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Original article

Scleroderma dermal microvascular endothelial cells exhibit defective response to pro-angiogenic chemokines

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Abstract

Objectives. Angiogenesis plays a critical role in SSc (scleroderma). The aim of this study was to examine the expression of growth-regulated protein-γ (Gro-γ/CXCL3), granulocyte chemotactic protein 2 (GCP-2/CXCL6) and their receptor CXCR2 in endothelial cells (ECs) isolated from SSc skin and determine whether these cells mount an angiogenic response towards pro-angiogenic chemokines. The downstream signalling pathways as well as the pro-angiogenic transcription factor inhibitor of DNA-binding protein 1 (Id-1) were also examined.

Methods. Skin biopsies were obtained from patients with dcSSc. ECs were isolated via magnetic positive selection. Angiogenesis was measured by EC chemotaxis assay.

Results. Gro-γ/CXCL3 and GCP-2/CXCL6 were minimally expressed in both skin types but elevated in SSc serum. Pro-angiogenic chemokine mRNA was greater in SSc ECs than in normal ECs. SSc ECs did not migrate to vascular endothelial growth factor (VEGF), Gro-γ/CXCL3, GCP-2/CXCL6 or CXCL16. The signalling pathways stimulated by these chemokines were also dysregulated. Id-1 mRNA in SSc ECs was lower compared with normal ECs, and overexpression of Id-1 in SSc ECs increased their ability to migrate towards VEGF and CXCL16.

Conclusion. Our results show that SSc ECs are unable to respond to pro-angiogenic chemokines despite their increased expression in serum and ECs. This might be due to the differences in the signalling pathways activated by these chemokines in normal *vs* SSc ECs. In addition, the lower expression of Id-1 also decreases the angiogenic response. The inability of pro-angiogenic chemokines to promote EC migration provides an additional mechanism for the impaired angiogenesis that characterizes SSc.

Key words: angiogenesis, scleroderma, chemokines.

Rheumatology key messages

- Despite their increased expression in SSc, endothelial cells in SSc are unable to respond to pro-angiogenic chemokines.
- The expression of CXCR2 is elevated in endothelial cells in SSc compared with healthy controls.
- The signalling pathways and transcription factor machinery activated by chemokines are impaired in endothelial cells in SSc.

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CLINICAL SCIENCE

Introduction

SSc (scleroderma) is a chronic autoimmune disease that is characterized by immune dysregulation, vascular abnormalities and extensive fibrosis of the skin and internal organs. Although SSc has been considered a connective tissue disease, vascular injury is believed to be first in the cascade of events that lead to fibrosis in SSc [1]. Indeed, peripheral microvascular abnormalities such as RP occur before the onset of clinical manifestations of SSc [2]. In addition to RP, clinical evidence for vascular involvement includes the development of digital ulcers, telangiectasias, gastric antral vascular ectasia and morphological changes on nailfold capillaroscopy. Microvascular injury in SSc occurs in nearly all organs, leading to pulmonary arterial hypertension and SSc renal crisis, which are the major causes of morbidity and mortality. The progression of the disease leads to loss of capillaries and arterioles, resulting in tissue hypoxia and ischaemia.

Angiogenesis is a highly regulated process. It involves endothelial cell (EC) activation, proliferation, migration and invasion into the extracellular space, followed by sprouting and lumen formation of the capillaries. The initiation of angiogenesis is triggered by upregulation of pro-angiogenic factors, which can be initiated by tissue ischaemia and hypoxia. In SSc, elevated pro-angiogenic mediators have been extensively documented, however, compensatory angiogenesis does not occur sufficiently to allow the vasculature to recover [3]. Interestingly, overexpression of angiostatic factors is also observed in SSc [1, 3], therefore it is possible that the increased anti-angiogenic mediators shift the vasculature to an anti-angiogenic state.

Chemokines are a class of small proteins that regulate leucocyte activation and migration. They are classified based on the location of conserved cysteine residues in the mature protein [4]. In addition to their role in the inflammatory response, some chemokines are also involved in modulating angiogenesis [5, 6]. Among them, CXC chemokines, such as growth-regulated oncogene-γ (Gro-γ)/CXCL3 and granulocyte chemotactic protein 2 (GCP-2)/CXCL6, contain the Glu-Leu-Arg (ELR) motif that is essential for their pro-angiogenic activities [6]. We have shown that CXCL16, which does not contain the ELR motif, also acts as a pro-angiogenic mediator [7, 8]. These chemokines activate CXC receptors (CXCRs; CXCR2 for Gro-γ/CXCL3 and GCP-2/CXCL6; CXCR6 for CXCL16) on the cell surface to initiate cellular signalling and activate transcription factors for EC activation and migration. Inhibitor of DNA-binding protein 1 (Id-1) is a class of transcription factors that is crucial in promoting angiogenesis. It is involved in vascular endothelial growth factor (VEGF)–induced angiogenesis, however, its role in chemokine-promoted cell migration is unknown.

