivatives like peroxynitrites. NO is produced by activation of iSON, whose substrate is L-arginine. In macrophages, this enzyme is induced by proinflammatory cytokines like TNFα, IFNγ and IL-12.

During the acute phase of the infection by *T. cruzi* and other protozoa like *Leishmania sp*[50] and *Plasmodium sp,* induction to the inflammatory response is necessary to be able to control parasitaemia[51]. However, if the classi-

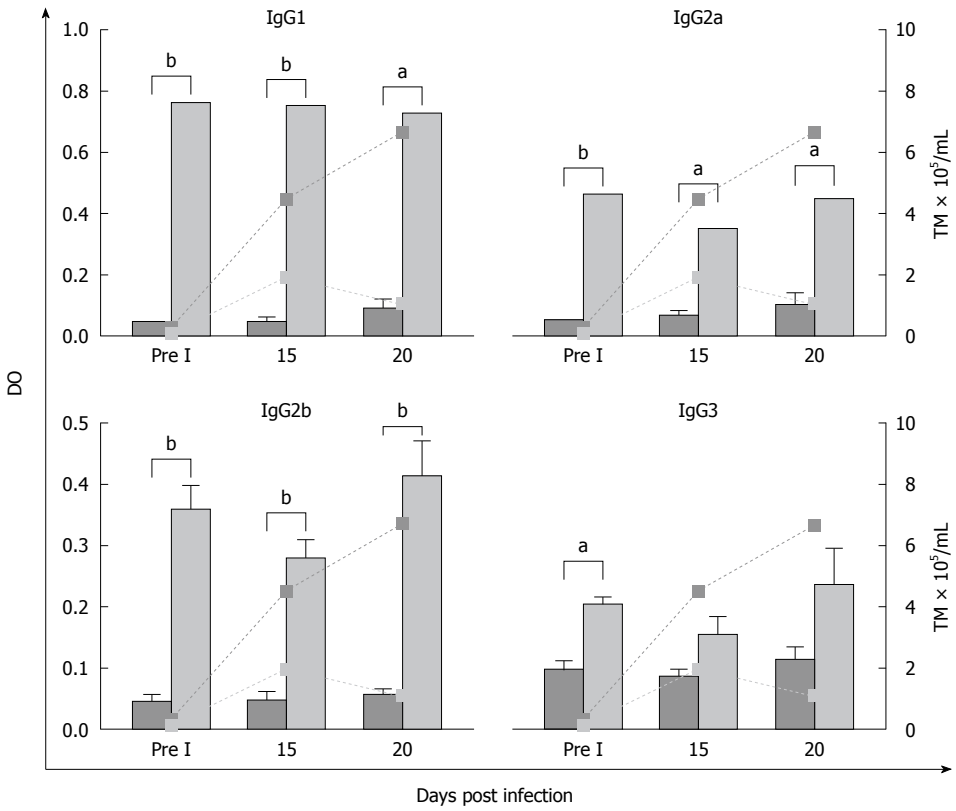

Figure 4 Specific IgG isotype levels in peritoneal fluid obtained from vaccinated, (light gray bars) or control mice, (dark gray bars) before and after infection determined by enzyme-linked immunosorbent assay (optical density mean ± SE). The doted line is the parasitemia level at different time of infection. Significant differences between both groups evaluated by Student's *t*-test ($^aP < 0.05$, $^bP < 0.001$).

cal activation of the macrophages is not regulated, it can cause severe harm to the host's tissue, which is why the production of IL-4, IL-10 and TGFβ is very important, because they modulate the action of NO, oxygen reactive metabolites and proinflammatory cytokines. In the early stages of the infection by *T. cruzi*, the action of soluble mediators like IL-12, IL-18, IFNγ and NO is crucial to inhibit the replication of the parasite.

Induction of arginase has been shown to inhibit the mechanisms involved in eliminating the parasite, among them the activation of T lymphocytes favouring their permanence in tissues. Therefore, evolution of the infection by *T. cruzi* depends on the magnitude of the TH1 - TH2 response and the macrophages activated classically *vs* those activated alternatively[52]. Moreover, it has been suggested that the increase in NK cells could act as a "bridge" between the innate and the early adaptive responses[53].

ADAPTIVE IMMUNE RESPONSE

To bring about protection against *T. cruzi*, CD4+ and CD8+ effector cells need to be generated, which are capable of migrating from lymph nodes to tissues and exert a strong immune response. As it was above mentioned, both types of cells secrete IFNγ, which activates the macrophage in order to exert trypanosomicide activity by means of NO. Antigen presenting cells like macrophages, dendritic cells and B lymphocytes play an essential role in generating effector T lymphocytes, which produce different cytokines involved in the polarization of the TH1 or TH2 immune response[32]. Despite this, *T. cruzi* is capable of surviving in the host for long periods, which contributes to the development of symptoms and chronic disease[54]. It initially produces immunosupression; however, during infection, large quantities of CD8+ are generated, which circulate towards places where the parasite persists in order to find the antigen; there they exert their effector functions, they acquire an activation phenotype and then become memory cells, responsible for perpetuating the immune response in the face of a second encounter with the antigen[55]. The importance of the function of CD8+ lymphocytes is based on the reduction of the parasite load observed in most of the tissues from which these cells are recruited. On the other hand, the presence of the parasite in tissues might be due to a lack of stimulation for the recruitment of CD8+ or to the fact that the functions of these cells might be inhibited by other populations like CD4+, CD25+ and producers of TGFβ[56].

At a second stage, the immune response mediated by antibodies is very important to control infection. Numerous experimental models with antibody or B cell deficiency have shown high parasitaemia and a low survival rate[57].

T. cruzi infection is known to induce polyclonal activa-

tion of B lymphocytes, and as a result hypergammaglobulinemia occurs. Recent studies have shown that most of the activated B lymphocytes do not synthesize specific antibodies during the first days of infection by *T. cruzi*[58], but they do produce a specific response at the end of the acute stage of the infection. However, this polyclonal activation may also be an important cause of the autoimmune phenomena mediated by autoreactive antibodies. In addition, the proliferation of B cell populations responding specifically to the antigen with poor polyclonal response is associated with resistant strains (C57Bl/6), with IFNγ production and a prevalence of the TH1 profile[59].

Different immunoglobulin isotypes, mainly IgG subclasses, are involved in the elimination of the parasite at the local and systemic levels by means of mechanisms such as complement fixation, agglutination and cytotoxicity. In this sense, Umekita *et al*[60] observed that IgG2 might contribute along with other mechanisms to the reduction of parasitaemia in mice infected with *T. cruzi* upon recognizing the parasitical antigen and acting as opsonin. We observed in our works, in accordance with these authors, that another factor involved in the clearance of the parasite might be associated with high levels of specific antibodies induced by vaccination, which might also act as an early mechanism for controlling the infection[61]. As it is shown the Figure 4, we observed the increase in IgG1 and IgG2 isotypes in peritoneal fluid (the site of the infection) related to different immune response patterns. The ratio IgG1/IgG2a in vaccinated group was 1.6 before infection, 2.1 at 15th *pi* and 1.6 at 20th *pi* day. In control mice the ratio was: 0.9; 0.8 and 0.9 respectively. These results showed the importance of TH2, related to antibody response, in vaccinated animals which, together with TH1 response observed through the patterns of cytokines, are both involved in the protection.

Brodskyn *et al*[62] could reveal the importance of the effector function at the infection site, induced before the challenge, contributing to the reduction of the parasite load. These results are in agreement with those obtained by Gruppi *et al*[63] who worked with an immunization model using exoantigens of *T. cruzi* and observed protection associated with increases of IgG1 and IgG2, with low levels of IgG3 at the systemic level. Other authors studying the acute period of the infection detected high levels of IgM, IgG and the isotypic variant IgG1 parallel to the reduction of parasitaemia[64,65]. In our work, IgM, responsible for the specific primary immune response, was high in both experimental groups, in both peritoneal fluid and in plasma. As was expected, the levels detected were always higher in vaccinated animals than in those belonging to the control group[61].

The protective role of the antibodies in the acute phase of the infection is mainly linked to the capacity to induce the elimination of the parasite from circulation, parallel to other cellular events, as we observed in our work, in agreement with others authors[60]. It has been shown that the specific IgG, particularly IgG2, recognizes an important number of parasitical antigens and is able to form microaggregates that fix complement, and increase opsonisation and cytotoxicity mechanisms[66]. In this sense, the neutralization studies developed in our research showed that antibodies and soluble mediators present in the peritoneal fluid of vaccinated mice might be involved in some of the mechanisms responsible for the lysis and reduction in the infectivity of trypomastigotes when these enter the host.

Our findings suggest that the immunogen used in this vaccination model induces an important modulation of the host's immune response, which are involved in the early clearance of the *T. cruzi* used in the challenge. Similar results were obtained by Paláu *et al*[67] and Zuñiga *et al*[68] when they immunized BALB/c mice with metacyclic trypomastigotes of *T. rangeli* and later challenged them with a virulent strain of *T. cruzi*, observing a reduction of the parasitaemia and of the severity of the progress of the infection, with high survival in relation to non immunized and infected mice.

There are also other modulators to the immune response, such as Actinomycetes. Treatment with these actinomycetes significantly reduces acute parasitemia, modifies cell infiltration during acute myocarditis and limits chronic myocarditis in comparison with the infected control group. Similar results were obtained for immunized pregnant mice and then challenged with live *T. cruzi*[69,70].

These findings are a stimulus to go further in the search for knowledge of immunological events, identifying target cells and molecules, with the goal of advancing in prophylaxis or immuno-intervention, directed towards the development of therapeutic approaches to Chagas disease.

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ORIGINAL ARTICLE

Effects of exercise on leukocytosis and blood hemostasis in 800 healthy young females and males

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Author contributions: Sand KL, Flatebo T and Andersen MB performed experiments, supervised the students and helped in withdrawing blood and performing activated partial thromboplastin time and D-dimer assays; Sand KL and Maghazchi AA designed experiments, interpreted results of experiments and analyzed data; Maghazchi AA supervised the entire work, performed analysis and wrote the manuscript.
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Abstract

AIM: To investigate the effects of exercise on healthy individuals of both genders.

METHODS: This study lasted 6 years and involved about 800 healthy people. Individuals were divided into females and males and further sub-divided into two groups; in the first group individuals run (or skied in the winter time) and then rested for 3 h, whereas individuals in the second group intensely cycled for 5 min. The status of health was determined by measuring the sedimentation rate and the intensity of exercises by measuring the heart rate. Blood samples were collected before and after exercise.

RESULTS: We observed that in the first group a significant increase of the total white blood cells, segmented neutrophils, band neutrophils, eosinophils and to a lesser extent lymphocytes but not monocytes in the blood circulation. However, all cell types were increased in the circulation after 5 min intense exercise. No differences in the pattern of cell increase were observed among the genders. Activated partial thromboplastin time (APTT) and D-dimer were also measured in the blood of individuals who cycled intensely for 5 min to determine the coagulation and fibrinolytic activities in the blood. APTT is reduced and D-dimer values significantly increased after intense exercise. However, APTT was statistically lower in males than females, whereas no differences in the D-dimer values were observed among the genders.

CONCLUSION: Our results indicate that exercise whether leisure or strenuous affects leukocytosis and hemostasis in both genders. A major advantage of this study is the high numbers of individuals involved and the inclusion of both females and males values.

Key words: Exercise; Leukocytosis; Activated partial thromboplastin time; D-dimer

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INTRODUCTION

Recreational exercise is now part of everyday life style because it is important for maintaining cardiovascular fitness. Emphasis is on improving cardiac input and activity as well as manipulating the immune system for a better and prolonged life. Hence, several studies were dedicated to examine the blood hemodynamic and hemostasis after exercise whether being leisure, intense or strenuous. The

total number of white blood cells (WBCs) was increased during and immediately after exercise[1], and both leukocytosis and thrombocytosis occurred in the first 10 min of high-intensity exercise[2]. In 16 male volunteers (average age 30 years), all leukocytes except basophils and eosinophils were increased very early after resistance exercise but declined after 15 and 30 min with the exception of neutrophils[3]. About 5 h after exercise, there was a marked increase in the numbers of blood granulocytes and monocytes along with several inflammatory cytokines and chemokines for both well-trained and untrained runners[4]. Another study examined leukocytosis in 10 female soccer players and observed neutrocytosis but not lymphocytosis after one bout of intense exercise "at 75% maximal heart rate"[5]. Upon comparing the immunological parameters in age and gender different groups it was observed that exercise induced significant increases in total leukocytes and lymphocytes in 11 girls as compared to 13 boys studied[6].

Neutrocytosis may depend on the duration rather than the intensity of the exercise, which also depends on the release of adrenocorticotropic hormone[7]. Intensive short-term exercise resulted in increased leukocytosis which included lymphocytes, granulocytes and monocytes concomitant with alterations in plasma catecholamine levels[8]. A 30 min exercise in 8 male volunteers resulted in increased catecholamine and leukocytosis, whereas another round of exercise 3 h later also resulted in enhanced leukocytosis "neutrophils and lymphocytes" and increased cortisol levels[9]. It was proposed that catecholamine increases the number of circulating leukocytes, whereas cortisol which has a later effect maintains this increase in the vascular compartments[10]. Similarly, a short period of recreational vigorous exercise induced significant leukocytosis, which could be due to the release of adrenalin[11]. In contrast to these results it was reported that neither the total increase nor the subsequent decline in plasma cortisol concentration after exercise in 7 healthy male volunteers is important for leukocytosis[12].

Physical exercise also affects blood hemostasis since blood coagulation cascade was activated as demonstrated by shortening the activated partial thromboplastin time "activated partial thromboplastin time (APTT)"[13]. In 10 healthy adolescents, APTT is shortened by about 15% immediately after exercise which returned to normal value after 1 h concomitant with a high increase of fibrinolytic activity[14]. Similarly, activation of the coagulation cascade is detectable after acute physical exercise in 10 healthy subjects, which led to increased thrombin generation[15]. It was also reported that in 11 healthy male subjects irrespective of the type of exercise they performed, alterations in markers of thrombin and fibrin formation were pronounced after 1 h exercise. In this study of high impact, it was suggested that prolonged exercise is necessary for exercise-induced activation of coagulation resulting in thrombin and fibrin formation[16].

In 15 healthy individuals who performed strenuous exercise for 15, 45 and 90 s, it was observed that this exercise did not induce blood coagulation, whereas fibrinolysis, *e.g.*, generation of tissue-plasminogen activator "t-PA" was increased after 15 s and remained high through the duration of exercise. The release of t-PA might be due to increased catecholamine concentrations and blood sheer-stress, whereas no increase in D-dimer generation was observed[17]. In contrast, Gunga *et al*[18] observed that PT and APTT were both increased in 15 healthy individuals after 30 s exercise, and that t-PA and D-dimer levels were also elevated after the same period suggesting that short-time intensive exercise shifts the homeostasis system into a higher equilibrium. The influence of moderate exercise was also studied and it was observed that after 30 min exercise there was an increase in t-PA[19]. On the other hand, the levels of APTT or D-dimer were not significantly increased during 15 min short-term extreme exercise, but APTT was decreased and D-dimer increased after termination of the exercise[20].

Although these studies are informative, drawbacks include the small numbers of samples included in each study (not more than 30 individuals at best). Therefore, it was difficult to draw a reasonable conclusion from these studies regarding the effects of exercise. In the current study we combined monitoring blood leukocytosis and hemodynamic in about 800 healthy individuals, a study that lasted about 6 years and included comparison of both males and females.

MATERIALS AND METHODS

Physical exercise protocols

Healthy volunteers (age 19-44 years, average 23 years) were divided into two groups with different experimental designs. Individuals in the first group were instructed to run or go cross-country skiing for at least 1 h without any pauses. Blood samples were collected before exercise and 3 h after exercise, and all samples were analyzed immediately after blood withdrawal. Individuals in the other group were instructed to use an exercise bike for 5 min at high resistance and with as high rpm as they could manage. Blood samples were collected before exercise and immediately after exercise. An overview of the numbers of individuals involved and the sort of exercise they performed are shown in Table 1.

Hematological tests

Duplicates of total white blood cell counts before and after exercise were performed using coulter counter Z1 from Beckman Coulter (Miami, FL, United states). Full blood was diluted with an isotonic salt solution and mixed with Zap-Oglobin II (Beckman Coulter) to lyse red blood cells. Blood smears were made from each EDTA-tube and stained using color rapid set from Lucerna-Chem (Lucerne, Switzerland). Differential counts of at least 100 WBCs were done on these smears. Triplicates of hematocrit were measured before and after exercise to determine the changes in packed cell volume (PCV). Determination of hematocrit was done by centrifuging the hematocrit capillary tubes in hematocrit centrifuges and then mea-

Table 1 Overview of individuals involved in the current study

Blood examinations	Subjects	Exercise	Gender
SR	795	-	F
	506	-	M
WBCs	241	Running	F = 121/M = 120
	273	Cycling	F = 133/M = 140
Differential counting	241	Running	F = 121/M = 120
	273	Cycling	F = 133/M = 140
PCV	241	Running	F = 121/M = 120
	273	Cycling	F = 133/M = 140
APTT	291	Cycling	F = 132/M = 159
D-dimer	279	Cycling	F = 125/M = 154

SR: Sedimentation rate; PCV: Packed cell volume; APTT: Activated partial thromboplastin time; WBCs: White blood cells; F: Female; M: Male.

suring on a circular micro capillary reader (Damon IEC division).

Coagulation tests

Vacuette sodium citrate tubes from before and immediately after 5 min intensive exercise were centrifuged for 15 min at 2000 × *g* to obtain platelet free plasma. Plasma samples were then tested for APTT and D-dimer values. APTT was measured using DG-APTT kit and a Thrombotrack coagulometer (Axis-Shield PoC AS Oslo, Norway). D-dimer test was done using NycoCard D-dimer test kit and NycoCard READER II (Axis Shield PoC AS). The purpose of this test was to determine the levels of D-dimer as an indirect measurement of plasma t-PA. Citrated plasma (500 μL) from before and after exercise was added to 0.1 U/mL thrombin (Sigma-Aldrich, St. Louis, MO, United states) and incubated for 5 min. A positive test was done from 500 μL plasma mixed with 0.01 mg/mL actilyse "recombinant t-PA" from Boehringer Ingelheim (Ingelheim am Rhein, Germany) and 0.1 U/mL thrombin. D-dimer concentration was measured using NycoCard READER. In these tests, the coagulation was performed on an independent set of individuals performing only intense exercise.

Heart rate and sedimentation rate

Heart rate was measured before and after exercise at the same time blood was collected for the two different groups. For sedimentation rate (SR), blood was drawn in BD Vacutainer glass seditainer tubes, and SR was measured in a seditainer manual ESR stand (BD Diagnostics, Plymouth, United Kingdom) from all individuals involved in these experiments.

Statistical analysis

Data was collected over a period of 6 years from anonymous individuals reporting only gender and physical condition. All statistical analyses were determined utilizing Graphpad Prism program (San Diego, CA, United States), and significant values were determined using the two-tailed Student's *t* test. A P value < 0.05 was considered to be statistically significant.

RESULTS

Hematological evaluations

Individuals were divided into females and males and each were further sub-divided into two groups; individuals in one group run or skied for 1 h and then rested for 3 h, whereas those in the other group cycled at a maximum intensity for 5 min. Fitness levels ranking from 1-4 were investigated, where 1 indicates lack of training and 4 indicates athletic "training sports at a national level" (Figure 1). Only 4 subjects in total reported fitness level 1, and that group was not further analyzed. Blood was withdrawn before and after each exercise. A total number of 795 females were examined for SR, and those who showed 20 mm/h or more were excluded from the evaluation (Figure 2A). Heart rate measurement was important to determine whether individuals optimally followed the instructions, particularly with the intense exercise (5 min cycling). As can be seen pulse (heart rate) was higher after 1 + 3 h exercise and was more pronounced after the 5 min intense exercise (Figure 2A). There was no effect of 1 + 3 h exercise on PCV of females, but an increase was observed after the 5 min intense exercise. Exactly similar pattern was observed with males; those who had SR 15 mm/h or more were excluded from the evaluation. In addition heart rate difference between before (B) and after (A) exercise was more pronounced with the 5 min intense cycling. Similar to females, PCV was not increased after the 1 + 3 h exercise but increased after the 5 min intense exercise (Figure 2B).

Leukocytosis examination after 1 + 3 h exercise

A significant increase in the number of WBCs was observed in the blood of females who run (or skied) for 1 h and then rested for 3 h (P < 0.0001 when compared to the number of WBCs before exercise, Figure 3A). Both segmented and stab (band) neutrophils numbers were also significantly increased after this mode of exercise (P < 0.0001 for both cell types, Figure 3A). There was a significant increase in the number of lymphocytes (P < 0.04), albeit it was much lower than neutrophils. Monocytes number was not increased, whereas the number of eosinophils in the blood circulation was significantly increased after exercise (P < 0.002). Similar findings were observed with males blood where the numbers of total WBCs, segmented neutrophils, stab neutrophils, lymphocytes and eosinophils, but not monocytes were increased as compared to the resting state (P < 0.0001, P < 0.0001, P < 0.0001, P < 0.03, P < 0.0001, and not significant, respectively; Figure 3B).

Leukocytosis examination after 5 min intense exercise

WBCs numbers significantly increased in the blood circulation of females 5 min after intense exercise (P < 0.0001; Figure 3C). In this category, the numbers of granulocytes, lymphocytes, monocytes and eosinophils were also significantly increased after intense exercise (P < 0.0001 for all cell types). Similar results were observed with males

Table 2 Comparison of the exercise intensity levels among the various groups (mean ± SD)

Fitness level	Exercise form (*n*)	WBC increase (× 10^9/L)	Change in heart rate right after 5 min cycling	APTT (after-before)(s)	D-dimer (after-before) (mg/L)
2	Running (78)	2.97 ± 0.24			
2	Cycling (130)	2.44 ± 0.20	59.82 ± 2.35	-2.11 ± 0.22	0.459 ± 0.22
3	Running (118)	4.93 ± 0.41			
3	Cycling (125)	3.54 ± 0.17	78.67 ± 2.34	-2.16 ± 0.19	0.631 ± 0.16
4	Running (31)	4.84 ± 0.27			
4	Cycling (25)	3.80 ± 0.41	86.80 ± 5.30	-2.05 ± 0.31	0.959 ± 0.67

Individuals were divided into groups according to physical activities ranging from 1 (no activity) to 4 (high intense activity). The numbers of increased WBCs after 1 h run and 3 h rest (Running group), and after 5 min intense cycling (Cycling group) are shown. Shown are changes in the heart rates among the various groups. Also shown are the differences in activated partial thromboplastin time (APTT) and D-dimer measurements. WBCs: White blood cells.

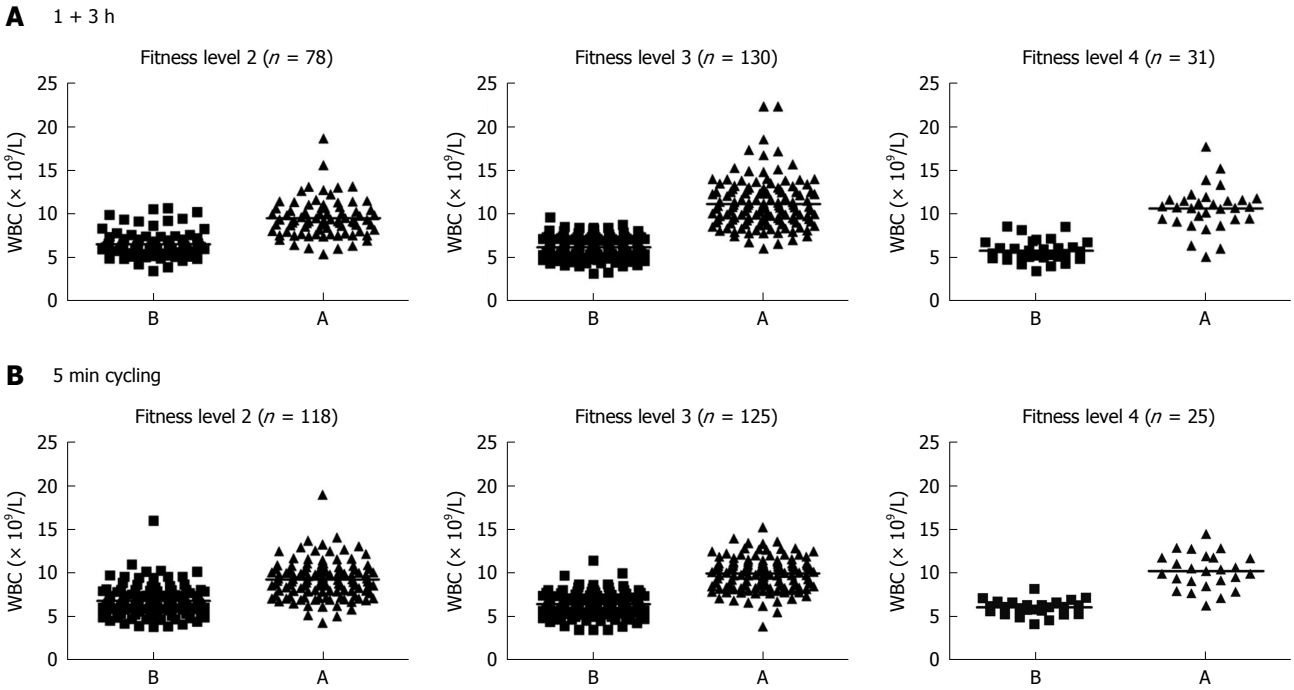


Figure 1 Fitness levels of individuals involved in this study. The numbers of white blood cells (WBCs) were counted according to the fitness levels of individuals (levels 1-4 from low to high intensity) in each group (those that run for 1 h and then rested for 3 h, and those that cycled for 5 min). The numbers of individuals in each category are shown. Also shown the numbers of WBCs before and after exercise. B: Before; A: After.

blood where the numbers of total WBCs, segmented granulocytes, stab neutrophils, lymphocytes, monocytes and eosinophils were increased when compared to the numbers of these cells in the blood of individuals at resting state ($P < 0.0001$ for all cell types; Figure 3D). Fold increases in the numbers of WBCs after both bouts of exercise are shown in Figure 4.

It should also be mentioned that data based on fitness levels showed no significant differences among the various groups on leukocytosis either after running or after cycling (Table 2). The only significant difference observed was the lower number of WBCs circulating at resting situation between members of group 4 as compared to those in group 2 (mean 5.8 × 10^9/L for group 4 and 6.6 × 10^9/L for group 2) and also a larger change "after-before" numbers of leukocytosis for members in group 4 (Table 2). For those who cycled significantly higher changes in heart rate was observed after exercise for those in groups 3 and 4 as compared to those in group 2. No significant differences were found when APTT and D-dimer were analyzed based on the fitness levels (Table 2). Further analysis showed that there was a significant ($P < 0.0001$) relationship between the number of WBC's and heart rate, *i.e.*, increased heart rates are correlated with increased leukocytosis (Figure 5).

Hemostasis shifts into a higher equilibrium after 5 min intense exercise

Next, we investigated the influence of short-term exercise on the hemodynamic of blood coagulation system. Both female and male healthy individuals cycled for 5 min at high intensity. Blood samples were withdrawn before and after exercise and APTT as well as D-dimer values were measured. Results shown in Figure 6A dem-

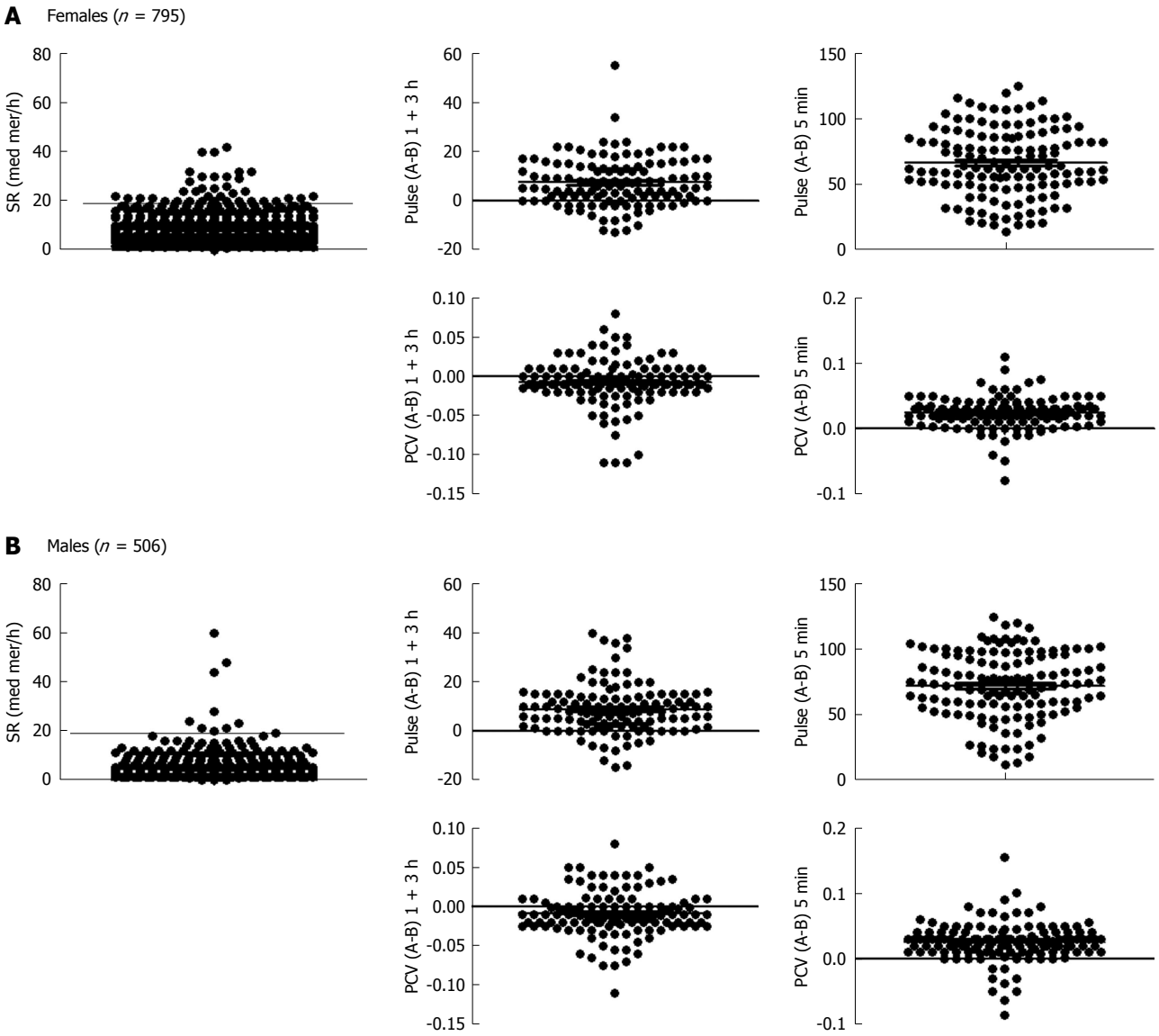
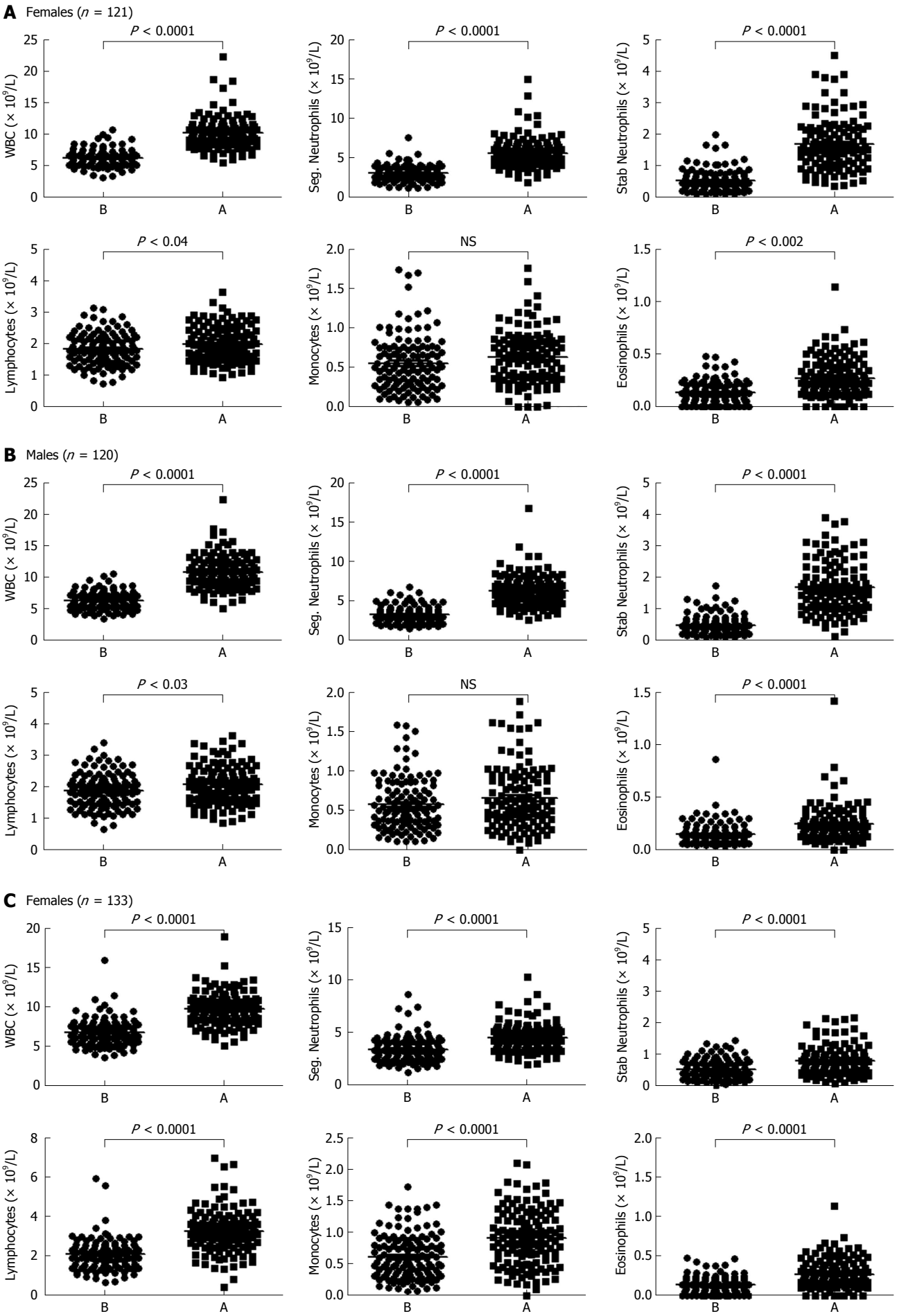


Figure 2 Evaluation of blood and hearts. A: Sedimentation rate (SR) was measured in 795 female students (age 19-44 years, average 23 years old). Females with SR more than 20 mm/h were excluded from the evaluation (above the thick line). Packed cell volume (PCV) and heart rate (pulse) changes were calculated after exercise minus before exercise in 120 females who run or skied for 1 h and then rested for 3 h (1+3 h). PCV and pulse were also measured in about 135 females who exercised for 5 min at a maximum intensity; B: This is similar to Panel A except that 506 males were examined for SR; those that have more than 15 mm/h were excluded (above the thick line). Lines in the other panels show the PCV or pulse after minus before exercise. Each dot represents one individual. B: Before; A: After.

onstrate that APTT was decreased in females, *i.e.*, shortened time after this type of exercise. APTT is decreased after exercise and was significantly lower than before exercise ($P < 0.0001$). From the same blood, plasma levels of D-dimer showed increased values after exercise ($P < 0.04$). A high D-dimer level was also observed when the plasma from females was supplemented with thrombin and recombinant t-PA. Similar pattern was observed with the blood of healthy males, where APTT was significantly shortened ($P < 0.0001$), and D-dimer significantly increased ($P < 0.007$) after 5 min intense exercise (Figure 6B). Curiously, the decrease in APTT found in males was significantly higher than those found in females (Figure 6C), whereas no significant difference was observed when D-dimer was compared among the genders (Figure 6C).

DISCUSSION

The numbers of granulocytes, lymphocytes and monocytes have been reported to be increased after exercise[1-12], but the numbers of samples in these studies were too small to come up with a consensus. In addition, effects of exercise on gender differences are surprisingly unclear. Avloniti showed that there is a significant increase in total WBCs of elite female national-team soccer players as well as increased their heart rates after exercise[5]. Also, a great weight loss occurred after running in both males and females[21]. However, no differences in the post-exercise systolic blood pressure or heart rates were detected among healthy young males and females after exercise[22]. These results were supported by the study of Fernandez-Fernandez *et al*[23], showing no differ-


A Females (n = 121)
P < 0.0001
WBC (× 10⁹/L)
Seg. Neutrophils (× 10⁹/L)
Stab Neutrophils (× 10⁹/L)
P < 0.04
NS
P < 0.002
Lymphocytes (× 10⁹/L)
Monocytes (× 10⁹/L)
Eosinophils (× 10⁹/L)
B Males (n = 120)
P < 0.03
C Females (n = 133)
B
A

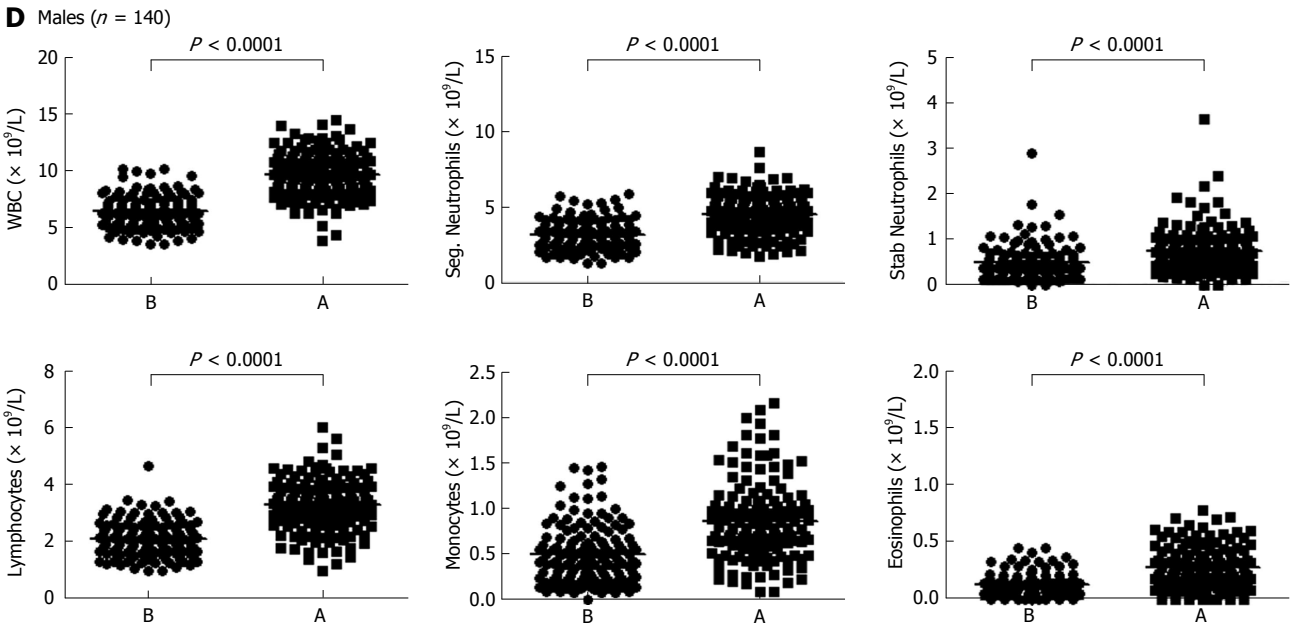


Figure 3 Evaluation of leukocytosis before and after leisure exercise. A: Total numbers of white blood cells, segmented (seg.) neutrophils, stab (band) neutrophils, total lymphocytes, monocytes and eosinophils were calculated from 121 healthy females before and after 1 h run (or ski) and 3 h rest. Lines indicate the mean values. *P* values compared the differences in the numbers between after and before exercise; B: This is similar to panel A, except that 120 healthy males were examined. Each dot represents one individual; C: Total numbers of white blood cells, segmented neutrophils, stab (band) neutrophils, total lymphocytes, monocytes and eosinophils were calculated from 133 healthy females before and after 5 min of intense exercise. Lines indicate the mean values. *P* values compared the differences in the numbers between after and before exercise; D: This is similar to panel A, except that 140 healthy males were examined. Each dot represents one individual. B: Before; A: After; WBC: White blood cell.

