Evidence for reduced angiogenesis in bone marrow in SSc: immunohistochemistry and multiparametric computerized imaging analysis

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Abstract

Objective. Dysfunctional angiogenesis is a pathogenetic marker of SSc. Circulating levels of endothelial progenitor cells are reduced, and mesenchymal stromal cells show a reduced differentiation into endothelial cells and capacity to form capillaries. This suggests that pathophysiologically relevant changes may already exist in SSc bone marrow (BM) stromal cells that may affect downstream angiogenesis. The aim of this study is to evaluate, in SSc BM, angiogenesis, cellular immune system and fibrosis.

Methods. Eight SSc patients affected by a severe dcSSc and screened for autologous haematopoietic stem cells transplantation (HSCT) underwent a BM biopsy. BM biopsies were compared with six healthy controls. To evaluate angiogenesis and cellular immunity, the following antibodies were used: vascular endothelial growth factor (VEGF), kinase insert domain-containing receptor/fetal liver kinase-1 (KDR/flk-1), MMP-9 and CD34. To evaluate fibrosis, silver impregnation for reticulum was used. The number of vessels, the mean area of vascularization, the perimeter and microvessel density (MVD) were measured with a multiparametric computerized imaging analysis.

Results. A significant reduction in BM vascularity was found, while VEGF expression was much higher in SSc BM samples. Two patients had a Grade 2, whereas another two had a Grade 1 fibrosis.

Conclusion. In SSc, BM is characterized by a reduction of microvascular density and number of vessels and a significant increase of VEGF. This indicates that BM may be involved in the process of loss of angiogenesis, despite the presence of high local and systemic levels of VEGF.

Key words: angiogenesis, systemic sclerosis, bone marrow, VEGF, fibrosis.

Introduction

SSc involves the microcirculation, the immune system and the connective tissue, eventually leading to fibrosis [1]. Vascular dysfunction is one of the earliest events in SSc pathogenesis: endothelial damage leads to a dysregulation of angiogenesis [2] and a loss of capillaries (desertification). The consequent chronic ischaemia provokes a diffuse sufferance of the tissues with formation of ulcers and eventually gangrene [3]. Endothelial progenitors have a reduced capacity to form capillaries even when stimulated with vascular endothelial growth factor (VEGF) [4], which is found in high concentration in the blood of SSc patients [5]. Low levels of circulating endothelial progenitor cells [6] and a reduced angiogenic potential of endothelial cells, like mesenchymal stem cells (MSCs) [7], have also been shown in SSc patients, outlining the role of endothelial cells in the SSc pathogenesis.

In SSc, fibrosis is the final step and is responsible for the most prominent clinical manifestations. It is due to an increased fibroblast production of collagen, especially Types 1 and 3, with Type 1 being the most abundant [8].
In the past decade, intense immunosuppression followed by autologous haematopoietic stem cell transplantation (HSCT) has emerged as a new therapeutic procedure for severe SSc refractory to conventional treatments. Preliminary results indicate that HSCT can significantly improve skin involvement and stabilize lung function, thus having a positive effect on quality of life [9, 10]. Increased bone marrow (BM) angiogenesis evaluated as BM microvessel density (MVD) or as immunohistochemical expression of angiogenic factors in BM biopsy has been demonstrated in a variety of haematological disorders [11, 12], but so far BM has not been investigated in SSc patients. BM biopsy is commonly performed before HSCT to screen clinically silent haematopoietic alterations, possibly related to previous chemotherapy. Indeed, severe BM fibrosis has been associated with prolonged engraftment, which has not been reported so far in SSc. We have analysed BM biopsies in a series of patients undergoing HSCT for severe, progressive SSc in order both to provide a systematic assessment of BM fibrosis and to clarify the association between modification of BM angiogenesis and clinical features of this disease.

