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Endothelin stimulates transforming growth factor-β1 and collagen synthesis in stellate cells from control but not cirrhotic rat liver

Chandrashekhar R. Gandhi [a,b,*], Ruhul H. Kuddus [a], Tadahiro Uemura [a], Abdul S. Rao [a]

[a] *Department of Surgery, Thomas E. Starzl Transplantation Institute, University of Pittsburgh, E-1540 BST, 200 Lothrop Street, Pittsburgh, PA 15213, USA*

[b] *Department of Pathology, Veterans Administration Medical Center, University of Pittsburgh, E-1540 BST, 200 Lothrop Street, Pittsburgh, PA 15213, USA*

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Abstract

Interactions between hepatic stellate cells and endothelin-1 are implicated in liver fibrosis. We determined endothelin-1, its receptors and its effects on the synthesis of a fibrogenic agent transforming growth factor (TGF)-β1 and collagen in stellate cells from control and CCl_4-induced cirrhotic rats. The basal synthesis of endothelin-1, TGF-β1 and collagen was much higher in cirrhotic stellate cells than in control cells. Endothelin-1 stimulated TGF-β1 and collagen synthesis via endothelin ET_A and endothelin ET_B receptors, respectively, in control stellate cells, but did not elicit these effects in the cirrhotic cells despite increased density of the respective receptor subtypes in them. These results indicate that the actions of endothelin-1 on stellate cells may be an important physiological mechanism in maintenance of hepatic architecture. However, inability of endothelin-1 to stimulate TGF-β1 and collagen synthesis in cirrhotic stellate cells suggests that it does not influence fibrogenic activity by direct action on them probably because the processes are already maximally activated.

Keywords: Stellate cell; Cirrhosis; Endothelin; Liver; Fibrosis, rat

1. Introduction

Physiologically, perisinusoidal stellate cells maintain the architecture of the liver by synthesizing components of extracellular matrix and the sinusoidal blood flow by contractile activity. During chronic liver injury, stellate cells lose their storage of retinoids, express α-smooth muscle actin, and transform into proliferating myofibroblast-like cells that are highly contractile and excessively fibrogenic. Thus, stellate cells play an important role in fibrogenesis during the development of liver cirrhosis (Blomhoff and Wake, 1991; Geerts et al., 1994; Nakata and Shibayama, 1987).

Hepatic concentrations of endothelin-1, a powerful constrictor of the hepatic vasculature (Gandhi et al., 1990; Tran-Thi et al., 1993), and its receptors increase progressively during the development of experimental cirrhosis (Gandhi et al., 1998). Furthermore, hepatic concentrations of endothelin-1 and its receptors increase in human cirrhosis (Gandhi et al., 1996a; Pinzani et al., 1996) and there is a direct relationship between endothelin-1 receptor gene expression and portal pressure in cirrhotic patients (Leivas et al., 1998). Endothelin-1 causes contraction of quiescent, (Zhang et al., 1995) as well as transformed stellate cells (Housset et al., 1993; Pinzani et al., 1996). Antagonism of endothelin-1 receptors has been reported to reduce the concentration of type I collagen and its mRNA in rat models of liver fibrosis (Rockey and Chung, 1996). However, whether endothelin-1 exerts this effect by direct actions on stellate cells is not known. Therefore, we investigated endothelin-1, its receptors and its effects on the fibrogenic activity of stellate cells isolated from the livers of carbon tetrachloride (CCl_4)-induced cirrhotic rats.

* Corresponding author. Department of Surgery, Thomas E. Starzl Transplantation Institute, University of Pittsburgh, E-1540 BST, 200 Lothrop Street, Pittsburgh, PA 15213. Tel.: +1-412-648-9316; fax: +1-412-624-6666.

E-mail address: gandhics@msx.upmc.edu (C.R. Gandhi).

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2. Materials and methods

The following chemicals and reagents were purchased from the indicated sources: protease type XIV, 5-[*N*-2,3-dihydroxypropylacetamido-2,4,6-troiodo-*N*,*N*′-bis(2,3-dihydroxypropyl)isophthalamide] (Nycodenz), CCl_4 (99.9% purity) and BQ-788 (*N-cis*-2,6-dimethylpiperidinecarbonyl-L-γMeLeu-D-Trp(COOMe)-D-Nle-ONa) (Sigma, St. Louis, MO); collagenase type IV (Worthington Biochemical, Freehold, NJ); transforming growth factor β1 (TGF-β1) (Life Technologies, Grand Island, NY); endothelin-1, sarafotoxin S6c and BQ-123 (cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu-)) (American Peptide, Sunnyvale, CA); [^{125}I] endothelin-1 (2200 Ci/mmol) (DuPont-New England Nuclear, Boston, MA); endothelin-1 enzyme linked immunosorbent assay (ELISA) kit (Peninsula Laboratories, Belmont, CA); and TGF-β1 ELISA kit and L-[2,3,4,5-^{3}H]proline (100 Ci/mmol) (Amersham-Pharmacia, Piscataway, NJ).

2.1. Induction of liver cirrhosis

The experimental protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee in accordance with the guidelines of the National Institutes of Health. Cirrhosis was induced in male Sprague–Dawley rats (230–250 g) by intraperitoneal injection of CCl_4 (0.15 ml/kg twice a week for 8 weeks) (Gandhi et al., 1996b). Control rats were injected with the carrier (peanut oil) alone. Rats were allowed access to sodium phenobarbital (0.4 g/l) containing water as the only drink source throughout the induction period, starting 4 days prior to the beginning of CCl_4 treatment. The rats were used in week 8 of CCl_4 treatment. As established previously (Gandhi et al., 1996b, 1998), cirrhosis is fully developed at this time of CCl_4 treatment, as characterized histologically by extensive fibrosis and gross nodulality of the liver tissue; the histological characteristics are accompanied by spelenomegaly, portal hypertension and ascites. Phenobarbital and CCl_4 treatments were terminated 3 days prior to the sacrifice.

