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Effects of Endothelin-1 and Angiotensin-II on Extracellular Matrix Metabolism in a Rat Mesangial Cell Line, CRL-2573

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The profibrogenic role of endothelin-1 (ET-1) and angiotensin-II (AT-II) in renal fibrosis remains disputed. We therefore studied the effect of ET-1 and AT-II on rat mesangial cells (CRL-2573), the major source of extracellular matrix proteins in the kidney. ET-1 stimulated DNA synthesis assessed by BrdU uptake in microtiter tetrazolium assay in a dose dependent manner, but AT-II did not. Proliferation of the mesangial cells by ET-1 was suppressed by 15% with BQ123, an endothelin receptor A antagonist, which suggested a significant role of the endothelin receptor A on growth stimulation. In the mesangial cells, ET-1 increased the mRNA expressions of procollagen α1 (I), transforming growth factor-β_1, connective tissue growth factor, tissue inhibitor of metalloproteinase-1 and matrix metalloproteinase-13 in dose dependent manners, whereas AT-II had exerted no effect on the expression of these mRNAs. These data suggested that ET-1 might be involved in renal fibrosis through the mesangial cell proliferation and/or the increase in fibrogenic cytokines.

Key words: angiotensin-II; endothelin-1; extracellular matrix metabolism; mesangial cell

Renal fibrosis and nephrosclerosis are pathological states common to nephropathy of various types, such as glomerulonephritis, diabetic nephropathy and hypertensive nephropathy. In various diseases, accumulation of extracellular matrix, that is, progression of renal fibrosis causes further deterioration of renal function (Sun et al., 2000; Boffa et al., 2003). It has become increasingly clear that various cytokines and vasoactive factors are involved in extracellular matrix accumulation (Eddy, 2000; Yao et al., 2001; Mishra et al., 2003). Among the vasoactive factors, endothelin-1 (ET-1) and angiotensin-II (AT-II) have potent vascular smooth muscle-contracting activity (Yanagisawa et al., 1988), and promote cell proliferation and protein synthesis. Indeed, overexpression of ET-1 in transgenic mice causes renal interstitial fibrosis (Hocher et al., 1997), glomerulosclerosis (Herman et al., 1998; Hocher et al., 1997), renal cyst formation (Hocher et al., 1997, 2003), and arteriolosclerosis (Boffa et al., 2001, 2003). By contrast, ET-1 antagonists attenuate progressive glomerulosclerosis in vivo (Boffa et al., 1999, 2001; Yao et al., 2001). Similarly, AT-II plays an important role in the development of renal fibrosis (Sun et al., 2000; Mezzano et al.,

Abbreviations: AT-II, angiotensin-II; BrdU, bromodeoxyuridine; CTGF, connective tissue growth factor; DMEM, Dulbecco's modified Eagle medium; ECE, endothelin-converting enzyme; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; ET-1, endothelin-1; ETAR, endothelin A receptor; ETBR, endothelin B receptor; FBS, fetal bovine serum; MMP-13, matrix metalloproteinase-13; MTT, microtiter tetrazolium; OD, optical density; PBS, phosphate buffered saline; TGF-β_1, transforming growth factor-beta 1; TIMP-1, tissue inhibitor of metalloproteinases-1

2001; Boffa et al., 2003). Rats chronically infused with AT-II develop tubulointestitial injuries with tubular atrophy and dilation, cast formation and intestinal fibrosis (Hocher et al., 1997; Mezzano et al., 2001). It has been shown that ET-1 and AT-II are involved in the progression of various pathological states, such as acute renal failure, chronic renal failure and chronic heart failure (Benigni et al., 2000; Muller et al., 2002). However, much of their association with renal matrix metabolism remains unclear. In this study, we examined the effects of ET-1 and AT-II on cell proliferation and matrix metabolism in mesangial cells, which are the main cells producing extracellular matrix in the kidney (Schnaper et al., 1996; Yao et al., 2001; Sorokin et al., 2003).