Materials and methods

Patients

This study was approved by the local ethics committee (Ethics Committee of University of Florence), and written informed consent was obtained from all participants. All patients were recruited from the Division of Rheumatology of the University of Florence and screened for the autologous stem cell transplantation international scleroderma (ASTIS) trial. BM biopsies were obtained from the posterior iliac crest under local anaesthesia (lidocaine) with Jamshidi needle, and collected at the Haematology Department. Systemic involvement was evaluated as follows: skin involvement with modified Rodnan skin score (mRSS), lung involvement with pulmonary function tests with carbon monoxide diffusion capacity (DLco), chest high-resolution CT (HRCT) and broncoalveolar lavage (BAL); heart involvement with Holter EKG and transthoracic Doppler-echocardiography and nail-fold videocapillaroscopy (NVC) to evaluate microvascular damage and renal function by creatinine clearance. Patients were referred for transplant and three of them underwent the procedure; the fourth patient failed twice to mobilize an adequate number of CD34+ cells.

Immunohistochemical analysis and histochemistry

Eight SSc BM biopsies were studied. BM biopsies from six age- and sex-matched adults diagnosed with lymphoma at staging without any evidence of marrow involvement were examined as controls [13]. A formalin-fixed, paraffin-embedded section of BM was deparaffinized in xylene and hydrate in graded alcohols. Antigen retrieval was performed with EDTA buffer, pH 8.0, in microwave. To evaluate angiogenesis, the following mAbs were used: VEGF (mouse mAb, 1:100 dilution, Santa Cruz Biotechnology), kinase insert domain-containing receptor/fetal liver kinase-1 (KDR/flk-1) (mouse mAb, 1:50 dilution; Santa Cruz Biotechnology), MMP-9 (mouse mAb, 1:25 dilution; Novocastra Laboratories) and CD34/QBEND10 (mouse mAb, pre-diluted; Ventana Medical Systems). Immunostaining was performed with NexES histostainer (Ventana Medical Systems) using a peroxidase detection kit with the chromogen 3,3'-diaminobenzidine tetrahydrochloride (Ventana Medical Systems). All staining procedures used a negative control sample. BM fibrosis was evaluated by silver impregnation for reticulum (Gomori’s silver impregnation; Bio-Optica), according to Bauermeister classification criteria [14], as follows:

- Grade 0 = no reticulin fibres demonstrable;
- Grade 1 = occasional fine individual fibres and foci of a fine fibre network;
- Grade 2 = fine fibre network throughout most of the section; no coarse fibres;
- Grade 3 = diffuse fibre network with scattered thick coarse fibres but no mature collagen; and
- Grade 4 = diffuse, often coarse fibre network with areas of collagenisation.

Microvessel detection and counting

To identify BM microvessels, anti-CD34 was used. Sections were observed at 400× magnification by two different blinded observers (R.A. and V.C.). Microvessels were defined as any endothelial cell or group of endothelial cells, distinct from other same cells, non-endothelial cells and connective tissue. The presence of a lumen was not considered necessary for defining a microvessel [15]. To calculate the number of vessels, vascular mean area expressed in square micrometres, vascular percentage mean area, perimeter and MVD (vessels per square micrometres), multiparametric, semi-automatic computerized imaging analysis was employed. Hardware is connected to a digital camera JVCC1380 placed on a light microscope. LEICA QWin Std (Leica) software reveals and quantifies stained vessels through brightness and colour properties. For each section, we evaluated eight consecutive areas and each area was 16 001.92 μm² (total area was 128 015.36 μm²).

VEGF, MMP-9 and KDR expression

The VEGF, MMP-9 and KDR expression was evaluated as percentage and absolute number of positive cells in a total of eight consecutive areas at 400× magnification. The cut-off value to distinguish sclerosis with low VEGF, MMP-9 and KDR expression from sclerosis with high expression was the median value of control cases, calculated in the same modality. Cut-off was established as follows: VEGF 25%, KDR 40% and MMP-9 30%.

Statistics

Data were analysed using SPSS 10.0 for Windows. Descriptive statistics were expressed as the mean (1 s.d.). Normal distribution of each examined parameter was verified by Kolmogorov–Smirnoff test. The statistical
significance of the differences between means of two groups was evaluated by the Student’s t-test for unpaired data. A $P \leq 0.05$ was considered statistically significant. Linear regression analysis to evaluate the relationship between vessel area and number of vessels was performed.

