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Full-length article

Similar effects on rat renal mesangial cells by expressing different fragments of adrenomedullin gene *in vitro*[1]

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Key words

adrenomedullin; transfection; glomerular mesangium; transforming growth factor-β1

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Abstract

Aim: To construct pEGFP-N3 recombinant vectors carrying adrenomedullin (*AM*) or fragments of the *AM* gene, and to express AM or fragments of AM from the pEGFP-N3 recombinant vectors (pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3) and study their biological properties on cultured rat renal mesangial cells (RMC). **Methods:** Total RNA of rat kidney was obtained using TriZol reagent. The cDNA was synthesized by reverse transcriptase using oligo-deoxythymidine as primer. The fragments of *AM* gene were then amplified by polymerase chain reaction (PCR) with specific upstream and downstream oligonucleotides. The PCR products were digested with *Eco*RI and *Bam*HI and subcloned into the plasmid pEGFP-N3. Facilitated by cationic liposomes, RMC were transfected with pEGFP-N3-AM1-2 or pEGFP-N3-AM1-3. After 24 h, green fluorescent protein (GFP) fluorescent images were examined with a fluorescence microscope. After 48 h, the proliferation of RMC was detected using the MTT assay, and the mRNA expression of *transforming growth factor-β1* (*TGF-β1*) was measured by semiquantitative PCR. **Results:** DNA sequence reports verified that pEGFP-N3-AM1-2, which carried the full length *AM* gene translation fragment (preproadrenomedullin preproAM$_{1–185}$), and pEGFP-N3-AM1-3, which carried the translation fragment of preproAM [without adrenotensin (ADT, preproAM$_{150–185}$)], were constructed successfully. After 24 h, green fluorescence was observed in RMC into which either pEGFP-N3-AM1-2 or pEGFP-N3-AM1-3 was transfected, while in the control cells no fluorescence was observed. Either pEGFP-N3-AM1-2 or pEGFP-N3-AM1-3 delivery inhibited the proliferation of RMC ($P<0.01$) and decreased the mRNA transcription level of *TGF-β1* in RMC ($P<0.05$). However, no significant difference was observed between the effects of pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3. **Conclusion:** pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3 were constructed successfully and were functionally expressed in RMC. Expressing the fragment of *AM* without ADT has similar inhibitory biological effects on RMS proliferation and *TGF-β1* transcription with full length preproAM.

Introduction

Adrenomedullin (AM) was discovered in tissue extracts of human pheochromocytoma by Kitamura *et al* in 1993[1]. Subsequently identified as a potent vasodilator, AM was detected to be expressed in a wide range of tissues, such as the adrenal gland, kidney, heart, lung, spleen, and brain[2]. AM has since been suggested to play important roles in a great number of disease areas, "ranging widely from heart failure through to oncology", especially in cardiovascular and renal diseases[3,4]. Previous studies revealed that plasma AM levels were increased in hypertension and renal failure[5,6]. Researches have also shown that AM can improve cardiac function, renal function, and survival rate in hypertensive rat models[7,8].

The gene encoding preproadrenomedullin (preproAM),

a precursor molecule of *AM*, is termed as the *AM* gene. Sequence analysis of cloned rat *AM* showed that this precursor consisted of 185 amino acids, including a signal peptide[9]. After synthesis, the signal peptide is cleaved between Thr21 and Ala22. The remaining fragment of preproAM, which is termed as proadrenomedullin (proAM), is cleaved specifically by endopeptidase at Lys43–Arg44, Lys92–Arg93 and Arg145–Arg149, resulting in the production of four proAM-derived peptides: PAMP (proadrenomedullin N-terminal 20 peptide, preproAM$_{22–41}$), preproAM$_{45–91}$, AM (preproAM$_{94–143}$) and ADT (adrenotensin or preproAM$_{150–185}$)[9,10] (Figure 1).

Finding out the appropriate expression pattern is one of the major topics in gene delivery. It is well known that peptides derived from a common polypeptide precursor may have different, even opposite biological properties. Previous studies suggest that ADT may play a role that is opposite in effect to AM[11,12]. The present study was carried out in order to explore the different effects of expressing full length preproAM and a fragment of preproAM without ADT on renal mesangial cells (RMC).

Materials and methods

Materials pEGFP-N3 vector was obtained from BD Biosciences Clontech (Bedford, MA,USA). Top10 *Escherichia coli* was obtained from Invitrogen (Carlsbad, CA, USA) and primary rat RMC was a gift from the Department of Pathology, Shanghai Medical College (Shanghai, China).

