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The TGF β Receptor Endoglin in Systemic Sclerosis

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SUMMARY Immunohistochemical, flow cytometric and ELISA studies were performed to examine the expression of endoglin (CD105, a TGF β receptor) on dermal endothelial cells, peripheral blood monocytes and free and bound serum levels in patients with systemic sclerosis as compared with appropriate controls. Endoglin was found to be significantly upregulated on dermal blood vessels in patients with scleroderma (and in patients with inflammatory skin disorders) as compared to healthy skin ($p < 0.05$). In contrast, there was no significant difference in endoglin expression on circulating blood monocytes between scleroderma patients and patients with a rheumatic disoder or healthy control subjects; however, endoglin expression was upregulated on monocytes in inflammatory joint fluid from patients with rheumatoid arthritis. Endoglin expression on monocytes was also influenced by isolation techniques and during whole blood culture. No differences were found in circulating free or bound endoglin levels between scleroderma patients and healthy controls. In conclusion, endoglin expression on dermal endothelial cells was significantly enhanced in scleroderma but levels on circulating monocytes and in the serum were within normal limits. The functional significance of this upregulation is uncertain but may reflect endothelial activation in scleroderma.

Previous studies have suggested that transforming growth factor β (TGF β) is a pivotal cytokine for fibrosis since its expression is increased in some diseases characterized by fibrosis such as keloid scarring, lichen sclerosus and systemic sclerosis/scleroderma.[1] It is suspected that the role of TGF β is to regulate the activation of a precursor form of TGF β (a latent polypeptide), to stimulate fibroblasts to proliferate and synthesize collagen and also to stimulate deposition of fibronectin into extracellular matrix.[1] Fibrosis is a prominent feature of systemic sclerosis but there is scarcity of information regarding the TGF β receptor in this disorder especially concerning the homolog of the TGF β receptor III which is called endoglin or CD105. Mutations of endoglin are found to underlie the pathogenesis of telangiectasia in hereditary haemorrhagic telangiectasia type I.[2] Furthermore, nailfold capillaroscopy and clinical examination have shown that the morphology and the distribution of these telangiectasia are very similar with those found in scleroderma.[3] So it is of considerable interest to examine whether endoglin has a role in the pathogenesis of telangiectasia in scleroderma.

Endoglin is expressed principally on endothelial cells, syncytiotrophoblast and cytotrophoblast (transiently during first trimester), blood monocytes and weakly on stromal fibroblast and stroma of

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some tissues.[4] The disregulation of endoglin on endothelial cells might be a link in the evolution from the early vascular pathology to the fibrosis prominent in the pathogenesis of scleroderma. If endoglin is involved in this pathogenesis, it might be a good entry point for therapeutic intervention since it is widely accepted that microvascular abnormalities are the initial event in the pathogenesis of this enigmatic immune disorder.

MATERIALS AND METHODS

Patients and controls

This was a cross sectional study involving ten skin samples from scleroderma patients (6 diffuse and 4 limited) and 6 from control subjects (3 inflammatory and 3 normal skin). The mean age of the diffuse scleroderma patients was 68 ± 8.9 years, limited scleroderma patients 65 ± 19.8 years, inflammatory control patients 79 ± 17.0 years and normal subject 62 ± 16.1 years.

Patients with diffuse, limited and overlap scleroderma were selected from the South Australian Scleroderma Register.[5] All patients fulfilled the American College of Rheumatology preliminary criteria for the classification of scleroderma.[6] Skin biopsies were obtained from involved skin of scleroderma and inflammatory controls during the diagnostic work up of the patients. The inflammatory controls were from patients with sub-acute eczema, lichen planus and papular urticaria. Normal healthy skins were obtained from patients undergoing plastic surgery.

Immunohistochemistry

Four micrometer sections of skin were cut with a microtome and placed on APTS (2% 3-aminopropyltriethoxysilane) coated slides as previously published.[7] Immunohistochemical staining was performed as previously described.[8]

The skin sections were incubated overnight at room temperature with the primary antibody against endoglin (CD105, clone 266, IgG1κ, PharMingen, Becton Dickinson Company, NJ, USA). Blocking serum (normal donkey serum) and secondary antibody (donkey anti-mouse IgG) were from Jackson Immuno Research (West Grove, Pensylvania, USA). ABC complex was purchased from Vector Laboratories (Burlingame, California, USA). Immunohistochemical staining was revealed using 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, Australia) as previously described.[8] Finally, the slides were counterstained with hemotoxylin and lithium carbonate. Positive (tonsil) and negative controls (omission of the primary antibody) were carried out with each immunohistochemical staining experiment.