**Results**

The main clinical features of the patients are reported in Table 1. The mean mRSS was 17 (14.3). In all patients, ANA and anti-topo I antibodies were positive. Two patients presented a mild restrictive syndrome at lung function tests, and in six patients a reduction in DLCO was detected. On chest HRCT, ground-glass areas in six patients and bibasal fibrosis in two patients were found. At BAL, lymphocytosis was found in five patients, and in three a mild increase of eosinophils was detected. The NVC pattern was late in five patients and active in three patients. Two patients had active ulcers. Sporadic isolated monomorphic extrasystolic beats were observed in two patients; in one patient an endocavitary defibrillator was implanted for asymptomatic episodes of ventricular tachycardia. Pulmonary pressure and creatinine clearance were normal in all patients. Acute-phase reactants were increased in five patients. Three patients underwent the transplant, were infused with a median of 6.59 $\times 10^6$ CD34+ (range 5.12–12.3) and showed a normal and sustained haematopoietic recovery (median days to PMN and platelets recovery were 13 and 10, respectively). One patient failed to mobilize peripheral HSCs: the histological BM pattern did not show any peculiarity as compared with the other mobilized patients.

**BM morphology**

Controls showed a mean cellularity of 45% (8.66) (range 35–50%), similar to patients [40% (5.24), range 30–45%]. All haemopoietic lineages were present at all maturation stages. Myeloid precursors were localized in paratrabecular areas, whereas megakaryocytes and erythroid cells were distributed in central areas of the BM (Fig. 1a and b). Four patients showed hyperplasia of megakaryocytes.

**Fibrosis**

No fibrosis was detected in controls (Fig. 1c). In four patients BM fibrosis was detected: two patients were classified as Grade 1 and two as Grade 2 (Fig. 1d).

**Angiogenesis**

In BM biopsy specimens from SSc patients, a substantial reduction in vascularity was detected (Fig. 2a and b). A multiparametric computerized analysis demonstrated that the mean MVD in control specimens was 1364.58 (44.20) (range 1124.88–1624.82), whereas in SSc BM it was significantly reduced [712.63 (392.03), range 124.98–1312.34] ($P = 0.006$) (Fig. 3a). The mean number of vessels are higher in controls [21.5 (0.70), range 18–22] than in SSc [12 (6.22), range 2–21] ($P = 0.004$) (Fig. 3b), as was the percentage vascular mean area [controls 6.89 (0.73), range 3.84–10.60; SSc 2.2112 (1.94), range 0.1–5.23]
The coefficient of determination, $r^2$, of the linear regression equation was 0.7, showing a positive correlation between vascular area and the number of vessels (Fig. 3d). A significant increased expression of VEGF was observed in myeloid cells, megakaryocytes and the histiocyte-macrophage system of SSc BM (Fig. 2c and d). The median VEGF rate in controls was 24.25% (range 20–35%), whereas in SSc the median VEGF rate was 48.75% (range 30–85%) ($P=0.002$) with expression $\geq 50\%$ in half the patients: in two advanced SSc patients this expression was $>70\%$. The median KDR rate was 42% (range 40–45%) and 13.25% (range 1–40%) in controls and SSc, respectively ($P=0.003$). In controls, the expression of MMP-9 in myeloid cell and rarely in histiocytes and endothelial cells was significantly higher than in patients, with a median rate of 30% (range 20–40%), whereas in SSc it was 13.06% (range 1–25%) ($P=0.0009$) (Fig. 4).