Reagents Trizol, AMV reverse transcriptase, *Taq* DNA polymerase, Silver Beads DNA Gel Extraction Kit, kits for total RNA extraction, endonucleases *Eco*RI and *Bam*HI, and T4 DNA ligase were purchased from Sangon (Shanghai, China). Lipofectamine^{TM} 2000 was from Gibco (Grand Island, NY, USA), and MTT, dimethyl sulfoxide (Me_2SO) and Dulbecco's Modified Eagle's Medium (DMEM)/F-12 were from Sigma (St Louis, MO, USA).

Construction and confirmation of expression of vectors pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3 Total RNA was isolated from rat kidney tissue samples using Trizol reagent. The full coding region of the *AM* gene was generated by reverse transcription-polymerase chain reaction (RT-PCR) using the following oligonucleotide primers: AM-1, 5'-TAC TGA ATT CGC CAC CAT GAA GC-3'; and AM-2, 5'-TTG CGG ATC CTA ACC TAG AGA C-3'. After an initial denaturation step at 94 °C for 3 min, PCR were carried out for 30 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, and then 72 °C for 10 min. The PCR products and pEGFP-N3 were digested with restriction endonucleases *Eco*RI and *Bam*HI (recognition sites in bold) and the purified digestion products were subcloned into the *Eco*RI and *Bam*HI sites of pEGFP-N3 with T4 DNA ligase. The recombinant plasmids were then transformed into competent Top10 *E coli* and amplified in the hosts. DNA sequencing was carried out to identify purified recombinant vectors. The recombinant vector carrying the full coding region of the rat *AM* gene was named pEGFP-N3-AM1-2. Using the same method (with oligonucleotide primers: AM-1, 5'-TAC TGA ATT CGC CAC CAT GAA GC-3'; and AM-3, 5'-TTG CGG ATC CAT AGC CTT GAG-3'), we developed another recombinant pEGFP-N3 vector (pEGFP-N3-AM1-3) with a fragment encoding a region of the rat *AM* gene in which the sequence encoding ADT was absent.

Cell culture and transfection Primary rat mesangial cells were cultured in DMEM/F-12 containing 10% heat-inactivated fetal calf serum (FCS) and incubated in a humidified 5% CO_2 incubator at 37 °C. Cells were growth-arrested in DMEM containing 1.0% FCS for 24 h prior to transfection. pEGFP-N3-AM1-2 or pEGFP-N3-AM1-3 was transfected into

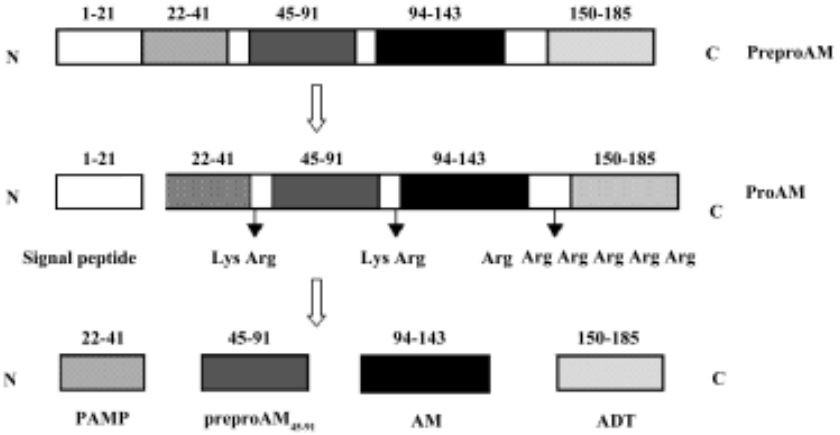

Figure 1. Schematic presentation of the biosynthesis of AM and ADT.

cultured RMC facilitated by cationic liposomes Lipofectamine™ 2000. At 24 h after transfection, phase contrast and fluorescence microscopy were carried out using a BX61olympus microscope (Melville, NY, USA)

Detection of cell proliferation by MTT assay Cellular proliferation was determined in triplicate with a colorimetric non-radioactive MTT proliferation assay. RMC were plated at 3×10^3 cells/well into 96-well plates and cultured in DMEM/F-12 medium supplemented with 10% FCS at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h. The cultures were replaced with medium containing 1.0% FCS for another 24 h, and the cells were then transfected with pEGFP-N3-AM1-2 or pEGFP-N3-AM1-3 for 48 h. The effects of pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3 on the proliferation of RMC were measured by MTT assay. Briefly, a 20 μL aliquot of 5 mg/mL MTT solution was added to each well. After 4 h of incubation, the absorbance of each well was measured at 490 nm.