Most studies have demonstrated that chemokine expression is upregulated in SSc. We previously assessed the expression of select angiostatic and pro-angiogenic chemokines in SSc and found that the expression of their receptors was differentially regulated [9]. In this study, we further examined the levels of pro-angiogenic chemokines in blood as well as dermal ECs isolated from SSc patients. We also determined if SSc dermal ECs have a dysregulated response to these pro-angiogenic chemokines. The mechanisms of the angiogenic response by SSc ECs, including the expression of chemokine receptor, the activation of signalling pathways and the involvement of Id-1, to these chemokines were then examined.

Materials and Methods

Detailed methods can be found in the supplementary data, methods section, available at *Rheumatology* Online. All SSc patients fulfilled the 1980 ACR criteria for classification of SSc [10]. The details of the enrolled subjects are summarized in Table 1. For biomarker analysis

Table 1 SSc patient and healthy volunteer characteristics

	SSc (n = 73)	Diffuse SSc (n = 48)	Limited SSc (n = 25)	Healthy subjects (n = 52)
Age, mean (S.E.M.), years	55.7 (1.3)	54.2 (1.5)	58.5 (2.2)	47.4 (2.0)
Sex, female/male, n/n	60/13	39/9	21/4	36/16
Disease duration, mean (S.E.M.), years	8.6 (1.2)	4.7 (0.8)	15.4 (2.4)	NA
mRSS, mean (S.E.M.)	13.9 (1.4)	19.1 (1.7)	4.7 (0.9)	NA
RP, n	69	43	25	NA
Early disease[a], n	39	33	6	NA
Deceased, n	6	5	1	NA
Immunosuppressives, MMF/MTX, n	16/12	16/7	0/5	NA
Digital ulcers, n	23	16	7	NA
Telangiectasias, n	39	24	15	NA
Gastrointestinal disease, n	60	37	23	NA
ILD, n	32	23	9	NA
PAH, n	17	10	7	NA
Renal involvement, n	3	2	1	NA

ILD: interstitial lung disease; mRSS: modified Rodnan skin score (0–51); NA: not applicable; PAH: pulmonary arterial hypertension.
[a]Early disease, <5 years.

using patient plasma, blood from healthy subjects and both dcSSc and lcSSc patients was collected. For EC isolation, skin biopsies from healthy volunteers and dcSSc patients were obtained. Two 4 mm punch biopsy specimens were taken from the distal forearm of SSc patients of involved skin. For immunohistological studies, punch biopsy specimens were also obtained from the proximal arm that represents uninvolved skin. The location of the punch biopsies was consistent among the patients. Normal skin tissue was obtained from recruitment as well as the tissue procurement service provided by the University of Michigan Hospital. Written informed consent was obtained for all subjects and the study was approved by the Institutional Review Board of the University of Michigan.

Cell culture

Dermal ECs were isolated from human skin in our laboratory. After the skin biopsy specimens were obtained in the clinic, they were maintained in RPMI at 4°C until use. The biopsies were cut into small pieces and digested using digestion solution containing 2.4 units/ml dispase, 650 units/ml type II collagenase and 10 000 Dornase units/ml DNase. After digestion, the cells were allowed to grow for 7–10 days. Dermal ECs were isolated using the CD31 MicroBead Kit and a MiniMACS Separator with an MS column (Miltenyi Biotech, Auburn, CA, USA). ECs, which were the positively selected cells, were maintained in EBM-2 media with growth factors (Lonza, Allendale, NJ, USA) on gelatine-coated plates. The morphology of the cells, the EC markers [i.e. CD31 and von Willebrand factor (vWF)] and the fibroblast markers [i.e. collagen I (Col I) and α-smooth muscle actin (αSMA)] were examined to ensure the purity of the cells. If needed, the cells were re-purified when they were expanded. Cell senescence was examined using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Danvers, MA, USA). Passages 3–6 were used for experiments. The ECs used in this study were from nine SSc patients and nine healthy volunteers.

Results

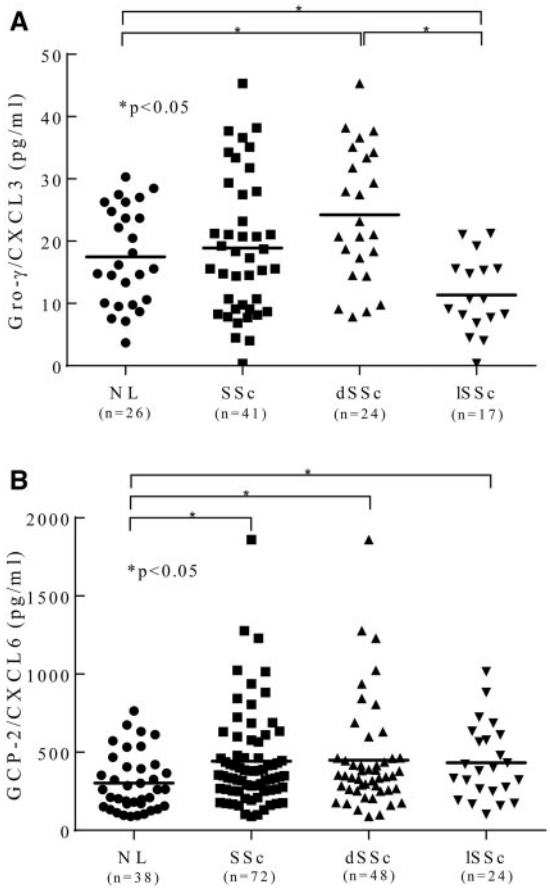
Chemokine expression was elevated in SSc patients

The expression of Gro-γ/CXCL3 was significantly elevated in dcSSc plasma compared with normal plasma, while plasma from lcSSc patients had significantly lower Gro-γ/CXCL3 levels ($P < 0.05$; Fig. 1A). GCP-2/CXCL6 was significantly increased in SSc plasma compared with normal plasma, with no difference observed between dcSSc and lcSSc ($P < 0.05$; Fig. 1B).