Materials and Methods

Materials

CRL-2573, a rat mesangial cell line was purchased from ATCC (Manassas, VA). BQ123, an antagonist for endothelin A receptor (ETAR), and BQ788, for endothelin B receptor (ETBR), as well as ET-1, were purchased from Alexis Biochemicals (San Diego, CA). A bromodeoxyuridine (BrdU) colorimetric cell proliferation enzyme-linked immunosorbent assay kit was purchased from (Roche Diagnostics, Mannheim, Germany). A cell count kit-8 was purchased from Dojindo Laboratories (Kumamoto, Japan). The primers for real-time polymerase chain reaction (PCR) were synthesized at Nihon Gene Research Laboratories (Sendai, Japan), and Superscript II RNaseH(–) reverse transcriptase was purchased from Invitrogen (Carlsbad, CA). Random hexamers and oligo (dT) primers were purchased from Promega (Tokyo, Japan).

Cell culture

A cell pellet was suspended in Dulbecco's modified Eagle medium (DMEM; Gibco-BRL; Grand Island, NY), containing 200 U/mL penicillin G sodium (Gibco-BRL), 200 μg/mL streptomycin sulfate (Gibco-BRL), 0.25 μg/mL amphotericin B (Gibco-BRL) and 10% fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS). Incubation was performed in a humidified atmosphere of air/CO_2 (95:5) in an incubator maintained at 37˚C. The cells were subcultured in DMEM with 10% FBS, penicillin G sodium (200 U/mL), streptomycin sulfate (200 μg/mL) and amphotericin B (0.25 μg/mL), and the medium was changed every 24 h.

Measurement of DNA synthesis and cell multiplication

Cell multiplication was measured by MTT assay. DNA synthesis was measured as the incorporation of BrdU into DNA. Cells were plated in 96-well plates at a density of 2×10^3 cells/well, 4×10^3 cells/well, 8×10^3 cells/well or 16×10^3 cells/well in DMEM with 15% FBS. After 24 h, the cells were washed with phosphate buffered saline (PBS; Gibco-BRL) (pH 7.2) and placed in DMEM with 0.125% FBS for 6 h. This medium was removed and the cells were placed in fresh DMEM with 0.125% FBS and BrdU (2×10^{-5} L/well). After 6 h of incubation with BrdU at 37˚C, the BrdU incorporated into DNA was measured by enzyme-linked immunosorbent assay (ELISA). After 2 h of incubation with the cell counting kit-8 solution (1×10^{-5} L/well), the absorbance of formosan, which is produced by WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt], the main component of cell counting kit-8, was measured at 450 nm using microplate reader. Subsequent experiments were performed at 4×10^3 cells/well. The cells were plated in 96-well dishes at a density of 4×10^3 cells/well in DMEM with 15% FBS for 24 h. After the cells were washed with PBS, they were placed in fresh DMEM with 0.125% FBS containing ET-1 or AT-II at various concentrations (10^{-6} M, 10^{-8} M or 10^{-10} M). After 6 h of incubation with BrdU (2×10^{-5} L/well) or cell counting kit-8 solution ($1 \times$

10^{-5} L/well) at 37˚C, the BrdU uptake or formosan was measured. To assess the effect of ETAR or ETBR on DNA synthesis and cell proliferation, the cells were incubated with ETAR antagonist BQ123 (10^{-6} M) or ETBR antagonist BQ788 (10^{-6} M).