Reverse transcription-polymerase chain reaction analysis of *transforming growth factor-β1* gene expression Expression of the *transforming growth factor-β1* (*TGF-β1*) gene was analyzed by semiquantitative PCR using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal standard. Total RNA was isolated from cultured RMC following the protocol of RNA extraction kits. The amount of RNA isolated was determined by measuring the specific absorption at 260 nm. The integrity of the RNA isolated was confirmed by agarose gel electrophoresis, run under denaturing conditions. cDNA was synthesised in 40 μL reaction mixtures using 1 μg of total RNA; 2 μL of the cDNA solution was used for PCR amplification. The primers for the *TGF-β1* and *GAPDH* genes were as follows: *TGF-β1* sense, 5'-AAG TGG ATC CAC GAG CCC AA-3'; *TGF-β1* antisense, 5'-GTC GCA CTT GCA GGA GCG CA-3'; *GAPDH* sense, 5'-ACC ACA GTC CAT GCC ATC AC-3'; *GAPDH* antisense, 5'-CCA CCA CCC TGT CAT GCC ATC AC-3'. After an initial denaturation step at 94 °C for 3 min, PCR was carried out for 30 cycles and 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and then at 72 °C for 10 min.

Statistical analysis Statistical analysis was carried out using the statistical program SPSS 11.0 (Chicago, Illinois, USA). The data were expressed as mean±SD and the one-way analysis of variance (ANOVA) *t*-test was used for statistical analysis. $P<0.05$ was accepted as statistically significant.

Results

Construction and identification of pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3 The full coding region of the rat *AM* gene and the fragment of coding region without ADT were subcloned separately into pEGFP-N3, producing pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3, respectively. The construction of pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3 was confirmed by DNA sequencing.

Expression of pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3 in renal mesangial cells Cultured RMC were transfected with pEGFP-N3-AM1-2 or pEGFP-N3-AM1-3. After 24 h, green fluorescence was detected using a fluorescence microscope. Both pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3 were expressed in RMC as bright green fluorescence was observed, indicating that the in-frame GFP-tagged *AM* sequences were expressed and translated successfully. The expression of GFP was observed in above 95% transfected RMC. No green fluorescence was detected in untransfected RMC (Figures 2–4).

Proliferation of renal mesangial cells in response to pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3 To determine the

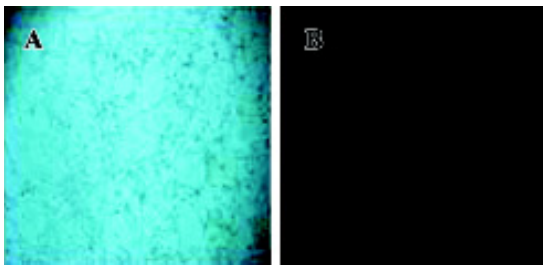

Figure 2. Phase contrast (A) and fluorescence (B) microscopy of normal cultured renal mesangial cells (×100).

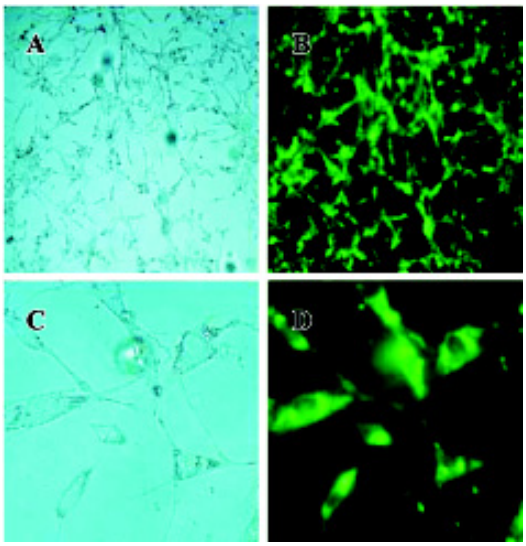

Figure 3. Phase contrast (A,C) and fluorescence (B,D) microscopy of renal mesangial cells transfected with pEGFP-N3-AM1-2. (A,B) ×100; (C,D) ×400.