Isolation of ECs

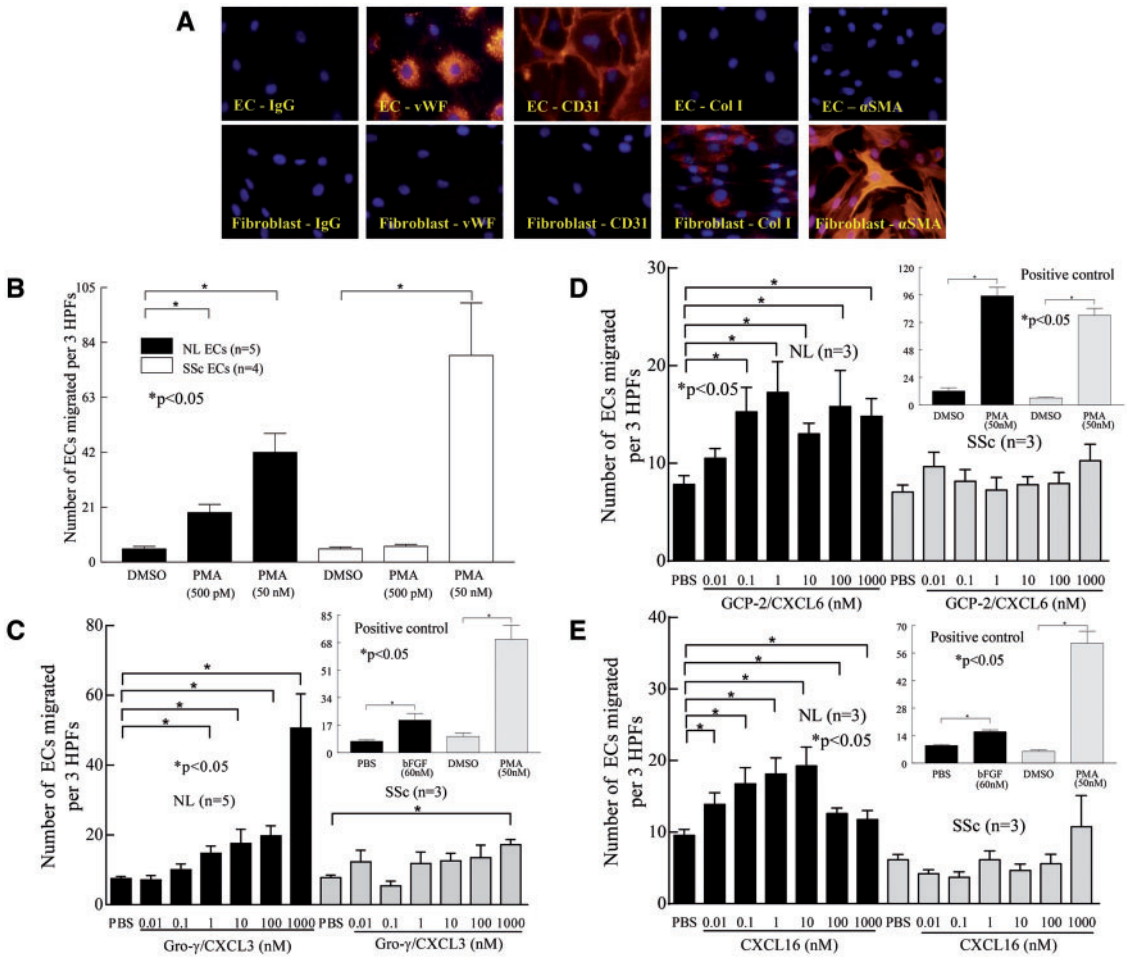
Dermal ECs were isolated from skin biopsies and characterized by immunofluorescence and flow cytometry. As shown in Fig. 2A and supplementary Fig. S1 and S2, available at *Rheumatology* Online, ECs showed the typical cobblestone phenotype and displayed positive EC markers (vWF and CD31) but no fibroblast marker (Col I and αSMA). The αSMA staining of fibroblasts showed the classic filamentous structures, suggesting that the isolated ECs showed different phenotypes compared with fibroblasts. On the other hand, fibroblasts isolated from the same subject stained negative for EC markers but positive for fibroblast markers. The purity of ECs was ~99% as measured by flow cytometry (supplementary Fig. S3, available at *Rheumatology* Online, both CD31 and CD144 positive). Among these cells, ~50% of the cells were CD34 positive. Cultured ECs are known to lose this marker, which is regulated by cell contact and proliferation as well as the external environment [11–13]. To determine whether the cells isolated were undergoing senescence, we stained the cells with β-galactosidase between passages 3 through 7. As shown in supplementary Fig. S4, available at *Rheumatology* Online, minimal senescent cells were observed at passage 4, while at passage 7, a lot more cells were undergoing senescence.

