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Junctional adhesion molecule-A is abnormally expressed in diffuse cutaneous systemic sclerosis skin and mediates myeloid cell adhesion

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ABSTRACT

Objective: To investigate the role of junctional adhesion molecule-A (JAM-A) in the pathogenesis of systemic sclerosis (SSc).

Methods: Biopsy specimens from proximal and distal arm skin and serum were obtained from patients with SSc and normal volunteers. To determine the expression of JAM-A on SSc dermal fibroblasts and in SSc skin, cell surface ELISAs and immunohistology were performed. An ELISA was designed to determine the amount of soluble JAM-A (sJAM-A) in serum. Myeloid U937 cell–SSc dermal fibroblast and skin adhesion assays were performed to determine the role of JAM-A in myeloid cell adhesion.

Results: The stratum granulosum and dermal endothelial cells (ECs) from distal arm SSc skin exhibited significantly decreased expression of JAM-A in comparison with normal volunteers. However, sJAM-A was increased in the serum of patients with SSc compared with normal volunteers. Conversely, JAM-A was increased on the surface of SSc compared with normal dermal fibroblasts. JAM-A accounted for a significant portion of U937 binding to SSc dermal fibroblasts. In addition, JAM-A contributed to U937 adhesion to both distal and proximal SSc skin.

Conclusions: JAM-A expression is dysregulated in SSc skin. Decreased expression of JAM-A on SSc ECs may result in a reduced response to proangiogenic basic fibroblast growth factor. Increased JAM-A expression on SSc fibroblasts may serve to retain myeloid cells, which in turn secrete angiogenic factors.

The pathogenesis of systemic sclerosis (SSc) is complex and remains incompletely understood; however, fibroblasts, monocytes and endothelial cells (ECs) seem to be key players. These cells facilitate excessive synthesis of extracellular matrix proteins and deposition of increased amounts of collagen, immune activation and vascular damage, all of which are known to be important in the development of this illness.[1]

Adhesion molecules have multiple roles in angiogenesis. Specific adhesion molecule expression can mediate angiogenesis indirectly by promoting the migration of monocytes.[2] These monocytes are then capable of becoming tissue macrophages and secreting angiogenic factors. Cellular adhesion molecules may have a role in the immunopathogenesis of SSc.[3] We and others have shown that several adhesion molecules are overexpressed in SSc skin.[4 5] A number of soluble adhesion molecules are also elevated in SSc.[3]

Junctional adhesion molecule-A (JAM-A) has been implicated in a variety of physiological and pathological processes involving cellular adhesion, tight junction assembly and leukocyte transmigration.[6 7] It facilitates leukocyte adhesion and transmigration through its interaction with lymphocyte function-associated antigen-1 (LFA-1). JAM-A has been shown to play a part in angiogenesis.[8]

As SSc is characterised by both inflammatory cell infiltration and vasculopathy, we hypothesised that JAM-A may have a role in its pathogenesis. Here we demonstrate aberrant expression of JAM-A in SSc skin and soluble JAM-A (sJAM-A) in SSc serum. Moreover, we show a novel role for JAM-A in mediating myeloid cell adhesion to SSc skin.

MATERIALS AND METHODS

Patients and controls

Skin punch biopsy specimens and peripheral blood samples were obtained from subjects with SSc (all with diffuse disease) and control subjects. All patients with SSc fulfilled the American College of Rheumatology criteria for SSc and also met the criteria for diffuse SSc.[1 9] Biopsy specimens were taken with full informed consent and this study was approved by the institutional review board.

Immunohistology

We performed immunohistological staining on cryosections from SSc and normal skin, as described previously.[10] Goat anti-human JAM-A antibody (R&D Systems, Minneapolis, Minnesota, USA) was used as a primary antibody. The slides were read by a pathologist under blinded conditions. For JAM-A staining, the percentage of positive cells was calculated semiquantitatively as stained cells in proportion to all cells of a distinctive subset. We also used a similar method with an anti-von Willebrand factor antibody to identify endothelial cells. Blood vessels were scored using a scale of 0–4: 0, avascular; 1, slight decrease; 2, normal; 3, slight increase; 4, marked increase.

Immunofluorescence

We performed immunofluorescence on cryosections from SSc and normal skin. Sections were fixed with 4% formalin and blocked with 20% fetal bovine serum and 5% donkey serum. Goat anti-human JAM-A antibody (R&D Systems) and mouse anti-human von Willebrand factor (Dako, Denmark) were used as primary antibodies. Fluorescent conjugated secondary antibodies were

purchased from Molecular Probes (Eugene, Oregon, USA). 4′,6-Diamidino-2-phenylindole was used to stain cell nuclei. Images were taken at 400×.