Expression of matrix related genes

Cell culture with ET-1 and AT-II

The cells were plated on 25 cm^2 dishes at a density of 4×10^3 cells/dish in DMEM containing 10% FBS. After 48 h of culture, the cells were washed with PBS and placed in DMEM with 0.125% FBS for 12 h. Cells were incubated for 12 h in fresh DMEM with 0.125% FBS containing ET-1 or AT-II at various concentrations (0, 10^{-8} and 10^{-6} M), and the total RNA was extracted. To assess the effect of ETAR or ETBR on expression of procollagen α1 (I), transforming growth factor-beta1 (TGF-β_1), connective tissue growth factor (CTGF), tissue inhibitor of metalloproteinases-1 (TIMP-1), matrix metalloproteinase-13 (MMP-13), ET-1 and endothelin-converting enzyme (ECE) mRNA, the cells were incubated using BQ123 (10^{-6} M) or BQ788 (10^{-6} M) with ET-1 (10^{-6} M).

RNA isolation and reverse transcription

Total RNA of the confluent CRL-2573 in 25 cm^2 dishes was extracted using the acid-phenol guanidium method. Total RNA (1×10^{-5} L) was reverse transcripted in a final volume of 2×10^{-5} L containing 1× RT buffer (5×10^{-4} M each dNTP, 3×10^{-3} M $MgCl_2$, 7.5×10^{-2} M KCl, 5×10^{-2} M Tris-HCl, pH 8.3), 10 units of Superscript II RNaseH(–) reverse transcriptase, 50 mg/ L random hexamers 1×10^{-6} L, 1×10^{-7} M oligo (dT) primer 5×10^{-7} L.

Real-time PCR with light cycler system

We used real-time PCR to quantify mRNAs of procollagen α1 (I), TGF-β_1, CTGF, TIMP-1, MMP-13, ET-1 and ECE mRNA. Real-time PCR was performed using a Light Cycler System (Roche, Tokyo) and a Light Cycler-FastStart DNA Master SYBR Green I kit (Roche). The appropriate size of each PCR product was verified on an ethidium bromide-stained 3% agarose gel. Conditions for real-time PCR were as follows: 10 min denaturing at 95˚C, 10 s annealing at 55˚C, and 3 s to 6 s amplification at 72˚C. Forty-five cycles were performed and then followed by melting-curve analysis to verify the correctness of the amplification. Amplification efficiency was determined for all primer pairs. The efficiency of the PCR was determined by analyzing a dilution series of control cDNA. Using the analysis mode of the Light Cycler software package, the slope of log concentrations of the control cDNA dilutions was calculated. Efficiency was calculated as follows: $Eff = 10^{-1/slope}$.

The measured efficiencies were used for the calculation of the mRNA values. β_2-Microglobulin, a stable housekeeping gene in this cell system, was analyzed in parallel with every PCR. The resulting β_2-microglobulin values were used as the standard for the presentation of the mRNA data of the different transcriptions. Analysis of the data was performed according to the manufacturer's instructions, using Light Cycler software version 3.5.3. Oligonucleotide primers were designed according to the published sequences (Table 1).

Statistical analysis

Statistical analysis was performed using the Statview Version 5.0 (SAS Institute, Cary, NC). Significance was calculated using the unpaired *t*-test. Statistical significance was regarded as $P < 0.05$.

Results

Effects of ET-1 and AT-II on DNA synthesis and multiplication of CRL-2573 cells

In the cells, BrdU incorporation into DNA increased with cell density: 1.04 ± 0.09 OD at 450 nm at 2×10^3 cells/well, 1.51 ± 0.16 OD at 4×10^3