Fig. 1 Expression of chemokines in plasma from healthy subjects and SSc patients



(**A**) Gro-γ/CXCL3 was significantly elevated in dcSSc patients but not in lcSSc patients. (**B**) GCP-2/CXCL6 was elevated in both diffuse and limited SSc patients. Means are given with S.E. Differences were determined using the Mann–Whitney *U* test and P values <0.05 were significant. dSSc: diffuse SSc; lSSc: limited SSc; NL: normal.

FIG. 2 Characterization of EC purity and chemotactic ability towards various chemokines



(**A**) ECs displayed positive EC markers but not fibroblast markers. (**B**) PMA induced normal EC migration, however, SSc ECs migrated only at the higher dose. (**C**) Gro-γ/CXCL3 induced SSc EC chemotaxis at 1 μM with a smaller number of cells migrated compared with normal ECs. (**D**) GCP-2/CXCL6 dose dependently induced normal EC chemotaxis but not in SSc ECs. (**E**) CXCL16 dose dependently induced normal EC chemotaxis but not in SSc ECs. Results are expressed as mean (S.E.) and $P < 0.05$ is considered significant. αSMA: alpha smooth muscle actin; Col I: collagen I; ECs: endothelial cells; HPF: high-power field; n: number of experiments; NL: normal; PMA: phorbol-12-myristate-13-acetate; vWF: von Willebrand factor.

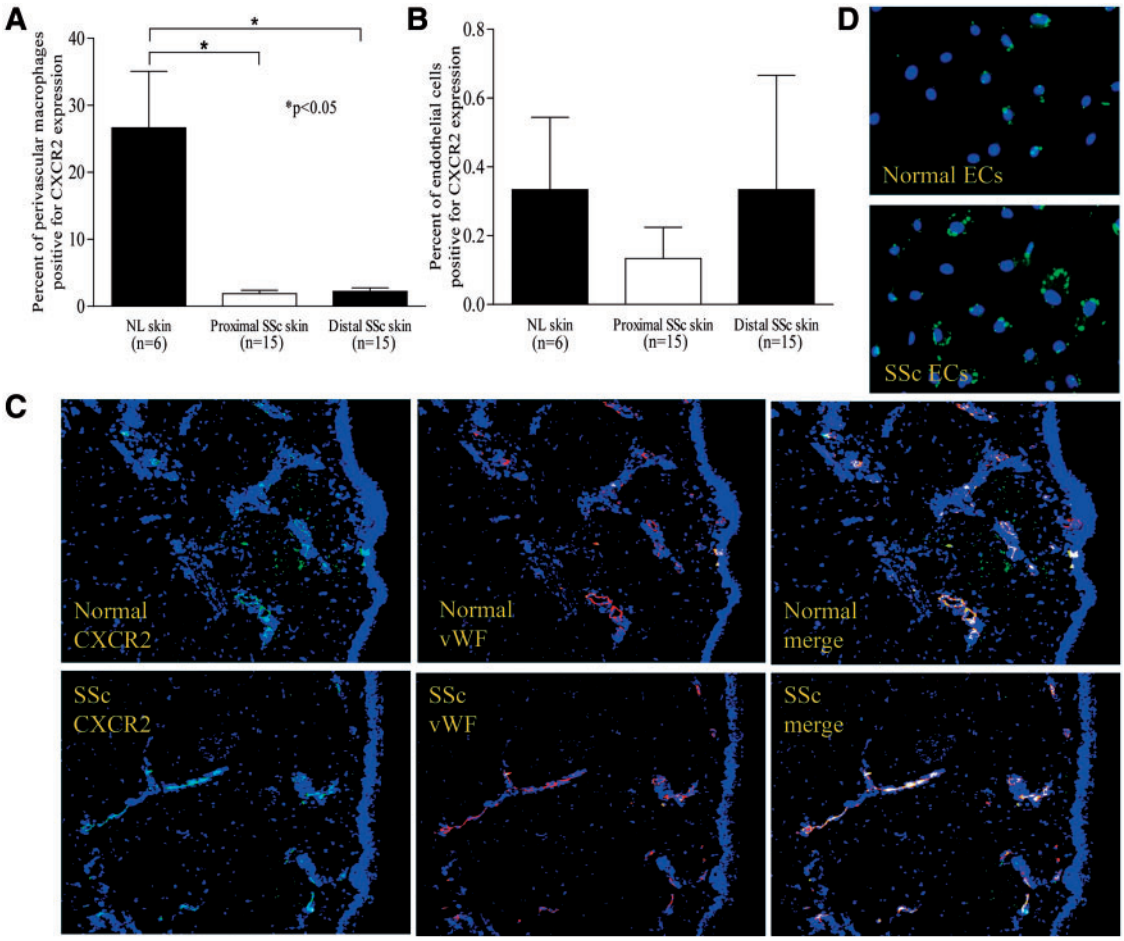
High doses of phorbol-12-myristate-13-acetate (PMA) induced SSc EC chemotaxis

It was shown that the ability to form tube-like structures is diminished using SSc ECs [14, 15]. In addition, these cells had a reduced response to basic fibroblast growth factor (bFGF) and VEGF [16]. We tested the response of the SSc ECs isolated in our laboratory towards bFGF and VEGF and they showed diminished response towards both (supplementary Fig. S5, available at *Rheumatology* Online). To determine a positive control for our cell migration assays, we used PMA as a stimulant. PMA induced dose-dependent migration in normal ECs, while it stimulated SSc EC migration only at the higher dose (50 nM; Fig. 2B). We therefore used 50 nM PMA as the positive control in the subsequent cell migration assays.

