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Full Length Research Paper

Inhibitory effect of human angiogenesis inhibitor arresten on TGF-β1 expression in biliary endothelial cell

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To investigate the inhibitory effect of arresten on TGF-β1 expression in biliary endothelial cells (BECs), BECs was collected by primary culture from human biliary scar tissue. The cells were cultured by different concentrations arresten. 3-(4,5-dimethythiazol-. 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to measure the proliferation rate of BECs, and the mRNA and protein expression of TGF-β1 were measured by semi-quantitative real time-polymerase chain reaction (RT-PCR) and western-blot, respectively. Proliferation of BECs was not inhibited by arresten. However, the mRNA and protein expression of TGF-β1 in BECs were decreased after it was incubated with arresten, with dose-dependent. Arresten cannot inhibit BECs proliferation, but it can inhibit the mRNA and protein expression of TGF-β1 of BECs, supposing it maybe play an important role in inhibiting biliary stricture.

Key words: Biliary endothelial cells (BECs), arresten, TGF-β1.

INTRODUCTION

Biliary stricture is a very common complication derived from varied iatrogenic bile duct injury, such as biliary tract exploration, biliary anastomosis and liver transplantation (Lillemoe et al., 2000; Pasha et al., 2007). It has been the complex and intractable difficult problem in biliary tract surgery field. Recently, there is evident that fibroblast over-proliferation is one main cause for occurrence and development of biliary stricture, and this proliferation may be induced and regulated by TGF-β (Xu et al., 2003; Simmons et al., 2002). Some other studies indicated that biliary endothelial cells (BEC), as major cell structure in biliary tract, could secrete TGF-β1 to participate biliary stricture development in an indirect manner (Geng et al., 2005). Therefore, the growth and development of biliary stricture may be inhibited through inhibiting the expression level of TGF-β1 in BECs. Arresten has been demonstrated as one endogenous angiogenesis inhibitor (Nyberg et al., 2005) and plays an inhibitory role in endothelial cell (Boosani et al., 2010). Hence, in this study, we aim to investigate the inhibitory role of arresten in BECs proliferation and TGF-β1 expression.

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Abbreviation: BECs, Biliary endothelial cells.

MATERIALS AND METHODS

The required reagents and instruments in our study were listed as follows: TGF-β1 and β-actin primers (Sangon, Shanghai); anti-human TGF-β1 monoclonal antibody, horseradish peroxidase (HRP) coupled goat anti-rabbit IgG (second antibody) and related antibodies with BECs detection (Santa Cruz Biotechnology, USA); ECFTM Western-blot kit (Amersham, USA); Light Cycler (Roche Diagnostics, Germany); 3-(4,5-dimethythiazol-. 2-yl)-2,5-diphenyl tetrazolium bromide MTT and Trizol (GIBCO, UK); real time-polymerase chain reaction (RT-PCR) kit (TAKARA, Dalian).

In addition, arresten was obtained in our previous study and stored in our laboratory; lesion samples were collected from extrahepatic bile duct scar caused by benign biliary stricture. All samples we used originated from Sun Yat-Sen Memorial Hospital.

Primary human BECs collection

BECs were collected from patients' tissue sample with benign biliary stricture in the Second Affiliated Hospital, Sun Yat-sen University.

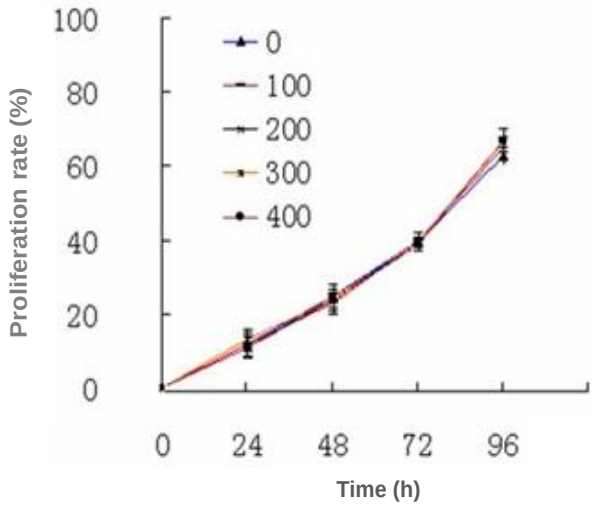

Figure 1. The effect of arresten on BECs proliferation rate.

BECs isolation procedure was performed as previously reported, with a 90% cell viability result (trypan blue exclusion) (Gigliozzi et al., 2004). The purity of BECs preparations was assessed by (1) γ-glutamyltransferase-positive staining, (2) glucose-6-phosphatase staining and RT-PCR for albumin (hepatocyte markers), (3) RT-PCR for fucose receptor (Kupffer cell marker), and (4) RT-PCR for von Willebrand factor (endothelial cell marker). All isolated cells were γ-glutamyltransferase-positive, whereas glucose-6-phosphatase-positive cells were absent and RT-PCR for albumin, fucose receptor and von Willebrand factor was negative in cell preparations, indicating absolute purity of BECs preparations.