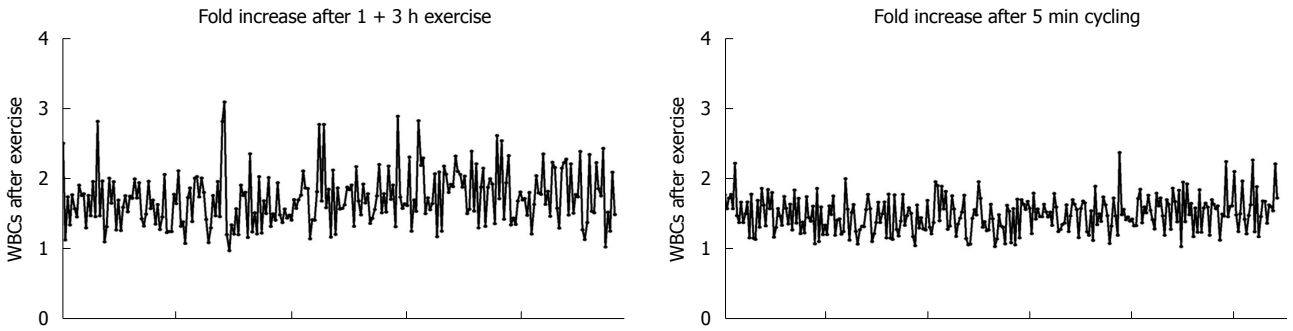


Figure 4 Fold increase in the numbers of white blood cells after exercise. Each dot represents one Individual. The increase in the numbers of leukocytes after exercise varies between 1-3 times. WBC: White blood cell.

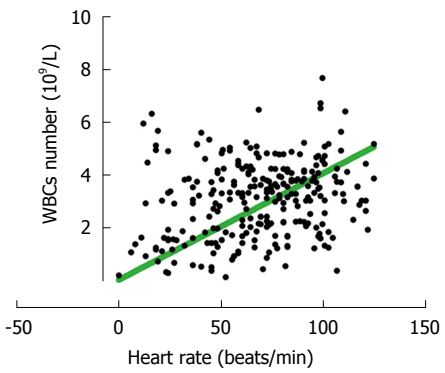


Figure 5 Correlations between the numbers of white blood cells and heart rate after intense exercise. The increase in the numbers of white blood cells accumulating in the blood after 5 min intense cycling is correlated with increased heart rate. WBCs: White blood cells.

ences among females and males heart rates during badminton match play. Here, we examined a large number of medical students whom we taught the blood course in the department of Physiology at the University of Oslo medical school between January 2007 and June 2012. The students were divided into two groups (males and females) and were further sub-divided into a group where individuals run (or skied during winter time) for 1 h and then rested for three h, whereas those in the other group cycled at a maximum intensity for 5 min. The intensity of the exercise was monitored by measuring the heart pulse after exercise and compared it to before exercise. PCV showed no difference in the group that run for 1 h and then rested for 3 h, but an increase in PCV occurred after 5 min exercise. This could be due to the pressure exerted on RBCs during intense exercise due to blood sheer

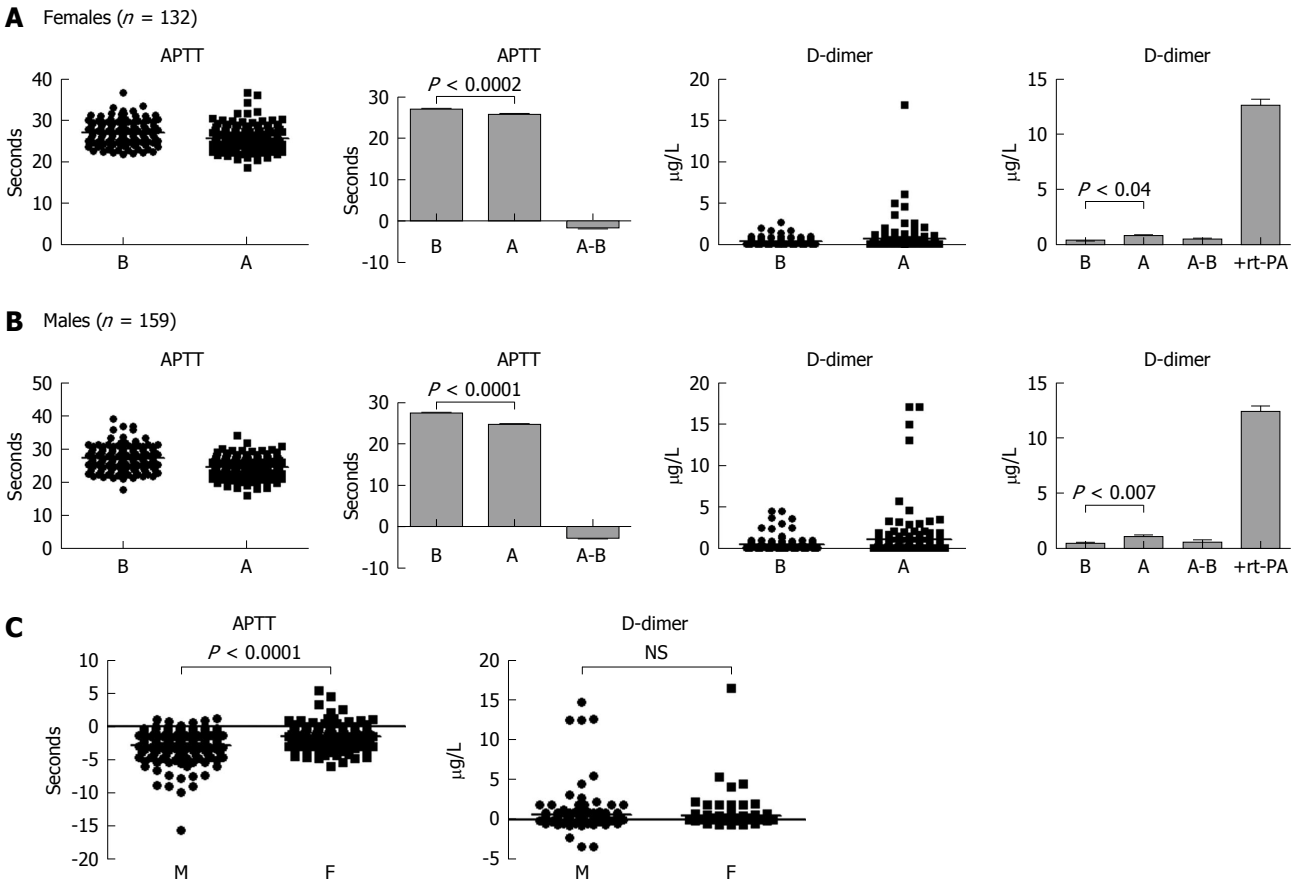


Figure 6 Evaluation of blood hemostasis. A: Activated partial thromboplastin time (APTT) and D-dimer were measured in 132 healthy females before and after 5 min intense exercise. *P* values show the difference between after and before exercise. In the last panel actilyse "recombinant tissue-PA = rt-PA" was added to the assay in the presence thrombin and plasma samples collected from these individual and represent positive control. Dot and column graphs are shown to indicate the differences between after and before exercise; B: This is similar to panel A except that plasma samples of 159 healthy males were examined; C: Comparison among males (M) and females (F) for differences in APTT or D-dimer values (before minus after exercise). B: Before; A: After; NS: Not significant.

stress and outward filtration of plasma from vasculature into tissue space, among other factors.

In the 1 + 3 h group, significant increases in the numbers of segmented neutrophils, band neutrophils and eosinophils were observed after this bout of exercise. Lymphocyte numbers were also increased, although to a much lower extent than the other cells, whereas no increase in the numbers of monocytes was observed. These results could be due to the recruitment of cells from the bone marrow. It was previously observed that CXCL1/interleukin (IL)-8 is increased in the blood circulation of female soccer athletes performing exercise[6]. This chemokine is also increased in the blood of 16 male volunteers 5 h after exercise[3]. Increased CXCL8/IL-8 concentration could explain the higher numbers of neutrophils observed after this bout of exercise. However, the numbers of eosinophils could not be explained by increased CXCL8/IL-8 levels, since these cells do not express CXCR1 or CXCR2, receptors that bind CXCL8/IL-8. Instead, the results may be due to increased cortisol levels which peaks after 3 h of exercise and which maintains cells in the vascular compartments, as shown by others[10]. Alternatively, the levels of eotaxin/CCL11 may also be increased which could be responsible for the recruitment of eosinophils into the circulation since these cells express CCR3 that binds this chemokine[24-26]. One can also exclude the possibility that MCP-1/CCL4 is released at this stage, since it is the major chemokine recruiting monocytes which express CCR2 that binds this chemokine[24-26]. Lastly, no differences were demonstrated in the patterns of cell accumulation in the blood circulation after 1+3 h exercise between females and males.

However, different patterns of cell accumulation in the blood circulation were observed after 5 min intense exercise. Blood samples collected from individuals that exercised intensely showed increased numbers of all cell types including segmented and stab (band) neutrophils, lymphocytes, monocytes, and eosinophils. This increase could be due to potentiating the sheer blood flow that occurs during this sort of exercise as previously described[17]. It could also be due to the release of catecholamine which was reported to be increased in plasma after intensive short-term exercise[8,11]. We also noted a similar pattern of cell accumulation in the blood circulation of males and females performing similar activity, suggesting that there are no gender differences in relation to neutrocytosis, lymphocytosis or monocytosis after this type of exercise. A correlation between increased heart rate and

enhanced leukocytosis was also observed.

Our work also combined leukocytosis with hemostasis. We observed that in both males and females a shift towards higher homeostasis occurred after 5 min intense exercise. Hence, both higher coagulation (shortened APTT) and D-dimer activation were observed. These results suggest that during exercise, the increase in coagulation is offset by enhanced fibrin degradation. To this end, we observed that increased coagulation was significantly higher in males than females, albeit no difference was observed in their D-dimer activity. This may suggest that males might be more prone to higher coagulation after intense short-term exercise than females. The impact of these findings on the exercise ability among genders has not yet been publicized but it might be important to distinguish the coagulation patterns among genders when one is planning exercise regiments. Collectively, our results support those of Gunga *et al*[18] where both coagulation and fibrinolytic cascades are increased in healthy individuals after short-term intense exercise.

D-dimer is increased in patients with deep venous thrombosis and pulmonary embolism[27]. In this regard, elevated plasma concentrations of von Willebrand factor, t-PA and PAI-1 could be predictors of myocardial infarction and stroke incidence in older or in cardiac patients[28]. However, fibrinolysis is increased during 2 h triathlon where it neutralizes the increase in thrombin generation[29], and increased D-dimer activity in healthy individuals is a marker of hydrolyzing the cross-linked fibrin by plasminogen which is generated from plasmin by t-PA.

The advantages of our study as compared to other published data are: (1) large numbers of samples were examined; (2) comparison was done among male and female healthy individuals performing two different kinds of exercise; and (3) both leukocytosis and hemostasis observations were included. Taken together, we observed that WBCs are accumulated in the blood circulation after 5 min intense exercise or after 1 h run plus 3 h rest and that the mechanism of accumulation is different among the two types of exercise. The results also suggest that in both types of exercise, changes in the hemodynamic of blood cells in the circulation took place, and the effects may not dampen the immune system but instead may potentiate it due to increased numbers of immune cells in the circulation. Hence, although exercise may resemble acute inflammation regarding recruitment of leukocytes, the effect of exercise could be beneficial since most cells are recruited into the blood circulation rather than into inflamed tissues such as what happens during infections. Regarding hemostasis, our results continue to support others demonstrating a shift of the hemostasis system into a higher equilibrium after exercise. It was previously suggested that changes in hemostasis during exercise may induced coronary ischemia and possibly sudden death[30]. However, based on the numbers of individuals examined in our study and their gender status, we support the consensus that short-term intense exercise or leisure exercise that may last up to 1 h is beneficial and should be continued particularly for healthy individuals.

COMMENTS

Background

Exercise is part of normal life. However, the effects of exercise in health and diseases are not clearly defined, basically due to the low numbers of individuals examined in most studies published in the literature.

Research frontiers

This is a large study that involves about 800 individuals. Consequently, the results can be regarded as standards for the effects of both intense and leisure exercises on the numbers of blood cells as well as blood coagulation and fibrinolytic components. In contrast to other reports which examined similar effects, this study included large sample size and showed comparison among healthy females and males. Further, this report combined leukocytosis and hemostasis in one study.

Applications

Although not all aspects of blood components were examined, the results may form a reference guide for any future study examining the effects of exercise on blood hemodynamic.

Terminology

Activated partial thromboplastin time and D-dimer are tests which measure blood hemostasis; one measures the time of blood coagulation and the other the degradation of fibrin. Hence, these tests are widely used to examine blood hemodynamic.

Peer review

The data from this paper strongly support the notion that exercise (independent of the intensity) affects leukocytosis and blood hemostasis in both genders. The present results are promising and very important to the research in this area of knowledge once this study includes a high number of individuals (both females and males). In general the paper is well written and the results are consistent, thus supporting the conclusions.

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Italics
Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
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MINIREVIEWS

Steroid resistance in leukemia

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Abstract

There are several types of leukemia which are characterized by the abnormal growth of cells from the myeloid or lymphoid lineage. Because of their lympholytic actions, glucocorticoids (GCs) are included in many therapeutic regimens for the treatment of various forms of leukemia. Although a significant number of acute lymphoblastic leukemia patients respond well to GC treatment during initial phases; prolonged treatments sometimes results in steroid-resistance. The exact mechanism of this resistance has yet not been completely elucidated, but a correlation between functional GC receptor expression levels and steroid-resistance in patients has been found. In recent years, several other mechanisms of action have been reported that could play an important role in the development of such drug resistances in leukemia. Therefore, a better understanding of how leukemic patients develop drug resistance should result in drugs designed appropriately to treat these patients.

Key words: Leukemia; Glucocorticoid; Steroid resistant; Mutations; Genes

Core tip: The exact mechanism of this resistance has yet not been completely elucidated, but a correlation between functional glucocorticoid receptor expression levels and steroid-resistance in patients has been found. In recent years, several other mechanisms of action have been reported that could play an important role in the development of such drug resistances in leukemia.

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INTRODUCTION

Leukemia affects a large population of individuals, both young and old; however, those over the age of 60 years are more at risk of developing the disease. Leukemia is characterized by the abnormal growth of cells of the myeloid or lymphoid lineage that can arise due to several factors including chromosomal abnormalities, transcription factor alterations, and/or chromosomal hyperploidies[1]. In addition to affecting either the myeloid or lymphoid lineage, leukemia can be classified as either acute or chronic. In acute leukemia, the white blood cells multiply very rapidly and are very immature, and therefore cannot function properly. In chronic leukemia, the blasts form more slowly, allowing the body to continue to producing normal, functional cells. This causes fewer symptoms for the patient but often results in splenomegaly. In all major forms of leukemia, as the leukemic bone marrow cells divide, they crowd the marrow and suppress production and function of other healthy cells. The rate of progression and replacement of normal bone marrow cells with cancerous ones is different with each type of leukemia[1].

TYPES OF LEUKEMIA

There are four major types of leukemia: chronic lymphoblastic leukemia (CLL), chronic myeloid leukemia (CML), acute myeloid leukemia (AML), and acute lymphoblastic leukemia (ALL). CLL is mainly caused by unregulated

proliferation of developing B-cells. The lack of growth regulation can be related to the developmental process where B-cells interact with T-cells in the germinal centers of lymph nodes to receive proliferative signaling. In CLL the B-cells express CD19, CD5, and CD23 and have lower levels of IgM, IgD, and CD79b. This phenotype is indicative of an activated mature B-cells[2]. CML is a cancer of the hematopoietic stem cell, in which 95% of cases arise from the formation of the Philadelphia chromosome. The Philadelphia chromosome is the result of a reciprocal translocation between chromosome 9 and 22, specifically t(9;22)(q34;q11)[3-5]. Currently, Imatinib or interferon α are used as the primary treatment for standard risk CML[6].

In AML, the hematopoietic progenitor cells lose their ability to differentiate normally due to a heterogeneous clonal disorder. The specific mutations associated in AML are very diverse but some common mutations are related to the *FLT3*, *NPM1*, *CEBPA*, *MLL*, *BAALC*, or *EV11* genes[7-12]. Regardless of the mutation(s), patients diagnosed with AML have difficulty producing mature erythrocytes, neutrophils, monocytes, and platelets. This is usually noticed in the patient by the inability to fight infections[13]. Induction therapy for AML includes a course of cytarabine following a course of antharcycline daunorubicin[14]. Due to different biological and clinical features compared to younger patients, treatment of older patients with AML has been hampered by uncertainties. There are suggestions that cytogenetic information is critical in order to facilitate treatment decisions for older AML patients. Such patients with adverse-risk cytogenetics derive little benefit from standard induction therapy, and an assessment of the percentage of bone marrow blasts may guide treatment decisions. Over the last few years large-scale genomic studies of patients with AML have also unveiled recurrent somatic mutations in genes involved in epigenetic regulation and the spliceosomal machinery. The identification of these mutations and their impact on prognostication has led to improvements in risk-stratification strategies and provided new potential targets for the treatment of these myeloid malignancies.

The causes of ALL include chromosomal translocations, hyperploidy of more than 50 chromosomes, and altered transcription factors. These alterations contribute to changes the cellular function of the hematopoietic stem cells[15]. The possible cellular processes damaged in ALL are the regulation of differentiation, proliferation, and cell programed death[1]. Some of the pathways affected include the expression of FLT3, a tyrosine receptor kinase, which regulates the retinoblastoma pathway and cells entrance into mitosis cycle[16]. Another mutation that commonly occurs in ALL is the formation of the TEM-AML1 fusion protein which causes deacetylation of histones. By deacetylating the histones, this inhibits differentiation of the hematopoietic stem cells by inhibiting gene transcription[17,18]. Regardless of the type of ALL, the standard induction therapy for all cases is the administration of glucocorticoids (GCs). The most commonly used steroids are dexamethasone and prednisolone. Unlike AML, and CML, ALL has a higher incidence rate in children than in adults giving it a median age of incidence of 39 years[19]. Given the younger median age of these patients, they tend to be very resilient to the treatment over the course of therapy, and in over 80% of cases go into full remission[1,20]. Because they are unable to tolerate chemotherapy regimens as intense as those administered to children, adult ALL patients present with higher-risk features, and therefore, the overall treatment plan for adult ALL is modeled after the pediatric paradigm. This includes multi-agent chemotherapy in the forms of induction, consolidation, and maintenance. Most patients will go into complete remission but often relapse. Wealth of new information regarding the genetic alterations involved in the development of lymphoid leukemias is likely to have a significant impact on patient care as well present several important challenges and opportunities. It is likely that some genetic alterations may have a complex and unexpected role in the development of malignancies. Further, different genetic lesions can affect the same cellular pathway in different cases. Proper understanding of these genetic variations may not only provide a framework for basic research but also could convert these results into a meaningful clinical outcome.

ROLE OF GLUCOCORTICOIDS IN LEUKEMIA

Most of the biological effects of GCs are regulated *via* the glucocorticoid receptor (GR) at the level of second messengers such as cAMP[20]. The two major GR-protein isoforms of interest in pro-inflammatory responses are the GR-α and GR-β. The GR-α is typically associated with steroid sensitivity and acts in an agonistic manner whereas the GR-β seems to be associated with GC mediated GR resistance and seems to act as an antagonist when bound to GC[21,22]. The *GR* gene lies on chromosome 5 (5q31). The GR protein contains four major functional domains: the N-terminal transactivation domain for AF1, A DNA binding domain (DBD) that contains two zinc fingers, and the C-terminal ligand binding domain (LBD)[23]. The N-terminal transactivation domain contains the AF1 domain and is responsible for transcriptional activation of target genes[24]. The first zinc finger binding domain contains the AF1 and nuclear factor κB binding domains and is involved in the transrepression of the receptor[25]. The second zinc finger domain contains the genes that regulate receptor dimerization and glucocorticoid response element mediated transactivation[26-29]. The LBD contains a pocket for steroid binding, a nuclear localization signal, and small but potent ligand-dependent transactivation region (AF2), which interacts with a specific set of co-activators or co-repressors. The determination of AF2 co-activation/co-repression activation depends on the orientation of the AF2 binding, which is based on whether the LBD is activated by an agonist or an antagonist to LBD[30,31]. Due to their lympholytic actions, GCs are included in many therapeutic regimens for

the treatment of various forms of leukemia and lymphoma[32,33]. It is clear that the presence of adequate quantities of properly functioning GR is a necessary but not solely sufficient component for lymphocytolysis[34,35]. In chronic lymphocytic leukemia, certain types of childhood ALL, and malignant lymphomas, a correlation between response to GC therapy and functional GR levels assayed by ligand binding has been found[36,37]. However due to the diversity of these diseases, coupled with their relative individual rarity, no simple overall correlation was found between GC activity and activation of lymphocytolysis.

STEROID-RESISTANCE

Although a significant number of ALL patients respond well to GCs, some reveal primary GC resistance, and those sensitive to GCs almost exclusively develop secondary resistance after prolonged GC therapy[38]. There are two forms of resistance, primary and secondary. Primary resistance is due to an inherent GC resistance. This can be related to the level of GR expression and the regulation of intracellular substrate availability[39]. The second form of steroid resistance is developed over the course of treatment and can take multiple forms. Within the developed resistance, B-cell lineages and T-cell lineages seem to have divergent mechanisms of resistance. Though the mechanism of this resistance has not been completely elucidated, it was once thought that the resistance was due to an alteration in GR expression that is induced by GC treatment. However, later studies found this not to be the sole cause of the resistance[40]. Another proposed mechanism of resistance involves the B-cell lymphoma family of proteins[41]. Large clinical surveys suggest a correlation between functional GR expression levels and primary GC sensitivity and prognosis[42,43]. GC-resistance is either a familial or sporadic condition that can be characterized by generalized end-organ inability to respond to normal GC levels[44]. Thus, steroid resistance in leukemia is a major problem in ALL as it is the primary induction therapy in childhood. Without GC sensitivity the leukemic cells are no longer induced into apoptosis by the treatment.

The BCL2 family of proteins is critical in the regulation of apoptosis induced by cellular stress. BCL2 family of proteins can have either pro-apoptotic or pro-survival properties. Though the signals control opposing functions, they are related through conserved sequence motifs. These motifs are the BCL2 homology domains (BH). The high affinity BH1 and BH2 domains, a pro-survival specific motif, interacts with the pro-apoptotic BH3 domains, inhibits the death signaling pathway allowing for cell survival[45]. The opposing pathway, induced by BH3, induces cell death *via* the BCL2 pathway. It is induced by the activation of the BCL2-associated X protein (BAX) and BCL2 homologous antagonist/killer (BAK). The activation of the BAX/BAK pathway leads to apoptosis *via* permeation of the mitochondrial membrane[46]. However, when BCL2 and BCL2 extra-large (*MCL-1*) knockdowns were induced, GC administration showed no effect on cell viability when cells from a child ALL patient were used. This indicated that the mechanism of GC resistance lies downstream of BCL2 and MCL-1 pathways[47].

Another possible cause of resistance in ALL patients could be due to an over expression of 11β-HSD. The function of this protein is to inactivate GC as they enter the cell. In rats that have been undergoing dexamethasone therapy and are sensitive to GCs, it has been found that their circulating levels of 11β-HSD1 are elevated. However, once they become resistant to GCs, their levels of 11β-HSD1 are significantly decreased even in the presence of dexamethasone[48]. As a result, inhibitors of 11β-HSD, such as carbenoxlone, have been shown to improve cell death in the T-cell leukemic cell line, CCRF-CEM[49]. This suggests that 11β-HSD inhibitor could be used as part of a combination therapy in ALL.

SUMMARY AND PERSPECTIVES

It has been reported that several point mutations in the LBD of the human GR develop in steroid-resistant leukemic patients, which interferes with GR's ability to bind GCs and subsequently interferes with gene regulation. GC-based therapy is still the most commonly used treatment to combat chronic and acute inflammation. Due to multiple physiological actions of GC/GR, a chronic exposure to pharmacological GC doses becomes a problem in therapeutic settings, causing undesirable, yet on-target effects. Therefore the real challenge is not only to develop more specific GR-ligands, but to change the spectrum of GR-mediated events and try to skew it more towards desired pathways. Therefore, the mainstay of research efforts must be focused on further characterizing the mechanisms of GR actions in detail and developing new therapeutic strategies to fight leukemia with a better benefit-to-risk-ratio. We must aim to dissect certain important determinants of GC/GR signaling in clinical contexts that can be applied for designing of more specific and better targeted therapies to combat leukemia.

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EDITORIAL

Interplay of adipokines and myokines in cancer pathophysiology: Emerging therapeutic implications

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Abstract

Excess body weight constitutes a worldwide health problem with epidemic proportions impacting on the risk and prognosis of several disease states including malignancies. It is believed that the metabolic changes associated with weight gain, particularly visceral obesity, and physical inactivity could lead to dysfunctional adipose and muscle tissues causing insulin resistance, low-grade chronic inflammation and abnormal secretion of adipokines and myokines. The complex paracrine and endocrine interconnection between adipokines and myokines reflects a yin-yang balance with important implications in processes such as lipolysis control, insulin sensitivity and prevention from obesity-driven chronic low-grade inflammation and cancer promotion through anti-inflammatory adipokines and myokines. Furthermore, the complex pathophysiology of cancer cachexia is based on the interplay between muscle and adipose tissue mediated by free fatty acids, various adipokines and myokines. The purpose of this editorial is to explore the role of the adipose and muscle tissue interplay in carcinogenesis, cancer progression and cachexia, and to examine the mechanisms underpinning their association with malignancy. Understanding of the mechanisms connecting the interplay of adipokines and myokines with cancer pathophysiology is expected to be of importance in the development of therapeutic strategies against cancer cachexia. Advances in the field of translational investigation may lead to tangible benefits to obese and inactive persons who are at increased risk of cancer as well as to cancer patients with cachexia.

Key words: Adipokine; Myokine; Cancer; Cachexia; Interleukin-15; Interleukin-6; Obesity; Myostatin

Core tip: The complex paracrine and endocrine interconnection between adipokines and myokines reflects a yin-yang balance with important implications in processes such as lipolysis control, insulin sensitivity and prevention from obesity-driven chronic low-grade inflammation and cancer promotion through anti-inflammatory adipokines and myokines. In addition, the complex pathophysiology of cancer cachexia is based on the interplay between muscle and adipose tissue mediated by free fatty acids, various adipokines and myokines. Advances in the field of translational investigation may lead to tangible benefits to obese and inactive persons who are at increased risk of cancer as well as to cancer patients with cachexia.

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INTRODUCTION

Excess body weight constitutes a worldwide health prob-

lem with epidemic proportions impacting on the risk and prognosis of several disease states including cardiovascular disease (CVD), type 2 diabetes mellitus (t2DM) and common forms of cancer, such as colon cancer, postmenopausal breast cancer, endometrial cancer, renal cell cancer and esophageal adenocarcinoma[1-10]. Globally, about 25% of cancer cases are due to overweight/obesity and sedentary lifestyle[11].

Obesity prevents muscle gain and the combination of obesity and loss of muscle mass could lead to elevated health risks including obesity-associated malignancies. It is believed that the metabolic changes associated with weight gain, particularly visceral obesity, and physical inactivity could lead to dysfunctional adipose and muscle tissues causing insulin resistance, low-grade chronic inflammation and abnormal secretion of adipokines and myokines[6,12,13]. Therefore, the adipose-muscle cross-talk plays a critical role in cancer promotion. On the other hand, in the context of cancer cachexia which characterizes cancer patients with advanced stage, the interplay between adipose tissue and skeletal muscle that occurs through adipokines and myokines is an exciting field of research with emerging novel therapeutic implications[14-16].

The purpose of this editorial is to explore the role of the adipose and muscle tissue interplay in carcinogenesis, cancer progression and cachexia, and to examine the mechanisms underpinning their association with malignancy. Understanding of the mechanisms connecting the interplay of adipokines and myokines with cancer pathophysiology is expected to be of importance in the development of preventive and therapeutic strategies against cancer.

INTERPLAY OF ADIPOKINES AND MYOKINES IN CANCER ETIOPATHOGENESIS

Adipose tissue, main adipokines and cancer

In addition to its inert lipid-storing capacity, adipose tissue represents the largest endocrine organ modulating energy homeostasis, metabolism, inflammation, immunity and endocrine balance[6]. Adipose tissue synthesizes and secretes more than fifty hormones and cytokines, known as adipokines[6]. As adipose tissue expands in obesity, the amount of anti-inflammatory adipokines, particularly adiponectin, decreases and the amount of pro-inflammatory adipokines with an oncogenic potential, such as leptin, resistin, visfatin and chemerin, and cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1 and IL-6 increases[6,17]. Obesity-driven chronic low-grade inflammation is also involved in insulin resistance (IR), which is characterized by hyperinsulinemia, increased levels of growth factors such as insulin-like growth factor-I (IGF-I) and activation of transcriptions factors participating in pro-inflammatory response and cell-cycle regulation, like nuclear factor kappa-B (NF-κB), which can promote carcinogenesis[6,17,18]. Important cancer-related adipokine effects are summarized below.

Adiponectin is a 30-kDa, 244-amino-acid adipokine exerting insulin-sensitizing, anti-inflammatory and anti-neoplastic effects[6]. The majority of epidemiologic evidence has connected *in vivo* hypoadiponectinemia with an increased risk for IR, metabolic syndrome (Mets), t2DM, CVD and obesity-associated malignancies[6,19] as well as with a more aggressive cancer phenotype characterized by higher histologic grade, large size of tumor, lymph node invasion, distal metastases or estrogen receptor negativity for breast cancer[6,20-25]. In summary, adiponectin presents anti-tumorigenic effects *via* two mechanisms: (1) it can act directly on cancer cells by modulating receptor-mediated signaling pathways, including mitogen-activated protein kinase (MAPK), AMP-activated protein kinase (AMPK), Wnt/β-catenin and estrogen receptor (ER) signaling; and (2) it can act indirectly by regulating insulin sensitivity, influencing tumor angiogenesis and modulating inflammatory responses by inhibiting NF-κB signaling[6,24,25]. On the contrary, leptin, a 167-amino acid pleiotropic adipokine that regulates food intake, energy expenditure, immunity, and inflammation[26,27], has been shown *in vitro* to promote growth and proliferation of neoplastic cells *via* activation of various growth and survival signaling pathways including canonical: Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3), phosphatidylinositol 3-kinase/v-Akt murine thymoma viral oncogene homolog/mammalian target of rapamycin (PI3K/Akt/mTOR), MAPK/Extracellular signal-related kinase 1/2 (ERK1/2) and non-canonical signaling pathways such as protein kinase C, c-Jun N-terminal kinase (JNK) and p38 MAPK[25-29]. Additionally, leptin may act indirectly by diminishing insulin tissue sensitivity causing hyperinsulinemia, by shifting inflammatory responses towards a T-helper 1 phenotype with oversecretion of pro-inflammatory cytokines and by influencing tumor angiogenesis; though such leptin effects were not seen *in vivo*[26,27]. Resistin, another pro-inflammatory adipokine synthesized predominantly in visceral macrophages in humans, is a 12 kDa cysteine-rich polypeptide[30-32]. Visfatin or nicotinamide phosphoribosyl-transferase (Nampt), a novel pleiotropic adipokine found in the visceral fat, acts as a pro-inflammatory cytokine, a growth factor and an enzyme in the cellular energy metabolism, particularly nicotinamide adenine dinucleotide (NAD) biosynthesis, which is required in a plethora of intracellular processes such as redox reactions, DNA repair, transcriptional regulation and activity of poly-ADP ribosyltransferases (PARPs) and deacetylases (sirtuins) modulating cell survival and cytokine responses[33-35]. The majority of epidemiologic studies has indicated that *in vivo* hyperresistinemia and hypervisfatinemia are associated with some obesity-related malignancies such as colon cancer, postmenopausal breast cancer and prostate cancer[7,31-34,36-42]; though their ontological role in the association between obesity and cancer needs to be clarified. Resistin and visfatin may: (1) upregulate pro-inflammatory cytokines *via* the NF-κB pathway[32,33]; (2) stimulate signaling pathways which are

important components of cancer-promoting machinery[32,33,41-43]; and (3) induct pro-angiogenic proteins such as the vascular endothelial growth (VEGF) and the expression of metalloproteases (MMPs) participating in tumor invasiveness and metastasis[32,33]. Much less is known about a novel pro-inflammatory adipokine, chemerin, which is found elevated in obese individuals[44]. Chemerin may cause IR in human skeletal muscle at the level of glycogen synthase kinase 3 (GSK3) and Akt phosphorylation, and glucose uptake. Finally, chemerin may activate signaling pathways pertinent to inflammation and cancer promotion, such as NF-κB, p38 MAPK and ERK 1/2[45].

Skeletal muscle, main myokines and cancer prevention

Skeletal muscle accounts approximately for 40% of body weight in non-obese individuals, constituting therefore the largest human organ[46]. There has been accumulating evidence that skeletal muscle is an important secretory organ producing several proteins and low molecular weight molecules[45,46]. Myokines are muscle-derived cytokines that exert autocrine/paracrine and endocrine effects. Myokines play a privotal role in metabolism as mediators of muscle-to-adipose tissue cross-talk and regulators of muscular glucose and fat homeostasis, and in cancer prevention as mediators of the beneficial effects of physical activity counteracting the harmful effects of pro-inflammatory adipokines[45,46]. It seems that the complex paracrine and endocrine interconnection between adipokines and myokines reflects a yin-yang balance with important implications in processes such as lipolysis control, insulin sensitivity and prevention from obesity-driven chronic low-grade inflammation and cancer promotion through anti-inflammatory adipokines and myokines. At the same time, skeletal muscle cells may secrete adipokines such as adiponectin, which can exert beneficial local metabolic effects enhancing insulin sensitivity and inhibiting inflammatory processes[47]. It is important to underscore that adipose tissue is not the exclusive source of adipokines. Although adipose tissue constitutes the primary site of adipokines production, several adipokines are synthesized by both fat and muscle, playing a critical role for autocrine/paracrine loops[45]. For example, IL-6 and IL-8 are considered adipokines but also myokines with different roles in inflammation, exercise, skeletal muscle development and insulin sensitivity.

It is well known that physical activity offers protection against a variety of chronic diseases including obesity, t2DM, CVD, osteoporosis, depression and cancer[45]. Recent meta-analyses and epidemiological studies have underscored the protective effect of physical activity on reducing colorectal, prostate and breast cancer risk by 20%-40%[45]. Interestingly, moderate-intensity physical activity after breast and colorectal cancer diagnosis may improve prognosis and reduce the risk of cancer-specific and overall mortality[48-51]. Below is discussed the role of major beneficial myokines.

IL-6 was the first described myokine produced in an exponential manner in response to muscle contraction after exercise in a strictly TNF-independent fashion[12,52]. IL-6 release from muscle is associated with exercise intensity and duration as well as muscle mass involved in the mechanical load[52]. Muscular IL-6 is involved in AMPK-mediated fat oxidation, skeletal muscle lipolysis and insulin-stimulated glucose uptake enhancing insulin sensitivity[12]. IL-6 also mediates some of the immunoregulatory and anti-inflammatory properties of regular exercise as it modulates TNF-α levels[52] and stimulates the secretion of classic anti-inflammatory cytokines such as IL-10 and IL-1ra[12]. In contrast to the beneficial effects of muscular IL-6, chronic elevated serum IL-6 levels synthesized by adipocytes and immune cells in the visceral adipose tissue are closely associated with *in vivo* IR, Mets, obesity and physical inactivity[12,45]. Interestingly, oncostatin M (OSM), a member belonging to the IL-6 superfamily, represents a pleiotropic myokine released by contracting myotubes[12]. OSM has been shown to exert *in vitro* important apoptotic effects on tumor cell lines by inhibiting proliferation in a variety of tissues comprising mammary epithelial cells, melanoma, ovarian and lung cells[12].

IL-15 is a 15 kDa myokine that is highly expressed in skeletal muscle especially after aerobic exercise and resistance, and acts as a myokine that inhibits adiposity[13]. Apart from its hypertrophic and anabolic effects on muscle tissue as an authentic myokine, IL-15 exerts many metabolic actions by enhancing glucose uptake and fat oxidation in muscle tissue, stimulating lipolysis and inhibiting preadipocyte differentiation and lipogenesis as part of the muscle-adipose cross-talk[13]. Obese individuals exhibit low plasma IL-15 levels[46]. Interestingly, IL-15 may stimulate the production of anti-inflammatory and anti-neoplastic adiponectin downregulating visceral obesity while it reduces white adipocyte size and serum leptin levels in male mice[12].

A new myokine, irisin, was recently discovered and named after the Greek messenger goddess Iris[46,53-55]. Physical activity increases the muscular expression levels of the transcriptional co-activator PGC-1α upregulating the expression of the type I membrane protein FNDC5, which is C-terminally cleaved and secreted into the circulation as irisin[53]. In turn, irisin increases the expression of uncoupled protein-1 (UCP-1) contributing to the "browning" of white adipose tissue characterized by enhanced mitochondrial density, oxygen consumption and non-shivering thermogenesis[55]. Therefore, the muscle-derived irisin exhibits beneficial metabolic actions by increasing energy expenditure, causing small weight loss and improving metabolic parameters such as insulin signaling and sensitivity[55]. Basal plasma irisin levels may increase in response to 10 wk of regular exercise in humans and correlate with physical activity levels both in

mice and humans[46,54,55].

INTERPLAY OF ADIPOKINES AND MYOKINES IN CANCER CACHEXIA

Almost 50% of patients suffering from advanced cancer stage present cachexia which is responsible for 25% of deaths due to cancer[14-16]. Cachexia is a complex metabolic state characterized by loss of skeletal muscle mass and adipose tissue leading to progressive functional impairment. Cachexia is usually associated with asthenia, anorexia, anemia, weight loss, hypoalbuminemia, IR and abnormal metabolism of carbohydrates, lipids and proteins[56]. Cancer cachexia may be caused by anorexia, dysphagia related to advanced esophageal cancer, an imbalance between protein synthesis and catabolism with an increase in energy expenditure, or a combination of the two[14-16]. However, the complex pathophysiology of cancer cachexia is based on the interplay between muscle and adipose tissue mediated by free fatty acids, various adipokines and myokines[14,15].