2.2. Preparation of stellate cells

Stellate cells were prepared and their purity ascertained as described previously (Gabriel et al., 1998). Briefly, the liver was digested with protease (0.02%) and collagenase (0.04%), and stellate cells purified on a Nycodenz (13.12%) gradient. The concentration of collagenase was doubled for the digestion of the cirrhotic liver. The cells were suspended in Dulbecco's modified Eagle medium (DMEM) containing antibiotics and 10% fetal bovine serum/10% horse serum at a density of 2×10^6 cells/ml. Aliquots (0.5, 1.0 and 8 ml) of the cell suspension were plated in 24- and 12-well culture plates, and 100-cm^2 culture flasks, respectively. The medium was renewed after 3 h and the cells were used following an overnight incubation. Stellate cells were characterized by light microscopy, vitamin A autofluorescence, and immunohistochemically using specific markers for endothelial cells (antibody for factor VIII related antigen; DAKO, Carpintoria, CA), stellate cells (antibody for desmin and α-smooth muscle actin; DAKO), Kupffer cells (clone ED2; Serotec, Indianapolis, IN) and epithelial cells (clone AE1/AE3; Boehringer Mannheim, Indianapolis, CA) as described previously (Kuddus et al., 2000). The purity of the cells was greater than 95%, and the plating efficiency was greater than 75%.

2.3. [^{125}I]endothelin-1 binding assay

The assay was performed as described previously (Gabriel et al., 1998). Cells were washed with Hank's balanced salt solution (HBSS) containing 10 mM HEPES, pH 7.4, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.1% bovine serum albumin (buffer A), and placed in this medium containing 0.3 mg/ml bacitracin and 5–800 pM [^{125}I]endothelin-1 ± 1 μM unlabeled ET-1 (saturation binding). In the competition binding assay, cells were incubated with 20 pM [^{125}I]endothelin-1 and 20 pM–1 μM unlabeled endothelin-1 (an endothelin$_A$ + endothelin$_B$ agonist), 100 μM–1 mM endothelin ET$_A$ receptor antagonist BQ-123 (Ihara et al., 1992) or 20 pM–10 μM endothelin$_B$ receptor agonist sarafotoxin S6c (Williams et al., 1991). After incubation at 22°C for 2 h, the cells were washed with buffer A and digested with 0.75 N NaOH for determination of radioactivity. Specific binding of [^{125}I]endothelin-1 was calculated as the difference between cell-associated radioactivity in the presence and absence of excess (1 μM) unlabeled endothelin-1.

2.4. Determination of the mRNA expression of preproendothelin-1 and endothelin receptors by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR)

The relative levels of the low abundance mRNA transcripts of preproendothelin-1, endothelin$_A$ receptor and endothelin$_B$ receptor were determined by semiquantitative RT-PCR essentially, as described previously (Gabriel et al., 1998, 1999). Total RNA was prepared from the cells using TRI reagent™ containing phenol and guanidine thiocyanate (Molecular Research Center, Cincinnati, OH), treated with RNase-free DNase (Life Technologies) and quantified spectrophotometrically. Complementary DNA (cDNA) was made using Superscript II cDNA synthesis kit (Life Technologies), and 1–3 μl of cDNA (equivalent of 75–225 ng total RNA) was used in the PCR reaction. Control reactions were performed in the absence of RT. The level of β-actin mRNA was determined to ascertain the efficiency of the cDNA synthesis and reverse transcription. The PCR primers used were — preproendothelin-1 cDNA: 5′CCAACTCTGGGCTCTCCATGCTGG3′ (F) and

5′GAATGGCACTGTGTCTCTGCTCTC3′ (R) [product size: 241 bp (Sakurai et al., 1991)]; endothelin$_A$ receptor cDNA: 5′CGAGGTCATGAGGCTTTTGG3′ (F) and 5′GTGTTTAAGCTGTTGGCGGG3′ (R) [product size: 787 bp (Tarada et al., 1992)]; endothelin$_B$ receptor cDNA: 5′AGCTGGTGCCCTTCATACAGAAGGC3′ (F) and 5′TGCACACCTTTCCGCAAGCACG3′ (R) [product size: 919 bp (Tarada et al., 1992)]; and β-actin cDNA: 5′TTCTACAATGAGCTGCGTGTG3′ (F) and 5′TTCATGGATGCCACAGGATTC3′ (R) [product size: 561 bp (Nudel et al., 1983)]. In preliminary experiments, each primer set was used to amplify equal amounts of cDNA derived from various samples for 20–35 cycles, and based on these findings, a predetermined number of cycles were chosen for each primer set so that the product accumulation stays in the linear range. PCR products were resolved in a 1.2% agarose gel and stained with 1X SYBR Green I (FMC Biproduct, Rockland, ME). The gels were scanned under blue fluorescence light using a phosphorimager and the band intensity was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Repeated PCR reactions showed that each primer set generated a single band of expected size. Authenticity of the PCR products were positively identified by Southern blot hybridization using radiolabeled probes with sequences within the PCR products as described previously (Gabriel et al., 1998, 1999) as well as by restriction enzyme analysis.

