Bosentan blocks the antiangiogenic effects of sera from systemic sclerosis patients: an in vitro study

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ABSTRACT

Objective. In systemic sclerosis (SSc), clinical evidence has shown that Bosentan may foster the regeneration of the peripheral microcirculatory network. The aim of this study was to verify in vitro the influence of Bosentan on the angiogenic performance of dermal microvascular endothelial cells (MVECs) and its possible capacity to counteract the antiangiogenic effects of SSc sera.

Methods. Healthy dermal MVECs were challenged with Bosentan at different concentrations (0.1 µM, 1 µM, 10 µM) or with sera from patients with diffuse cutaneous SSc (n=8) and healthy subjects (n=8), alone or in combination with Bosentan (10 µM). Cell viability and chemoinvasion were determined by WST-1 and Boyden chamber assays, respectively. Angiogenesis was evaluated by capillary morphogenesis on Matrigel.

Results. Challenge of dermal MVECs with SSc sera induced a significant reduction in angiogenesis (p<0.005 vs. basal condition; p<0.001 vs. healthy sera). The addition of Bosentan could significantly restore angiogenesis in the presence of SSc sera (p<0.01 vs. SSc sera alone). Healthy sera promoted cell viability which was, instead, significantly reduced with SSc sera (p<0.005 vs. healthy sera). The addition of Bosentan to MVECs challenged with SSc sera significantly increased cell viability (p<0.005 vs. SSc sera alone), reaching levels similar to MVECs treated with healthy sera. Co-incubation of MVECs with Bosentan and SSc sera significantly increased chemoinvasion (p<0.005 vs. SSc sera alone) which was inhibited by SSc sera (<0.001 vs. healthy sera).

Conclusion. Bosentan effectively counteracts the antiangiogenic effects of SSc sera on dermal MVECs and fosters the restoration of a proangiogenic environment.

Introduction

Systemic sclerosis (SSc) is characterised by injury to vascular wall, fibrosis and autoimmunity affecting the skin and internal organs (1). Endothelial cell dysfunction is the earliest pathological event favouring an unbalance between vasocostricthing (e.g. endothelin-1 (ET-1)) and vasodilating (e.g. nitric oxide) factors. The loss of the endothelial-dependent vasoregulation triggers a vicious circle of endothelial damage, tissue ischaemia and fibrosis (2, 3). Increasing evidence indicates that ET-1 overexpression is involved in the fibrotic and vasculopathic aspects of SSc (4, 5). Elevated ET-1 expression has been reported in the skin, lungs, kidneys and bronchoalveolar lavage fluid of SSc patients (5). Circulating levels of ET-1 are significantly increased both in limited and diffuse cutaneous SSc and correlate with the severity of skin involvement and pulmonary fibrosis (5, 6). Cultured SSc fibroblasts display enhanced ET-1 expression, and the exposure of human fibroblasts to ET-1 promotes the fibroblast-myofibroblast transition mirroring the SSc profibrotic phenotype (5, 7). Moreover, ET-1 stimulates vascular smooth muscle cell proliferation and contraction, contributing to remodeling of the vascular wall and vasculopathic manifestations characteristic of SSc, such as pulmonary arterial hypertension (PAH) and digital ulcers (8-10). ET-1 acts through the endothelin receptors type A (ETA) and type B (ETB) that are expressed on various cell types (11). The vasconstrictive properties of ET-1 are mediated primarily by ETA receptors on vascular smooth muscle cells, whereas ETB receptors promote the release of nitric oxide by endothelial cells (8-10). A dysbalanced expression of ETA and ETB receptors has been reported in SSc patients (5, 11, 12).

