Fibroblast Matrix Gene Expression and Connective Tissue Remodeling: Role of Endothelin-1

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This study examines endothelin-induced modulation
of extracellular matrix synthesis and remodeling by
fibroblasts, and its potential role in the pathogenesis
of systemic sclerosis (scleroderma). Endothelin-1
promoted fibroblast synthesis of collagen types I and
III, but not fibronectin, by a mechanism dependent
upon both ET_A and ET_B receptors. Conversely,
endothelin-1 inhibited both protein expression of
matrix metalloproteinase 1 and zymographic activity
exclusively via ET_A receptors. A dual regulatory role
for endothelin-1 in transcriptional regulation was
suggested by the ability of endothelin-1 to enhance
steady-state levels of collagen mRNA and activate
the proα2(I) collagen (Col1a2) promoter, but in con-
trast to reduce matrix metalloproteinase 1 transcript
expression and suppress transcription of a human
matrix metalloproteinase 1 promoter reporter con-
struct in transient transfection assays. Although
endothelin-1 significantly enhanced remodeling of
three-dimensional collagen lattices populated by
normal fibroblasts, this was not observed for lattices
populated by systemic sclerosis fibroblasts.
Promotion of matrix remodeling was dependent
upon ET_A receptor expression and was blocked by
specific inhibitors of tyrosine kinases or protein
kinase C. Reverse transcriptase polymerase chain
reaction, S1 nuclease, and functional cell surface
binding studies showed that normal and systemic
sclerosis fibroblasts express both ET_A and ET_B recep-
tors (predominantly ET_A), but that ET_A receptor
mRNA levels and ET_A binding sites on fibroblasts
cultured from systemic sclerosis skin biopsies are
reduced by almost 50%. Endothelin-1 is thus able to
induce a fibrogenic phenotype in normal fibroblasts
that is similar to that of lesional systemic sclerosis
fibroblasts. Moreover, reduced responsiveness to
exogenous endothelin-1 in systemic sclerosis suggests
that downstream pathways may have already been
activated in vivo. These data further implicate dysre-
gulated endothelin-receptor pathways in fibroblasts
in the pathogenesis of connective tissue fibrosis. Key
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Endothelin-1 (ET-1) is a 21 amino acid peptide, first
characterized as a potent endothelial cell-derived
vasoconstrictor. It is synthesized as an inactive
precursor polypeptide (preproendothelin) and pro-
cessed to mature active peptides (big endothelin and
endothelin) by zinc metalloproteinases, known as endothelin-
converting enzymes (Shao *et al*, 1999). Three isoforms of
endothelin are recognized (ET-1, ET-2, ET-3), exhibiting
substantial sequence homology although the extent of functional
overlap is uncertain. In addition to effects on vascular tone (Barton
et al, 1998), endothelins modulate survival, growth, and differ-
entiation of a number of different cell types (Rubanyi and Polokoff,

1994). Elevated circulating levels of ET-1 have been reported in a
wide variety of vascular and inflammatory disease states (Levin,
1995), and in the fibrosing connective tissue disease systemic
sclerosis (scleroderma) (Vancheeswaran *et al*, 1994a). Moreover,
increased endothelin binding has also been demonstrated by
autoradiography in skin and lung biopsies from scleroderma
patients (Vancheeswaran *et al*, 1994b; Abraham *et al*, 1997)
suggesting an overall increase in ET receptor expression in disease.
These observations provide evidence that this peptide is involved in
the systemic sclerosis pathogenesis, but do not precisely define its
role in regulating fibroblast properties, nor the pattern of receptor
subtype expression on these cells.

Lesional fibroblasts from biopsies of clinically active systemic
sclerosis skin demonstrate increased matrix biosynthesis (LeRoy,
1974; Jimenez *et al*, 1986) and reduced degradation (Strehlow and
Korn, 1998). Other fundamental fibroblast properties are also
altered, including proliferative capacity (Ichiki *et al*, 1995) and
downregulation of collagen gene expression, which normally
occurs in three-dimensional collagen gel cultures (Ivarsson *et al*,
1993; Shi-Wen *et al*, 1997). The process by which fibroblasts
acquire this altered phenotype is uncertain, but chronic exposure to
profibrotic cytokines or growth factors *in vivo* is postulated. The

occurrence of vascular damage and endothelial dysfunction coexisting with, or even preceding, dermal and visceral fibrosis in systemic sclerosis implicates endothelial cell-derived factors (Carvalho et al, 1996; Denton et al, 1996) and makes ET-1 a plausible mediator. We have investigated endothelin-induced modulation of extracellular matrix turnover and remodeling by normal fibroblasts, to address the hypothesis that ET-1 is an activator of fibroblasts with respect to extracellular matrix synthesis, and to delineate the endothelin receptors and signaling pathways involved.

MATERIALS AND METHODS

Cells and cell culture   Fibroblasts were obtained from biopsies of lesional areas of the skin of patients with early (less than 3 y duration) diffuse cutaneous systemic sclerosis ($n = 8$) and from age, sex, and anatomical site matched healthy volunteers ($n = 5$). All patients fulfilled the criteria of the American College of Rheumatology for the classification of systemic sclerosis (1980). None was receiving immunosuppressive medication or corticosteroids at the time of biopsy. Fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco), 100 U per ml penicillin, and 100 mg per ml streptomycin, and cultured in a humidified atmosphere of 5% CO_2 in air. Fibroblasts were subcultured at confluence and used between passages 2 and 5.

Measurement of collagen type I and III, and matrix metalloproteinase 1 (MMP-1) production   Total collagen type I and III synthesis by cultured fibroblasts was measured in the absence and presence of ET-1 (concentration range 10^{-8}–10^{-12} M), by inhibition enzyme-linked immunosorbent assay (ELISA) (Shi-wen et al, 1997). Culture supernatants were collected after incubating the cells in the presence of ascorbate (50 μg per ml) for 48 h. Interstitial collagenase (MMP-1) concentration in culture supernatants in the absence of ascorbate was measured using a commercial ELISA kit (Amersham, Buckinghamshire, U.K.). For inhibition experiments, cells were preincubated in the presence of endothelin receptor antagonist (100-fold molar excess) for 30 min prior to initiation of the assay. The specific receptor antagonists used were as follows: ET_A receptor antagonist (ETA-RA) PD 156707 (sodium 2-benzo [1,3]dioxol-5-yl-4-(4-methoxy-phenyl)-4-oxo-3-(3,4,5-trimethoxy-benzyl)-but-2-enoate), ET_B receptor antagonist (ETB-RA) BQ-788 (N-cis-2,6-dimethylpiperidimocarbonyl-L-gMeLeuD-Nle-ONa), and the mixed $ET_{A/B}$ receptor antagonist (Bosentan) Ro 47-0203 (4-tert-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzenesulfonamine).

