Growth Inhibitory Properties of Endothelin-1 in Human Hepatic Myofibroblastic Ito Cells

An Endothelin B Receptor-mediated Pathway

Ariane Mallat,* Laura Fouassier,* Anne-Marie Préaux,* Claudine Serradeil-Le Gal,‡ Danièle Raufaste,‡ Jean Rosenbaum,* Daniel Dhumeaux,* Catherine Jouneaux,* Philippe Mavier,* and Sophie Lotersztajn*

**Unité INSERM 99, Hôpital Henri Mondor, 94010 Créteil, France; and ‡SANOFI Recherche, 31036 Toulouse, France*

Abstract

Ito cells play a pivotal role in the development of liver fibrosis associated with chronic liver diseases. During this process, Ito cells acquire myofibroblastic features, proliferate, and synthesize fibrosis components. Considering the reported mitogenic properties of endothelin-1 (ET-1), we investigated its effects on the proliferation of human Ito cells in their myofibroblastic phenotype. Both ET receptor A (ETA: 20%) and ET receptor B (ETB: 80%) binding sites were identified, using a selective ETA antagonist, BQ 123, and a selective ETB agonist, sarafotoxin S6C (SRTX-C). ET-1 did not stimulate proliferation of myofibroblastic Ito cells. In contrast, ET-1 inhibited by 60% DNA synthesis and proliferation of cells stimulated with either human serum or platelet-derived growth factor -BB (PDGF-BB). PD 142893, a nonselective ETA/ETB antagonist totally blunted this effect. SRTX-C was as potent as ET-1, while BQ 123 did not affect ET-1–induced growth inhibition. Analysis of the intermediate steps leading to growth-inhibition by ET-1 revealed that activation of mitogen-activated protein kinase by serum or PDGF-BB was decreased by 50% in the presence of SRTX-C. In serum-stimulated cells, SRTX-C reduced c-*jun* mRNA expression by 50% whereas c-*fos* or krox 24 mRNA expression were not affected.

We conclude that ET-1 binding to ETB receptors causes a potent growth inhibition of human myofibroblastic Ito cells, which suggests that this peptide could play a key role in the negative control of liver fibrogenesis. Our results also point out that, in addition to its well known promitogenic effects, ET-1 may also exert negative control of growth on specific cells. (*J. Clin. Invest.* 1995. 96:42–49.) Key words: liver • fat storing cells • fibrosis • proliferation • mitogen-activated protein kinase • c-*fos* • c-*jun* • krox 24.

Introduction

Hepatic Ito cells (lipocytes, fat-storing cells, or perisinusoidal cells) are currently recognized as a key element in the development of liver fibrosis (1). In normal liver, these cells exhibit a quiescent phenotype and are mainly involved in the storage and metabolism of retinoids (2). During chronic liver diseases, Ito cells undergo transdifferentiation to a myofibroblastic phenotype, characterized by the disappearance of vitamin A droplets, an hypertrophic rough endoplasmic reticulum and the expression of specific markers, such as smooth muscle α-actin (3). Experimental models and studies of cirrhotic human liver have clearly shown that during chronic liver injury, myofibroblastic Ito cells proliferate, accumulate, and secrete components of fibrosis, namely collagens (such as type I, III, IV, and VI), glycoproteins (such as laminin, fibronectin, tenascin, undulin, etc.) and several proteoglycans (1). Parallel studies using cultured cells have demonstrated that proliferation of myofibroblastic Ito cells is controlled by various cytokines and growth factors (1, 4).

Endothelins (ET)[1] are a family of three potent vasoconstrictor peptides which share a high homology with toxins from snake venom, sarafotoxins (5, 6). ET-1 was originally isolated from the culture supernatant of porcine aortic endothelial cells (7). Two other forms of endothelins have subsequently been characterized, ET-2 and ET-3, which differ from ET-1 by two and six amino acids, respectively (5, 6). Pharmacological studies have demonstrated that endothelin peptides bind to three G protein–coupled receptors, which have been cloned. The ETA receptor binds ET-1 with a higher affinity than ET-3, the ETB receptor displays similar high affinity for both peptides (8) and the ETC receptor exhibits a higher affinity for ET-3 than ET-1 (9). These receptors, originally identified in vascular tissues, have also been found in many non vascular tissues, including heart, kidney, brain, and liver (8). In keeping with the wide distribution of endothelin receptors, endothelins elicit a wide variety of biological effects, including positive inotropism and chronotropism, inhibition of sodium reabsorption, neuroendocrine functions, etc. (6, 8). Importantly, ET-1 demonstrates a growth promoting activity towards various cell types such as fibroblasts, mesangial cells, endothelial cells, melanocytes, etc. (10).

Several recent reports have stressed that the liver is a major target of endothelins. It has been shown that at least two populations of liver cells, Ito cells, and sinusoidal endothelial cells secrete ET-1 (11, 12). Endothelin peptides have been demonstrated to induce cholestasis (13), to increase portal pressure (14), and have been involved in the pathogenesis of ischemia-reperfusion liver injury (15). We have identified a high number

Address correspondence to Dr. Ariane Mallat, Unité INSERM 99, Hôpital Henri Mondor, 94010 Créteil, France. Phone: 1 49 81 35 30; FAX: 1 48 98 09 08.

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1. *Abbreviations used in this paper:* ET, endothelins; MAP kinase, mitogen-activated kinase; MBP, myelin basic protein; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; p44 MAP-kinase, 44 kD isoform of MAP kinase; SRTX-C, sarafotoxin S6C.

of ET receptors in liver plasma membranes, with ETB receptors predominating over ETA (16, 17). In a recent study, assessment of the distribution of ET receptors in the different populations of normal rat liver cells indicated that only ETB receptor mRNA is detected in endothelial and Kupffer cells, while both ETA and ETB receptor mRNAs are present in Ito cells, with the ETB subtype predominating over ETA (11). In addition, ETB receptors also predominate over ETA receptors in rat hepatocytes (Lotersztajn et al., unpublished observations). Accordingly, distinct biological activities of endothelins have been ascribed to the various liver cell populations. We have reported that ET-1 causes a protracted calcium-dependent activation of glycogenolysis in hepatocytes (16, 17). In addition, endothelins stimulate the biosynthesis of prostaglandin E2 in rat Kupffer cells (18) and provoke calcium-dependent contractility of human (19) and rat myofibroblastic Ito cells (11, 20).