Table 1. Primer nucleotide-sequence

β_2-Microglobulin	Forward primer	*5′-CCGATGTATATGCTTGCAGAGTTAA-3′*
	Reverse primer	*5′-CAGATGATTCAGAGCTCCATAGA-3′*
Procollagen $\alpha 1$(I)	Forward primer	*5′-TCCGGCTCCTGCTCCTCTTA-3′*
	Reverse primer	*5′-GTATGCAGCTGACTTCAGGG-3′*
Transforming growth factor-beta 1 (TGF-β_1)	Forward primer	*5′-TTGCCCTCTACAACCAACAG-3′*
	Reverse primer	*5′-GGCTTGCGACCCACGTAGTA-3′*
Connective tissue growth factor (CTGF)	Forward primer	*5′-TCCCTGCGACCCACACAA-3′*
	Reverse primer	*5′-TGCAACTGCTTTGGAAGGAC-3′*
Tissue inhibitor of metalloproteinases-1 (TIMP-1)	Forward primer	*5′-TGCTCTTGTTGCTATCATTGATAGCTT-3′*
	Reverse primer	*5′-CGCTGGTATAAGGTGGTCTCGAT-3′*
Matrix metalloproteinase-13 (MMP-13)	Forward primer	*5′-GGAAGACCCTCTTCTTCTCA-3′*
	Reverse primer	*5′-TCATAGACAGCATCTACTTTGTC-3′*
Endothelin-1 (ET-1)	Forward primer	*5′-CAAGCTGGGAAAGAAGTGT-3′*
	Reverse primer	*5′-ATGTGCTCGGTTGTGTATC-3′*
Endothelin-converting enzyme (ECE)	Forward primer	*5′-AGCTCTTCTTCCTGGGATT-3′*
	Reverse primer	*5′-CCAGACTTCGCATTTGTG-3′*
Angiotensin-II (AT-II) type 1a receptor	Forward primer	*5′-TGTTCTGACCTTCTTGGATG-3′*
	Reverse primer	*5′-AAACACACTACGGAGCTTCT-3′*
Endothelin A receptor (ETAR)	Forward primer	*5′-ACCAGTCGAAAAGCCTCA-3′*
	Reverse primer	*5′-TCTGCACAGGGTTAGTTCA-3′*
Endothelin B receptor (ETBR)	Forward primer	*5′-AACTTCCGCTCGAGCAAT-3′*
	Reverse primer	*5′-TCCCGAGGCTTCATTCAT-3′*

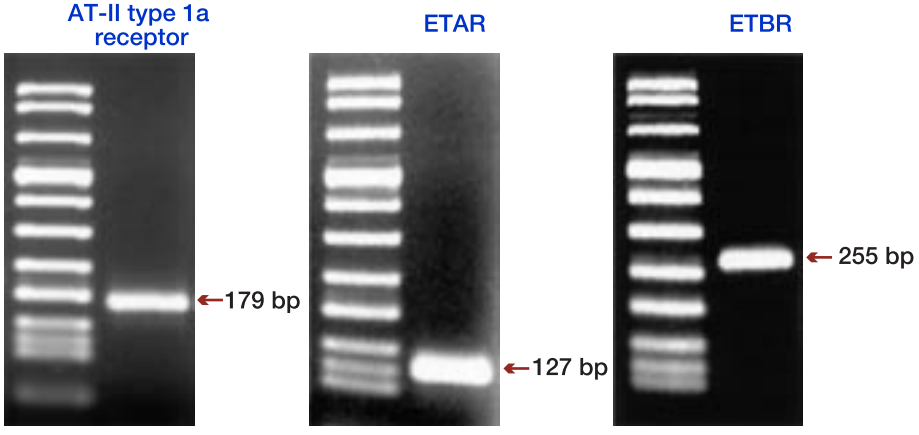

Fig. 1. Reverse transcription-PCR. Expressions of mRNA for AT-II type 1a receptor, ETAR and ETBR were found by 3% agarose gel electrophoresis.

cells/well, 1.81 ± 0.09 OD at 8 × 10^3 cells/well and 2.08 ± 0.06 OD at 16 × 10^3 cells/well. Subsequent experiments were performed at 4 × 10^3 cells/well. The expression of mRNA for the AT-II type 1a receptor, for ETAR and for ETBR in mesangial cells was confirmed by electrophoresis on 3% agarose gels (Fig. 1). The addition of ET-1 to CRL-2573 cells increased DNA synthesis by

