Cardiac myofibroblasts isolated from the site of myocardial infarction express endothelin de novo

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Abstract

Recently it was demonstrated that treatment with a nonselective endothelin (ET) receptor antagonist significantly reduces myocardial infarct size, which suggests a major role for ET in tissue repair following myocardial infarction (MI). Tissue repair and remodeling found at the site of MI are mainly attributed to myofibroblasts (myoFbs), which are phenotypically transformed fibroblasts that express α-smooth muscle actin. It is unclear whether myoFbs generate ET peptides and consequentially regulate pathophysiological functions de novo through expression of the ET-1 precursor (prepro-ET-1), ET-converting enzyme-1 (ECE-1), a metalloprotease that is required to convert Big ET-1 to ET-1 and ET receptors. To address these intriguing questions, we used cultured myoFbs isolated from 4-wk-old MI scar tissue. In cultured cells, we found: *1*) expression of mRNA for ET precursor gene (*ppET1*), ECE-1, and ET_A and ET_B receptors by semiquantitative RT-PCR; *2*) phosphoramidon-sensitive ECE-1 activity, which converts Big ET-1 to biologically active peptide ET-1; *3*) expression of ET_A and ET_B receptors; *4*) elaboration of Big ET-1 and ET-1 peptides in myoFb culture media; and *5*) upregulation of type I collagen gene expression and synthesis by ET, which was blocked by bosentan (a nonselective ET_A- and ET_B receptor blocker). These studies clearly indicated that myoFbs express and generate ET-1 and receptor-mediated modulation of type I collagen expression by ET-1. Locally generated ET-1 may contribute to tissue repair of the infarcted heart in an autocrine/paracrine manner.

Keywords

converting enzyme; receptors; type I collagen; bosentan; *ppET1* gene

Tissue remodeling is a common response to various insults or injuries; it can be the outcome of any perturbation in the cellular function of any tissue and is characterized by excessive expression and accumulation of type I collagen (α, RI). Often following myocardial infarction (MI), the remodeling process (extracellular matrix deposition) stands as an obstacle to normal physiological functioning of the heart. Studies have identified a deleterious role for endothelin (ET) after MI (7, 8, 16, 22). In a clinical study that consisted of 142 cardiac patients, plasma ET levels 3 days post-MI were strongly correlated to survival probability, with high ET levels being associated with an unfavorable prognosis (23). Additional correlations between ET and negative survival probability after MI were observed in rats with left coronary artery ligation, where blockade of ET_A receptors with BQ-123 significantly increased the survival rate of animals (29).

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In vivo and in vitro studies in several organ systems and cell cultures using ET receptor antagonists strongly indicate a correlation between endogenous ET and fibrous tissue formation. In the liver, studies demonstrated that antagonism of ET after the establishment of fibrosing injury reduced hepatic stellate cell activation and matrix production, which suggests a role for ET in the development of fibrogenesis (26). Addition of ET-1 to cultured cells led to increased collagen synthesis of adult rat cardiac fibroblasts (10) and neonatal bone organ cultures (41).

It has been shown that treatment with bosentan, an ET_A and ET_B receptor blocker, attenuated deoxycorticosterone acetate-induced cardiac fibrosis in rats (11). Furthermore, treatment with TAK-044, a nonselective ET_A and ET_B receptor antagonist, significantly reduced rat heart infarct size, which suggests a role for ET in tissue repair following MI (16).

Although several studies support the concept that administration of ET antagonists mitigates ET-mediated adverse remodeling (27, 30), some reports demonstrate that antagonizing ET action may aggravate remodeling after MI (7). However, the time of treatment with these receptor antagonists and the choice of antagonist used have been shown to play important roles in alleviating the ET-induced adverse remodeling (19, 22, 29, 39). In rats, subsequent to MI, ET was elevated in plasma and heart interstitial fluid (3, 8). To determine areas of ET-1 production in the infarcted heart, Oie et al. (21) examined infarcted regions of rat heart with immunohistochemistry and found heavy immunostaining for ET-1 in the granulation tissue at the site of infarction. Fibroblasts and endothelial cells also displayed marked immunoreactivity, and interestingly, the time course of the immunoreactivity correlated with wound healing and was consistent with ET production by the wound-healing cells that produce the granulation tissue (21).

Myofibroblasts (myoFbs) are the predominant wound-healing fibroblast-like cells and have morphological features of both smooth muscle cells and fibroblasts. They express extracellular matrix and contractile proteins and play major roles in matrix remodeling and wound contraction in diverse tissues (9, 15, 37). The contribution of myoFbs to tissue repair in the injured heart is of considerable interest. Recently it was demonstrated that ET along with vascular endothelial growth factor (VEGF) is a major player in angiogenic response and gastric ulcer healing processes (1). Our previous and recent studies on cardiac myoFbs demonstrated that these cells express ANG II (12), transforming growth factor (TGF)-β_1 (4), and VEGF (5), which play major roles in tissue regeneration and angiogenesis (4, 5, 12, 15). However, it is unclear whether these cells also produce ET de novo. To study the local production of ET at the site of MI during tissue regeneration and/or remodeling, we isolated, cultured, and examined myoFbs from the site of MI for various components involved in de novo generation of ET. These components are composed of the ET precursor (prepro-ET-1) gene *ppET1*, Big ET-1, ET-converting enzyme-1 (ECE-1, a metalloprotease that converts Big ET-1 to ET-1), and stereospecific ET_A and ET_B receptors. Furthermore, we also investigated the influence of ET on type I collagen expression in this study.