Evaluation of staining intensity

Staining intensity were examined manually and semiquatitative scoring (SQA) was performed as previously described.[9] A score for the number of positive cells was expressed as a percentage ratio of the total number of vessels.[10] Digital image analysis was performed on six representative regions using high power fields (HPF, x 400 magnification) as previously described.[10] Measurements included area of staining integrated optical density (IOD) and mean optical density (MOD) as previously published.[11]

Flow cytometry

Whole blood monocytes were examined by flow cytometry. Samples were obtained from 10 patients with scleroderma (3 diffuse, 5 limited, 2 overlap), 17 healthy subjects, 23 patients with rheumatoid arthritis (RA) and 6 patients with other rheumatic diseases.

Purified monocytes from the same patients were also examined for comparison using two separation techniques (monocytes obtained through lysis of red cells and through isolation with Ficoll gradient). Six synovial fluids from RA patients were examined as examples of fluid from a local site of inflammation. Six samples of whole blood were also examined to investigate the influence of cell culture on the expression of endoglin on peripheral blood monocytes (PBMC).

Heparinized whole blood (100 µl) was stained with CD14 PE (Silenus) and CD105 FITC (Diaclone Research) (both antibodies were pretitrated prior to the application) in 3 ml tube and incubated for 30 minutes, at 4°C. Then the red cells were lysed with ammonium chloride (NH_4Cl) for 10 minutes, at room temperature. After centrifugation at 400 x *g*, the supernatant was decanted, and the cells were washed with PBS pH 7.2-7.3 containing 0.2% azide plus 1% BSA. Finally the cells were fixed and were analyzed on a FacScan (Becton Dickinson, San Jose, USA). Monocytes were identified on side

and forward scatter characteristics and analyzed for fluorescence intensity. For each sample 10,000 monocytes were collected. As a negative control the cells were stained with CD14 PE and X 63 FITC instead of CD105 FITC. All results were expressed as mean fluorescence intensity (MFI).

Purified monocytes were obtained from isolation using Ficoll/Hipopaque (Lymphoprep™, Nycomed). Diluted heparinized whole blood was layered over the lymphoprep. After centrifugation at 800 x *g* for 20 minutes, the interface layer between the Lymphoprep and plasma was collected and the cells resuspended in PBS containing azide and then centrifuged at 400 x *g* for 10 minutes. The preparation was then adjusted to 10^7 cells/ml and stained with CD14 PE and CD105 FITC as above but without the lysing solution step.

Cells from 6 blood samples were cultured in hybridoma serum free medium (Ex-Cell™) supplemented with penicillin, streptomycin and glutamine (Sigma Chemical Co., USA). The cells were placed in a CO_2 incubator for 72 hours and were harvested every 24 hours and stained with CD14 PE and CD105 FITC as above. As a control, patient's samples were stained on day 0 prior to culture.

Synovial fluids were stained with CD14 PE and CD105 FITC after they were washed and the cells were adjusted to 10^7 cells/ml in PBS azide.

ELISA

Sera from 49 scleroderma patients (12 diffuse, 24 limited, 13 overlap) and 31 healthy controls were examined for circulating CD105 and circulating TGF β1. ELISA for measurement of the circulating CD105-TGFβ1 complex was performed according to the method as previously published.[4] The capture antibody used was Mab E9, which recognizes the epitope between amino acids 276-331 of the extracellular domain of CD105. One hundred microliters of Mab E9 at a concentration of 1 μg/ml diluted in PBS was added to each well of the ELISA plates and incubated overnight at 4°C. The coated plates were blocked with 1% BSA and 0.1% Tween-20 in PBS (PBS-Tween-BSA) for 2 hours at room temperature. After incubation overnight with the serum samples, polyclonal chicken anti TGF β1 antibody (diluted in PBS containing 0.01% Tween-20, PBS-Tween) was added to each well and incubated for 3 hours at 4°C, followed by HRP (horse radish peroxidase) conjugated rabbit anti-chicken IgG, diluted in PBS-Tween-BSA. The plates were washed three times between each procedure. Light emissions were measured at 420 nm.