Considering the reported mitogenic properties of ET-1, the aim of the present study was to investigate its effect on the proliferation of human Ito cells in their myofibroblastic phenotype. We show here that, in contrast to other cell types, binding of ET-1 to ETB receptors causes potent growth inhibition of human Ito cells. Analysis of the intermediate steps leading to growth inhibition indicates that the antiproliferative effects of ET-1 are consecutive to inhibition of the MAP kinase cascade and inhibition of c-*jun* expression. These results point out the dual function of ET-1 on cell proliferation.

Methods

Materials. ET-1 and ET-3 were from Novabiochem Ltd (UK), BQ 123 from Neosystem (France), SRTX-C from Bachem (Switzerland) and PD142893 was from Alexis Corp (Switzerland). Human recombinant PDGF-BB, culture media and reagents were from GIBCO (Scotland). ^{125}I-ET-1 (2,000 Ci/mmol), [methyl-^{3}H]thymidine (25 Ci/mmol), [^{3}H]leucine (69 Ci/mmol), [α^{32}P]dATP (3,000 Ci/mmol) and [γ^{32}P]-ATP (5,000 Ci/mmol) were from Amersham Corp. (France). Fetal calf serum was from JBio Laboratories (France). Pooled human AB positive serum was supplied by the National Transfusion Center. cDNAs encoding mouse c-*fos* and c-*jun* were kindly provided by Dr. Yaniv (Institut Pasteur, France). The human krox 24 probe was a generous gift of Dr. Casellas (Sanofi Recherche, France). Bovine myelin basic protein (MBP) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (France).

Cell isolation and culture. Human Ito cells were obtained in their myofibroblastic phenotype by outgrowth from explants of normal liver obtained after surgery of benign or malignant liver tumors. This procedure is in accordance with ethical regulations imposed by French legislation. Explants were incubated, as described previously (21), in Dulbecco's modified Eagle's medium (DME) containing 10% serum (5% fetal calf serum /5% pooled human serum), thereafter referred to as DME 5%/5%. Growing Ito cells were recovered by trypsinization after 2–3 wk, and subsequently subcultured weekly at a density of 5.10^5 cells per 10-cm dish in DME 5%/5%. Myofibroblastic Ito cells obtained from explants were characterized both by electron microscopy and by immunocytochemistry as previously described (21, 22). Electron microscopy of the cells showed typical features of myofibroblast-like cells, such as abundant rough endoplasmic reticulum and large bundles of microfilaments. Immunocytochemical studies indicated that all cells were positive for smooth muscle α-actin and desmin, two markers of human Ito cells in their myofibroblastic phenotype. In addition, absence of contamination by mononuclear/macrophagic cells, endothelial cells, or epithelial cells was demonstrated by negative staining with lysozyme, von Willebrand factor, and cytokeratin, respectively (22). All experiments were performed between passage 3 and 8, without any noticeable difference in results observed with cells from various passages, or obtained from various livers.

In some experiments, we used human Ito cells isolated as described in (19), kindly provided by Dr. M. Pinzani (Universita di Firenze, Florence, Italy). Using this procedure, human Ito cells are isolated in their "lipocyte" phenotype. After the first passage, they acquire a myofibroblastic phenotype by culture on plastic (19). Cells were routinely grown as described by Pinzani et al. (19), and used between passage 2 and 4.

Rat mesangial cells, kindly provided by Dr. Dunn (Case Western Reserve University, Cleveland, OH), were cultured as described in (23) and used between passages 27 and 30.

Binding of ^{125}I-ET-1 to myofibroblastic Ito cell particulate fraction. Human myofibroblastic Ito cells were subcultured in 10-cm dishes up to confluence, and further incubated in serum-free Waymouth 705/1 medium for 24 h. Cells were washed twice with Hanks medium containing 20 mM Hepes, pH 7.4, scraped in 1 mM $NaHCO_3$ at 4°C, homogenized three times with a Polytron homogenizer for 10 s, and subsequently centrifuged for 30 min at 30,000 *g*. The pellet was resuspended in Hanks medium containing 20 mM Hepes, pH 7.4, at a final concentration of 4 mg/ml, and used for binding experiments. Storage of this fraction in liquid nitrogen was possible for up to 1 mo.

Binding of ^{125}I-ET-1 to myofibroblastic Ito cell particulate fraction was performed as previously described (16). Briefly, myofibroblastic Ito cell particulate fraction (10–20 μg/ml) was incubated for 90 min at 22°C in a 200 μl Krebs-Ringer medium containing 20 mM Hepes, pH 7.4, 1% (wt/vol) bovine serum albumin, 300 μg/ml bacitracin, with either 40 pM ^{125}I-ET-1 and varying concentrations of peptide (competition experiments), or varying concentrations of ^{125}I-ET-1 (4.5–750 pM, saturation experiments). Nonspecific binding was determined by incubating with 0.1 μM unlabeled ET-1. Data from saturation and competition experiments were analyzed using the nonlinear regression program LIGAND.