Materials and Methods

Materials

Protease inhibitors (leupeptin, aprotinin, pepstatin A, and PMSF) were purchased from Sigma Chemical (St. Louis, MO). Phosphoramidon, bestatin, and actinonin were obtained from Peptide International (Louisville, KY). The ^{125}I-labeled ET-1 was purchased from DuPont-NEN (Boston, MA). Rat ET-1 and Big ET-1 were purchased from Peninsula Laboratories (Belmont, CA). Bosentan was a generous gift from Roche Pharmaceuticals. Enzymes and other reagents required for RT-PCR were obtained from Promega Biotech

(Madison, WI). The β_2-microglobulin (β_2-MG) oligonucleotide primers were purchased from ClonTech (Palo Alto, CA), and other oligonucleotide primers were synthesized in the DNA Core Facility at the University of Missouri Health Sciences Center (Columbia, MO). Rat-specific anti-ET_A and anti-ET_B receptor antibodies were obtained from Alomone Laboratories (Jerusalem, Israel). All other chemicals used in this study were of reagent grade.

Isolation and Culture of MyoFbs

Left ventricular MI was created in 8-wk-old male Sprague-Dawley rats (250–300 g body wt) via ligation of the left coronary artery. Rats were euthanized 28 days following ligation, hearts were removed, and scar tissue from the infarcted area was isolated for harvesting the myoFbs. Rat MI models were created using a protocol approved by the Institutional Animal Care and Use Committee. Previous in vivo studies confirmed the presence of an abundant population of myoFbs at the site of infarction at 28 days post-MI (37). The border region was removed by microdissection to avoid possible contamination of blood vessels and cardiac myocytes from this region. Recovered MI scar tissue was washed four times with 5 ml of saline. Microdissected scar tissue was sliced into smaller pieces and placed in a six-well plate that contained 20% FCS in DMEM with amphotericin-B (2 μg/ml), gentamycin (20.5 μg/ml), and penicillin-streptomycin (20 U/ml and 20 μg/ml, respectively). After 6 h, additional medium with 10% FCS was added. Cells were washed after 24 h with DMEM that contained 10% FCS, and medium was subsequently replaced every 2–3 days. Cells were grown and maintained under the conditions previously described (4, 12). After 2 wk, cells reached confluence and were routinely split at a 1:3 ratio. Early passages of cells (*passages 3* and *4*) were frozen in 10% DMSO, stored in liquid nitrogen, and used as needed.

Morphological and phenotypical characterization of cultured myoFbs were studied by immunohistochemical and confocal microscopy as previously reported (4). Briefly, myoFbs were found to be positive to α-smooth muscle actin, vimentin, and desmin and negative to anti-factor VIII. Vimentin staining is specific for fibroblast-like cells and excludes the presence of vascular smooth muscle cells from our preparations. Absence of a positive factor VIII result indicated that these cells were nonendothelial. Therefore, cultured myoFbs were neither endothelial nor vascular smooth muscle cells. We observed little or no contamination of these cells in culture, and these cells were stable up to 12 passages. We used cells from early passages for these studies to avoid possible phenotypic instability.

RT-PCR Analysis

We used previously established semiquantitative RT-PCR assays (5, 12) for prepro-ET-1, ECE-1, and ET_A and ET_B receptor expression. To monitor cDNA synthesis efficiency, β_2-MG was used as an internal control.

RNA extraction and cDNA synthesis—As reported previously (4, 12), total RNA was extracted from myoFbs using the guanidine thiocyanate/phenol method. Briefly, confluent myoFbs were washed with PBS, and total cellular RNA was extracted with RNAzol B solution (Biotecx; Houston, TX), which was followed by chloroform extraction and precipitation with isopropanol. RNA pellets were washed with 75% ethanol, air dried, and dissolved in distilled water. cDNA was synthesized by reverse transcription of cellular RNA as previously described (5).

Oligonucleotide primer design—PCR primers for amplifying rat prepro-ET-1 (31), ECE-1 (43), and ET_A (18) and ET_B receptor cDNA were selected from the rat gene sequence as previously reported (32). The β_2-MG cDNA was PCR amplified using primers obtained from ClonTech. The specificity of these primers was confirmed by purifying the

respective PCR products (QiaQuick gel extraction system, Qiagen; Chats-worth, CA) and sequencing them. The sequences of the oligonucleotide primers used and the expected PCR product sizes are summarized in Table 1.

Polymerase chain reaction—With the use of primer-specific sequences of β_2-MG, prepro-ET-1, ECE-1, and ET_A and ET_B receptor genes were PCR amplified as described previously (5, 12). Briefly, 25-µl PCR reaction volumes that contained 20 mmol/l Tris·HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.01% (wt/vol) gelatin, 200 µmol/l of each dNTP, 40 pmol of the appropriate oligonucleotide primers, 1 unit of *Taq* DNA polymerase, and 5–10 µl of the diluted myoFb cDNAs were amplified in a GeneAmp model 9600 thermocycler (Perkin-Elmer; Norwalk, CT) using the following conditions: after an initial denaturation at 94°C for 1 min, β_2-MG sequences were PCR amplified using 25 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 1 min, and primer extension at 72°C for 2 min with the denaturation time increasing an additional 1 s/cycle. A final extension at 72°C for 5 min was performed to ensure that all reactions were completed. Pre-pro-ET-1, ECE-1, and ET_A and ET_B receptor sequences were PCR amplified using a similar procedure except that the number of cycles was increased to 30 and the annealing temperature was increased to 58°C. PCR products were elec-trophoretically size fractionated on 1.5% agarose gels that contained ethidium bromide and were visualized with UV light.