2.5. Northern analysis of TGF-β1, and collagen α type-I and type-III mRNA

RNA was isolated from cultured stellate cells in 10-cm dishes by TRI reagent™. Twenty micrograms of total RNA from each sample was denatured at 55°C for 15 min and electrophoresed on a 1% agarose/formaldehyde gel containing ethidium bromide and transferred to a Hybond N + membrane (Amersham-Pharmacia). The membrane was hybridized with ^{32}P-labeled cDNA probes in a solution of 50% formamide, 5X standard saline citrate (SSC), 10 mM sodium phosphate (pH 6.8), 0.5% sodium dodecyl sulfate (SDS), 5X Denhart and 20 μg/ml herring sperm DNA at 42°C overnight. The membrane was then washed twice with 2X SSC containing 0.1% SDS at room temperature for 30 min, twice with 0.2X SSC containing 0.1% SDS at 55°C for 30 min, and then exposed to Kodak XOmat film (Eastman Kodak, Rochester, NY). The probes were radiolabeled by random priming method using multiprime DNA labeling kit (Amersham-Pharmacia) in the presence of [^{32}P]dCTP (NEN). A 0.99-kb fragment of rat TGF-β1 cDNA was purchased from ATCC (MD, USA). To construct plasmids pRcol I and pRcol III, rat cDNA was used as template to PCR amplify a fragment of collagen α type-I and collagen α type-III cDNAs prepared from rat skin. The primers used for PCR amplification were 5′GGTTCTCGGACTATTGAAGGAGC 3′ (F) and 5′AGACAAGAACGAGGTAGTCTTTC 3′ (R) for collagen α type-I (product size: 245 bp) and 5′CGAGGTAACAGAGGTGAAAGA 3′ (F) and 5′AACCCAGTATTCTCCGCTCTT 3′ (R) for collagen α type-III (product size: 349 bp), and 5′AGGTCGGTGTGAACGGATTT3′ (F) and 5′CAGCATCAAAGGTGGAAGAA3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (product size: 881 bp). The amplified products were purified after resolving in 1.2% agarose gel using Qiaex II kit (Qiagen, Valencia, CA) and cloned in PCR 2.1 TOPO vector using TOPO TA cloning kit (Invitrogen, Carlasbad, CA). The authenticity of the insert was confirmed by DNA sequencing using Sequenase™ kit (USB, Cleveland, OH). The plasmids were amplified in *Escherichia coli*, and DNA purified by CsCl gradient centrifugation [(Sambrook et al., 1989)]. The DNA was cut with appropriate restriction enzymes, resolved in 1% agarose gel and the insert portion was purified using Qiaex II kit and subsequently used for the synthesis of the probe.

2.6. Determination of endothelin-1 and TGF-β1

Cells were washed three times, and placed in DMEM containing 0.1% bovine serum albumin and the test agents. After incubation for 24 h at 37°C, the medium was aspirated for the extraction and determination of endothelin-1 and TGF-β1 by ELISA as described previously (Gabriel et al., 1999).

2.7. Collagen synthesis

The synthesis of collagen was determined by a previously described procedure (Shina et al., 1989) with some modifications. Cells were washed and placed in DMEM/0.1% bovine serum albumin containing 5 μCi/ml [^{3}H]proline and various agents at concentrations indicated in the figure legends. After incubation for 24 h at 37°C, the medium was aspirated, mixed with chick embryo extract (GibcoBRL) (50 μl/ml) and proteins were precipitated with 10% trichloroacetic acid. After centrifugation, the pellet was washed twice with 10% trichloroacetic acid followed by HBSS and dissolved in 0.6 ml 0.2 N NaOH. An aliquot (0.2 ml) of this solution was neutralized with HCl, mixed with 0.1 ml 50 mM Tris–Cl, pH 7.4, containing 5 mM $CaCl_2$ and 10 mM *N*-ethylmaleimide, and the reaction was stimulated with 20 units/ml collagenase (type VII from *Clostridium histolyticum*, Sigma). The total volume of the reaction mixture was 0.5 ml. After incubation for 3 h at 37°C, 50 μl of 10 mg/ml bovine serum albumin and 50 μl of 100% trichloroacetic acid were added to each tube. After centrifugation, an aliquot of the supernatant was aspirated for determination of radioactivity. Negative controls (i.e. without enzyme) were used in each assay for determination of nonspecific release of [^{3}H]proline.

2.8. Statistical analysis

Results are averages of duplicate or triplicate determinations with S.E.M. shown for triplicates. Each experiment was repeated at least three times using separate cell preparations. Paired samples were compared using Student's *t*-test. A P value of < 0.05 was considered statistically significant.

3. Results

We use phenobarbital to enhance the toxicity of CCl_4 and establishment of uniform cirrhosis (Gandhi et al., 1996b, 1998). However, considering the effects of phenobarbital alone on the liver tissue, we compared various parameters described in this paper between the cells isolated from untreated rats (these cells are routinely used in our laboratory) and rats treated with phenobarbital. No difference was observed in the morphological and immunohistochemical characteristics, as well as endothelin biology between these cells.

3.1. Morphologic characteristics of cirrhotic stellate cells

Fig. 1 shows control and cirrhotic stellate cells after overnight culture. Control stellate cells (A) are roundish and loaded with fat droplets, whereas the cirrhotic cells (B) are already flattened and demonstrate morphologic characteristics of myofibroblasts.

3.2. Synthesis of endothelin-1 by cirrhotic stellate cells

The expression of preproendothelin-1 mRNA was two-fold higher in cirrhotic stellate cells as compared to the control cells (Fig. 2A,B). Consistent with this observation, the basal release of endothelin-1 by cirrhotic stellate cells (422 ± 51 pg/mg protein) was greater than by the control cells (271 ± 42 pg/mg protein) (Table 1). The release of endothelin-1 by both control and cirrhotic stellate cells increased by 2.5–3-fold upon stimulation with TGF-β1 (Table 1).