Western blot analysis   For protein studies fibroblasts were grown to confluence in DMEM with 10% fetal bovine serum, serum starved in DMEM containing 0.5% bovine serum albumin (BSA) (Sigma, St. Louis, MO) for 24 h, and then stimulated with ET-1. Culture medium aliquots were adjusted to 20% (vol/vol) ammonium sulfate and incubated at 4°C with rotation overnight. Samples were then centrifuged (14,000g for 30 min) at 4°C, and the pellet was resuspended in Laemmli sample buffer containing β–mercaptoethanol. The cell layer was washed twice with Tris-buffered saline and cells were directly lyzed by the addition of 1 × sample buffer. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% polyacrylamide gels, and the separated proteins were transferred onto nitrocellulose membranes at 30 V for 90 min. Membranes were blocked by incubation for 1 h with 5% nonfat milk in phosphate-buffered saline (PBS) containing 0.2% Tween-20 and antigens were detected using the following mono-specific antibodies: rabbit antiproα1(I) collagen antibody (directed against the proα1 chain), rabbit anticollagen type I (directed against collagen type I), and rabbit anticollagen type III (all from Fibrogen, CA); mouse monoclonal antibody against human fibronectin (NovoCastra Laboratories, Newcastle-upon-Tyne, U.K.); mouse antihuman MMP-1 (Oncogene Research Products, Boston, MA); goat antihuman actin (Santa Cruz Biotech, CA), all diluted in PBS. Primary antibody was visualized using a species-specific secondary IgG biotin (Vector Labs, Burlingame, CA) conjugate. Resultant antigen-antibody complexes were detected by incubation with ABC reagent (Vector Labs) using the enhanced chemiluminescence substrate kit (Amersham). Films were analyzed by laser scanning densitometry on an Ultroscan XL (LKB–Wallac, U.K.), with correction of densitometric units according to actin signal, as a surrogate for cell number.

Zymography   Gelatin and casein zymography was performed using fibroblast culture supernatants from fibroblast cultures grown in the absence and presence of ET-1 (100 nM) for 48 h. Briefly, culture medium (15 μl)

was diluted in SDS loading buffer and applied to precast 10% polyacrylamide gel Zymogram (Novex, Frankfurt, Germany). Following electrophoresis at 125 V, 4°C, gels were washed twice with renaturing buffer at room temperature for 60 min. Zymograms were then transferred into activity buffer and developed at 37°C for between 4 and 12 h. Following fixation and staining with Coomassie Brilliant Blue R-250 (0.25%), the zymograms were destained with 10% (vol/vol) acetic acid.

Transient transfection   The following promoter reporter DNA constructions were used: a Col1a2-luciferase construction containing −3.5 kb of the human collagen type I alpha 2 gene promoter (from Francesco Ramirez, Mount Sinai School of Medicine, New York); the hFNLuc construction, containing a −1.3 kb fragment of the human fibronectin promoter (from Noelynn Oliver, FibroGen, San Francisco, CA); and a −4.3 kb MMP-1 luciferase plasmid (–4372hMMP1Luc) containing the human MMP-1 promoter fragment (from Constance Brinckerhoff, Dartmouth Medical School, New Hampshire). Transient cell transfection of fibroblasts was carried using FuGene6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. Briefly, fibroblasts were grown to subconfluence, serum starved for 12 h, and then transfected with the indicated DNA constructions (0.5–1.0 μg) mixed with FuGene6 and medium in a standard 50–100 μl volume. Following transfection, cells were washed and further cultured in medium in the absence and presence of ET-1 (100 nM). After 48 h of incubation the cells were rinsed once with PBS and lyzed in 200 μl of reporter lysis buffer (Promega, Madison, WI). Reporter plasmids and pCMV-βGal, pGL3-promoter, and pTK-renilla–Luc used as internal control for transfection efficiency were transfected in a 5:1 ratio. Luciferase activity was measured by luminometry (Turner Designs, Sunnyvale, CA) using the Dual-Luciferase Reporter Assay System (Promega), and β-galactosidase activity was measured according to the manufacturer's instructions (Tropix, Bedford, MA). Values given are the means ± SEM of triplicate assays from three individual experiments.

Preparation of collagen gels and measurement of matrix remodeling   To study collagen gel contraction, fibroblasts were cultured within three-dimensional collagen lattices (fibroblast-populated collagen lattices, FPCL). These were prepared as previously described (Ivarsson et al, 1993; Shi-wen et al, 1997). In brief, 24-well tissue culture plates (Costar) were precoated with sterile 2% BSA in PBS (2 ml per well) by incubation at 37°C overnight, and were then washed three times with sterile PBS. For FPCL, neutral collagen solution (containing one part of 0.2 M HEPES, pH 8.0, four parts collagen (Vitrogen-100, 3 mg per ml, Celltrix, Santa Clara, CA), and five parts of MCDB 104 medium (Sigma, two times concentrated) was prepared and mixed with fibroblasts that were resuspended in two times MCDB 104 medium, to bring the final concentrations to 80,000 cells and 1.2 mg collagen per ml. The collagen–cell suspension (1 ml) was added to each well and allowed to gel for 1 h. After polymerization, 1 ml of MCDB medium was added to each well, causing detachment of the FPCL from the tissue culture plastic. ET-1 was added at defined concentrations and gel contraction was measured up to 48 h. Endothelin receptor antagonists were used as described above, except that gel contraction was measured at 12 h. To examine the downstream mediators of ET-1 induced gel remodeling, specific inhibitors of signal transduction pathways were employed. Each inhibitor was added to the culture medium prior to seeding fibroblasts. Control experiments using monolayer fibroblast culture and fibroblasts in collagen gel assays demonstrated that these inhibitors had an effective range of 20–300 μM. This range was determined by the retention of cell viability of fibroblasts cultured as monolayers in the presence of increasing concentrations of inhibitors and the observed optimal gel retraction. Blocking experiments were performed with inhibitors at a concentration of 200 μM. Thus, protein kinase C (PKC) activity was inhibited using calphostin C; genistein and herbimycin A were used to block tyrosine kinases and vanadate to inhibit phosphatase activity. Fibroblast viability following exposure to inhibitors was assessed by exclusion of Trypan Blue vital dye (0.4% in phosphate-buffered saline). Rates of gel contraction were measured by ocular micrometry, and results are presented as the percentage of initial gel area or, upon treatment, as the percentage of the retraction rate observed where the retraction rate in the absence of treatment was taken as 100%.