Separation and Determination of ET-1 in MyoFb Culture Media

Serum-free culture media (each dish contained 5–10 ml media/≈2 million cells) from four separate experiments was utilized in triplicate, and the medium was collected in the presence of protease inhibitor cocktail (final concentrations were 1 µM each of pepstatin, aprotinin, leupeptin, bestatin, actonin, and phosphoramidon, and 0.1 mM PMSF), separated on C-2 columns, and concentrated before performance of ELISA for ET-1 or Big ET-1 (Amersham; Arlington Heights, IL).

Preconditioned solid-phase C-2 extraction columns were equilibrated with Tris·HCl buffer (0.1 mol/l, pH 7.3), and the samples (5–10 ml/sample) were drawn through the cartridge by vacuum. ET peptides were eluted with 0.1% trifluoroacetic acid in acetonitrile, evaporated to dryness in a Speed Vac concentrator equipped with a cold-finger trap, dissolved in ELISA buffer, and then stored at −20°C until further analysis by ELISA. ET peptide extraction efficiency in myoFb culture media ranged from 70 to 83%, which suggests a possible 13–30% underestimation of these peptides.

Protein Analysis

MyoFb lysate protein content was determined using a modified Lowry method (Bio-Rad protein assay kit, Bio-Rad Laboratories; Hercules, CA).

Collagen ELISA

Collagen ELISAs were performed using previously reported methods (24, 25) with minor alterations. Briefly, 96-well Costar EIA/RIA (enzyme immunoassay/radioimmunoassay) plates were coated with 100 µl of 100 mM carbonate-bicarbonate buffer. Collagen standards (type I collagen, Sigma Chemical) and 100-µl media samples were added directly to the buffer in triplicate, and plates were incubated at 4°C overnight. Wells were washed three times with PBS that contained 0.05% Tween 20 (PBS-Tween 20) buffer. Nonspecific binding sites were blocked by 1 h of incubation with 250 µl/well of 1% BSA in Tris-buffered saline (TBS, 100 mM Tris·HCl with 150 mM NaCl). After the wells were washed with PBS three times, 100 µl of primary antibody (1:1,000 dilution, rabbit anti-rat type I collagen polyclonal antibody, Bio Design International; Kennebunk, ME) in TBS was added and wells were incubated for 1 h at room temperature. Plates were washed five times before

addition of 100 µl of secondary antibody (1:20,000 dilution, goat anti-rabbit monoclonal antibody with alkaline phosphatase conjugate, Sigma Chemical) in TBS and incubated at room temperature for 1 h. Wells were then washed five times with PBS-Tween 20 and twice with distilled water before 100 µl (1 mg/ml) of the color-development agent *p*-nitrophenyl phosphate in 1.0 M diethanolamine buffer was added. Optical density readings at 405 nm were taken at half-hour intervals with the Vmax microplate reader (Molecular Devices; Sunnyvale, CA).

Conversion of Exogenous Big ET-1 to ET-1 by MyoFbs

Confluent myoFbs (100-mm dishes in triplicate; ≈2 million cells/dish) were washed three times (3×10 ml/dish) with serum-free media and then incubated in serum-free media (5 ml/ dish) with and without Big ET-1 (10 nM) in the absence and presence of the ECE-1 inhibitor phosphoramidon (0.1 µM) for 20 h. Media were collected, processed as above, and analyzed for ET-1 contents.

Western Immunoblotting for ET receptors

Confluent myoFbs were washed with PBS, and cell lysates were prepared (12). Aliquots with 50 µg of protein were electrophoretically fractionated on 10% SDS-PAGE minigels and were electroblotted onto a nitrocellulose membrane. The membrane was sequentially incubated in TBS (pH 7.4) that contained 5% dry milk for 30 min and diluted (1:200) rat anti-ET_A and anti-ET_B receptor primary antibody (Alomone Laboratories) in 5% Blotto (Bio-Rad nonfat dry milk powder in TBS) for 12 h on a shaker at 4°C. The blot was washed with TBS that contained 0.1% Tween 20 and was incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma Chemical) in 5% Blotto for 1 h at room temperature, washed with TBS, and incubated with the peroxidase substrate. Immunoreactive bands were visualized within 10 min using an ECL detection kit (Amersham Pharmacia Biotech; Piscataway, NJ).

Statistical Analysis

Results are reported as means ± SE for a minimum of five determinations, each of which was performed either in duplicate or triplicate. Statistical analysis was performed using one-way ANOVA with P 0.05 considered significant.

Results

Expression of Prepro-ET-1 and ECE-1 by MyoFbs

Expression of the prepro-ET-1 gene (*ppET1*, the only known precursor gene that encodes Big ET-1) and ECE-1 were examined in myoFbs by semiquantitative RT-PCR technique. RT-PCR studies resulted in PCR products of predicted sizes for *ppET1* (334 bp) and ECE-1 (529 bp) (Fig. 1). These results demonstrated that myoFbs express *ppET1* and the key enzyme ECE-1 that converts Big ET-1 to the active peptide ET-1. The PCR products for ET-1 and ECE-1 were of predicted sizes, and their identities were confirmed by cycle sequencing.