Chemokines were unable to induce SSc EC chemotaxis

To examine whether SSc ECs respond to chemokine stimulation, we treated ECs with pro-angiogenic chemokines at different concentrations. As shown in Fig. 2C, Gro-γ/CXCL3 induced normal EC migration dose-dependently from 1 nM to 1 μM. In contrast, Gro-γ/CXCL3 induced significant SSc EC migration at 1 μM, and the degree of migration was significantly lower compared with normal ECs at those concentrations. In terms of GCP-2/CXCL6 and CXCL16, they both dose-dependently induced normal EC migration, however, this was not seen using SSc ECs (Fig. 2D and E). These results suggest that SSc ECs show an impaired angiogenic response towards Gro-γ/CXCL3, GCP-2/CXCL6 and

FIG. 3 CXCR2 expression in skin and ECs



(**A**, **B**) Minimal CXCR2 were detected in SSc and normal skin sections done by immunohistochemistry. (**C**) Immunofluorescent staining of vWF and CXCR2 showed co-localization of the two on skin sections, suggesting CXCR2 was expressed on ECs. (**D**) Expression of CXCR2 was higher on SSc ECs compared with normal ECs. ECs: endothelial cells; vWF: von Willebrand factor.

CXCL16, which could contribute to the altered angiogenesis observed in SSc.

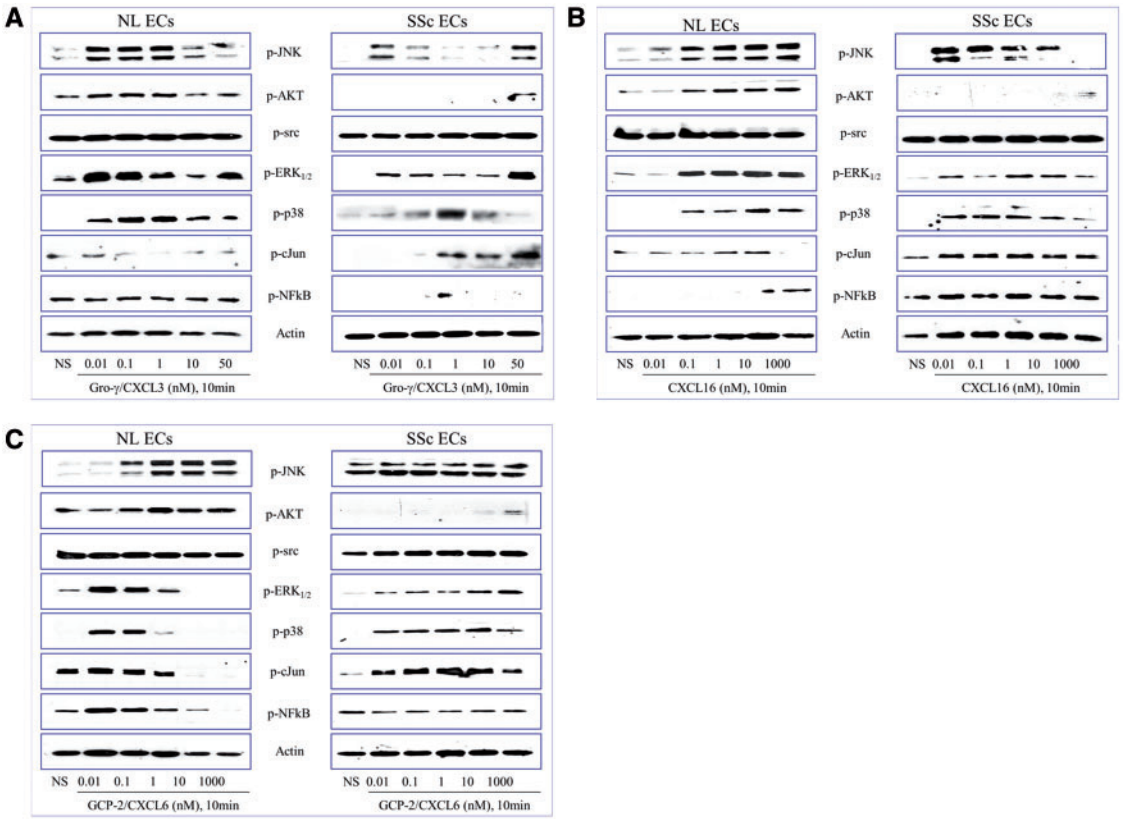
Increased CXCR2 expression was observed in SSc ECs