RNA extraction: northern blots, reverse transcriptase polymerase chain reaction (RT–PCR), and S1 nuclease analysis   Total RNA was isolated from fibroblasts using the isothiocyanate/caesium chloride method (Chomczynski and Sacchi, 1987). Levels of specific transcripts were determined by northern blotting, following separation of RNA on 1% agarose gels containing 2.2 M formaldehyde and capillary transfer to Hybond N+ membrane (Amersham). Filters were hybridized and probed

Figure 1. Endothelin-1 modulates matrix synthesis by fibroblasts. (*A*) Time course of the effects of ET-1 on upregulation of proα1(I) collagen production in normal fibroblasts. Fibroblasts were incubated with ET-1 (100 nM) for between 0 and 72 h and proα1(I) collagen was determined by Western blot analysis. Densitometric units are corrected according to signals for actin. (*B*) Collagen (I and III) secretion by normal (*n* = 6) fibroblasts is increased by incubation with ET-1 (10^{-7} M) for 48 h. Under these culture conditions, fibronectin production and secretion is unchanged, whereas MMP-1 secretion is significantly reduced. Conditioned medium from fibroblasts was examined using SDS-PAGE followed by Western blot. These effects could be completely abolished by coincubation with the mixed $ET_{A/B}$ receptor antagonist bosentan. Data are representative of three separate experiments.
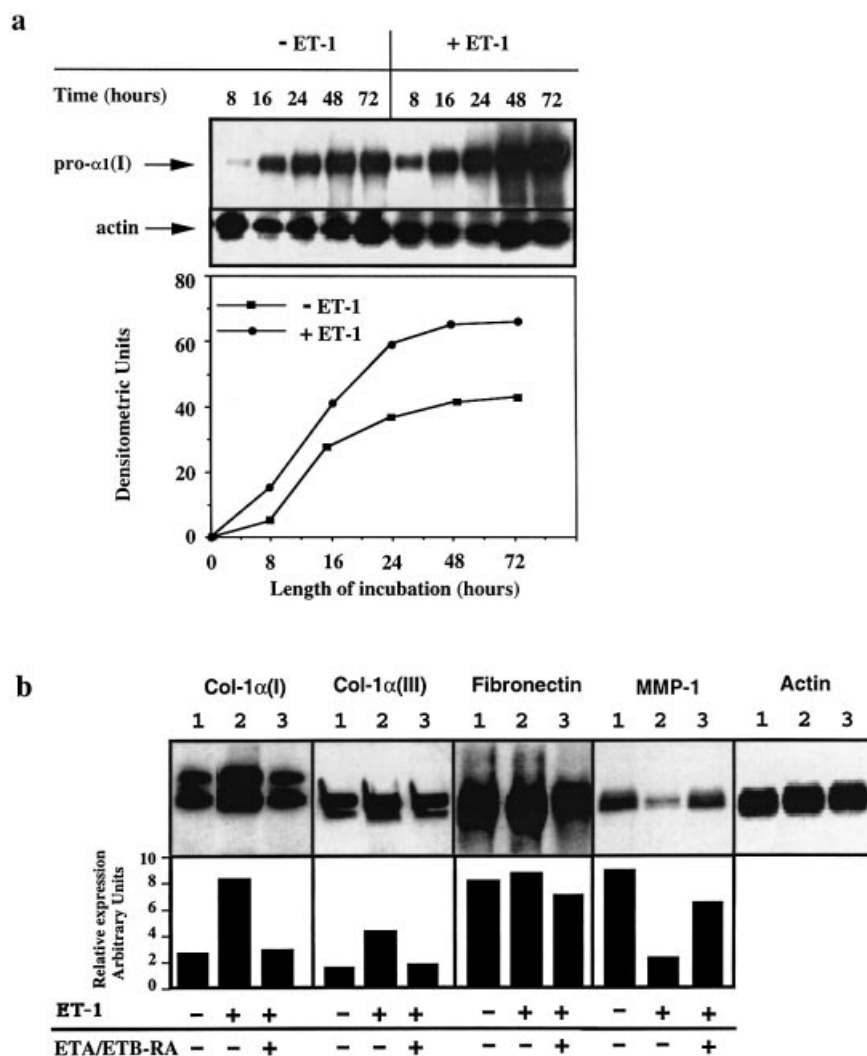
with cDNA fragments specific for proα1(I) and proα1(III) collagen, fibronectin, and MMP-1 mRNAs as previously described (Shi-wen *et al*, 1997). A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific probe was used as an internal standard to allow for differences in amounts of RNA. Probes were labeled with [α^{32}P]-dCTP to a specific activity of 10^9 dpm per μg, using the Megaprime random priming method (Amersham). Levels of transcripts were determined from signal intensities after quantitation performed by Phosphorimager analysis (Molecular Dynamics, Chesham, Buckinghamshire, U.K.) and adjusted relative to GAPDH signal intensity. Experiments in the presence of ET-1 and endothelin receptor antagonist were performed as described above. For RT-PCR, 5 μg RNA was reverse transcribed using the SUPERSCRIPT Choice system (Gibco BRL, Paisley, U.K.) and amplified by nested PCR for ET_A and ET_B receptor mRNAs as previously described (Pagotto *et al*, 1995). The PCR consisted of 30 cycles, each cycle consisting of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min. Specific amplified products (ET_A, 299 bp; ET_B, 428 bp; and GAPDH, 605 bp) were analyzed on 1.8% agarose gel and stained with ethidium bromide. GAPDH control primers (sense 5'-CTTCACCACCATGGAGAAGG-3'; antisense 5'-AGGGCA-ATGCCAGCCCCAG-3'; positions cDNA 363–968) were used as normalization controls for RNA.

Receptor mRNA levels were measured using S1 nuclease analysis as previously described (Abraham *et al*, 1997). The PCR fragments for ET_A and ET_B receptors were cloned into pGEM-T vector (Promega) and sequenced, and S1 probes were generated by restriction enzyme and phosphatase digestion followed by end-labeling with T4 polynucleotide kinase using [γ^{-32}P]-ATP (Abraham *et al*, 1997). Probes were hybridized to 20–60 μg total RNA for 16 h at 53°C, after which samples were digested with S1 nuclease (100 U) by incubation at 23°C for 1 h. Following incubation, digestion was terminated by addition of stop buffer (5 mM Tris-

HCl, 50 mM ethylenediamine tetraacetic acid, 1.0% SDS, 10 mg tRNA, pH 7.4) and protected fragments were resolved on 8% denaturing polyacrylamide gel. Transcript levels were determined from signal intensities by phosphorimager analysis (Molecular Dynamics), and are presented relative to levels of the control β-actin RNA transcript. Data summarize three scleroderma and three normal fibroblast strains.

Endothelin A and B receptor binding kinetics Receptor binding studies were performed on confluent fibroblasts cultured in 96-well plates. Washing fibroblasts in 50 mM Tris-HCl, pH 7.4, buffer three times at room temperature reduced endogenous peptide levels. Cells were then incubated for 2 h in binding buffer (PBS containing 5 mM MgCl$_2$, 100 kIU per ml aprotinin, and 1% BSA) containing [^{125}I]-ET-1, or the ET_A selective ligand ([^{125}I]-PD151242), or the ET_B selective ligand ([^{125}I]-BQ3020), over the concentration range 0.3–1000 pM. Non-specific binding was established in the presence of 1 mM unlabeled ET-1. After incubation, cells were rinsed with buffer at 4°C, harvested by treatment with cell lysis buffer for 10 min (0.25 M NaOH containing 0.5% SDS), and counted using a Packard gamma counter. Specific binding was determined by subtracting nonspecific from total binding, and receptor density and affinity [B_{MAX} (sites per cell) and K_D] were calculated using GraphPad InPlot software (Graph Pad, San Diego, CA).