Determination of Big ET-1 and ET-1 Contents in MyoFb Culture Media

The elaboration of Big ET-1 and the production of ET-1 in the media of serum-deprived myoFbs were studied in a time-dependent manner. The results indicated that myoFbs generate both Big ET-1 and ET-1 (Figs. 2 and 3). The time-dependent pattern of Big ET-1 elaboration (0.5, 2, 4, and 8 h) indicated maximum levels of Big ET-1 in myoFb culture media at 2 and 4 h. In another set of experiments with an 8-h incubation in the presence of phosphoramidon (a specific inhibitor for ECE-1), Big ET-1 levels were increased two- to

threefold (Fig. 2, fifth bar), which suggests that the gradual drop seen in Big ET-1 concentration at 4 and 8 h is due to its conversion to ET-1 by ECE-1 (Fig. 2, third and fourth bars). The production of ET-1 was time dependent (6, 12, 24, and 36 h) with the highest levels of ET-1 found at 24 h. ET-1 concentrations after 24 h in the presence of bosentan (10^{-6} M) were highly elevated (90 pg/ml) compared with 24 h without bosentan (Fig. 3, fifth bar), because a significant portion of ET-1 loss due to its binding to cellular ET receptors was prevented by bosentan.

Protease Involved in Conversion of Big ET-1 to ET-1 and Effects of Exogenous Big ET-1 on MyoFb ET-1 Production

ECE-1 is a membrane-bound, phosphoramidon-sensitive, zinc metalloprotease that converts Big ET-1 to ET-1. The presence of ECE-1 was detected in the myoFb cell preparation, which converted exogenous Big ET-1 to ET-1 and was blocked by 100 μM phosphoramidon (Fig. 4).

The ability of confluent myoFbs to generate ET-1 was significantly (P 0.05) increased in the presence of exogenous Big ET-1 (Fig. 4, second bar) and was significantly reduced in the presence of the ECE-1 inhibitor phosphoramidon (Fig. 4, third bar), which suggests possible involvement of an active metalloprotease ECE-1 in generating ET-1 de novo.

Expression of ET$_A$ and ET$_B$ Receptors by RT-PCR

Using a standard semiquantitative RT-PCR method (5, 12, 14), we examined the expression of both ET$_A$ and ET$_B$ receptors in myoFbs. The results clearly demonstrated the amplification of a single predicted band of PCR product for ET$_A$ (787 bp) and ET$_B$ (919 bp) receptors, respectively (Fig. 5), which suggests that myoFbs express both ET$_A$ and ET$_B$ receptors.

Western Immunoblotting for ET$_A$ and ET$_B$ Receptors

Cultured myoFb and coronary endothelial cell (as positive control) pellets were used to detect the presence of ET receptors by Western blot analysis using ET$_A$ and ET$_B$ receptor-specific antibodies. Results obtained from Western immunoblotting revealed two major bands of ~60- and 39-kDa protein for the ET$_A$ receptor and a single band of ~40 kDa for the ET$_B$ receptor (Fig. 6). These protein bands were within the predicted range as specified by the commercial supplier of these antibodies. These results confirm that cultured myoFbs express both ET$_A$ and ET$_B$ receptors.

ET-1-Receptor Binding Studies

The presence of ET receptors was also confirmed by ET-1-receptor binding assays using ^{125}I-labeled ET-1 as the radioligand (13). ET-1 receptor densities as determined at the saturation of binding for fibroblasts and myoFbs were 31.80 ± 1.03 and 35.89 ± 1.93 fmol/mg of protein, respectively, which suggests that these were the active target cells for the autocoid influence of ETs on the induction of type I collagen gene expression.

Type I Collagen mRNA Induction by ET-1

The influence of ET on type I collagen mRNA expression was evaluated by Northern blot analysis, and the results indicated an induction of type I collagen mRNA by ET at the concentrations studied (10^{-7} to 10^{-11} M; Fig. 7). As shown in Fig. 7, the upregulation of type I collagen was effectively blocked by the ET$_A$ and ET$_B$ receptor antagonist bosentan (10^{-6} M), which suggests that induction of α_1-type I collagen mRNA is an ET receptor-mediated mechanism.

Type I Collagen Synthesis by ELISA

The influence of ET-1 on type I collagen protein synthesis was evaluated by ELISA, and the results indicated a dose-dependent increase in type I collagen synthesis induced by ET-1 (Fig. 8). The upregulation of type I collagen synthesis by ET-1 was significantly higher (P 0.05 at 10^{-10} to 10^{-8} M), which was attenuated with bosentan treatment (Fig. 8, seventh bar; 10^{-9} M ET-1 plus 10^{-6} M bosentan). These results clearly indicate that induction of type I collagen expression by ET-1 was at both transcriptional (mRNA; see Fig. 7) and translational (protein; see Fig. 8) levels.

Discussion

Several in vivo studies using ET-receptor antagonists implicated a role for ET in modulating extracellular matrix turnover, tissue repair, and remodeling (7, 8, 11, 16, 19, 21, 22, 27, 30, 39). ET-1 production in the failing heart as examined immunohistochemically revealed heavy ET-1 immunostaining that was confined to granulation tissue and proliferating fibroblasts (21). In many organs including heart, tissue repair in response to injury involves myoFbs, a phenotypically transformed cell type that is commonly referred to as a wound-healing cell. Studies from our laboratory and others have shown that myoFbs are the predominant wound-healing cells that appear at various sites of injury and express ANG II and are responsible for collagen turnover and tissue repair (6, 9, 12, 15, 34–37). These studies have consistently shown the presence of these cells at the site of injury and regeneration and also at sites of high collagen turnover such as MI (6, 12), skin-pouch tissue regeneration (15), and heart-valve leaflets (as valvular interstitial cells, a normal site of high collagen turnover; Ref. 14), where they have demonstrated generation of ANG II and regulation of collagen turnover. MyoFbs have morphological features of both smooth muscle cells and fibroblasts (4, 9). In our study, we isolated myoFbs from rat MI scar tissue and verified that these cells express ET peptides. RT-PCR experiments demonstrated that myoFbs express mRNA for the ET precursor prepro-ET-1. Furthermore, we demonstrated the presence of both Big ET-1 and ET-1 in the culture media by ELISA. The findings of Oie et al. (21) support our results that myoFbs isolated from the site of infarction generate ET-1 and could serve as a local source of ET at the site of MI.