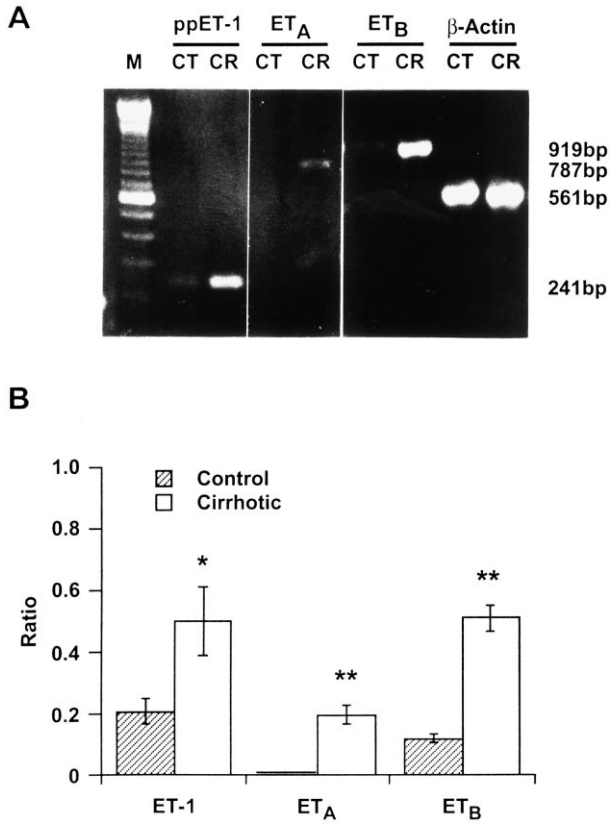

Fig. 2. Preproendothelin-1 (ppET-1), endothelin$_A$ (ET$_A$) receptor and endothelin$_B$ (ET$_B$) receptor mRNAs in control and cirrhotic stellate cells. (A) PCR products resolved in agarose gel. The level of β-actin expression is similar in various samples indicating similar quantities of mRNA in them. (B) Graphical presentation of the ratios of the intensity of ppET-1, ET$_A$ or ET$_B$ mRNA and β-actin mRNA. M, 100-bp DNA ladder; CT, control stellate cells; CR, cirrhotic stellate cells. Values shown are averages of six determinations ± S.E.M. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control.

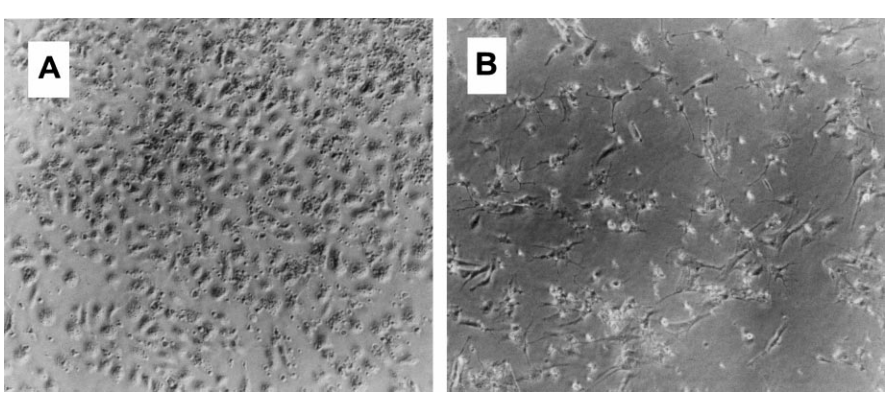

Fig. 1. Morphology of control and cirrhotic stellate cells. Phase contrast micrographs of control (A) and cirrhotic (B) stellate cells on day 2 of culture are shown. Magnification: ×100.

Table 1
Release of endothelin-1 in response to TGF-β1 by cultured control and cirrhotic stellate cells

	Endothelin-1 released (pg/mg protein)	
	Vehicle	TGF-β1
Control cells	271 ± 42	663 ± 23[a]
Cirrhotic cells	422 ± 51[a,b]	1276 ± 101[a,b]

The cells were placed in serum-free medium ± TGF-β1 (2 ng/ml). After 24 h, the medium was aspirated and endothelin-1 concentration was determined. Values shown are means ± S.E.M. of triplicate determinations from three experiments.
[a] $P < 0.01$ vs. vehicle.
[b] $P < 0.01$ vs. control stellate cells.

3.3. Endothelin-1 receptors and their mRNA expression in cirrhotic stellate cells

Scatchard analysis of the saturation binding data revealed about four-fold increase in [^{125}I]endothelin-1 binding capacity in cirrhotic stellate cells as compared to the control cells (6350 ± 656 vs. 1700 ± 202 fmol/mg) (Fig. 3A). The affinity of the receptors was not altered by the cirrhotic condition (K_d of 24 ± 5 pM for the control and 28 ± 6 pM for the cirrhotic cells).

Competition binding assay demonstrated that the increase in the endothelin-1 receptor density in control stellate cells occurred both in endothelin ET_A and endothelin ET_B receptor subtypes although their relative proportion (approximately 20% endothelin ET_A and 80% endothelin ET_B) did not change from that in control stellate cells (Fig. 3B).

Endothelin$_A$ receptor mRNA expression was very low in control stellate cells and was 20-fold higher in cirrhotic cells (Fig. 2A,B). On the other hand, significant expression of endothelin ET_B receptor mRNA was found in control stellate cells, with a five-fold increase in its expression in the cirrhotic cells (Fig. 2A,B).

