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ANGIOGENIC ACTIVITY OF SERA FROM PATIENTS WITH SYSTEMIC AUTOIMMUNE DISEASES IN RELATION TO CLINICAL, RADIOLOGICAL, AND FUNCTIONAL PULMONARY STATUS

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Systemic autoimmune diseases, such as vasculitis and collagen diseases, are characterized by chronic inflammation. Mutual interrelationship between angiogenesis and chronic inflammation has already been demonstrated. The aim of the study was to examine the effect of sera from patients with systemic autoimmune diseases on angiogenesis induced by human mononuclear cells. The study population consisted of 43 patients with a systemic autoimmune disease associated with pulmonary manifestations, divided into three groups: 14 with Wegener’s granulomatosis (WG), 13 with systemic sclerosis (SS), and 16 with collagen vascular diseases (CVD) such as rheumatoid arthritis, systemic lupus erythematosus, and dermatomyositis. The control group consisted of 15 healthy volunteers. Clinical status was evaluated using a questionnaire. Standard chest radiographs were performed in all patients. Pulmonary function tests were performed according to the ERS standards. An animal model of a leukocyte-induced angiogenesis assay was used as an angiogenic test. Sera from WG and CVD patients significantly stimulated angiogenesis compared with healthy subjects (P<0.001). On the other hand, sera from healthy donors exerted a proangiogenic effect compared with PBS. In contrast, sera from SS patients significantly (P<0.001) inhibited angiogenesis compared with sera from healthy subjects and PBS. Proangiogenic effect of sera from systemic diseases patients depended on radiological changes. No significant correlation between a degree of dyspnea or functional pulmonary tests and the number of new vessels or angiogenesis index was found. Sera from patients with systemic autoimmune diseases and healthy people constitute the source of mediators modulating angiogenesis. These modulatory effects differ depending on the disease entity.

Key words: angiogenesis, collagen diseases, systemic sclerosis, Wegener’s granulomatosis
INTRODUCTION

Systemic autoimmune diseases constitute a large group of disorders, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), systemic sclerosis (SS), and Wegener’s granulomatosis (WG) (1-3). Pulmonary manifestation is typical for many of them. The etiology of these diseases remains unknown, but vascular alteration has been suggested to be an important factor in their pathogenesis (1, 4). SS is a generalized autoimmune disorder characterized by immunological abnormalities, microvascular dysfunction, and tissue fibrosis involving the skin and various organs (5). The mechanism leading to a selective microvascular injury in SS is not completely known. Histopathological hallmarks of SS are perivascular infiltrates and a reduced capillary density, which precede the excessive accumulation of extracellular matrix proteins in the later stages of disease (5). Despite a reduced blood flow, there is no evidence for a sufficient angiogenesis in the skin of patients with SS (3). Chronic inflammation involves proliferation, migration, and recruitment of inflammatory cells, which can be extremely damaging to a normal tissue like pannus in RA (6). There are zones of a relative hypoxia, which induces capillary development in RA (7). Necrotizing vasculitis and granuloma formation are predominant features of WG (1). Antineutrophil cytoplasmic antibodies probably play a pathogenic role in WG and other vasculitis (8). Adhesive interactions of leukocytes with endothelial cells and with the extracellular matrix have been described in chronic inflammatory disorders (4, 9) Antiendothelial antibodies are present in SLE, SS, WG, and participation of autoantibodies in a vascular injury in systemic immune diseases is postulated (10). The co-dependence of angiogenesis and chronic inflammation is demonstrated and inflammatory mediators can directly or indirectly promote angiogenesis (11). An increase in the endothelial surface area creates an enormous capacity for the production of cytokines, adhesion molecules, and other inflammatory stimuli. Under hypoxic conditions, activated macrophages release large quantities of angiogenic factors (12).

The aim of the present study was to examine the effect of sera from different type of autoimmune systemic disease on angiogenesis induced by human mononuclear cells (MNC) in relation to clinical, radiological, and functional pulmonary status.

MATERIAL AND METHODS

Patients

The study protocol was approved by an institutional Ethics Committee. Sera from 43 patients with systemic autoimmune disease associated with pulmonary manifestations were divided into three groups: 14 with WG, 13 with SS, and 16 with collagen vascular diseases (CVD). The population of WG patients consisted of 12 women who had never smoked tobacco and 2 men (smokers) aged 21-68 years (49 ± 18). The population of SS patients consisted of 11 women who
had never smoked tobacco and 2 men (1 smoker) aged 32-67 (51 ± 12). The population of CVD patients consisted of 7 women (non smokers) and 9 men (4 smokers) aged 29-70 (55 ±11). The group of CVD consisted of 7 patients with RA, 4 with SLE, 2 with dermatomyositis, 2 with a mixed connective tissue disease (MCTD), and 1 with psoriatic arthritis. All patients fulfilled the diagnostic criteria of ACR or ARA for SS, RA, SLE, and WG (2, 5, 8, 13). Standard chest radiographs and lung function measurements were performed. Lung function tests, which included lung volumes measured by body plethysmography (MasterLab Jaeger, Germany), forced expiratory flows, single breath carbon monoxide transfer factor (TLCO), and static lung compliance, were performed according to the ERS standards (14). As a control, sera from 15 healthy nonsmoking volunteers were used (9 women and 6 men), mean age 35 ± 9, (range 20-52 years). No one of the healthy volunteers presented with abnormalities on physical examination or chest radiography.