Key words: systemic sclerosis, Bosentan, dermal microvascular endothelial cells, angiogenesis

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Bosentan is a dual antagonist of ETA and ETB receptors which is effective in the treatment of SSc-related PAH and in the prevention of digital ulcers (13, 14). The beneficial effects of Bosentan on SSc proliferative vasculopathy are likely due to the inhibition of ET-1-mediated vascular smooth muscle cell proliferation. However, it is most important to consider that in SSc there are parallel vascular pathologies that may require different and specific therapeutic approaches. In fact, the SSc vascular pathophysiology consists of a vascular remodeling process with intimal proliferation and vessel obliteration, as well as a progressive loss of dermal capillaries and lack of compensatory angiogenesis following peripheral ischaemia (2, 3). Therefore Bosentan, although displaying beneficial effects on SSc proliferative vasculopathy aspects, might have side effects on the peripheral vascular disease component by interfering with microvascular endothelial cell (MVEC) functions. Recent data from our group showed that Bosentan improves peripheral microcirculation in SSc patients, as demonstrated by the shifting from the “late” to the “active” nailfold videocapillaroscopy (NVC) pattern (15). In another study, long-term treatment with Bosentan in combination with iloprost reduced the progression of nailfold microvascular damage in SSc patients assessed by NVC, over a 3-year follow-up period (16). These findings suggest that in SSc Bosentan may have a beneficial effect on peripheral microvascular damage.

On these premises, we hypothesised that treatment with Bosentan might modulate endothelial cell function, thus fostering angiogenesis. Therefore, the aim of the present study was to evaluate in vitro the possible influence of Bosentan on the angiogenic performance of dermal MVECs and its capacity to counteract the antiangiogenic effects of SSc sera.

Materials and methods

Isolation of dermal MVECs

Dermal MVECs were isolated from forearm biopsy samples of 6 healthy subjects as previously reported (17). Briefly, skin biopsy samples were mechanically cleaned of epidermis and adipose tissue to obtain a pure specimen of vascularised dermis. Clusters of round-shaped cells were squeezed from microvessels and formed colonies composed of polygonal elements. Such colonies were detached with ethylenediaminetetraacetic acid, and CD31-positive cells were subjected to immunomagnetic isolation with Dynabeads CD31 (Dynal ASA, Oslo, Norway). Isolated cells were further identified as MVECs by labelling with anti-factor VIII-related antigen and anti-CD-105 (endoglin) antibodies, and by reprobing with anti-CD-31 antibodies. Cells were maintained in complete MCDB medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 30% heat-inactivated fetal bovine serum (FBS), 20 μg/ml endothelial cell growth supplement (ECGS; Calbiochem, Nottingham, UK), 10 μg/ml hydrocortisone, 15 U/ml heparin, and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml amphotericin). MVECs were used between the third and seventh passages in culture.

Serum samples

Serum samples were obtained from 8 patients with diffuse cutaneous SSc (1) and from 8 age- and sex-matched healthy individuals. Patients were not on immunosuppressive medications, corticosteroids or other disease-modifying drugs. Before blood sampling, they were washed out for 10 days from oral vasodilating drugs and for 2 months from intravenous prostanoids. Fresh venous blood samples were drawn, left to clot for 30 minutes before centrifugation at 1,500 g for 15 minutes, and serum was collected and stored in aliquots at -80°C until used. The study was approved by the local institutional review board at the Azienda Ospedaliero-Universitaria Careggi (AOUC), Florence, Italy, and all subjects provided written informed consent.

Cell viability assay

Dermal MVECs were seeded onto 96-well plates (40 x 10³ cells per well) in complete MCDB medium with 30% FBS and were left to adhere overnight. Cells were then washed three times with serum-free medium and incubated in 2% FBS–MCDB medium for additional 24 hours. Then, MVECs were incubated for 24 hours in basal MCDB medium with 2% FBS and Bosentan at different concentrations (0.1 μM, 1 μM, 10 μM) or 30% serum from SSc patients (n=8) and healthy subjects (n=8), alone or in combination with Bosentan (10 μM). The proliferative effect with 30% FBS was defined as the optimal growth. Cell viability was determined by WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedi sulfonate) assay (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. All measurements were performed in triplicate and the results were expressed as the percentage increase/decrease in cell viability over the basal response.

