Effects of adrenomedullin gene overexpression on biological behavior of hepatic stellate cells

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Abstract

AIM: To investigate the effects of adrenomedullin (AM) gene overexpression on the biological characteristics of human hepatic stellate cells (hHSCs) by stable transfection.

METHODS: hHSCs which express low basal levels of AM were stably transfected with an expression construct containing rat AM gene or with an empty expression vector. Expression of AM in hHSCs was determined by reverse transcription (RT)-polymerase chain reaction (PCR) and radioimmunoassay (RIA). Cell proliferation was evaluated by 5-bromo-2'-deoxyuridine (BrdU) incorporation and immunocytochemistry. RT-PCR and Western blot were used to test the expression of procollagen types I and III. Protein expressions of interstitial collagenase (MMP-1), gelatinase (MMP-2) and tissue inhibitors of matrix metalloproteinases-2 (TIMP-2) were assessed by Western blot.

RESULTS: Two cell clones (A-2, A-8) transfected with the AM gene expressed higher levels of AM mRNA (non-transfected group: 0.86±0.11, empty vector group: 1.01±0.11, A-2 clone group: 1.44±0.08 and A-8 clone group: 1.36±0.05) and protein (12.31±0.17, 12.35±0.12, 12.56±0.06 and 12.62±0.07) (P<0.05). AM gene overexpression had inhibitory effects on cell proliferation of hHSCs (29.6%, 30.9%, 18.9% and 21.8%, respectively. P<0.05) and expression of procollagen type I (0.58±0.1, 0.48±0.11, 0.3±0.06 and 0.31±0.07 at mRNA level) (0.27±0.07, 0.3±0.06, 0.14±0.05 and 0.13±0.05 at protein level) (P<0.05) and procollagen type III (0.17±0.04, 0.15±0.03, 0.1±0.02 and 0.09±0.02 at mRNA level) (0.22±0.04, 0.2±0.03, 0.11±0.04 and 0.13±0.03 at protein level) (P<0.05). Compared with cells non-transfected (TIMP-2: 2.77±0.03, MMP-2: 0.5±0.04, MMP-1: 0.49±0.07) and transfected with empty vector (TIMP-2: 2.79±0.04, MMP-2: 0.48±0.03, MMP-1: 0.45±0.09), these two clones had lower expression levels of TIMP-2(A-2 clone group: 2.7±0.02 and A-8 clone group: 2.71±0.02) (P<0.05) and MMP-2(A-2 clone group: 0.15±0.05 and A-8 clone group: 0.13±0.04) (P<0.05) but displayed a higher expression level of MMP-1(A-2 clone group: 0.68±0.06 and A-8 clone group: 0.81±0.09) (P<0.05).

CONCLUSION: AM gene exerts negative influence to some extent on hHSCs by inhibiting proliferation and production of extracellular matrix (ECM) in addition to inducing MMP-1 expression.

INTRODUCTION

Liver fibrosis is the common pathological consequence of chronic hepatic diseases. Many factors may lead to hepatic fibrosis[10,11]. HSCs represent up to 15% of the resident cells of the liver and play a pivotal role in hepatic fibrosis[9]. In response to liver injury, quiescent HSCs can be activated and differentiated into hepatic myofibroblast cells characterized by elevated proliferation rate, increased secretion of extracellular matrix (ECM) proteins and TIMPs, declined expression of MMPs and secretion of a wide range of cytokines. Therefore, it is generally accepted that HSCs are the target of anti-fibrotic strategies of the liver.

AM, a peptide isolated from human pheochromocytoma, is a member of the calcitonin gene-related peptide (CGRP) family[4,5]. AM is produced by a variety of cells such as vascular endothelial cells, smooth muscle cells, fibroblasts and mesangial cells[6-9]. It was reported that hHSCs express the receptor CRLR for AM and their associated proteins RAMP-1 and RAMP-2 in addition to spontaneously secreting AM[10,11]. AM acts as a circulating hormone and elicits multiple biological activities in a paracrine or autocrine manner[12]. Several reports have demonstrated that AM inhibits proliferation of glomerular mesangial cells and cardiac fibroblasts[13,14], antagonizes the secretion of collagen and lightens fibrosis degree[14-16].