In order to study ET receptor binding characteristics, confluent normal and systemic sclerosis fibroblasts were incubated (in triplicate) at a fixed concentration (approximate K_D value, 150 pM) of specific ligand, and binding kinetics were determined as above. Experiments using radiolabeled ET-1 and receptor selective ligands characterized fibroblast endothelin receptor binding kinetics. Dissociation constant values (K_D) were determined using saturation binding studies where the radiolabeled ligands (over a 4-fold log range of concentrations) were incubated with fibroblast
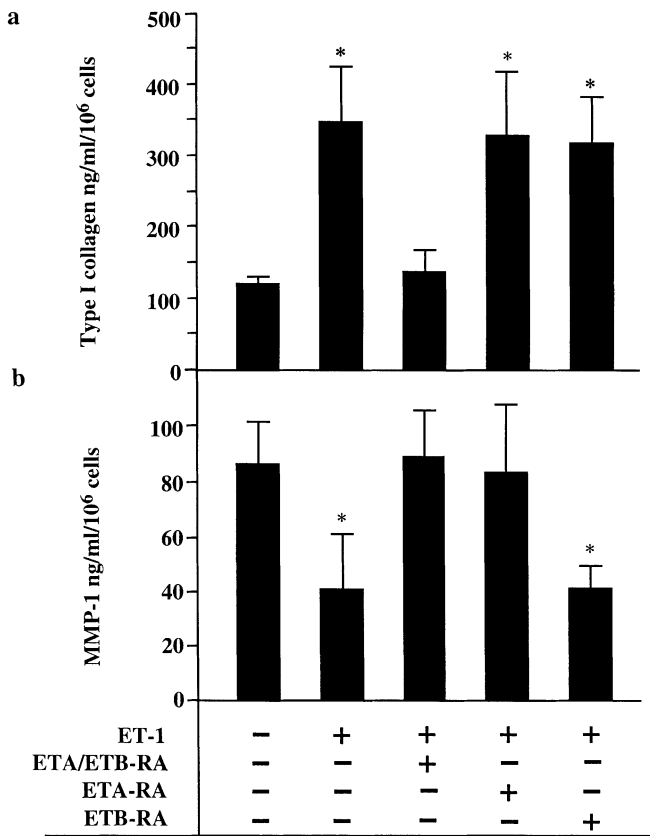
Figure 2. ET-1 induced changes in secretion of collagen depend upon both ET_A and ET_B receptor subtypes. (*A*) Collagen secretion by normal fibroblasts (*n* = 6) was increased by incubation with ET-1 (10^{-7} M) for 48 h. PD 156707 and BQ 788 (10 mM) alone did not prevent the increase in collagen type I synthesis by ET-1. Collagen induction can be effectively abolished by coincubation with the mixed $ET_{A/B}$ receptor antagonist bosentan (10 mM), however. (*B*) Incubation of fibroblasts with ET-1 resulted in a decrease in interstitial collagenase (MMP-1) production. This suppression could be abolished by coincubation with either the mixed $ET_{A/B}$ receptor antagonist bosentan or the ET_A selective receptor antagonist PD 156707. Data presented are means (± SEM) of four independent experiments. *Significant (*p < 0.05, Student's unpaired *t* test) with respect to control values.

monolayers as described above. Values for the number of endothelin receptor binding sites per cell are given as means ± SEM for five individual cell lines.

Statistical analysis All results are expressed as means ± SEM unless otherwise stated. Student's *t* test was used for statistical analyses. p-values less than 0.05 were considered statistically significant.

RESULTS

ET-1 enhances synthesis of collagen types I and III and inhibits the production of interstitial collagenase (MMP-1) Normal dermal fibroblasts treated with ET-1 showed a dose- and time-dependent increase in proα1(I) collagen chain production (**Fig 1*A***). This was apparent at 1 nM and maximal induction was at 100 nM ET-1 (data not shown). Elevated collagen type I production above basal levels was observed from 8 h post-treatment and was maximal at 72 h. ET-1 also significantly enhanced fibroblast production of collagen type III. The induction of type III collagen was dose dependent (data not shown), again reaching maximal levels after 48 h of culture (**Fig 1*B***). Treatment of normal fibroblasts with ET-1 had no significant influence on the production or secretion of fibronectin (**Fig 1*B***). In contrast, there was suppression of MMP-1 secretion,

determined by Western blot analysis, in response to ET-1 (**Fig 1*B***). Blocking studies showed that the mixed selective ligand bosentan (ET_A and ET_B receptor antagonist) blocked ET-1 induced collagen type I synthesis (p < 0.01). Neither an ET_A selective receptor antagonist (PD156707) nor an ET_B receptor antagonist (BQ-788) alone blocked induction of collagen secretion, however, even at concentrations that saturate subtype-specific binding, suggesting that signaling via both receptor types was necessary for this effect (**Fig 2*A***). Similar results were obtained for type III collagen (data not shown). In contrast to the inductive effects of ET-1 on collagen type I and III synthesis, incubation with ET-1 resulted in a significant decrease in MMP-1 expression by normal fibroblasts (p < 0.01) (**Fig 2*B***). The suppression of interstitial collagenase by ET-1 was not influenced by the presence of ET_B receptor selective antagonists, but could be prevented by the addition of either the mixed (ET_A and ET_B) or ET_A receptor antagonist (**Fig 2*B***). Examination of MMP-1 activity using zymography (data not shown) also confirmed that the influence of ET-1 on MMP-1 was mediated by the ET_A receptor.

ET-1 modulates extracellular matrix gene transcription and expression To determine whether changes in extracellular matrix protein levels were due to altered gene expression, collagen α1(I), α1(III), collagenase (MMP-1), and fibronectin genes were studied in fibroblasts grown in monolayers in the presence of ET-1. The expression profiles of these genes in normal fibroblasts are compared with those of lesional systemic sclerosis fibroblasts in **Fig 3(*A*)**. The latter exhibit increased steady-state levels of transcripts of proα1(I), proα1(III) collagen, and fibronectin, but substantially reduced levels of MMP-1 mRNA (**Fig 3*A***). Normal fibroblasts treated with ET-1 showed a significant upregulation of proα1(I) and proα1(III) collagen mRNA but downregulation of collagenase (MMP-1). Consistent with the protein data, there was little effect on fibronectin mRNA level (**Fig 3*B***). Incubation of normal fibroblasts with ET-1 in the presence of bosentan blocked the inductive influence of ET-1 on proα1(I) and proα1(III) collagen mRNA (**Fig 3*B***). Inhibition of MMP-1 mRNA level by ET-1 could be blocked by an ET_A receptor selective antagonist alone (**Fig 3*B***), but both ET_A and ET_B receptor blockade was necessary to prevent collagen I or III mRNA induction. To explore further the ET-1 mediated changes in steady-state levels of matrix gene transcripts we undertook a series of experiments to examine changes in gene transcription. Using promoter-reporter constructions we observed that ET-1 induced the transcription of the luciferase reporter gene driven by the Col1a2 promoter, but conversely exerted a significant suppressive influence on the human MMP-1 promoter sequence (**Fig 4**). In contrast, the fibronectin promoter was found to be nonresponsive to ET-1.