Our concurrent objective of this investigation was to demonstrate the expression of the key enzyme ECE-1 by myoFbs. The data showed the presence of mRNA for ECE-1 in myoFbs, and addition of exogenous Big ET-1 to myoFb culture media significantly increased ET-1 levels. This suggests the presence of ECE-1 activity, which was significantly reduced in the presence of the ECE-1 inhibitor phosphoramidon. Expression of phosphoramidon-sensitive ECE-1 by myoFbs strengthens the fact that these cells have all the necessary elements to produce biologically active ET-1 from ET precursors.

Earlier reports have shown that ET-1 is mitogenic and that it stimulates fibroblast proliferation (40). Media obtained from bovine aortic endothelial cells induced collagen accumulation and protein formation by human fibroblasts, which suggests that an endothelial cell-derived factor can stimulate collagen production by fibroblasts (42). We have previously demonstrated a positive influence of ET-1 on cardiac fibroblasts (10) and vascular smooth muscle cell (25) collagen expression. To determine whether myoFbs, a cell type that is isolated from infarcted rat heart, is responsive to ET-1, we investigated the presence of specific receptors for ET-1 for an autocrine influence. Through RT-PCR and Western blot analyses, we found the presence of both ET_A- and ET_B-receptor subtypes and also demonstrated a significant ^{125}I-labeled ET binding to myoFb cell membranes. In our studies, we have demonstrated that myoFbs isolated from the site of infarction not only express ANG II (12) and transforming growth factor (TGF)-β_1 (4), but also express $ppET1$, ECE-1, and ET_A and ET_B receptors thus implicating de novo expression of ET by myoFbs

and suggesting a possible role for locally generated ET-1 in tissue regeneration and remodeling at the site of MI.

As stated earlier, when Oie et al. (21) examined infarcted regions of rat heart with immunohistochemistry, they found heavy immunostaining for ET-1 in the granulation tissue at the site of infarction, which indicated that wound-healing myoFbs found at this site may be responsible for producing ET. Apart from the vascular endothelial cells, the possibility of myocytes, smooth muscle cells, and fibroblasts expressing ET cannot be ruled out, but these are not the classical wound-healing cells that are found at the site of tissue repair or remodeling. However, these cells can be the target cells for the paracrine action of ET as they express ET receptors (13, 25).

The regulation of ET-1 expression in these cells is unclear and it may be similar to the autoregulation that is reported for endothelial cells (28), or it could be influenced by TGF-β_1 and/or other cytokines. Understanding the regulatory aspects of ET generation by myoFbs at the site of infarction is important and has become the subject of our ongoing study. A study by Boffa et al. (2) on renal vasculature showed ET as a mediator of fibrogenic action of ANG II. The fibrotic response induced by ANG II was mitigated by bosentan, a nonselective ET-receptor antagonist. Similarly, it was shown that TGF-β_1 is known to upregulate ET-1 expression and production in several cell types (17, 20, 33, 40). Studies by Sun et al. demonstrated that ANG II plays a vital role in collagen turnover and remodeling in several tissue-injury models including myocardium (36, 37). Our earlier study (15) on tissue regeneration in response to injury in a rat-pouch tissue model showed the production of ANG II, which regulates TGF-β_1. Previous studies from our group conclusively showed that myoFbs produce ANG II, TGF-β_1, and VEGF (4, 5, 12), and the present study confirms the de novo production of ET and its receptors. From these studies, it is evident that myoFbs play a unique role in tissue repair and regeneration by expressing ANG II, ET, VEGF, and TGF-β_1, which modulate collagen turnover in response to injury in different organs.

Collectively, results from the present study demonstrate the generation of Big ET-1 and ET-1 de novo by cultured myoFbs isolated from infarct scar tissue and the influence of ET on type I collagen expression. Such locally generated ET-1, ANG II, TGF-β_1, and VEGF could influence collagen turnover and tissue repair and remodeling at and remote to the site of injury in an autocrine/paracrine manner as well as in cell-cell and cell-matrix contractions, which promote matrix remodeling and scar thinning.