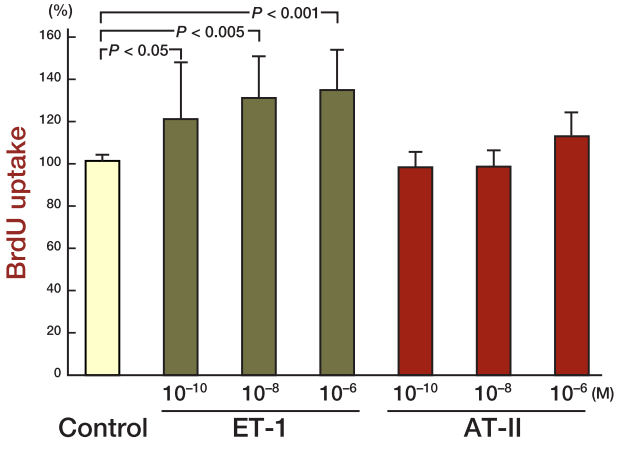

Fig. 2. Effects of ET-1 or AT-II on DNA synthesis of CRL-2573. BrdU incorporation into DNA was measured in 4.0 × 10^3 cells/well over 6 h at various concentrations of ET-1 or AT-II. The mean value of absorbance for controls was set at 100%. Data represent mean ± SD (8 wells).

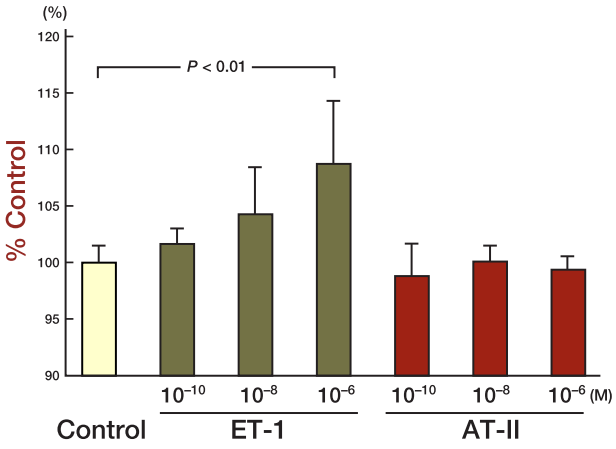

Fig. 3. Effects of ET-1 or AT-II on CRL-2573 multiplication. Cell number was measured in 4.0 × 10^3 cells/well over 6 h at various concentrations of ET-1 or AT-II, using a cell count kit-8. The mean value of absorbance for was set as 100%. Data represent mean ± SD.

20% at 10^{-10} M, 29% at 10^{-8} M and 33% at 10^{-6} M (Fig. 2) and the number of cells by 2% at 10^{-10} M, 4% at 10^{-8} M and 9% at 10^{-6} M (Fig. 3) in a dose-dependent manner. AT-II did not affect DNA synthesis (–2% at 10^{-10} M, –2% at 10^{-8} M and 10% at 10^{-6} M) or multiplication (–2% at 10^{-10} M, 0% at 10^{-8} M and –1% at 10^{-6} M) of CRL-2573 (Figs. 2 and 3). The ETAR antagonist, BQ123 inhibited the ET-1 enhanced multiplication of CRL-2573 by 11%. Similarly, the ETBR antagonist BQ788, inhibited the ET-1 enhanced multiplication of CRL-2573 by 8% (Fig. 4).

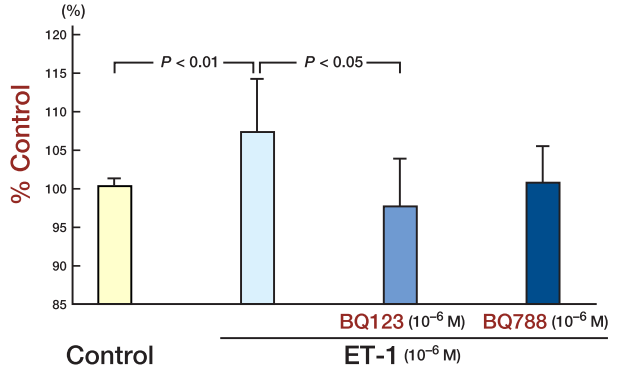

Fig. 4. Effects of endothelin receptor antagonists on cell number of CRL-2573. Cell number was measured in 4.0 × 10^3 cells/well over 6 h at 10^{-6} M of ET-1. The cells were treated with BQ123, ETAR antagonist, or BQ788, ETBR antagonist, at a concentration of 10^{-6} M in the presence of 10^{-6} M of ET-1.