We next attempted to determine the expression of the pro-angiogenic receptor CXCR2 in SSc skin. We found virtually absent expression of CXCR2 on perivascular macrophages in proximal [2% (S.D. 1)] and distal SSc skin [2% (S.D. 1)] compared with normal skin [27% (S.D. 8); Fig. 3A]. Moreover, CXCR2 was barely detectable on ECs in either normal skin [0.3% (S.D. 0.2)] or proximal [0.1% (S.D. 0.1)] and distal SSc skin [0.3% (S.D. 0.3); Fig. 3B]. To better visualize CXCR2 staining on skin biopsies, we stained the skin sections with fluorescent-tagged antibodies and showed that CXCR2 was expressed on ECs, as CXCR2 staining was co-localized with vWF, which is a marker for ECs (Fig. 3C). Next, we measured mRNA levels of CXCR2, Gro-γ/CXCL3 and GCP-2/CXCL6 in normal and SSc ECs. CXCR2 mRNA was present in both diffuse SSc and normal ECs and the expression was elevated in SSc ECs. We also found that mRNA levels of Gro-γ/CXCL3 and GCP-2/CXCL6 were elevated in SSc ECs (supplementary Table S1, available at *Rheumatology* Online), consistent with the observation in patient blood (Fig. 1). The protein expression of CXCR2 was examined in both normal and SSc ECs. Similar to the mRNA data, we observed significantly increased CXCR2 expression in SSc ECs compared with normal controls (Fig. 3D and supplementary Fig. S6, available at *Rheumatology* Online).

SSc ECs have impaired chemokine-induced signalling compared with normal ECs

To examine the mechanism of the impaired response in SSc EC chemotaxis by these chemokines, we stimulated the cells with different concentrations of chemokines and examined induced signalling pathways by Western

FIG. 4 Signalling pathways stimulated by various chemokines



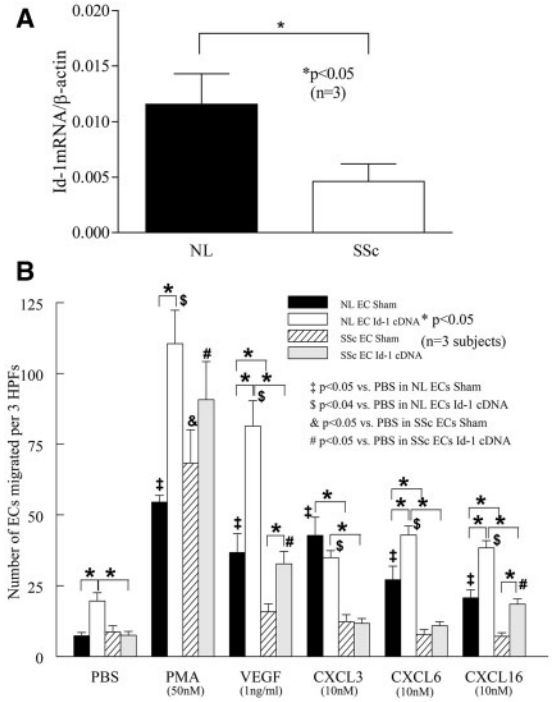
(**A**) Gro-γ/CXCL3 stimulated JNK, AKT, ERK1/2 and p38 phosphorylation in normal ECs, while in SSc ECs it stimulated JNK, ERK1/2, p38 and cJun phosphorylation. (**B**) GCP-2/CXCL6 stimulated JNK, AKT, ERK1/2, p38, cJun and NF-κB phosphorylation in normal ECs, while in SSc ECs it stimulated ERK1/2, p38 and cJun phosphorylation. (**C**) CXCL16 stimulated JNK, AKT, ERK1/2 and p38 in normal ECs, while in SSc ECs it stimulated JNK, ERK1/2, p38, cJun and NF-κB phosphorylation. Total protein expression of each signalling molecule was evaluated by Western blotting and there were no differences in expression (data not shown). ECs: endothelial cells.

blotting. In normal ECs, Gro-γ/CXCL3 stimulated JNK, AKT, $ERK_{1/2}$ and p38 phosphorylation, while in SSc ECs it stimulated JNK, $ERK_{1/2}$, p38 and cJun phosphorylation (Fig. 4A and supplementary Fig. S7A, available at *Rheumatology* Online). GCP-2/CXCL6 stimulated JNK, AKT, $ERK_{1/2}$, p38, cJun and NF-κB phosphorylation in normal ECs, while in SSc ECs it stimulated $ERK_{1/2}$, p38 and cJun (Fig. 4B and supplementary Fig. S7B, available at *Rheumatology* Online). CXCL16 stimulated JNK, AKT, $ERK_{1/2}$ and p38 in normal ECs, while it stimulated JNK, $ERK_{1/2}$, p38, cJun and NF-κB phosphorylation in SSc ECs (Fig. 4C and supplementary Fig. S7C, available at *Rheumatology* Online). Taken together, it appears that the AKT pathway is abolished in SSc ECs when stimulated by the chemokines used in this study, except for the highest doses used. We confirmed that the total expression of the signalling molecules examined was not altered in all samples (data not shown, supplementary Fig. S8, available at *Rheumatology* Online, using AKT as an example), therefore the lack of phosphorylation of AKT is not due to differential expression of the basal protein. It also appears that the src pathway is not induced by Gro-γ/CXCL3, GCP-2/CXCL6 or CXCL16 in either normal or SSc ECs.