3.4. Endothelin-1-induced TGF-β1 synthesis in stellate cells

Stellate cells are known to be a major source of TGF-β1 during liver cirrhosis (Pinzani and Abboud, 1991). Since endothelin-1 stimulates TGF-β1 synthesis in cultured vascular smooth muscle cells (Hahn et al., 1991), whether similar response to endothelin-1 occurs in stellate cells was determined. The basal release of TGF-β1 by the cirrhotic cells was nearly eight-fold higher than by the control cells (2561 ± 106 vs. 333 ± 12 pg/mg protein) (Table 2). This effect was consistent with increased TGF-β1 mRNA transcript in the cirrhotic cells (Fig. 4). Endothelin-1 caused an increase in TGF-β1 mRNA transcript (Fig. 4) and stimulated the release of TGF-β1 in control stellate cells. This

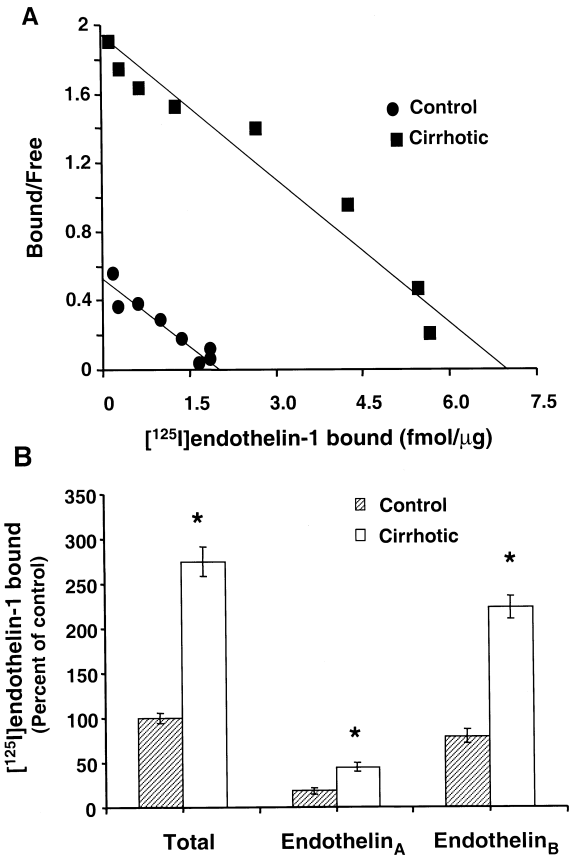

Fig. 3. [^{125}I]Endothelin-1 binding to control and cirrhotic stellate cells. (A) Saturation binding. Scatchard plots of the data derived from specific binding of 5–800 pM [^{125}I]endothelin-1 as described in Section 2. (B) Competition binding. Binding assay was performed in the presence of 20 pM [^{125}I]endothelin-1 ± increasing concentrations of unlabeled endothelin-1, BQ-123 or sarafotoxin S6c as described in Section 2. Specific binding of [^{125}I]endothelin-1 to endothelin-1 receptor subtypes is shown. * $P < 0.01$ vs. control.

effect was mediated via endothelin$_A$ receptor as evident by its inhibition with endothelin ET_A receptor antagonist BQ123 (Table 2). However, endothelin-1 did not affect

Table 2
Endothelin-1-induced release of TGF-β1 from control and cirrhotic stellate cells

	TGF-β1 released (pg/mg protein)	
	Control cells	Cirrhotic cells
Vehicle	333 ± 12	2561 ± 106[a]
Endothelin-1 (50 nM)	777 ± 40[b]	2698 ± 68[a]
BQ123 (10 μM) + endothelin-1	370 ± 9	2559 ± 54[a]
BQ788 (10 μM) + endothelin-1	666 ± 40[b]	2275 ± 51[a]

The cells were placed in serum-free medium ± the indicated agents. After 24 h, the medium was aspirated and TGF-β1 was determined. Values shown are means ± S.E.M. of triplicate determinations from three experiments.
[a] $P < 0.001$ vs. control stellate cells.
[b] $P < 0.01$ vs. vehicle.

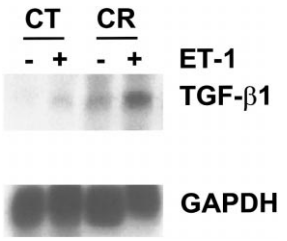

Fig. 4. Effect of endothelin-1 on the expression of TGF-β mRNA in control and cirrhotic stellate cells. Total RNA was extracted after a 24-h stimulation of the cells without (−) or with (+) 50 nM endothelin-1. Northern analysis was performed as described in Section 2. The experiment was performed on three separate cell preparations with similar results. CT, control stellate cells; CR, cirrhotic stellate cells.

TGF-β1 synthesis in the cirrhotic cells (Table 2) although it caused increase in TGF-β1 mRNA expression in them (Fig. 4).

3.5. Synthesis and mRNA expression of collagen

The basal level of synthesis and release of collagen (as determined by [^{3}H]proline incorporation) was more than six-fold higher in cirrhotic stellate cells than the control cells (38 835 ± 2955 vs. 5917 ± 412 c.p.m./mg protein) (Table 3). This effect was consistent with a great increase in the mRNA expression of both type-I and type-III collagen α in the cirrhotic cells (Fig. 5). The expression of the mRNA transcripts of both types of collagen was barely detectable in the control cells, and endothelin-1 did not appear to cause any noticeable change in their expression. However, endothelin-1 stimulated collagen synthesis only in control stellate cells via endothelin$_B$ receptor as illustrated by its inhibition with endothelin$_B$ antagonist BQ-788 (Table 3) without having any effect on the mRNA expressions of collagen α type-I and type-III (Fig. 5). Similar results were also obtained by semiquantitative RT-PCR assay (data not shown).

Table 3
Endothelin-1-induced synthesis of collagen in control and cirrhotic stellate cells

	[^{3}H]proline released (c.p.m./mg protein)	
	Control cells	Cirrhotic cells
Vehicle	5917 ± 412	38 835 ± 2955[a]
Endothelin-1 (50 nM)	12 672 ± 1739[b]	41 829 ± 1085[a]
BQ123 (10 μM) + endothelin-1	11 211 ± 1222[b]	38 572 ± 4124[a]
BQ788 (10 μM) + endothelin-1	6175 ± 531	43 154 ± 5145[a]

The cells were placed in serum-free medium containing 5 μCi/ml [^{3}H]proline and indicated concentrations of the various agents. After 24 h of incubation, the medium was aspirated and proteins precipitated with trichloroacetic acid. The precipitated proteins were reconstituted and collagenase-induced release of [^{3}H]proline was determined as described in Section 2. Values shown are means of triplicate determinations ± S.E.M. The experiment was repeated three times with essentially similar results.
[a] $P < 0.001$ vs. control stellate cells.
[b] $P < 0.01$ vs. vehicle.