The inhibitory effects of AM on mesangial cells and cardiac fibroblasts lead to a hypothesis that AM may have
the same influence on HSC. To test it, we investigated the effects of AM gene overexpression on cell proliferation, synthesis of procollagens and MMPs/TIMPs system in activated hHSCs by stable transfection, in pursuing the possible novel clue for therapeutics of liver fibrosis.

**MATERIALS AND METHODS**

**Cell culture**

Immortal activated human hepatic stellate cells were generously gifted indirectly by a member in the group of Professor David A. Brenner (Columbia University, NY). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, GIBCO, USA) containing 4 mmol/L L-glutamine and 10% fetal calf serum (GIBCO, USA) at 37 °C in a humidified atmosphere of 5% CO₂.

**Stable transfection**

PcDNA3/AM was recombined with PcDNA3 vector and rat AM gene cDNA gifted by Professor Kazuo Kitamura (Miyazaki Medical College, Japan) between the site of restriction endonucleases EcoRI and XhoI. hHSCs were seeded into 6-well plates at a density of 1.5×10⁵ cells/mL in DMEM containing 10% FCS and incubated at 37 °C in a CO₂ incubator until the cells reached 70-80% confluence. Transfection of PcDNA3/AM and PcDNA3 empty vector was performed with lipofectamine™2000 reagent (Life Tech, USA) following the manufacturer’s instructions. Stably transfected cells were selected after growing in DMEM containing 600 μg/mL G418 (GIBCO, USA) for 5-6 wk. Individual clones of stable transfectants bearing PcDNA3/AM and PcDNA3 were isolated and screened for AM mRNA expression by RT-PCR analysis, and tested for AM protein secretion by RIA. Two clones named A-2 and A-8 expressing the highest level of AM gene were chosen for further analysis.

**Radioimmunoassay**

Control and transfected cells grown on 6-well plates to confluence were washed twice with PBS and incubated in DMEM with 0.2% FCS for 24 h at 37 °C. Viability of cells after 24 h incubation was estimated to be more than 97% by trypan blue staining. Culture media were collected, acidified with acetic acid (final concentration, 1 mol/L), heated at 100 °C for 10 min to inactivate protease and lyophilized. The lyophilizates were dissolved in RIA buffer and submitted to RIA for AM (AM (1-50), rat RIA kit, Phoenixpeptide, USA) and submitted to RIA for AM protein secretion by RIA. Two clones named A-2 expressing the highest level of AM gene were chosen for further analysis.

**Detection of cell proliferation**

DNA synthesis of different groups of cells were assessed by BrdU (Sigma, USA) incorporation and immunocytochemistry. In brief, different groups of cells (1×10⁶ cell/mL) were grown to subconfluence on the 6-well plates. During the last 4 h of incubation, BrdU was added to the culture medium at a final concentration of 15 μg/mL. Cultures were fixed in methanol: acetic acid (3:1) for 15 min, and BrdU-positive cells were detected by immunocytochemistry. The percentage of positive cells was evaluated by counting 1 000 cells per dish.

**Western blot**

Cells were washed twice with ice-cold PBS, and lysed on ice by addition of lysis buffer (30 mmol/L HEPES, 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid, 0.2 mmol/L PMSF, 1.0 μg/mL pepstatin A, 3.5 μg/mL aprotinin and 10 μg/mL leupeptin) for 20 min. Cell lysates were cleared by centrifugation at 20 000 g for 10 min at 4 °C, and the quantity of total protein was evaluated by BCA protein assay (Pierce, USA). Protein samples with the addition of 5X SDS-PAGE sample buffer were heated at 100 °C for 5 min, subjected to 6% SDS-PAGE (procollagen types I and II) and 10% SDS-PAGE (MMP1, MMP2, TIMP2), and transferred by electroblotting onto 0.45 μm PVDF membranes (Millipore, USA). The membranes were blocked overnight with 5% non-fat milk in Tris-buffer plus 0.1% Tween-20, probed with rabbit anti-collagen types I and III (produced by our laboratory) at 1:100 dilution, mouse MMP-2 and TIMP2 monoclonal IgG and rabbit anti-MMP-1 (Oncogene, CA) at 1:200 dilution overnight at 4 °C, incubated with biotinylated secondary antibodies and ABC kit (1:1500) (vector, USA) at room temperature for 1 h and visualized with DAB (Sigma, USA). β-actin was used as internal control. Densitometric analysis was carried out using Kodak Scientific Imaging System (Eastman Kodak, USA), and the values were depicted as the ratio to those of GAPDH.