Chemoinvasion assay

The capacity of dermal MVECs to migrate was evaluated by the Boyden chamber assay. A 48-well microchemotaxis chamber (Neuro Probe, Gaithersburg, MD, USA) was used. The 2 compartments were separated by a polyvinylpyrrolidone-free polycarbonate filter with 8 μm pore size (Neuro Probe). To evaluate chemoinvasion, the filter was coated with Matrigel (50 μg/filter; BD Biosciences, Bedford, MA, USA). Fifty microliters of cell suspension (6.25 x 10³ cells) in 2% FBS–MCDB medium were placed in the upper compartment of the Boyden chamber. Test solutions were dissolved in serum-free medium and placed in wells of the lower compartment. Healthy sera (n=8) and SSc sera (n=8) were tested. Basal MCDB medium was used to verify the specificity of the effect. In some experimental points, Bosentan (0.1 μM, 1 μM, 10 μM) was added to the cell suspension. To verify the possible effects of Bosentan on chemoinvasion induced by healthy and SSc sera placed in the lower compartment, Bosentan (10 μM) was incubated with cells in the upper compartment. The chamber was incubated at 37°C for 6 hours. The filter was then removed and fixed with methanol. Non migrating cells on the upper surface of the filter were removed by a cotton swab. Cells were stained with
Diff-Quick (Mertz-Dade/Dade International, Milan, Italy) and counted using a microscope (×40 magnification) in 10 random fields. The number of cells moving across the filter was used as the measure of mobilisation. Experiments were performed in triplicate. Migration values were expressed as the mean ± SD number of total cells counted per filter, or as a percentage of the basal response value.

In vitro capillary morphogenesis assay

In vitro capillary morphogenesis assay was performed in 96-well plates covered with Matrigel (BD Biosciences). Matrigel (50 μl; 10–12 mg/ml) was pipetted into culture wells and polymerised for 30 minutes to 1 hour at 37°C, as described elsewhere (17). MVECs (30 x 10^3 cells/well) were incubated in basal MCDB medium with 2% FBS and Bosentan at different concentrations (0.1 μM, 1 μM, 10 μM) or 30% serum from SSc patients (n=8) and healthy subjects (n=8), alone or in combination with Bosentan (10 μM). Stimulation with recombinant human vascular endothelial growth factor-A165 (10 ng/ml; R&D Systems, Minneapolis, MN, USA) was used as positive control of angiogenesis. Plates were photographed at 6 and 24 hours. Results were quantified at 24 hours by measuring the percent field occupancy of capillary projections, as determined by image analysis. Six to nine photographic fields from 3 plates were scanned for each experimental point.

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) software for Windows, version 20.0 (SPSS, Chicago, IL, USA). Data are shown as mean ± SD. Student’s t-test or non-parametric Mann-Whitney U-test were used where appropriate to test the probability of significant differences between groups. A p-value less than 0.05 was considered statistically significant.

Results

Healthy sera significantly increased dermal MVEC viability when compared to basal condition (p<0.005), but cell viability was significantly suppressed when cells were matched with SSc sera (p<0.005 vs. healthy sera) (Fig. 1A). In MVECs, Bosentan induced a significant increase in cell proliferation at all concentrations tested compared with the corresponding untreated cells, reaching a maximum at 10 μM (p<0.005 for Bosentan 0.1 μM and 1 μM, p<0.001 for Bosentan 10 μM) (Fig. 1A). These results indicated that 10 μM Bosentan achieved the maximal proliferative effect. Therefore, this dose was subsequently used in combination
Bosentan blocks SSc serum antiangiogenic activity / E. Romano et al.

with SSc and healthy control sera. Co-incubation of MVECs with Bosentan and healthy sera further promoted cell proliferation compared to healthy sera alone (p<0.01) (Fig. 1A). Similarly, the addition of Bosentan (10 μM) to MVECs matched with SSc sera induced a significant increase of cell viability compared to MVECs challenged with SSc sera only (p<0.005), reaching levels comparable to those of cells treated with healthy sera (Fig. 1A).