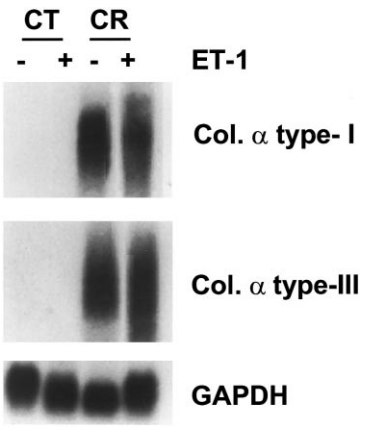

Fig. 5. Effect of endothelin-1 on the expression of collagen α type-I and type III in control and cirrhotic stellate cells. Total RNA was extracted after a 24-h stimulation of the cells without (−) or with (+) 50 nM endothelin-1. Northern analysis was performed as described in Section 2. The experiment was performed on three separate cell preparations with similar results. CT, control stellate cells; CR, cirrhotic stellate cells.

4. Discussion

The present investigation was performed considering the evidence that interactions between endothelin-1 and stellate cells can be of significant importance in physiology and pathology of the liver. Although all of the hepatic cell types synthesize endothelin-1 (Gandhi et al., 1996b; Housset et al., 1993; Kuddus et al., 2000; Laura et al., 1998; Pinzani et al., 1996) and react to its agonistic activities in cell culture (Caligiuri et al., 1998; Gandhi et al., 1992a,b; Housset et al., 1993; Pinzani et al., 1992, 1996), the physiological role of endothelin-1 in the liver is not clear. Particularly, the role of endothelin-1 in the regulation of hepatic vascular tone, despite its potent vasoconstrictor action (Gandhi et al., 1990; Tran-Thi et al., 1993), was questioned by the failure of the antagonism of endothelin-1 receptors to affect portal resistance in normal rats (Gandhi et al., 1998). Our results demonstrate that endothelin-1-stimulates synthesis of TGF-β1 as well as collagen in control stellate cells via distinct endothelin-1 receptor subtypes. Furthermore, TGF-β1 stimulates endothelin-1 synthesis in control stellate cells (Table 1) and hepatic endothelial cells (Rieder et al., 1991). Thus, autocrine as well as paracrine actions of endothelin-1 on control stellate cells appear to be important in the maintenance of hepatic architecture.

Transdifferentiation of stellate cells into highly contractile and excessively fibrogenic myofibroblast-like cells is a hallmark of the development of liver cirrhosis and its

complications (Blomhoff and Wake, 1991; Geerts et al., 1994; Nakata and Shibayama, 1987). Endothelin-1 has been reported to enhance transdifferentiation of stellate cells (Rockey and Chung, 1996), to modulate their proliferative activity (Mallat et al., 1996; Pinzani et al., 1996), and to exert potent contractile actions on them (Housset et al., 1993; Kawada et al., 1993; Pinzani et al., 1992). Therefore, augmentation of the already enhanced synthesis of endothelin-1 by TGF-β1 in cirrhotic stellate cells (Table 1), and increased endothelin-1 receptor density in them, (Fig. 3) suggest that a mechanism of increased vascular resistance in the cirrhotic liver may involve autocrine actions of endothelin-1 on stellate cells. Indeed, endothelin-1 receptor antagonism ameliorates portal hypertension in cirrhotic rats (Gandhi et al., 1998).

The molecular mechanisms of hepatic fibrosis are not fully described, although overexpression of TGF-β1 may play a critical role in the progression of fibrosis (Blomhoff and Wake, 1991; Friedman, 1993). TGF-β1 mRNA expression as well as protein concentration (as determined by immunohistochemistry) were reported to increase during CCl_4-induced liver injury (Armendariz-Borunda et al., 1993). Kupffer cells were shown to be the main producers of TGF-β1 during the acute inflammatory phase (Armendariz-Borunda et al., 1993). During the chronic phase of liver injury, stellate cells also produce large quantities of TGF-β1 (Pinzani and Abboud, 1991). Consistent with these findings, increased concentration of TGF-β1 mRNA transcript and nearly eight-fold higher basal release of TGF-β1 were observed in cirrhotic stellate cells than in the control cells. However, endothelin-1 caused an increase in TGF-β1 mRNA expression, but not the protein synthesis in the cirrhotic cells. These results suggest that TGF-β1 synthesis in cirrhotic stellate cells may be impervious to an additional stimulus because it is already maximally stimulated.

The higher level of basal synthesis of collagen in cirrhotic stellate cells also was consistent with robust increases in the collagen α type-I and type-III mRNA transcripts in them as compared to the control cells. These results indicate that stellate cells may be the major cell type responsible for the positive correlation observed between TGF-β1 mRNA expression and mRNAs encoding procollagens in animal models of liver fibrosis (Armendariz-Borunda et al., 1993; Czaja et al., 1989) and humans with chronic liver disease (Castilla et al., 1991). However, similar to its inability to stimulate TGF-β1 synthesis in cirrhotic stellate cells, endothelin-1 did not affect the collagen synthesis in these cells. Again, the mRNA expression and the synthesis of collagen are greatly increased in unstimulated cirrhotic stellate cells relative to the control cells, and this may be the reason why endothelin-1 is unable to produce a further enhancement of this process. A previous study reported decreased mRNA expression and protein concentration of collagen α type-I in stellate cells isolated from CCl_4-treated rats, that received a mixed endothelin $ET_{A/B}$ receptor antagonist bosentan (Rockey and Chung, 1996). Contrarily, chronic treatment of rats with bosentan during CCl_4-induced liver injury was shown to cause an increase in procollagen type-I mRNA expression in rats (Poo et al., 1999). These observations and our results, showing inability of endothelin-1 to stimulate the synthesis of collagen in stellate cells isolated from CCl_4-induced cirrhotic rats, indicate that the effect of endothelin-1 reported in vivo (Rockey and Chung, 1996) is indirect, possibly through fibrogenic mediators such as TGF-β1 and platelet-derived growth factor.