Cell lysis and western blotting

Normal and SSc dermal fibroblast cell lines were established as described previously.[11] Cell lysis and western blotting were performed as described.[10] Membranes were probed with anti-human JAM-A antibody (R&D Systems). Densitometric analysis of the bands was performed using UN-SCAN-IT software, version 5.1 (Silk Scientific).

Cell surface ELISA

Cell surface ELISAs were performed as previously described.[10] Dermal fibroblasts were plated in 96-well plates, stimulated with tumour necrosis factor α (TNFα), or interleukin 1β (IL1β), or interferon γ (all 25 ng/ml, R&D Systems) or incubated in serum-free RPMI for 24 h. The fibroblasts were incubated with anti-human JAM-A antibody (R&D Systems) or goat IgG.

Human dermal microvascular endothelial cell (HMVEC) cell culture

HMVECs were obtained from Lonza (Basel, Switzerland) and cultured using EBM complete media (Lonza). The cells were serum starved overnight and then stimulated with either TNFα or IL1β. Supernatants were collected and concentrated using Amicon ultra filters (Millipore, Billerica, Massachusetts, USA).

Serum JAM-A ELISA

Ninety-six-well microplates were coated with anti-human JAM-A antibody (R&D Systems) and blocked with 1% bovine serum albumin in phosphate-buffered saline. A sample or standard (R&D Systems) was added, followed by the addition of mouse anti-human JAM-A antibody (Santa Cruz, Santa Cruz, California, USA), anti-mouse biotinylated antibody (Vector Laboratories, Burlingame, California, USA), streptavidin-horseradish peroxidase (R&D Systems), tetramethylbenzidine substrate solution and 2N H_2SO_4. The absorbance of each well was read using a microplate reader at 450/570 nm. The diction limit of the sJAM-A ELISA was 0.3 ng/ml.

U937 cell–fibroblast adhesion assay

Adhesion assays were performed as previously described using myeloid U937 cells.[10] These cells are human histiocytic lymphoma cells that are myeloid. Combinations of JAM-A neutralising antibody (Santa Cruz), mouse antibody to human CD11a (GeneTex, Irvine, California, USA), neutralising mouse anti-human intercellular adhesion molecule-1 (ICAM-1) antibody (R&D Systems), or mouse IgG were used. The inhibitory effect of neutralising antibody treatment was given as the percentage of maximal binding, which was defined as the number of adherent cells in the control antibody-treated sections.

Stamper–Woodruff adhesion assay

In situ assays were performed as previously described.[10] JAM-A neutralising antibody (R&D Systems) or goat IgG control was added to the skin sections. U937 cells were labelled with Calcein-AM fluorescent dye (5 μM, Invitrogen, Carlsbad, California, USA), added to the sections and incubated for 1 h in the dark. Non-adherent cells were washed off. The total number of fluorescence-labelled U937 cells was counted by a blinded observer using a fluorescence microscope. The inhibitory effect

of the anti-JAM-A antibody was given as the percentage of maximal binding, which was defined as the number of adherent cells in the control antibody-treated sections.

Statistical analysis

Student t tests were performed and p values <0.05 were considered significant. All values presented were the mean (SEM).

RESULTS

Patient characteristics

The SSc group comprsied 18 women and two men (52.5 (1.8) years), while the normal control group consisted of seven men and three women (51.2 (4.4) years). The mean disease duration of the SSc group was 3.7 (0.8) years. Punch biopsy specimens were taken from clinically less affected proximal arm skin (mean skin score 1.2 (0.2)) and affected distal forearm skin (2.0 (0.2)). The proximal biopsy site was far away from the leading edge of the distal area.

SSc distal arm skin had significantly fewer blood vessels than normal skin

Our work confirmed that in our patient population SSc distal arm skin has significantly fewer blood vessels (blood vessel score = 1.7) than normal skin (blood vessel score = 2.0, p<0.05) (fig 1).[12] In addition, we found that SSc proximal arm skin (blood vessel scale score = 1.9) had a blood vessel score between that of SSc distal skin and normal skin.

