Increased endothelin-1 production in fibroblasts derived from patients with systemic sclerosis

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Abstract

**Objectives**—To determine whether fibroblasts from patients with systemic sclerosis (SSc) produce excessive amounts of endothelin-1 (ET-1), which is recognized as having vasoconstrictive properties and as having a potent mitogenic effect on fibroblasts.

**Methods**—Dermal fibroblasts were removed from 11 patients with SSc and from five normal controls (NC). The assay of ET-1 protein was measured by an ELISA that used two anti-ET-1 antibodies. The gene expression of prepro ET-1 mRNA was evaluated by a reverse-transcriptase polymerase chain reaction (RT-PCR) method.

**Results**—Levels of ET-1 protein were significantly higher in SSc fibroblast cultures than in those of normal fibroblasts (p < 0.01). The expression of prepro ET-1 mRNA was also higher in SSc fibroblasts than in normal fibroblasts. The addition of interleukin-1β (IL-1β) increased the production of ET-1 by fibroblasts.

**Conclusion**—The findings indicate that the overproduction of ET-1 is a novel abnormal function in SSc fibroblasts, and that ET-1 induced by fibroblasts may play a role in the fibrosis and Raynaud’s phenomenon of SSc.


Systemic sclerosis (SSc, scleroderma) is a connective tissue disease characterised mainly by fibrosis and endothelial damages. Although it is well known that Raynaud’s phenomenon is an initial symptom in SSc patients, the mechanism underlying Raynaud’s phenomenon remains to be clarified. Endothelin-1 (ET-1) is a vasoactive peptide isolated from the supernatant of serum-free cultured endothelial cells, and is believed to play an important role in the regulation of blood flow in microvascular beds. Because of its prolonged vasoconstrictor effect, it has been suggested that ET-1 could also stimulate the vasoconstrictive episodes involved in Raynaud’s phenomenon. Recently, an increased plasma level of ET-1 has been observed in patients with Raynaud’s phenomenon complicated with SSc. Increased production of ET-1 could also stimulate proliferation of fibroblasts, and it is possible that it may be associated with the fibrosis in SSc.

SSc fibroblasts have been reported to be associated with cytokine production, growth factor production, proliferation, production of extracellular matrix components and adhesion molecule expression. However, ET-1 production by SSc fibroblast cultures has not as yet been examined. The purpose therefore of the present study is: (1) To determine if ET-1 production by fibroblasts from SSc patients is the same as that in healthy subjects, and (2) to examine the effect of interleukin-1β (IL-1β), which is a potent regulator of ET-1 production by endothelial cells, on ET-1 production in human dermal fibroblasts.

**Patients and methods**

**FIBROBLAST SOURCES AND CULTURING**

Dermal fibroblasts were explanted from 11 patients with SSc (table) and from five normal controls. Following the criteria established by the American Rheumatism Association, six of the 11 SSc patients were diagnosed as having diffuse SSc and the other five as having limited SSc. The explants were derived by 5 mm punch biopsy from affected skins of patients and dorsal forearm skin of normal controls. The control group was approximately matched for sex and age with the patient group. A lower portion of biopsied tissue was minced and placed in plastic dishes (Corning Glass Works, Corning, NY). After attachment had occurred, Dulbecco’s modification of essential medium (DMEM, Flow Laboratories, McLean, VA) with 10% fetal calf serum (FCS, Flow Laboratories), 10 units/ml of penicillin, and 10 µg/ml of streptomycin (Gibco Laboratories, Grand Island, NY) was added to the dishes.

**Clinical characteristics of the systemic sclerosis patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Sex</th>
<th>Duration (months)</th>
<th>Systemic involvements</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Diffuse</td>
<td>54/F</td>
<td>12</td>
<td>Pulmonary fibrosis</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Diffuse</td>
<td>27/F</td>
<td>6</td>
<td>Pulmonary fibrosis</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Diffuse</td>
<td>63/M</td>
<td>12</td>
<td>Reflux esophagitis</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Diffuse</td>
<td>57/F</td>
<td>6</td>
<td>Pulmonary fibrosis</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Diffuse</td>
<td>23/M</td>
<td>60</td>
<td>Reflux esophagitis</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Diffuse</td>
<td>32/F</td>
<td>7</td>
<td>Pulmonary fibrosis</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Limited</td>
<td>44/F</td>
<td>12</td>
<td>Reflux esophagitis</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Limited</td>
<td>35/F</td>
<td>6</td>
<td>Reflux esophagitis</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Limited</td>
<td>46/F</td>
<td>4</td>
<td>Reflux esophagitis</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Limited</td>
<td>48/F</td>
<td>12</td>
<td>Reflux esophagitis</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Limited</td>
<td>67/M</td>
<td>3</td>
<td>Telangiectasia</td>
<td></td>
</tr>
</tbody>
</table>

All patients were complicated with Raynaud’s phenomenon.