In conclusion, our work has demonstrated that endothelin-1 plays an important regulatory role in maintaining hepatic architecture by stimulating TGF-β1 and collagen synthesis in stellate cells. These effects of endothelin-1 are mediated via distinct receptors (endothelin$_A$ and endothelin$_B$, respectively). However, inability of endothelin-1 to exert similar actions in stellate cells from cirrhotic liver suggests an indirect mechanism of its involvement in fibrosis.

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References

Armendariz-Borunda, J., Katai, H., Jones, C.M., Seyer, J.M., Kang, A.H., Raghow, R., 1993. Transforming growth factor β gene expression is transiently enhanced at a critical stage during liver regeneration after CCl_4 treatment. Lab. Invest. 69, 283–294.

Blomhoff, R., Wake, K., 1991. Perisinusoidal stellate cells of the liver: important roles in retinol metabolism and fibrosis. FASEB J. 5, 271–277.

Caligiuri, A., Glaser, S., Rodgers, R.E., Phinizy, J.L., Robertson, W., Papa, E., Pinzani, M., Alpini, G., 1998. Endothelin-1 inhibits secretin-stimulated ductal secretion by interacting with ET_A receptors on large colangiocytes. Am. J. Physiol. 275, G835–G846.

Castilla, A., Prieto, J., Fausto, N., 1991. Transforming growth factors β1 and α in chronic liver disease. N. Engl. J. Med. 324, 933–940.

Czaja, M.J., Weiner, F.R., Flanders, K.C., Giambrone, M.-A., Wind, R., Biempica, L., Zern, M.A., 1989. In vitro and in vivo association of transforming growth factor-β1 with hepatic fibrosis. J. Cell Biol. 108, 2477–2482.

Friedman, S.L., 1993. The cellular basis of hepatic fibrosis: mechanisms and treatment strategies. N. Engl. J. Med. 328, 1828–1835.

Gabriel, A., Kuddus, R.H., Rao, A.S., Watkins, W.D., Gandhi, C.R., 1998. Superoxide-induced changes in endothelin (ET) receptors in hepatic stellate cells. J. Hepatol. 29, 614–627.

Gabriel, A., Kuddus, R.H., Rao, A.S., Gandhi, C.R., 1999. Down-regulation of endothelin receptors by transforming growth factor β1 in hepatic stellate cells. J. Hepatol. 30, 440–450.

Gandhi, C.R., Stephenson, K., Olson, M.S., 1990. Endothelin, a potent peptide agonist in the liver. J. Biol. Chem. 265, 17432–17435.

Gandhi, C.R., Behal, R.H., Harvey, S.A.K., Nouchi, T.A., Olson, M.S., 1992a. Hepatic effects of endothelin: receptor characterization and endothelin-induced signal transduction in hepatocytes. Biochem. J. 287, 897–904.

Gandhi, C.R., Stephenson, K., Olson, M.S., 1992b. A comparative study of endothelin- and platelet-activating factor-mediated signal transduction and prostaglandin synthesis in rat Kupffer cells. Biochem. J. 281, 485–492.

Gandhi, C.R., Kang, Y., De Wolf, A., Madariaga, J., Aggarwal, S., Scott, V., Fung, J., 1996a. Altered endothelin homeostasis in patients undergoing liver transplantation. Liver Transplant. Surg. 2, 362–369.

Gandhi, C.R., Sproat, L.A., Subbotin, V.M., 1996b. Increased hepatic endothelin-1 levels and endothelin receptor density in cirrhotic rats. Life Sci. 58, 55–62.

Gandhi, C.R., Nemoto, E.M., Watkins, S.C., Subbotin, V.M., 1998. An endothelin receptor antagonist TAK-044 ameliorates carbon tetrachloride-induced acute liver injury and portal hypertension in rats. Liver 18, 39–48.

Geerts, A., DeBleser, P., Hautekeete, M.L., Niki, T., Wisse, E., 1994. Fat-storing (Ito) cell biology. In: Arias, I.M., Boyer, J.L., Fasuto, N., Jakoby, W.B., Schachter, D.L., Shafritz, D.A. (Eds.), The Liver: Biology and Pathobiology. Raven Press, New York, pp. 819–838.

Hahn, A.W.A., Resink, T.J., Bernhardt, J., Ferracin, F., Buhler, F.R., 1991. Stimulation of autocrine platelet-derived growth factor AA-homodimer and transforming growth factor β in vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 178, 1451–1458.

Housset, C., Rockey, D.C., Bissell, D.M., 1993. Endothelin receptors in rat liver: lipocytes as a contractile target for endothelin 1. Proc. Natl. Acad. Sci. U. S. A. 90, 9266–9270.

Ihara, M., Noguchi, K., Saeki, T., Fukuroda, T., Tsuchida, S., Kimura, S., Fukami, T., Ishikawa, K., Nishikibe, M., Yano, M., 1992. Biological profiles of highly potent novel endothelin antagonists selective for the ETA receptor. Life Sci. 50, 247–255.