JAM-A is abnormally expressed in SSc skin

JAM-A expression in normal and SSc skin was evaluated using immunohistology and immunofluorescence. JAM-A is expressed on dermal ECs, fibroblasts, macrophages and in the epidermis (fig 2). Moreover, dual immunofluorescence using anti-JAM-A and anti-von Willebrand factor antibodies further indicated that JAM-A is expressed on dermal ECs. As shown in fig 2I, quantification of JAM-A immunohistology demonstrated that SSc dermal ECs exhibited significantly decreased expression of JAM-A (mean of 71%) compared with normal controls (mean of 93%, p<0.05). In addition, JAM-A was less expressed in the stratum granulosum of the epidermis of distal SSc skin (mean of 50%) than in normal skin (mean of 98%, p<0.05, fig 2J). In contrast, SSc dermal perivascular macrophages expressed increased levels of JAM-A (12% in distal skin and 18% in proximal skin) compared with normal
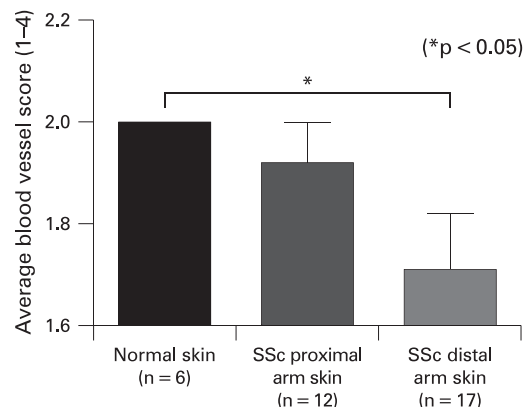

Figure 1 Distal systemic sclerosis (SSc) skin has fewer blood vessels than normal skin. n = the number of patients.
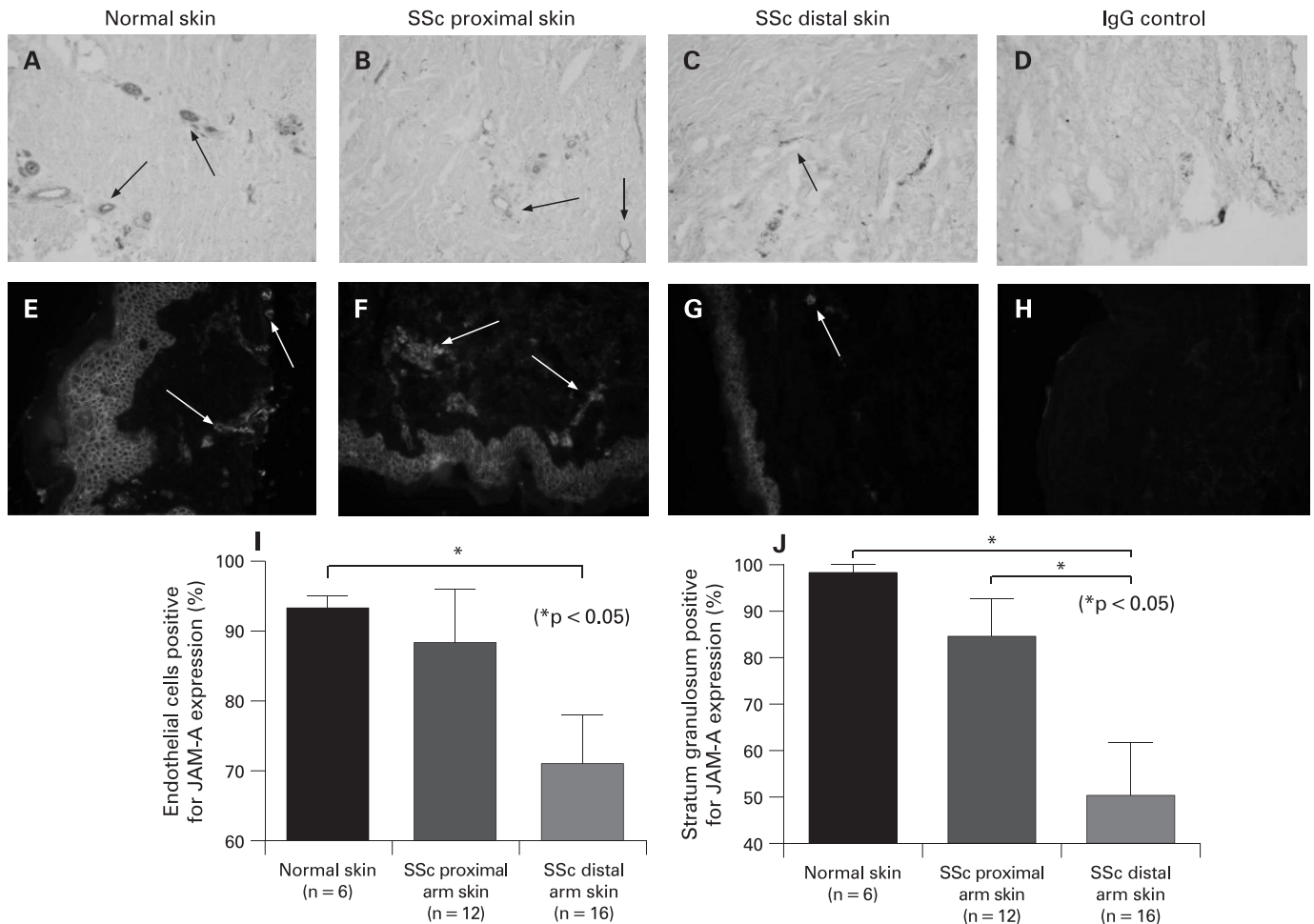
Figure 2 Immunohistological and immunofluorescence analysis of junctional adhesion molecule-A (JAM-A) on normal and systemic sclerosis (SSc) skin. Representative photos of JAM-A immunohistological staining in endothelial cells of normal skin (A), proximal SSc skin (B), distal SSc skin (C) and of the isotype control (D) are shown, all at ×200. Arrows indicate positive JAM-A staining of vasculature. Representative photos of dual immunofluorescence staining of JAM-A and von Willebrand factor (vWF) in normal skin (E), proximal SSc skin (F), distal SSc skin (G) and of the isotype control (H) are shown, all at ×400. Arrows indicate dermal blood vessels. (I) Dermal endothelial cells from proximal and distal SSc skin exhibited decreased expression of JAM-A compared with normal skin. (J) JAM-A was less expressed in the stratum granulosum of SSc skin than in normal skin. n = the number of patients.