Angiogenesis assay

Angiogenic activity of patient sera was measured using a cutaneous leukocyte-induced angiogenesis assay according to Sidky and Auerbach (15), with modification (16). The study was performed in two-month old female inbred Balb/c mice, weighing ±20 g, from a local laboratory colony. MNC isolated from peripheral blood of healthy donors were preincubated for 60 min at 37°C in PBS supplemented with 25% of serum from the examined patient, serum from healthy volunteers, or only in PBS free of serum. Following the incubation, cells were suspended in Parker medium (5x10⁶ cells/ml) and injected intradermally (multiple 0.05 ml per inoculum). Three days after the mice were sacrificed with a lethal dose of Morbital, scoring of all skin reaction areas was carried out at the same magnification (6x) by a dissection microscope (Nikon, Japan). The result was evaluated blindly by the same person, based on the previously described criteria (15, 16).

Statistical analysis

Serum angiogenic activity was expressed as a mean (±SD) number of new vessels formed after the injection of MNC preincubated with sera of patients or controls, and as an angiogenic index (AI) representing the mean number of new blood vessels after the injection of MNC preincubated with serum of patients divided by the mean number of new blood vessels after the injection of MNC preincubated simultaneously in identical conditions with serum of healthy volunteers. An unpaired t-test and a Pearson’s test were used for statistical analysis (Statistica 6 for Windows).

RESULTS

Sera from WG and CVD patients significantly (P<0.001) stimulated angiogenesis compared with sera from healthy subjects (Fig. 1A). The mean amount of new vessels formed after the injection of MNC preincubated with sera from the WG (16.9 ± 1.6) and CVD patients (16.2 ± 0.9) were similar. However, sera from the healthy donors exerted a stimulating effect on angiogenesis compared with PBS (P<0.001). Sera from the SS patients exerted an inhibitory effect on angiogenesis. The mean amount of vessels created after the injection of MNC preincubated with sera from SS (10.0 ± 0.6) was significantly (P<0.001) lower than that after the injection of MNC preincubated with sera from healthy donors (14.1 ± 0.5) or preincubated only with PBS (12.2 ± 0.9). The highest AI was observed for CVD patients (1.2 ± 0.1) and WG patients (1.2 ± 0.1); it was
lower for PBS (0.9 ± 0.04) and the lowest for SS patients (0.7 ± 0.04). The differences between the examined groups were significant (Fig. 1B).

No relation between the serum angiogenic activity and the presence of cough or general symptoms manifested by evaluated patients was found. The patients were divided into three groups: without dyspnea (11 patients), with moderate dyspnea (20 cases), and severe dyspnea (12 cases). The differences between the amount of new vessels created after the injection of MNC preincubated with sera from patients without dyspnea (15.7 ± 2.8), moderate dyspnea (14 ± 3.3), and severe dyspnea (15.2 ± 3.2) were not significant (Fig. 2A). However, proangiogenic effect of sera from systemic diseases patients depended on radiological changes (Fig. 2B). The amount of new vessels created after the injection of MNC preincubated with sera from patients with fibrotic changes (14.8 ± 3.2; n=20) was significantly higher (P<0.05) compared with patients without or with only small reticular changes (12.8 ± 1.5; n=12). However, the amount of new vessels in a group of patients with fibrotic changes was significantly lower (P<0.05) compared with that in the patients with parenchymal changes (16.9 ± 3.1; n=11). No significant correlation between VC, FEV₁, TLCO, Cst, and the amount of new vessels or angiogenesis index was found (Fig. 3).

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**Fig. 1.** A - Number of new vessels created after injection of MNC preincubated in sera from CVD (n=16), SS (n=13), WG patients (n=14), and from healthy donors (n=15) or PBS (n=15); B - Angiogenesis index after preincubation of MNC in sera from CVD, SS, and WG patients compared with angiogenesis index for PBS. The mean values are indicated by horizontal bars; significant differences between the groups are indicated.
Fig. 2. A - Number of new vessels in relation to dyspnea. Patients without or small (n=11), moderate (n=20), and severe dyspnea (n=12); B - Number of new vessels in relation to radiological stage of the disease. Patients with reticular pulmonary radiological changes or without radiological changes (n=12), with fibrotic (n=20), and parenchymal pulmonary radiological changes. The mean values are indicated by horizontal bars; significant differences between the groups are indicated.