As cancer progresses, a variety of cytokines (IL-6 and TNF-α) and tumor-derived mediators such as proteolysis-inducing factor (PIF) and parathyroid hormone-related protein (PTHrp), activate the pro-inflammatory catabolic cytokine cascade and deactivate the anti-inflammatory anabolic network (IL-4, IL-10, IL-12 and IL-15) leading to a systemic, chronic inflammation in cancer patients[16]. Pro-inflammatory and pro-cachectic cytokines, mainly TNF-α, IL-6 and interferon-α, and a lipid mobilizing factor (LIF), which is homologous to the soluble plasma protein Zinc-α2-glycoprotein (ZAG), activate adipose triglyceride lipase (Atgl) triggering lipolysis which results in net mobilization of white adipose tissue and an augmentation of plasma free fatty acids levels[14,15]. Interestingly, ZAG, a recently identified 43-kDa adipokine, acts as a lipid-mobilizing factor stimulating lipolysis in adipocytes, and is enhanced in mice and humans with cancer cachexia[57,58]. Based on its lipid-mobilizing role, ZAG could also contribute to adipose tissue atrophy associated with cancer cachexia[58]. At the same time, the process of protein catabolism in cachexia starts and may be regulated by the cross-talk between adipose and muscle tissue mediated by free fatty acids, adipokines, cytokines and myokines. Interestingly, in cancer-bearing mice in which the *Atgl* gene is ablated, lipolysis is not activated and both adipose tissue mass and skeletal muscle mass are preserved[59]. TNF-α, named originally cachectin, presents a critical mediatory role in cancer cachexia. IL-6 and leptin may also inhibit synthesis and enhance lipid and protein catabolism in adipocytes and myocytes respectively[60]. Nevertheless, hypoleptinemia and hyperadiponectinemia characterize the cancer cachectic state in human studies[61,62]. Our group has shown that low leptin and elevated adiponectin levels were seen in pancreatic cancer cases compared to controls[62]. Hyperadiponectinemia may be a compensatory response to inflammation, IR and/or the disease-induced weight loss possibly through altering the size of adipocytes[62]. Besides, cachectic patients exhibit frequently a relative glucose intolerance and IR due to alterations in fat metabolism, hypoleptinemia, a pro-inflammatory state and an increased activity of the Cori cycle[16]. Muscle wasting in cancer cachexia mediated by free fatty acids, adipokines, cytokines and myokines results in: (1) an activation of the ATP-dependent ubiquitin-proteasome pathway which targets not only structural and sarcomeric proteins such as myosin, troponin and titin but also important myogenic transcription factors such as calcineurin and Myo D[63,64]; (2) a defective muscle regeneration capacity due to an abnormal regulation of satellite cells in skeletal muscle[14]; and (3) a hyperexpression of myokines that play an important role in muscle atrophy such as myostatin[65]. Muscle regeneration may be further compromised in cachexia due to the reprogramming of protein metabolism toward an increased production of acute phase response proteins sustained by the aminoacids secreted by skeletal muscle catabolism. In agreement with this concept, there is also evidence that TNF-α inhibits skeletal muscle regeneration *in vivo via* a caspase-dependent stem cell response[66]. Besides its role as a potent cachexia inducer, TNF-α may be a potent inhibitor of *in vivo* myogenesis[67].

Myostatin is a protein belonging to the transforming growth factor-β (TGF-β) superfamily, playing a pivotal role in the negative regulation of muscle growth and determining the size and mass of skeletal muscle[68]. Myostatin is an authentic myokine as it is exclusively produced by skeletal muscle and to a lesser extent by adipose tissue[65]. Deletion of myostatin in mice results in an increased number of satellite cells that are involved in muscle growth[65] leading to an enhanced muscle regeneration and skeletal mass hypertrophy and a reduction in total adipose tissue[46]. Physical activity attenuates myostatin expression, whereas myostatin deactivation may stimulate the beneficial effects of exercise on metabolism[46]. High myostatin gene expression and signaling enhancement have been associated with cancer cachexia[68]. In blood, myostatin is inhibited by its propeptide or other binding proteins such as follistatin, a hepatokine which belongs to the TGF-β superfamily[68].

Other myokines that could play a role in cachexia are Leukemia inhibitory factor (LIF), IL-7 and IL-8[46]. LIF, an IL-6 cytokine superfamily member that affects cell growth by inhibiting differentiation, represents a contraction-induced myokine acting in a an autocrine/paracrine manner to promote satellite cell proliferation for muscle regeneration. IL-7 and IL-8 are novel myokines participating in the regulation of skeletal muscle development[46]; however, their exact biologic functions remain unknown.

EMERGING PREVENTIVE AND THERAPEUTIC IMPLICATIONS

High-fat diet, weight gain and physical inactivity may

lead to visceral obesity and muscle loss, and consequently to the enhancement of a network of inflammatory pathways promoting the development of IR, Mets and malignancy growth. Physical activity offers protection against metabolic disorders and obesity-associated malignancies[45].

The capacity of adiponectin to stimulate insulin sensitivity synergistically with its apoptotic properties has rendered this adipokine a promising diagnostic and prognostic biomarker as well as a novel therapeutic tool in the pharmacologic armamentarium for treating cancer[3]. However, since adiponectin is extremely difficult to synthesize, research should be conducted in identifying pathways to augment endogenous circulating adiponectin levels in order to attenuate the obesity/physical inactivity-cancer connection[6].

Modulating adipokines and myokines could be a particularly attractive goal for cancer prevention, specifically in overweight/obese and physical inactive individuals. Regular moderate exercise, adoption of a balanced diet, weight reduction and bariatric surgery for morbidly obese persons may increase plasma adiponectin, irisin, IL-15 and the hepatokine follistatin[65], and decrease plasma leptin, resistin, visfatin, chemerin and myostatin concentrations, reducing thus the risk of developing cancer. Very recently, L-4F, an apolipoprotein peptide mimetic used for the pharmacologic upregulation of adiponectin, decreased multiple myeloma (MM) tumor burden through induction of apoptosis, increased survival of myeloma-bearing mice and provided protection against myeloma destructive osteolytic bone disease, an important clinical feature of MM[69]. Interestingly, MM as well as monoclonal gammopathy of undetermined significance which may subsequently progress to MM are characterized by hypoadiponectinemia[69,70]. ADP 355, a new adiponectin-based short peptide mimicking adiponectin action, decreased proliferation in several adiponectin receptor-positive cancer cell lines, modulated several key adiponectin signaling pathways and suppressed the growth of orthotopic human breast cancer xenografts by 31% *in vivo*[71]. Additionally, anti-Nampt (anti-visfatin) agents such as FK866, CHS-828 and APO866 inhibited tumor growth in a broad range of tumor cell lines by diminishing NAD levels, enhanced apoptosis or autophagy, and abrogated tumor growth in animal models of hematological malignancies without significant toxicity[33,72,73].

The pathway of IL-15 and irisin could be explored as a potential therapeutic avenue to combat disease states such as obesity and muscle loss, Mets and obesity-associated malignancies. Increased formation of brown fat instead of white fat has been shown to exhibit beneficial metabolic effects by improving glucose homeostasis and insulin sensitivity in multiple murine models[53-55]. Through regular physical activity, irisin and other myokines could ameliorate insulin sensitivity and attenuate the link between IR and cancer[46,52]. Exercise-induced myokines have been found to inhibit mammary tumor cell growth[12]. There is accumulating evidence that hyperinsulinemia, the hallmark of IR, and the increase of bioavailable IGF-I may promote cancer. Insulin exerts its oncogenic potential through enhancing growth factor-dependent cell proliferation and through abnormal stimulation of multiple cellular signaling cascades[74]. Recent data have consistently underscored the strong link between anti-diabetic treatment, which improves insulin sensitivity and adiponectin production, and decrease in cancer incidence and mortality[6,75].

Finally, the long-term beneficial effects of physical exercise on cancer prevention may be ascribed to the anti-inflammatory actions of myokines and adipokines[11,52]. The upregulation of pro-inflammatory cytokines *via* the NF-κB pathway in a chronic low-grade inflammatory disease state such as obesity is a significant component of the cancer-promoting machinery[6].

Regarding cancer cachexia, the metabolic dysfunction precludes the accretion of skeletal muscle mass, even if additional proteins and calories are provided. Furthermore, the use of anti-TNF (and anti-IL-6) antibodies against the main cachectic factor (TNF-α) in reversing cachexia has led to moderate results[16]. Due to the complex pathophysiology of cachexia, combined approaches to deactivate various pathways implicated in cachexia may open up a new era of significant therapeutic progress. In particular, targeting myostatin may represent a novel therapeutic strategy by using potential myostatin inhibitors such as soluble myostatin receptors, follistatin-related proteins, myostatin propeptide, anti-myostatin antibodies and small interfering RNAs[68]. Anabolic factors such as insulin-like growth factor I enhancing muscle precursor cell proliferation and regeneration are at the forefront of future therapeutic modalities for cachexia[16].

Nevertheless, more intensive basic research studies, *in vivo* animal studies, observational human studies, and larger prospective and longitudinal studies are needed in order to fully clarify the mechanisms underlying the effects of adipokines and myokines on cancer pathophysiology. Further studies are required for the development of reliable laboratory techniques (*e.g.*, enzyme-linked immunosorbent assays) to assess adipokines and myokines as well as their physiologic relevance. Which levels of adipokines and myokines should be considered abnormal needs also to be determined along with standardization of levels and assay procedures. Proteomics will identify new adipokines, myokines and the extent of the "adipo-myokinome".

Whilst understanding the interplay of adipokines and myokines with cancer might provide potential therapeutic targets, lifestyle amelioration remains the most important component in preventing obesity-related malignancies. Reduction of body weight, daily physical exercise and a balanced diet with fruit and vegetables consumption may improve energy balance and reduce the risk of developing IR, Mets, t2DM, CVD and obesity-associated malignancies. Advances in the field of translational in-

vestigation may lead to tangible benefits to obese and inactive persons who are at increased risk of cancer as well as to cancer patients with cachexia.

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EDITORIAL

Obesity, insulin resistance, adipocytokines and breast cancer: New biomarkers and attractive therapeutic targets

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Abstract

Worldwide, breast cancer (BC) represents the most common type of non-skin human malignancy and the second leading cause of cancer-related deaths amid women in Western countries. Obesity and its metabolic complications have rapidly become major global health issues and are associated with increased risk for cancer, especially BC in postmenopausal women. Adipose tissue is considered as a genuine endocrine organ secreting a variety of bioactive adipokines, such as leptin, adiponectin, resistin and nicotinamide phosphoribosyltransferase/visfatin. Recent evidence has indicated that the constellation of obesity, insulin resistance and adipokines is associated with the risk and prognosis of postmenopausal BC. Direct evidence is growing rapidly supporting the stimulating and/or inhibiting role of adipokines in the process of development and progression of BC. Adipokines could exert their effects on the normal and neoplastic mammary tissue by endocrine, paracrine and autocrine mechanisms. Recent studies support a role of adipokines as novel risk factors and potential diagnostic and prognostic biomarkers in BC. This editorial aims at providing important insights into the potential pathophysiological mechanisms linking adipokines to the etiopathogenesis of BC in the context of a dysfunctional adipose tissue and insulin resistance in obesity. A better understanding of these mechanisms may be important for the development of attractive preventive and therapeutic strategies against obesity-related breast malignancy.

Key words: Breast cancer; Obesity; Insulin resistance; Adipokines; Adiponectin; Resistin; Leptin; Nicotinamide phosphoribosyl-transferase; Visfatin

Core tip: Recent evidence has shown that the constellation of obesity, insulin resistance and adipokines is associated with the risk and prognosis of postmenopausal breast cancer (BC). Direct evidence is growing rapidly supporting the stimulating and/or inhibiting role of adipokines in the process of development and progression of BC. Recent studies support a role of adipokines as novel risk factors and potential diagnostic and prognostic biomarkers in BC. This editorial aims at providing important insight into the potential pathophysiological mechanisms linking adipokines to the etiopathogenesis of BC in the context of a dysfunctional adipose tissue and insulin resistance in obesity. Understanding of these mechanisms may be important for the development of attractive preventive and therapeutic strategies against obesity-related breast malignancy.

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INTRODUCTION

Worldwide, breast cancer (BC) represents the most common type of non-skin human malignancy and the second lead-

ing cause of cancer-related deaths amid women in Western countries[1,2]. The prevalence of BC increases with age and, therefore, BC is more common in postmenopausal than premenopausal women. Despite substantial progress in BC treatment, metastatic disease, occurring in 50% of patients following radical surgery, remains incurable[3-7].

Apart from risk factors such as germline mutation in BRCA1, family history of BC or diagnosed carcinoma *in situ*, common well-established risk factors for BC, particularly postmenopausal BC (PBC), are hormone-associated reproductive factors such as earlier age at menarche, later age at menopause, older age at first birth, decreased parity and use of hormone therapy (HT); anthropometric features such as increased final height, weight, body mass index (BMI), waist circumference; atypical hyperplasia of the mammary gland; and high breast density on mammographic screening[4-6]. Alcohol consumption is considered a modest risk for BC risk, possibly by enhancing estrogen levels[4-9]. Nevertheless, more than 50% of BCs arise in the absence of known common risk factors[4-9].

OBESITY, INSULIN RESISTANCE AND BC

A meta-analysis and systematic review in conjunction with other evidence have linked obesity to excess risk for many cancers, including BC[1-3]. Obesity represents a growing global public health issue in industrialized countries affecting a significant part of the population across all age, gender and ethnic groups[2]. There is accumulating evidence that overweight/obesity constitutes a risk factor for BC in postmenopausal women[2-5]. The excess of body weight significantly increases PBC risk by 30%-50%[10]. However, based on epidemiological data, obesity has been associated with decreased or neutral BC risk in premenopausal women[6,10,11]. Whilst increased birthweight is associated with premenopausal BC, weight gain acquired later in life, after the age of 40 and mainly during the perimenopausal period, presents the most deleterious effects[9]. The effect of the BMI increase on BC risk is particularly observed in tumors with positive estrogen (ER) and progesterone receptor (PR) tumors and in HT non-users[9]. Obesity is also associated with increased tumor burden and histopathological grade, and a higher incidence of lymph node metastasis in BC patients. In addition to an increased risk of developing BC, overweight/obese and physically inactive patients appear to be at increased risk for BC progression and BC-related mortality regardless of menopausal status[1-3,9].

The mechanism connecting obesity with PBC is not completely elucidated. After menopause, adipose tissue is the main site of peripheral aromatization of androgens to estrogens, which may induce mitogenic activity in mammary epithelial cells[3,12]. Excess adiposity is associated with elevated estrogen levels. Obese postmenopausal women with BC present significantly higher total and free estradiol levels as well as increased local estrogen levels within breast tumors compared to healthy women[6,7].

Additionally, increased adiposity and, in particular increased visceral fat may cause hyperinsulinemia, insulin resistance and dyslipidemia. In turn, hyperinsulinemia leads to higher insulin-like growth factor-I (IGF-I) levels exerting a mitogenic effect on both normal and neoplastic breast epithelial cell as well as lowers the hepatic synthesis of sex hormone binding globulin resulting in an increase of the bioavailable fraction of both estradiol and testosterone[3,12]. Epidemiological evidence has indicated that both pre- and postmenopausal women with insulin resistance, metabolic syndrome and type 2 diabetes (t2DM) have an increased BC risk[3,13]. According to the International Diabetes Federation, the metabolic syndrome is defined as a cluster of conditions that include central (abdominal) obesity based on ethnicity specific values for waist circumference associated with any two of the following four factors: (1) hypertriglyceridemia [⩾ 150 mg/dL (⩾ 1.7 mmol/L) or specific treatment for this lipid abnormality]; (2) reduced HDL cholesterol [< 40 mg/dL (< 1.03 mmol/L) in males, < 50 mg/dL (< 1.29 mmol/L) in females, or specific treatment for this lipid abnormality]; (3) hypertension (systolic blood pressure ⩾ 130 or diastolic blood pressure ⩾ 85 mmHg or treatment of previously diagnosed hypertension); and (4) raised fasting plasma glucose levels [⩾ 100 mg/dL (⩾ 5.6 mmol/L) or previously diagnosed type 2 diabetes][14]. The metabolic syndrome is associated with increased risk of t2DM and cardiovascular disease[14].

The insulin-IGF-I pathway may lead to the activation of various intracellular pathways, including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling cascade affecting tumor growth[3,12]. Moreover, estrogen and the insulin-IGF-I pathways intersect at the G_1-S phase of cell-cycle progression and synergistically induce mitogenic effects on breast epithelium. The insulin-IGF-I pathway may activate ER-α transcriptional activity in BC cell lines even in the absence of estradiol[6,12].

Visceral adipose tissue plays a pivotal role in the development of a systemic inflammatory state contributing to obesity-related metabolic diseases[2,3]. Excess body weight is considered a subclinical chronic low-grade inflammatory and prothrombotic state involved in obesity-associated insulin resistance and cancer[2,3]. The activation of proinflammatory adipocytokines and the suppression of anti-inflammatory adipocytokines such as adiponectin increase the hepatic synthesis of acute phase reactants, establishing therefore a positive feed-back loop and enhancing the systemic inflammatory state which promotes cancerinogenesis[3]. At the same time, lipid accumulation increases demand on the endoplasmic reticulum resulting in an uncontrolled production of reactive oxygen species (ROS) which stimulate inflammatory signaling pathways and induce endoplasmic reticular stress, oxidative stress and DNA damage leading to genomic instability[15]. It is well known that oxidative stress which reflects an

imbalance between the systemic manifestation of ROS and the biological system's ability to detoxify the reactive intermediates or to repair the resulting damage, may cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including DNA[15]. Moreover, oxidative stress may cause disruptions in normal mechanisms of cellular signaling. As the adipose tissue expands in obesity, the vasculature is not sufficient to oxygenate adequately the adipocytes leading to hypoxia. The resultant hypoxia, mediated by the hypoxia-inducible factor- I , in conjunction with endoplasmic reticular stress and oxidative stress initiate a pro-inflammatory cascade with overproduction of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6, through nuclear transcription factor-κB (NF-κB) activation, stimulating the systemic inflammatory state which further promotes tumor growth[3].

ADIPOSE TISSUE, ADIPOKINES AND BC

Apart from its lipid storage function, adipose tissue constitutes an active endocrine organ secreting several bioactive adipocytokines or adipokines as well as inflammatory cytokines, regulating physiological and pathological processes, such as appetite, insulin sensitivity and resistance, inflammation, immunity, hematopoiesis and angiogenesis[3]. The mechanisms connecting excess adiposity in overweight/obesity with molecular and cellular pathways critical for cancerinogenesis involve innate and acquired immune activation, exposure to protumorigenic adipokines and growth factors as well as increased substrate availability to breast neoplastic cells. Adipocytes represent the majority of the breast tissue, with epithelial cells accounting for only 10% of breast volume[7]. A recent hypothesis places adipocytes along with their autocrine, paracrine and endocrine functions at center stage in breast tumorigenesis[3,7]. The deregulated expression of adipokines may thus be involved in the association of obesity with BC. Although the exact interplay between adipokines is not yet well clarified, this editorial presents the role of main adipokines in breast carcinogenesis and examines the pathophysiological mechanisms that underlie the association between adipokines and breast malignancy in the context of a dysfunctional adipose tissue in obesity. Understanding of the mechanisms linking adipokines to BC is expected to be of importance in the development of preventive and therapeutic strategies.

Leptin and BC

Leptin, a 167-amino acid peptide that is primarily produced in adipose tissue, is a pleiotropic adipokine that regulates food intake, energy expenditure, immunity, inflammation, hematopoiesis, cell differentiation and proliferation[16,17]. Circulating leptin is directly proportional to the amount of body fat and fluctuates with acute changes in caloric intake, signaling the amount of energy stored in adipose tissue[17]. Common forms of obesity, insulin resistance and metabolic syndrome are associated with hyperleptinemia and leptin resistance[16].

Leptin gene expression was found in normal breast epithelium, in BC cell lines as well as in solid tumors[7]. In the majority of cases with breast carcinoma, leptin was found to be overexpressed[7]. A growing body of evidence suggests that leptin exerts BC neoplastic effects *via* two mechanisms[16]. Firstly, leptin may act directly on BC cells by stimulating receptor-mediated signaling pathways leading to tumor cell growth, migration and invasion. Recently, *in vitro* studies have shown that leptin is involved in mammary tumorigenesis by stimulating tumor growth, cell survival and transformation, by amplifying ERα signaling that plays an critical role in hormone-dependent BC growth and progression and by upregulating the aromatase transcription which results in increased estrogen synthesis[18,19]. Leptin, through its receptor LepR, may promote growth and proliferation of BC cells *via* activation of various growth and survival signaling pathways including canonical: Janus Kinase 2/Signal Transducer and Activator of Transcription 3 (JAK2/STAT3), PI3K/v-Akt murine thymoma viral oncogene homolog/mammalian target of rapamycin (PI3K/Akt/mTOR), mitogen-activated protein kinase/extracellular signal-related kinase 1/2 (ERK1/2) and non-canonical signaling pathways such as protein kinase C, c-Jun N-terminal kinase (JNK) and p38 MAPK[16,19,20]. Interestingly, this leptin activity is reinforced through entangled crosstalk with insulin, multiple oncogenes, cytokines and growth factors. For example, insulin *via* the PI3K and MAPK signaling pathways has induced leptin and LepR overexpression in human BC cells contributing to an autocrine stimulation of BC cell[12]. Leptin has been shown *in vitro* to stimulate JNK in human BC cells in both a time- and a dose-dependent manner, with greater phosphorylated JNK levels after long-term exposure. JNK stimulation by leptin led to an upregulation of matrix metalloproteinase (MMP)-2 activity, which promotes cancer cell invasion[16,18-20]. It should be noted, however, that most *in vitro* studies have used extremely elevated leptin levels[16]. Secondly, leptin may act indirectly by decreasing tissue sensitivity to insulin causing hyperinsulinemia, by regulating inflammatory responses and shifting the T helper (TH) balance towards a TH1 phenotype with overproduction of cytokines such as IL-6, IL-12 and TNF-α, and by influencing tumor angiogenesis; though such leptin effects were not seen *in vivo*[16].

Nevertheless, in contrast to many *in vitro* studies, epidemiological studies have reported inconsistent and conflicting associations between circulating leptin levels and risk of BC[3,16]. Many studies have documented an association of hyperleptinemia with the risk for BC and advanced disease state[21]. In a recent prospective study, elevated prediagnostic leptin levels were associated with an increased risk of PBC independently from BMI[22]. However, other studies found no association of leptin levels with premenopausal or postmenopausal BC[7]. In

addition, serum leptin levels did not appear to increase substantially the risk of pre-menopausal BC *in situ* and invasive pre- and post-menopausal BC[16,23]. So far, based on the available evidence, the utility of leptin as a BC biomarker is not clear. A possible association of BC with leptin needs to be analyzed further with larger prospective, longitudinal and mechanistic studies in order to prove causality and provide further insights into the paracrine and endocrine mechanisms underlying leptin's role in breast malignancy.

Adiponectin and BC

Adiponectin is a 244-amino-acid, 30-kDa protein secreted predominantly by white adipose tissue, sharing homology with collagen Ⅷ, Ⅹ, complement factor C1q, and tumor necrosis factor-α (TNF-α)[3]. Adiponectin exerts insulin-sensitizing, anti-inflammatory, anti-atherogenic, anti-neoplastic and cardioprotective effects as well as distinct effects on lipid metabolism[3,24]. Adiponectin may be found in different configurations presenting different biological effects: full-length, globular, low molecular weight, medium molecular weight and high molecular weight (HMW) adiponectin[3,24]. The HMW isoform represents the biologically active form of adiponectin, being strongly related with insulin resistance, metabolic syndrome and cardiovascular disease[3,24]. Adiponectin acts through its three receptors that have been identified; two main receptors: AdipoR1 and AdipoR2, and one receptor similar to the cadherin family[3,24]. Adiponectin stimulates several intracellular signaling pathways after binding to its receptors, mainly adenosine monophosphate (AMP)-activated protein kinase (AMPK), but also mTOR, NF-κB, JNK and STAT3[3,24].

Circulating adiponectin levels are generally determined in the range of 2 to 20 μg/mL[3,25]. Based on the assay methodology, race and gender, median adiponectin levels in healthy individuals with a BMI between 20 and 25 kg/m^2 are approximately 8 μg/mL for men and 12.5 μg/mL for women[25]. Hypoadiponectinemia is the common pathodenominator of the constellation of risk factors that synthesize the metabolic syndrome such as hypertension, dyslipidemia, obesity, hyperglycemia and insulin resistance[24]. Furthermore, *in vivo* hypoadiponectinemia has recently been found inversely associated with the risk of insulin resistance and obesity-associated malignancies, that is BC, endometrial cancer, colon cancer, renal cancer, some hematologic malignancies of myeloid origin as well as gastric and prostate cancer[3].

The majority of epidemiologic evidence has linked lower total or HMW adiponectin levels to an increased risk for BC independently of classical risk factors including leptin and the IGF-I system in both premenopausal and postmenopausal women[3,23,26]. Macis *et al*[27] identified lower plasma circulating adiponectin levels in premenopausal women as a risk biomarker for progression from intraepithelial neoplasia to invasive cancer independently of age, BMI, and treatment group[3]. Adiponectin could play a role in BC etiopathogenesis, particularly in the low-estrogen environment observed in postmenopausal women[3]. Because adipocytes constitute the predominant breast stromal element, adiponectin may exert a major paracrine and autocrine influence in mammary epithelium. Since AdipoR1/R2 are expressed in BC lines and tissues samples, adiponectin may act not only through altering the hormonal milieu but directly through inhibition of BC cells proliferation[28]. In addition, some but not all studies have pointed out that breast tumors arising in women with hypoadiponectinemia may present a more aggressive phenotype (higher histologic grade, large size of tumor and ER negativity)[3]. Low adiponectin levels were associated with lymph node metastases and increased mortality in BC survivors after adjustment for parameters including obesity and insulin resistance[3]. Finally, some studies focusing on adiponectin genetic variants (ADIPOQ) and adiponectin receptor genes (ADIPOR1) and BC risk reported associations of ADIPOQ single nucleotide polymorphisms (SNPs) and ADIPOR1 SNP with BC risk[3]. However, other studies did not find such associations[3].

Adiponectin exerts BC anti-neoplastic effects *via* two mechanisms: (1) it can act directly on BC cells by modulating receptor-mediated signaling pathways, including MAPK, AMPK, Wnt/β-catenin and ER signaling; and (2) it can act indirectly by modulating insulin sensitivity at breast epithelium, influencing tumor angiogenesis and regulating inflammatory responses[3]. *In vitro* studies have indicated that adiponectin suppresses growth and promotes apoptosis of MCF-7 and MDA-MB-231 BC cell lines, and reduces the invasion of BC cells[3,29]. Adiponectin decreases also the secretion of proinflammatory cytokines (TNF-α and IL-6) which are responsible for aromatase enhanced production in adipose tissue[3]. The role of adiponectin in tumor angiogenesis remains to be defined as both proangiogenic and anti-angiogenic activities toward mammary tumor growth have been described[3].

Resistin and BC

Resistin, also known as adipose tissue-specific secretory factor or found in inflammatory zone 3, is a 12-kDa cysteine-rich polypeptide belonging to a small family of secreted proteins characterized by a unique spacing of 10-11 cysteine residues, the resistin-like molecules[30,31]. In contrast to mouse resistin, human resistin is synthesized in cells other than adipocytes, predominantly in macrophages and monocytes particularly in the visceral adipose tissue characterized by a high metabolic turnover[31]. Elevated resistin levels caused by genetic or environmental factors such as obesity, inflammation and diet may play a pivotal role in the pathogenesis of insulin resistance, metabolic syndrome, t2DM, gestational diabetes, atherosclerosis, hypertension, cardiovascular disease and several malignancies such as breast, gastric, colorectal and esophageal cancers[31,32].

The majority of epidemiologic studies studying the association of serum resistin with BC have shown that

hyperrestinemia *in vivo* is linked to the risk of BC, particularly in postmenopausal women[4,33]. Our group has shown that mean serum resistin level was significantly higher in postmenopausal women suffering from BC than in age-matched control participants and women with benign breast lesions (11.2 ± 6.4 *vs* 7.7 ± 4.8 *vs* 8.22 ± 6.1 ng/mL respectively, $P < 0.05$) both in univariate and multivariable analyses adjusting for age, date of diagnosis, education, family history of cancer, use of exogenous hormones, alcohol consumption, smoking status, physical activity, reproductive, metabolic, anthropometric, inflammatory markers and adipokines (OR = 1.17, 95%CI: 1.03-1.34, $P = 0.02$)[4].

In vitro findings have shown that resistin induced cancer cell proliferation through the PI3K/Akt signaling pathway[34]. A histopathological study has also indicated an association between tumor tissue resistin expression and malignant cancer behavior and prognosis in BC[35]. Serum resistin was reported as a good biomarker of malignant potential and stage progression in breast, esophageal, gastric, and colorectal adenocarcinoma, correlating positively with tumor size, cancer stage, histological grade and tumor markers[33]. In particular, circulating resistin levels may be an interesting biomarker for PBC reflecting advanced stage and inflammatory state[33]. Of interest, our group has shown that resistin level correlated with the tumor markers CA 15-3 and CEA, cancer stage, tumor size, histopathologic grade and lymph node invasion which are all associated with BC poor prognosis but not with anthropometric, metabolic parameters and hormone receptor status[30]. Although resistin's diagnostic performance was low based on receiver operating characteristic (ROC) curve analysis (0.72, 95%CI: 0.64-0.79), it may constitute a BC biomarker reflecting advanced disease stage and inflammatory state[33]. Hence, resistin may represent a biomarker for BC development and progression, but may also act as a molecular mediator linking adipose tissue to breast carcinogenesis.

Possible mechanisms associating resistin with BC pathogenesis may involve: (1) Upregulation of pro-inflammatory cytokines *via* the NF-κB pathway, an important component of cancer-promoting machinery. Resistin may also promote a pro-thrombotic state *via* mediating the lipoprotein metabolism and inducing inflammation in a hypercoagulable environment observed in BC[32]; (2) Activation of signaling pathways playing an important role in inflammation and tumorigenesis. Resistin phosphorylated both MAPKs, such as Erk or p38, and Akt, a downstream substrate of PI3K, in several cell lines[36]; (3) Induction of the proangiogenic protein: vascular endothelial growth (VEGF) and formation of endothelial cell tubes contributing to metastasis; and (4) Induction of the expression of MMPs and reduction of MMPs tissue inhibitors participating in tumor invasiveness and metastasis[32]. Further mechanistic, larger prospective and longitudinal studies are required to confirm these findings and determine whether resistin may play a role as a BC tumor marker. More studies are needed to clarify resistin's ontological role in the association between obesity and BC.

Nampt/visfatin and BC

Visfatin, also known as nicotinamide phosphoribosyltransferase (Nampt) or pre-B cell colony-enhancing factor, constitutes a novel pleiotropic adipokine acting as an adipocytokine, a growth factor and an enzyme, found in the visceral fat, playing an important role in a variety of metabolic and stress responses as well as in the cellular energy metabolism, particularly nicotinamide adenine dinucleotide (NAD) biosynthesis[37]. Nampt plays a significant role in the enzymatic activity of an array of NAD-dependent enzymes which influence a variety of biological responses essential in cell survival and inflammation such as TNF-α biosynthesis[37]. Serum Nampt concentrations are elevated in obese women, obese children and adolescents, in patients with metabolic syndrome, t2DM, non-alcoholic fatty liver disease and coronary heart disease[37-39]. So far, results have been conflicting regarding associations of Nampt with metabolic parameters[40] underscoring the potential role of Nampt in the pathogenesis of low-grade chronic vascular inflammation in obesity and t2DM as a pro-inflammatory adipocytokine. Nampt was introduced as an insulin-mimetic molecule enhancing insulin signaling by binding insulin receptor at a different site than insulin but this was questioned later[37,41]. Increased Nampt expression has been shown in primary colorectal cancer and prostate cancer, malignancies that are related to overweight/obesity[37-39]. Interestingly, our group has reported that serum Nampt is significantly elevated in patients with PBC[39], and may be a promising biomarker for BC[40]. Indeed, mean serum Nampt was significantly higher in PBC patients than in age-matched healthy controls and women with benign breast lesions (57.9 ± 31.2 *vs* 43.6 ± 28.1 *vs* 42.9 ± 18.1 ng/mL respectively, $P < 0.05$). Postmenopausal women in the highest quartile of Nampt concentration (> 44.8 ng/mL) present significantly higher risk for PBC adjusting for age, date of diagnosis, education, body mass index, waist circumference, years with menstruation, parity/age at first full term pregnancy, breastfeeding, family history of cancer, use of exogenous hormones, alcohol consumption, smoking status, homeostasis model assessment score, serum leptin and adiponectin concentrations (OR = 7.93, 95%CI: 2.52-24.9)[39]. Moreover, in patients, Nampt was significantly associated with CA 15-3, hormone-receptor status, lymph node invasion but not with metabolic and anthropometric variables[40]. Circulating Nampt levels outperformed serum CA 15-3 only in discriminating between PBC cases with early cancer stage than those with late stage, and in differentiating particularly patients with ER-PR- breast malignancies[41].

Elevated Nampt expression in BC tissues was reported to be associated with more malignant cancer behavior as well as adverse prognosis[42]. Pharmacologic inhibition of Nampt has been shown effective in a broad range of

cancer cell lines and in mouse carcinoma models; however the definite role of Nampt in malignant diseases has yet to be elucidated[37-39].

Pleiotropic Nampt may play a role in mammary epithelium tumorigenesis and provide an important link between obesity and PBC *via* the following mechanisms: (1) Nampt represents an essential enzyme in energy metabolism, circadian clock and cell longevity through intracellular NAD generation. NAD, a universal energy- and signal-carrying molecule, is required in a plethora of intracellular processes such as redox reactions, DNA repair, G-protein coupled receptor signaling, intra-cellular calcium-mobilizing molecules, transcriptional regulation and activity of poly-ADP ribosyltransferases (PARPs) and sirtuins which modulate cell survival and cytokine responses[37,38]. In particular, sirtuins constitute a conserved family of NAD-dependent protein deacetylases and/or ADP-ribosyltransferases, being involved in longevity, metabolism, and stress and cytokine responses by deacetylating transcription regulators[43]. Seven sirtuins are expressed in mammals (SIRT 1-7), three of which (SIRT 3-5) are located in mitochondria[43]. Recent data has shown that SIRT1 overexpression suppresses apoptosis, promotes cell proliferation and angiogenesis, and contributes to the oncogenic potential of the ERα on estrogen-induced breast cancer growth[44]. On the contrary, SIRT3, a mitochondrial localized NAD-consuming tumor suppressor protein, may repress the Warburg effect on human breast cancer cell lines, a metabolic hallmark of many tumors characterized by a glycolytic switch even in the presence of oxygen providing cancer cells all the necessary substrates for biomass generation[45]. Nampt activity prods cellular proliferation, shifts the balance toward cellular survival following a genotoxic insult and regulates the circadian clock machinery of some key transcriptions factors[37]. Further mechanistic studies using a metabolomics approach are needed in order to clarify the regulation of NAD biosynthesis and its temporal-spatial dynamics in BC cell metabolism. Therapeutic normalization of the NAD +/NADH balance may inhibit metastasis and prevent BC progression[46]. Indeed, the combination of a Nampt small molecule inhibitor, FK866, with olaparib, a PARP inhibitor, inhibited triple-negative breast tumor growth *in vivo* to a greater extent than either single agent alone[47]. A clear understanding of the interplay of NAD-consuming sirtuins and PARPs, Nampt and ER/PR at the molecular level may potentially open up new therapeutic avenues for BC treatment; (2) Nampt is a proliferative and anti-apoptotic factor. Nampt stimulated the proliferation and DNA synthesis rate of MCF-7 human BC cells[37]. Nampt may play a role in BC development by enhancing the cell proliferation rate through stimulation of cell cycle progression. In prostate carcinogenesis, Nampt augmented PC3 cell proliferation by activating the MAPKs ERK-1/2 and p38 signaling pathways[48]. It is intriguing to identify the Nampt receptor and its signaling mechanism in BC pathogenesis; (3) Nampt may play a pro-angiogenic, invasive and metastatic role. Increased Nampt concentrations were seen in PBC patients with advanced stage and worse prognosis[40], and high Nampt expression in BC tissues was related to poor survival[42]. Nampt was shown to promote angiogenesis *via* activation of MAPK ERK-dependent pathway through endothelial fibroblast growth factor-2, and to enhance the VEGF factor *via* MAPK and PI3K/Akt signaling pathways[37,38]. By increasing the expression of *MMP 2* and *9*, and *VEGF* genes, Nampt may contribute to angiogenesis and metastasis in BC[37,38]; and (4) Nampt constitutes a pro-inflammatory adipocytokine linking obesity to PBC. Nampt stimulated several pro-inflammatory cytokines in human mononuclear cells and upregulated the production of IL-1α, IL-6, TNF-α, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 through the pro-inflammatory transcription factor NF-κB[37,38]. Therefore, Nampt may contribute to the pathogenesis of vascular inflammation linking obesity-a state of low grade inflammation- to PBC.

FUTURE DIRECTIONS AND CLINICAL IMPLICATIONS

Obesity, its metabolic complications and BC have become major global health issues. Obesity increases the risk of BC incidence and mortality[7-9]. Imbalanced expression of adipokines could be involved in the association between obesity and BC, mainly postmenopausal. While understanding the connection of adipokines with BC might provide potential preventive and therapeutic strategies, lifestyle amelioration remains the most important component in preventing obesity-related PBC. Modulating adipokines might be a particularly attractive goal for BC prevention, specifically in overweight/obese women. A number of behavioral and drug interventions are associated with favorable modulation of main adipokines. Physical exercise, adoption of a balanced diet, weight reduction and bariatric surgery for morbidly obese women may augment plasma adiponectin and lower plasma leptin, resistin and Nampt concentrations reducing thus the risk of developing BC. Pharmacologic agents such as metformin or PPAR-γ agonists that increase adiponectin and decrease circulating resistin could be at the forefront of therapeutic modalities for BC[3]. Indeed, diabetic BC women using metformin experience a higher rate of pathological complete response to neoadjuvant chemotherapy than those taking other anti-diabetic treatments[49]. Lipid-lowering drugs such as statins and niacin, vitamin C and D supplementation, folic acid, oleic acid, calcium-channel blockers may also significantly modulate serum adipokines levels[3]. Some nutraceuticals such as curcumin have also been reported to decrease resistin and Nampt[5].

Adiponectin use as a direct therapeutic drug in not available due to the difficulty in converting the full-size protein into a viable drug[3]. Nevertheless, ADP 355, an adiponectin-based short peptide mimicking adiponectin's action sup-

pressed *in vivo* the growth of orthotopic human breast cancer xenografts by 31%[3]. AdipoR1/R2 agonists but also strategies to increase adiponectin receptors and upregulate adiponectin signaling pathway may provide novel therapeutic approaches for insulin resistance, t2DM and BC[3]. Targeting the inhibition of adipokines that are elevated in BC, either by antibody neutralization, antisense oligonucleotides or by antagonism of their receptor, could be an effective therapeutic strategy in BC, particularly in downregulating the tumor inflammatory microenvironment. In addition, if Nampt and resistin receptors, and their induced signaling pathways are clearly mapped out, inhibition of downstream targets may be further evaluated in BC therapeutics.