Statistical analysis

Demographic data were analyzed using descriptive statistics. The SQA scores were compared between groups by using non-parametric ANOVA (Kruskall-Wallis test). Because IODs were not normally distributed, they were log transformed. The log IOD results were compared between groups using ANOVA. Correlation between IOD and SQA was analyzed by Spearman rank test. Differences between mean fluorescence intensity of endoglin staining monocytes in different patient groups and RA synovial fluid were analyzed using one way ANOVA and post- hoc Tuckey HSD. The mean fluorescence intensities of endoglin staining monocytes in whole blood were compared to purified monocytes using paired students' *t*-test. The changes of expression of endoglin staining monocytes over time were analyzed using the repeated measurement analysis of variance options available within the SPSS statistical package release 10 (SPSS Chicago, IL). Circulating levels of endoglin were compared using Kruskall-Wallis. Circulating levels of TGF β1 and circulating TGF β1-CD105 complex in the 4 groups were compared using ANOVA. A *p*-value less than 0.05 was considered statistically significant.

RESULTS

Immunohistochemistry

Clinical and histological features of samples for immunohistological examination are shown in Table 1. There was an upregulation of endoglin expression on endothelial cells of scleroderma and inflammatory controls compared to normal skin. The endoglin expression on dermal endothelial cells in scleroderma and inflammatory skin was strong, but the staining was very weak or negative in the endothelial cells of normal skin. Quantitation of endoglin expression by SQA demonstrated a significant difference between scleroderma/inflammatory controls and normal skin (mean rank diffuse scleroderma is 9.75, limited 9.75, inflammatory controls 10.83, and normal 2.00, $p < 0.05$). Quantitation by video image analysis (VIA) measuring the IOD of endoglin staining in endothelial

Table 1 Clinical and histological features of skin biopsies

Patient	Diagnosis	Age	Sex	SQA of CD105	IOD of CD105 (pixel unit)
1	lSSc	51	male	2	467
2	dSSc	76	male	3	3,634
4	lSSc	46	female	3	2,132
5	dSSc	61	male	2	1,428
5	lSSc	80	female	2	499
6	dSSc	81	male	2	278
7	lSSc	85	male	3	3,577
8	dSSc	72	female	3	3,024
9	dSSc	61	female	3	4,757
10	dSSc	62	female	2	435
11	inflammatory (lichen planus)	67	male	3	1,307
12	inflammatory (papular urticaria)	ni	female	3	1,223
13	inflammatory (subacute exzema)	91	female	2	546
15	normal	77	male	1	922
16	normal	64	female	1	935
17	normal	45	female	1	487

SQA, semi quantitative analysis (graded 1-3)
DSSc, diffuse scleroderma
LSSc, limited scleroderma
IOD, Integrated Optical Density
Ni, not identified

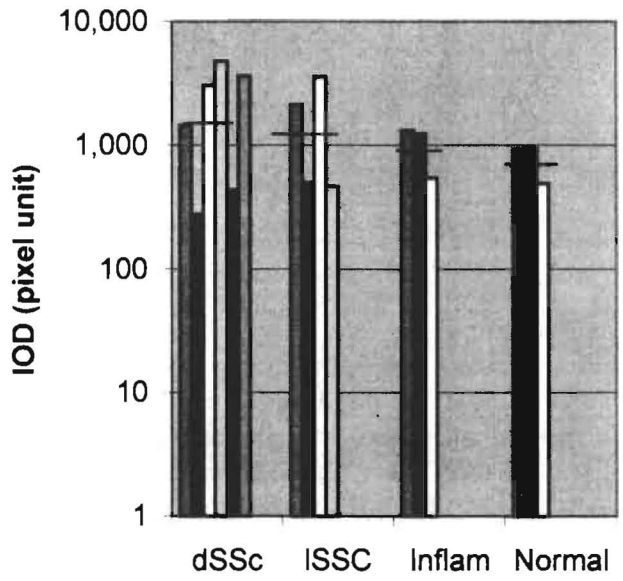

Fig. 1 Integrated optical density (IOD) after video image analysis of endoglin staining of dermal endothelial cells in each patient and controls; dSSc = diffuse scleroderma, lSSc = limited scleroderma, inflam = inflammatory control; bars show IODs; ——— = geometric means.