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The dishes were incubated at 37°C in 5% CO₂-95% air. Fibroblasts from all biopsied subjects were rapidly frozen with 7% dimethylsulfoxide in liquid N₂ and defrosted as needed. Cells in the third through fifth passages were used in this study.

ENDOTHELIAL CELL CULTURING

Human umbilical vein endothelial cells (HUVEC), isolated from one fresh human umbilical cord, were purchased from Sanko Junyaku (Tokyo, Japan). The cells were cultured in DMEM with 10% FCS and antibiotics. Cells in the second passages were used in this study.

MEASUREMENT OF ET-1

Monolayer confluent fibroblasts or HUVEC were cultured in 24-well culture plates with ASF-301, a serum-free media46 (Ajinomoto, Tokyo, Japan) in the presence or absence of recombinant interleukin-1β (IL-1β, Otsuka Pharmaceutical, Tokushima, Japan). ET-1 was measured in the supernatants of fibroblast cultures using an ELISA kit (Takeda Chemical Industries, Osaka, Japan) developed by Suzuki et al.17 This assay uses two antibodies which recognise different epitopes of the human ET-1 and is sensitive to as little as 0.4 pg/ml. The data were adjusted in accordance with cell counts after the various incubation periods.

ISOLATION AND ANALYSIS OF TOTAL RNA FROM HUMAN SKIN FIBROBLASTS

For isolation of total RNA, six SSC and three normal fibroblast cultures were allowed to reach confluency in 100 mm plastic culture dishes, after the medium was replaced with ASF-301. Seventy two hours later, the total RNA was isolated using RNA Zol™ (Biotex Laboratories, Houston, TX). Analysis of gene expression at the RNA level was performed using a GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT). Briefly, total RNA samples (1 μg) were incubated at 42°C for 15 minutes in reverse transcription (RT) reaction mixture containing 50 U of Moloney murine leukaemia virus reverse transcriptase. After the RT reaction, the products were held at 95°C for 5 minutes. DNA amplification by polymerase chain reaction (PCR) was performed in PCR buffer containing 0.15 μM primers (sense and antisense) and 2.5 U AmpliTaq DNA polymerase. The sequences of the sense and antisense primers for prepro ET-1 are 5'-TGGCATTGCGAACGAGCTCTCA-3' and 5'-CTCTGAAGCCTCTTCGGAC-3', respectively. Amplification of the same RNA with β-actin primers confirmed that equal amounts of RNA were reverse-transcribed. The sequences of the primers for β-actin are 5'-AAGAGAGCATCACCTACCCCTT-3' and 5'-TGACATGGCTGGGTGTTGAA-3', respectively. The mixture was amplified with the Perkin-Elmer Cetus thermal cycler. The amplification profile was 25, 30, or 35 cycles of denaturation at 95°C for 1 minute primer annealing and extension at 60°C for 1 minute. At the end of each cycle the reaction was continued with an extension incubation at 60°C for 7 minutes. The PCR products were electrophoresed in 3% NuSieve/1% Seakem agarose gel stained with ethidium bromide and visualised under ultraviolet light.

STATISTICAL ANALYSIS

All results were expressed as mean (SD). Student's t test was used to compare the differences between the means of the groups. A p value equal to or less than 0.05 was regarded as significant.

Results

ET-1 PRODUCTION BY SSC AND NORMAL FIBROBLAST CULTURES AND ENDOTHELIAL CELL CULTURE

Examination of the kinetics of ET-1 production by fibroblast monolayer cultures (3–5 passages) revealed that ET-1 production increased more rapidly and achieved higher levels in patients with SSC than in healthy controls. Data from 11 patients with SSC and five normal controls (NC), representing the levels of ET-1 production in the supernatants after 12, 24, 48 and 72 hours of culturing, are shown in figure 1A.

The mean (SD) ET-1 production levels at 24 hours of culturing were significantly higher in fibroblast cultures from the 11 SSC patients than in cultures from five normal controls [8.4 (4.4) versus 1.6 (0.2) pg/10⁵ cells, p < 0.01] (fig 1B). The 11 SSC cultures stemmed from five patients with limited SSC and six with diffuse SSC; there was no significant difference in mean ET-1 production levels between the two groups.