**Semi-quantitative RT-PCR**

Total RNA was extracted from cells using TRIzol reagent (Shanghai Sangon, China) following the manufacturer’s instructions. RNA concentration and purity were determined with GeneQuant II RNA/DNA calculator (Pharmacia Biotech, UK). The quality of RNA was checked by formaldehyde agarose gel electrophoresis. RT was performed with oligo (dT) primer using a complementary DNA (cDNA) synthesis kit (Promega, German). Specific primers for PCR were as follows: AM: sense 5’ TGGCAGC-ATTGAAACGTGGG 3’ and antisense 5’TATAGGGG-GGCAAGGGGA 3’, procollagen type I: sense 5’AAAGGT-GTTGTCGGATGACG 3’ and antisense 5’GGAGAC-CAGGAGGACCAGAG 3’, procollagen type III: sense 5’GCCGAGAATACTGGGTTG 3’ and antisense 5’TGGTATGTAATGTCTCGGGAG 3’, GAPDH: sense 5’CCATGGGAAGCTGCGGG 3’ and antisense 5’CAAAGTTGCTATGGATGACC 3’. In preliminary experiments, we determined the optimal number of cycles within the linearity of reactions for each PCR product. The cycle number was 28 cycles for AM, 23 cycles for GAPDH and 30 cycles for procollagen types I and III. PCR was carried out at 95 °C for 2 min followed by individual cycles at 95 °C for 60 s, at 59 °C for 60 s and at 72 °C for 30 s with an extension step at 72 °C for 10 min at the end of the last cycle. The products were separated on 1.5% agarose gel. The intensity of PCR products was calculated with Kodak Scientific Imaging System (Eastman Kodak, USA), and the values were depicted as the ratio to those of GAPDH.

**Statistical analysis**

All values were expressed as mean±SD, ANOVA and χ² test were used to determine the significance of differences among the four groups. P<0.05 was considered statistically significant.
The percentage of BrdU-positive cells in non-transfected group, empty vector group, A-2 clone group, and A-8 clone group was 29.6%, 30.9%, 18.9% and 21.8%, respectively ($\chi^2 = 58.492, P<0.05$). Cells overexpressing AM gene showed a statistically lower incorporation of BrdU than cells non-transfected or transfected with empty vector. No difference was detected between non-transfected group and empty vector group.

### Effects of AM gene overexpression on expression of procollagen types I and III

In non-transfected group, empty vector group, A-2 clone group, and A-8 clone group, the ratio of procollagen type I/GAPDH at mRNA level was 0.58±0.1, 0.48±0.11, 0.3±0.06 and 0.31±0.07, respectively ($P<0.05$); the ratio of procollagen type III/GAPDH at mRNA level was 0.17±0.04, 0.15±0.03, 0.1±0.02, and 0.09±0.02, respectively ($P<0.05$) (Figure 3); the ratio of procollagen type I/β-actin at protein level was 0.27±0.03, 0.2±0.03, 0.14±0.05 and 0.13±0.05, respectively ($P<0.05$); the ratio of procollagen type III/β-actin at protein level was 0.22±0.04, 0.2±0.03, 0.11±0.04 and 0.13±0.03, respectively ($P<0.05$) (Figure 4A). The expression levels of procollagen types I and III in cells transfected with pcDNA3/AM were lower than those in cells non-transfected and transfected with empty vector.

### Influence of AM gene overexpression on MMPs/TIMPs

We used Western blot to detect the protein expression levels of MMP-1, MMP-2 and TIMP2 in different groups of cells. In non-transfected group, empty vector group, A-2 clone group, and A-8 clone group, the ratio of MMP-1/
β-actin was 0.49±0.07, 0.45±0.09, 0.68±0.06 and 0.81±0.09, respectively (P<0.05); the ratio of MMP-2/β-actin was 0.5±0.04, 0.48±0.03, 0.15±0.05 and 0.13±0.04, respectively (P<0.05); and the ratio of TIMP2/β-actin was 2.77±0.03, 2.79±0.04, 2.7±0.02 and 2.71±0.02, respectively (P<0.05) (Figure 4B). Cells transfected with pcDNA3/AM showed a significantly higher protein expression of MMP-1 but a lower protein expression of MMP-2 and TIMP2 than cells non-transfected and transfected with empty vector.