DNA synthesis and cell proliferation assays. DNA synthesis was measured in triplicate wells by incorporation of [^{3}H]thymidine. Human myofibroblastic Ito cells were subcultured in 96-well plates, grown to confluency in DME 5%/5%, and allowed to achieve quiescence over 3 d in serum-free Waymouth medium. Quiescent Ito cells were subsequently stimulated for 30 h with either 2 or 5% human serum, or 20 ng/ml PDGF-BB as indicated, in the presence of ET-1 and/or ET receptor agonists or antagonists. [^{3}H]Thymidine (0.5 μCi/well) was added during the last 22 h of incubation. Radioactivity incorporated into trichloroacetic acid-insoluble material was recovered and measured by scintillation counting. DNA synthesis in rat mesangial cells was assayed as previously described (21).

Cell proliferation was assessed in triplicate 35-mm multiwell plates. Myofibroblastic Ito cells were seeded at low density (10^5 cells/well) in DME 5%/5%, and allowed to attach overnight. Cells were made quiescent by a 3 d incubation in serum-free Waymouth medium, and stimulated with 2% human serum in the absence or presence of ET-1. The complete medium was renewed every 24 h and the incubation was stopped after 3 d. Cells were subsequently trypsinized and counted with a hemocytometer.

Assay of cellular protein synthesis. Cells were processed as described in the DNA synthesis assay, except that [^{3}H]thymidine was replaced by [^{3}H] leucine (1 μCi/well).

Mitochondrial reduction of the tetrazolium salt MTT to formazan. Cells were incubated in the same conditions as for the DNA synthesis assay, except that the isotope was omitted. Upon termination of incubation, reduction of MTT (1 mg/ml) to formazan was assessed according to Denizot et al. (24).

Stability of ET-1. Stability of ET-1 was assessed over the time course of binding experiments and thymidine incorporation assays. The binding assay medium, containing 60 pM ^{125}I-ET-1 and 10–20 μg/ml of particulate fraction was incubated under standard binding conditions at various times (0–90 min) at 22°C and filtered through 0.8-μm Millex AA (Millipore Continental Water systems, Bedford, MA). Stability of ET-1 in cell cultures was also assessed under standard thymidine incorporation

conditions. Quiescent, confluent cells were incubated for various times (0, 3, 9, and 30 h) with 100 nM ET-1 and ^{125}I-ET-1 (250,000 cpm/ml), in the presence of 2% human serum, at 37°C. At the end of the incubation period, the cell medium was centrifuged for 15 min at 12,000 rpm. In both cases, the ^{125}I-ET-1 in the resulting supernatants was analyzed by reverse-phase HPLC on a Syncropack RP-4 column (Synchrom, Inc., Linden, IN) using a 18–60% aqueous gradient of acetonitrile in 0.1% trifluoroacetic acid over 20 min at a 1 ml/min rate. Degradation of the peptide did not exceed 10% during the first 3 h of incubation, represented 50% after 9 h, and 90% after 30 h.

RNA preparation and Northern blot analysis. Confluent quiescent cells were stimulated with 5% human serum, in the absence or in the presence of sarafotoxin S6C (SRTX-C), as indicated. Total RNA was extracted in guanidium isothiocyanate, according to Chomczynski and Sacchi (25), and quantified by A_{260} spectrophotometry. RNA samples (20 μg/lane) were denatured, fractionated by electrophoresis through a 0.8% agarose/formaldehyde gel, and subsequently transferred to a Hybond N membrane (Amersham, Les Ulis). Prehybridization was performed at 42°C in 5× SSC (1× SSC = 150 mM NaCl, 15 mM Na citrate), 50% formamide, 1× Denhardt's, 50 mM NaH_2P0_4 and 250 μg/ml salmon sperm DNA. cDNA probes for mouse c-*fos* (0.5-kb NCO1 fragment), mouse c-*jun* (1-kb SmaI fragment), human glyceraldehyde-3-phosphate dehydrogenase (G3PDH, 1.3-kb EcoRI fragment), and human krox 24 (prepared from a PCR product obtained from amplification with specific primers for krox 24 [(5′ ATTGTGAGGGACATGCTCAC 3′ and 5′ ACAAAAATCGCCGCCTACTC 3′, krox 24, 246 bp)] were labeled with α-^{32}P-dATP by nick translation with a commercially available kit (Promega, Madison, WI). Blots were subsequently hybridized overnight at 42°C with the ^{32}P-labeled probes, washed once at 22°C for 15 min with 1× SSC, 0.1% SDS (*fos* and *jun* mouse probes) or 0.1× SSC, 0.1% SDS (G3PDH and krox 24 human probes), and twice in the same medium for 45 min at 52°C (*fos* and *jun*) or 68°C (G3PDH or krox 24). Blots were then exposed to x-ray film (Hyperfilm, Amersham) at −70°C. Hybridization signals were quantified at 633 nm by scanning densitometry. Results are relative to glyceraldehyde-3-phospho-dehydrogenase (G3PDH) expression, which was used as an internal standard to correct for variations in loading and transfer. Autoradiograms show representative experiments and densitometric data are the mean±SEM of three independent experiments.

In situ MAP kinase assays in MBP-containing polyacrylamide gels. Confluent quiescent cells were incubated for various periods of time (5–60 min) with the indicated effectors. The reaction was terminated by washing the cell cultures twice with 6 ml of ice-chilled phosphate-buffered saline. Cells were lysed for 30 min at 4°C in 0.3 ml of Hepes 50 mM, pH 7.4, containing 1% Triton-X 100, 10% glycerol, 137 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin and 40 mM β-glycerophosphate. Lysates were centrifuged at 4°C for 15 min at 12,000 *g*. The supernatant was either used directly, or frozen at −70°C for a maximum of 3 d without any modifications. Protein concentration was estimated according to Peterson (26).