Healthy sera significantly increased cell invasion compared with basal condition (p<0.001), while chemoinvasion was significantly suppressed after the addition of SSc sera (p<0.001 vs. healthy sera) (Fig. 1B). At all concentrations, Bosentan was effective in favouring Matrigel invasion reaching the maximum effect at 10 μM (p<0.005 for Bosentan 0.1 μM, p<0.001 for Bosentan 1 μM and 10 μM vs. basal condition) (Fig. 1B). In the presence of Bosentan and healthy sera, MVEC chemoinvasion was significantly higher than in the presence of healthy sera alone (p<0.005) (Fig. 1B). Similarly, the addition of Bosentan to MVECs matched with SSc sera increased the percentage of invading cells as compared with stimulation with SSc sera alone (p<0.005) (Fig. 1B).

To investigate whether Bosentan was able to induce in vitro angiogenesis, we performed capillary morphogenesis on Matrigel matrix. In this assay, MVECs usually produce elongated processes that eventually form anastomosing cords of cells mimicking a tubular capillary plexus. Dermal MVECs stimulated with healthy sera produced an abundant network of branching cords (p<0.05 vs. basal condition) (Fig. 2). On the contrary, capillary morphogenesis was significantly reduced upon challenge with SSc sera compared both with basal and healthy sera-treated cells (p<0.005 vs. basal condition, p<0.001 vs healthy sera) (Fig. 2). When MVECs were incubated with different concentrations of Bosentan, only the higher concentration tested (10 μM) showed a significant proangiogenic effect (p<0.01 vs. basal condition) (Fig. 2). Co-incubation of MVECs with Bosentan and healthy sera further promoted capillary morphogenesis compared to cells stimulated with healthy sera only (p<0.01) (Fig. 2). Moreover, the addition of Bosentan was
able to significantly counteract the antiangiogenic effect of SSc sera (p<0.01 vs. SSc sera alone) (Fig. 2).

Discussion
In this in vitro study we evaluated for the first time the effects of Bosentan on the capacity of human dermal MVECs to perform angiogenesis and its possible ability to counteract the antiangiogenic effects of sera from SSc patients. Vascular damage is a primary event in the pathogenesis of SSc (3). The progressive vascular injury includes persistent endothelial cell activation/damage and apoptosis, intimal thickening, vessel narrowing and obliteration (2, 3). These profound vascular changes lead to vascular tone dysfunction and reduced capillary blood flow, with consequent tissue ischaemia and severe clinical manifestations (2, 3). The resulting tissue hypoxia induces complex cellular and molecular mechanisms in the attempt to recover endothelial cell function and tissue perfusion. Nevertheless, in SSc there is no evidence of significant angiogenesis and the disease evolves towards chronic tissue ischaemia, with progressive and irreversible structural changes culminating in the loss of peripheral capillaries (2, 3). Increasing evidence indicates that a dysregulated expression of a large array of circulating proangiogenic and antiangiogenic (angiostatic) factors may be mostly responsible for the impaired angiogenic response found in SSc (2, 3). Indeed, in vitro studies demonstrated that serum from SSc patients is toxic for MVECs and may suppress MVEC proliferation, migration and vascular tube formation (2, 18). Currently, few therapeutic options are available to promote effective angiogenesis and capillary regeneration in the course of SSc (3, 19).

Our data clearly show that Bosentan exerts a pro-proliferative and pro-angiogenic effect on dermal MVECs in a dose-dependent manner, and that it is able to increase both cell viability and migration even in the presence of SSc sera. Moreover, Bosentan is effective in fostering MVEC angiogenic properties and may significantly block the inhibitory effects of SSc sera on the formation of capillary-like structures. These findings are consistent with recent SSc clinical studies reporting that Bosentan treatment may foster the regression of avascular areas detected by NVC, thus significantly improving peripheral microcirculation (15, 16). In this context, substantial evidence also indicates that Bosentan is effective in the treatment of SSc-related PAH and in the prevention of the occurrence of new digital ulcers in SSc patients with a past history of digital vasculopathy (13, 14, 20). Nevertheless, further investigations will be necessary to provide a mechanistic explanation for the in vitro effects reported herein.

In summary, together with previous clinical studies (13-16, 20), our in vitro findings suggest that, in SSc, Bosentan treatment may represent a therapeutic approach effective not only in contrasting proliferative vasculopathy (i.e., PAH and digital ulcers), but also in promoting angiogenesis and fostering regeneration of the peripheral capillary network.

References