Fig. 3. Correlations between the angiogenesis index and FEV$_1$ (A), VC (B), Cst (C), and TLCO (D); r - Pearson’s coefficient.
DISSCUSSION

The results demonstrate that sera of patients with a systemic autoimmune disease contain mediators modulating angiogenesis. Angiogenesis is important in the pathogenesis of RA and other inflammatory diseases, such as SLE, SS, polymyositis/dermatomyositis (PM/DM), Sjogren’s syndrome, and MCTD (17). In RA, synovium is rich in newly formed vessels (6). An increase in neovascularization promotes the inflow of inflammatory cells into the synovium and thus perpetuates progression of disease (18). In RA, an excess of various angiogenic factors in relation to angiogenesis inhibitors is apparent. The serum level of VEGF in patients with RA increases, and blockade of VEGF reduces the disease severity in murine collagen-induced arthritis (19). From the proinflammatory cytokines and other molecules, which play a role in the pathogenesis of RA, TNF-α, IL-1, IL-8, IL-13, IL-15, VCAM-1, integrins, and matrix metalloproteinases have all been implicated in angiogenesis (20). Koch et al (21) have demonstrated that C-X-C chemokines and epithelial-neutrophil activating protein 78 (ENA-78) play a role in the pathogenesis of RA synovitis and promote angiogenesis. Some of antirheumatic drugs used in the treatment of RA inhibit angiogenesis (20).

Our results confirm that sera of patients from RA with pulmonary manifestations stimulate angiogenesis. In contrast, less information is available on the role of angiogenesis in other autoimmune inflammatory collagen disorders, such as SLE, MCTD, PM/DM, and psoriasis (22-25). Levels of soluble VCAM-1 correlate with a disease activity in patients with SLE, being significantly higher during the active disease process and normalizing with clinical remission (22). The overproduction of other chemokines and receptors, such as IP-10, MCP-1, and RANTES has been demonstrated in SLE patients (23), and also C-C chemokines in PM/DM (25). Overexpression of VEGF and its receptors in psoriasis has been demonstrated (24). Thalidomide, a potent angiogenesis inhibitor, has clinical effects in SLE (26). In the present study, we also observed that sera from SLE, PM/DM, MCTD, and psoriatic arthritis stimulated angiogenesis.

In contrast to other collagen diseases, such as RA or SLA, sera from SS patients with pulmonary manifestations exert inhibitory effect on angiogenesis compared with the PBS and healthy control. Previously, Majewski et al (27) have demonstrated a decrease in angiogenic activity in sera from chronic diffuse scleroderma patients (27). Late stages of SS are characterized by the loss of dermal papillae, subepidermal fibrosis, and hypovascularity subunit expression (28). However, concentration of VEGF is increased in the serum of SS patients in the earliest stages of the disease (29). A correlation of VEGF level with the radiological and functional pulmonary changes has been shown in SS patients (30). However, Mackiewicz et al (31) have suggested that the imbalanced expression of VEGF and its vascular receptors, due in part to insufficient local...
production of VEGF which was low compared with VEGFR expression, is responsible for angiogenesis failure in SS. Since microvascular angiogenic stimuli normally induce VEGF followed by VEGFR, these authors’ results also suggest that the angiogenic cascade is turned on, but there is a defect in the finalization of its effects. Our present results suggest an increase in angiostatic factors in serum from SS patients. From the extracellular matrix-derived angiostatic growth factors, endostatin has been characterized as a potent inhibitor of VEGF-induced angiogenesis (32). A production of endostatin may result from tissular sclerosis and could contribute to the development of ischemic manifestations (33). D’Alessio et al (9) have suggested that overproduction of matrix metalloproteinase - 12 by SS microvascular endothelial cells accounts for the cleavage of urokinase-type plasminogen activator and may contribute to decreased angiogenesis in these patients (9). Angiogenesis seems impaired in SS and this could result from excessive angiostatic factors or disrupted VEGF signaling (34). A reduction of tissue kallikreins 9, 11, and 12 may be relevant to the reduced angiogenesis in SS patients (35). Recently, Giusti et al (36) have demonstrated that microvascular endothelial cells of patients with SS show abnormalities in a variety of genes that are able to account for defective angiogenesis. Normalization of the angiogenic cascade in SS could provide a future therapeutic target.

Vascular injury plays a pathophysiological role in vasculitis, but the role of angiogenesis in those diseases is unclear. Regarding systemic vasculitis, most data on angiogenesis concern the Kawasaki disease, where an increased production of VEGF and TGFβ is observed (37). Our present results suggest that angiogenesis plays a role in the pathogenesis of WG. Li et al (38) have demonstrated that VEGF levels are raised in WG patients compared with normal controls and may be a marker of a disease activity (38). Soluble serum thrombomodulin, a marker of endothelial cell injury and proangiogenic factor, is also elevated in active WG (39). In WG, cANCA can bind to the endothelial cells and provoke a deterioration of endothelial cells functions (40). cANCA plays a pivotal role in inducing monocyte IL-8 release (41). This cytokine is an important proangiogenic factor that mediates chemotaxis, stimulates proliferation and migration of endothelial cells, and also recruits and activates neutrophils. Finally, haptoglobin has been identified as an angiogenic factor which is involved in vasculitis associated neovascularization (42).

We conclude that sera from patients with systemic autoimmune diseases associated with pulmonary manifestation constitute the source of mediators modulating angiogenesis. Sera from WG, RA patients, and some other inflammatory rheumatic diseases stimulate neovascularization, but sera from SS patients exert an inhibitory effect on angiogenesis.

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REFERENCES


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