ETA receptor activation promotes collagen matrix contraction by fibroblasts Within the environment of a three-dimensional collagen lattice (FPCL) fibroblasts interact with the collagen fibers and contract the collagen framework into a compact tissue-like matrix (Grinnell, 1994) (**Fig 5*A*, *B***). Addition of ET-1 resulted in a pronounced dose-dependent increase in FPCL contraction over a 24 h period (**Fig 5*C***), with maximum contraction elicited by ≥ 10 nM ET-1. Under these conditions contraction was first apparent at 4 h, and was maximal by about 48 h of culture (**Fig 5*D***). Comparison of FPCL contraction rates (ET-1 treated or untreated) showed that, whereas the final extent of contraction was similar, ET-1 enhanced the contraction at earlier time points, to an extent that was apparent after 4 h and became significant at 12 h (p = 0.04) (**Fig 5*D***). This time point was later used to investigate receptor activation and downstream signaling pathways involved in promotion of collagen lattice contraction. Using subtype-specific antagonists the promotion of lattice retraction was shown to be mediated via ET_A receptors as an ET_A antagonist, or a combined ET_A/ET_B receptor antagonist, but not ET_B receptor blockade, prevented ET-1 effects although it did

Figure 3. ET-1 induces matrix mRNA expression in control fibroblasts. (*A*) Total cellular RNA from normal or scleroderma fibroblast strains (*n* = 2) were analyzed by northern hybridization. Transcripts for proα1(I) collagen, proα1(III) collagen, fibronectin, MMP-1, and GAPDH were localized using radiolabeled cDNA probes. *Lanes 1*, *2*, normal fibroblasts; *lanes 3*, *4*, scleroderma fibroblasts. (*B*) RNA from confluent skin fibroblasts was analyzed by Northern hybridization as described above. *Lanes 1*, normal cells culture alone; *lanes 2*, normal cells cultured in the presence of ET-1 (100 nM for 48 h) and, *lanes 3*, in the presence of ET-1 plus endothelin receptor antagonist(s) as indicated; *lane 4*, representative transcript expression from scleroderma fibroblasts. Transcript levels were quantitated by phosphorimager analysis in comparison with the control internal GAPDH mRNA.
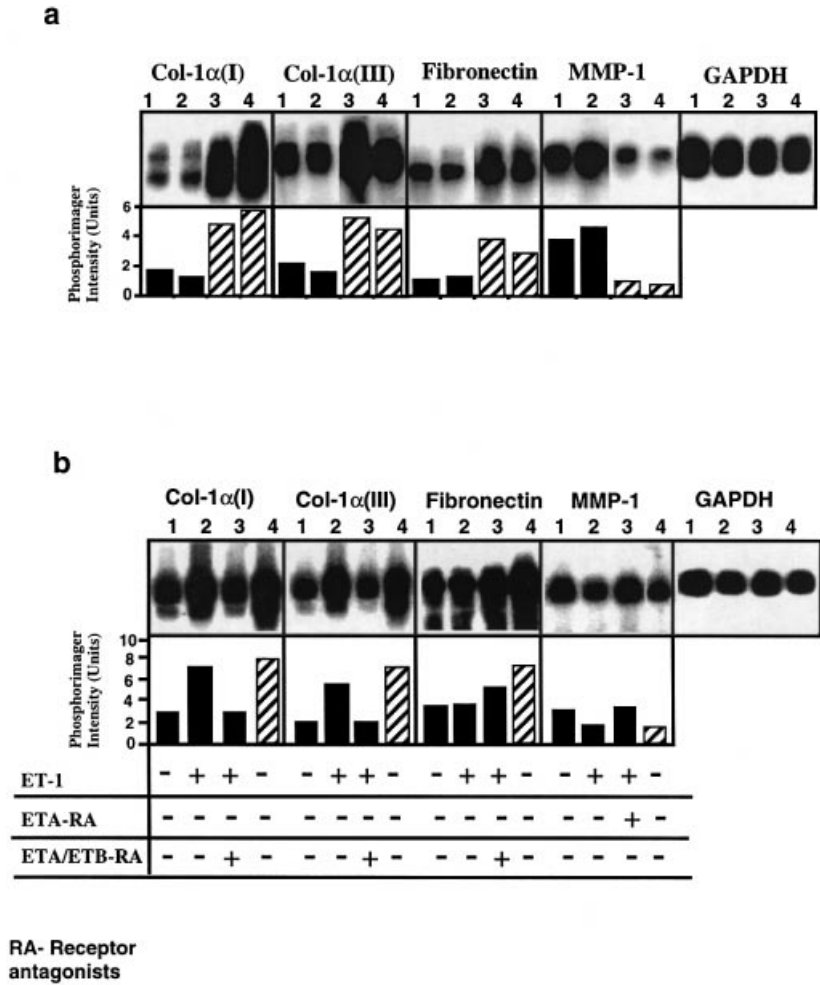
Figure 4. ET-1 modulates transcriptional activity of matrix gene promoters. Fibroblasts were transiently transfected with the DNA reporter constructs containing the Col1a2, fibronectin, and MMP-1 human promoter sequences inserted upstream of a luciferase reporter gene. Results shown are for three individual experiments and the values given are expressed as mean ± SEM (*significant at p < 0.05).
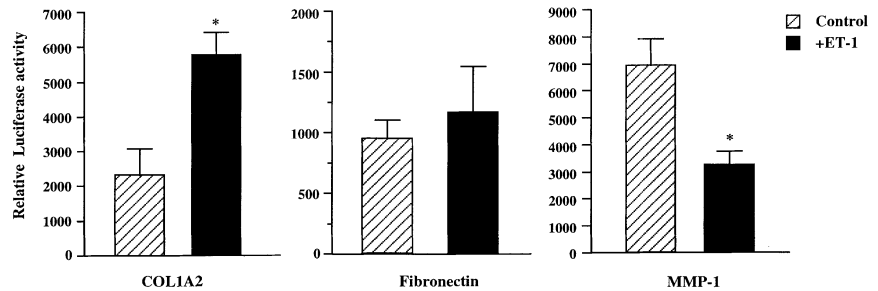
not influence basal contraction (**Fig 6*A***). Comparison of collagen gel matrix contraction by normal and scleroderma fibroblasts confirmed our previous findings that over a 48 h period both fibroblast populations induced contraction of the collagen gel lattice of around 80%–90% (Shi-wen *et al*, 1997). When time course and ET-1 dose-dependent gel contraction experiments were also performed using scleroderma fibroblasts, however, differences in the kinetics of gel contraction were revealed. Normal cells were significantly more responsive than systemic sclerosis fibroblasts (**Fig 6*B***). The mixed receptor antagonist bosentan reduced ET-1 mediated FPCL contraction, although the response by systemic sclerosis cells did not reach significance.