MAP kinase activity was analyzed as described by Chao et al. (27). Briefly, cell extracts (30 μg protein) were electrophoresed on an 10% SDS polyacrylamide gel copolymerized with 0.5 mg/ml myelin basic protein (MBP). After electrophoresis, SDS was removed by washing the gel at room temperature twice with 20%-propan-2-ol in buffer A (50 mM Hepes, pH 7.4, 5 mM β-mercaptoethanol) for 45 min, then once in buffer A for 30 min. Denaturation of the proteins was achieved by two changes of buffer A containing 6 M guanidine-HCl, for 60 min at room temperature, and renaturation was performed by incubating the gel in buffer A containing 0.04% Tween 40, for 16 h at 4°C. After preincubation of the gel for 30 min at 30°C in buffer B (25 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 90 μM sodium orthovanadate and 5 mM β-mercaptoethanol), the phosphorylation assay was conducted in 5 ml of buffer B containing 50 μM ATP and 125 μCi [γ^{32}P]ATP for 1 h at 30°C. The reaction was stopped by immersing the gel in 5% trichloroacetic acid and 10 mM sodium pyrophosphate, and the gel was extensively washed with this solution until the radioactivity of the bath became negligible. The gel was subsequently dried and subjected to autoradiography. Quantitation of MBP phosphorylation by MAP kinase was performed by densitometry.

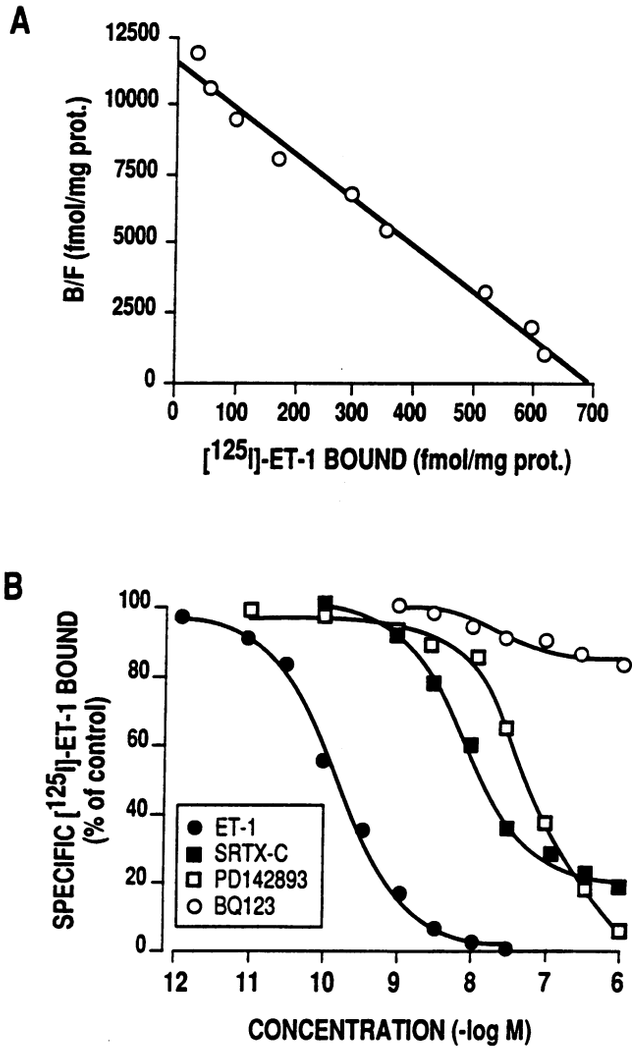

Figure 1. Characterization of ^{125}I-ET-1 binding to human myofibroblastic Ito cells. (*A*) Scatchard plot of the specific binding of ^{125}I-ET-1 to a human Ito cell homogenate. Proteins (3 μg/assay) were assayed for ^{125}I-ET-1 binding with increasing concentrations (4.5 to 750 pM) of ^{125}I-ET-1 as described in Methods. (*B*) Inhibition of specific ^{125}I-ET-1 binding to human hepatic Ito cell by endothelin agonists and antagonists. Proteins (3 μg/assay) were incubated with ^{125}I-ET-1 (40 pM) and increasing concentrations of either ET-1 (●), the ETB agonist SRTX-C (■), the ETA/ETB antagonist PD 142893 (□) or the ETA antagonist BQ-123 (○). Results show a typical experiment performed in triplicate which was repeated three times with similar results.

Results

Binding of ^{125}I-ET-1 to a human myofibroblastic Ito cell particulate fraction. Binding of ^{125}I-ET-1 to a myofibroblastic Ito cell particulate fraction was saturable and showed high affinity. Scatchard analysis revealed the presence of a single class of high affinity binding sites with an apparent kD of 53±12 pM and a maximal binding capacity (B_{max}) of 815±150 fmol/mg protein (n = 3), that is ~ 75,000 sites/cell (Fig. 1 *A*). Competi-