skin (mean of 4%, both $p<0.05$) (data not shown). Similarly, SSc subepidermal macrophages expressed increased levels of JAM-A (mean of 8% in distal skin and mean of 8% in proximal skin) compared with normal skin (mean of 3%, both $p<0.05$) (data not shown).

JAM-A was more highly expressed on dermal SSc fibroblasts than on normal dermal fibroblasts

JAM-A was more highly expressed on dermal SSc fibroblasts than on normal fibroblasts (fig 3A). Western blotting resulted in similar results (fig 3B). However, the expression of JAM-A on either SSc or normal dermal fibroblasts was not inducible by TNFα, interferon γ, or IL1β (data not shown).

Elevated sJAM-A in SSc serum

A sJAM-A ELISA was designed and serum JAM-A was detected in all normal volunteers and patients with SSc. The concentration of sJAM-A in the serum of patients with SSc was 2.4 (0.4) ng/ml, whereas the concentration for normal controls was 1.0 (0.2) ng/ml ($p<0.05$) (fig 4A).

sJAM-A is secreted by ECs

sJAM-A was detectable in the culture supernatant of HMVECs (fig 4B). Moreover, stimulation with TNFα resulted in a significant increase of sJAM-A in HMVEC culture supernatants ($p<0.05$).

JAM-A mediates myeloid U937 cell binding to SSc dermal fibroblasts

We found that SSc dermal fibroblasts bound a greater number of myeloid U937 cells than normal dermal fibroblasts ($p<0.05$) (data not shown). Moreover, JAM-A accounted for a significant portion of U937 binding to SSc dermal fibroblasts. U937 binding to SSc dermal fibroblasts was inhibited by neutralising anti-JAM-A antibody treatment (86% of maximal binding, $p<0.05$) (fig 5). Neutralising antibody against the JAM-A ligand LFA-1 also inhibited U937 cell binding to SSc dermal fibroblasts (90% of maximal binding, $p<0.05$). Similarly, neutralising antibody against the LFA-1 receptor ICAM-1 inhibited U937 cell binding to SSc dermal fibroblasts (83% of maximal binding). A combination of neutralising antibodies against JAM-A, LFA-1
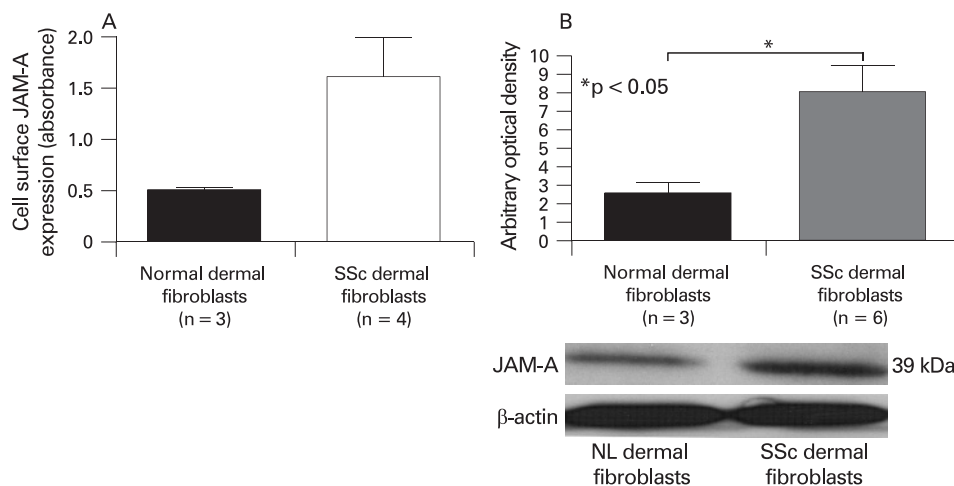
Figure 3 Junctional adhesion molecule-A (JAM-A) is overexpressed on systemic sclerosis (SSc) dermal fibroblasts than on normal (NL) dermal fibroblasts (A). A representative western blot is shown in (B); expression of JAM-A protein is higher in SSc dermal fibroblasts. n = the number of different SSc patient or normal volunteer derived fibroblast cell lines.

and ICAM-1 resulted in the greatest inhibition of U937 cell binding to SSc dermal fibroblasts (73% of maximal binding).

JAM-A contributes to U937 cell adhesion to SSc skin

We found that similar to the results of the U937-fibroblast in vitro adhesion assay, anti-JAM-A neutralising antibody decreased U937 cell binding to SSc skin (fig 6). U937 cell binding to SSc proximal arm skin (44% of maximal binding, $p<0.05$) and distal forearm skin (61% of maximal binding, $p<0.05$) was inhibited by anti-JAM-A antibody treatment. Collectively, these results indicate that JAM-A plays an important role in myeloid cell adhesion to SSc skin.

DISCUSSION

The aetiology and pathogenesis of SSc remains unknown. Nonetheless, signs of vascular injury and devascularisation of affected organs in association with evidence of profound endothelial dysfunction are well documented. Adhesion molecules, molecules known to promote both inflammatory cell infiltration and angiogenesis, may have a role in the pathogenesis of SSc.[3]

Our results demonstrated that dermal ECs from SSc skin exhibit decreased expression of JAM-A compared with ECs in normal skin. Decreased JAM-A expression increases permeability. Blocking JAM-A expression caused a decrease in neutrophil

and monocyte extravasation in several models, including inflammatory meningitis, peritonitis and ischaemia-reperfusion injury.[13–16] These findings suggest that the downregulation of JAM-A expression on SSc dermal ECs may effect the influx of leukocytes into SSc skin.

In addition, JAM-A is a proangiogenic adhesion molecule. It forms a complex with integrin $\alpha_v\beta_3$ and mediates basic fibroblast growth factor (bFGF)-induced angiogenesis.[8] JAM-A overexpression on ECs induces both EC proliferation and migration on vitronectin. In addition, inhibition of JAM-A signalling blocks bFGF-induced EC proliferation, tube formation and in vivo angiogenesis.[8 17] Our results demonstrate that JAM-A is downregulated on SSc dermal ECs and therefore may not be able to respond to bFGF and mediate angiogenesis.