Studies using specific inhibitors of intracellular signaling pathways suggest that gel contraction is dependent upon PKC and tyrosine kinase activity. Thus ET-1 induced (**Fig 7**) collagen lattice contraction was blocked by calphostin C, an inhibitor of PKC

signaling pathways, and by genistein, a broad spectrum kinase inhibitor, although not by the more selective tyrosine kinase inhibitor herbimycin A, or by an inhibitor of intracellular phosphatases (vanadate) (**Fig 7**). This suggests that the pathways stimulated by ET-1 that control extracellular matrix contraction and remodeling involve both PKC signaling and specific tyrosine kinases.

Altered endothelin receptor expression by systemic sclerosis fibroblasts   Normal and systemic sclerosis fibroblasts expressed both ET_A and ET_B receptor mRNA transcripts (**Fig 8*A***). S1 nuclease analysis demonstrated a significant reduction in expression of the ET_A receptor subtype mRNA in systemic sclerosis fibroblasts compared with control cells (**Fig 8*B***) but no difference between ET_B receptor mRNA levels. Ligand binding experiments allowed endothelin receptor K_D values to be determined and the number of
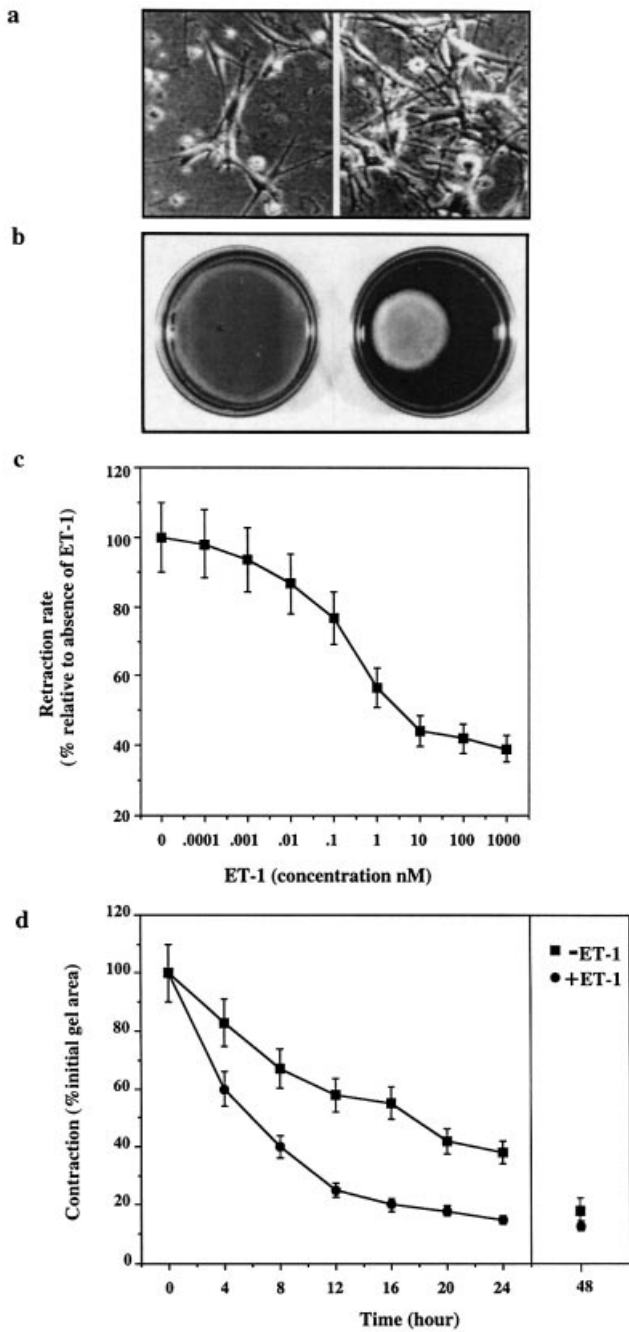
Figure 5. ET-1 stimulates collagen matrix remodeling by fibroblasts. (*A*) Morphology of fibroblasts in relaxed three-dimensional collagen gel pre (*left panel*) and post (*right panel*) gel contraction. (*B*) Appearance of relaxed three-dimensional collagen gel pre (*left panel*) and post (*right panel*) gel contraction. (*C*) The effect of different concentrations of ET-1 on the contraction of collagen gels containing normal fibroblasts (*n* = 4). Contraction of gel lattice was assessed by measurement of the maximum gel diameter. The results are presented as the retraction rate defined as the percentage of contraction observed taking the retraction in the absence of endothelin as 100%. (*D*) The influence of ET-1 (100 nM) on gel contraction by normal fibroblasts. Collagen-cell suspensions were polymerized in the absence and presence of ET-1 and the rates of subsequent gel contraction were measured over 48 h.
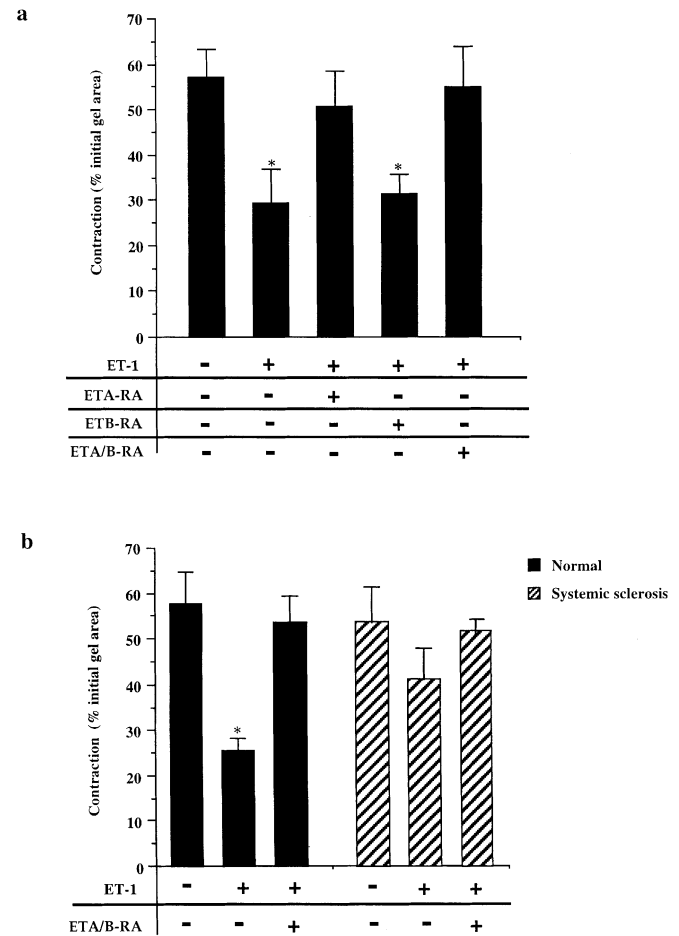