Id-1 contributes to VEGF and CXCL16-induced chemotaxis in SSc ECs

To further dissect the mechanism of impaired angiogenesis by chemokines, we examined the expression of Id-1, a transcription factor required for ECs to mount an angiogenic response [17]. Via quantitative PCR, the expression of Id-1 was significantly lower in SSc ECs compared with normal ECs (Fig. 5A). We then sought to determine whether it plays a role in the impaired angiogenic response following chemokine stimulation. Transfecting ECs with 1 μg Id-1 cDNA for 24 h resulted in significant overexpression of Id-1 (data not shown). Under this condition, we examined whether ECs, both from normal and SSc skin, change their response towards different stimuli. In normal ECs, Id-1 overexpression increased EC chemotaxis under basal conditions (PBS control; Fig. 5B). In addition, the overexpression also increased PMA, VEGF,

FIG. 5 Expression of Id-1 and its impact on chemokine-induced chemotaxis



(**A**) In SSc ECs, Id-1 mRNA was significantly lower. (**B**) In sham transfected normal ECs, PMA, VEGF, Gro-γ/CXCL3, GCP-2/CXCL6 and CXCL16 stimulated significant EC chemotaxis. Id-1 overexpression resulted in a significant increase in EC chemotaxis when stimulated with PBS, PMA, VEGF, GCP-2/CXCL6 or CXCL16. In contrast, in sham transfected SSc ECs, only PMA induced significant EC migration. Id-1 overexpression resulted in a significant increase in chemotaxis after VEGF and CXCL16 stimulation. Results are expressed as mean (S.E.) and $P < 0.05$ is considered significant. ECs: endothelial cells; HPF: high-power field; n: number of experiments; NL: normal; PMA: phorbol-12-myristate-13-acetate; VEGF: vascular endothelial growth factor.

GCP-2/CXCL6 and CXCL16-induced EC migration. In contrast to sham-treated normal ECs, which responded to all the stimuli, sham-treated SSc ECs only responded to PMA, the positive control. The lack of response towards VEGF, Gro-γ/CXCL3, GCP-2/CXCL6 and CXCL16 in SSc ECs agrees with the literature [14] and confirms our findings in Fig. 2 in that these cells show impaired angiogenic responses towards these pro-angiogenic chemokines. Overexpression of Id-1 in SSc ECs increased their ability to move toward VEGF and CXCL16 (Fig. 5B) despite the smaller numbers of cells migrated compared with the corresponding Id-1 transfected normal ECs, which were stimulated with VEGF or CXCL16. The lack of improvement in SSc EC migration in response to Gro-γ/CXCL3 and GCP-2/CXCL6 after Id-1 overexpression suggests that Id-1 is not the only factor required for an efficient response to these chemokines.

Discussion

In this study, we showed that the levels of pro-angiogenic chemokines Gro-γ/CXCL3 and GCP-2/CXCL6 were significantly elevated in dcSSc patients compared with healthy controls. In a previous study we also showed increased levels of CXCL16 in patient blood and ECs [9]. We next examined whether dermal ECs isolated from SSc patients respond to these pro-angiogenic chemokines. Compared with healthy ECs, SSc ECs showed an impaired response towards all three pro-angiogenic chemokines. It should be noted that the inability of the SSc ECs to respond to the chemokines in this study is not due to the senescent phenotype of these cells, since these cells do respond to other chemokines or cytokines, such as interleukin-8 (IL-8; data not shown), to the same extent as healthy ECs. In addition, β-galactosidase staining showed that the cells were healthy up to passage 6. To further explore the possible mechanisms, we examined the expression of their receptors, their downstream signalling pathways, as well as a pro-angiogenic transcription factor, Id-1. Since both CXCR2 and CXCR6 [9] were elevated in SSc ECs, it suggests that these pro-angiogenic chemokine receptors were regulated to promote angiogenesis. However, when we examined the signalling pathway activated by these chemokines, it appears that SSc ECs were not activated the same way as the normal ECs and that the AKT pathway seemed to be impaired in all cases. The inability of these cells to respond to pro-angiogenic chemokines, at least for CXCL16, can be further explained by the lower expression of Id-1. Overexpression of Id-1 in SSc ECs restored CXCL16's ability to attract EC migration. Taken together, we showed that SSc ECs exhibit defective angiogenesis towards pro-angiogenic chemokines, and this adds to the possible causes of SSc vasculopathy.

Pro-angiogenic chemokines have been shown to play crucial roles in SSc [3]. This is the first report to show that Gro-γ/CXCL3 and GCP-2/CXCL6 are elevated in SSc. We and others have shown that other pro-angiogenic CXC chemokines such as Gro-α/CXCL1, IL-8/CXCL8, stromal cell–derived factor 1 (SDF-1)/CXCL12 and CXCL16 were also upregulated in SSc [9, 18–20]. In addition, angiostatic CXC chemokines such as platelet factor 4 (PF-4)/CXCL4, monokine induced by interferon-γ (MIG)/CXCL9 and interferon-inducible protein 10 (IP-10/CXCL10) were found to be elevated in SSc [9, 21]. When CXC receptors were examined, the anti-angiogenic CXCR3 was found to be downregulated [9]. In contrast, we showed that the pro-angiogenic CXCR2 and CXCR6 were upregulated in SSc ECs [9]. CXCR4, the pro-angiogenic chemokine receptor for SDF-1/CXCL12, was found to be highly expressed in SSc ECs and decreased as the disease progressed [20]. Collectively the dysregulated expression of the CXC receptors could contribute to impaired angiogenesis in SSc.