Recent data suggest that adipokines could be promising BC biomarkers in conjunction with other tumor markers, that reflect advanced stage, adverse prognosis and inflammatory state. Nevertheless, further studies are needed for the development of reliable and "user friendly" laboratory techniques (*e.g.*, enzyme-linked immunosorbent assays) to assess adipokines, their isoforms and other adipokine-like molecules[3], as well as their pathophysiologic relevance. There are still a number of unanswered practical questions in the clinical laboratory. What circulating levels of adipokines should be considered adnormal and what are their optimal levels for BC prevention? In the future, international standardization of levels and methodology procedures is also needed before full commercialization of adipokines as potential monitoring tools in BC.

Further evidence from mechanistic, larger prospective and longitudinal studies is required to determine exactly if and when adipokine concentrations are altered in BC and whether adpokines *per se* and/or other hormonal parameters connecting obesity with BC may be associated with BC etiopathogenesis. The hypothesis could also be tested by determining whether adipokines genetic polymorphisms are associated with BC prevalence. Moreover, the epigenetic regulation of the adipokine genes remains a *Terra incognita*.

In summary, there is evidence for a strong link between obesity-driven chronic inflammation, insulin resistance, adipokines and BC. Advances in adipokine research may hold promise for the use of adipokines as potential prognostic markers and therapeutic targets. At the same time, several issues remain to be clarified in order to unmask the ontological role of some adipokines in BC pathophysiology. Reversing obesity-associated inflammation and dysfunction of the adipose tissue by lifestyle interventions such as weight reduction, physical activity and dietary modifications may present a clinically relevant contribution to decreasing BC risk or progression. Advances in the field of translational investigation may lead to tangible benefits to overweight/obese women who are at increased risk for BC.

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BRIEF ARTICLE

Expression of matrix metalloproteinases 9 and 12 in actinic cheilitis

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Abstract

AIM: To investigate the role of matrix-degrading metalloproteinases 9, 12 (MMPs), as mediators of functional connective tissue damage in actinic cheilitis.

METHODS: Thirty five formalin-fixed, paraffin embedded specimens of actinic cheilitis, and twelve specimens of normal lower lip vermillion, which were obtained by the archives of the Department of Oral Medicine and Maxillofacial Pathology, were examined. From each block, 5 μm thick sections were cut and routinely stained with Hematoxylin and Eosin. Immunohistochemical studies were performed on 4-μm thick sections of formalin-fixed paraffin embedded actinic cheilitis lesions and of normal lower lip vermillion, for MMP-9 and MMP-12 in serial sections of our specimens. Appropriate positive and negative controls were performed to confirm the specificity of the staining reaction. MMP immunohistochemistry was evaluated using a semiquantitative immunoreactive score.

RESULTS: Haematoxylin and eosin staining revealed in actinic cheilitis lesions atrophic stratified squamous cell epithelium, or focally and irregularly hyperplastic of variable thickness, in some areas was observed marked keratin production. Varying degrees of epithelial dysplasia were noticed with a wide spectrum of change within the same specimen. Characteristic was the appearance of chronic inflammatory infiltration, and a band of amorphous acellular, basophilic change like solar elastosis (elastin replacement of collagen). In normal lower lip specimens weak and scanty positive expression of MMP-9 and MMP-12 was observed. Anti-MMP-9 antibody showed a weak reaction, in actinic cheilitis lesions, focal in the elastotic material, in chronic inflammatory cells and mostly in macrophages and neutrophils. Strong and in some cases diffused immunohistochemical expression of MMP-12 was detected in actinic cheilitis lesions in the areas of the fragmented, distorted and thickened elastic fibers. MMP-12 was also expressed in chronic inflammatory cells and mostly macrophages. MMP-12 was significantly higher in actinic cheilitis specimens compared with the normal lower lip specimens (P = 0.0029).

CONCLUSION: Our results suggest that especially MMP-12 may play an important role in remodeling events occurring in the connective tissue during long-term exposure to sunlight in the actinic cheilitis lesions.

Key words: Actinic cheilitis; Metalloproteinase-9; Metalloproteinase-12; Immunohistochemistry

Core tip: Actinic cheilitis is a chronic inflammatory disorder affecting mainly the lower lip, and it is caused by chronic and excessive exposure of the lips to the ultraviolet radiation in sunlight. Histologic features for actinic cheilitis include epithelial and connective tissue alterations. The matrix metalloproteinases (MMPs) are a large family of zinc-dependent endopeptidases, which are responsible for a wide range of proteolytic

events. The aim of this study was to investigate the role and the expression of MMP-9, -12 as mediators of functional connective tissue damage in actinic cheilitis.

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INTRODUCTION

The matrix metalloproteinases (MMPs) are a large family of zinc-dependent endopeptidases, which are capable of digesting extracellular matrix and basement membrane components. The MMPs are responsible for a wide range of proteolytic events. Under physiological conditions, the MMPs are involved in many processes including cell proliferation, differentiation, migration, apoptosis, and angiogenesis. Moreover the MMPs are often up-regulated in groups forming activation cascades both in the inflammatory and malignant diseases[1].

MMP-9 is a member of the matrixin family of metallo-endopeptidases. MMP-9 is historically referred as gelatinase B because of its ability to cleave gelatin, a denatured form of collagen, *in vitro*[2]. MMP-9 differs from other MMPs because it contains three fibronectin type Ⅱ repeats that have high binding affinity for collagen. These repeats are thought to mediate binding of MMP-2 and -9 to collagen[3]. This binding interaction brings the catalytic pocket of the MMP in proximity to collagen, thereby enhancing its rate of hydrolysis[4]. Despite the above mentioned biochemical interactions, MMP-9 is also able to cleave a number of other proteins and may have a rather wide range of physiologic substrates[5].

Much of our understanding of the biological function of MMP-9 comes from the study of mice lacking this gene[6]. Studies on this mice indicated that MMP-9-deficient mice are resistant to dermal blistering in a bullous pemphigoid model, an effect that has been attributed to the inability of these mice to cleave the SEPRIN a-1 proteinase inhibitor[7]. Other work in the same model for multistage carcinogenesis indicated that MMP-9 is part of the angiogenic "switch" that is essential for tumor growth[8]. Furthermore other reports suggested that MMP-9 may play a role in inflammation in the nervous system[9].

Human macrophage metalloelastase (MMP-12) is an MMP that was initially found in alveolar macrophages of cigarette smokers[10]. On a molar basis, it is clearly the most active MMP against elastin[11]. MMP-12 has a broad substrate specificity, however being able to degrade also type Ⅳ collagen, laminin, fibronectin vitronectin entactin, heparan and chondroitin sulfates[12]. *In vivo* MMP-12 has been shown to participate in the degradation of elastic fibers in the pathogenesis of atherosclerosis and in emphysema[13]. MMP-12 mRNA and protein are also expressed by accumulations of macrophages in granulomatous skin disorders[14].

In addition to degrading elastic tissue, MMP-12 has been shown to aid macrophage migration in other tissues; macrophages from MMP-12 deficient mice are unable to penetrate reconstituted basement membranes (BMs) *in vivo* and *in vitro*[15]. Additional findings on MMP-12 expression in macrophages under the shedding intestinal epithelium in inflammatory bowel diseases support its role in the degradation of BM components. Furthermore, MMP-12 expression may also be associated with macrophage migration through BM in certain inflammatory skin diseases such as dermatitis herpetiformis or pityriasis lichenoides[14].

Long term sun exposure causes to the lower lip vermillion a lesion known as actinic cheilitis (AC), the histologic examination of which reveals loss of collagen with concomitant accumulation of elastotic material[16].

The aim of this study was to investigate the role and the expression of MMP-9, -12 as mediators of functional connective tissue damage in actinic cheilitis.

MATERIALS AND METHODS

Sample collection

Biopsies of the lower lip vermilion from 35 patients with AC (32 males and 3 females, mean age 54.7 ± 12.1) were obtained from the archives of the Department of Oral Medicine and Maxillofacial Pathology. Normal lip vermilion biopsies from 12 patients (11 males and 1 females, mean age 53.6 ± 14.8) were used as controls. Cases of squamous cell carcinoma of the lower lip with previous diagnosis of AC were excluded. The demographic information of the patients, their occupation (sun exposure), as well as their habits of smoking and drinking are presented in Table 1.

The tissue specimens were fixed in 10% buffered formalin for a maximum of 24 h, followed by paraffin embedding. From each block, 5-μm thick sections were cut and routinely stained with hematoxylin and eosin (HE). Histopathological characteristics that were evaluated included the presence and the degree of epithelial keratinization, the thickness of the spinous cell layer, the presence and the degree of epithelial dysplasia, connective tissue changes, such as inflammation and elastosis. All study participants gave informed consent, the whole study was approved by the appropriate ethics committee and was performed according to Helsinki Ⅱ Declaration.

Immunohistochemistry and semiquantitative analysis

Immunohistochemical studies were performed on 4-μm thick sections of formalin-fixed paraffin embedded actinic cheilitis lesions and of normal lower lip vermillion, which were mounted on 3-amino-propyltriethoxy-silane (organo-silane; Sigma, United Kingdom)-coated slides dewaxed in xylene and rehydrated in graded ethanols according to standard procedures. Endogenous peroxidase

Table 1 Demographic data of patients with actinic cheilitis

	n (%)
Males	32 (91.42)
Females	3 (8.57)
Mean age (yr)	54.7 ± 12.1
Cigarette smokers	23 (65.7)
Drinking	15 (42.85)
Out-door occupation	24 (68.57)
Out-door occupation + smoking	18 (51.42)
Out-door occupation + drinking	13 (37.14)
Out-door occupation + smoking + drinking	22 (62.85)

Table 2 Immunoreactive score for matrix metalloproteinases immunohistochemistry

Score	Percentage of immunopositive cells (PS)	Staining intensity
0	0	Negative
1	< 10%	Weak
2	10%-50%	Moderate
3	> 50%	Strong

PS: Positive stained.

Table 3 Applied primary antibodies and staining conditions

Antibody	Source	Dilution	Antigen retrieval	Incubation time
Anti-MMP-9 NCL-MMP9-439	Novocastra /Leica	1:50	Microwave	Room temperature 1 h
Anti-MMP-12 ab52897	Epitomics	1:50	Microwave	Room temperature 1 h

MMP: Matrix metalloproteinase.

activity was blocked by pretreatment of slides with 3% hydrogen peroxidase for 10 min. Lyophilized Mouse Monoclonal Antibody Matrix Metalloproteinase-9 (NCL-MMP9-439) (Novocastra) was used as a primary antibody for the MMP-9, and Rabbit Monoclonal Antibody Matrix Metalloproteinase-12 (ab52897) for the MMP-12 respectively. Slides were incubated with the primary antibodies diluted in Tris -buffered solution (50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.4) under conditions as described in Table 2 followed by incubation with Dako Envision™ Peroxidase (Dako Diagnostica), for 30 min at room temperature. For visualization of the antigen 3,3'-diaminobenzidine tetrahydrochloride (Dako) was added for 20 min at room temperature. Slides were counterstained with Mayer's haematoxylin. The applied antibodies and the staining conditions are summarized in Table 3. Appropriate positive and negative controls, including the use of an irrelevant antibody, and the omission of various layers, were performed to confirm the specificity of the staining reaction.

MMP immunohistochemistry was evaluated using a semiquantitative immunoreactive score from 0 to 6 based on the percentage of positive stained cells (PS) (0-3 points) and their staining intensity (SI) (0-3 points), as described in previously established protocols[17], and shown in Table 2. The total score (TS) was calculated by adding the PS and SI scores, and the mean of the TS was used for the statistical analysis.

Proportion scoring was performed only if the intensity of the cells staining was more than that of the internal controls limiting errors in semiquantitation as a consequence of nonspecific background staining. Sections were examined by two of the authors independently of each other. The slides then were reviewed by the examiners as a group and discussion was occasionally necessary to establish uniformity.

Statistical analysis was performed on the immune scores derived, by using the Mann-Whitney test and the statistical package SPSS 20.0 (Statistical Package for the Social Sciences) (Chigago Illinois, United States) for Windows 7.

Table 4 Histopathological characteristics in 35 patients with actinic cheilitis *n* (%)

Histopathological characteristics	Negligible	Mild	Moderate	Severe
Increased thickness of keratin layer	4 (11.42)	13 (37.14)	11 (31.42)	7 (20)
Parakeratosis /orthokeratosis	12 (34.28)	2 (5.71)	5 (14.28)	16 (45.71)
Increased thickness of spinous cell layer	8 (22.85)	12 (34.28)	9 (25.71)	6 (17.14)
Atrophy of the spinous cell layer	0	0	2 (5.71)	0
Epithelial dysplasia	3 (8.57)	11 (31.42)	13 (37.14)	8 (22.85)
Connective tissue inflammation	3 (8.57)	11 (31.42)	12 (34.28)	9 (25.71)
Perivascular inflammation	17 (48.57)	7 (20)	6 (17.14)	5 (14.28)
Elastosis	1 (2.85)	9 (25.71)	15 (42.8)	10 (28.57)

RESULTS

HE staining revealed in actinic cheilitis lesions atrophic stratified squamous cell epithelium, or focally and irregularly hyperplastic of variable thickness, in some areas was observed marked keratin production (Figure 1). Varying degrees of epithelial dysplasia were noticed with a wide spectrum of change within the same specimen. Characteristic was the appearance of chronic inflammatory infiltration, and a band of amorphous acellular, basophilic change like solar elastosis (elastin replacement of collagen). The histopathological findings of actinic cheilitis patients are presented in Table 4.

In normal lower lip specimens minimal to negative expression of MMP-9 (Figure 2A) and MMP-12 (Figure 2B) was observed.

The negative controls confirmed the specificity of the staining reaction (Figure 3).

Anti-MMP-9 antibody showed a weak reaction, in actinic cheilitis lesions, focal in the elastotic material, in chronic inflammatory cells and mostly in macrophages and neutrophils (Figure 4A).

Table 5 Immunoscores of matrix metalloproteinases expressed as mean total score ± SD

	MMP-9	MMP-12
Normal lower lip	1.11 ± 0.35	1.56 ± 0.69
Actinic cheilitis	1.33 ± 0.47	4.89 ± 0.86
Normal lower lip/actinic cheilitis	NS	[1]P = 0.0029

[1]Significant over-expression in actinic cheilitis lesions compared with normal lower lip. NS: Not significant; MMP: Matrix metalloproteinase.

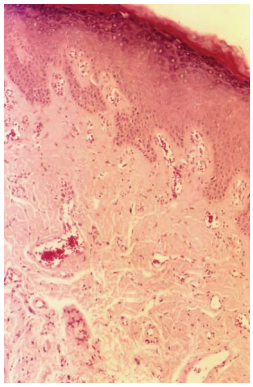

Figure 1 Hematoxylin and eosin staining of actinic cheilitis lesion presenting epithelial hyperplasia, drop-shaped rete ridges, mild inflammatory infiltrate, vasodilatation, and elastosis (Original magnification × 100).

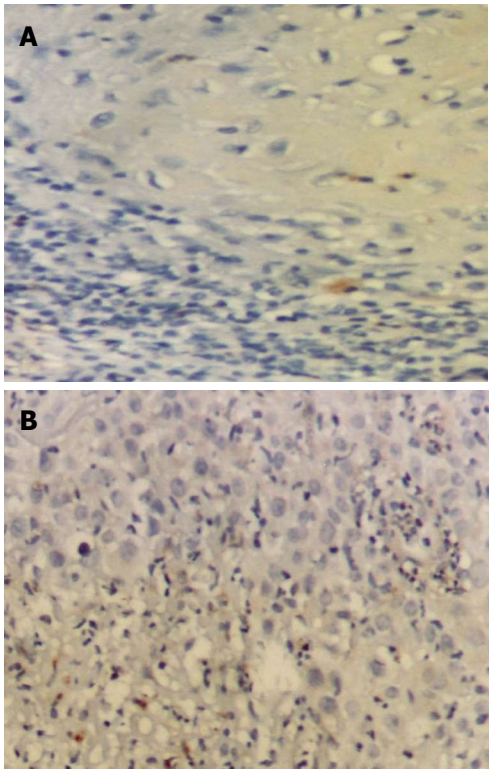

Figure 2 Minimal to negative immunostaining (Original magnification × 200). A: Matrix metalloproteinase (MMP)-9 in normal lower lip specimen; B: MMP-12 in normal lower lip specimen.

Furthermore strong and in some cases diffused immunohistochemical expression of MMP-12 was detected

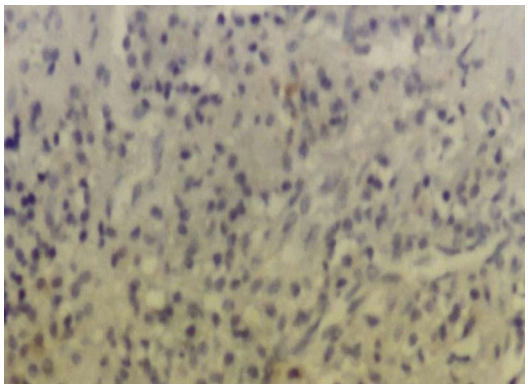

Figure 3 Negative control specimen of the lower lip specimen (Original magnification × 200).

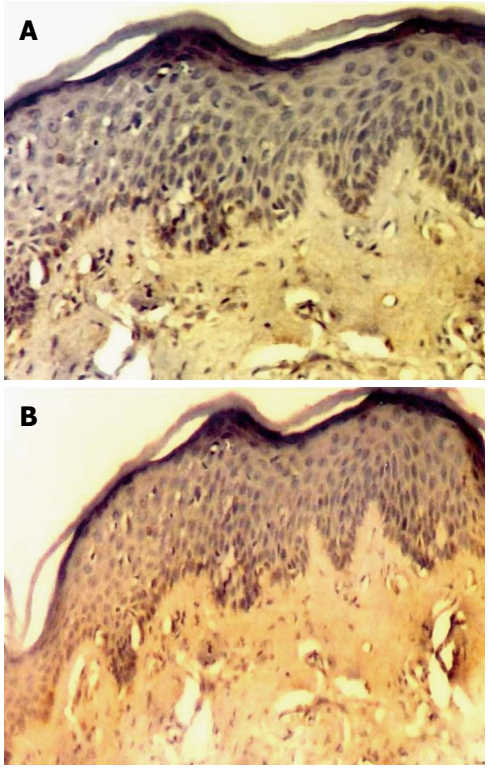

Figure 4 Weak immunostaining of matrix metalloproteinase-9 (A, Original magnification × 100), and strong immunoexpression of Matrix metalloproteinase-12 (B, Original magnification × 100) in actinic cheilitis lesion.

in actinic cheilitis lesions (Figure 4B), in the areas of the fragmented, distorted and thickened elastic fibers. MMP-12 was also expressed in chronic inflammatory cells and mostly macrophages (Figure 5).

MMP-12 was significantly higher in actinic cheilitis specimens compared with the normal lower lip specimens (P = 0.0029). The immune scores of MMPs expressed as mean total score are presented in Table 5.

DISCUSSION

Chronic exposure to ultraviolet (UV) radiation causes degenerative alterations to the lower lip vermillion, clini-

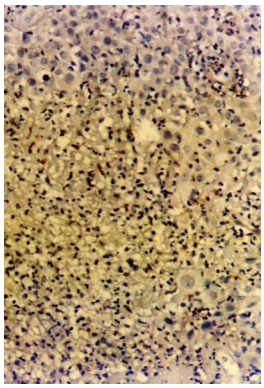

Figure 5 Strong immunostaining of matrix metalloproteinase-12 in chronic inflammatory cells in actinic cheilitis lesion (Original magnification × 200).

cally characterized by erosions and atrophy[18]. The major histological changes in actinically damaged lip are the accumulation of basophilic fibers in the upper part of the connective tissue, referred to as basophilic degeneration or as actinic elastosis[19]. Previous histological and biochemical studies on the nature of the accumulated fibers have demonstrated that altered elastin is the primary component of actinic elastosis[20].

Disappearance of normal elastic fibers is a feature of many skin diseases and actinic cheilitis[21]. Little research has been done, however, to reveal the pathomechanisms behind this phenomenon. Elastin is critical to the structural integrity of a variety of connective tissues. Elastin is highly resistant to proteolytic degradation, but several enzymes capable of solubilizing this matrix constituent have been found. Because of their role in tissue remodelling at sites of inflammation and injury, inflammatory cells have been of particular interest for their elastase activity[20].

The participation of several proteases in photoaging has been reported. It has been reported that in neutrophil elastase -deficient mice, elastic fibers in the skin are unaffected by UVB irradiation, whereas an increase in elastic fibers occurs in normal mice[22]. It has been demonstrated that multiple exposures of the skin to UV radiation result in elevated levels of MMPs *in vivo* and *in vitro*[23]. These previous findings suggest that the elevated expression of certain MMPs may be associated with the elastotic material in sun-damaged skin.

Histologically, photoaging causes accumulation of so-called elastotic material, composed of elastin and versican, in the upper and mid connective tissue[24]. This is accompanied by degeneration of surrounding collagenous meshwork, but due to the inability of MMP-12 and MMP-7 to degrade fibrillar collagens, they probably do not participate in that event.

We found abundant immunostaining for MMP-12 in the areas of abnormal elastotic fibers in actinic cheilitis lesions. Furthermore we have not find any immunoreactivity for MMP-12 in normal lower lip vermillion specimens, suggesting that MMP-12 does not bind to normal elastin or that in healthy lower lip vermillion no abnormal accumulations of macrophages exist, as possible sources of MMP-12. Our findings are in accordance to previous immunohistochemical study for the accumulation of MMP-12 in actinic damage skin[25].

It has been proposed that the elastotic material accumulating in photoaged skin results from direct UV-mediated damage to elastotic fibers and fibroblasts[26]. MMP-12 possibly takes part in a reparative remodeling process by trying to cleave this abnormal elastotic material or fibrillin[27]. Granulocyte-macrophage colony stimulating factor, induced at least in keratinocytes by UV light, is able to upregulate MMP-12 production by macrophages[28], and could thus be one of the candidate cytokines to stimulate MMP-12 in solar damage. UV irradiation causing abnormal changes in elastin might lead to accumulation of macrophages that try to cleave both abnormal elastin by secreting elastases as well as activate elastin/collagen synthesis by releasing, *e.g.*, transforming growth factor (TGF)-β[29]. Interestingly, abundant staining for latent TGF-β binding protein-1 and TGF-β colocalize with MMP-12 staining in solar elastosis and keratosis[30]. TGF-β is not likely to upregulate MMP-12 expression, as it usually downregulates MMP function. We cannot however exclude that MMP-12 participates in the proteolytic release of this growth factor from the extracellular matrix. TGF-β could further augment the deposition of abnormal elastin, which is unable to assemble into functional elastic fibers due to influence of proteolytic enzymes and UV radiation.

Elevated expression of MMP-9 in UV irradiated skin has been demonstrated previously[31]. Recently it has been reported that MMP-9 was strongly expressed in SCCs of the lip and moderately expressed in ACs and control samples[32]. Whereas another study detected positive expression of MMP-9 in actinic cheilitis lesions with no statistical differences in the pattern of expression in comparison with squamous cell carcinomas[33]. Despite the above mentioned previous findings, in our study we were not able to detect strong immunoreactivity for MMP-9 in elastotic material in actinic lesions. Our findings are in accordance with similar findings for MMP-9 in actinic elastosis of sun damaged skin[21]. This may possibly be because enhanced expression of MMP-9 does not lead to the accumulation or the incorporation of MMP-9 in the elastotic material. MMPs are secretary enzymes and cannot be detected in the extracellular spaces in normal tissue. In addition, cells potentially producing MMP-9 are limited to keratinocytes, monocytes, alveolar macrophages, PMN leukocytes and SV40-transformed human lung fibroblasts, and secretion of this enzyme is not detected in any normal cell strain of fibroblast origin[34]. This restricted distribution of potential MMP-9 producing cells may explain the diminished immunoreactivity of MMP-9 in elastotic materials in actinic cheilitis.

In conclusion, our findings suggest that enhanced MMP-12 expression was detected in abnormal elastic fibers in actinic cheilitis lesions. Thus MMP-12 may play a

role in the remodeling events occurring in the connective tissue during long term exposure to sunlight in actinic cheilitis.

COMMENTS

Background

Actinic cheilitis is a common inflammatory disorder caused by solar ultraviolet radiation that affects the lip vermilion. The lesion is potentially malignant and may transform into squamous cell carcinoma. The progression of actinic cheilitis is associated with the expression of matrix metalloproteinases (MMPs).

Research frontiers

The MMPs are a large family of zinc-dependent endopeptidases, which are often up-regulated in groups forming activation cascades both in the inflammatory and malignant diseases. However, the role of MMPs is not entirely clear in actinic cheilitis lesions. In this study, enhanced MMP-12 expression was detected in actinic cheilitis lesions.

Innovations and breakthroughs

Recent and previous reports investigated the presence of Metalloproteinases in a spectrum of preinvasive and invasive neoplastic epithelial lesions that included actinic cheilitis and squamous cell carcinoma. This is the first study to focus specifically in actinic cheilitis lesions and to demonstrate that the alterations in MMP-12 expression may play a role in the remodeling events occurring in the connective tissue during long term exposure to sunlight in actinic cheilitis.

Applications

By understanding how metalloproteinases and especially MMP-12 are induced and by preventing extracellular matrix damage and activation of MMPs, and inhibition of MMP expression (*e.g.*, by retinoids) and activity (*e.g.*, by natural and synthetic inhibitors), this study may represent a future strategy for therapeutic intervention in the treatment of actinic cheilitis.

Terminology

MMP-9 is a member of the matrixin family of metallo-endopeptidases. MMP-9 is historically referred as gelatinase B because of its ability to cleave gelatin, a denatured form of collagen, *in vitro*. MMP-12 is able to degrade extracellular matrix components such as elastin and is involved both in inflammatory and malignant diseases.

Peer review

The authors investigated the role and the expression of MMP-9, -12 in actinic cheilitis lesions. Enhanced immunohistochemical expression of MMP-12 was detected in actinic cheilitis lesions. The results are interesting and suggested that MMP-12 may play a role in the remodeling events occurring in the connective tissue during long term exposure to sunlight in actinic cheilitis.

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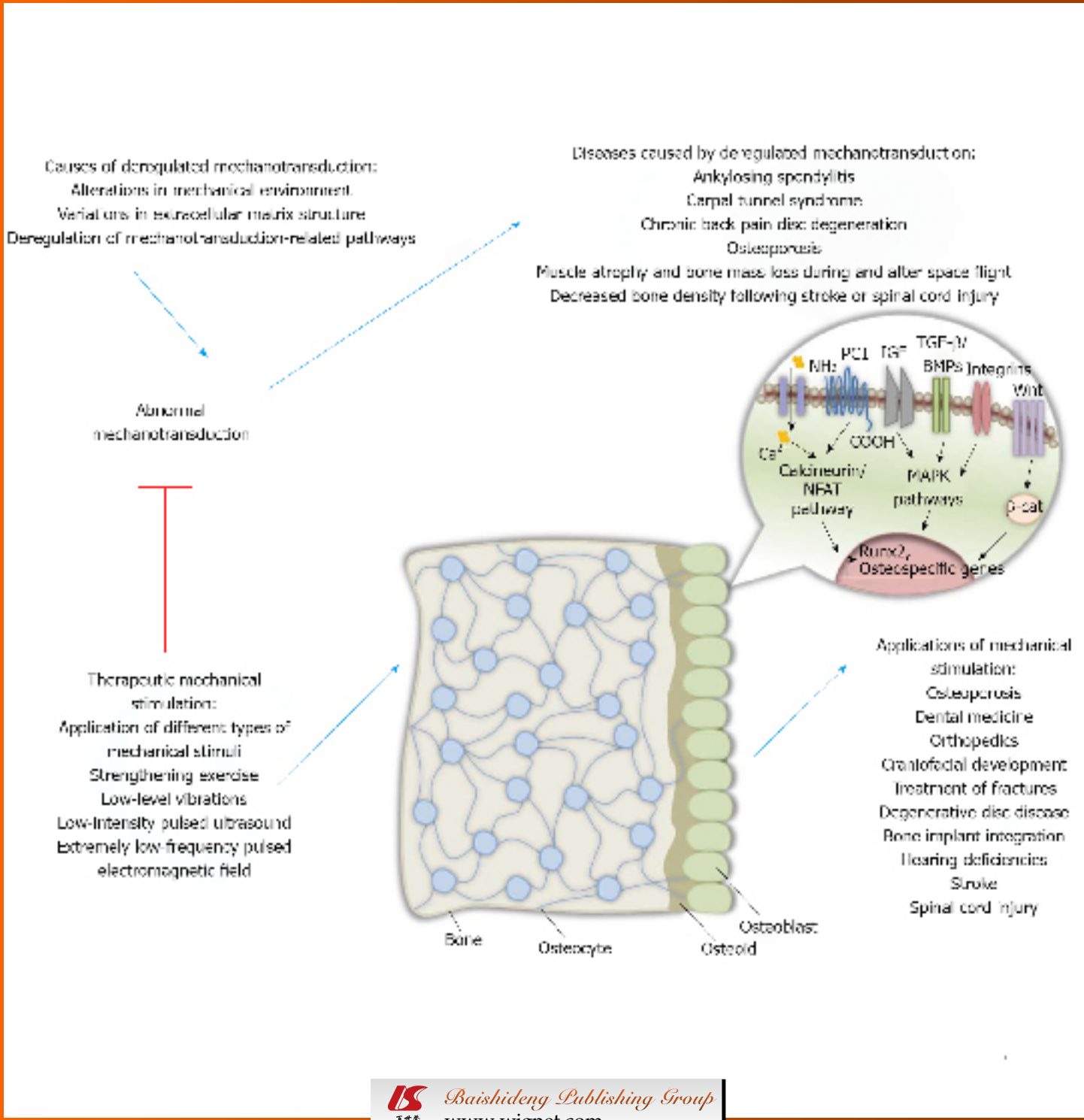

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EDITORIAL

Why not change classical treatments for glioblastoma in elderly patients?

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Abstract

In consideration of the poor results obtained with conventional treatments, a review of alternative treatments for elderly patients with glioblastoma was researched in this study. The proposal considers the elimination of human cytomegalovirus, modifying the immune response, arresting growths, blocking some signaling pathways, and modulating the effects of oxygen reactive species.

Key words: Glioblastoma; Treatment; Elderly patients; Glioma; Glioblastoma

Core tip: It is necessary to reconsider the treatment of elderly patients with glioma, focusing on their life quality when conventional treatment is used, such as, chemotherapy and radiotheraphy, in review of the fact that these treatments cause the patient further suffering. This is a review of othe therapeutic options, including some phase Ⅰ vaccine trials.

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INTRODUCTION

Glioblastoma (GBM) is the most aggressive and frequent of all brain tumors. Glioblastoma usually appears between the ages of 45 and 70 years[1]. The goal of surgery is to confirm the diagnosis and reduce the effect of the tumor mass[2]. Survival of elderly patients of more than 71 years, with glioblastoma GBM, is poor. Temozolomide (TMZ) and radiotherapy (RT) improves the average patient survival rate by as much as 10 to 13 mo, in patients of more than 71 years[3].

Stereotactic radiosurgery, whole brain radiation therapy, and surgery, in isolation or in combination[4] with gamma knife, cyberknife, LINAC, stereotactic brachytherapy, boron neutron capture therapy and hadrontherapy, amongst other treatments, have been used as ther-

apy[5,6]. Although all these methods have been reported to improve prognostic indices, when under constant observation, the statistical analyzes of survival are not effective. Radiation therapy has considerable limitations, mainly infiltrating glioma characteristics and neuronal damage.

Although the survival rate is greater when using chemotherapy than when not used[7], the difference in the number of months survived is very low, and the quality of life is much lower when radiation is used rather than with no chemotherapy.

Gliomas show tumor-associated antigens, which should be detectable by the immune system, however, there are shortcomings in the elimination of a tumor.

Gliomas lack clear-cut tumor-rejection antigens for immune targeting by $CD8^+$ T-cells and indicate cancer/testis antigens as NY-ESO-1[8,9]. However, other antigens from human cytomegalovirus (HCMV) have been found.

The question is, how can tumor growth be limited without chemotherapy or radiotherapy? Here, some therapeutic options are reviewed, including some phase I vaccine trials, supporting treatment of elderly patients with glioma (Figure 1).

TREATMENT WITH ANTIVIRAL DRUGS

It has been suggested that glioma in elderly patients should be treated as a tumor associated with viral infection[10], this would mean treatment for the elimination of the infectious agent, arresting growths and apoptosis, modifying the immune response and blocking some signaling pathways, which involve metastasis and the modulation of reactive oxygen species (ROS).

Glioma tissues indicate the change in a cascade of a viral protein, typical of replicative HCMV[11], the virus is trophic in glial cells and the HCMV infection remains in between 50% to 90% of adults. The HCMV can be reactivated when there is inflammation and immunosuppression and plays an active role in the pathogenesis of a glioma[12].

In order to clarify the controversy surrounding glioma and HCMV, some researchers have shown the close relationship between HCMV and glioma in the context of mutations related to their existence[13].

In order to reduce the effects of HCMV on the glioma it is possible to use valganciclovir, which targets the DNA polymerase, or the Cox-2 inhibitor celecoxib, and averts HCMV replication by decreasing PGE2 levels[14]. Moreover, infiltrating gliomas in microglia, have been found to be an important source of PGE2, Cox-2 inhibitors and are an alternative, as opposed to glucocorticoids, in peritumoral edema of malignant gliomas[15]. In addition to its anti-inflammatory properties, celecoxib is able to exert a pro-apoptotic effect *in vitro* and *in vivo* in the absence of the action of Cox-2 in malignant glioma cells. In fact, it has developed a variant of this substance, 2,5-dimethyl-celecoxib, which is more potently cytotoxic against these cells[16]. The effect of celecoxib is dependent on the existence of p53[17].

One mechanism that could be used in the treatment of gliomas, is the induction of autophagy. Valproic acid is a potent histone deacetylase inhibitor which induces cell differentiation, growth arrest and apoptosis in gliomas and other cancers. Valproic acid induces autophagy in glioma cells, independently from apoptosis[18].

Chloroquine and quinacrine bind tightly to nucleic acids, in particular CG sequences, and reinforce its structural configuration and preventing mutagenesis[19]. Chloroquine also acts as an immunomodulator through the inhibition of phospholipase A2 and the tumor necrosis factor-α (TNF-α)[20]. Chloroquine improves survival in patients with GBM when added to conventional therapy[21,22].

TREATMENT BY IMMUNOMODULATION

Gliomas show a sequence of events that increase immunosuppressant cytokines, such as interleukin-10 (IL-10), transforming the growth factor-β (TGF-β), prostaglandin E2, inducing regulatory T cells (Treg), and decreasing costimulatory molecules, all results lose the function of effector T cells. Moreover, GBM cells show human leukocyte antigen (HLA) class I molecule mutation. Loss of HLA class I correlate with the grade of tumor and show little response to immunotherapy. NK cells do not have a histocompatibility complex (MHC) restriction. In patients with GBM, the NK cells are depressed, and it has been observed that when NK cells increase there is tumor regression in a recurrent glioma[23]. Glioblastoma stem cells suppress T cell responses in different ways: producing immunosuppressive cytokine that suppress T cell responses and inducing regulatory T cells, which act as a brake on the immune response and eliminate T cells through apoptosis. This is accomplished through the immunosuppressive protein B7-H1 from stem cells, or soluble galectin-3[24]. Gamma-delta T cells (γδ T-cells) are the primary effector cells in the immune response of a high grade glioma[25].

Some GBM subjects have responded similarly in autoimmune diseases, showing anergy to common bacterial antigens, lymphopenia, defective production of antibodies, and abnormal delayed hypersensitivity [26].

In order to modify the immune response in gliomas, the quantity of Tregs, a subclass of lymphocytes with immunosuppressive properties, is increased. It has been noted that indoleamine 2,3-dioxygenase (IDO), which converts tryptophan to kynurenine, increases the activity of Treg, and could be modified by aciclovir[27]. Also, in order to reduce Tregs and improved antitumoral immunity in other tumors, denileukin diftitox is used, which is a recombinant fusion protein of IL-2 and the diphtheria toxin targeting IL-2 receptors (CD25)[28].

Dendritic cells (DCs) have an antigen presentation function, their maturation is critical for the induction of the T cell response. Glioma cells suppress the matu-

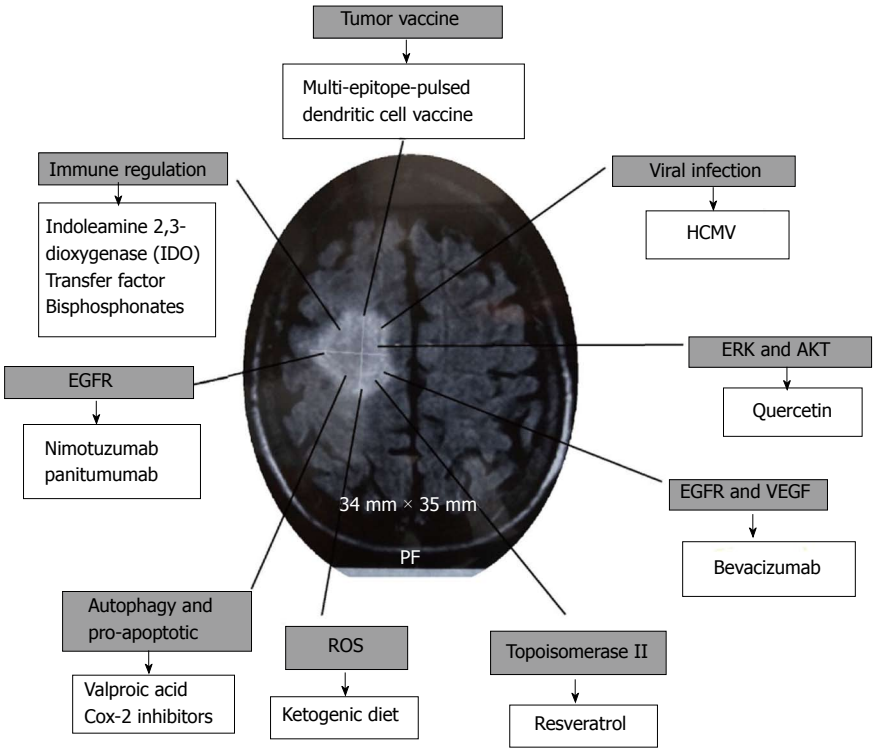

Figure 1 Molecular targets propose for glioblastoma. EGFR: Epidermal growth factor receptor; ERK: Extracellularregulatedproteinkinases; ROS: Reactive oxygen species; VEGF: Vascularendothelialgrowthfactor.

ration of DCs[29], Inmunoferon (AM3) promotes the maturation of DCs derived from human monocytes[30], and reduces the concentration of TNF-α and IL-6[31], IL-6 promoting the invasiveness of glioma cells *via* up-regulation of the STAT3 pathway and fascin-1[32].