Effects of ET-1 and AT-II on mRNA expression of CRL-2573 cells

ET-1 at concentration of 10^{-8} M and 10^{-6} M increased the expression of mRNA for procollagen α1 (I) (1.27- to 1.57-fold), TGF-β_1 (1.83- to 1.90-fold), TIMP-1 (1.60- to 1.80-fold) and MMP-13 (1.76- to 2.62-fold), compared with that of controls (Figs. 5a to e). Meanwhile, AT-II had no affect on these mRNA expressions (Figs. 5a to e).

Both BQ123 and BQ788 suppressed the ET-1 enhanced expression of mRNA for procollagen α1 (I), TGF-β_1 and MMP-13 by 54%, 73% and 74% respectively. While, BQ123 completely blocked CTGF mRNA expression enhanced by ET-1, but BQ788 exerted no effect. BQ123 did not affect the expression of mRNA for TIMP-1, but BQ788 tended to suppress ET-1 enhanced TIMP-1 mRNA

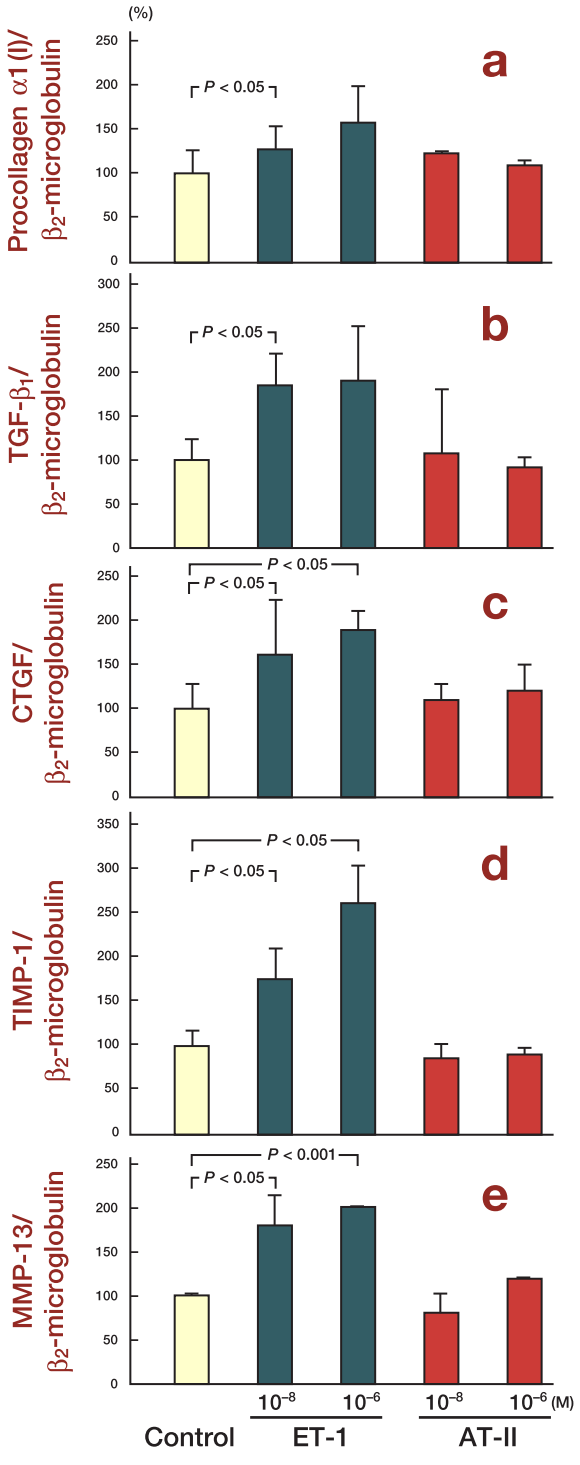

Fig. 5. Effects of ET-1 or AT-II on CRL-2573 matrix-related gene expression. The expressions of mRNA for procollagen α1 (I) (**a**), TGF-β1 (**b**), CTGF (**c**), TIMP-1 (**d**) and MMP-13 (**e**) were determined by real-time quantitative PCR. Data were normalized to β2-microglobulin mRNA levels. Error bars represent mean ± SD of 4 independent experiments.

expression (Table 2). ET-1 at a concentration of 10^{-6} M significantly increased ET-1 mRNA expression 1.77 fold and ECE mRNA expression 1.38 fold, suggesting that it may mediate biological action through an autocrine loop (Fig. 6).

Discussion

In this study, we investigated the expression of ETAR and ETBR in rat mesangial cells (CRL-2573). ET-1 has been reported to promote the contraction, hypertrophy and proliferation of cells, and the synthesis of the extracellular matrix (Herman et al., 1998; Duan et al., 1999; Chen et al., 2001). The addition of ET-1 to CRL-2573 cells increased DNA synthesis and cell numbers, but the rates of increase were not very high. ET-1 stimulates mesangial cell