Figure 6. ET-1 induced gel remodeling is blocked by ET$_A$ subtype specific receptor antagonists. (*A*) The promotion of FPCL contraction induced by ET-1 is blocked by a specific ET$_A$ receptor antagonist or by bosentan (ET$_A$/ET$_B$ antagonist) but not by an ET$_B$ specific antagonist at concentrations that saturate ET-1 binding via these receptors. (*B*) At 12 h post gel polymerization, the extent of three-dimensional gel contraction by normal and systemic sclerosis fibroblasts was measured in the absence and presence of ET-1 and in the presence of ET-1 following pretreatment with the mixed receptor antagonist (bosentan) as indicated. *Solid bars*, normal fibroblasts; *hatched bars*, systemic sclerosis fibroblasts.

receptors, normal and systemic sclerosis fibroblast cell lines were incubated in 150 pM radioligand (the average of the K_D values obtained) for subsequent experiments in the absence or presence of unlabeled ET-1. Quantitative measurement of the endothelin receptor levels revealed that both cell types expressed similar functional levels of ET$_B$ receptors (602 ± 156 and 645 ± 232 binding sites per cell for normal and systemic sclerosis fibroblasts, respectively). In contrast the number of ET$_A$ binding sites on systemic sclerosis fibroblasts were shown to be dramatically reduced to almost half the number found on normal cells (4145 ± 126 and 2299 ± 68.9 binding sites per cell for normal and systemic sclerosis fibroblasts, respectively; $p < 0.05$). Nonetheless, ET$_A$ receptors were expressed at a level at least 10–20-fold greater than the ET$_B$ subtype.

DISCUSSION

Previous experimental studies suggest that ET-1 stimulates fibroblast collagen synthesis (Kahaleh, 1991; Guarda *et al*, 1993) and promotes collagen gel matrix contraction (Guidry and Hook, 1991), an *in vitro* model for extracellular matrix remodeling. More recent data from animal experiments confirm the fibrogenic effect of ET-1 *in vivo*. Thus, ET-1 overexpression under the control of an
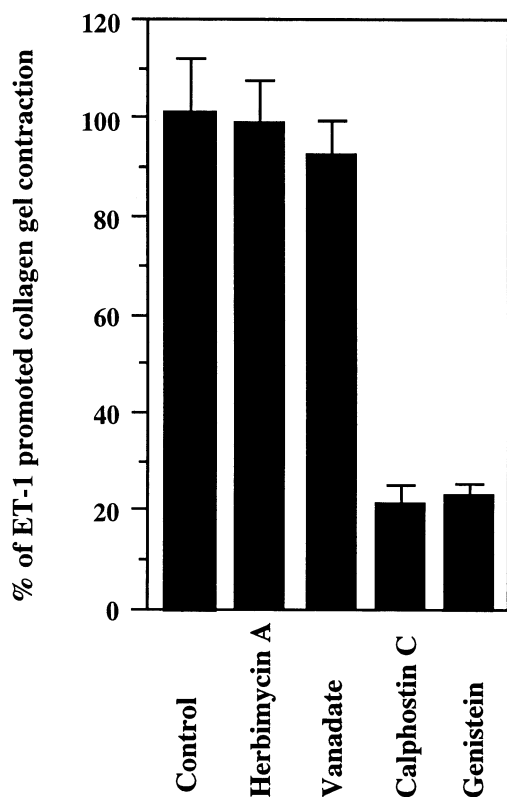
binding sites per cell calculated for normal fibroblasts. Dissociation constant values (K_d) as determined using saturation binding studies with radiolabeled ligands as described in *Materials and Methods* were found to be 149 ± 23 pM and 168 ± 38 pM for the ET$_A$ and ET$_B$ receptors, respectively. In order to compare the levels of endothelin

Figure 7. Inhibitors of intracellular signaling pathways block ET-1 induced gel remodeling. The extent of ET-1 induced contraction (100 nM) in the presence of each inhibitor was compared for a 12 h time point from lattice seeding as described in *Methods*. Calphostin C and genistein blocked the promotion of fibroblast-mediated gel contraction by ET-1.

Figure 8. ET$_A$ receptor mRNA expression and binding sites are reduced in systemic sclerosis fibroblasts. (*A*) Agarose gel showing products of PCR amplification of ET$_A$ (299 bp), ET$_B$ (428 bp), and GAPDH (605 bp) cDNA from reverse transcribed mRNA derived form three separate fibroblast cultures. *Lanes 1–3*, normal fibroblast strains; *lanes 4–6*, systemic sclerosis fibroblast strains. (*B*) S1 analysis was used for quantitative examination of endothelin receptor subtype expression. S1 probes were hybridized to total fibroblast RNA, and following digestion the protected fragments corresponding to ET$_A$ and ET$_B$ receptors and actin transcripts were separated on 8% denaturing polyacrylamide gels and visualized by autoradiography. *Lane 1*, ssDNA standards OX174 HaeIII digest; *lanes 2, 3*, normal fibroblasts; *lanes 4, 5*, scleroderma fibroblasts; *lanes 2, 4*, ET$_B$ probe; *lanes 3, 5*, ET$_A$ probe. (*C*) ET$_A$ receptor mRNA expression in scleroderma fibroblasts was determined by phosphorimager and normalized to β-actin.

endogenous promoter induces interstitial fibrosis of internal organs, which develops despite only slight increases in plasma and tissue ET-1 levels (Hocher *et al*, 1997). Moreover, experimentally induced models of both hepatic and pulmonary fibrosis are characterized by elevated ET-1 expression at early stages of the disease (between days 3 and 7 postinjury), prior to an increase in collagen deposition (Mutsaers *et al*, 1998; Rocky *et al*, 1998). In addition, liposome-mediated ET-1 gene transfer into rat lungs results in an obliterative fibrotic disease characterized by appearance of hyperplastic connective tissue plaques (Takeda *et al*, 1997).