Transfer factors are dialyzable products of low molecular weight extracted from the cells involved in the immune system. It has been reported that the transfer factor, in combination with carmustine in experimental malignant glioma, reduces the tumor and increases the $CD2^+$ $CD4^+$, $CD8^+$, NK lymphocytes, and apoptotic tumor cells[33]. γδ T-cells recognize unprocessed non-peptide compounds known as phospho-antigens and are involved through the mevalonate pathway or 1-deoxy-D-xylulose-5-phosphate, in activating the cytotoxic response and releasing cytokine and chemokines[34,35]. γδ T-cells activation can be induced *in vivo* by molecules such as zoledronic acid, which induce the accumulation of the T cell Vγ2. The zoledronic acid induces an effective antitumor response. Aminobisphosphonates play dual roles, apparently acting directly against GBM cells and enhancing antitumor activity from Vγ2 T-cells, which is present up to 75% in γδ T-cells[36]. Otherwise bisphosphonates, such as alendronate, increase γδ T-cell activation by interaction with monocytes circulating or macrophage associated tissues[37].

INHIBITION OF SIGNALING WITH ANTIBODIES AND KINASE INHIBITORS

In glioblastomas there are many genomic alterations especially RTK amplification/mutation, NF1 mutation/loss, NFK1B loss, PIK3R1/PIK3CA mutation, PTEN mutation/loss, TP53 loss, CDK2N2A loss, CDKN2B loss, RB1 mutation/loss, and CDK4 amplification[38].

Heterogeneity in glioblastoma suggests that no therapy can be generalized in different types of GBM. In neural/classical type GBM there are mutations in the epidermal growth factor receptor (*EGFR*) gene. In proneural type GBM frequent mutations occur in p53, in platelet-derived growth factor receptor A, and in isocitrate dehydrogenase 1. Mesenchymal type GBM causes frequent mutation, observed in neurofibromatosis type 1 gene (NF-1)[39].

In neural/classical type GBM, Nimotuzumab could be used, it is a humanized antibody that recognizes EGFR[40] or panitumumab, originally approved for treating colorectal cancer, and it has been used with good results in glioma[41]. In proneural types which have IDH1 mutation, bevacizumab[42] could be used. Bevacizumab is a humanized monoclonal IgG1 antibody that selectively binds with great affinity to human vascular endothelial growth factor. This antibody is being used in phase Ⅲ randomized trials in combination with temozolomide and radiotherapy, and has also been reported to be of benefit in phase Ⅱ studies in recurrent glioblastoma [43]. In mesenchymal type NF-1, therapeutic targets use Ras antagonists and ERK antagonists. Also, mTOR dysregulation and PI3K/PKB/mTOR are central regulators of cell proliferation, growth, differentiation, and survival[44], they could logically be used with resveratrol or quercetin[45]. Treatment with low doses of resveratrol inhibits mono-ubiquitination of histone H2B at K120 in senescent glioma cells[46]. Resveratrol reduces TNF-α induced

NF-κB, and reduces the effect of urokinase plasminogen activator[47]. Resveratrol acts over topoisomerase Ⅱ on one of the enzymes found in highly proliferating cells[48]. Quercetin causes a rapid reduction in phosphorylation regulated to kinase (ERK) and Akt signaling. With quercetin the death of human glioma cells is brought about with a mechanism that involves caspase-dependent down-regulation of ERK, Akt, and survivin[49].

ROS are regulators of mitogen-activated protein kinase (MAPK), a family of serine/threonine kinases. An increase in intracellular ROS participates in autophagic execution[50]. The ketogenic diet reduces oxygen reactive species (ROS) in tumor cells, it also induces a total pattern of reversal in gene expression, compared with non-tumorous tissues[51].

TREATMENT WITH TUMOR VACCINES

Considering that there is a poor immune response to tumor associated antigens (TAAs) various strategies have been proposed to increase the immune response. Amongst them are new experimental options for treatment, for example, cytokine like IL-4, which facilitates an immune response against glioma[52] in a similar way to toll-like receptor (TLR) agonists. One example of this TLR agonist is Imiquimod, which could enhance T-cell responses to intracranial tumors, apart from reducing the number CD4(+)Foxp3(+) cells[53]. Costimulation of B7 molecule[54], blocks the B7-H1/PD-1 pathway with antagonistic antibodies to protect T cell responses[55].

Most immunotherapy attempts have had limited clinical success, with the exception of cellular immunotherapy using dendritic cell vaccines[56]. The multi-epitope-pulsed dendritic cell vaccine can be used for treatment. Dendritic cells are the most potent antigen-presenting cells for naive T cells, and can be obtained ex vivo from blood monocytes[57]. Monocytes are matured with the granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4, or IL-6, prostaglandin E2 (PGE2), IL-1β and the TNF-α[58], to obtain denditric cells. Mature DC (mDC) induces antigen-specific T-cell responses when mDC is pulsed with tumor lysate, cancer stem cells, or peptides from TAAs, as reported by Phuphanich *et al*[59]. These pulsed dendritic cells increase the immune response against tumor cells[59]. Amongst the various TAAs used for pulsed cells are antigens from gliomas or cancer stem cells which are HER2/N, TRP-2, AIM-2 or peptides.

It is more effective if multiple epitopes are used to target and enhance cancer vaccines[60]. The peptides used in the autologous vaccine mDC, could be a combination of peptides, for example, six synthetic class I peptides AIM-2, MAGE1, TRP-2, gp100, HER2/neu, and IL-13Ra2. These were named ICT-107 and were selected from a glioma[58]. This combination of enhanced epitopes is clearly recognised by HLA class I -restricted T cells. This multi-epitope-pulsed dendritic cell vaccine can be administered intradermally at multiple sites.

In the treatment of patients with glioblastoma the use of many forms of therapeutic drugs could cause three main reactions, firstly increasing the brain edema which was a problem for the patient. Secondly, it is believed that brain tumor capillaries could limit the delivery of therapeutic drugs to the brain, and finally, the sum of many therapeutic drugs may easily lead an elderly patient into a delirious state.

There are many regulatory edema molecules in the brain. In the environment of the brain tumor, PGE2, aquaporins, aquaporin 1 (AQP1) and 4 (AQP4) exist. The glioma that infiltrate microglia are an important source of PGE2 and Cox-2, so Cox-2 inhibitors are proposed as an alternative to the use of glucocorticoids in peritumoral edema of malignant gliomas[15].

In short, in order to improve the quality of life in elderly patients with brain tumors, such as glioblastoma, many new treatment options should now be tested.

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EDITORIAL

Anti-cancer potential of litchi seed extract

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Abstract

Polyphenol-rich fruit are believed to be healthy food for humans. Traditional Chinese Medicines (TCMs) from fruit are rich sources of polyphenols and exhibit antioxidant and anti-inflammatory activities, and have been shown experimentally to overcome some chronic diseases, including cancer. The litchi seed is one of the TCMs traditionally used for relieving pain and sweating, and has been revealed in our recent report and other studies to possess rich amounts of polyphenolic species, including flavonoids and proanthocyanidines, and exhibits strong anti-oxidant activity, and could be applied for the treatment of diabetes and cancer. Herein, we review the recent findings regarding the benefits of this TCM in the treatment of human cancer and the possible cellular and molecular mechanisms of the litchi seed.

Key words: Litchi seed; Cancer; Cell cycle; Apoptosis; Traditional Chinese Medicine

Core tip: Litchi seeds possess rich amount of polyphenols and anti-cancer activity, which could be a potential cancer prevention or treatment agent.

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INTRODUCTION

Cancer is one of the most prevalent diseases worldwide, with high morbidity and mortality. It has been accepted that cancer is a progressive disease requiring slow and stepwise development for several years to become a life-threatening disease. Therefore, it is regarded largely as a preventable disease[1-3]. Recent advances in medical techniques have rendered some types of cancer curable, but other cancers are still difficult to cure, even under advanced treatment. Novel detection methods and treatment strategies must be developed[4]. Traditional Chinese Medicine (TCM) has been developed in China for more than two thousand years. TCMs comprise various forms of therapies, such as acupuncture, massage (Tui na), exercise (qigong), and dietary therapy, and the main part of these therapies is herbal medicines. A substantial amount of information from human, animal and cell line studies has provided evidence that consumption of certain herbal products used in TCM can exert chemopreventive effects[5]. Recent studies have revealed that some TCMs or their components exhibit anti-tumor activities towards several types of cancer, such as liver[6], lung[7], gastric[8], nasopharyngeal[9] and colorectal cancer[10]. Several clinically-used chemotherapeutic drugs are derived from TCMs, such as camptothecin, isolated from the "happy tree" (*Camptotheca accuminata*); etoposide, semi-synthesized from

a compound of *Podophyllum emodi var. chinense*; vincristin and vinblastin, isolated from the Madagascar periwinkle (*Catharanthus roseus*); and paclitaxel, purified from *Taxus chinensis*[11,12]. However, severe side effects and drug resistance always lead to therapy failure when using these chemotherapeutic drugs. Other types of substances need to be discovered to overcome these problems. Phenolic compounds have been accepted to be possible chemopreventive and treatment agents for cancer[13-16]. Polyphenols are obtained mainly from plants, and some have been regarded as forming part of a healthy diet for many years, such as tea, soybean, pomegranate, and pine nuts[17]. Litchi seeds have been analyzed and were found to possess rich amounts of polyphenols and exhibit strong antioxidant and inflammatory activities[18,19]. Recently, several studies by our research group and others have further revealed that litchi seed extract exhibits anti-cancer activity towards colorectal, liver, lung, and cervical cancer[19,20]. Herein, we review the recent findings regarding the benefits of this TCM in the treatment of human cancer and the possible cellular and molecular mechanisms of this substance.

LITCHI SEEDS IN TCM

The litchi (*Litchi chinensis*, Sapindaceae) is a tropical fruit tree that originates from southern China and is cultivated in semi-tropical areas world-wide for the delicious taste of the fruit[21]. A TCM pharmacopoeia named the *Compendium of Materia Medica* revealed that litchi seeds could be used to release or loose stagnant complexion, decadent colicky and the woman angry blood pain. Another TCM pharmacopoeia named *Ben-Cao-Yan-Yi* also recorded the analgesic effects of litchi seeds for heartache and intestinal pain. Yet another TCM pharmacopoeia named *Ben-Cao-Bei-Yao* described that the pharmacologic effect of litchi seeds could affect the liver and kidney and remove the stagnant humor, pathogenic cold and the woman angry blood pain. In Chinese folk remedies, Li-Ho-San, the mixture of litchi seeds, cumin and peel, can relieve the pain of a hernia or testicular swelling. Li-Shang-San, the mixture of litchi seeds and the root powder of *Aucklandia* lappa Decne., can treat gastralgia, period pain and postpartum abdominal pain. In summary, litchi seeds are used in China to release stagnant humor and remove chilling, and serve as an analgesic agent that can relieve the symptoms of coughing, gastralgia, neuralgia, and testicular swelling. However, scientific studies to prove the effects of the litchi seeds are still ongoing.

Evidence-based pharmacologic effects of litchi seed extract

In recent decades, several experimental studies have been performed in China on the pharmacologic effects of litchi seeds. Present pharmacological studies are mainly focused on the anti-hyperglycemic effect of litchi seeds. Pan and colleagues indicated that litchi seed extract or its components could repress blood sugar and liver glycogen in a rat non-insulin diabetes mellitus model[22]. Guo *et al*[23] reported that litchi seed water extract could increase insulin sensitivity and reduce the concentrations of blood fasting glucose, triglyceride, leptin and tumor-necrosis factor in a type-2 diabetes mellitus rat model. Li *et al*[24] revealed that litchi seed extract could decrease fasting blood glucose of alloxan induced diabetes mellitus rat to a level equal to that of normal rats. Indeed, many other Chinese reports have demonstrated that litchi seed extract can reduce hyperglycemia and restore the sensitivity to insulin in both type 1 or type 2 diabetes mellitus models. Litchi seeds also contain anti-hyperlipidemic agents. Pan and colleagues reviewed some Chinese studies and reported that litchi seed oil could prevent blood triglyceride and low density lipoprotein in a high-fat-fed rat model[22]. Zheng *et al*[25] revealed that litchi seed extract could inhibit the expression of the surface antigen of the hepatitis B virus. Zhang *et al*[26] found that litchi seed extract showed the protective effect in rat with nonalcoholic steatohepatitis, indicating litchi seed extract could overcome the liver damage from inflammation. In India, the seeds are powdered as an herbal medicine owing to their astringency, and after oral intake they have the reputation of relieving neuralgic pain[27]. These reports together indicated that the litchi seeds exert antihyperlipidemic, hypoglycemic and pain-relieving effects, implying multiple pharmacologic uses in TCM.

RECENT ADVANCES RELATED TO LITCHI FRUIT

Polyphenols in litchi and their pharmacologic effects

Recent studies have revealed that the litchi is a polyphenol-rich fruit. Litchi pericarp is composed of significant amounts of flavonoids and anthocyanins, including procyanidin B2, B4, epicatechin, cyanidin-3-retinoside, cyanidin-3-glucoside, quercetin-3-retinoside and quercetin-3-glucoside, *etc.*[27]. These components carry high free radical scavenging properties and could be used as anti-inflammation, anti-oxidation or anti-cancer agents[28,29]. Wang and colleagues showed that litchi pericarp ethanol extract inhibited the *in vitro* and *in vivo* growth of mouse hepatocellular carcinoma and both estrogen-dependent and -independent human breast carcinoma cells[30,31]. In recent reports, polyphenol compounds from litchi seeds were identified and found to be composed of a variety of proanthocyanidins and flavonoid glycoside[18,20,32]. Xu *et al*[32] revealed that litchi seeds contain litchitannin A1, litchitannin A2, aesculitannin A, epicatechin-(2βfOf7,4βf8)-epiafzelechin-(4Rf8)-epicatechin, proanthocyanidin A1, proanthocyanidin A2, proanthocyanidin A6, epicatechin-(7,8-bc)-4β-(4-hydroxyphenyl)-dihydro-2(3H)-pyranone, and epicatechin. All of these compounds exert strong anti-oxidant activity with ferric reducing antioxidant power values of 3.71-24.18 mmol/g and IC_{50} values of 5.25-20.07 μmol/L toward 2,2-diphenyl-1-picrylhydrazyl radicals. Litchitannin A2 exerts an anti-viral activity against coxsackie virus B3. Aesculitannin A and proanthocyanidin A2 exhibit anti-herpes simplex virus 1 activity[32]. The same research group also identified some flavonoid glycosides in the litchi seed,

Table 1 Sensitivity of various types of carcinoma cells to litchi seed extract (mean ± SD)

Cancer type	Cell line	IC50[1] (μg/mL)
Lung adenocarcinoma	A549	22.49 ± 1.02
Duke'C CRC	Colo 320DM	23.91 ± 2.25
Cervical carcinoma	C33A	24.45 ± 3.36
Duke'B CRC	SW480	26.33 ± 2.80
Oral carcinoma	SCC-25	36.80 ± 3.03
Breast carcinoma	MDA-MB-231	43.70 ± 2.76
Ovarian carcinoma	ES-2	45.46 ± 4.33
Lung large cell carcinoma	H661	52.47 ± 2.83

[1]Cells were cultured in complete medium and then treated with different concentrations of litchi seed extract at 37 ℃ for 24 h. Cells were trypsinized and the viable cells were counted using a hemocytometer under a microscope. The viability was calculated and the concentration with 50% viability was defined as the IC_{50}. CRC: Colorectal carcinoma.

including litchioside D, (-)-pinocembrin 7-*O*-neohesperidoside, (-)-pinocembrin 7-*O*-rutinoside, taxifolin 40-*O*-β-*D*-glucopyranoside, kaempferol 7-*O*-neohesperidoside, tamarixetin 3-*O*-rutinoside, and phlorizin[20]. Some of these compounds appear to exhibit anti-neoplasm activities in lung cancer, cervical cancer and hepatocellular carcinoma cells[20]. Another report from the same group also showed the anti-neoplastic activity of a cyclopropyl-containing fatty acid glucoside from the litchi seed[33]. In our report, rich amounts of flavonoids and condensed tannins [195.3 ± 6.7 and 230.2 ± 3.6 mg catechin equivalent/g of dry mass litchi seed extract (LCSP)] in LCSP were obtained by heating litchi seeds to 70 ℃ followed by 70% ethanol extraction[19]. The LCSP potently inhibits colorectal carcinoma (CRC) cell proliferation. According to these results, the litchi seed could be developed as a potent anti-tumor agent.

Anti-tumor activity of litchi seed: Over the last decade, the researchers were focused on litchi seed and its active components for the anti-tumor activity [34]. Chen and colleagues treated litchi seed water extract or granules to mouse xenograft of mouse Ehrlich ascites tumor cells, sarcoma S180 tumor cells and liver tumor cells and found the reduced tumors[35]. Chen and colleagues found that litchi seed could enhance both innate and acquired immunity in S180 cell xenograft[36]. Lv *et al*[37] demonstrated that litchi seed extract could reduce Bcl-2/Bax ratio in tumor tissues of sarcoma S180 mouse xenograft. Xu *et al*[20] isolated 7 different compounds from the litchi seeds and tested their cytotoxic activity towards human lung (A549), pulmonary (LAC), liver (Hep G2), and cervical (HeLa) cancer cell lines *in vitro* using the MTT colorimetric assay after 72 h. They found that kaempferol 7-*O*-neohesperidoside represented significant cytotoxicity towards all of the test cell lines, with IC_{50} values of 0.53, 7.93, 0.020 and 0.051 μmol/L, respectively. Litchioside D exhibited cytotoxic activity toward LAC and Hep-G2 cells (IC_{50} = 0.79 and 0.030 μmol/L). Taxifolin 40-*O*-β-*D*-glucopyranoside exerted cytotocic effects towards all four cell lines, with IC_{50} values ranging from 1.82 to 17.58 μmol/L. Compared with adriamycin, kaempferol 7-*O*-neohesperidoside represented more cytotoxic effect to these four cell lines[34]. Although the active components of litchi seeds against cancer have been revealed, Weber *et al*[38] suggested that the treatment approaches combined with an overall treatment protocol for the tumor microenvironment and chronic systemic inflammation are likely to provide a more successful outcome than a single tactical approach. According to these findings, they concluded that kaempferol 7-*O*-neohesperidoside, litchioside D and Taxifolin 40-*O*-β-*D*-glucopyranoside might be involved in the anti-tumor activity of litchi seeds.

Our recent report revealed that LCSP exhibits inhibitory effects on two colorectal cancer cell lines, SW480 and Colo 320DM[19]. Recently, we also tested the inhibitory effect of LCSP towards human lung adenocarcinoma cell line A549, lung large cell carcinoma cell line NCI-H661, cervical carcinoma cell line C33-A, breast carcinoma cell line MDA-MB-231, oral carcinoma cell line SCC-25, and ovarian carcinoma cell line ES-2, with IC_{50} values as shown in Table 1. The most sensitive cell lines were A549 cells, CRC cell line Colo 320DM, SW480 and C33A cells, with IC_{50} values of 22.49, 23.91, 26.33 and 24.45 μg/mL, respectively. SCC-25, MDA-MB-231, ES-2 and NCI-H661 were less sensitive towards LCSP treatment, with IC_{50} values of 36.8, 43.7, 45.46 and 52.47 μg/mL, respectively. These results further indicate the anti-neoplastic activity of the litchi seeds. However, the exact cellular and molecular mechanisms of LCSP or its components in the inhibitory effect of cancer cell growth require further investigation. Two possible mechanisms may be the induction of cell-cycle arrest and apoptosis. We reviewed recent evidence showing that LCSP could arrest cancer cells in the G_2/M phase and induce mitochondria-mediated apoptosis in CRC cells.

Possible mechanisms of the litchi seeds

LCSP arrests CRC cells in G_2/M: Our recent study revealed that LCSP-treated Colo 320DM and SW480 cell lines are partly arrested at the G_2/M phase. Cyclins are the key regulatory factors controlling the cell-cycle progression in cancer cells. According to our results, LCSP may disturb cyclin expression to arrest CRC cells at the G_2/M phase. Cyclin D1 is an important regulator of G1 phase progression in many different cell types, including CRC cells[39]. In our study, LCSP treatment decreased the level of cyclin D1 in Colo 320DM and SW480 cells, which was correlated with the cell cycle analysis showing G_2/M phase arrest. Moreover, disruption of cyclin A, a cyclin expressed during the S phase, can block DNA replication during the S phase[40]. Cyclin B is expressed in the G_2 and M phases of the cell cycle. A decrease in cyclin B blocks the cell cycle from progressing into mitosis[41]. Together with alteration of cyclin D1, these findings suggest that the effect of LCSP on the cell division cycle is mainly due to disturbance of G_2/M progression. Our previous studies demonstrated that flavonoids and proanthocyanidin-rich substances such as grape seeds, longan seeds or longan flower extract could increase the numbers of G_1- or S-phase cells in cancer cells[19,42-45].

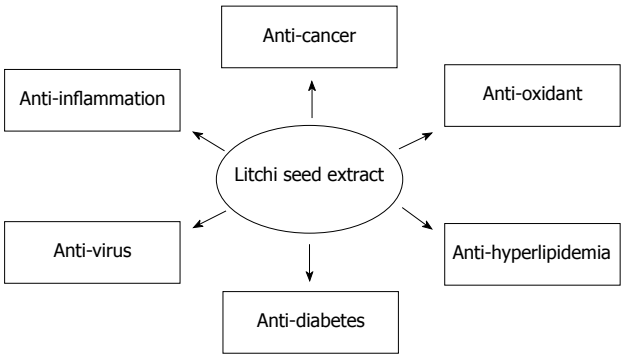

Figure 1 The multiple pharmacologic activities of litchi seeds on anti-cancer, anti-oxidant, anti-inflammation, anti-diabetes, anti-hyperlipidemia and anti-virus.

LCSP-treated CRC cells exhibited significant increases in the number of G_2/M-phase cells, which differed from previous reports. These findings suggested that the anti-proliferative effect induced by flavonoids and proanthocyanidin from naturally-occurring products could occur through a different cell-cycle-controlling mechanism. The different compositions of flavonoids and proanthocyanidin in each natural product might induce different expressions of cyclin proteins to control the cell cycle in CRC cells. Whether the alteration of cyclin D and A levels by LCSP treatment is the only molecular mechanism responsible for the perturbation of the M to G_1 phase of the cell cycle in CRC cells needs further investigation.

LCSP induces apoptosis toward CRC cells: Apoptosis is the elimination process to remove unwanted or damaged cells during development or maintenance of tissue homeostasis in multiple cellular organisms[46,47]. Dysfunction of apoptosis has been implicated as the main mechanism causing many human chronic diseases, such as neural degeneration, autoimmune disease, AIDS and cancer[48]. Many anti-cancer drugs and chemopreventive natural products possess activity to induce cancer cells into apoptosis and concomitantly suppress cancer cell growth[47]. In our recent study, we demonstrated that LCSP could induce CRC cells to undergo apoptosis[19]. The evidence came from the phosphatidylserine translocation to the outer leaflet of the plasma membrane, which was detected using annexin V analysis and activation of the caspase pathway in treated CRC cells. Caspase 3 expression and activation plays a crucial role in polyphenolic compound-induced apoptosis in CRC cells[42,44,49-51]. In our study, the active form of caspase 3 was increased in LCSP-treated CRC cells, further indicating that LCSP-induced apoptosis is mediated by caspase 3 activation. The subsequent increase in cleavage of caspase 3 substrate PARP in LCSP-treated CRC cells confirmed the activation of caspase 3. Involvement of the Bcl-2 family of proteins may play an important role in LCSP-induced apoptosis. The Bcl-2 family members are important mediators of mitochondria-induced apoptosis in cancer cells[46,52,53]. These proteins form multimers, which act as pores in cell membranes, controlling the flow of molecules[54]. Bcl-2 proteins are important mediators of apoptosis in CRC cells[46,47]. Some family members promote apoptosis (*e.g.*, Bax and Bad), while others inhibit it (*e.g.*, Bcl-2 and Bcl-x)[55,56]. Bcl-2 inhibits apoptosis by inhibiting the release of cytochrome c (Apaf 2) and apoptosis inducing factor from the mitochondria to the cytoplasm, and by limiting the activation of caspase 3 by inhibiting its activator protein, Apaf 1[57]. Some studies have suggested that the ratio of Bax:Bcl-2 proteins is the determining factor in transmission of the apoptotic signal[54,58-60]. Previously, proanthocyanidine-rich grape seed extract has been found to suppress the expression of Bcl-2 protein in breast and skin carcinoma cells[61,62]. Additionally, in our previous reports, we also confirmed that longan seed extract increases the Bax:Bcl-2 ratio in CRC cells[44,63]. The Bax:Bcl-2 ratio in LCSP-treated CRC cells increased significantly, indicating the importance of the Bax:Bcl-2 ratio in cancer cell life and death[54,58]. Taken together, our results demonstrated that LCSP-induced apoptosis in CRC cells was mediated by an increasing Bax:Bcl-2 ratio, by which LCSP induced mitochondria-mediated apoptosis in CRC cells. Although the anti-cancer activity of Litchi seed extract has been revealed, the toxicity to normal cells and the possible side effect has not yet been studied. Wan and his coworkers found that oral administration of the maximum dosage of litchi seed water or ethanol extract could not cause acute toxicity to mouse[64]. However, in our recent unpublished result, litchi seed extract exhibited suppression effect on normal small intestinal cells and lung fibroblast cells at more than 50 μg/mL. These results implicated the usage of litchi seed extract at lower dose and the possible toxicity may occur in gastrointestinal and lung system.

CONCLUSION

The litchi is one of the most important fruits in China, economically speaking. The seeds of the litchi were regarded as waste for a long time, and failed to be utilized. However, according to TCM pharmacopoeia, litchi seeds possess multiple pharmaceutical applications. Recent advanced biotechnology and pharmacology techniques have allowed us to gain deeper insight into the functions of this TCM using scientific methods. Litchi seed extract could overcome metabolic diseases such as diabetes mellitus, decrease triglycerides and suppress oxidation and inflammation. Some components of the litchi seed have been identified to be anti-cancer agents against lung, liver, pulmonary and cervical cancer. We further provide data to demonstrate that LCSP is also capable of inhibiting the growth of colorectal carcinoma, lung adenocarcinoma, lung large cell carcinoma, breast carcinoma, oral carcinoma, cervical carcinoma, and ovarian carcinoma cells. All of the pharmacologic effects of litchi seed extract are summarized in Figure 1. The main mechanisms of LCSP are the induction of cell-cycle arrest and apoptosis, at least in colorectal cancer cells, with the molecular mechanisms acting through decreased levels of cyclin D1, A and B1 and alteration of the Bax:Bcl-2 ratio and

activation of caspase 3. However, upstream factors mediating LCSP induction of cell-cycle arrest and apoptosis need further investigation. We found that LCSP treatment could inhibit proliferation in various cancer cells and induce cell-cycle arrest and apoptosis in CRC cells, suggesting its potential as a novel chemoprevention agent for cancer in the future.

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REVIEW

RNA vaccines for anti-tumor therapy

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Abstract

The immune system is able to recognize tumor antigens and this has been the basis for the development of cancer immunotherapies. The immune system can be instructed to recognize and attack tumor cells by means of vaccination strategies. One such strategy involves the delivery of tumor antigen as genetic material. Herewith we describe the use of RNA encoding tumor antigens for vaccination purposes in tumor settings. RNA has features that are interesting for vaccination. Upon transfection, the RNA has no possibility of integration into the genome, and the tumor translated proteins enter the intrinsic antigen processing pathway thus enabling presentation by MHC-I molecules. This can specifically activate cytotoxic CD8 T cells that can attack and kill tumor cells. RNA can be delivered as a naked molecule for vaccination purposes or can be used to transfect dendritic cells. The combination of RNA technology with dendritic cell vaccination provides a powerful tool for cancer immunotherapies.

Key words: RNA; Vaccine; Dendritic cells; Immunization; Cytotoxic T cells; Tumors

Core tip: In this review we discuss the use of RNA encoding tumor antigens for anti-tumor vaccination. RNA has several features that makes it relevant for vaccination purposes. Importantly, the RNA has no possibility of integration into the genome, and the tumor translated proteins enter the intrinsic antigen processing pathway thus enabling presentation by MHC-I molecules thus specifically activating cytotoxic CD8 T. Further, RNA can be delivered as a naked molecule f or can be used to transfect dendritic cells. This combination of RNA technology with dendritic cell vaccination provides a powerful tool for cancer immunotherapies.

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TUMOR IMMUNOLOGY

Cancer is one of the leading causes of mortality in humans and most of the successes obtained battling this disease rely on early prevention even though a gamut of treatments such as chemotherapy, radiotherapy and surgery are available to patients. In view of this situation it becomes necessary to generate innovative approaches for the treatment of this disease. One such strategy entails educating the immune system to recognize and destroy tumor cells. To this end, several immunotherapeutic strategies have been designed and tested in preclinical studies and clinical trials.

Tumors are composed not only by cancer cells, but also by other cellular types such as fibroblasts, endothelial

cells and infiltrating leukocytes that together with extracellular matrix components constitute the microenvironment of the tumor[1]. In recent years the relevance of the tumor microenvironment as a key player in cancer progression has been highlighted and the role of its cellular populations and extracellular matrix components examined. In this context, immune cells play a double edge sword role[2].

On one hand, the protective role of the immune system against tumors has been widely described and indeed the presence of tumor-infiltrating lymphocytes (TILs) has been reported in numerous studies involving melanoma[3], colorectal[4-7], breast[8,9], ovarian[10-16], prostate[17], renal[18], and esophageal carcinoma[19]. These TILs are able to recognize tumors as demonstrated by their capability to get activated by tumor antigens and kill cancer cells *ex vivo*[10,20-22]. Notably, several reports showed that the prevalence of certain T cell populations is associated with a better outcome in different types of cancers. Particularly, studies involving ovarian, non-small cell lung, mesothelioma, colon, and urothelial cancers showed that a high CD8/regulatory T cell ratio among TILs is usually associated with a better prognostic or a better response to antitumor treatment[14,23-28].

On the other hand, the presence of a robust number of regulatory T cells within the TILs, or a CD4/CD8 ratio that favors CD4 T cells, has been associated with a worse outcome or tumor growth in various studies[29-33]. These studies highlight the ability of the immune system to recognize tumors and provide a rationale for pursuing immunotherapeutic approaches, but also underscore the hurdles for its success. Similarly, other tumor-associated leukocytes such as myeloid-derived suppressor cells (MDSCs) can promote tumor growth by modulating the immune response[34]. Indeed, we have previously demonstrated the relevance of the tumor microenvironment in attracting MDSCs by a complement-mediated process[35]. Further, the presence of a subset of splenic dendritic cells (DCs) with the ability to suppress antitumor T cells responses *via* indoleamine 2,3-dioxigenase expression highlights the immunosuppressive role of antigen presenting cells (APCs) in some tumor settings[36]. Notably, leukocyte infiltration can precede the development of a neoplasm, being chronic inflammation a risk factor for the development of cancer[37-39]. Further, inflammatory conditions such as caused by certain types of infections can be involved in the pathogenesis of many human malignancies. For example, gastric carcinomas can arise in a *Helicobacter pylori*-induced gastritis environment[38] or hepatitis B virus/hepatitis C virus can induce hepatocellular carcinomas[39]. Also, chronic but non-infective inflammatory conditions as in the case of smoking-related bronchial cancer can induce carcinogenesis[40]. In the same way, chronic pancreatitis is considered a risk factor for the development of pancreatic cancer, and many of the growth factors involved in tissue remodeling and regeneration in chronic pancreatitis are present in pancreatic cancer[41]. In addition, there is strong evidence that tumor-associated leukocytes can also promote tumor angiogenesis. In particular, infiltrating inflammatory cells secrete a diverse repertoire of growth factors and proteases that potentiate tumor growth by stimulating angiogenesis. We and others have described the capability of APCs such as DCs or macrophages, to collaborate with neoangiogenesis in human cancers and in different mouse tumor models[42-47]. Thus, tumors exhibit an arsenal of mechanisms in order to inhibit an effective immune response.

Collectively, these data indicate that in some settings immunoablative procedures must precede immunotherapeutic treatments. To this end, some studies have suggested that depletion of regulatory T cell populations or tumor-associated leukocytes can enhance the effectiveness of a subsequent immunotherapy[3,48].

TUMOR IMMUNOTHERAPY

The ability of the immune system to recognize and attack tumors relies on the presence of tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs). As recently reviewed by Aly[49], TSAs are expressed only by tumor cells due to mutations in normal cellular genes, or to the expression of viral antigens or normally suppressed oncogenes in cancer cells. On the other hand, TAAs are molecules expressed both in normal and cancer cells but expressed at higher levels by tumors, or expressed by normal cells only during the embryonic state differentiation. For the purposes of the present review, tumor antigens will be named generically as TAAs.

Pioneering studies performed by Rosenberg *et al*[50] in melanoma aimed to activate lymphocytes *in vivo* by treating cancer patients with IL-2. The rationale being that the patients' T cells have the ability to recognize and attack tumors. Indeed, this is the basis for immunotherapies using TILs. To carry out these T cell adoptive therapies, upon purification from tumor tissues, TILs are expanded and activated *ex vivo* using TAAs and are subsequently re-infused into patients[51]. Recent advances in this area involve the generation of TAA-specific T cells by means of genetic recombination. As previously described in detail, chimeric antigen receptor (CAR) T cells are engineered to express the portion of an antibody that recognizes an antigen fused to the T cell receptor signaling region[52]. Thus, they recognize TAA on tumor cells with the specificity of an antibody and they kill them using the cytotoxic machinery of T cells[52]. This circumvents the problem of isolating TILs, which might not be present in all patients or present at very low numbers in tumor samples. Recently, by using CAR T cells, Kalos *et al*[53] were able to completely eradicate cancer cells in patients with advanced leukemia.

Additional immunotherapeutic strategies have been proposed and investigated based on the ability of the immune system to recognize TAAs. One such strategy involves inducing immune responses against TAA by means of vaccination. To this end, TAAs are used as tumor lysates, proteins purified from these lysates, or pep-

tides (derived from tumor protein digests or synthesized *in vitro*). Furthermore, as described below, tumor vaccination strategies also involve the use of apoptotic or necrotic tumor cells as way of delivering the TAAs. These molecules will be recognized *in vivo* by resident APCs, which are key components of the innate immune system. The innate immunity is the first line of defense against pathogens. Cells of the innate immune response include macrophages, granulocytes, DCs, and natural killer cells. Macrophages, B lymphocytes and DCs are generally described as APCs. After ingesting a pathogen, APCs are able to eliminate it through various mechanisms involving enzymatic degradation and the use of reactive oxygen or nitrogen species. APCs detect pathogens through the expression of pattern recognition receptors (PRRs) which are able to recognize conserved pathogen associated molecular patterns (PAMPs). Some of the main PRRs include membrane associated toll-like receptors (TLRs) and cytoplasmic NOD-like receptors[54,55]. DCs are highly effective APCs distributed throughout the body, particularly in immunological organs such as thymus, spleen, lymph nodes and Peyer's patches[56-58].

DC ACTIVATION PROCESS

Immature (non-activated) DCs present in peripheral tissues can detect PAMP-bearing microorganisms through their high expression of cell surface, vesicular and cytoplasmic PRRs[59]. This process leads to the activation of the DCs, which can degrade pathogenic proteins (both recovered from the extracellular space, or from the cytoplasmic pool) and process them into peptides[58]. Antigenic peptide fragments derived from the processed pathogen molecules are the exposed on the surface of the DCs in the context of MHC I or Ⅱ molecules. During this process, an immature DC will undergo "maturation" due to presence of inflammatory cytokines generated by the DC itself, or by other surrounding cells in response to the pathogen or tissular damage. This maturation process entails upregulation of MHC class Ⅱ molecules, costimulatory molecules such as CD40, CD80, CD86; OX40L and the chemokine receptor CCR7. This receptor recognizes the chemokines CCL19 and CCL21 which are constitutively expressed at high levels by lymph nodes[60]. Thus, mature DCs migrate from the sites of antigen capture to the T-cell regions of draining lymph nodes, where they contact naïve or memory T cells. Through interaction with specific cell receptors for antigen on the surface of T lymphocytes, DCs select and activate specific T cell clones with the capability to recognize the presented antigen[58,61,62]. In this way, DCs tie the innate and adaptive immunity, being keystones for the development of antigen specific immune responses.

APCs have different ways of processing and presenting antigens. Typically, antigens that are captured by the phagocytosis or endocytosis are degraded in the lysosomal compartment and peptides are presented by MHC-Ⅱ molecules on the surface of the cells thus interacting and activating CD4 T cells. On the other hand, antigens generated within the cells for example as a result of a viral infection, can be degraded by the proteosome and the peptides presented on the surface of the cell in the context of MHC I molecules[55,61,63]. This strategy selects and activates antigen specific CD8 T cells[55, 61,63]. Notably, DCs have the capability to cross-present antigens[64]. This means that DCs can acquire extracellular antigens, like for example apoptotic or necrotic tumor cells, or tumor lysates and also present them to CD8 T cells in the context of MHC I molecules.

DCS AND ANTI-TUMOR THERAPY

A multitude of preclinical studies and clinical trials have been designed in order to determine the anti-tumor efficacy and safety of DC-based vaccines[65]. The development of a successful DC-based tumor vaccination depends heavily on generating robust and long lasting specific CD4 and CD8 T cell responses[66]. To accomplish this, DCs have been generated from bone marrow precursors in the mouse and mostly from monocytes in humans as we previously reviewed[1]. Different steps in the antigen presentation process have been evaluated such as antigen loading, DC maturation, and delivery route and dose scheme as we have recently reviewed[1]. One strategy for loading DCs with TAAs in the mouse model involves pulsing the cells with peptides derived from tumor antigens[67]. In addition, since TAAs are not well characterized for the majority of tumors, vaccines can be prepared with whole tumor antigens[68,69]. To this end, DCs have been loaded with whole tumor lysates[70], apoptotic or necrotic cells[71] alone or conjugated with TLR ligands[72], antigens coated with antibodies to target them to Fcγ receptors[73] or peptides encapsulated in biodegradable polymers[74]. We have showed that inducing the expression of danger signals in tumor cells by means of replication-deficient or replication-restricted virus appears also to be an efficient method to pulse DCs for vaccination purposes, probably by upregulating danger signals in the tumor cells[71]. Finally, other strategies such as fusing DCs with tumor cells have also been successfully pursued[75]. These fused cells express tumor antigen but had the machinery of the DCs to present these antigens to T cells.

This information regarding DC-based antitumor vaccines pulsing has been translated to the human, where clinical trials have involved, among others, DCs pulsed with peptides[76], whole tumor lysates[77], or fused with tumor cells[78-80]. Other strategies involved pulsing human DCs with apoptotic or necrotic cells[81-90]. As we have previously reviewed[91] controversy exists regarding whether necrotic or apoptotic cells are better for pulsing DCs for tumor vaccination purposes[90,92-94]. Nevertheless, inducing tumor cell death by exposure to ultraviolet-B radiation seems to provide a mixture of apoptotic and necrotic cells suitable for vaccination purposes DCs[95,96].

TAA AS GENETIC MATERIAL

Another vaccination strategy entails delivering TAAs as

the genetic material that encodes their synthesis. Thus, either DNA or RNA carrying the information to synthesize TAAs can be administered to laboratory animals in preclinical studies or to patients under clinical trials with the aim to induce local synthesis of TAAs. In contrast to delivery of TAAs as protein/peptide formulations, the recombinant antigens synthesized in the cytosol of the cells may enter the degradation process of intracellular molecules, yielding peptides that can be directly presented by MHC I molecules hence inducing a robust CD8 (cytotoxic) T cell immune response. To this end, numerous studies have been performed in order to determine the effectiveness of DNA vaccination in tumor settings[49,97,98]. The genetic material can be administered *in vivo* by using different techniques such gene gun, ultrasound, electroporation, cationic liposomes, and nanoparticles[99]. Alternatively, viral vectors can deliver DNA encoding for TAAs directly to the DCs. Viral vectors used to transduce human DCs[100] include recombinant adenoviruses[101-103], poxviruses[104], and retrovirus[100]. Lentiviruses have also been used to induce stable transduction of human hematopoietic stem cells or DCs[105,106]. These vectors have the advantage of infecting non-dividing cells, therefore being excellent tools to express different molecules in terminally differentiated DCs which have lost the capability to duplicate. Moreover, hematopoietic stem cells have been transduced with lentiviruses and then differentiated into antigen-expressing DCs[107]. The full scope of DNA vaccination has been extensively reviewed in the literature and will not be discussed here.