Acknowledgments

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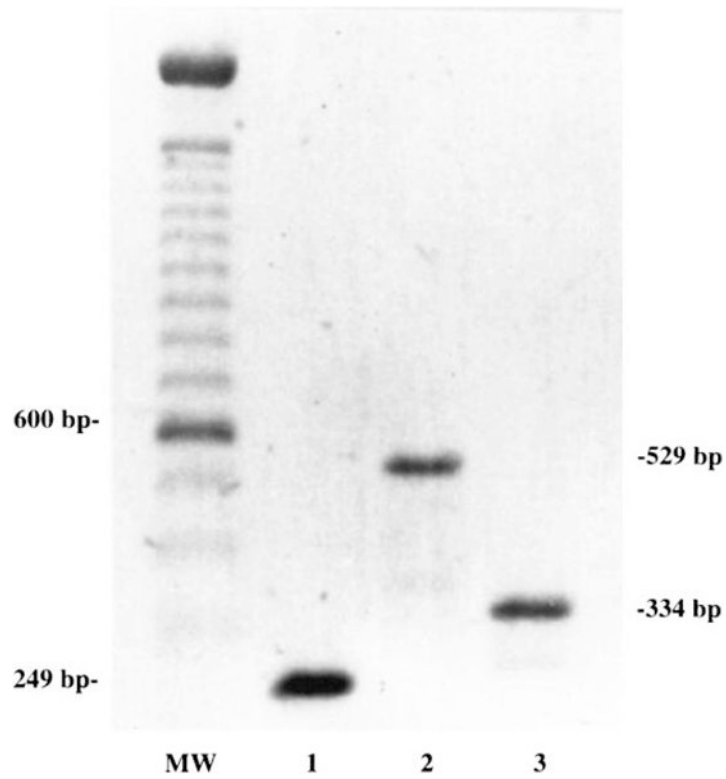
Fig. 1.
Myofibroblasts (myoFbs) express endothelin (ET) precursor gene *ppET1* and ET-converting enzyme-1 (ECE-1); RT-PCR results demonstrate expression of transcripts for *ppET1* and ECE-1 genes. *Lane 1*, internal control β_2-microglobulin; *lane 2*, ECE-1; *lane 3*, *ppET1*. MW, mol wt marker.
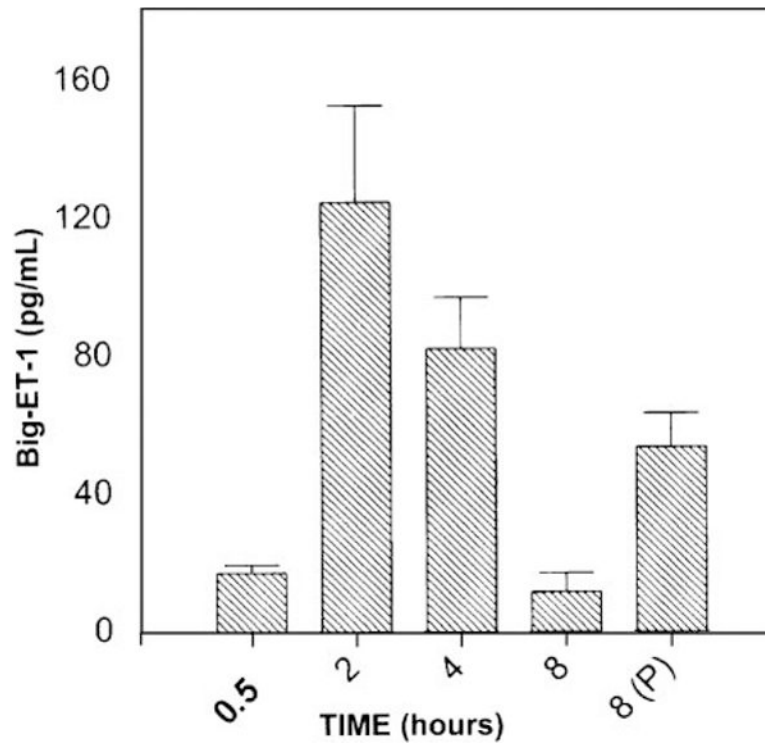
Fig. 2.
Time-dependent elaboration of Big ET-1 by myoFbs. Big ET-1 levels in serum-free culture media were analyzed by ELISA. Big ET-1 levels were elevated at 8 h in the presence of 0.1 mM phosphoramidon (P), which is an ECE-1-specific inhibitor (fifth bar). Values are means ± SE.
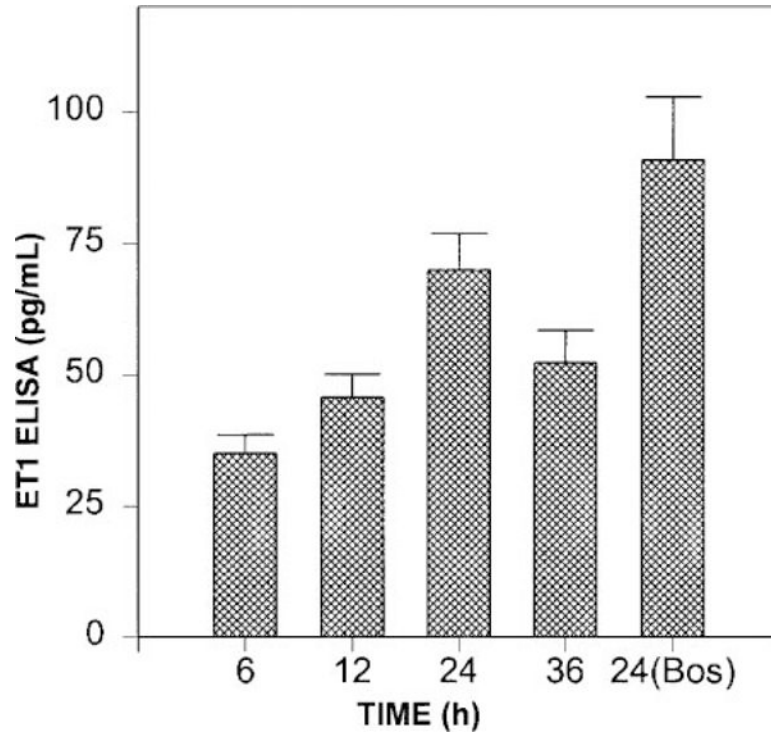
Fig. 3.
Time-dependent production of ET-1 by myoFbs. ET-1 levels in serum-free culture media determined at different time points by ELISA are shown. Fifth bar represents 24-h incubation values in the presence of 10^{-6} M bosentan (Bos), a nonselective ET_A and ET_B receptor agonist. Values are means ± SE.
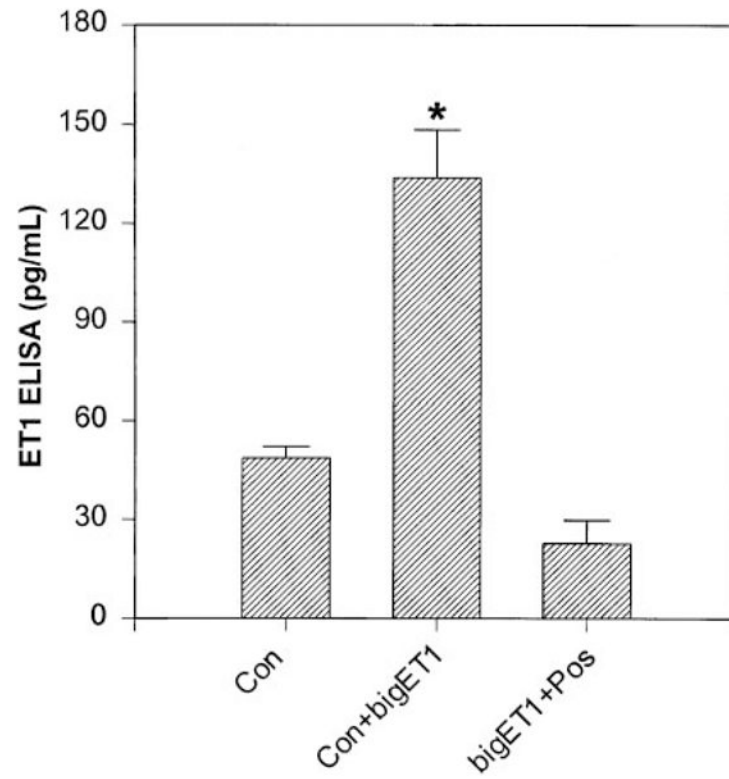
Fig. 4.