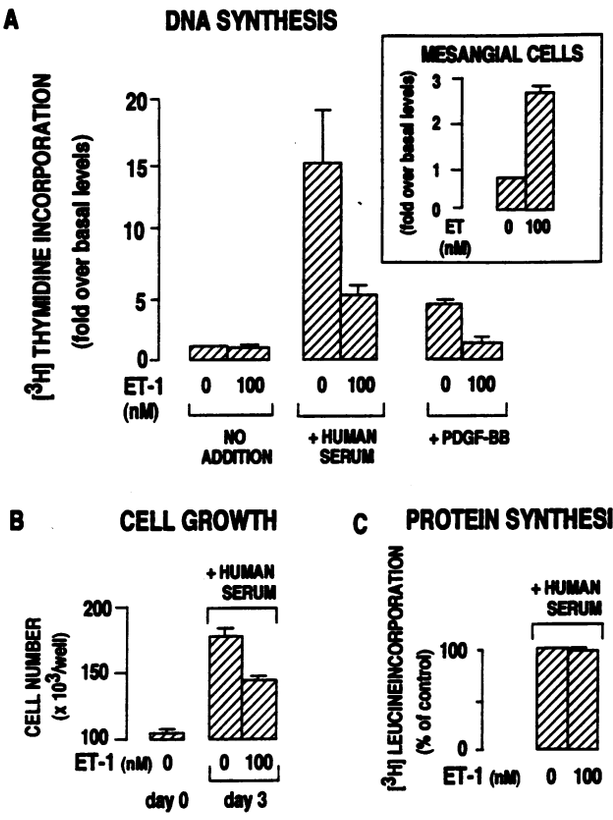

Figure 2. ET-1 inhibits proliferation of human myofibroblastic Ito cells but activates DNA synthesis in rat mesangial cells. (*A*) Effect of ET-1 on [^{3}H]thymidine incorporation into DNA in human myofibroblastic Ito cells. Quiescent cells were incubated for 30 h with endothelin-1, either alone, or together with 2% human serum or 20 ng/ml PDGF-BB. (*Inset*) Effect of ET-1 on DNA synthesis by rat mesangial cells. [^{3}H]-Thymidine incorporation was assayed as described in Methods. Results represent the mean±SEM of three experiments and are expressed relative to basal incorporation (620±104 cpm/well). (*B*) Effect of ET-1 on human Ito cell proliferation. Quiescent cells (triplicate wells) were incubated over 3 d with 2% human serum in the absence or the presence of 100 nM ET-1. Cell number was determined at day 0 and day 3. Results show a typical experiment which was repeated three times. (*C*) Effect of ET-1 on [^{3}H]leucine incorporation into proteins in the presence of 2% human serum. Quiescent cells were incubated during 30 h with 2% human serum either alone or together with 0.1 μM ET-1. Results are expressed as percent of serum-stimulated [^{3}H]leucine incorporation (13,122±828 cpm/well) and are the mean±SEM of four experiments. [^{3}H]leucine incorporation in unstimulated cells was 5,930±612 cpm/well (not shown).

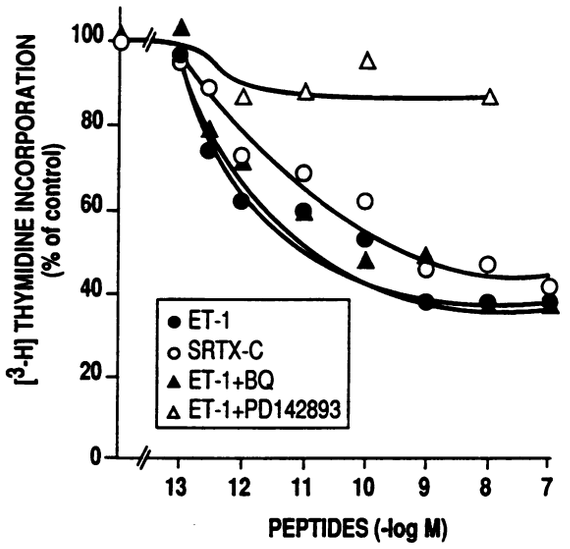

Figure 3. Antiproliferative effects of ET-1 are mediated by ETB receptors. Quiescent cells were stimulated over 30 h with 2% human serum, in the presence of varying concentrations of either ET-1 (●), the ETB agonist SRTX-C (○), ET-1 and the ETA antagonist BQ 123 (1 μM) (▲), or ET-1 and the ETA/ETB antagonist PD 142893 (5 μM) (△). BQ 123 and PD 142893 were added to quiescent cells 30 min before stimulation with human serum and ET-1. [^{3}H]Thymidine incorporation was determined as described in Methods. BQ 123 and PD 142893 alone had no effect on [^{3}H]thymidine incorporation in serum-stimulated cells. Results (mean of 3 to 5 experiments) are expressed as percent of control (22,200±7,600 cpm/well).

tion experiments of ^{125}I-ET-1 binding with agonists and antagonists of ET receptors clearly demonstrated the preponderance of ETB over ETA binding sites in our preparation. About 80% exhibited similar high affinity for ET-3 (IC50 = 0.20±0.01 nM) and ET-1 (IC50 = 0.084±0.010 nM), while the remainder (~ 20%) displayed low affinity for ET-3 (IC50 = 370±210 nM) (not shown). SRTX-C, a selective ligand of ETB receptors (28), displaced 80% of the ^{125}I-ET-1 bound, with an IC50 of 3.0±1.5 nM, whereas BQ 123, a selective ETA receptor antagonist (29) inhibited the 20% remaining ^{125}I-ET-1 binding (Fig. 1 *B*). The non selective ETA/ETB antagonist PD 142893 (30) did not discriminate between ETA and ETB receptors in our preparation and totally displaced ^{125}I-ET-1 bound in a dose-dependent manner with a 1,000-fold lower potency than the unlabeled natural ET-1 (IC50 = 58±3 nM for PD 142893). Collectively, these data indicate the presence of a large amount of ET binding sites in human myofibroblastic Ito cells, both ETA and ETB receptors being expressed in a ratio of 1:4.

ET-1 inhibits DNA synthesis and cell growth in human myofibroblastic Ito cells. Addition of ET-1 alone to quiescent myofibroblastic Ito cells did not affect [^{3}H]thymidine incorporation into DNA. Stimulation of cells with either 2 or 5% (not shown) human serum, or 20 ng/ml PDGF-BB caused a 15- and a 4-fold increase in DNA synthesis, respectively (Fig. 2 *A*), which was reduced by 60 to 70% in the presence of 100 nM ET-1 (Fig. 2 *A*). ET-1 dose-response curve in serum-stimulated cells indicated that DNA synthesis was decreased dose-dependently, a maximal 60% inhibition being observed at 1 nM with an IC50 of 22±15 pM (Fig. 3). Using myofibroblastic Ito cells obtained after passaging of vitamin-A laden lipocytes (19), a similar inhibition by ET-1 was observed in serum- or PDGF-stimulated cells (not shown). These antiproliferative properties of ET-1 in Ito cells contrast with the mitogenic effects of the peptide in rat mesangial cells (Fig. 2 *A, inset*). Growth inhibitory activity of ET-1 was also demonstrated by cell counting. Serum-stimulated cell growth was reduced by 50% in the presence of 100 nM ET-1 over 3 d, as compared with control cells (Fig. 2 *B*).