Although the expression of CXCR2 and CXCR6 increased in SSc ECs, the downstream signalling pathways stimulated by the three pro-angiogenic chemokines

showed different activation patterns when compared with normal ECs (Fig. 4 and supplementary Fig. S7, available at *Rheumatology* Online). It appears that in most cases, SSc ECs required a higher chemokine concentration to activate the signalling pathways, agreeing with the chemotaxis results that showed more cell migration at higher concentrations (Fig. 2). Interestingly the AKT pathway was inactivated in all cases except for the highest doses used. This pathway is involved in cell survival, cell migration and protein synthesis [22], and AKT is also a well-known signalling intermediate that is necessary for angiogenesis [23]. With regard to SSc, Zhu *et al.* [24] previously showed that SSc serum shuts down AKT signalling in normal endothelial progenitor cells. Their results, together with our data, further suggest that the impaired angiogenic response in SSc ECs could be due to inactivation of the AKT pathway.

The Id family consists of Id1–4 and belongs to the helix–loop–helix family of transcription factors [25]. Among these, Id-1 has been shown in several reports to be critical in cell proliferation, differentiation, senescence and tumourgenesis. It is also identified as a direct target of bone morphogenetic protein signalling [26, 27]. In $Id1^{+/-}Id3^{-/-}$ mice, Id-1 expression was required for angiogenesis during mouse development [28]. In addition, in adult mice, reduction of Id-1 resulted in decreased vascularization, growth and metastasis of tumour xenografts. In synovial tissues of RA, Id-1 was overexpressed compared with OA tissues and was predominantly localized to ECs [29]. In a subsequent study the authors examined the role of Id-1 in VEGF-induced activation and angiogenic processes in ECs and found that Id1 was not only induced by VEGF, it was also involved in EC transmigration and MMP expression, as well as VEGF-induced angiogenesis [17]. Interestingly, it appears that Id-1 induces VEGF expression through hypoxia-inducible factor 1α [30, 31]. The role of Id-1 in promoting blood vessel formation is further supported by the study by Volpert *et al.* [32]. They showed that Id-1 was a potent repressor of thrombospondin 1, which is an extracellular matrix protein that inhibits angiogenesis [33]. They also showed that Matrigel injected with either bFGF or VEGF in Id-1 null mice induced significantly lower angiogenic activity compared with the wild-type mice, and that the presence of anti-thrombospondin 1 antibody increased angiogenesis. It was suggested that the angiogenic defect in Id-1 null mice was due to downregulation of pro-angiogenic genes including MMP-2, FGF receptor 1 and α6 and β4 integrins [34]. Similar results were obtained in tumour ECs, as decreased expression of Id-1 resulted in downregulation of several pro-angiogenic genes such as intergrin α6 and β4 [34].

In this study, we showed that Id-1 expression is lower in SSc ECs (Fig. 5A). The mechanism of the lower Id-1 levels is not clear. However, it has been shown that Id-1 expression in ECs is stimulated by bone morphogenetic protein [35]. Since the bone morphogenetic protein type II receptor is downregulated in SSc ECs [36], it is possible that this pathway plays a key role in controlling Id-1 expression in SSc ECs. As shown in Fig. 5B, overexpression of Id-1 led to an increase in VEGF activity in both normal and SSc ECs. This is not unexpected, as it has been shown that knockdown of Id inhibited VEGF-induced proliferation and activation, as well as angiogenesis processes [17]. It is evident that the Id family is critical for VEGF activity. An increase in Id expression led to elevation of ICAM-1, E-selectin and MMP-2 and -9, which could be a possible mechanism for the increased cell migration seen here.

We also showed that the pro-angiogenic effect of CXCL16 is due in part to Id-1, as overexpression of Id-1 in both normal and SSc ECs resulted in increased EC migration (Fig. 5B). Id-1 also appears to play a role in GCP-2/CXCL6-mediated angiogenesis in normal ECs, however, overexpression of Id-1 did not affect SSc EC migration after GCP-2/CXCL6 stimulation, suggesting other factors might play a more significant role. In a recent study by Isozaki *et al.* [37], the expression of secreted Id-1 in RA synovial fluid significantly correlated with the levels of CXCL16. Interestingly, in addition to being a transcription factor, it appears that Id-1 itself is an angiogenic mediator, as it induces EC chemotaxis and tube formation [37]. We have preliminary data showing that Id-1 secreted from SSc dermal fibroblasts was significantly lower compared with normal dermal fibroblasts [38]. Therefore the significantly lower expression and production of Id-1 may act as an additional mechanism for impaired angiogenesis in SSc ECs.

In summary, this is the first study to show that dermal ECs isolated from diffuse SSc patients are unable to respond to pro-angiogenic chemokines. The possible mechanisms include dysregulated expression of CXCRs, impaired AKT signalling and lower cellular Id-1 expression. The combination of these mechanisms may serve to explain the impaired angiogenesis seen in SSc patients.

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Supplementary Data

The full guideline is available as supplementary data at *Rheumatology* Online.

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