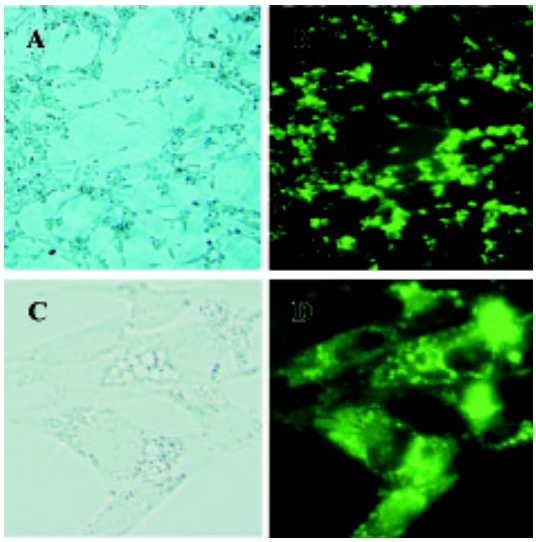

Figure 4. Phase contrast (A,C) and fluorescence (B,D) microscopy of renal mesangial cells transfected with pEGFP-N3-AM1-3. (A,B) ×100; (C,D) ×400.

biological activities of the recombinant vectors, proliferation of RMC was analyzed using the MTT assay 48 h after pEGFP-N3-AM1-2 or pEGFP-N3-AM1-3 delivery. Significant changes in optical density values at 24 h after transfection was observed with recombinant plasmids compared with the controls, indicating that pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3 elicited a decrease in RMC proliferation (P<0.01) (Figure 5).

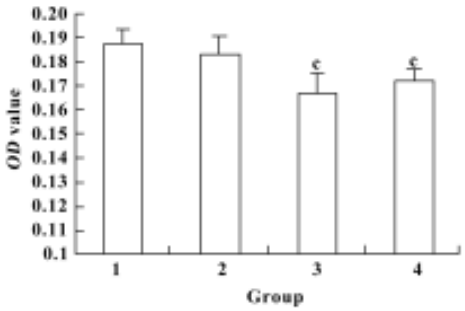

Figure 5. Effect of pEGFP-N3-AM1-2 or pEGFP-N3-AM1-3 on the proliferation of renal mesangial cells (RMC). (1) Normal cultured RMC (48 h); (2) RMC transfected with pEGFP-N3 (48 h); (3) RMC transfected with pEGFP-N3-AM1-2 (48 h); (4) RMC transfected with pEGFP-N3-AM1-3. n=12. Mean±SD. ^{c}P<0.01 *vs* group 2.

Analysis of the effects of pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3 on *TGF-β1* gene expression Along with the control experiments, which indicated that product accumulation did not reach a plateau at 30 cycles under the assay conditions used, our results for semiquantitative PCR indicate that, compared with the control group, transfection of pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3 suppressed *TGF-β1* mRNA expression in RMC (Figure 6).

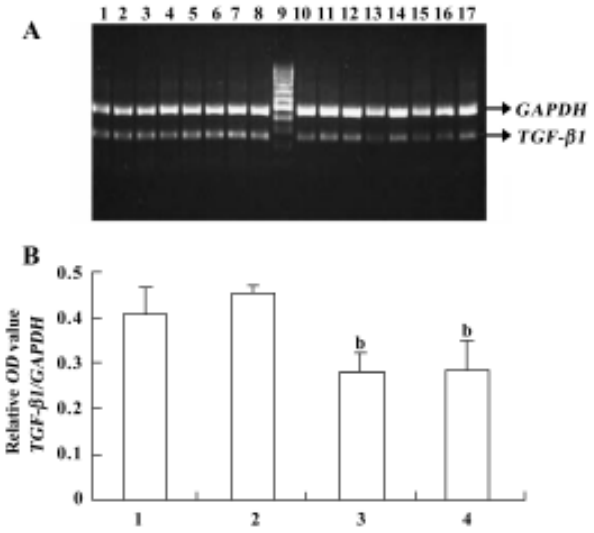

Figure 6. Semiquantitative analysis of the effect of pEGFP-N3-AM1-2 or pEGFP-N3-AM1-3 on *transforming growth factor-β1* (*TGF-β1*) mRNA expression in renal mesangial cells (RMC). (A) Electrophoresis of *TGF-β1* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): 1–4, normal cultured RMC; 5–8, RMC transfected with pEGFP-N3 (48 h); 9, DNA molecular weight marker; 10–13, RMC transfected with pEGFP-N3-AM1-2 (48 h); 14–17, RMC transfected with pEGFP-N3-AM1-3 (48 h). (B) Statistical results of *TGF-β1* mRNA expression in RMC. 1, normal cultured RMC (48 h); 2, RMC transfected with pEGFP-N3 (48 h); 3, RMC transfected with pEGFP-N3-AM1-2 (48 h); 4, RMC transfected with pEGFP-N3-AM1-3 (48 h). n=4. Mean±SD. ^{b}P<0.05 *vs* group 2.