ET-1 did not modify either [^{3}H]leucine incorporation into cellular proteins (Fig. 2 *C*), or reduction of MTT to formazan (1 nM ET-1 = 107±4% of control, 100 nM ET-1 = 113±11% of control, n = 4), indicating that the peptide does not behave as a general inhibitor of cell function.

ETB receptor mediates inhibition of cell growth by ET-1.

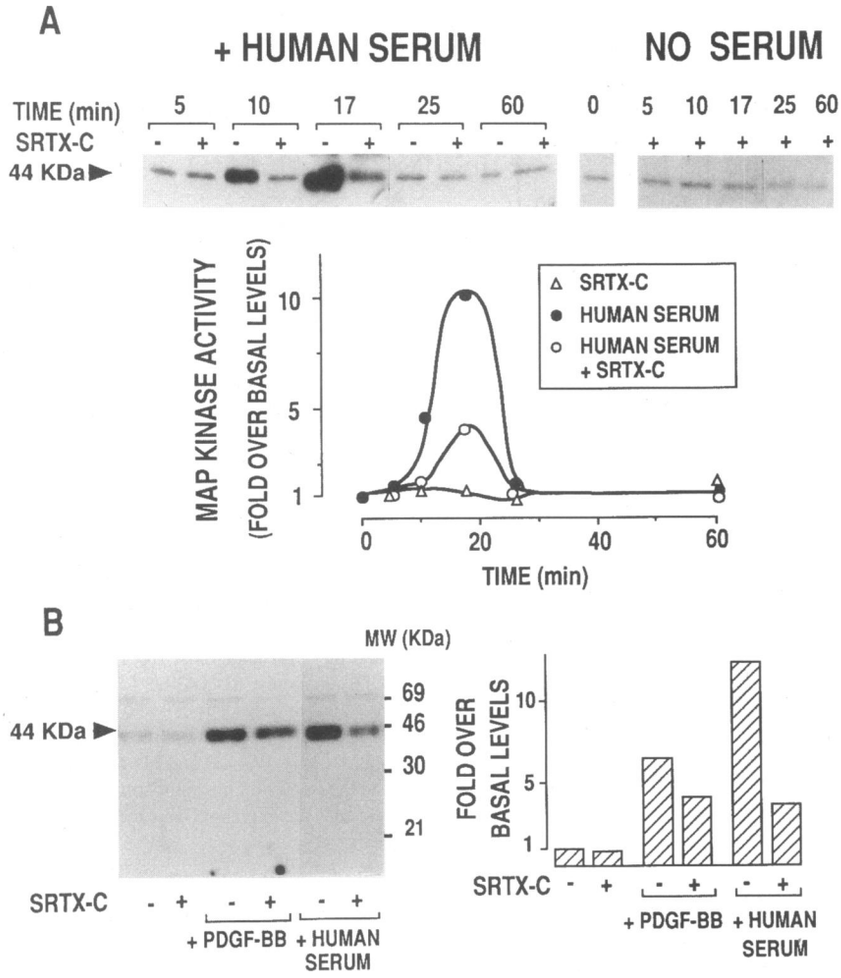

Figure 4. SRTX-C reduces activation of p44 MAP kinase by human serum or PDGF-BB. (*A*) Time course: quiescent myofibroblastic Ito cells were stimulated for the indicated times with 5% human serum alone, 5% human serum and 100 nM SRTX-C, or 100 nM SRTX-C alone. MAP kinase activity was determined by in situ phosphorylation in an SDS-polyacrylamide gel containing myelin basic protein, as described in Methods. Results show a typical experiment which was repeated three times. (*Top*) Autoradiogram of the gel showing the renaturable p44 MAP kinase. As described by others, phosphorylation of myelin basic protein by proteins of higher molecular weight was also observed (see *B*), but is not shown for clarity. (*Bottom*) Quantification of the autoradiographic p44 MAP kinase signal by scanning densitometry. (*B*) SRTX-C reduces activation of MAP kinase by PDGF-BB: Quiescent myofibroblastic Ito cells were incubated for 17 min in the absence or in the presence of PDGF BB (20 ng/ml) or 5% human serum, with or without the ETB receptor agonist, SRTX-C, in the conditions described in A. The left panel shows an autoradiogram of the gel. The right panel represents quantification of the autoradiographic p44 MAP kinase signal by scanning densitometry. Results show a typical experiment which was repeated twice.

We next investigated the nature of the endothelin receptor involved in growth inhibition elicited by ET-1 in myofibroblastic Ito cells, and we tested a series of agonists and antagonists of ETA and ETB receptor subtypes. SRTX-C, a specific ETB agonist (28) and ET-3 (not shown), were as potent as ET-1 in inhibiting DNA synthesis (IC50 = 26±15 pM for SRTX-C, 43±5 pM for ET-3 and 22±14 pM for ET-1), eliciting a 55% inhibition at 1 nM (Fig. 3). The selective ETA receptor antagonist (29) BQ 123, had no effect on ET-1–induced inhibition of DNA synthesis, while a nonselective ETA/ETB antagonist, PD 142893 (30), blunted the ET-1–induced growth inhibition (Fig. 3). Taken together, these data indicate that inhibition of cell growth by ET-1 is mediated by ETB receptors.