RNA VACCINES

An alternative approach for delivering TAAs as genetic material is the use of RNA for vaccinations. The advantage of RNA vaccination in comparison to DNA vaccination is that there is no danger of genome integration with the latent possibility of oncogene activation, and that there is no need to engineer expression vectors for delivery. On the contrary the expression of the antigens in the context of RNA delivery is transient, and then RNA is very labile as compared to DNA. Both DNA and RNA vaccines in addition to carrying TAAs have the potential to non-specifically stimulate the immune response upon recognition of CPG sequences by TLR9 (DNA) or by activation of TLR3 (RNA). RNA vaccination strategies involve naked RNA delivery or the pulsing of DCs with RNA molecules. Further, both whole tumor RNA or TAA specific RNA have been used as inducers of antitumor immunity.

VACCINES WITH NAKED RNA

Murine studies

Several murine studies describe the use of naked RNA for vaccination purposes. The naked RNA can be administered by injection or delivered intradermally through electroporation[108]. In order to decrease degradation, the RNA has been complexed with histidine-rich cationic polymers and histidylated cationic lipids. In this case, systemic injections of specific synthetic messenger(m) RNA encoding the human melanoma MART-1 TAA complexed with polyethylene glycol ylated histidine-rich polylysine and histidylated liposomes (termed lipopolyplexes) were able to delay the growth of B16F10 melanoma in the mouse model[109]. Notably, intravenous injection of mannosylated liposomes containing mRNA encoding for the EGFP protein proved to be taken up by spleen DCs. Further, when mRNA for MART-1 was complexed into these mannosylated liposomes, a decrease in the growth of B6F10 murine melanoma tumors was observed[110].

Another strategy is to deliver naked RNA that could simultaneously activate the immune response by way of TLR signaling. These kind of vaccines are called "two component" since they deliver TAAs while simultaneously activating the immune response. It has been reported that two component OVA-encoding RNA vaccines containing free and protamine-complexed mRNA induced specific immune responses activating both humoral and cellular immune responses against OVA-expressing tumors[111]. In addition, naked RNA can be injected systemically, or can be administered directly to sites harboring high concentration of immune cells by means of intranodal injection[112,113]. This strategy aims to directly target APCs in the site where they interact with T cells.

An innovative approach to RNA vaccine immunotherapy has been the developing of self-replicating RNA vectors (replicons). These vectors encode for a RNA-dependent RNA polymerase derived from alphaviruses which has the capability to amplify a plasmid-encoded TAA RNA[114]. This increases the availability of TAA RNA and consequently, TAA protein availability. In addition, this counteracts the high degradation that naked RNA is subjected to upon injection. Immunization with RNA replicons encoding for HPV antigens was able to decrease the growth of aggressive TC1 tumors, which carry HPV E6 and E7 antigens[115].

Human studies

Naked RNA vaccinations have been assayed in clinical settings. In particular, naked RNA encoding for several TAAs has been delivered intradermally inducing expression of cytotoxic T cells in cancer patients, together with an improve on the clinical response in some individuals[116,117]. In order to enhance the effectiveness of the transfection process while protecting the RNA from degradation, naked RNA has also been delivered complexed with liposomes in human clinical studies[118]. Further, both in mouse and human studies, adjuvants that target APCs such as FLT3 and GM-CSF have been co-delivered in their protein state or as RNA together with the naked RNA vaccines in order to further activate these cells locally[118]. This strategy aims to induce a robust activation of the transfected DCs *in vivo*, thus potentiating their migratory potential and their ability to induce the activation of T cells capable of recognizing TAAs of interest.

USE OF RNA-PULSED DCS FOR ANTITUMOR THERAPIES

Mouse studies

Foundational studies evaluating the effectiveness of DC-based RNA vaccination in the mouse model and in humans were performed by Dr Eli Gilboa. In 1996 his group was able to demonstrate that murine DCs pulsed with whole tumor RNA were able to induce a robust antitumor immune response in a mouse model of melanoma[119]. Shortly after, they were able to demonstrate the feasibility of this approach in a preclinical setting, inducing specific T cell responses *in vitro* by pulsing human monocyte-derived DCs with the carcinoembryonic antigen (CEA) antigen[120]. Since then, a multitude of studies have built on these successes in order to generate efficient DC-based RNA vaccines.

In animal experimental models, the efficacy of RNA-pulsed DC vaccination has been extensively tested. Collectively, vaccinated animals showed a decrease in tumor growth together with the activation of tumor specific cell-mediated immunity. In particular, murine DCs have been pulsed with whole tumor RNA as a source of TAAs[121-125]. Interestingly, we have previously reported that DCs pulsed with whole tumor RNA are more effective in inducing antitumor immune responses than DCs loaded with equivalent amounts of apoptotic tumor cells[126]. In order to enhance antigen presentation by DCs and the consequent efficacy of the vaccination procedure, DCs have also been pulsed with specific TAA mRNA replicons[127]. As described above, these constructs aim to increase the amount of TAA RNA present in the APCs with the consequent increase in the levels of expression of the antigen.

Other strategies designed to increase the effectiveness of DC-based RNA vaccination entailed pulsing DCs with TAA mRNA together with mRNA of cytokines such as GM-CSF and particularly IL-12[128-131], the rationale being that these cytokines will potentiate the degree of activation of the pulsed DCs.

Alternative strategies focused on enhancing the processing of the nascent TAA in the transfected DCs. To this end, studies pulsing DCs with RNA encoding for TAAs fused with molecules that augment the delivery of the synthesized proteins to the endoplasmic reticulum, TAAs RNA linked with ubiquitin RNA to target the ubiquitin-proteosome pathway, MHC I and Ⅱ pathways by fusion with LAMP1 or DC. LAMP sequences, or with immunogenic helper proteins such as EGFP have been used[97,132-134]. In this way, cytoplasmic TAAs will be more efficiently processed by the ER, increasing the levels of TAAs peptides presented in the context of MHC I molecules on the surface of the DCs.

Finally, others strategies to potentiate the efficacy of DC-based RNA vaccines entail the use of different maturation cocktails or immunostimulatory factors to activate the RNA-pulsed cells. For example, soluble CD40 has been shown to act as an adjuvant for cytokine treatment of RNA-pulsed DCs increasing the generation of cytotoxic T cells in a an experimental model of melanoma[135].

Human preclinical

In order to optimize the likelihood of effective translation into the clinic, human DCs have been prepared from monocytes recovered from apheresis products or by differentiation of $CD34^+$ hematopoietic precursors[136,137]. As above, whole tumor RNA or mRNA can be used to transfect these cells by electroporation or lipofection[137,138]. In addition, RNA recovered from tumor cells lines can be used to pulse human DCs. For example, whole RNA from KL562 leukemia cells was delivered to monocyte-derived DCs by electroporation and lipofection being the transfected RNA degraded within 24 h. Notably, the translated TAA proved to be processed through the MHC-I presentation pathway rather than the endosomal-phagocytic pathway indicating that these DCs could be able to activate CD8 cytotoxic T cells[139]. Interestingly, not only monocyte or hematopoietic $CD34^+$ derived DCs have been tested in RNA vaccination studies. Indeed, DCs directly recovered from hepatocellular carcinoma patients could be efficiently pulsed with whole RNA recovered from hepatic cancer cell lines[140].

It has been determined that better expression of TAAs after transfection with whole tumor RNA is achieved when antisense RNAs are eliminated from the whole tumor RNA preparation[141]. This highlights the need to prepare high quality RNA for transfection studies. Further, although most of DC protocols (both in mouse and human) propose to induce maturation of these cells after RNA transfection, a study suggests that RNA transfection of DCs can also be performed after maturation of these cells[142]. Taking into account studies indicating the viability of cryopreserved mature human DCs[143], this opens the possibility of transfecting DCs right before administration to patients.

Human DCs transfected with tumor RNA have been shown to elicit specific T cell responses *in vitro*. This was demonstrated by their ability to generate TAA specific T cell lines, or by activating *ex vivo* TILs recovered from cancer tissues. For example, DCs transfected with survivin or TERT RNA were used to generate CD8 cytotoxic cell lines with the capability to eliminate tumor cell lines and primary tumors *in vitro*[144,145]. Further, RNA recovered from prostate tumor samples by laser capture microdissection was amplified and used to transfect DCs generated from blood precursors. It was shown that these DCs were able to induce cytotoxic T cells *in vitro*[146].

As described above, mouse studies determined that RNA encoding for TAAs can be engineered to enhance the capability of the DCs to process the nascent antigens. To translate these results into the human setting, DCs generated from human monocytes were transfected with mRNA encoding for the TERT antigen fused with LAMP in order to augment the processing of the TAA upon translation. This strategy induced a robust activation of CD4 T cells specific for TERT as determined in

in vitro studies[147].

Human clinical studies

Several clinical trials have been conducted in order to evaluate the efficacy of DC-based RNA vaccines in cancer patients. In these clinical trials, the vaccines were generated by pulsing monocyte-derived DCs either with whole tumor RNA or specific TAA RNA. Altogether, human clinical studies highlight that the administration of DC-based RNA vaccines is safe and does not induce adverse reactions. For example, in a phase I clinical trial involving acute myeloid patients aiming to generate clinical grade DC vaccines, monocyte-derived DCs were pulsed with *in vitro* transcribed RNA encoding the Wilm's tumor. Then, these cells were injected repeatedly into patients by the intramuscular route[148]. The results of this study indicated that the vaccination scheme was well tolerated by the patients[148]. This was also observed in a clinical trial involving stage Ⅳ malignant melanoma patient[149]. In this case, DCs were pulsed with whole tumor RNA expanded *in vitro* but no positive effect of the vaccination was observed. This is no surprising taking into account the advanced stage of the illness, but nevertheless the study highlights the safety of using this procedure for antitumor therapies. Other studies showed that DC-based RNA vaccination is able to induce specific T cell responses in cancer patients. In particular, in a clinical trial involving relapsed metastatic ovarian cancer patients, DCs pulsed with mRNA specific for folate receptor α were able to induce a large population of effector memory CD8 cytolytic T cells reactive to the antigen upon repeated injections[150]. Similarly, specific T cell responses were observed in colorectal cancer patients receiving several injections of DCs harboring CEA mRNA[151]. In addition, it has been shown that patients vaccinated with DCs transfected with mRNA recovered from autologous melanoma tumor cells were capable of initiating T cell responses specific to antigens encoded by the pulsed APCs[152]. Finally, in order to ensure a robust activation of T cells, strategies designed to deliver the transfected DCs directly to the lymph nodes have been tested. In a phase Ⅰ/Ⅱ clinical trial with melanoma patients it has been shown that upon intranodal administration, DCs electroporated with mRNA encoding for gp100 or tyrosinase migrate towards T cells areas of the lymph node [153].

CONCLUSION

In closing, in the last 15 years, a growing body of literature has argued for the use of RNA for vaccination purposes. Importantly, RNA is safer than DNA vaccine approaches taking into account that no possibility of genomic integration exists. Furthermore, the combination of RNA technology with DC-based vaccines has made available a powerful strategy for antitumor therapies. Advances in RNA technology (*i.e.*, strategies to increase stability, use of replicons), together with the development of more effective protocols for generating activated DCs (*i.e.*, use of better inflammatory cocktails) and an increase in our knowledge of tumor immunology (*i.e.*, the use of immunoablative therapies to eliminate suppressor populations) will guide further pursuit of tumor immunotherapies using DC-based RNA vaccines. This offers the potential to advance the outcome of cancer immunotherapies for the benefit of patients.

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REVIEW

Mechanotransduction in bone: Intervening in health and disease

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Abstract

Mechanotransduction has been proven to be one of the most significant variables in bone remodeling and its alterations have been shown to result in a variety of bone diseases. Osteoporosis, Paget's disease, orthopedic disorders, osteopetrosis as well as hyperparathyroidism and hyperthyroidism all comprise conditions which have been linked with deregulated bone remodeling. Although the significance of mechanotransduction for bone health and disease is unquestionable, the mechanisms behind this important process have not been fully understood. This review will discuss the molecules that have been found to be implicated in mechanotransduction, as well as the mechanisms underlying bone health and disease, emphasizing on what is already known as well as new molecules potentially taking part in conveying mechanical signals from the cell surface towards the nucleus under physiological or pathologic conditions. It will also focus on the model systems currently used in mechanotransduction studies, like osteoblast-like cells as well as three-dimensional constructs and their applications among others. It will also examine the role of mechanostimulatory techniques in preventing and treating bone degenerative diseases and consider their applications in osteoporosis, craniofacial development, skeletal deregulations, fracture treatment, neurologic injuries following stroke or spinal cord injury, dentistry, hearing problems and bone implant integration in the near future.

Key words: Mechanotransduction; Bone remodeling; Bone disease; Bone health

Core tip: Mechanotransduction has been shown to be of major significance in modulating bone remodeling under physiological and pathological conditions. Therefore the study of the underlying mechanisms is of major importance and necessary step towards the better understanding of bone biology as well as the development of therapeutic strategies against conditions characterised by deregulated mechanotransduction. This review will consider the molecular mechanisms behind mechanotransduction as well as the scientific models currently used for its better understanding. It will also focus on mechanostimulatory techniques that could be used against a variety of deregulated mechanotransduction-related diseases.

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INTRODUCTION

Bone tissue biology

The importance of bones for a living organism is undeniable and goes far from just providing structural support for the body, protecting vital organs and exchanging minerals. Bones also comprise a multi-functional system that

interacts with other systems and abnormalities in bone tissues may result in mild or severe diseases.

Bone tissue is composed of the bone matrix and five different cell types. The bone matrix contains an inorganic (carbonated hydroxyapatite) and an organic phase (mainly type I collagen and several growth factors) whilst the cellular content of the bone tissue comprises of osteoprogenitors, osteoblasts, osteocytes, osteoclasts and lining cells[1]. Osteoprogenitor cells comprise pluripotent cells of mesenchymal origin, localised on bone surfaces[1] which have the ability, under the appropriate conditions, to commit and differentiate towards osteoblasts[1]. On the same bone osteoblasts, the bone forming cells, are cited. They are responsible for the protein synthesis of the bone matrix as well as its calcification[1]. The cavities of the calcified bone matrix bear osteocytes which comprise entrapped inactive osteoblasts forming a net of communicating cells inside the calcified matrix[1]. Osteoclasts are large multinucleate cells of blood monocyte origin, settled inside bone resorption lacunae and they are responsible for bone resorption in bone remodeling areas[1]. Lining cells comprise inactive osteoblasts with the ability to protect bone surfaces from bone resorption[1].

Runt-related transcription factor 2 transcription factor in bone biology

Runt-related transcription factor 2 (Runx2) or core-binding factor subunit alpha-1 (Cbfα1), the major osteospecific transcription factor[2] is responsible for the regulation of osteoblast differentiation as well as for hypertrophic cartilage synthesis[2,3]. Its expression is necessary and sufficient for the commitment of mesenchymal cells towards the osteoblastic cell line[4].

Abnormalities in Runx2 expression are indicative of its importance in bone biology. When Runx2 is expressed ectopically it has been shown to lead to increased expression of osteocalcin, alkaline phosphatase, collagenase-3, bone sialoprotein and collagen type Iα1[5]. Osteoblast maturation in mice bearing a mutant *runx2* gene is inhibited and thus so are the procedures of intramembranous and endochondral ossification[6,7]. Furthermore, it has been shown that differentiation of stem cells in adipocytes and chondrocytes in *runx2* knockout mice has not been impaired. In addition, heterozygous mice (*runx2*$^{-/+}$) developed characteristic skeletal abnormalities similar to human heritable skeletal disorder cleidocranial dysplasia (CCD) abnormalities[8]. On the other hand, tissue-specific Runx2 over-expression in transgenic mice results in decreased bone density, bone fractures and osteopenia[7,9,10].

Bone remodeling

Bone remodeling, the continuous bone reconstruction is of major importance for conserving bone structural integrity as well as for the bone to perform its metabolic role by modulating calcium and phosphorus levels in the body[1].

Shortly, bone remodeling activation depends mostly on local factors and their effects on mesenchymal progenitor cells. Bone reconstruction initiates with osteoclasts performing bone resorption and forming cavities inside the bone. At the end of this phase, osteoclasts produce the appropriate signals for the initiation of bone synthesis[1]. Osteoblasts quickly cover the cavity surfaces and synthesize new bone. Those two bone remodeling phases, bone formation and resorption are closely correlated and interconnected. This means that under normal conditions, the newly formed and the reabsorbed bone quantities are equal[11]. Impaired bone remodeling may lead in pathophysiological bone conditions like osteoporosis, Paget's disease, orthopedic disorders and osteopetrosis among others[1].

Research has shown that the GH-IGF-1 axis may also be of significance in the modulation of bone mass quantity and quality. More specifically, growth hormone (GH) is suggested to potentially play a role on bone remodeling[12]. However, the exact mechanisms through which GH acts on osteoblast biology have not been elucidated[12].

Role of RANK/RANKL/OPG pathway in bone remodeling

The receptor activator for nuclear factor κb (RANK)/receptor activator for nuclear factor κb ligand (RANKL)/osteoprotegerin (OPG) system comprises the main modulator of bone remodeling[13]. More specifically, pre-osteoclasts express RANK in their surface. Its ligand, RANKL, is produced in osteoblasts, stromal cells as well as activated T cells[14]. In osteoblasts and under steady-state conditions, vitamin D, parathyroid hormone and prostaglandins lead in induced RANKL expression. The binding of RANK and RANKL leads in osteoclast differentiation[15,16]. More specifically, during normal bone remodeling, RANKL is produced by cells of the bone marrow- supporting tissue and osteoblasts. RANKL binds to RANK on pre- osteoclasts resulting in their maturation and activation. Nuclear-factor κB (NF-κB), which is of importance in inflammation response, also plays a central role in osteoclast activation. NF-κB performs both aforementioned functions through regulation from interleukin-6 (IL-6). Pro-inflammatory cytokines play an important role in bone remodeling as indicated by the presence of interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α) receptors on pre-and mature osteoclasts[17]. OPG is produced by osteoblasts and has the ability to bind to RANKL and block its functions resulting in decreased bone resorption[17,18].

MECHANOTRANSDUCTION

Bone remodeling and mechanotransduction

Bone remodeling is a strictly regulated process, largely modulated by the application of different mechanical stimuli or by metabolic stress on the bone[3].

More specifically, local mechanical stress leads in bone resorption as an initial response[19]. The nature of the mechanical stimulus is of importance in the regulation of bone remodeling since different types of mechanical stimuli result in different responses. For example, con-

stant repetitive application of mechanical force inducing high stress levels or unusual load distribution result s in elevated bone synthesis and high bone mass. Furthermore, short pauses between long periods of mechanical loading have been shown to enhance bone strength and structure[20]. However, static load, slow rates of pressure rotation as well as "predictable" pressure application, lead in decreased bone synthesis, enhanced bone resorption and thus low bone mass[21,22].

Bone remodeling and mechanostimulation have been shown to roughly follow these rules: Bone synthesis is promoted by dynamic and not static loading application. Short-term load applications are sufficient for adaptive response initiation and lead in increased bone formation whereas long-term load applications result in decreased bone synthesis and enhanced resorption[23,24]. In addition, the repentance of the same mechanical stimulus results in decreased response due to signaling prediction[25]. The application of these rules is evident in the effects of space microgravity, osteoporosis or paralysis on bone tissues, where bone loss is observed[20,26], as well as in the effects of tennis at a professional level on bone tissues, where bone growth is observed[27].

Mechanisms in mechanotransduction

Signals of mechanical nature induce in osteoblasts and osteocytes the production and secretion of different types of molecules, which modulate osteoblast differentiation and proliferation[3]. Such mechanical stimuli can include flow of fluids, strain of the substrate, membrane deformation or stimulation of integrins, vibration, altered gravity forces and compressive loading[3]. Bone remodeling functions, after the application of different mechanical stimuli, are locally regulated by cytokines and growth factors among other molecules. More specifically, IL-1β, TNF-α, prostaglandin E2 (PGE2)[26,28], IL-6, IL-8, RANKL, OPG[27,29-31], insulin-like growth factor (IGF), transforming growth factor β-1 (TGFβ-1) and fibroblast growth factor (FGF)[32,33] have been demonstrated to be induced after application of mechanical stimuli. Additionally, it has been shown that mechanical stimulation in osteoblasts results in increased mRNA levels of osteopontin, osteocalcin, platelet derived growth factor and collagen types I and Ⅲ[34,35].

Although some of the molecules taking part in mechanotransduction are known, the mechanisms behind it have not been fully elucidated.

The stage of osteoblast differentiation is shown to be of importance in osteoblast proliferation, apoptosis and translation of mechanical cues[36]. Furthermore, it has been shown that undifferentiated mesenchymal stem cells seem to respond more successfully to load application than mesenchymal stem cells that have already started to differentiate[37].

A diversity of molecules have been considered to play the role of mechano-sensors in differentiated osteoblasts: mechanical stimulation has been shown to lead in enhanced sensitivity and elevated open cation channels number[38,39], increased communication through gap junctions between osteoblasts as well as increased integrin production in osteoblasts[39]. Actin cytoskeleton abnormalities have been shown to prevent mechanical signaling and therefore the integrin network has been considered as the main candidate for transduction of mechanical signals[39]. On the other hand, a considerable number of research groups argue that cytoskeletal components involved in mechanotransduction differ depending on different types of stress or the response under study[39].

Integrins comprise transmembrane receptors connecting the extracellular matrix to the cytoskeleton[40]. Under mechanical signal application, integrins form complexes with molecules of the cytoskeleton with the help of the Rho family of Ras-related GTPases[40]. Rho family members also induce multiple kinase cascades and particularly mitogen-activated protein kinase (MAPK) cascades[40]. Rho and other Ras-related GTPases have been shown to play a role in osteoblast response after application of mechanical pressure[41]. More specifically, it has been shown that the continuous application of mechanical forces leads in deregulation of Rab and Rho GTPases activity in osteoblast-like cells[41].

Recently, another molecule, Polycystin-1 (PC1), was suggested to provide a link between environmental mechanical signals and their transformation towards biochemical signals. It has been shown that PC1 not only functions as a mechanosensor but that also conveys mechanical signals through the calcineurin/nuclear factor of activated T-cells (NFAT) signaling pathway and thereby regulates osteoblast- specific gene transcription as well as osteoblast differentiation[42].

The primary cilium, a cellular sensory system, has also been demonstrated to be of importance in the transfer of mechanical signals as well as in mesenchymal stem cell differentiation. Additionally it was shown that the cilium modulates fluid flow mechanotransduction in human mesenchymal stem cells by maintaining fluid flow-induced osteogenic gene expression elevation and preventing fluid flow-induced increased proliferation[43].

Following the reception of mechanical cues, the signal conveying the mechanical conditions of the extracellular environment is carried towards the nucleus through MAPK kinases and more importantly through extracellular signal-regulated kinases (ERKs) and c-Jun N-terminal kinases (JNKs)[44,45]. ERKs, which in human osteoblasts seem to be induced by growth factors, estrogen and fluoride among others[45], have been shown to play a significant role in osteoblast maturation and in osteoblast biology in general[45-49]. Furthermore, duration and strength of JNK/ERK signaling is indicated to be significant in gene expression[50].

Following ERK/JNK activation, the signal is transmitted to transcription factors that alter gene expression, like Jun and Fos family members[51]. In their turn, c-Jun and Fos family members interact to form activator protein-1 (AP-1) transcription factor, which has been shown to be of major importance in osteoblast differentiation[52] since it regulates the expression of collagen type I , osteocalcin, osteopontin and osteonectin[52].

Application of continuous mechanical pressure in osteoblast-like cells as well as osteoblasts resulted in increased production of AP-1 components through activation of MAPK cascades[41,53,54]. However, data on c-Jun expression after mechanical stimulation are inconclusive with some research groups arguing that human osteoblast-like cells after mechanical loading over-express c-Jun[53] whereas others have opposing results[55,56]. However, the above mentioned differences could be attributed to application of different stress type or usage of different cell system. Finally, different types of mechanical pressure applied on osteoblasts seem to result in different composition AP-1 and therefore regulate gene transcription accordingly depending on the extracellular signal applied[57].

Application of short-term mechanical pressure activates both JNK2 and ERK2, with following activation of downstream molecules, like c-Jun, which alter the expression of osteoblastic genes[54]. More specifically, it has been demonstrated that short-term continuous mechanical stimuli of physiological intensity in osteoblast-like cells activates JNK and ERK members resulting in enhanced AP-1 DNA binding activity on the human *L/B/K ALP* gene and thus osteoblast differentiation[54]. This is further evidenced by the observation that osteoblast-like cells receiving mechanical stimuli synthesized increased quantities of type 1 collagen and osteocalcin, markers of early osteoblast differentiation[58].

PGE2 production has been shown to be induced in osteoblast-like cells after mechanical stimulation[59] and in osteoblasts under the effect of physiological stress, growth factors, hormones, trauma or inflammatory cytokines and its production leads in cAMP-dependent IGF-1 induction in osteoblasts[3]. IGF-1 and IGF-2, in turn, induce osterix (Osx) transcription factor expression in osteoblasts[60], induce osteoblast function *in vitro* as well as lead in increased bone mass *in vivo*[61]. PGE2 is also shown to lead in increased Runx2 expression *in vivo*[62]. Downstream of PGE2, TGF-β expression, which leads in proliferation of osteoblasts and extracellular matrix synthesis[63], has been found increased in human osteoblast-like cells under mechanical stimulation. Furthermore, TGF-β receptor 1 comprises a Runx2 target in osteoblasts[64]. Those two observations combined explain why Runx2 knockout mice demonstrate characteristic abnormal extracellular matrix formation due to decreased number of mature osteoblasts[65,66].

Nitric oxide (NO) production in osteoblasts is another response to mechanical stimulation. NO functions through the MEK/ERK cascade by binding to a regulatory site on Ras leading in cell proliferation and extracellular matrix production[67]. Following, cyclooxygenase 1 (Cox1), Cox2, ERK1 and ERK2 are activated and result in bone matrix formation[68].

Additionally, signals of mechanical nature have been shown to promote vascular endothelial growth factor-, bone morphogenetic protein 2 (BMP-2)- and BMP-4- dependent and PGE2- independent increased expression of IGF-1[69]. BMPs result in bone synthesis in osteoblasts[70] and BMP-2 expression promotes Runx2, Osx and Dlx5 expression[71].

Mechanical cues also promote the expression of genes that encode for c-Fos, early growth response factor 1 (Egr-1) and basic fibroblast growth factor (bFGF) which have been shown to promote cell growth in MC3T3-E1 osteoblasts[22].

The nature the mechanical signal determines whether bone or cartilage formation will occur[72]. More specifically, application of pressure of high frequency and low intensity in bone cells *in vitro*, results in elevated extracellular matrix (ECM) disposition and thus increased bone formation[73]. On the contrary, mechanical loading of high intensity on osteoblasts leads in BMP extracellular antagonists expression and therefore results in inhibition of osteoblast development[74]. In addition, the application of continuous mechanical forces on osteoblastic cells *in vitro* promotes inflammatory cytokines and their receptors expression[75]. More specifically, IL-1b production is found elevated under such mechanical stimuli, and is accompanied by RANK-RANKL signaling pathway activation and thus bone resorption[76]. Stimuli from short periods of fluid flow or cyclic substrate tension at physiological intensity levels promote osteoblast proliferation and survival[77]. Mechanical signals of physiological intensity levels are associated with survival of human osteoblasts and several studies suggest that pro-survival proteins promote the production of survival factors like IGF-1 or IGF-2 and activate estrogen receptor[78]. It has also been shown that gravitational force maintains osteoblast survival whereas when gravitational force is not taking place, osteoblasts are led to apoptosis through reduced DNA binding of an important for survival transcriptional factor[18]. *In vivo*, the absence of mechanical signals promotes osteoblast apoptosis and thus osteoporosis[72]. The application of excessive mechanical force *in vitro* leads in cell detachment from their adhering surface[79] as well as in a form of programmed cell death called anoikis[80].

Mechanical stimulation in osteocytes has also been under investigation since it may lead in better mechanotransduction understanding and may represent a potent therapeutic target against bone degenerative diseases. Recent studies have underlined the role of osteocytes in bone remodeling since their absence in mice led in fragile bones, microfractures, deregulated osteoblast functions, bone loss in the trabeculae as well as adipose tissue proliferation in the marrow indicating an aging skeleton. In addition, these mice could not experience bone loss due to unloading, an event that indicates osteocytes' importance in the procedure of mechanotransduction[81] (Figure 1).

Runx2 in mechanotransduction

Runx2 which is known to play a significant role in osteoblast differentiation has been shown to be the recipient of mechanical signals in human osteoblast-like cells[82]. As it has been demonstrated, continuous mechanical stimuli of low intensity in human osteoblast-like cells of the periodontal ligament (PDL) result in elevated Runx2

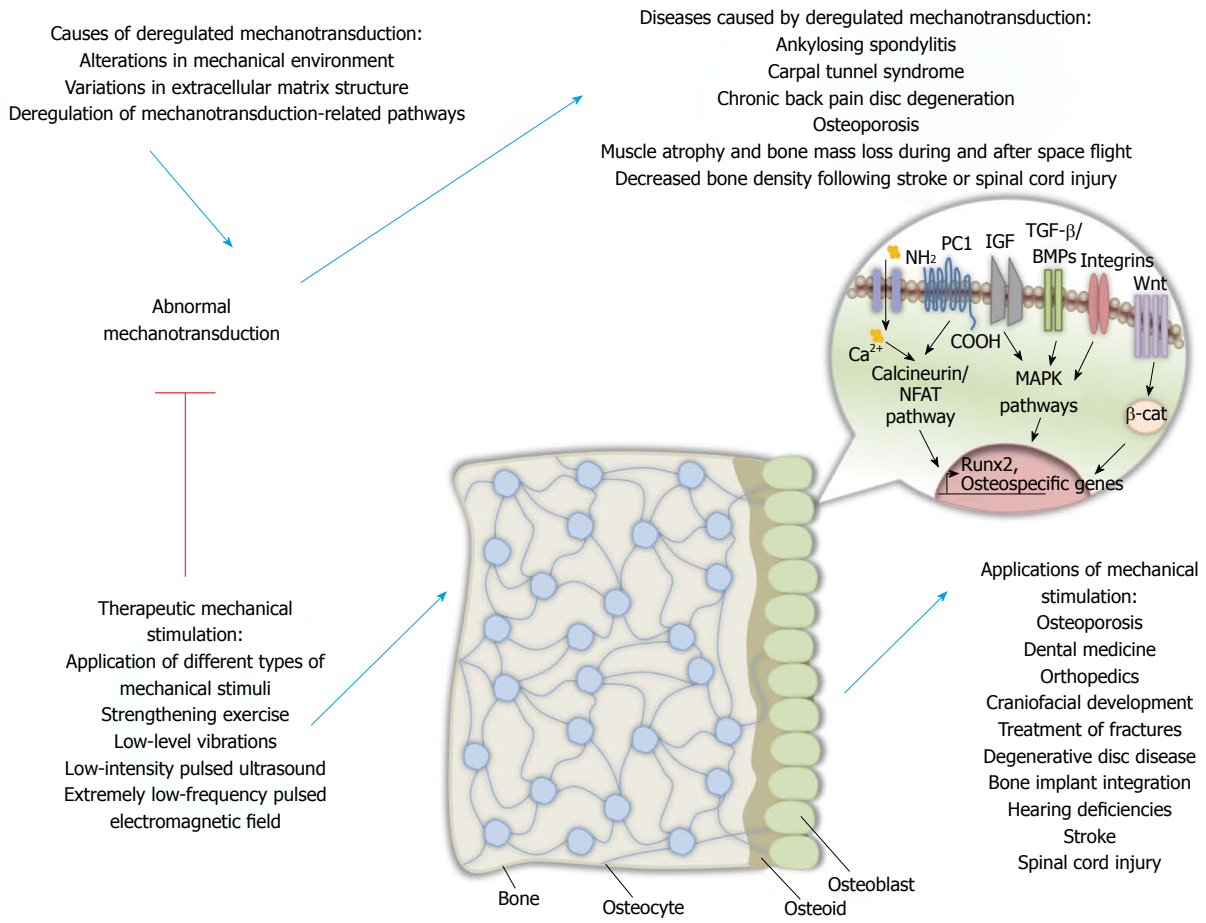

Figure 1 Mechanotransduction: Deregulation, associated disorders and therapeutic implications. Causes and effects of distorted mechanotransduction and the role of mechanical stimulation in the treatment of various pathophysiologies. PC1: Polycystin-1; IGF: Insulin-like growth factor; TGF-β: Transforming growth factor β; BMP: Bone morphogenetic protein. NFAT: Nuclear factor of activated T-cells; MAPK: Mitogen-activated protein kinase; Runx2: Runt-related transcription factor 2.

expression and DNA- binding capacity. The mechanical signal, according to the researchers, initiates at the plasma membrane and more specifically from integrins and travels towards the nucleus through MAPK cascades. In the nucleus, the signal targets Runx2 and induces its expression[82]. More specifically, Runx2 demonstrates increased expression at both mRNA and protein levels as well as elevated DNA binding activity. During this process, ERK1 and ERK2 are activated in a parallel manner with the Runx2 DNA- binding capacity elevation. After their activation, ERKs interact, phosphorylate and activate Runx2 *in vivo* causing osteoblast maturation[7,82].

Runx2 expression depends on an autoregulatory mechanism[83]. More specifically, activated by mechanical stimuli ERKs phosphorylate and activate already existing Runx2 molecules. Those activated Runx2 molecules bind to Runx2 promoter inducing Runx2 expression[82]. In addition, a canonical AP-1 binding site has been found in Runx2 promoter which potentially plays a role in the regulation of Runx2 expression. AP-1 and Runx2 proteins have also been shown to interact and regulate collagenase-3 expression[84].

NF-κB transcription factor in mechanotransduction

NF-κB transcription factor which is implicated in inflammatory response signaling[31] also plays a crucial role in osteoclast formation and thus bone resorption[85]. NF-κB, which is activated either through the RANK-RANKL system or potentially through integrins that transmit signals of mechanical nature to src-kinases[86], besides its role in osteoclast maturation, may be implicated in osteoblast differentiation under mechanical stimulation. This is indicated by the fact that NF-κB is found to be activated and then translocated in the nucleus of osteoblasts that receive mechanical stimuli[26,87] where it has been hypothesized to promote the transcription of osteoblast-specific genes.

MODEL SYSTEMS IN MECHANOTRANSDUCTION STUDY

The *in vitro* study of mechanostimulation in osteoblasts, has been made possible with the usage of osteoblast-like cells that are acquired either from healthy tissue (human PDL or mouse MC3T3-E1 calvaria cells) or from osteosarcomas (MG-63, SaOs cells). Different types of mechanical stimulation are applied on the aforementioned cell models, each causing a different response in osteoblast-like cells[3]. Such types of mechanical stimulation include fluid flow, four-point bending and substrate

stretch, gravity force, vibration, magnetic bead twisting and atomic force or shockwaves among others[88].

Periodontal ligament (PDL) cell system is a helpful model for the study of mechanotransduction signaling cascades in osteoblasts[89]. More specifically, PDL cells are undifferentiated mesenchymal fibroblasts[90] that bear all the characterized properties of osteoblasts. Furthermore, these cells are adapted to receive mechanical pressure, either because of physiological conditions or orthodontic treatments. Under specific conditions, PDL cells have the ability to differentiate towards more specialized cells capable of taking part in the regeneration and repair of the periodontal ligament as well as its surrounding hard tissue[91].

Furthermore, three dimensional (3-D) constructs, like polydimethylsiloxane microdevices and human trabecular 3-D bone scaffolds, have been used to investigate the effects of mechanical stimulation on osteoblasts[92].

Scientists are trying to develop an effective way to monitor the levels and characteristics of mechanical pressure applied as well as a way to measure the rates of tissue regeneration. In order to achieve the first part, scientists have made either fixation devices with different mechanical pressure characteristics and then monitor their effects *in vivo* or custom-made devices that accurately control the mechanical stimulation characteristics. With the first type of devices they are able to study bone tissue regeneration under more physiological conditions while with the second they assess the effects to a specific loading signal[93]. In order to study the effect of mechanical signals on healing processes at organs, it is necessary to develop techniques to assess their mechanical environment *in vivo*. Today, we have found ways to determine loading applied on the affected limb[94], load distribution between implant and bone[95-97], and assess interfragmentary movements[94,98] but the development of techniques to study the intermediate steps and not only the final outcome of loading are imperative.

MECHANOTRANSDUCTION IN BONE DISEASE

As mentioned before, deregulated bone remodeling is the main cause of a number of bone diseases. Bone remodeling abnormalities may be due to genetic alterations. For example, a mutant *runx2* gene can result in human heritable skeletal disorder CCD[99,100]. A mutation in *runx2* gene may also lead in cancer metastasis to bone tissues since Runx2 is responsible for the expression of genes that are implicated in cancer development and more specifically, in cell metastasis in bone. Among those genes regulated by Runx2 are those encoding matrix metalloproteinases (MMPs) MMP-9 and MMP-13 as well as osteopontin and bone sialoprotein[101]. Abnormal mechanotransduction due to lack of mechanical loading or other causes may result in bone remodeling deregulations like ankylosing spondylitis, carpal tunnel syndrome, chronic back pain disc degeneration and osteoporosis.

Recent studies have shown that annulus fibrosus (AF) cells that originate from non degenerative tissue respond to cyclic tensile strain through IL-1 and IL-4 dependent mechanisms, something that does not apply in AF cells coming from degenerative tissue[102]. Furthermore, annulus fibrosus cells from degenerative discs have been found to have little capacity to successfully respond to application of mechanical stimuli and exhibit an intense response to inflammatory stimuli. The above observations may explain the different responses observed in patients with intervertebral disc degeneration after specific therapies[103].

During space flight, astronauts are exposed to microgravity and thus altered mechanical stimuli are applied on their skeletons. As a result, their muscles atrophy and their bones experience bone mass loss. Short exposure to microgravity has been shown to result in increased bone resorption evidenced by the urinary calcium excretion observed[104]. Under long periods of microgravity, the structural alterations occurring in bones have even more crucial effects on bone strength than was previously thought while counteracting measurements like exercise seem to have little or no effects[104]. The mechanism behind bone loss is not yet clarified but probably is a result of decreased hydrostatic pressures and thus decreased intramedullary pressure which may lead in reduced fluid flow shear stresses on osteocytes and thus enhanced bone loss. Since exercise does not seem to prevent bone loss, it has been suggested that the decreased hydrostatic pressure may result in impaired mechanosensitivity in the bone tissue. Furthermore, other physiologic alterations on the body under reduced gravity conditions may contribute to the observed bone loss in co-operation with the reduced hydrostatic pressures like low vitamin D levels, oxidative stress, radiation exposure and acidosis[105-109].