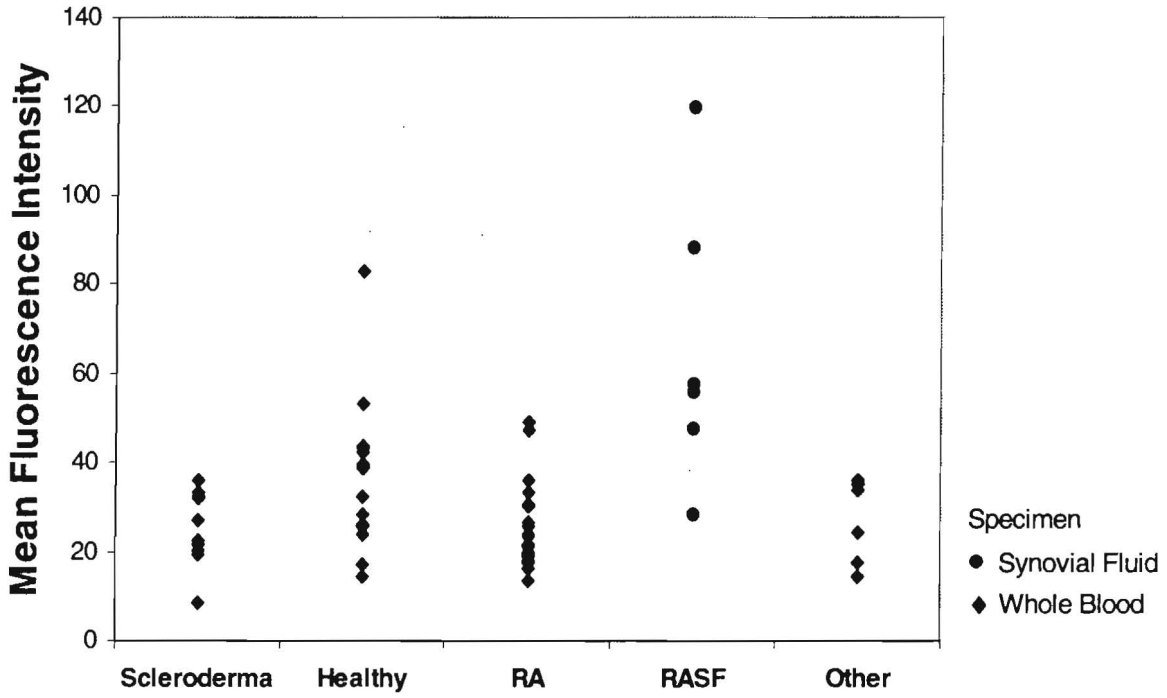

Fig. 2 Mean fluorescent intensity of endoglin staining monocytes (CD14 PE+/CD105 FITC+) in scleroderma and control groups. RA, whole blood monocytes of rheumatoid arthritis patients; RASF, synovial fluid monocytes of rheumatoid arthritis patients.

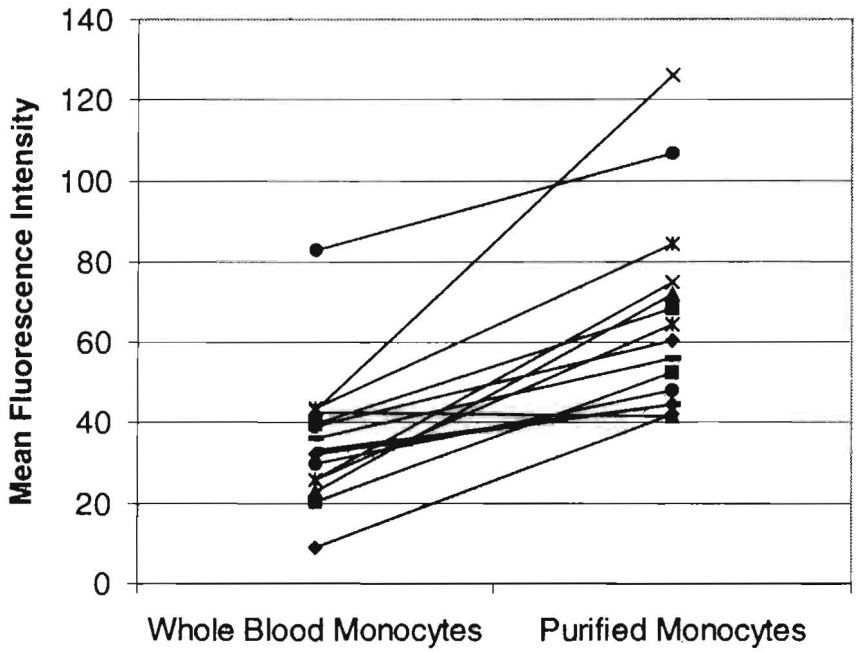

Fig. 3 Comparison between mean fluorescent intensity of endoglin staining monocytes in whole blood and purified monocytes. WB, whole blood monocytes; purified monocytes = purified monocytes after isolation with Lymphoprep.