Soluble adhesion molecules have also been shown to be elevated in patients with SSc. Recently, Cavusoglu et al observed significantly higher levels of plasma sJAM-A in patients with advanced coronary artery disease and indicated that JAM-A may be an important mediator of the effects of inflammation on the vessel wall.[18] Our results show that the concentration of sJAM-A in the serum of patients with SSc is elevated compared with normal serum. Moreover, we demonstrated that sJAM-A can be secreted by cultured ECs. This is the first study to suggest a link between serum sJAM-A concentration and SSc and further study is needed to determine if the
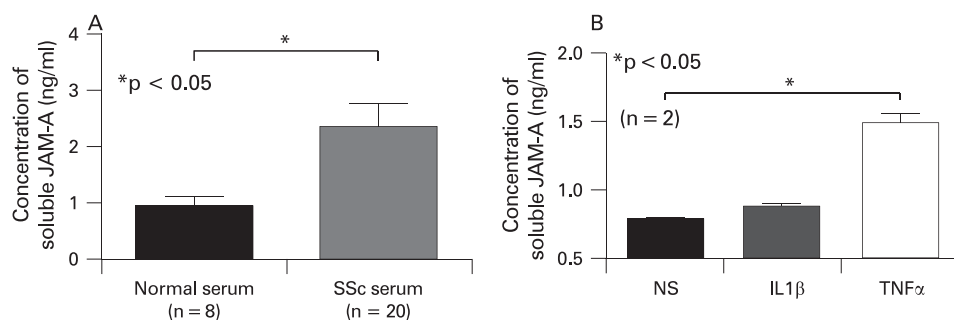

Figure 4 Soluble junctional adhesion molecule-A (sJAM-A) is elevated in the serum of patients with systemic sclerosis (SSc) and secreted by human dermal microvascular endothelial cells (HMVECs). The concentration of sJAM-A in SSc serum was significantly