In addition, ET-1 production by human umbilical vein endothelial cell (HUVEC) was examined using the same methods. The kinetics study of ET-1 production indicated that ET-1 production increased more rapidly and achieved higher levels (approximately 25-fold) in HUVEC than in human normal skin fibroblasts (fig 2).

PREPRO ET-1 MRNA EXPRESSION IN SSC FIBROBLASTS AND NORMAL CONTROLS

Fibroblasts from six SSC patients (three diffuse and three limited SSC) and three normal controls (NC) expressed prepro ET-1 mRNA constitutively. The levels of ET-1 mRNA in SSC fibroblasts were higher than those in NC at all cycles of amplification, whereas the levels of β-actin mRNA in SSC fibroblasts were approximately equal to those in NC (fig 3). There was no difference between fibroblasts derived from diffuse and limited SSC patients.

EFFECT OF IL-1β ON ET-1 PRODUCTION IN FIBROBLASTS

ET-1 production by fibroblasts from five normal controls and from five patients with
SSc was enhanced by the presence of IL-1β in a dose-dependent manner (fig 4). At IL-1β concentrations of 1 and 10 nM (normal controls) or 10 nM (SSC patients), ET-1 production was significantly higher (p < 0.01) than at spontaneous levels.

Discussion

The results of the present study demonstrate that the amount of ET-1 produced by SSC fibroblasts in serum-free media is much greater than that by normal controls in vitro (fig 1). Although it has been reported that normal fibroblasts were able to produce ET-1,8,18 the present study is the first attempt to evaluate the amount of ET-1 produced by SSC fibroblasts. It is possible that ET-1 may mediate the vasoconstrictive events of Raynaud’s phenomenon because of its prolonged vasoconstrictor effect.9 In fact, several reports indicate that the plasma level of endothelin is increased in the patients with Raynaud’s phenomenon complicated with SSC.4,5 These reports suggest that the increased levels of endothelin might result from endothelial cell damage. However, there is no evidence to date that endothelial cells in patients with SSC can produce ET-1 protein in vitro or in vivo. In our study it seems clear that ET-1 derived from SSC fibroblasts is augmented and that the increased levels of ET-1 could have vasoconstrictor effects, irrespective of whether or not endothelial cell damage exists in SSC patients.

To clarify the relationship between ET-1 production and the extent of fibrosis, we examined the difference in ET-1 production between diffuse and limited SSC. As indicated in the table, all diffuse SSC patients were complicated with pulmonary fibrosis. The mean (SD) levels of ET-1 in diffuse SSC were higher than those in limited SSC [10.7 (4.8) v 5.7 (1.9)], although there was no significant difference between the two groups. This result may support the hypothesis that fibroblasts derived from SSC patients with extensive fibrosis could constitutively produce higher levels of ET-1. To evaluate this hypothesis, further studies are required.

The largest source of ET-1 seems to be the endothelial cells. More specifically, the level of ET-1 production in human umbilical vein endothelial cells (HUVEC) in vitro is reportedly approximately 20–100 times more than that in normal human fibroblast cells.19 20 We also examined the level of ET-1 produced by HUVEC in serum-free media and found that our data agreed with the previous reports. The increased level of ET-1 production in SSC fibroblasts found in the present study was lower than the spontaneous level of ET-1 production in normal HUVEC. This fact suggests that the increased serum level of ET-1 in SSC patients is little affected by the overproduction of ET-1 in SSC fibroblasts. In sclerodermatous skin, however, there is little doubt that ET-1, which is constitutively overproduced by SSC fibroblasts, can stimulate the fibroblasts themselves as well as endothelial and vascular smooth muscle cells in an
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patients produce high levels of IL-1β. These observations strongly suggest that SSc fibroblasts may be exposed to IL-1β in vivo. Thus far it seems reasonable to hypothesise that IL-1β may stimulate ET-1 production in SSc fibroblasts in vivo, but the mechanisms whereby increased ET-1 production can be prolonged in SSc fibroblast culture (serum-free media) in vitro remain unknown.

Taken together, our results show that the production of ET-1 in SSc fibroblasts is constitutive and augmented, and that the increased production of ET-1 might be implicated in the pathogenesis of Raynaud’s phenomenon and the fibrosis in SSc. At present, studies of endothelin receptor antagonists are in progress as a result of a pharmacological approach to treating vasoconstrictive and vasoplastic diseases. We expect that in the near future an endothelin receptor antagonist will be a novel valid strategy for the treatment of patients with SSc.

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