**DISCUSSION**

Liver fibrosis represents the final common pathological outcome for the majority of chronic liver insults. HSCs, previously termed as fat or vitamin A-storing cells or Ito cells are the most pathogenetically relevant cell type for development of liver fibrosis. Activated HSCs show an elevated proliferation rate, synthesize and secrete some extracellular matrix components and contribute to diminished degradation of ECM by expressing TIMPs. It is generally accepted that HSCs are important target cells for the treatment of liver fibrosis.

AM was discovered from human pheochromocytoma tissue in 1993. The function of AM is mainly mediated by intracellular adenylate cyclase coupled with cyclic adenosine monophosphate (cAMP) and nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathways through its specific receptor. Many functions have been ascribed to AM aside from vasodilation and hypotension effects. AM can act as a regulator of cell growth, but its paradoxical effect on cell growth depends on the cell type. It was reported that AM/AMBP-1 exerts anti-apoptotic effects on vascular endothelial cells. In glomerular mesangial cells, AM exerts its anti-proliferative effect mainly through a cAMP-dependent mechanism. Also in the cardiovascular system, AM has an inhibitory effect on proliferation of cardiac fibroblasts. In our study, HSCs were stably transfected with pcDNA3/AM and two clones with high expression of AM were selected. Compared with cells non-transfected and transfected with empty vector, these two clones showed a significantly lower incorporation of BrdU, indicating that AM gene overexpression exerts an inhibitory effect on proliferation of HSCs.

Among several functions of AM, its negative effect on synthesis of collagen and fibrosis interests us most. Several reports implicated that AM dose-dependently inhibits collagen synthesis in cardiac fibroblasts and human tubular epithelial cell line (HK-2) stimulated by TGF-β. In vivo study, AM gene delivery attenuates interstitial fibrosis and ECM formation within the heart. In agreement with the data reported above, our results showed that the expression levels of procollagen types I and III in pcDNA3/AM group were significantly lower than those in non-transfected group and empty vector group, indicating the antagonistic effect of AM on synthesis of ECM.

One characteristic of liver fibrosis is the excessive accumulation of ECM, which results from the imbalance between its secretion and degradation. MMPs/TIMPs are a family of proteins which participate in and regulate the process of ECM degradation. Collagen types I and III cover about 60-70% and 20-30% of the total collagen of fibrotic liver, respectively, both MMP-1 and MMP-2 can degrade these two types of collagen. We found that cells transfected with PcDNA3/AM had lower expression levels of MMP-2 and TIMP2 but higher expression level of MMP-1 than cells non-transfected and transfected with empty vector by Western blot.

We hypothesize that AM may exert effects on MMP-1 and MMP-2 gene transcription and expression through different signal transduction pathways. Transcriptional regulation of MMPs is dependent on signal transduction pathways and the functional significance of such regulation remains to be elucidated.
upon multiple DNA binding elements in their promoters. Multiple PEA3 sites which bind to members of the Ets family of transcription factors such as AP-1 site present in promoters of all the MMPs except for MMP-2 [27,30]. In HSC, stimulation of AM gene overexpression is likely to be transduced by various signal pathway cascades which interact with specific transcription factors, and the binding of these specific transcription factors to different DNA sites in promoters of MMP-1 and MMP-2 may result in divergent effects of AM on MMP-1 and MMP-2 expression. Higher expression level of MMP-1 and lower expression level of TIMP2 in PCDNA3/AM group benefit the degradation of ECM, suggesting that AM gene overexpression may reduce deposition of ECM in liver. AM gene overexpression may indirectly affect the expression of procollagen type I, because some reports indicate that overexpression may indirectly affect the expression of collagen I in a dose-dependent manner [29,31]. In our study, AM gene overexpression inhibited the expression of procollagen type I, which is likely to reduce the expression of MMP-2.

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