Neurologic injury results in bone loss in the affected paretic limb whereas the other limb is characterized either by reduced or increased bone mass. Those effects are probably due to alterations in muscle mass and strength and load pressure applied. More specifically, strokes result in decreased bone density mostly in the paretic limb and its effects are more intense in the upper extremities. The pattern of bone loss observed in stroke patients is generally limited to the paretic side and is more evident in the upper extremities than in the lower extremities. The pathogenesis of the observed bone loss after stroke probably depends between others on immobilization, duration of paresis, loss of muscle activity, endocrine disorders, nutritional deficiencies as well as medications[110].

Following spinal cord injury, bone loss is observed in pelvis and lower extremities of paraplegics and in the upper and lower extremities of tetraplegics after spinal cord injury[111]. Those effects are predominantly observed in trabecular bone. Recent data indicate the presence of endocortical resorption without periosteal synthesis[112]. Absence of mechanical stimulation, muscle contraction, neuroendocrine alterations as well as neural innervation alteration are probably responsible for the observed bone

loss after those types of injuries[113,114] (Figure 1).

MECHANOSTIMULATION IN THERAPY OF BONE DISEASE

Pharmaceutical treatments like anabolic treatments or treatments with anti-resorptive agents have been the norm in order to achieve increased bone density until now[3]. Nowadays, mechanical stimulation is considered to be of great importance in designing new therapies for bone diseases, avoiding this way the unwanted side effects of pharmaceutical products.

A number of studies demonstrate the role of mechanostimulation in acquiring a higher bone mass quantity and thus its role in treatment of bone diseases. For example, it has been shown that low intensity mechanical signals result in bone remodeling activation and increased bone mass and that following a period of time confer regenerative abilities to bone tissues[115]. It has also been observed that mechanical signal application on PDL and osteoblast cell lines leads in enhanced OPG expression[116,117] and therefore in RANK-RANKL signaling interruption which results in decreased osteoclastogenesis. Furthermore, mechanical stimulation has been shown to activate Cox enzymes and prostaglandins which reduce RANKL production and thus block bone resorption *in vitro*[77,118]. Mechanical stimuli have also been demonstrated to activate the Wnt-b-catenin pathway on osteoblasts resulting in enhanced osteoblast differentiation and bone synthesis[119]. Studies on three dimensional models have showed that osteoblasts receiving dynamic application of mechanical pressure, expressed elevated ALP, Runx2 and osteocalcin levels[120,121]. Additionally, application of mechanical pressure resulted in increased mineralized matrix production in 3-D, partially demineralized bone scaffold-cultured human bone marrow stromal cells[122].

Considering the aforementioned and other results, researchers have turned to mechanical stimulation in order to design treatments against bone diseases which will avoid the undesirable effects of pharmacological treatments[115]. Application of mechanostimulation has already a variety of applications in dentistry, orthopedics, the craniofacial development and treatment of fractures.

More specifically, strengthening exercises in osteoporotic patients has been shown to result in increased bone mineral content[123] and physical exercise has been observed to prevent post-menopausal and age-related ECM bone mineral decrease[124]. Moreover, other types of mechanical stimulation like low-level vibrations at intensity safe for the bone integrity may play a protective role in osteoporosis[125]. A functional mechanical environment seems to be of importance in the treatment of degenerative disc disease as well as other skeletal deregulations[126]. Mechanical signals of specific ratio[127], form[128] and intensity in osteoblasts have also been shown to be beneficial in bone fracture treatment[128]. Additionally, low-intensity pulsed ultrasound has been indicated to promote osteoblast differentiation and bone formation in bone fractures[129]. Extremely low-frequency pulsed electromagnetic field has been demonstrated to result in osteoblast proliferation and maturation[130].

In addition, mechanostimulation was found to have positive effects in bone implant integration by modulating osteoblast differentiation through regulation of Cbfα1 as well as osteocalcin levels. Cbfα1 and osteocalcin levels were shown to be frequency-, magnitude-, and duration of mechanical application- dependent. Furthermore, osteoblast cells under strain in the implant seem to produce factors that have the ability to activate DNA synthesis and thus cell proliferation in a larger scale than non-strained cells[131].

Mechanical stimulation has also its applications in the treatment of hearing problems. For example, SPAHA, which comprises a novel bone conduction hearing device, whose effects are accomplished through elastic bending of the bone and not the application of a point force which results in cochlea vibration as previous devices used to do[132].

Exercise has not been shown to meliorate bone loss in space flights until now[104]. Furthermore, there is no indication that osteoporosis drug therapies would be successful during or following space flight. Exercise seems to be helpful in increasing bone density after stroke or spinal cord injury according to a recent study[133,134]. Bisphosphonates have been shown to be able to prevent bone loss after a stroke[134]. Mechanical stimulation may have some positive effects on preventing bone loss after spinal cord injury, with early application demonstrated to bear better results[135,136]. Furthermore, bisphosphonate early administration after spinal cord injury may be able to prevent bone loss[137].

Researchers have investigated whether sympathetic nervous system inhibition could be beneficial against bone loss in osteopenia induced by absence of mechanical signals. They found that its inhibition led in blockade of neurectomy-induced bone resorption but further studies need to be conducted[138].

Although mechanical loading is thought to be an anabolic beneficial procedure against osteoporosis, abnormal mechanotransduction in conjunction with age seem to counteract its beneficial effects in elderly people. Recently, a research group presented an agent-based model of real-time Ca^{2+}/NFAT signaling in bone cells that successfully described periosteal bone synthesis induced by different types of mechanical stimulation in young and aged animals. The model demonstrated age-related pathway changes being responsible for the decrease in bone synthesis during senescence. This way the group managed to identify important pathway alterations that comprise potent therapeutic targets. In accordance, the researchers applied an *in vivo* intervention and showed that application of mechanical stimuli along with Cyclosporin A can prohibit the decrease in bone synthesis in the bones of elderly people. This study not only provided a potent inexpensive treatment for osteoporosis in the elderly but also demonstrated the significance of real-time cellular

signaling and *in silico* techniques in studying, intervening and treating bone diseases like osteoporosis[139].

The primary cilium was shown to modulate fluid flow mechanotransduction in human mesenchymal stem cells by maintaining fluid flow-induced osteogenic gene expression elevation and preventing fluid flow-induced increased proliferation[43]. Therefore, fluid flow systems may be effective in designing techniques to develop bone-like tissues for bone regenerative purposes. Furthermore, the role of cilium in developing techniques that imitate loading in order to treat bone loss in bone diseases needs to be investigated. Last but not least, studying the events taking place during acute proliferation of mesenchymal stem cells with not functional cilia receiving mechanical cues could help in understanding the mechanisms behind ciliopathies and cystic diseases[43] (Figure 1).

CONCLUSION

Bone remodeling is of major importance for the proper structure and metabolic functions of the bone. Deregulations in bone remodeling can result in a variety of bone diseases like osteoporosis, hyperparathyroidism, hyperthyroidism, Paget's and osteopetrosis among others. Therefore, the investigation of mechanisms and pathways behind bone remodeling and mechanotransduction, which comprises of the most important variables of bone remodeling, is of great significance.

There is a lot that we don't know about bone biology and bone diseases as well as the implication of mechanical signals in the aforementioned procedures. The better understanding of the underlying mechanisms will potentially result in designing a successful strategy for treating bone diseases, avoiding the unpleasant side effects of conventional treatments like the administration of pharmaceutical substances. Furthermore, it will help us design techniques to successfully predict and prevent bone diseases when possible.

Undeniable is the necessity of innovative new ways to monitor bone density, to identify hormonal or metabolic risk factors for bone loss, to develop effective ways to apply mechanical stimulation with successful results against reduced bone density, to assess the effect of newly developed anabolic drugs against osteoporosis and their effects on bone loss characterizing bone diseases due to absence of mechanical stimuli, as well as to develop trials investigating the improvement of bone health under the afore mentioned conditions. In addition, the study on the effects of mechanostimulation on bone tissue and organ healing is of great significance for future interventions. In order for this to be achieved, we need to develop an effective way to monitor the levels and characteristics of mechanical pressure applied on bone tissue, a way to measure the rates of tissue regeneration as well as techniques to assess mechanical environment of organs *in vivo*[106].

Currently, researchers have started using mechanostimulation with encouraging results for certain bone conditions but further study is required. Mechanostimulation is considered to comprise the future in treating bone diseases that have their origin in absence of mechanical cues. Further investigation of the molecular players and pathways involved in mechanotransduction and bone remodeling will amplify our knowledge and understanding of these processes and help us build successful prevention, prediction and treatment strategies for a variety of bone diseases.

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REVIEW

Role of oral-fluid based measles diagnostic methods for measles global elimination

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Abstract

Measles eradication is biologically feasible. There is an availability of a safe, effective and inexpensive vaccine; a proven elimination strategy; high Local demand; and an effective global partnership and initiative to support vaccination. Measles eradication is a cost-effective scenario and a good investment to avoid expensive epidemics and save those children die due to measles. Laboratory investigations are indispensable to monitor the progress of measles elimination. This role will require the development of more sensitive diagnostic methods suitable for diagnosis and surveillance, genetic analysis of measles strains and a technology which is transferable worldwide. Measles diagnosis relies increasingly on serological tests. The practical utility of oral-fluid methods (antibody and genetic) in evaluating and refining measles immunization programs would, additionally, provide support for a global surveillance initiative. The utility of in a population survey, in a vaccine sero-conversion study and application in molecular epidemiological use is demonstrated in this review. It is to be hoped that this review will assist in the wider uptake and acceptance of methodology in both developed and developing country situation. More research needed for further evaluation of a recently developed point-of-care test for measles diagnosis: detection of measles-specific IgM antibodies and viral nucleic acid for wider use oral-fluid methodology. There is a strong case and imperative for the promotion of methods by World Health Organization in its global program of control/eradication of measles over the coming decade.

Key words: Measles elimination; Oral-fluid test methods; Laboratory diagnosis

Core tip: Laboratory investigations play a critical role in monitoring the success of measles elimination strategies. The role requires the development of more sensitive diagnostic which is transferable worldwide. Measles diagnosis relies increasingly on serological tests. Promotion of the use of oral fluid as viral diagnostic alternative to serum may be of advantage in communities where reliable age-specific notification and vaccination data are unavailable or in groups that are "hard to reach". This review will assist in the wider uptake and acceptance of oral-fluid methodology in both developed and developing country situation for global measles elimination.

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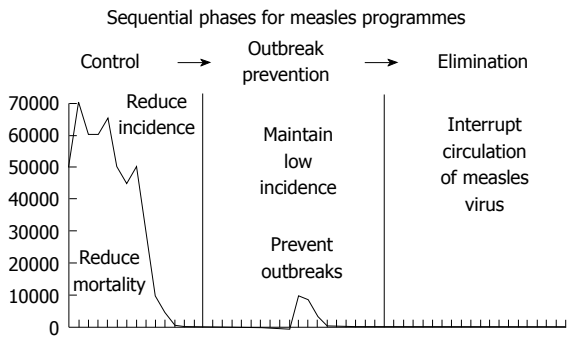

Figure 1 Phases for measles control/eradication programmes.

PHASES OF MEASLES CONTROL AND ELIMINATION

Measles is a highly contagious disease caused by a virus. It is one of the leading causes of death among young children. In 1980, before widespread vaccination, measles caused an estimated 2.6 million deaths each year. It remains one of the leading causes of death globally, despite the availability of a safe and effective vaccine. It is estimated that approximately 158000 people died from measles in 2011, mostly children under the age of five[1].

Based on implementation of a combination of vaccination and surveillance strategies, countries are considered to be in 1 of 3 stages: control, outbreak prevention, or elimination[2,3] (Figure 1).

MEASLES CONTROL

Control is defined as the reduction of disease incidence and/or prevalence to an acceptable level as a result of deliberate efforts, requiring continued interruption measures. In the control stage, the objective is to achieve high routine coverage with 1 dose of measles vaccine among infants to reduce measles morbidity and mortality. To accelerate measles control in large urban and other high-risk areas with a substantial proportion of unvaccinated children and measles associated deaths, mass vaccination campaigns targeting children aged 9 mo to 3-14 years have been recommended[4,5]. Countries in all regions have committed to the mortality reduction goal. Global measles deaths are decreased by 78% between 2000 and 2008, averting an estimated 4.3 million deaths[6,7]. The Southeast Asia region is already exceeding 90% measles mortality reduction[8].

MEASLES OUTBREAK PREVENTION

Measles outbreak prevention aims to maintain low incidence and prevent outbreaks by the administration of supplemental doses of measles vaccine through mass vaccination campaigns. As programmes plan for elimination of measles, a high coverage of single dose vaccine with supplementary immunization is assumed be sufficient to interrupt transmission[9]. A second dose is required to eliminate susceptibles from the population and interrupt measles transmission[10]. The Africa region is adopting to raise routine vaccine coverage to at least 80% and using supplemental campaigns in all non-polio-reservoir countries by 2003[11]. The Western Pacific Region from 1996 to 2009, 235 million persons received measles vaccine during 94 immunization campaigns in 30 countries and areas[12]. In the same region during 2009, 32 countries and areas provided 2 routine doses of measles vaccine[12]. The steady increase in routine measles coverage is shown from 71% to 82% globally between 2000 and 2009[8]. Between 2000 and 2008, administration of more than 600 million doses of measles vaccine in mass vaccination campaigns were made globally[7,13].

MEASLES ELIMINATION

Elimination is defined as the reduction of endemic incidence of a disease to zero as a result of deliberate efforts, requiring continued control measures. An alternative approach to documenting measles elimination are molecular evidence to confirm the lack of a circulating endemic genotype for at least one year and maintenance of 95% coverage of one dose of measles-containing vaccine, with an opportunity for a second dose[14]. It is well understood that laboratory testing and confirmation of suspected measles infection is crucial in countries that are in elimination phase of measles[15-17]. Generally, all 6 World Health Organization (WHO) regions have committed to measles elimination, and 5 (except Southeast Asia) set target date to move from regional measles mortality reduction to the regional elimination of indigenous transmission[6].

GLOBAL PROGRESSES TOWARDS ELIMINATION

Although there has been tremendous success in the reduction of measles endemic incidence in many countries with measles elimination, the total interruption of measles transmission remains a major challenge due to importation of measles cases to America and Europe regions[18-20]. For example, the ongoing transmission of endemic measles was declared eliminated in the United States in 2000[21]. However, within five months period starting from January to May 2011, 118 cases were reported in the United States in which 46% of the cases were imported[18]. The elimination of measles deaths in Southern Africa in 2000 joins the region of the Americas to be free from measles deaths[22,23]. However, from July 2003 to November 2005, 1676 laboratory-confirmed measles cases were reported in South Africa[24] and silent causalities' the disease was also reported [25].

The WHO Europe strategic plan for measles 2010-15 sets targets of 90% measles vaccination coverage, and reductions in the number of cases to fewer than five per million and in mortality by 95% compared with 2000 levels[26,27]. The 37 countries and areas of the WHO Western Pacific Region have targeted measles for elimination by 2012[12].

Between 1997 and 2011, the goal of interrupting measles transmission was adopted toward the elimination of measles in the Eastern Mediterranean Region (EMR). For the 22 EMR member countries, routine coverage with the first dose of a measles-containing vaccine increased from 70% in 1997 to 82% in 2009. Reported measles cases decreased by 86% during 1998-2008, and estimated measles mortality decreased by 93% during 2000-2008, accounting for 17% of global measles mortality reduction during that period. Despite these successes, EMR was not being able to achieve measles elimination by the end of 2010[28].

Many Progresses have been achieved toward measles elimination in the People's Republic of China between 2000-2009 and in the Russian Federation between 2003-2009[29,30]. Globally, the number of measles deaths worldwide fell by 78% between 2000 and 2008, from an estimated 733000-164000[31]. Despite the efforts measles elimination, measles remains a disease still endemic in many parts of Europe[32]. For instance, between 2009 and 2011, Austria, France, Germany, Ireland, Italy, Greece, the Netherlands, Spain, Bulgaria, Norway and United Kingdom have all seen outbreaks[32-41].

Estimates indicate that almost a quarter of all lives saved annually towards achieving Millennium Development Goal 4 are the result of progress towards achieving a 90% reduction in measles deaths [7,10,13].

STRATEGIES FOR MEASLES CONTROL AND ELIMINATION

Key strategies for the local elimination/total eradication of measles as a disease are as follows. The spread of measles infection through a population requires that a chain of invectives should be maintained. Protection against this spread of infection can be taken at two points. First, the route from susceptible to recovered (return to immunized state after vaccine uptake) can be short-circuited by the establishment of immunization[42-45]. Second is to interrupt the mixing of invectives (carrier of the infections) and susceptible with protective barriers (*e.g.*, isolation)[46]. Incidence rises as susceptible individuals enter the population. Acquisition of immunity through exposure to the wild virus or vaccination decreases the number of susceptible individual in the population and measles incidence falls [47]. The greatest potential is with vaccination.

Acquired immunity after measles illness is permanent. Live attenuated measles virus, when administered at recommended ages, produces about 85% immunity after one dose and greater than 90% immunity after two doses[5,48,49]. Vaccine-induced immunity is long lasting and protective to all the diverse geographic origin strains. Widespread vaccination has resulted in interruption of measles virus transmission in a number of countries. For instance, the Gambia in 1968-1969, the English speaking Caribbean islands, Cuba, Chile, United States over short periods in 1993, 1995, and 1996[50,51]. Similar achievements were obtained in England and Wales through 1995-2000[20]. Estimate indicates increase in routine measles coverage from 71% to 82% globally between 2000 and 2009, and from 56% to 73% in the 47 countries with the greatest burden of measles deaths[7,8].

The success of recent mass vaccination campaigns in these countries has suggested that global eradication of measles is possible biologically, technically, and operationally[19,52]. Reaching this goal will require continued commitment to increase vaccination coverage levels with a co-coordinated global effort.

Vaccine investments rose from donors in United Kingdom, Japan, United States, *etc.,* to provide additional funding to the Global Alliance for Vaccines and Immunization (GAVI) for its childhood immunization program save many children's lives. Vaccination is one of the most cost-effective health interventions[53]. Studies show that measles eradication by 2020 was found to be the most cost-effective scenario globally[54].

Programmatic and technological innovation will be needed to sustain recent successes in reduction of the global burden of measles. Delivery of the measles vaccine through the respiratory tract could help this effort[55]. It has many advantages compared to the injectable vaccines in which the major one can be stated as follows[55-58]. Respiratory delivery generates robust local and systemic immune responses which resulted in superior and longer lasting protection and boosting better responses in seropositive people than are injectable vaccines[57,58]. This route is less likely to be blocked by maternal antibodies in infants than is a subcutaneous measles vaccine. Aerosol administration of vaccines needs fewer skills than injectable vaccines. Use of non-injectable vaccines reduces the likelihood of unsafe disposal and reuse of syringes in immunization program.

An important component of the measles control and elimination strategy is information obtained from laboratory. Currently the WHO Global Measles and Rubella Laboratory Network (LabNet) include 690 laboratories serving 183 countries[59].

MEASLES VACCINATION

Different factors affect the response to immunization, such as, age and maternal antibody level[60]. Wesley *et al*[61] reported that the response to measles immunization was delayed among malnourished children. The optimal age at delivery of measles vaccine depends upon the relationship between the average age at infection and the rate of loss (average duration) of maternal antibodies specific to measles[62,63]. Maternal antibodies typically provide protection during the first 6 mo of life, but often longer[63,64]. Interference with the replication of vaccine virus is frequently still seen at the age of 12 mo[65]. As a consequence vaccination in the first year of life gives inadequate immunity to measles, meaning the earlier at the age of vaccination the lower the sero conversion rate[63,64]. The requirement for delay until maternally derived antibodies vanish is an impediment for early vaccination.

The duration of maternally derived immunity in a child depends on the mother's antibody titer, the efficiency of transfer across the placenta and the rate of catabolism in the child[66,67]. A child exposed to many infections makes a large variety of immunoglobulin G (IgG); in order to keep the total blood IgG level in the normal range, catabolism is accelerated and passively acquired antibodies are swept out at an accelerated pace. In this way, early susceptibility to measles is strongly correlated with low economic status[67]. To meet this challenge age cross-sectional sero-epidemiological surveys and sero conversion studies are important for recommending the proper age for vaccination. An evaluation of the routine immunization program in Ethiopian children, reported here[68], gives support for the WHO recommended age for measles vaccination at 9 mo[69]. The ability of a measles vaccine to induce an immune response, particularly in the presence of maternal antibody, varies according to the strain and the dose of vaccine[63].

Acquired immunity after measles illness is permanent. Live attenuated measles virus, when administered at recommended ages, produces about 85% immunity after one dose and greater than 90% immunity after two doses[5,48,49]. Vaccine-induced immunity is long lasting and protective to all the diverse geographic origin strains.

Developed and developing countries of the world have different measles vaccination policy. In developed countries children are immunized at the age between 12-18 mo (depending up on the policy of different countries), as part of a three-part mumps and rubella (MMR)-vaccine. The vaccination is not given earlier than this because children younger than 12 mo usually retain anti-measles immunoglobulin's transmitted from the mother during pregnancy. A second dose is usually given to children between the ages of four and five. In developing countries where measles is highly endemic, it is recommend that two doses of vaccine be given at six months and at nine months of age. Serological studies in developing countries have shown sero-conversion rates following immunization at age 9 mo of 80%-90%[70,71]. Generally, the two-dose schedule is beneficial when there is a need to increase net vaccine efficacy, after coverage has been maximized with a one-dose schedule[64,72,73].

Role of laboratory for measles control and elimination

Laboratory investigation will play a critical role in monitoring the success of measles control strategies[15,16,59,74]. This role will require the development of more sensitive diagnostic methods suitable for diagnosis and surveillance, genetic analysis of measles strains and a technology which is transferable worldwide. Measles diagnosis relies increasingly on serological tests[75]. Serum based diagnosis can be made by virus isolation, by demonstration of a significant increase in specific IgG titers, or by the detection of anti-measles virus (MV) IgM antibodies by using radio-immunoassays (RIA), enzyme-linked immune sorbent assays (ELISAs) and direct or indirect fluorescence-antibody techniques[10,76,77]. Genetic characterization of wild-type measles viruses from different types of specimen sources provides a means to study the transmission pathways of the virus and is an essential component of laboratory-based surveillance[78-82].

The effectiveness of an immunization program can be evaluated though serological survey methods. Using dried blood spot (DBS) sample-drops of whole blood collected on filter paper from a simple finger prick-provides a minimally invasive method for collecting blood samples in nonclinical settings for serological and genetic analysis of measles[83-87]. The measles laboratory network required the use of alternative sampling techniques for surveillance[88]. The need for techniques that obviate the requirement for blood sampling promotes the application of oral-fluid based methods to the evaluation of the immunization programs. Oral-fluid based testing has an advantage of convenience, avoidance of inadvertent transmission of blood-borne pathogens, ease of use in pediatric and geriatric populations; as well as the potential for blood-free home and work place collection of patient samples.

Of the "ten elements of surveillance" summarized by WHO[89] at least in six of them visa a VIS, morbidity reporting, epidemic reporting, laboratory investigations, individual case investigations, epidemic field investigations and surveys, the laboratory has a role in providing serological results for measles surveillance. This indicates that the general advantages of measles surveillance data depend in large part on laboratory results[59,74]. The requirement of blood specimens for laboratory results can limit the yield of data for measles surveillance. In this respect we need another source of human biological material for measles surveillance, which is inexpensive and simple to collect, acceptable to donor and collector and provides accurate representation of serological status. Oral fluid has been explored as a source of human biological material for surveillance of viral diseases[90,91]. It has clear advantages over venipuncture in surveillance and epidemiology of viral diseases. In the United Kingdom, oral-fluid sampling and screening has been used for the surveillance of measles, MMR since 1994[20]. This has permitted the impact of MMR vaccination program to be monitored and evaluated in a way which may not have been possible through blood collection alone. Measles serological surveys could play a role in the evaluation of immunization programs[92,93]. Immuno-serological cross-sectional measles surveys have particular importance to determine immunization program strategy in relation to age groups, geographic areas, socio-economic groups and risk population groups. Follow-up serological measurements in measles immunized persons has importance to determine the proportion developing immune responses, quality and extent of response, duration of response and level of protection against measles infection. Periodic measles serological surveys have advantage to identify groups who are not receiving measles vaccines or who have inadequate responses. The importance of sero epidemiology for such purposes is paramount although the necessity for vein puncture reduces the ease

Table 1 Samples for laboratory diagnosis of measles virus infections

Virus disease	Samples for virus isolation for detection of antigen	Samples for serology	Remarks
Acute measles	Blood (leukocytes), throat secretions (saliva/oral-fluid), conjunctival secretions, urine; skin biopsies	Acute and convalescent serum	Period of infectivity; prodromal stage until 1-2 d after rash; antibody rises occur at appearance of rash; in tropical measles, possibly prolonged virus excretion also in stools
Measles pneumonia	Blood (leukocytes), throat secretions, conjunctival secretions, urine	Acute and convalescent serum	Frequently no rash; prolonged period of infectivity
Acute measles encephalitis	Brain specimen (biopsy or autopsy specimen), cells in CSF	Serum and CSF	In most cases, no infectious virus is detectable; occasional local production of antibodies in the CNS
SSPE	Brain specimen (biopsy or autopsy specimen), cells in CSF, lymph node biopsy (?)	Serum and CSF	Virus antigen detected in CSF cell; virus isolation requires propagation of explants cultures and cocultivation with susceptible cells; hyper-immune antibody response; local production of antibodies in the CNS

Modified from Norrby *et al*[137]. CSF: Central spinal fluid; CNS: Central nervous system.

and acceptability of this method. New methods that obviate the requirement for blood sampling could further encourage the application of measles serological surveys for the evaluation of measles immunization program. To achieve the aforementioned roles at better performance work was undertaken for measles vaccination program evaluation and surveillance based on oral-fluid collection and screening methods[94]. The purpose of this review is, therefore, to explore the development and evaluation of oral fluid as a diagnostic specimen for measles virus with particular reference to the developing country setting. The technologies developed[68,77,81] have increased the level of sensitivity and specificity where salivary examination for measles IgG and IgM is practical and convenient. Using polymerase chain reaction (PCR) technology we found oral-fluid from measles cases to be useful in the molecular characterization of measles virus. Success of the measles vaccination program can be assessed using oral fluid specimens as markers of sero-conversion.

ORAL-FLUID AS CLINICAL SPECIMENS

Laboratory investigation will play a critical role in monitoring the success of measles elimination strategies. As we shall see in this review the role will require the development of more sensitive diagnostic methods suitable for diagnosis and surveillance, genetic analysis of measles strains and a technology which is transferable worldwide.

Measles virus can be detected from various clinical samples by using serological methods, cell cultures techniques or molecular techniques. Samples that can be collected at different stages of the measles infection for virus isolation and serological tests are outlined in Table 1.

The concentration of antibody in saliva was found at much lower levels compared to plasma[95]. This has limited its use as diagnostic specimen for viral immunological assays. However, research demonstrated that salivary antibody has two sources, the parotid and crevicular crevice, with different concentration levels of immunoglobulin's[95]. The transudate that comes from the gingival crevice, whilst being lower in concentration, closely reflects the immunoglobulin class and specificities of antibody found in plasma[91,96]. The major reason for this is that the majority of the antibody present in the transudate comes from the small capillary bed beneath the margin that separates the teeth and gum. These properties of crevicular fluid lead investigators for measurements of virological markers of immune activation as an alternative to serum.

The other problem associated to the use of saliva as a viral diagnostic fluid is the need of immunological assays that have higher sensitivity. The development of antibody capture assays, ^{125}I labeled (RIA) or ELISA, that are able to generate higher signals by capturing a higher proportion of the total immunoglobulin (present in the oral fluid) specific for the antigen under test, enabled saliva to be used for successful immunological assays[97,98]. Presently the production of purified nucleoprotein through Baculovirus expression[99] increases the utility of saliva in diagnostic enzyme immunoassays.

The value of oral-fluid in screening for human immunodeficiency virus infection is now well established with the use of IgG captures radioimmunoassay[100,101]. The methodology has been applied to oral-fluid diagnosis of measles, mumps, rubella, Epstein-Barr virus and hepatitis A and B infection[77,100-104]. Veterinarians found it useful for detecting feline immunodeficiency virus[105], and feline leukemia virus[106]. Hepatitis C virus antibodies can be able detected from oral fluid[107]. Its potential application in bacteria was demonstrated with the measurement of specific IgA antibody to *Bordetella pertussis* antigens in saliva for diagnosis of whooping cough[108]. Other possibilities were seen in the diagnosis of cysticercoids by measuring specific salivary antibody to *Taenia solium* larvae[47]. Measuring of specific IgA antibodies to gliadin is used as a screening marker for coeliac disease[109,110]. Methods that can detect microbial antibodies in oral fluid such as *Helicobacter pylori* antibodies have been developed[111]. The potential to use oral fluid as Porcine Reproductive and Respiratory Syndrome virus in swine, cardiac diagnostics, oral cancer, systemic diseases, water-borne diseases, alcohol and drug testing specimen has been the subject of considerable scientific interest[112-118]. Generally oral-fluid as diagnostic fluid has the following advantages: (1) humanitarian-the patients are spared the discomfort of

repeated venipunctures; (2) clinical- with less stress, non-risk of anemia, infection or thrombosis; (3) for children-saliva sampling is the technique of choice; (4) economic-patients can collect themselves, thereby saving technicians' time, samples may also be mailed, eliminating travel time; and (5) eliminates the issue of protection of privacy and adulteration during sample collection; the ease and low cost of collection are major benefits in large-scale studies.

STUDIES PERFORMED ON MEASLES ORAL-FLUID BASED TEST METHODS

The works so far done can be specifically summarized as follows: (1) The development of a GACELISA for the detection of measles specific IgG in oral-fluid, with performance (sensitivity and specificity) that makes it suitable for replacement of serum assays, particularly for estimating population immunity[77]. By comparison with the serum measles IgG assay, the oral fluid GACELISA had a sensitivity of 97.4% (95%CI: 95.9-98.2) and a specificity of 90.0% (95%CI: 81.9-94.3), with no significant differences observed by age group. It is concluded that the overall performance of the GACELISA was satisfactory, showing close agreement to the serum ELISA, and has potential to serve as an easily transferable tool for large scale epidemiological studies as required for the World Health Organization's program for the global control of measles; (2) The development of a MACELISA for the detection of measles specific IgM in oral-fluid, suitable in performance to replace serum assays[68]; Screening of sera was undertaken using commercial indirect ELISA kits, and of oral fluids using an in-house IgM-capture ELISA. Pre-vaccination serology showed 1.4% IgM positive, 2.0% IgG positive, and 97.0% sero negative; Post-vaccination seroprevalence of IgM and IgG was 91.3% and 85.0%, respectively, and 92.9% overall. The seroconversion rate was 92.6% (95%CI 88.2-95.7); Based on oral fluid results, 87.3% (95%CI: 82.0-91.4) of children showed specific IgM antibody conversion. These results are in support of the recommended age for measles vaccination in Addis Ababa, and show the merit of oral-fluid IgM screening as a non-invasive alternative to blood for assessing vaccine immunogenicity; (3) Demonstration of the use of these assays in the estimation of measles antibody (immunity) prevalence in the vaccine-targeted population and in monitoring the outcome of a measles vaccination program (routine and campaign) in a developing country setting[68,96-98]; (4) Demonstrate the utility of oral fluid to study the molecular epidemiology of measles virus in both developed and developing country situations in a period of accelerated measles control[81,82,109]; (5) Oral fluid for the serological and molecular diagnosis of measles in a developed country setting[109,119]. These studies demonstrate the use of oral fluid samples for the detection of measles virus in the United Kingdom and the Belgian measles surveillance system and other studies in the framework of the WHO elimination program; (6) Technical refinements of sample collection and laboratory screening of oral fluid, and, importantly, comparisons with existing methods based on serum prior to wider adoption of non-invasive methods. This work includes the evaluation oral-fluid relative to serum and DBS for the detection of measles specific IgM in suspected measles cases in relation to assay type and sample timing post onset of rash. Works done to assess the performance (sensitivity and specificity) of a commercial IgG antibody capture method for oral fluid in relation to currently used assays for serum/blood spots is in preparation for publication (Dr. Nigatu W personal communication); (7) The studies emphasize the potential and suitability of oral-fluid to substitute serum in estimating and monitoring measles IgG antibodies, during community surveys[118-120]; (8) Applicability of oral fluid collected onto filter paper for detection and genetic characterization of measles virus strains[119,121]. The former study showed molecular nested RT-PCR using oral fluid was validated against the standard assay on nasopharyngeal secretions and gave a sensitivity of 100% and specificity of 100%. The latter study demonstrate that oral fluid dried onto filter paper can be used for the detection and characterization of MV strains. Using this approach, an MV-positive sample by reverse transcriptase PCR could be obtained from 67% of serologically confirmed acute measles cases; (9) Determination of measles immunization status using oral-fluid samples [122]. The presence of antibodies in oral fluid specimens correlated with that in serum with sensitivity and specificity: measles, 97% and 100%, respectively. This study assessed protective antibodies to measles by means of an oral fluid sample with good reliability; and (10) Evaluation of the performance of a newly developed point-of-care test (POCT) for the detection of measles-specific IgM antibodies in serum and oral fluid specimens and to assess if measles virus nucleic acid could be recovered from used POCT strips[123]. With oral fluids POCT showed sensitivity and specificity of 90.0% (63/70) and 96.2% (200/208), respectively. Both *H* and *N* genes were reliably detected in POCT strips and the *N* genes could be sequenced for genotyping. Measles virus genes could be recovered from POCT strips after storage for 5 wk at 20-25 ℃. The POCT has the sensitivity and specificity required of a field-based test for measles diagnosis. However, its role in global measles control programs requires further evaluation.

PRESENT AND FUTURE APPLICATIONS OF MEASLES ORAL-FLUID METHODS

Present applications

Community surveys of measles specific IgG/IgM are useful to guide the design of measles control programs. For example these help in (1) defining levels of immunity to measles pre- and post-vaccination efforts, *i.e.,* assessing the effectiveness of the vaccination program; (2) identifying age groups in which a significant susceptible proportion remain; and (3) assessing sero-conversion rates following vaccination. Analysis of the genetic characteristics

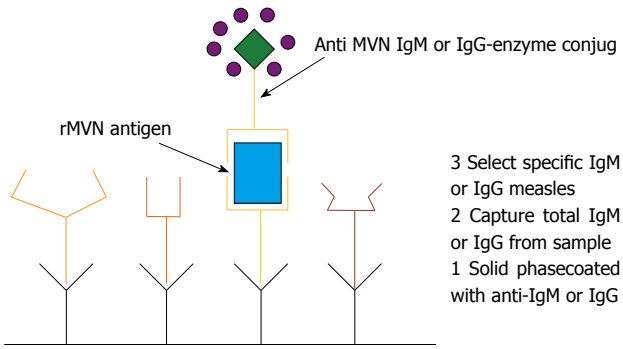

Figure 2 Principle of the"Microimmune" measles IgM or IgG Capture Enzyme Immuno-assay methodology. IgG: Immunoglobulin G; IgM: Immunoglobulin M; MVN: Medial vestibular nucleus.

of wild-type measles helps to elucidate the origin and transmission pathways of measles virus [124]. The genetic data when analyzed with other epidemiological data provides a means to assess the efficacy of measles control programs. For such molecular studies measles RNA can be detected by RT-PCR from isolates, oral fluid, blood, throat-swabs, urine collected from acute cases. There have been no systematic studies made to evaluate the relative sensitivity of these different samples. The primary role of this review is in the demonstration of the use of oral fluid as a clinical specimen for detecting IgG/IgM antibody for evaluating measles control strategies and the virus genome for molecular epidemiological studies.

Future technical development

The low concentration of IgG/IgM antibodies in oral fluid relative to other diagnostic specimens such as plasma[95] demanded the development of an enhanced immuno assays and of diagnostic techniques based on nucleic acid amplification.

Promotion of the use of oral fluid as viral diagnostic fluid requires that immunological assays have higher sensitivity. The development of antibody capture assays, either ^{125}I labeled (RIA) or ELISA, that are able to generate higher signals by capturing the proportion of specific to the total immunoglobulin (present in the oral fluid), enabled oral fluid to be used for successful immunological assays[97-99]. The RIA have associated problems in disposing of radioactive waste from aspect of health may decrease its acceptability. Capture ELISA is better for wide-scale use in many laboratories. A study also showed that the capture ELISA with saliva was more sensitive than the radioimmunoassay for specific rubella IgG[102]. Hence sensitivity enhancement is required to make best advantage of ELISA. This review shows that FITC/anti-FITC enhanced capture ELISA that can be used for population and vaccine surveys [68,77].

The production of measles antigen for measles diagnosis, such as the one we used for GAC- and MAC-ELISA, benefited from tissue culture. However, production of purified measles antigens in tissue culture can be difficult. The capture format has been revolutionized by the raising of purified antigen and monoclonal antibodies for use in oral-fluid measles diagnostics. Cloning and expression of measles genes provides a relatively straightforward alternative approach[99,125], simplifying purification and enabling large-scale production for improvement in measles oral-fluid diagnostic assays.

Kits based on the use of recombinant antigens such as the Light Diagnostic kit (Chemicon Temecula, CA, United States) benefited from the cloning and expression approach. More recently IgG and IgM kits specific to measles have been developed based on such an alternative approach by Microimmune Ltd (Brentford, Middlesex, United Kingdom) for both oral-fluid and serum samples. However, there are problems associated with the use of recombinant antigens associated with the production of "incorrectly" processed antigens by most expression systems[99,125] and the problem of using a single cloned antigen to detect a measles antigen that may vary between isolates. This may be resolved by cloning and expressing the most conserved region of the measles gene identified from sequence data of different isolates. Notwithstanding this problem measles antibody assays that are increasingly based on the use of cloned proteins will continue to play a prominent role in oral-fluid diagnostic development. Such immuno-assays may be useful in the future when they become better suited to use with automated systems that are capable of handling all stages of testing from specimen preparation to issuing of diagnostic results.

Microimmune assays are observed to be easy to use, but have not yet been evaluated under a wide range of conditions such as in highly vaccinated populations. The procedure and principle of the oral-fluid Microimmune EIA methodology is described in Figure 2.

Studies of rubella revealed problems of sensitivity in enhanced GACELISA in older age groups. This appears to be due to decay in the level of specific antibody in serum and in oral fluid[98,102,126]. Age-related variation in sensitivity was not seen as a big problem in measles assays[77,98]. However, low-level measles antibodies resulting from vaccine-induced immunity is a feature of many communities, particularly those with high-level routine immunizations coverage. Future work is required to evaluate the performance of newly developed kit assays in such settings.