proliferation in an autocrine fashion as well as through the paracrine loop, where our data indicates that ET-1 increased the expression of ET-1 and ECE. The proliferating effects of ET-1 in mesangial cells are primarily via ETAR (Benigni, 2000; Hocher et al., 2003), although there are data suggesting that ETBR mediates human mesangial cell proliferation (Herman et al., 1998; Benigni, 2000). In the present study, although antagonist administration apparently did not change DNA synthesis, the administration of the ETAR antagonist suppressed cell proliferation, strongly suggesting that ETAR mediates cell proliferation. More interestingly, the addition of ET-1 also increased the expression of mRNA for ET-1 and ECE, suggesting that ET-1 functions in an autocrine and paracrine manner. This indicates that much higher levels of locally produced ET-1 than those in circulating plasma promote tissue fibrosis.

A major role of ET-1 in mediating renal fibrosis has recently been pointed out. Antagonism of ET-1 receptors delayed the evolution of renal failure and increased survival rate in rats with renal mass reduction (Hocher et al., 2003; Boffa et al., 2001; Goddard et al., 2004). Moreover, the use of an endothelin receptor antagonist improved

Table 2. Effects of ET-1 and endothelin receptor antagonist on CRL-2573 matrix-related gene expression

mRNA	Control	ET-1 (10^{-6} M)	ET-1 (10^{-6} M)	
			BQ123 (10^{-6} M)	BQ788 (10^{-6} M)
Procollagen α1 (I)	1.01 ± 0.23	2.16 ± 0.17	1.18 ± 0.24	0.91 ± 0.17
	$P < 0.01$	$P < 0.05$		
		$P < 0.01$		
TGF-β_1	0.52 ± 0.25	1.02 ± 0.22	0.75 ± 0.09	0.68 ± 0.27
	$P < 0.05$		$P < 0.05$	
CTGF	0.19 ± 0.08	0.36 ± 0.04	0.19 ± 0.08	0.29 ± 0.19
	$P < 0.05$	$P < 0.05$		
TIMP-1	0.35 ± 0.11	1.53 ± 0.09	1.57 ± 0.12	1.22 ± 0.23
	$P < 0.05$			
MMP-13	0.51 ± 0.16	0.95 ± 0.15	0.70 ± 0.16	0.71 ± 0.12
	$P < 0.05$	$P < 0.05$		
		$P < 0.05$		