ECE-1 in myoFbs. ET-1 levels in 24-h serum-deprived media were detected after 20-h pretreatment with 10 μM Big ET-1 (*middle*) and Big ET-1 with 0.1 M phosphoramidon (*right*). Values are means ± SE. *P < 0.05.
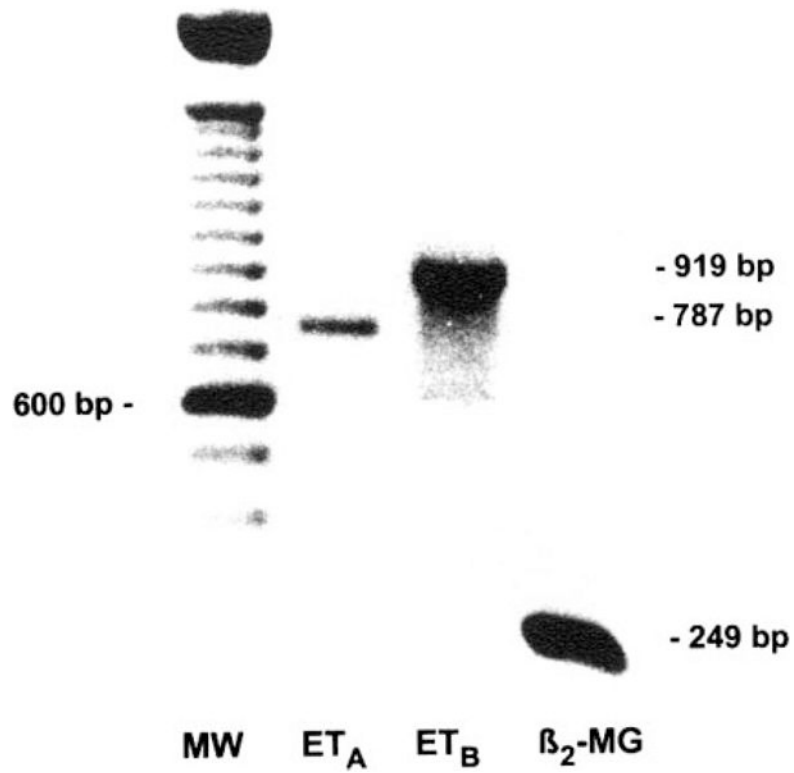
Fig. 5.
RT-PCR results demonstrate the expression of both ET_A and ET_B receptor genes in myoFbs. *Lane 1*, MW (100-bp ladder); *lane 2*, ET_A receptors (787 bp); *lane 3*, ET_B receptors (919 bp); *lane 4*, internal control, β_2-microglobulin (β_2-MG, 249 bp).
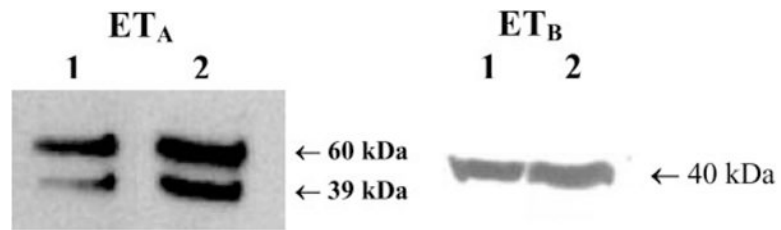
Fig. 6.
Identification of ET_A and ET_B receptor protein by Western blot using ET_A (*left*) and ET_B (*right*) receptor-specific antibodies. *Lanes 1* and *2* represent myoFbs and coronary endothelial cells, respectively. ET_A immunoblot with anti-ET_A-specific antibody resulted in two bands of ≈60- and 39-kDa proteins, whereas immunoblot with anti-ET_B receptor antibody identified a protein band of ≈40 kDa. Preimmune serum served as a control (not shown).
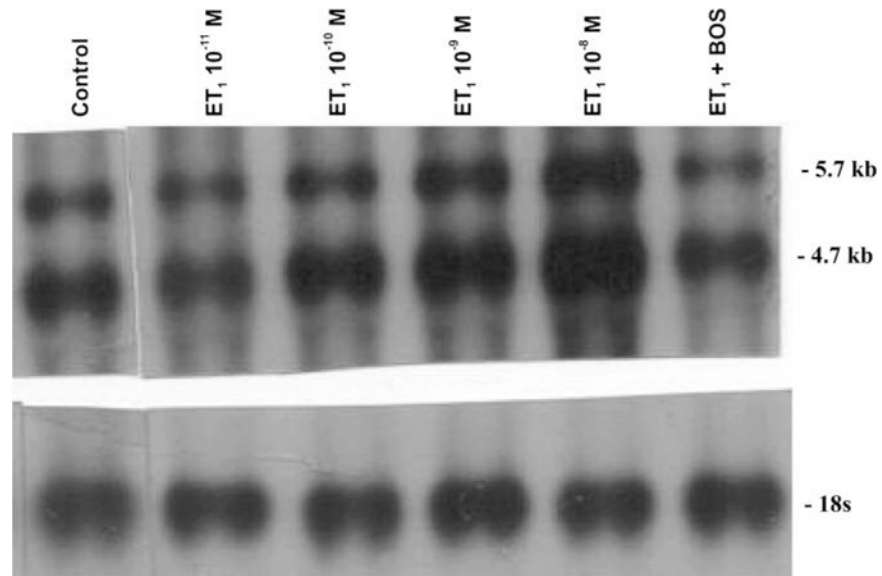
Fig. 7.
Type I collagen mRNA analysis by Northern blot. ET-1 has a concentration-dependent effect on type I collagen gene expression. ET induction of myoFb type I collagen mRNA was determined by Northern hybridization (band sizes: *top*, 5.7 kb; *bottom*, 4.7 kb). *Lane 1*, untreated control. *Lanes 2–5*, treated with ET-1 (10^{-11} to 10^{-8} M). *Lane 6*, treated with ET-1 (10^{-9} M) and bosentan (10^{-6} M).