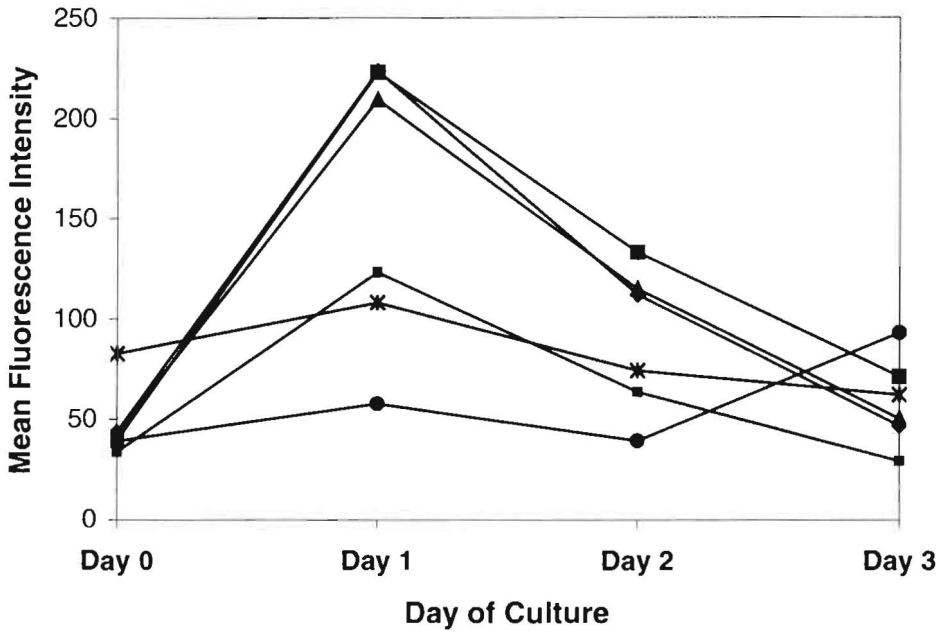

Fig. 4 Expression of endoglin staining monocytes during period of whole blood culture. Six subjects studied.

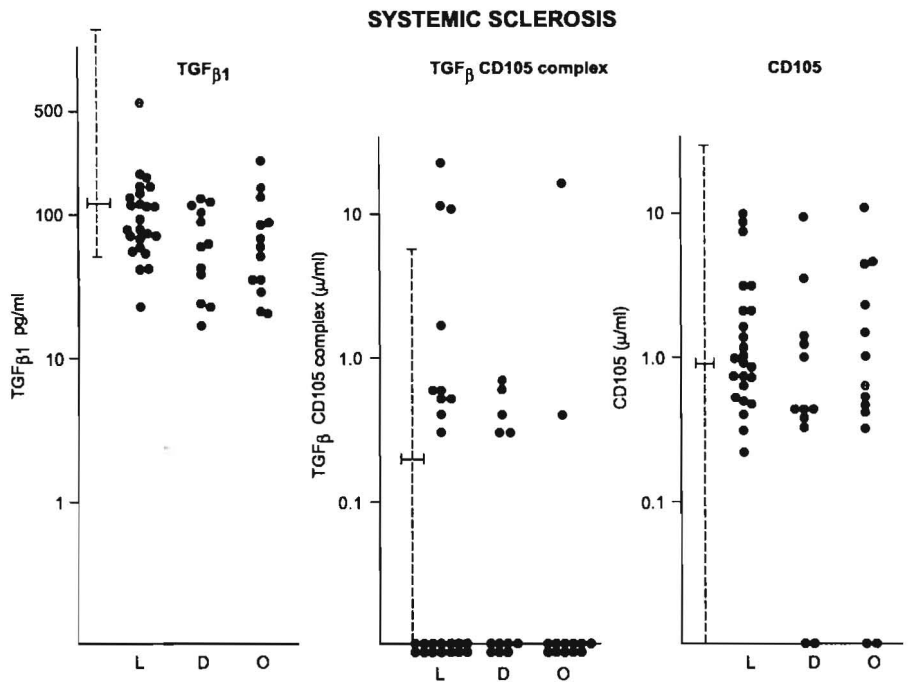

Fig. 5 Circulating levels of TGF β1, TGF β1-CD105 complex and circulating CD105 in scleroderma. L, limited scleroderma; D, diffuse scleroderma; O, overlap; The mean and 2 SD range obtained from 31 healthy subjects is indicated by the dashed line adjacent to the y axis.

cells in each group is shown in Fig. 1. The increased amount of endoglin staining especially in the patient group with diffuse scleroderma was consistent with SQA results but the difference between diffuse scleroderma and other groups was not statistically significant ($p = 0.79$). There was a good correlation between SQA and VIA ($r = 0.694$, $p < 0.01$).