Kawada, N., Tran-Thi, T.-A., Klein, H., Decker, K., 1993. The contraction of hepatic stellate (Ito) cells stimulated with vasoactive substances: possible involvement of endothelin 1 and nitric oxide in the regulation of the sinusoidal tonus. Eur. J. Biochem. 213, 815–823.

Kuddus, R.H., Subbotin, V.M., Nalesnik, M.A., Rao, A.S., Gandhi, C.R., 2000. Enhanced synthesis and reduced metabolism of endothelin-1 (ET-1) by hepatocytes — an important mechanism of increased endogenous levels of ET-1 in liver cirrhosis. J. Hepatol., in press.

Laura, F., Chinet, T., Robert, B., Carayon, A., Balladur, P., Mergey, M., Paul, A., Poupon, R., Capeau, J., Barbu, V., Housset, C., 1998. Endothelin-1 is synthesized and inhibits cyclic adenosine monophosphate-dependent anion secretion by an autocrine/paracrine mechanism in gallbladder epithelial cells. J. Clin. Invest. 101, 2881–2888.

Leivas, A., Jimenez, W., Bruix, J., Boix, L., Bosch, J., Arroyo, V., Rivera, F., Rodes, J., 1998. Gene expression of endothelin-1 and ET_A and ET_B receptors in human cirrhosis: relationship with hepatic hemodynamics. J. Vasc. Res. 35, 186–193.

Mallat, A., Preaux, A.-M., Serradeil-Le Gal, C., Raufaste, D., Gallois, C., Brenner, D.A., Bradham, C., Maclouf, J., Fouassier, L., Dhumeaux, D., Mavier, P., Lotersztajn, S., 1996. Growth inhibitory properties of endothelin-1 in activated human hepatic stellate cells: a cyclic adenosine monophosphate-mediated pathway. J. Clin. Invest. 98, 2771–2778.

Nakata, K., Shibayama, Y., 1987. Hepatic vascular resistance in liver cirrhosis. In: Tsuchiya, M., Asano, M., Mishima, Y., Oda, M. (Eds.), Microcirculation — An Update vol. 2 Elsevier, New York, pp. 339–344.

Nudel, U., Zakut, R., Shani, M., Neuman, S., Levy, Z., Yaffer, D., 1983. The nucleotide sequence of the rat cytoplasmic beta-actin gene. Nucleic Acids Res. 11, 1759–1771.

Pinzani, M., Abboud, H.E., 1991. Liver fat-storing cells, polypeptide growth factors and the progression of chronic liver inflammation and fibrosis. In: Gentilini, P., Dianzani, M.U. (Eds.), Experimental and Clinical Hepatology. Elsevier, pp. 63–75.

Pinzani, M., Failli, P., Ruocco, C., Casini, A., Milani, S., Baldi, E., Giotti, A., Gentilini, P., 1992. Fat-storing cells as liver-specific pericytes: spatial dynamics of agonist-stimulated intracellular calcium transients. J. Clin. Invest. 90, 642–646.

Pinzani, M., Milani, S., DeFranco, R., Grappone, C., Caligiuri, A., Gentilini, A., Tosti-Guerra, C., Maggi, M., Failli, P., Ruocco, C., Gentilini, P., 1996. Endothelin 1 is overexpressed in human cirrhotic liver and exerts multiple effects on activated hepatic stellate cells. Gastroenterology 110, 534–548.

Poo, J.-L., Jiminez, W., Munoz, R.M., Bosch-Marce, M., Bordas, N., Morales-Ruiz, M., Perez, M., Deulofeu, R., Sole, M., Arroyo, V., Rodes, J., 1999. Chronic blockade of endothelin receptors in cirrhotic rats: hepatic and hemodynamic effects. Gastroenterology 116, 161–167.

Rieder, H., Ramadori, G., Meyer zum Buschenfelde, K.H., 1991. Sinusoidal endothelial liver cells in vitro release endothelin — augmentation by transforming growth factor β and Kupffer cell-conditioned media. Klin. Wochenschr. 69, 387–391.

Rockey, D.C., Chung, J.J., 1996. Endothelin antagonism in experimental hepatic fibrosis. J. Clin. Invest. 98, 1381–1388.

Sakurai, T., Yanagisawa, M., Inoue, A., Ryan, U.S., Kimura, S., Mitsui, Y., Goto, K., Masaki, T., 1991. cDNA cloning, sequence analysis and tissue distribution of rat preproendothelin-1 mRNA. Biochem. Biophys. Res. Commun. 175, 44–47.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning — A Laboratory Manual. 2nd edn. Cold Spring Harbor Laboratory Press, New York, NY.

Shina, S., Shiratori, Y., Kawase, T., Sugimoto, T., 1989. Increased collagen synthesis by fat-storing cells exposed to superoxide — possible mechanism of hepatic fibrosis during inflammation. In: Wisse, E., Knook, D.L., Decker, K. (Eds.), Cells of Hepatic Sinusoids vol. 2 Kupffer Cell Foundation, pp. 52–56.

Tarada, V., Tomita, K., Nonoguchi, H., Marumo, F., 1992. Different localization of two types of endothelin receptor mRNA in microdissected rat segments using reverse transcription and polymerase chain reaction assay. J. Clin. Invest. 90, 107–112.

Tran-Thi, T.A., Kawada, N., Decker, K., 1993. Regulation of endothelin-1 action on the perfused rat liver. FEBS Lett. 318, 353–357.

Williams, D.L. Jr., Jones, K.L., Pettibone, D.J., Lis, E.V., Clineschmidt, B.V., 1991. Sarafotoxin S6c: an agonist which distinguishes between endothelin receptor subtypes. Biochem. Biophys. Res. Commun. 175, 556–561.

Zhang, J.X., Bauer, M., Clemens, M.G., 1995. Vessel- and target-specific actions of endothelin-1 and endothelin-3 in rat liver. Am. J. Physiol. 269, G269–G277.