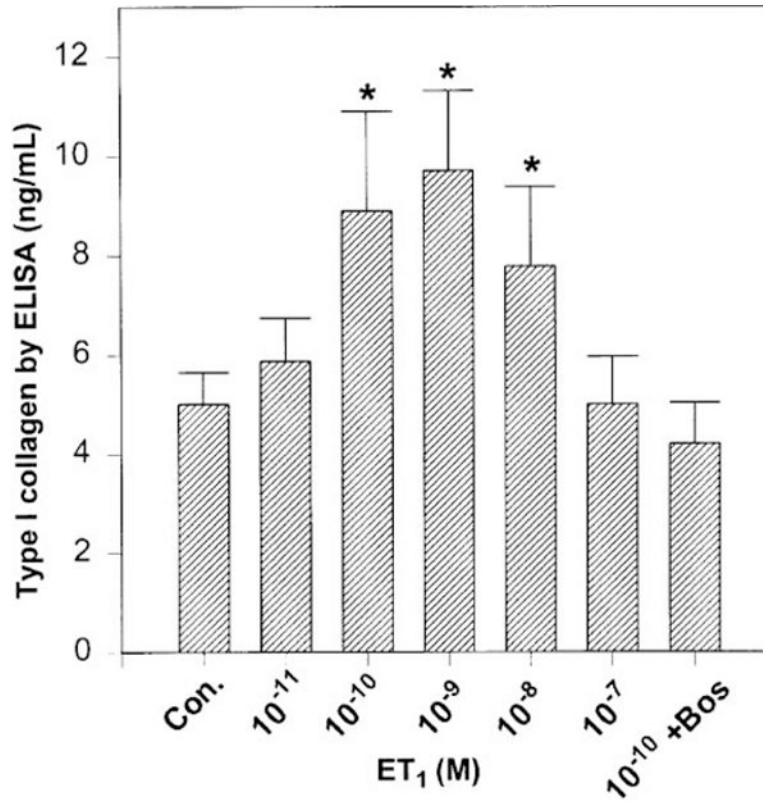
Fig. 8.
Quantitation of soluble type I collagen by ELISA. A concentration-dependent effect of ET-1 on myoFb type I collagen protein expression was determined by ELISA using rat type I collagen-specific antibody. First bar, untreated control; second through sixth bars, treated with ET-1 (10^{-11} to 10^{-7} M); seventh bar, treated with ET-1 (10^{-9} M) plus bosentan (10^{-6} M). Values are means ± SE. *$P < 0.05$.

Table 1

Oligonucleotide primers for PCR amplification

Gene	GenBank Accession No.	Primer Name	Primer Sequence 5′ → 3′	Product Size, bp
$ppET1$	M64711	ET-1-5′	GAAGTGTATCTATCAGCAGC	334
		ET-1-3′	GGAACACCTCAACCTCTCTTGG	
$ECE-1$	U29196	ECE-5′	CGTAGCGATAGTCTTAGCAC	529
		ECE-3′	GTGCCACACCAAAACTACAG	
ET_A	M60786	ET_A-5′	CGAGGTCATGAGGCTTTTGG	787
		ET_A-3′	GTGTTTAAGCTGTTGGCGGG	
ET_B	AF074963	ET_B-5′	TGCACACCTTTCCGCAAGCACG	919
		ET_B-3′	AGCTGGTGCCCTTCATACAGAAGGC	
$\beta_2\text{-}MG$	Y00441	β_2-MG-5′	CTCCCCAAATTCAAGTGTACTCTCG	249
		β_2-MG-3′	GAGTGACGTGTTTAACTCTGCAAGC	

β_2-MG, β_2-microglobulin (used as internal control); ET, endothelin.