**Discussion**

Our data show that the morphology of the SSc BM is normal but also that the number of microvessels is severely reduced despite the striking increase of VEGF. In half of the patients, BM fibrosis was associated with megakaryocyte hyperplasia. This observation could be explained by the production of pro-fibrotic cytokines (TGF-$\beta$, PDGF) by megakaryocytes [16]. In SSc, the derangement of microcirculation with abortive neoangiogenesis leads to eventual vessel loss [17]. In SSc BM, a reduction in the number of vessels was detected, indicating that BM stem cells share a reduced capacity to respond to angiogenic factors. The levels of circulating endothelial progenitor cells (EPCs) that differentiate into endothelial cells were found significantly lower in SSc, despite the higher concentration of most angiogenic factors. These results may reflect an inadequate response of EPCs, and their stem cells in the BM to angiogenic stimuli, suggesting an intrinsic dysfunction [18]. A reduced number of functionally impaired EPCs have also been found in SSc BM; since EPCs and stromal cells express VEGF receptor, a defective differentiation into the endothelial lineage may be hypothesized. However, EPCs from early SSc gave rise to some degree of in vitro endothelial differentiation, and their circulating levels were significantly higher than in patients with a late disease, suggesting that an altered differentiation may be a later step of the disease pathogenesis [19]. In addition, in SSc MSCs, a decreased percentage of VEGFR2$^+$, CXCR4$^+$, VEGFR2$^+$/CXCR4$^+$ cells has been found. Indeed, the angiogenic
Fig. 2 MVD by CD34 immunostaining [diaminobenzidine tetrahydrochloride (DAB) method, 400×] in BM biopsies of controls and SSc patients. Normal vascularization in control subject (a) and reduction of vascularization in SSc patients (b). Immunohistochemical stain of BM specimens using a mouse mAb specific for VEGF (DAB method, 400×). Specimen from a control subject (c). Specimen from a patient with SSc (d).

Fig. 3 MVD in controls [1364.58 (44.20)] and SSc [712.63 (392.03)] (P = 0.006) (a). Vessel number in controls [21.5 (0.70)] and SSc [12 (6.22)] (P = 0.004) (b). Percentage vascular area in controls [6.89 (0.73)] and SSc [2.2112 (1.94)] (P = 0.0009) (c). Data in (a–c) are presented as means (s.d.). Correlation of vascular area with vessel number. Linear regression shows a positive correlation (R² coefficient = 0.7) (d).
potential of endothelial cells like MSCs was reduced after being stimulated with VEGF and stromal cell-derived factor-1, suggesting that endothelial repair may be affected in SSc starting from the BM [7]. In our SSc patients, a significant increase of VEGF expression was found along with a reduction of KDR on the cell surface. In fact, Dor et al. [20] showed that VEGF chronic stimulation induced alteration in vascular morphology in experimental models, and Distler et al. [4] support these data in SSc skin lesions, suggesting an insufficient angiogenic response with the formation of irregularly shaped sac-like megacapillaries. Besides this, VEGF gene expression is potentiated both by hypoxia, due to vascular damage, and TGF-β, a pro-fibrotic cytokine that is increased in SSc sera [21]. This suggests that fibrosis might contribute to VEGF elevation [5] and may explain the correlation between VEGF levels and the frequency of lung fibrosis and skin involvement [22]. In our BM biopsies, no correlation between VEGF expression and BM fibrosis has been observed. Only four patients were referred to the transplant procedure, due to either clinical reasons or to trial randomization. One out of four failed to adequately mobilize the HSC, and no correlation between fibrosis and mobilization/engraftment was found, possibly because of the low numbers.

MMPs are also involved in angiogenesis through a breakdown of extracellular matrix component. The low levels of MMP-9 found in SSc BM may be due to a hyperproduction of MMP inhibitors [tissue inhibitor of metalloproteinases (TIMPs)], as detected in SSc sera [23], thus contributing to a reduction in vascularity. These data are in contrast with another study in which high circulating levels of MMP-9 and TIMPs were found [24]. Further studies are necessary to define the potential role of MMP-9 and their inhibitors in BM SSc biopsies. In conclusion, our data demonstrate that in SSc the BM angiogenic potential is reduced, mirroring the systemic loss of capillaries and desertification despite the increase of VEGF [4]. The amount of reticular fibres detected in BM suggests that the fibrotic process may affect the BM, contributing further to the reduction of angiogenic potential.

**Rheumatology key messages**

- In SSc BM, the number of microvessels is severely reduced despite the increased expression of VEGF.
- In SSc, endothelial repair may be affected starting from the BM.

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