The ETB receptor agonist, SRTX-C, inhibits MAP kinase activity. MAP kinases, a family of Tyr/Thr-regulated kinases play a central role in the transduction of growth-factor signaling (31, 32). We studied MAP kinase activity in order to clarify the mechanism whereby ET-1 inhibits the growth of myofibroblastic Ito cells. These experiments were conducted with SRTX-C, to induce selective activation of the ETB receptor. MAP kinase activity was assayed by an in situ kinase assay, after electrophoresis in an SDS gel containing the MAP kinase substrate, myelin basic protein (MBP). Under these conditions, MAP kinases, as well as other MBP kinases (see Fig. 4 *B*), are renatured to active forms and located by the phosphorylation of the substrate. In serum-stimulated cells, the phosphorylation of MBP by the 44-kD isoform of MAP kinase (p44 MAP-kinase) was time-dependent and showed a maximal 10-fold stimulation at 17 min, followed by a rapid drop to basal levels at 25 min (Fig. 4 *A*). The ETB receptor agonist, SRTX-C, decreased by 60–70% the activation profile of MAP kinase by serum at 10 and 17 min (Fig. 4 *A*). Addition of SRTX-C alone (in the absence of serum) did not affect p44 MAP kinase activity (Fig. 4 *A*). Stimulation of cells with PDGF-BB revealed the same pattern of MBP kinase activation as that obtained with human serum (Fig. 4 *B*). PDGF-BB elicited a sevenfold stimulation of p44 MAP kinase at 17 min, which was reduced by 40% in the presence of SRTX-C. It should be noted that the 42-kD isoform of MAP kinase was not reproducibly observed; however, when visualized, serum stimulation of the 42-kD isoform was also inhibited by SRTX-C (not shown). We also observed the phosphorylation of MBP by other MBP kinases with an apparent molecular weight of 72 kD (Fig. 4 *B*) and of 58 and 80 kD, as revealed by longer exposures of the film (not shown). The presence of these other MBP kinases has previously been mentioned in other studies (27, 33, 34). Phosphorylation of these MBP kinases was unaffected by serum or SRTX-C treatment (Fig. 4 *B*).

The expression of serum-stimulated early genes is differentially regulated by the ETB agonist, SRTX-C. Induction of immediate–early genes plays a pivotal role in cell proliferation (35). We investigated the effects of SRTX-C on steady-state

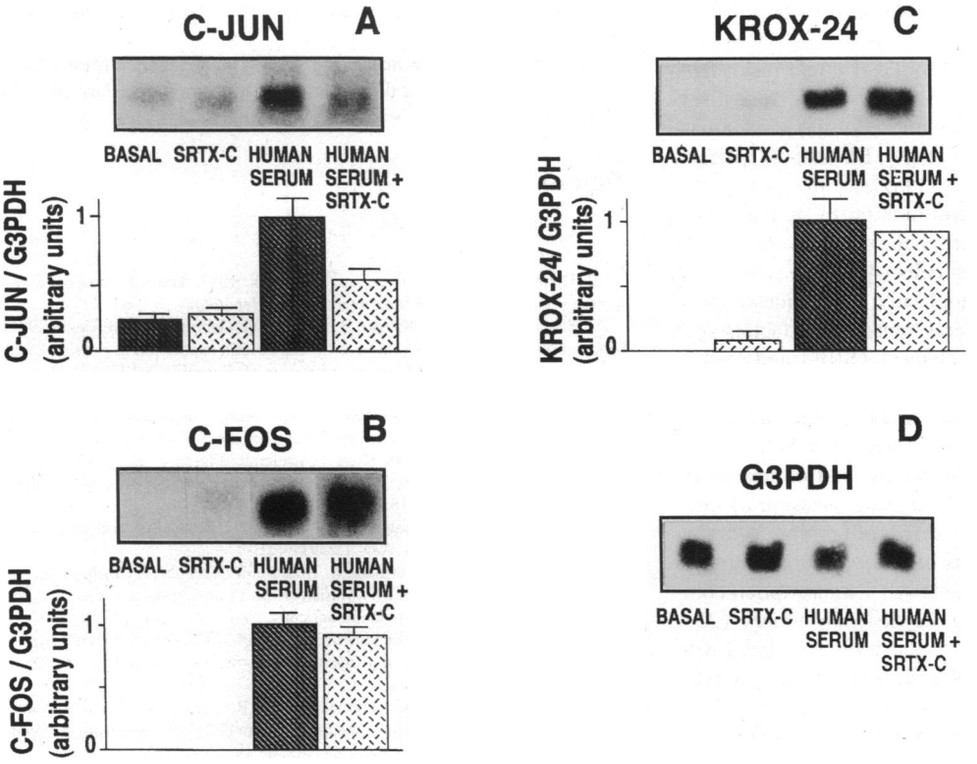

Figure 5. Northern blot analysis of the effects of SRTX-C on serum-stimulated mRNA expression of c-*jun*, c-*fos*, and krox 24. Quiescent cells were incubated for 60 min in medium alone or in medium containing 5% human serum, in the absence or the presence of 100 nM SRTX-C. Total RNA (20 μg/lane) was separated on a 0.8% agarose gel, transferred to a nitrocellulose membrane and hybridized with ^{32}P-labeled cDNA probes encoding c-*jun* (*A*), c-fos (*B*), krox 24 (*C*), and G3PDH (*D*), as described in Methods. The upper part of each panel shows a typical autoradiogram. The lower parts of *A*, *B*, and *C* show quantification of c-*jun*, c-*fos*, and krox 24 signals relative to G3PDH. Results represent mean±SEM of three independent experiments.

expression of c-*jun*, c-*fos*, and krox 24 mRNAs. As described in other cells (23, 35), quiescent myofibroblastic Ito cells showed basal expression of c-*jun*, but not of c-*fos* or krox 24 mRNAs (23, 35, 36) (Fig. 5). Stimulation of quiescent myofibroblastic Ito cells by human serum resulted in the induction of 2.9-, 2.3-, and 3.8-kb mRNAs, corresponding to the expected sizes of c-*jun*, c-*fos*, and krox 24 mRNAs, respectively (Fig. 5). Maximal induction of c-*jun*, c-*fos*, and krox 24 was observed after a 30–60-min stimulation and was followed by a rapid decline thereafter (not shown). Maximal serum-induced expression of c-*jun* mRNA was reduced by 50% in the presence of SRTX-C (Fig. 5 *A*). By contrast, SRTX-C did not affect serum-stimulated expression of c-*fos* (Fig. 5 *B*) and krox 24 (Fig. 5 *C*) mRNAs. Taken together, these data indicate that ETB receptor agonists selectively down-regulate serum-induced expression of c-*jun* in myofibroblastic Ito cells.