Assays of measles nucleic acid are fundamentally different from those of measles antibodies, since they detect a component of the measles virus itself, rather than serological evidence of its past presence. Among the several techniques used to detect viral nucleic acids the PCR is the one widely used for detection of measles nucleic acid[127-129]. In contrast to direct hybridisation, whose application is restricted to where high concentration of the virus is present, PCR amplifies the probe signal by means of a sequential series of secondary, tertiary, *etc.* stages. The signal amplification thus increases the sensitivity of detection to a range where it can detect viruses at low concentration in various specimens[129]. PCR is suitable

for the detection of the low concentration of measles virus present in oral fluid. Actually oral fluid is better for nucleic acid extraction than serum or blood because of the absence of PCR inhibitors, such as haem or porphyrin, in the oral fluid[129]. In addition, oral fluid specimens do not need pre-treatment for nucleic acid extraction. In future developments of measles oral-fluid diagnosis based on the nucleic acid amplification systems are likely to play an increasing part. The new tool developed by Roche Molecular Biochemicals, MagNA Pure LC DNA isolation kit, for the isolation of nucleic acid from various types of specimen including oral fluid, is a breakthrough that has shortened the tedious manual RNA extraction process in measles nucleic acid detection. This is now practiced in many laboratories of industrialized countries but may be restricted to laboratories that have specialised requirements and too costly for most developing countries. Recently Health Protection Agency has developed point-of-care test (POCT) for the detection of measles-specific IgM antibodies in serum and oral fluid specimens and to assess if measles virus nucleic acid could be recovered from used POCT strips[123]. Further evaluation of this test method under different scenario will refine the technique for wider future application.

IgG can be measured in terms of its functional binding avidity. The binding strength between the IgG and the virus antigen is supposed to be low in primary infection and changes to high in past infection. This avidity can be measured by disrupting the interaction using protein denaturants such as urea or diethylamine[130]. Diagnosis of primary infection by IgG avidity assay using serum samples has got relevance for the diagnosis of viral infection such as rubella[131-132]. The detection of antibody with low or high avidity enables a more accurate diagnosis in differentiating primary infection from past infection.

IgG avidity is useful when the IgM assay result is indeterminate. It may also help in distinguishing primary and secondary ("boosting") response to measles vaccine. Future development of IgG avidity in oral-fluid measured by GACELISA may allow specific, sensitive and accurate diagnosis of primary infection. Our study[68] show the problem of MACELISA in detecting IgM in oral-fluid samples collected at early onset of measles rash. The future development of IgG avidity that can determine IgM in early-collected oral fluid samples makes MACELISA better use.

Another interesting area to look at in the future is the differentiation between antibodies resulting from vaccine strain and wild type measles virus. This assists in defining vaccine uptake and estimating continued measles transmission. It may be difficult to explain the technical development at this stage. However, it is an area for future research.

Evaluation of a new diagnostic test has the potential sources of bias introduced by the study design. The test's discriminatory ability, sensitivity, and specificity depend upon the composition of the study population. The study design we used for evaluation of the present measles oral- fluid diagnostic assays[68,77] is an area that can be followed in the future for other viral diagnostic test evaluation.

Future wider applications of oral-fluid methods

The application of oral-fluid methods to population surveys, vaccine surveys, diagnosis of clinical cases, and case surveillance for different vaccine uptake settings of a country/district are illustrated in Table 2. The following is a description of some of the applications of oral-fluid methods.

Population surveys: Measles antibody population surveys can used to define the proportions of susceptible and immune in the population. Population immunity may result from natural measles infection or/and measles routine and campaign vaccination. Current methods cannot distinguish between the two whether the immunity is induced by wild or vaccine virus. Population immunity surveys can identify in which age groups large pockets of susceptibles remain in unvaccinated populations, in a population with routine immunization, and before and after a vaccine campaign. This would provide valuable information on the age groups to target for vaccination and effectiveness of the routine or campaign vaccination, and clues to where future outbreaks might arise. Similarly, through such surveys hard-to-reach groups in rural/urban under different geographical settings can be reached.

Population surveys may be appropriate at all stages of vaccination programmes, in country/settings of vaccine uptake for low to high, with or without campaigns/accelerated measures. Predominantly, such surveys could assess specific antibody status. However, post-campaigns there might be a role for IgM testing in community survey to establish what proportion of the population actually responded to vaccine. Based on surveys cluster sampling techniques, as for EPI vaccine cluster sampling, and using of the-shelf EIA kits, the surveys would be rapid and simple to effect.

Vaccine surveys: Vaccine surveys assess the level of population immunity attending vaccine clinic to a measles routine vaccination. It can identify the responses to routine vaccine in pre- and post-vaccinated children. The widespread use of serological determinants of vaccine responsiveness is limited by the need to carry out follow up of vaccinees at 2 (IgM) and < 4 (IgG) wk after vaccination. Oral-fluid sampling will not improve greatly up on this situation, except that compliance for second samples is likely to be greater than if blood samples are required. However, the future development of oral-fluid IgG avidity measurement cannot be ruled out that may improve this situation.

Diagnosis: Measurement of measles antibody present in oral-fluid samples provides information on the status of current and past infection by use of tests for IgG and IgM antibody. Laboratory diagnosis of suspected measles clinical cases can assist in (1) confirmation of the occurrence of measles clinical illness (2) capability of physicians to diagnose illness and (3) reporting of the infection to health department. The usefulness of

Table 2 Application of oral-fluid methods under different countries settings

Applications of oral-fluid methods	Setting for country/district		
	Low/Med uptake routine	High uptake routine	Campaign
Population survey	Methods: community surveys of IgG across wide age range. Including hard-to-reach groups, informal settlements. Purpose: immunity profiles. Identifies susceptibility gaps and age range for campaigns. Implications: increase in coverage, need for and age range for campaigns	As previous	As previous plus. Methods: Post-campaign surveys of IgG and perhaps IgM. Purpose: IgG-Identify immunity levels post-campaign. Susceptibility in target age group and outside target group. IgM-indicator of impact, *i.e.*, proportion responding to vaccine. Implications: Age-range for future campaigns; locate problems of vaccine efficacy
Vaccine surveys	Methods: Vaccine clinic samples pre- and post-vaccination. IgM and/or IgG testing. Purpose: Assess efficacy of routine vaccination. Implications: Identify cause of low efficacy.	As previous	As previous plus. Methods: IgG survey of individuals attending vaccine clinics. Purpose: Identify proportion able to respond to vaccine. Implications: Assess potential effectiveness, and suggest alternative method for delivery eg hard-to-reach groups.
Diagnosis	Not indicated while measles incidence remains high	Method: IgM testing on demand. Purpose: Confirmation of clinical diagnosis	As previous
Case surveillance: serological and genetic	Not indicated while measles transmission remains high	Method: System of reporting and oral fluid sampling from sporadic cases and outbreaks. IgM and Genotyping Purpose: Verify cases, and monitor distribution of virus and endemicity Implications: Need for additional control measures	As previous

IgG: Immunoglobulin G; IgM: Immunoglobulin M.

oral fluid in this capacity is at present hindered by the relatively low sensitivity of IgM assays in samples taken early after onset of rash. A study showed the oral fluid measles IgM detection rate increased from 63%-67% at 2 d and 3%-100% at days 6 and 7[82]. Delay in collecting a sample may be impractical. Improved sensitivity of assays remains a need.

Case surveillance: The recognition and identification of measles outbreaks and sporadic cases using a system of reporting and oral-fluid sampling is established in the United Kingdom[20,76,133]. For measles epidemic investigation in Ethiopia, where infrastructure is poor and locations of the remote, oral-fluid sampling was found to be appropriate. Especially in the situations where community beliefs or attitudes like "measles sick should not get injection" are present, in which communities declined to give blood specimens, oral-fluid specimens are preferable. Provided reasonable storage conditions while in transit or awaiting transit to the laboratory are made, oral-fluid is a robust sample for IgG testing, IgM testing and viral genome detection (United Kingdom surveillance and in these studies in Ethiopia)[77,134]. However, further stability studies of oral-fluid at different temperature in field conditions are required in the future.

Another area of increasing importance is the application of sequence data obtained from oral-fluid nucleic acid amplification techniques. Genetic information is valuable, in combination with other traditional epidemiological data, to enhance the ability to determine measles transmission pathways and to assess the success of measles control strategies[79,124,135,136].

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ORIGINAL ARTICLE

Epigallocatechin-3-gallate suppresses transforming growth factor-beta signaling by interacting with the transforming growth factor-beta type Ⅱ receptor

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Abstract

AIM: To investigate the (-)-epigallocatechin-3-gallate (EGCG) binding to transforming growth factor-β (TGF-β) type Ⅱ receptor (TGFRⅡ).

METHODS: The expression of α-smooth muscle actin (α-SMA) was used as a marker for fibrotic change in human lung fibroblast MRC-5 cells. The α-SMA expression level was determined by western blotting and immunohistological analysis. We examined whether the anti-fibrotic effects of EGCG on MRC-5 cells was dependent on antioxidant mechanism by using edaravone and *N*-acetylcysteine (NAC). The suppression effects of EGCG on Smad2/3 activation were studied by confocal fluorescence microscopy. The binding of EGCG to recombinant TGFRⅡ protein was analyzed by immunoprecipitation and affinity chromatography.

RESULTS: When MRC-5 cells were treated with TGF-β, EGCG decreased the expression of α-SMA in a dose dependent manner, whereas catechin did not influence the α-SMA expression in the cells. Except for EGCG, antioxidant compounds (*e.g.,* edaravone and NAC) had no effects on the TGF-β-induced α-SMA expression. Nuclear localization of phosphorylated Smad2/3 was observed after TGF-β treatment; however, EGCG treatment attenuated the nuclear transportation of Smad2/3 in the presence or absence of TGF-β. After a TGFRⅡ expression vector was introduced into COS-7 cells, cell lysates were untreated or treated with EGCG or catechin. The immunoprecipitation experiments using the lysates showed that EGCG dose-dependently bound to TGFRⅡ and that catechin did not at all. Affinity chromatography study indicated that EGCG would bind to TGFRⅡ.

CONCLUSION: Our results demonstrate that EGCG interacts with TGFRⅡ and inhibits the expression of α-SMA via the TGF-β-Smad2/3 pathway in human lung fibroblast MRC-5 cells.

Key words: Epigallocatechin-3-gallate; Transforming

growth factor-β; Myofibroblast; α-smooth muscle actin; Fibrosis

Core tip: (-)-Epigallocatechin-3-gallate (EGCG) binds to transforming growth factor-β (TGF-β) type Ⅱ receptor (TGFRⅡ) and inhibits TGF-β action by interfering with the interaction between TGF-β and TGFRⅡ. Because TGF-β is considered to be the strongest inducer of tissue fibrosis, the obtained data from this investigation suggest that EGCG may be a new therapeutic agent for organ fibrosis.

Tabuchi M, Hayakawa S, Honda E, Ooshima K, Itoh T, Yoshida K, Park AM, Higashino H, Isemura M, Munakata H. Epigallocatechin-3-gallate suppresses transforming growth factor-beta signaling by interacting with the transforming growth factor-beta type II receptor. *World J Exp Med* 2013; 3(4): 100-107 Available from: URL: http://www.wjgnet.com/2220-315X/full/v3/i4/100.htm DOI: http://dx.doi.org/10.5493/wjem.v3.i4.100

INTRODUCTION

(-)-epigallocatechin-3-gallate (EGCG), the most biologically active constituent in green tea, has been recognized as a component that provides the beverage with potential benefits for human health[1]. The reported health-promoting properties of green tea include anti-cancer[1-3], anti-obesity[4], anti-diabetic[5,6], anti-atherosclerotic[7], anti-viral[8-10], anti-bacterial[11-13] and neuroprotective[14-16] effects. The anti-fibrotic effects of green tea and its constituents, especially EGCG, on liver fibrosis[17-19], pancreatic fibrosis[20] and pulmonary fibrosis[21] have been also reported.

Activation of myofibroblasts is the one of the critical events during fibrosis development. Transforming growth factor-beta (TGF-β) is a multifunctional cytokine that is pivotal in the regulation of myofibroblast activation, differentiation, migration, and extracellular matrix production; it also plays an important role in the initiation and progression of fibrosis[22]. However, the mechanisms by which EGCG influences TGF-β action on myofibroblast activation remain incompletely defined.

Tachibana *et al*[23] identified a catechin receptor for EGCG, and showed that this receptor partially mediates the function of EGCG. It is also known that EGCG shows its biological action by interacting with receptors other than the catechin receptor[24,25]. In the present study, we investigated the possibility that EGCG might bind to the TGF-β type Ⅱ receptor (TGFRⅡ).

MATERIALS AND METHODS

Cell culture

The MRC-5 and COS-7 cell lines were obtained from the Riken Cell Bank (Tsukuba, Japan), and were maintained in Dullbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, United States) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, United States) at 37 ℃ under 5% carbon dioxide and 95% air.

Chemicals

Catechin and EGCG were obtained from Funakoshi Co. (Tokyo, Japan) and dissolved in PBS. *N*-acetylcysteine (NAC) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and dissolved in dimethyl sulfoxide. Edaravone was the product of Mitsubishi Tanabe Pharma (Osaka, Japan). TGF-β was obtained from R&D Systems (Minneapolis, MN, United States).

Antibodies

The following antibodies were used in this study: monoclonal anti-FLAG antibody produced in mouse (anti-Flag) (Sigma); monoclonal anti-α-smooth muscle actin antibody (anti-α-SMA) produced in mouse (Sigma); monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (anti-GAPDH) produced in mouse (Sigma); rabbit anti-Smad2/3 antibody (anti-Smad2/3) (Cell Signaling Technology, Danvers, MA, United States); and goat anti-human TGFRⅡ antibody (anti-TGFRⅡ), which recognizes extracellular domain of the receptor (R and D).

Western blotting

After washing with ice-cold phosphate buffer saline (PBS), cells were treated with 0.25% trypsin-EDTA solution (Invitrogen, Carlsbad, CA, United States), suspended in growth medium and collected by centrifugation at 700 *g* for 5 min. The pellets were washed with PBS, resuspended in lysis buffer (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate), which contained a cocktail of protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany) on ice, and centrifuged at 18000 *g* at 4 ℃ for 10 min.

Protein concentration was determined by a BCA protein assay kit (Pierce, Rockford, IL, United States). Cell lysates were suspended in SDS electrophoresis sample buffer and boiled for 5 min. Samples (2.5 μg of protein per lane) were separated on 10% polyacrylamide gels and then transferred to an Immobilon P membrane (Millipore, Billerica, MA, United States). Antibody binding was detected by ECL Plus (GE Healthcare, Buckinghamshire, United Kingdom).

Immunohistological studies

Cells were seeded on BD Falcon 8-well CultureSlide. Cells were cultured under indicated conditions. Medium was removed, and cells were washed with PBS, fixed by 3% paraformaldehyde in PBS for 10 min. After washing with PBS, cells were permeabilized by 0.1% Triton X-100 in PBS. Fixed cells were sequentially treated with anti-α-smooth muscle actin (SMA) antibody (1/100, 37 ℃, 1 h), and fluorescein isothiocyanate conjugated goat anti-mouse immunoglobulin G (37 ℃, 30 min). Actin stress fibers were visualized by rhodamine-labeled phalloidin (1/50,

37 ℃, 10 min). For staining the nuclei, cells were treated with 4',6-diamidino-2-phenylindole (DAPI) (1 μg/mL) for 20 min. Cells were examined with a fluorescence microscope (Nikon ECLIPSE E-800, Nikon Corporation, Tokyo, Japan) equipped with a fluoresecence digital microscope camera controller (VB-7000; Keyence Co., Osaka, Japan).

Plasmid construction

Plasmid was constructed according to standard recombinant DNA techniques. The fragment encoding the human TGFRⅡ cDNA (Met1-Lys567, GenBank accession no. M85079) was amplified from a human fetal liver cDNA library (OriGene Technologies, Rockville, MD, United States) by polymerase chain reaction (PCR) with KOD Plus DNA polymerase (Toyobo Co., Ltd., Osaka, Japan) using the primers 5'-TTTGAATTCGCCATGGGTCGGGGGCTGCTC-3' (forward) and 5'-TTTGGATCCTTTGGTAGTGTTTAGGGAGCC-3' (reverse). The forward and reverse primers were designed to introduce an *Eco*R Ⅰ and a *BamH* Ⅰ restriction site (underlined), respectively, for subcloning purposes. The PCR product was cloned into the pFlag-CMV-5a vector (Sigma). The construct was verified by DNA sequencing.

Transfection

COS-7 cells, grown to 50%-70% confluence, were transfected using Lipofectamine plus (Invitrogen) according to the manufacturer's instructions. The transfectants were grown in DMEM containing 10% FBS. After 3 d, the medium was removed and expression of TGFRⅡ in the cells was examined by western blotting.

Immunoprecipitation

Cell lysates were treated with Protein G Sepharose (GE Healthcare) for 30 min at 4 ℃ to remove proteins nonspecifically bound to Protein G Sepharose. Anti- TGFR Ⅱ antibody was then added to the above lysate, and incubated for 2 h at 4 ℃. Next, Protein G Sepharose was added and incubated for 1 h at 4 ℃. Protein G Sepharose was recovered by centrifugation and washed three times with PBS. The immunoprecipitated proteins were removed from the Protein G Sepharose by boiling in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for 5 min and then separated by electrophoresis.

Affinity chromatography

EGCG was coupled to CNBr-activated Sepharose 4B (GE Healthcare) at a concentration of 5 mg/mL of wet gel. Cell lysate was applied to a column of EGCG-Sepharose 4B and washed with PBS. Bound proteins were eluted with 4 mol/L urea, 1 mol/L NaCl in PBS, and fractions of 0.25 mL were collected. An aliquot of each fraction was spotted onto polyvinylidene difluoride (PVDF) membrane and stained with Coomassie Brilliant Blue. A portion of each fraction was also examined by western blot analysis after SDS-PAGE using anti-TGFRⅡ antibody.

RESULTS

Effects of EGCG on the expression of α-smooth muscle actin

The MRC-5 cell line, which is derived from human fetal lung fibroblasts, expresses α-SMA and is considered to be a myofibroblast cell line[26,27]. Therefore, this cell line was used in this study.

MRC-5 cells were grown to 85% confluence, and then serum-starved (0.5% FBS) for 48 h. After serum starvation, cells were treated with TGF-β. We and others usually use 1-2 ng/mL of TGF-β in culture media[27-31]. A representative and frequently used marker of myofibroblast activation is α-SMA[32,33]. Western blot analysis and immunohistological examination showed that expression of α-SMA was increased by TGF-β (Figure 1). Whereas a catechin control showed no effects on α-SMA expression, EGCG dose-dependently abolished the increase in expression of α-SMA induced by TGF-β (Figure 1B). The EGCG concentration used in this study was reasonable[34]. The expression of GAPDH also seemed to be decreased by a high dose of EGCG. The band densities of α-SMA and GAPDH were compared (Figure 1B), and the result clearly showed the effects of EGCG on α-SMA.

Because EGCG is an antioxidant compound, we examined whether edaravone and NAC, two well-known antioxidant compounds, have similar effects. Neither treatment with edaravone (Figure 2A) nor treatment with NAC (Figure 2B) affected the increase in expression of α-SMA induced by TGF-β.

EGCG suppresses SMAD activation

The effects of TGF-β are largely mediated by Smad proteins. TGF-β causes phosphorylation of Smad2/3, and then phosphorylated Smads enter into the nucleus. After treatment with TGF-β, MRC-5 cells were examined by confocal fluorescence microscopy. Nuclear localization of phosphorylated Smad2/3 was observed after TGF-β treatment, whereas EGCG treatment clearly decreased the nuclear transportation of Smad2/3 (Figure 3).

EGCG binds to TGFRⅡ

Next, we examined the possibility that EGCG interferes with binding of TGF-β to the TGFRⅡ. To this end, cells expressing large amounts of the receptor are preferable. Because COS-7 cells showed high transformation efficiency and marked expression of exogenous cDNA, these cells were used for transformation experiments. A TGFRⅡ expression vector was introduced into COS-7 cells. Cell lysates were untreated or treated with EGCG or catechin, and then subjected to immunoprecipitation with anti-TGFRⅡ antibody. In untreated lysate and lysate treated with catechin, TGFRⅡ was precipitated by the antibody. When lysate was treated with EGCG, however, anti-TGFR did not precipitate TGFRⅡ (Figure 4).

To confirm the binding of EGCG to TGFRⅡ, we next performed affinity chromatography. Namely, cell ly-

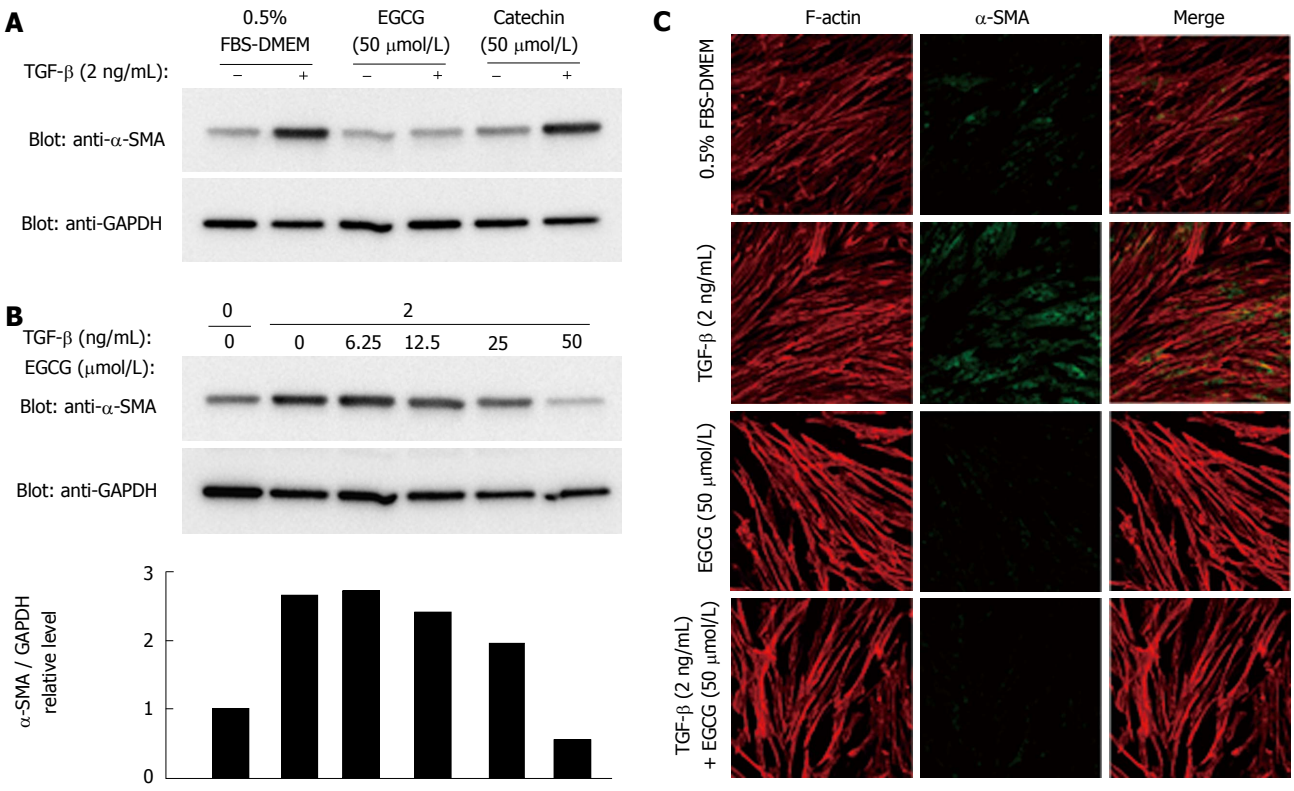

Figure 1 Effects of (-)-epigallocatechin-3-gallate on expression of α-smooth muscle actin. A: Lysates of MRC-5 cells were obtained from cells treated with 0.5% FBS in DMEM alone, (-)-epigallocatechin-3-gallate (EGCG) (50 μmol/L), or catechin (50 μmol/L) for 24 h. After SDS-PAGE, proteins were blotted onto Immobilon, and probed with anti-α-smooth muscle actin (α-SMA) antibody. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control; B: MRC-5 cells were treated with the indicated amounts of EGCG. α-SMA was detected in the same manner as in (A). The expression levels of α-SMA were normalized to those of GAPDH; C: Expression of α-SMA in cells treated with EGCG (50 μmol/L) in the absence (-) or presence (+) of transforming growth factor-β (TGF-β) (2 ng/mL) were examined by confocal microscopy. Green: α-SMA (fluorescein isothiocyanate conjugated goat anti-mouse IgG); Red: Actin stress fiber (rhodamine-labeled phalloidin); blue: Nuclei (DAPI). FBS: fetal bovine serum.

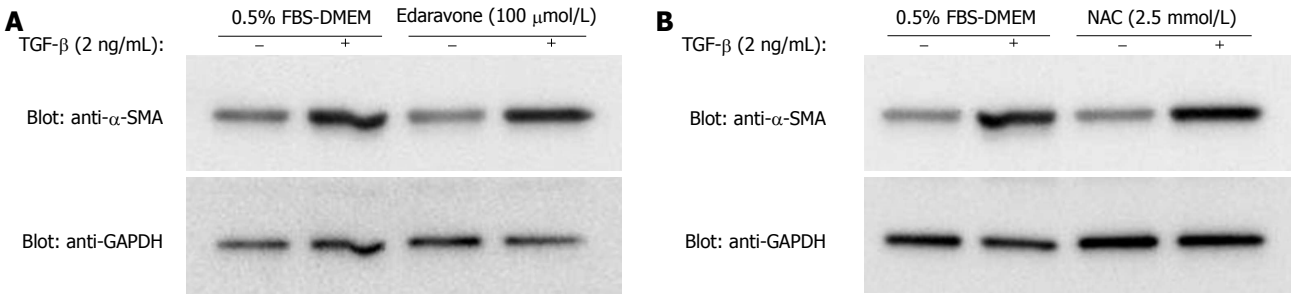

Figure 2 Effects of scavenging compounds on expression of α-smooth muscle actin. MRC-5 cells were treated with edaravone (100 μmol/L) (A) or *N*-acetylcysteine (NAC) (2.5 mmol/L) (B) for 1 h, and then stimulated with transforming growth factor-β (TGF-β) for 24 h. Cell lysates were electrophoresed, blotted onto Immobilon, and probed with anti-α-smooth muscle actin (α-SMA). GAPDH: Glyceraldehyde 3-phosphate dehydrogenase. FBS: Fetal bovine serum.

sates were applied to an EGCG-conjugated agarose column and proteins bound to the column were examined by western blotting. Figure 5 shows that TGFR Ⅱ bound to the column, indicating that EGCG binds to TGFR Ⅱ.

DISCUSSION

In this study, we have demonstrated that EGCG both inhibits the signal transduction of TGF-β by binding to TGFR Ⅱ and attenuates the expression of α-SMA in MRC-5 cells, which is a myofibroblast cell line, when it is stimulated by TGF-β. Myofibroblasts play crucial roles in the pathogenesis of tissue fibrosis[35]. Stimulation by TGF-β and other cytokines leads myofibroblasts to an activated state[36]. Activated myofibroblasts then secrete collagen and other components of the extracellular matrix, which can result in fibrosis[37].

TGF-β is the most potent cytokine causing fibrosis. Both Smad-dependent and Smad-independent TGF-β signaling pathways are known. Initiation of both pathways takes place via binding of TGF-β to its receptor. TGF-β binds to a type Ⅱ receptor, which then phosphorylates a TGF-β type I receptor. Subsequently, the type I receptor phosphorylates R-Smads (receptor-

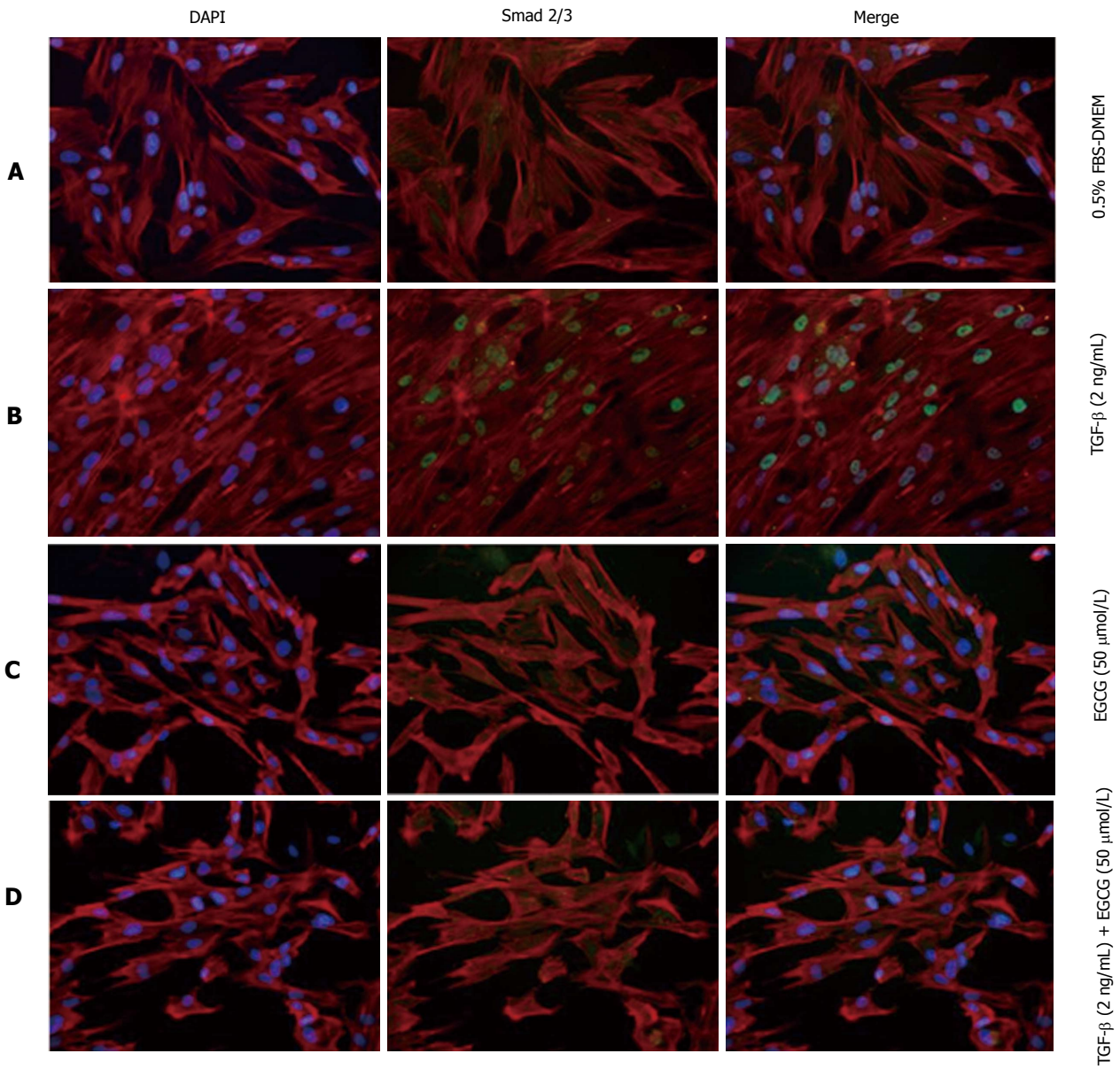

Figure 3 Effects of (-)-epigallocatechin-3-gallate on activation and localization of Smad2/3. MRC-5 cells were treated with transforming growth factor-β (TGF-β) and/or (-)-epigallocatechin-3-gallate (EGCG). Cells were examined by confocal microscopy. Subcellular localization of Smad2/3 (green) and actin stress fibers (red) are shown. Nuclei were stained by DAPI (blue). A: Control; B: Treated with TGF-β; C: Treated with EGCG; D: Treated with TGF-β and EGCG. DAPI: 4',6-diamidino-2-phenylindole.

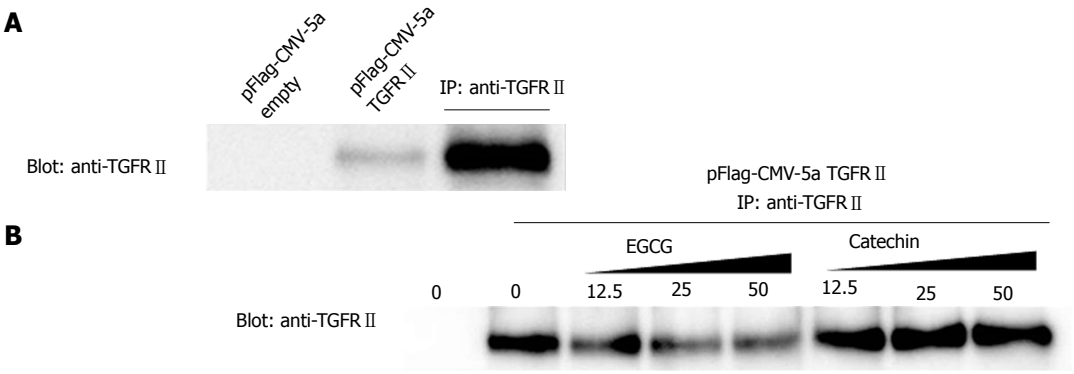

Figure 4 (-)-Epigallocatechin-3-gallate interferes with binding between transforming growth factor-β and its type Ⅱ receptor. A: Positive control of the immunoprecipitation experiment. Cell lysates from transfected COS-7 cells were treated with anti-transforming growth factor-β type Ⅱ receptor (TGFR Ⅱ). TGFR Ⅱ was recovered in the immunoprecipitation product of the lysate; B: Effects of (-)-epigallocatechin-3-gallate (EGCG) and catechin on the antigen-antibody interaction. After cells were treated with EGCG or catechin, anti-TGFR Ⅱ bound to Protein G was added to each lysate. Western blotting was performed using anti-TGFR Ⅱ.

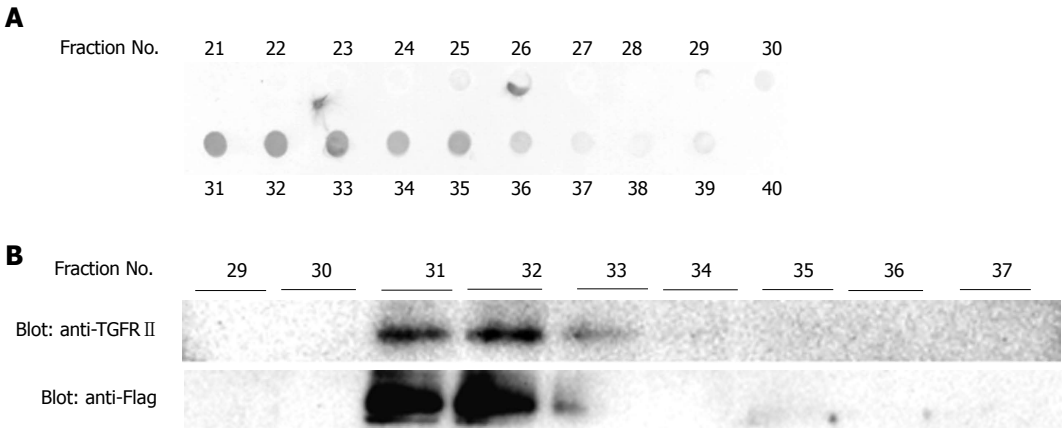

Figure 5 Transforming growth factor-β type Ⅱ receptor binds to (-)-epigallocatechin-3-gallate. A: Proteins in the fractions were detected by staining with Coomassie Brilliant Blue. An aliquot of each fraction was blotted on polyvinylidene difluoride membrane and stained; B: The protein-containing fractions revealed in (A) were electrophoresed and Western blotting was performed using anti-Flag antibody. TGFR Ⅱ: Transforming growth factor-β type Ⅱ receptor.

regulated Smads), and phosphorylated R-Smads bind to Co-Smad (common-mediator Smad). R-Smad/Co-Smad complexes translocate into the nucleus, where they act as transcription factors[38]. In this manner, regulation of TGF-β target gene expression is carried out. Expression of many proteins in MRC-5 cells changes after stimulation by TGF-β[39]. A frequently used marker of the activation of myofibroblast is α-SMA; therefore, this protein was also used as a marker in this study. Expression changes after TGF-β stimulation in cells other than MRC-5 has been observed, for example, in IMR-90 human lung fibroblasts[33] and WI38-VA13 cells[40]. Besides α-SMA, upregulation of collagen I [41], fibronectin[27]and CTGF[42] has been reported when human lung fibroblasts are treated with TGF-β.

The expression of α-SMA is regulated by Smad[43]. TGF-β increases the nuclear translocation of Smad and expression of α-SMA. We examined the influence of EGCG treatment on Smad2/3 appearance in MRC-5 cells. Immunohistological experiments indicated that EGCG inhibits the nuclear transportation of Smad2/3.

Moreover, we found that EGCG had suppressive effects on the expression of α-SMA in MRC-5 cells, whereas catechin did not. These data suggest that the effects are dependent on the gallate or pyrogallol moiety of EGCG.

Next, we investigated the mechanism of the inhibitory effect on the Smad2/3 pathway. EGCG is a potent antioxidant and a lot of its health benefit effects are thought to be due to its antioxidative action[44-46]. EGCG attenuated the increase in α-SMA expression brought about by TGF-β, whereas edaravone and NAC did not. These results indicate that the inhibitory effect of EGCG on α-SMA expression is independent of its antioxidative action.

We thought that part of the EGCG's effects on α-SMA expression might arise through interference with receptor-ligand binding. Indeed, EGCG treated cell lysate containing TGFR Ⅱ showed no immunoprecipitation with anti-TGFR Ⅱ antibody. The interaction between EGCG and TGFR Ⅱ was also confirmed by the affinity chromatography experiment. A likely explanation for this observation is that EGCG binds to TGFR Ⅱ, thereby blocking the antibody from binding to TGFR Ⅱ. Similarly, if EGCG binds to the TGF-β receptor, TGF-β would not be able to bind to its receptor and downstream signaling pathways would be ineffective.

In conclusion, we have shown that EGCG interacts with TGFRII and inhibits the expression of α-SMA via the TGF-β-Smad2/3 pathway in MRC-5 cells, which are human lung fibroblasts. These results suggest that EGCG has anti-fibrotic effects that are crucial for the control of myofibroblast differentiation and extracellular matrix deposition, which are involved in fibrosis. The evidence that EGCG is effective in the suppression of fibrosis may lead not only to better understanding of the biological roles of EGCG but also to clinical applications of this flavonoid.

COMMENTS

Background

Fibrosis is an intractable disease. Effective treatments for it have not been developed yet. Catechin is a substance with a variety of physiological effects. However, the investigation on the antifibrotic effect of catechin has not been fully performed.

Research frontiers

Various physiological effects of catechin have been intensely studied. It has been reported that catechin has a variety of physiological activity (*e.g.,* regulation of blood pressure, blood cholesterol, blood sugar; antioxidant, anti-aging, anti-cancer effects).

Innovations and breakthroughs

Many studies have been performed about (-)-epigallocatechin-3-gallate (EGCG) relationship with transforming growth factor-β (TGF-β) and its antifibrotic properties. We demonstrated that EGCG inhibits the TGF-β activity through its binding to TGF-β type Ⅱ receptor (TGFR Ⅱ).

Applications

TGF-β is believed to be the strongest inducer of tissue fibrosis. EGCG inhibits TGF-β activity by interacting with TGFR Ⅱ. Therefore, EGCG may become an antifibrotic agent.

Terminology

Green tea contains four main catechin substances: Epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate, all of which are

inclusively called catechin. Organ fibrosis is a clinical condition caused by an excessive deposition of extracellular matrix. The progression of fibrosis resulted in a loss of normal function.

Peer review

This paper reports a novel, interesting and important study. This is a basic work which shows that EGCG could bind to the TGFRⅡ abolishing myofibroblast activation. The original point in this work is the analysis that is done on the cytokine receptor. The authors soundly demonstrated the binding EGCG to TGFR Ⅱ by immunoprecipitation and affinity chromatography experiments.

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