Role of arresten in BECs proliferation

The BECs were randomly divided in to five groups based on the different arresten concentration gradient (0, 100, 200, 300 and 400 ng/ml). The BECs in exponential growth phase were suspended in complete medium and plated at a concentration of 1.0×10^4 cells/well in 96-well plates (each group with 15 replicated well). After incubation for 0, 24, 48, 72 and 96 h, cell proliferation rate was then detected by MTT assay. The procedure was as follows (Zheng et al., 2007). Briefly, 20 µl MTT (5 mg/ml) was added to each well, and the cells were incubated for 4 h. The culture medium was then removed, and 150 µl dimethyl sulfoxide (DMSO) was added to each well. After thorough shaking for 10 min, the absorbance of each well was read in an enzyme immunoassay instrument at a wavelength of 570 nm. Because absorbance is in proportion to the number of living cells in a sample, the MTT assay reflects the extent of cell proliferation. Growth curves were drawn according to the absorbance (each experiment was repeated for three times).

Semi-quantitative RT-PCR quantification of TGF-β1 mRNA

Total cell RNA was extracted from BECs in different concentration arresten culture using Trizol kit according to the manufacturer's instructions. RNA purity and concentration were determined by spectrophotometric absorbance at 260 and 280 nm. One-step RT-PCR was performed to quantify the TGF-β1 mRNA expression. The 50 µl PCR reaction mixture contained Rnase free H_2O 9 µl, 1.5 × reaction buffer 10 µl, 2.5 mM dNTPs 6 µl, 25 mM $Mn(Oac)_2$ 2 µl, P1 2 µl, P2 2 µl, β-actin-1 2 µl, β-actin-2 2 µl, 10 U/µl Rnase inhibitor 2 µl, 2.5 U/µl rTth DNA polymerase 2 µl and total RNA 2 µl. The PCR condition was incubated at 60°C for 30 min; initial denaturation at 94°C for 2 min; 40 cycles of amplification (94°C for 10 min and 60°C for 1.5 min) and finally 60°C for 7 min. The PCR primers used were as follows: TGF-β1, sense primer (P1) 5'-GCTTGAACCCAAGGAGACCGGAATA-3' and anti-sense (P2) 5'-ACCTCGACGTTGGGACTGA-3'. β-actin is an internal control to adjust the TGF-β1 amplification. The sense primer (β-actin-1) is 5'-CCTCTATGCAACACAGTGC-3' and the anti-sense primer (β-actin-2) is 5'-ATACTCCTGCTTGCTGATCC-3'. The amplification product was 310 and 410 bp, respectively. PCR products were separated by electrophoresis in 2% agarose gels, and were quantified by Eagle Eye II Video Imaging System (Stratagene, CA). TGF-β1 mRNA level was normalized to β-actin expression (relative expression).

Western blot quantification of TGF-β1 protein

BECs were lysed in Laemmli lysis buffer (NaCl 20 mmol/L, KCl 10 mmol/L, $MgCl_2$ 1.5 mmol/L, pH 7.4 Hepes 10 mmol/L and 1% Triton X-100). Protein lysates (50 mg/lane) were electrophoresed on 10% SDS-polyacrylamide gels and were transferred to nitrocellulose membranes. The filters were blocked with 5% nonfat dried milk in 1 × phosphate-buffered saline (PBS)/0.5% Tween 20 and were then incubated with the anti-human TGF-β1 antibody at 4°C for 24 h. HRP coupled goat anti-rabbit IgG was used as the secondary antibody, and the bands were developed using the enhanced chemiluminescence. Protein expression was detected by densitometric scanning on autoradiographs using the GD8000 gel image analysis system.

Statistics analysis

All data were analyzed by SPSS 10.0 and the results were measured by average ± standard deviation ($\bar{x} \pm s$). Two non-parametric tests, Kruskal-Wallis or Man-Whitney Test, were used for the comparison of different treatment groups. $P < 0.05$ was considered as statistically significant.

RESULTS

Effect of arresten on human BECs proliferation

MTT assay results showed that BECs proliferation rate was almost the same, although with different concentrations of arresten treatment and different times. There was no significant difference, which suggested that arresten has no inhibiting or stimulating effect on BECs proliferation (Figure 1).

Effect of arresten on TGF-β1 mRNA expression in BECs

RT-PCR results revealed that TGF-β1 mRNA expression was decreased upon arresten treatment, and with the arresten concentration raised, the TGF-β1 mRNA expression was gradually decreased (Figure 2). This indicated that arresten has an inhibitory effect on TGF-β1 mRNA expression and it is dose-dependent.