Data are mean ± SD of 4 independent experiments.
Each mRNA was normalized to β_2-microglobulin mRNA.

renal structural damage and reduced extracellular matrix formation (Benigni, 2000; Boffa et al., 2001; Hocher et al., 2003; Chade et al., 2003). In the present study, ET-1 increased procollagen α1 (I) gene expression in a dose-dependent manner. In addition, ET-1 dose-dependently increased the expression of TGF-β_1, as well as CTGF. TGF-β_1 promotes fibroblast proliferation and increases extracellular matrix production (Gomez-Garre et al., 1996; Boffa et al., 2003; Sorokin and Kohan, 2003). TGF-β_1 is thought to play a central role in the progression of renal fibrosis. CTGF is located downstream from TGF-β_1 and acts profibrogenically. As clearly indicated in these findings, ET-1 increases the production of the collagen-synthesizing system. According to one study, ET-1 increases the expression of the fibronectin and procollagen α1 (I) and (IV) genes in mesangial cells (Gomez-Garre et al., 1996). ET-1 also increased the collagen-degrading system inhibitor TIMP-1, suggesting that collagen degradation may be inhibited, thereby promoting fibrosis, although increased MMP-13 mRNA gene expression was observed.

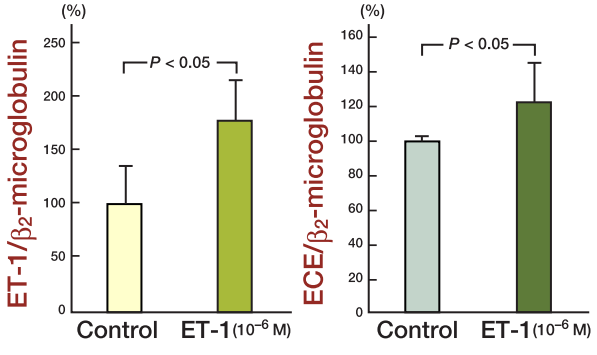

Fig. 6. Effects of ET-1 on ET-1 and ECE transcript levels of CRL-2573. The expressions of mRNA for ET-1 and ECE were determined by real-time PCR. The data were normalized by β_2-microglobulin mRNA. Error bars represent mean ± SD of four independent experiments.

In rat models of mesangial proliferative glomerular nephritis, ETAR blocker reduced mesangial cell proliferation, and bosentan (ETAR/ETBR blocker) improved renal function and reduced mesangial cell ET-1 mRNA levels. There is abundant evidence in vivo that ET-1 antagonists ameliorate glomerular injury in animal models with diabetic nephropathy (Nakamura et al., 1995; Benigni, 2000). Bosentan reduced albuminuria, increased glomerular filtration

rate and ameliorated the mesangial matrix in diabetic rats (Benigni et al., 1998, 2000). Our findings also indicated that the increased expression of procollagen α1 (I) induced by ET-1 was attenuated by both ETAR and ETBR blockers (BQ123 and BQ788). However, the ET-1 enhanced expression of TGF-β_1 and TIMP-1 was reduced by ETBR blocker. The causes of these receptor-related differences are not clear. Since nephropathy is improved by the administration of ETAR antagonists, ETAR has been considered responsible. As the data we obtained in the present study suggest, ETBR is also involved in increased production of ECM. These results may reflect our culture conditions for the cell line CRL-2573, necessitating further study. To use ET-1 antagonists as therapeutic agents, it is necessary to compare the effects of an ETAR antagonist and a combination of ETAR and ETBR antagonists.

On the other hand, the addition of AT-II did not change the cell growth or the gene expression of procollagen α1 (I) or TGF-β_1, although the expression of AT-II type 1a receptor mRNA was observed in the cell line. Therefore, there may be abnormalities in the receptors or the signal transduction system, and this cell line would not be suitable for the study of the angiotensin system.

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