Flow cytometry

Results of flow cytometric analysis are shown in Fig. 2. There was no difference in expression of endoglin on peripheral blood monocytes from scleroderma patients compared with controls ($p = 0.06$). The mean fluorescence intensity of endoglin staining monocytes in synovial fluids of RA patients was higher than whole blood. There was an increased expression of endoglin when purified monocytes were used (compared to the expression in whole blood of the same patients, $p = 0.02$) (Fig. 3). This increased expression was found both in healthy controls and scleroderma patients. During culture, the expression of endoglin on PBMC increased on day 1 compared to the other days ($p = 0.02$) shown in Fig. 4.

ELISA

The levels of soluble CD105, soluble TGF β1 and CD105-TGF β 1 complex are shown in Fig. 5. There was no difference ($p = 0.465$) between the CD105 levels in scleroderma patients and healthy controls (presented in dashed line for the range and horizontal line for the mean in Fig. 5). There was also no difference between TGF β1 levels and TGF β1-CD105 complex in scleroderma and controls ($p = 0.232$ and $p = 0.291$, respectively).

DISCUSSION

Fibrosis and telangiectasia are characteristic pathological features of scleroderma although the pathogenesis of each is unclear. TGF β, a pivotal fibrogenic cytokine has been implicated in the fibrosis with previous studies suggesting enhanced activity in the skin from patients with scleroderma.[14,15,19] Telangiectasia of the hands and face is also prominent in the disorder hereditary haemorrhagic telangiectasia and recent studies[2] have shown that this genetic disorder is due to mutation in ALK-1 or endoglin (CD105), a protein that acts as a part of the receptor complex for TGF β. Furthermore we have recently observed a close similarity in the spatial distribution and morphology of telangiectasia in hereditary haemorrhagic telangiectasia and systemic sclerosis.[3] We therefore hypothesized that disorders of endoglin (or its transduction proteins) may underlie the pathogenesis of telangiectasia in scleroderma and undertook the present study to investigate endoglin in scleroderma.

Endoglin was noted to be upregulated on the endothelium in the involved skin in the scleroderma patients compared to normal skin with higher expression in the diffuse scleroderma subgroup. The majority of these biopsies were obtained in the acute/early phase of the disease. Furthermore, endothelial endoglin was also upregulated in patients with inflammatory skin disorders in keeping with a previous publication[4] suggesting that this protein can be considered as an endothelial activation marker.

Endoglin was also detectable on circulating monocytes and we therefore assessed its expression in our scleroderma patients and in patients with other rheumatic disorders, but no differences were seen. However, levels on monocytes were elevated in inflammatory joint fluids obtained from patients with rheumatoid arthritis, again consistent with its upregulation in chronic inflammation. This higher expression of endoglin in inflammatory joint fluid is similar to previous published results.[16] This study found that the expression of some adhesion molecules needed for interaction with T cells (CD54, VLA-4) and constitutive tissue receptors (CD44) were increased on synovial fluid monocytes compared with whole blood.[16] Furthermore, it was apparent that purification of mononuclear cells from whole blood also led to enhanced expression of endoglin as did culturing whole blood with a peak expression being noted at 24 hours of culture. These observations suggest that endoglin expression can be modulated by a number of variables, possibly in response to inflammatory or immunoregulatory cytokines. Another possibility might be the influence of temperature that can change the functional or structural epitope of a surface antigen on monocytes.[12,17]

Endoglin can also be enzymically released from the cell membrane into the circulation either in a free form or bound to TGF β. Measurement of both these forms of circulating endoglin revealed no differences when scleroderma were compared with control sera or between the three subsets of scleroderma patients. However, circulating receptor or cytokines levels do

not necessarily reflect functional activity or alterations at tissue levels.[18]

In conclusion, endoglin expression on dermal endothelial cells was enhanced in scleroderma patients but levels on circulating monocytes and in the serum were within normal limits. The functional significance of this upregulation is uncertain but may reflect endothelial activation in scleroderma.

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