greater than in normal control serum (A). n, the number of patients. sJAM-A is found in the culture supernatants of HMVECs (B) and its expression is increased with stimulation by tumour necrosis factor α (TNFα). IL1β, interleukin 1β; n = the number of replicates.
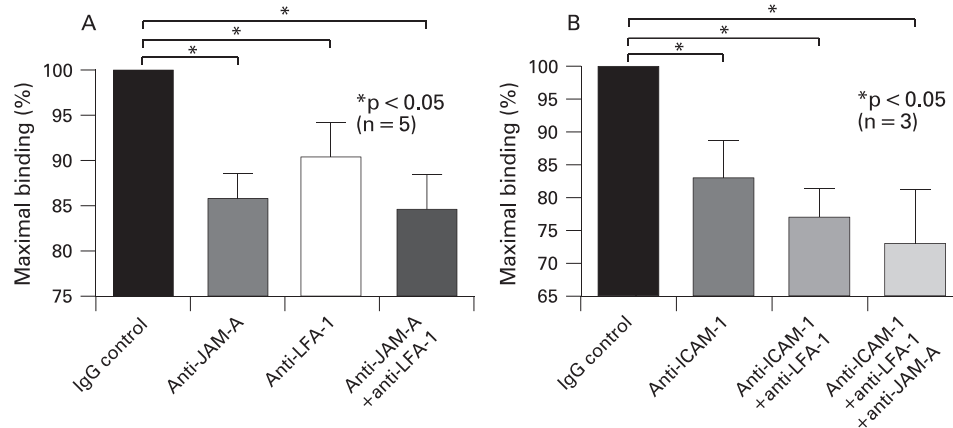
Figure 5 Junctional adhesion molecule-A (JAM-A) mediates adhesion of U937 cells to systemic sclerosis (SSc) dermal fibroblasts. U937 binding to SSc dermal fibroblasts was inhibited by anti-JAM-A antibody and anti-LFA-1 antibody (A). U937 cell binding to SSc dermal fibroblasts was inhibited by anti-ICAM-1 antibody and a combination of anti-ICAM-1, LFA-1 and JAM-A antibodies (B). LFA-1, lymphocyte function-associated antigen-1 ; ICAM-1, intercellular adhesion molecule-1; n = the number of different fibroblast cell lines from patients with SSc.
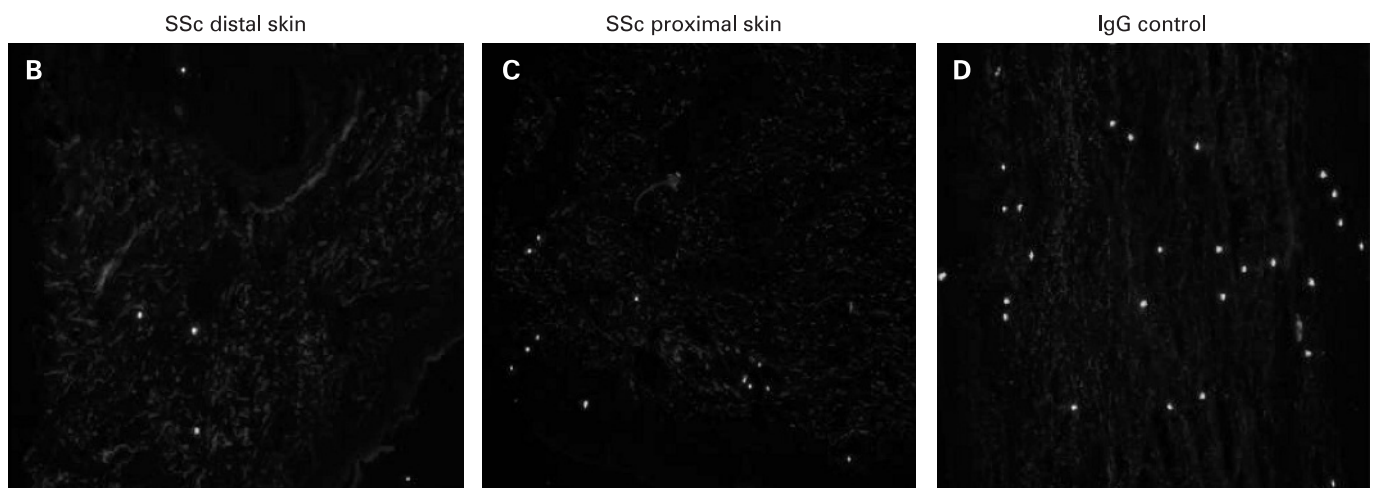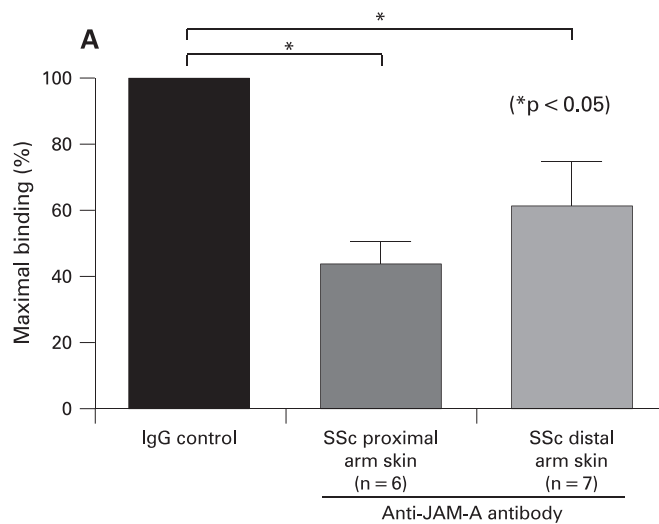