Discussion

As an endogenous vasodilator and natriuretic peptide, AM is thought to have great potential in the treatment of cardiovascular and renal diseases[13,14]. However, there are still some impediments to understand AM's biological functions and the promising clinical uses of AM. One of the obstacles is the short half-life of AM. The plasma half-life of AM is estimated to be about only 22 min[15]. Gene transfer is a novel means of providing sustained and localized delivery of the required therapeutic protein. In order to achieve long-term expression of AM, *AM* gene delivery has been used in recent years. The studies show that *AM* gene delivery has multiple functions in protection against hypertension, renal damage, cardiac fibrosis and cardiac hypertrophy[16–20].

Currently, both non-viral and viral vectors are used to express the heterogeneous genes, each of them with their

own advantages and disadvantages. The advantages of non-viral vectors are: (i) non-vial vectors do not integrate the heterogeneous genes into recipient-cell genome, therefore it is considered safe for researchers and will have higher clinical acceptability; (ii) there are minimal or no immune effects, and none of the safety concerns regarding infection is found with viral vectors[21]; (iii) manipulate and delivery of plasmids into cells is much easier; and (iv) although plasmid-delivered DNA does not integrate into the recipient-cell genome, delivery of plasmids *in vivo* can maintain very prolonged effects[22]. In the present study, we chose plasmid pEGFP-N3 as the vector.

In our experiment, for the first time, the recombinant vector pEGFP-N3-AM1-3, which carries the DNA fragment encoding the AM protein without the ADT sequence, was designed and constructed successfully. It is well known that peptides derived from a common polypeptide precursor may have different, even opposite biological properties. As prior studies have demonstrated, ADT has some quite opposite vascular effects to those of AM[11,12]. We therefore hypothesized that the absence of ADT might have more effective effects in protecting heart and kidney.

The pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3 plasmids were constructed successfully. As the fluorescent images showed, both of these vectors were expressed efficiently in RMC. Our data analysis showed that either pEGFP-N3-AM1-2 or pEGFP-N3-AM1-3 delivery inhibited the proliferation of RMC ($P<0.01$) and decreased the mRNA transcription of *TGF-β1* in RMC ($P<0.05$). However, no significant difference was observed between the effects of pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3.

In kidney, mesangial cells have a central function in maintaining structural and functional integrity of the glomerulus, including "structural support of the capillary tuft, modulation of glomerular hemodynamics, and a phagocytic function allowing removal of macromolecules and immune complexes", and the proliferation of mesangial cells is a pivotal feature of glomerular disease[23]. Our results are in agreement with prior studies showing that AM can suppress RMC proliferation[24–26], and this may be one of the mechanisms by which *AM* gene delivery protects renal function. So far, no data about the effects of ADT on the proliferation of mesangial cells have been reported.

Transforming growth factor-β1 is a multifunctional peptide that regulates manifold cellular functions including cell proliferation, differentiation and apoptosis[27]. As a potent inducer of extracellular matrix synthesis leading to progressive glomerular fibrosis, TGF-β1 is also thought to be the major pathogenic factor in the development of renal fibrotic disorders[28]. A previous study showed that chronic AM infusion significantly reduced the *TGF-β/GAPDH* mRNA levels in the renal cortex[7]. In the present study, we demonstrated that either pEGFP-N3-AM1-2 or pEGFP-N3-AM1-3 delivery could decrease the mRNA transcription level of *TGF-β1* in RMC ($P<0.05$). This may contribute to the renal protective effects of *AM* gene delivery; the function of ADT in the regulation of *TGF-β1* mRNA expression requires further study.

In the present experiment, a very interesting phenomenon was observed in that there was no difference between pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3 in their effects on the proliferation of RMC and the mRNA transcription level of *TGF-β1* in cultured RMC. Although ADT plays some opposite vascular effects to AM, previous studies indicate that there are complicated mutual regulation interactions between ADT and AM. In incubated blood vessels, AM could inhibit the synthesis and release of ADT, whereas ADT could enhance the synthesis and release of AM[29]. Therefore, in order to understand the mechanism of why pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3 had similar effects on cultured RMC, further investigations are indispensable.

In conclusion, recombinant vectors pEGFP-N3-AM1-2, expressing preproAM, and pEGFP-N3-AM1-3, expressing a fragment of preproAM without ADT, were constructed successfully. The biological activity studies of pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3 on cultured RMC showed that expressing the fragment of preproAM without ADT and the full-length preproAM had similar inhibitory effects on cell proliferation and *TGF-β1* gene transcription. As for the mechanism of why pEGFP-N3-AM1-2 and pEGFP-N3-AM1-3 had similar effects on cultured RMC, and what may be the effects of the 2 vectors on the kidney *in vivo*, further investigations are required.

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