Discussion

A major finding of this study is that ET-1 is a potent antiproliferative agent in human myofibroblastic Ito cells, a finding which contrasts with the mitogenic properties of the peptide in other cell types (10).

Characterization of endothelin receptors in human myofibroblastic Ito cells indicates the presence of a high number of endothelin binding sites (815±150 fmol/mg prot). A majority (80%) are of the ETB type, while 20% are ETA binding sites (Fig. 1), in agreement with data obtained with rat Ito cells (11). In human myofibroblastic Ito cells, these preponderant ETB receptors are responsible for the growth inhibitory properties of ET-1, based on the use of selective agonists and antagonists (Fig. 3). This contrasts with the mitogenic effects of ET-1 in numerous cell types, which are transduced by the ETA receptor (10). Together, these data indicate that ET-1 can exert both positive and negative control of cell proliferation. Dual regulation of cell growth by a single ligand in different tissues or cell lines is not restricted to ET-1. Transforming growth factor-β1 induces growth inhibition in several epithelial cells (37) but exhibits mitogenic properties for human myofibroblastic Ito cells (21), fibroblasts or some tumor cells (37). Somatostatin and lysophosphatidic acid may also regulate cell proliferation positively or negatively, through activation of distinct receptors and second messenger pathways (38, 39). Therefore, it is tempting to speculate that growth regulatory effects of ET-1 could depend on the endothelin receptor subtype, cell proliferation being linked to ETA receptor, and growth inhibition to ETB receptor. Consequently, modulation of the distribution of ETA and ETB receptors would be a key regulatory parameter, under normal or pathophysiological conditions.

To analyze the intermediate steps leading to growth inhibition by ET-1, we first studied the regulation of MAP kinase activity. MAP kinases are a family of cytosolic protein kinases which play a central role in the cytosolic transmission of mitogenic signals to the nucleus (31, 32). Activation of MAP kinases occurs following stimulation of tyrosine kinase receptors or G protein–coupled receptors. Both types of stimuli initially use distinct signaling pathways, which converge to activate MAP kinase. It has been shown that mitogenic effects of ET-1 involve stimulation of MAP kinases in myocytes (34), mesangial cells (40), or astrocytes (33). By contrast, we show here that stimulation of ETB receptors in myofibroblastic Ito cells reduces activation of MAP kinase by serum and PDGF-BB, and results in growth inhibition. Activation of distinct second messenger systems could be responsible for these opposite effects of ET-1 on MAP kinases in different cell types. In fact, ET-1 can use both the Ca^{2+}/protein kinase C and the cAMP

pathways (6, 8), and stimulation of MAP kinases by ET-1 has been linked to activation of protein kinase C (33, 34, 40). In contrast, inhibition of MAP kinase by ET-1 could result from production of cAMP, since growth inhibitory properties of several hormones are consecutive to inhibition of the MAP kinase cascade by cAMP (31, 41). Additionally, ET-1 could interfere with the inactivation process of MAP, kinases. In this hypothesis, ET-1 would inhibit Ito cell proliferation by activating the family of specific MAP kinase phosphatases that were recently identified in yeast and mammalian cells (42).

After mitogen stimulation, cytoplasmic MAP kinases translocate to the nucleus, activate several transcription factors such as c-*jun* or the serum response element (SRE) and lead to induction of immediate–early gene expression (32). These events are sequentially linked and critical in the process of cell proliferation, as recently demonstrated with MAP kinase antisense oligonucleotides (43). In the last part of this study, we investigated the effects of ET-1 on the expression of three immediate–early genes crucial for cell proliferation, c-*fos,* c-*jun,* and krox 24 (35, 36). Proteins of the *fos* and *jun* families regulate gene transcription by forming AP-1 transcription complexes which consist in *jun–jun* or *fos–jun* dimers (35). Among krox proteins, krox 24 has often been associated with regulation of cell growth (36). We show here that an ETB agonist reduces serum-stimulated expression of c-*jun* mRNA but does not affect c-*fos* or krox 24 mRNA expression (Fig. 5) indicating that ET-1 elicits differential regulation of immediate-early gene expression. Such an observation is not unique to these cells since in mesangial cells, stimulation of ETA receptor induces specific expression of c-*fos* and is linked to cell proliferation (23). Therefore, positive and negative control of cell growth by ET-1 may involve distinct pathways of early gene expression. Differential regulation of immediate–early gene expression has also been observed with other growth regulatory factors. Activation of rat Ito cell proliferation by vitamin D3 involves induction of krox 24 expression but not that of c-*fos* (44). Growth inhibition of astrocytes by atrial natriuretic factor has been linked to specific inhibition of krox 24 expression with no modification of c-*jun* or c-*fos* expression (45). Interestingly, antiproliferative effects of cAMP in NIH 3T3 fibroblasts are associated with selective down-regulation of c-*jun* mRNA expression (46) and inhibition of MAP kinase activity (47)

Experimental models of liver fibrosis as well as human studies indicate that proliferation of myofibroblastic Ito cells is a key feature of chronic liver injury (1). Consequently, our findings raise the question of the role of ET-1 during liver fibrogenesis. It has been shown that expression of ET-1 is markedly increased in cirrhotic human liver (48). In addition, secretion of ET-1 has been demonstrated in myofibroblastic Ito cells, isolated either from human liver (48), or from rats with experimental liver fibrosis (11). In light of these observations, demonstration of an antiproliferative effect of ET-1 in human myofibroblastic Ito cells suggests the possibility of a negative autocrine loop which could counteract development of liver fibrogenesis.

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