Figure 6 Junctional adhesion molecule-A (JAM-A) mediates adhesion of U937 cells to systemic sclerosis (SSc) skin. (A) U937 binding to SSc proximal arm skin and distal arm skin sections was inhibited by anti-JAM-A antibody treatment. Representative photos of the effect of anti-JAM-A on U937 cell adhesion to distal SSc skin (B), proximal SSc skin (C) and in the presence of an IgG control in place of anti-JAM-A (D) are shown, all at ×100. n = the number of patients.

Extended report

concentration of serum sJAM-A correlates with additional clinical manifestations of SSc.

Previous studies have shown that SSc peripheral blood mononuclear cells and SSc dermal fibroblasts are hyperadhesive.[19] Here we found that JAM-A is overexpressed on SSc dermal fibroblasts and mediates the adhesion of myeloid U937 cells to both SSc dermal fibroblasts and proximal and distal SSc skin. As myeloid cells mature into monocytes and macrophages that have the potential to secrete a variety of angiogenic factors, our results have particular importance to the pathogenesis of SSc.

Our study suggests that JAM-A plays multiple roles in the pathogenesis of SSc. The reduced expression of JAM-A on the surface of ECs may contribute to dysregulated angiogenesis in SSc skin, as JAM-A EC expression is required for bFGF-induced angiogenesis.[8 20 21] Moreover, the elevated SSc serum levels of sJAM-A could be the result of the characteristic EC injury in SSc, further strengthening the suggestion of JAM-A as a vascular disease marker.[18] In contrast, JAM-A exemplifies the dual nature of an adhesion molecule, as our results demonstrate its importance in mediating myeloid cell retention in SSc skin. Collectively, these results suggest that JAM-A is dysregulated on multiple cell types in SSc and that further study of its role in SSc skin angiogenesis is warranted.

Competing interests: None.

Ethics approval: Ethics committee approval from the University of Michigan.

Patient consent: Patient consent received.