Our data show that exogenous ET-1 induces types I and III collagen synthesis, in a dose- and time-dependent manner. In contrast to its effect on collagen biosynthesis, ET-1 was found to decrease the level of interstitial collagenase (MMP-1) produced by fibroblasts. As extracellular degradation of native type I collagen requires initial cleavage by MMP-1, the influence of ET-1 on interstitial collagenase expression could have substantial effects on matrix turnover. Interestingly, this inhibition appears to be dependent only upon ET$_A$ receptor activation, in contrast to the effect on collagen biosynthesis, which could only be prevented by blocking both ET$_A$ and ET$_B$ receptor subtypes. Transient transfection assays using promoter reporter constructions suggested that the influence of ET-1 on the level of collagen type I, and MMP-1, was primarily at the transcriptional level. The ability of ET-1 to activate the Col1a2 promoter yet suppress transcription from the human MMP-1 suggests a specificity of ET-1 action that may be dependent on distinct signaling pathways evoked through binding to the specific receptors. Although we were able to demonstrate increased fibronectin production by systemic sclerosis fibroblasts, ET-1 induced no change in fibronectin expression in normal cells. This contrasts with the report by Marini *et al* (1996),
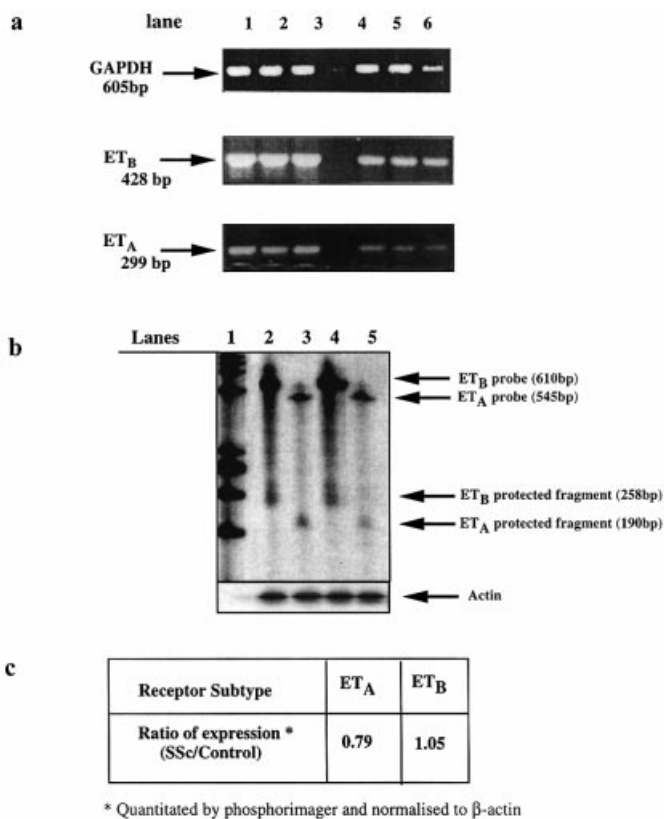
showing an induction of fibronectin synthesis in bronchial epithelial cells following ET-1 stimulation, mediated via an ET$_A$ receptor type. The discrepancy may reflect different endothelin receptor expression profiles, or that distinct responses to ET-1 are evoked in different cell types via the same receptors.

FPCL represent a more physiologic environment than standard monolayer culture, and interactions between fibroblasts and the extracellular collagen modulate properties of fibroblasts within these lattices. For example, when cultured in FPCL, fibroblasts show altered collagen and interstitial collagenase expression, and initiate gel lattice contraction (Guidry and Hook, 1991). In many respects these processes are thought to be analogous to those that take place *in vivo* during wound healing and tissue regeneration (Langholz *et al*, 1995). Contraction of gels is dependent upon engagement of collagen-binding integrins (Grinnell, 1994), and involves re-organization of the cytoskeleton and redistribution of these cell surface receptors. ET-1 accelerated the rate of gel contraction in a dose-dependent manner. Studies performed in the presence of specific endothelin receptor antagonists confirmed that this promotion was via activation of ET$_A$ receptors. Although ET-1 signaling has previously been shown to involve G-protein

linked activation of phospholipase C, the role of PKC in ET-1 induced gel contraction suggested by our data is consistent with previous reports that basal contraction is PKC dependent (Langholz *et al*, 1995). From these studies it appears that fibroblast–collagen interaction via cell surface integrin receptors leads to activation of PKC, either by direct effects on integrin expression (Racine-Samson *et al*, 1997) or by convergence of downstream signaling pathways. Future experiments should elucidate this interplay between independently triggered signaling events, and whether these pathways are perturbed in fibrotic cells.

We have demonstrated that both ET_A and ET_B receptor subtypes are functionally expressed and their mRNA transcripts are present in human fibroblasts. Downregulation of ET_A receptor expression on scleroderma fibroblasts appears to underlie reduced responsiveness to ET-1 by these cells. This is consistent with earlier studies showing that exogenous ET-1 provokes a smaller increase in DNA synthesis in scleroderma than in normal fibroblasts (Kikuchi *et al*, 1995). Although these authors observed reduced ET_A receptor protein on scleroderma fibroblasts, using Western blot analysis, our data considerably extend their studies. We have demonstrated expression of functional ET_A and ET_B receptors on fibroblasts in tissue culture by ligand binding assays, although there is around a 20-fold excess of ET_A receptors. These expression data are confirmed by analysis of mRNA levels for receptor transcripts, and detection of ET_B receptor transcript contrasts with previous reports (Kikuchi *et al*, 1995). Although total ET_B expression does not appear to be different between control and scleroderma fibroblasts, the relative ET_A:ET_B ratio will be perturbed by virtue of the lower ET_A levels. Thus, some of the differences in effects mediated via both receptor subtypes may arise through this relative difference rather than the absolute reduction in ET_A expression. Our confirmation of reduced ET_A receptor expression on scleroderma fibroblasts is noteworthy in view of previous data derived directly from tissue sections that demonstrated increased ET-1 binding sites in lesional skin or lung biopsies. This suggests that cells other than fibroblasts, such as vascular or inflammatory species, may be responsible for increased ligand binding. The basis for reduced ET_A receptor expression on scleroderma fibroblasts is not known, but potential mechanisms include cell selection resulting in the expansion of fibroblast populations with reduced ET_A receptor expression; ligand-induced downregulation of receptor expression as a consequence of elevated ET-1 *in vivo*; or a reduction in receptor expression provoked by the presence of elevated levels of cytokines and growth factors within a fibrotic environment (Smith *et al*, 1998).

In conclusion, our study demonstrates that ET-1 stimulates fibroblast collagen (types I and III) production, downregulates MMP-1, and promotes contraction of FPCL. From our studies on mRNA expression and promoter reporter constructs it appears that ET-1 can act to both enhance and suppress transcription. In the context of fibrosis, this dichotomy may have a significant impact, on the one hand promoting matrix production and on the other inhibiting matrix turnover, the net result being enhanced matrix deposition and accumulation. Modulation of MMP-1 and collagen gel remodeling were mediated exclusively via ET_A receptors whereas promotion of collagen gene expression appears to require both receptor subtypes. In view of the abnormal endothelin receptor profiles in systemic fibroblasts, these findings suggest that ET-1 signaling is altered in scleroderma and that healthy fibroblasts treated with ET-1 develop a fibrogenic phenotype *in vitro*. Overall, our data support the hypothesis that ET-1, possibly derived from activated or damaged endothelial cells, may be important not only in the control of vascular tone and abnormal blood vessel function but also as an activator of fibroblasts in this systemic sclerosis, and perhaps a potential target for therapeutic modulation.

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