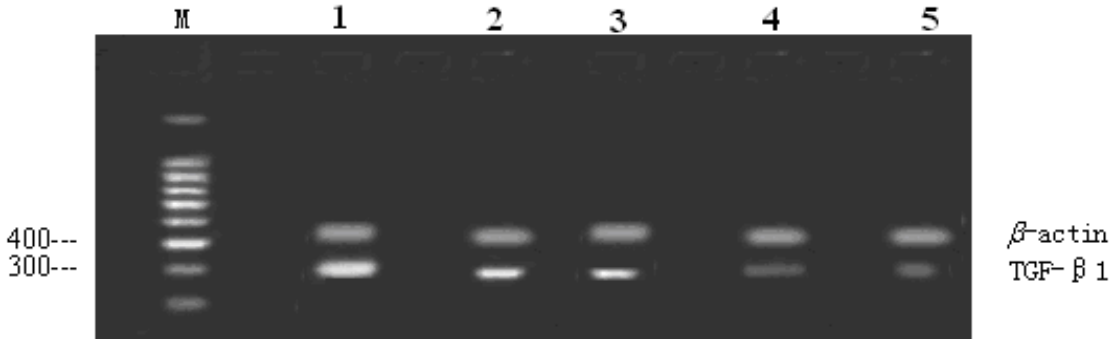

Figure 2. The effect of arresten on TGF-β1 mRNA expression. M: marker (bp); 1: 0 ng/ml arresten group; 2: 100 ng/ml arresten group; 3: 200 ng/ml arresten group; 4: 300 ng/ml arresten group; 5: 400 ng/ml arresten group.

Figure 3. The effect of arresten on TGF-β1 protein expression. 1: 0 ng/ml arresten group; 2: 100 ng/ml arresten group; 3: 200 ng/ml arresten group; 4: 300 ng/ml arresten group; 5: 400 ng/ml arresten group.

Effect of arresten on TGF-β1 protein expression in BECs

Western-blot analysis demonstrated that TGF-β1 protein expression was also decreased upon arresten treatment, and with the increase of arresten concentration, the TGF-β1 protein expression displayed a gradual decline illustrating that arresten has an inhibitory effect on TGF-β1 protein expression and it is dose-dependent (Figure 3).

DISCUSSION

Benign biliary stricture is a serious complication of biliary duct surgery and liver transplantation (Sharma et al., 2008), and if untreated, it would turn to repeated cholangitis, biliary cirrhosis, biliary colic and icterus (Tocchi et al., 1996; Scheiman et al., 2001).

TGF-β is a multifunctional cytokine involved in the cell growth, proliferation, differentiation, migration, adhesion and extracellular matrix formation (Chan et al., 2009; Van Geest et al., 2010). Almost all cells can express TGF-β and TGF-β receptor (Shevach, 2006; Hold et al., 2009). Recently, it has been demonstrated as one of the important growth factors associated with scar formation (Zhu and Li, 2011). TGF-β1, a member of TGF-β family, could exert a strong mitogenic effect to promote many cells division, reproduction and migration. Importantly, it is also a potent chemoattractant to induce angiogenesis and fibroblasts (Salib and Howarth, 2009). In fibroblasts, TGF-β1 could stimulate synthesis of extracellular matrix proteins, procollagen I and fibronectin to increase intasome and other membrane receptors (Campaner et al., 2006), on the other hand, it could strengthen cell-matrix interaction to recruit inflammatory related cells, such as monocytes-macrophages migration to wound sites (Barrientos et al., 2008). In addition, Desmouliere et al. (2005) found that TGF-β1 could induce α-smooth muscle actin expression to promote fibroblast-to-myofibroblast differentiation and collagen matrix contraction. Meanwhile, TGF-β1 decrease matrix degradation by inhibiting collagenase formation, increasing collagen enzyme inhibitors (including metalloprotease) and alpha-2 macroglobulin tissue inhibitor (Garrett et al., 2004). In a word, fibroblast correlated with a-smooth muscle actin (SMA) high expression stimulated by TGF-β1 migrates into the wound and contribute to the formation

of the myofibroblastic population of granulation tissue. They readily contract collagen gels *in vitro*. But if fibroblast over proliferates, it would turn to scar tissue. Geng et al. (2008) reported that the expression of TGF-β1 in stenotic bile duct is significantly higher than that in normal bile duct in autocrine or paracrine fashion. It was followed by the activation of TGF-β/Smad/CTGF signal transduction pathway to result in fibroblasts proliferation. Therefore, inhibition of TGF-β1 expression in bile duct cell would prevent stenotic bile duct formation. In our work, TGF-β1 was identified as high expression in BECs of benign biliary stricture scar tissue, which may play an important role in the progress of biliary stricture.

Arresten, as an angiogenesis inhibitor, is supposed to inhibit the proliferation of vascular endothelia cells. However, in our work, MTT assay results showed that BECs proliferation rate was almost the same, although with different concentration of arresten treatment and at different times, suggesting that arresten has no inhibiting or stimulating effect on BECs proliferation. Further study indicated that arresten inhibited TGF-β1 mRNA and protein expression in BECs, raising the possibility that it may decrease TGF-β1 expression by inhibiting BECs secretion. BEC is the main source of TGF-β1. Hence, arresten may inhibit fibroblasts proliferation induced by TGF-β1, with the expectation to inhibit the progression of biliary stricture. Our study also revealed that with the increase of arresten concentration, the inhibitory effect on TGF-β1 mRNA and protein expression increased and was dose-dependent, supposing that arresten could become a narrow-spectrum biological agent to cure biliary stricture. In conclusion, based on the aforementioned, arresten could be an effective biological agent to inhibit biliary stricture by decreasing TGF-β1 expression in BECs and further fibroblasts proliferation.

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