REFERENCES

1. **LeRoy EC,** Black C, Fleischmajer R, et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1988;**15**:202–5.
2. **Kumar P,** Hosaka S, Koch AE. Soluble E-selectin induces monocyte chemotaxis through Src family tyrosine kinases. J Biol Chem 2001;**276**:21039–45.
3. **Sato S.** Abnormalities of adhesion molecules and chemokines in scleroderma. Curr Opin Rheumatol 1999;**11**:503–7.
4. **Abraham D,** Lupoli S, McWhirter A, et al. Expression and function of surface antigens on scleroderma fibroblasts. Arthritis Rheum 1991;**34**:1164–72.
5. **Koch AE,** Kronfeld-Harrington LB, Szekanecz Z, et al. In situ expression of cytokines and cellular adhesion molecules in the skin of patients with systemic sclerosis. Their role in early and late disease. Pathobiology 1993;**61**:239–46.
6. **Liu Y,** Nusrat A, Schnell FJ, et al. Human junction adhesion molecule regulates tight junction resealing in epithelia. J Cell Sci 2000;**113**:2363–74.
7. **Ostermann G,** Weber KS, Zernecke A, et al. JAM-1 is a ligand of the beta(2) integrin LFA-1 involved in transendothelial migration of leukocytes. Nat Immunol 2002;**3**:151–8.
8. **Naik MU,** Mousa SA, Parkos CA, et al. Signaling through JAM-1 and alphavbeta3 is required for the angiogenic action of bFGF: dissociation of the JAM-1 and alphavbeta3 complex. Blood 2003;**102**:2108–14.
9. **Anonymous.** Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Arthritis Rheum 1980;**23**:581–90.
10. **Rabquer BJ,** Pakozdi A, Michel JE, et al. Junctional adhesion molecule-C mediates leukocyte adhesion to the rheumatoid arthritis synovium. Arthritis Rheum In press.
11. **Louneva N,** Huaman G, Fertala J, et al. Inhibition of systemic sclerosis dermal fibroblast type I collagen production and gene expression by simvastatin. Arthritis Rheum 2008;**58**:3020–9.
12. **Fleming JN,** Schwartz SM. The pathology of scleroderma vascular disease. Rheum Dis Clin North Am 2008;**34**:41–55; vi.
13. **Corada M,** Chimenti S, Cera MR, et al. Junctional adhesion molecule-A-deficient polymorphonuclear cells show reduced diapedesis in peritonitis and heart ischemia-reperfusion injury. Proc Natl Acad Sci USA 2005;**102**:10634–9.
14. **Del Maschio A,** De Luigi A, Martin-Padura I, et al. Leukocyte recruitment in the cerebrospinal fluid of mice with experimental meningitis is inhibited by an antibody to junctional adhesion molecule (JAM). J Exp Med 1999;**190**:1351–6.
15. **Martin-Padura I,** Lostaglio S, Schneemann M, et al. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. J Cell Biol 1998;**142**:117–27.
16. **Woodfin A,** Reichel CA, Khandoga A, et al. JAM-A mediates neutrophil transmigration in a stimulus-specific manner in vivo: evidence for sequential roles for JAM-A and PECAM-1 in neutrophil transmigration. Blood 2007;**110**:1848–56.
17. **Cooke VG,** Naik MU, Naik UP. Fibroblast growth factor-2 failed to induce angiogenesis in junctional adhesion molecule-A-deficient mice. Arterioscler Thromb Vasc Biol 2006;**26**:2005–11.
18. **Cavusoglu E,** Kornecki E, Sobocka MB, et al. Association of plasma levels of F11 receptor/junctional adhesion molecule-A (F11R/JAM-A) with human atherosclerosis. J Am Coll Cardiol 2007;**50**:1768–76.
19. **Rudnicka L,** Majewski S, Blaszczyk M, et al. Adhesion of peripheral blood mononuclear cells to vascular endothelium in patients with systemic sclerosis (scleroderma). Arthritis Rheum 1992;**35**:771–5.
20. **Naik MU,** Naik UP. Junctional adhesion molecule-A-induced endothelial cell migration on vitronectin is integrin alpha v beta 3 specific. J Cell Sci 2006;**119**:490–9.
21. **Naik MU,** Vuppalanchi D, Naik UP. Essential role of junctional adhesion molecule-1 in basic fibroblast growth factor-induced endothelial cell migration. Arterioscler Thromb Vasc Biol 2003;**23**:2165–71.