# Endothelin-1 Antisense Oligonucleotide Suppresses the Proliferation of Glomerular Mesangial Cells Stimulated with Angiotensin-II

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Antisense oligonucleotide (AON) has been applied to modern molecular pharmacology. We have previously demonstrated that angiotensin-II (Ang-II) is an active stimulator of endothelin-1 (ET-1) production in glomerular mesangial cells. This study was designed to investigate the specific effect of ET-1 AON on inducing proliferation of cultured rat mesangial cells stimulated with Ang-II. ET-1 was measured by radioimmunoassays. The results were: (1) Ang-II enhanced ET-1 production of mesangial cells; (2) ET-1 production of mesangial cells was significantly suppressed by ET-1 AON, and this production was not affected by either ET-1 sense or scramble oligonucleotide in different concentrations; (3) Ang-II increased [$^3$H]-thymidine uptake of mesangial cells, which was suppressed to 25% by ET-1 AON but not by ET-1 sense or scramble oligonucleotide. Our results indicate that ET-1 AON effectively suppresses the ET-1 production and the Ang-II-stimulated proliferation of mesangial cells, and therefore may offer treatment for proliferative glomerulonephritis.

**Key Words:** angiotensin-II, antisense oligonucleotide, endothelin-1, mesangial cell
(*Kaohsiung J Med Sci* 2007;23:170–5)

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide originally isolated and purified from the conditioned medium of cultured porcine aortic endothelial cells [1], and it has been shown to process a wide spectrum of biologic activity in the kidneys [2]. Recent studies indicate that ET-1 may also play an important role in glomerular diseases [3]. ET-1 induces mesangial cell proliferation and the production of extracellular matrix [4]; both are important in the pathogenesis of glomerulosclerosis.

Received: May 30, 2006 Accepted: September 29, 2006
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Antisense oligonucleotide (AON) is usually a single-stranded synthetic nucleic acid polymer consisting of 15–25 nucleotides, and was initially used for research. Their sequence-specific (antisense) effects are due to the hybridization with the target mRNA in a sequence-dependent complimentary manner. AON can be used to disrupt gene function in a variety of *in vitro* culture systems and *in vivo*, and they have proven potential in clinical use [5]. AON as therapeutic agents was proposed earlier in the 1970s when the antisense strategy was initially developed. AONs have been applied to modern molecular pharmacology. In the emerging or expanding areas of therapeutic intervention, transcription and translation are the target events of interest for modifying gene expression [6]. The direct effect of ET-1 AON on glomerular mesangial cells, however, is not clear. In this study, we observed the direct

effects of specific ET-1 AON on ET-1 production and [$^3$H]-thymidine uptake of cultured rat glomerular mesangial cells stimulated with angiotensin-II (Ang-II), the important vasoactive peptide involved in the formation of glomerulosclerosis.

## Materials and Methods

### *Culture of mesangial cells*

Mesangial cells were isolated and cultured from rat renal glomeruli according to the methods described previously [7]. Briefly, glomeruli were harvested from six to eight male Sprague-Dawley rats (150–200 g) by sieving the renal cortices, which were then digested with 0.25% trypsin and 0.05% collagenase. The digested glomeruli were incubated at 37°C in RPMI 1640 medium containing 20% fetal calf serum (FCS), penicillin (100 U/mL), streptomycin (100 μg/mL) and insulin (0.6 U/mL). After 2–3 weeks, mesangial cells appeared and were characterized. On phase contrast microscopy, the cells were stellate or spindle shaped in appearance, and in postconfluent culture they piled and formed small nodules. The nodules were sensitive to mitomycin C, but not to aminonucleoside of puromycin, and they contracted in response to Ang-II. In addition, the cells failed to stain for Ia and leukocyte common antigen. The cell passage was performed every 4–6 days. Cells were starved by incubation in a medium containing 0.5% FCS for 24 hours before adding the reagents. The animal experiment was approved by the animal committee of Kaohsiung Medical University.

### *Experimental protocol*

ET-1 production of cultured rat glomerular mesangial cells was measured. Mesangial cells were stimulated with Ang-II after obtaining a dose-response curve [8]. Cells were also preincubated with ET-1 AON for 24 hours before stimulation with Ang-II. Ang-II was purchased from Sigma Chemical Co. (St Louis, MO, USA).

### *Thymidine uptake*

Mesangial cells were brought to confluent density, trypsinized, and counted. The medium was aspirated and the cells were washed twice with Dulbeccos-PBS and treated with 1 mL of 0.2% trypsin–0.02% EDTA solution for 10 minutes at 37°C. Cells were then resuspended in RPMI 1640 containing 20% FCS and were adjusted to a concentration of $2 \times 10^4$ cells/mL. An aliquot of 200 μL of cells was placed in each of 96 well plates. The plates were cultured for 48 hours in a 5% $CO_2$ humidified atmosphere at 37°C, and then the medium was replaced with RPMI containing 0.5% FCS. After adding Ang-II for 3 hours, the plates were pulsed with 0.5 μCi of [$^3$H]-labeled thymidine per well for 18 hours [9]. The cells were harvested onto glass fiber filters, and washed with distilled water using a semiautomatic cell harvester. The incorporated radioactivity was counted with a β-scintillation counter. All assays were performed in triplicate.

### *Radioimmunoassay (RIA) for ET-1*

The ET-1-like immunoreactivity of supernatant was determined by a specific ET-1 RIA (Peninsula Laboratories, Inc., Belmont, CA, USA) after extraction. The supernatant was applied to a Sep-Pak $C_{18}$ cartridge (Waters Associates, Milford, MA, USA) and eluted with 5 mL of 60% acetonitrile in 0.1% trifluoroacetic acid. The eluate was lyophilized and reconstituted for RIA. The antibody used cross-reacted with ET-1 (100%), big ET-1 (17%), ET-2 (7%) and ET-3 (7%) and did not react with Ang-II, vasoactive intestinal peptide or α-ANP 1–28. The recovery rate of ET-1, extracted through a Sep-Pak $C_{18}$ column by adding radiolabeled ET-1 to the medium, was 61.2 ± 1.2%. The sensitivity for ET-1 RIA was 0.4 pg/tube, and the 50% intercept was 20 pg/tube. The intra- and interassay coefficiency of variation was 9.7% and 10.5%, respectively, over a range of concentration between 0.1 and 64 pg/tube [10].

### *Preparation of ET-1 AON*

Mesangial cells were grown to 80% confluence on coverslips and transiently transfected with either antisense or scrambled phosphorothioate-modified oligonucleotide according to the manufacturer's instructions (Genosys Company, Woodlands, TX, USA). The sequence was as follows: 5′-ATCACGGGAAAATAATC-CAT-3′. The sense oligonucleotide had the sequence 5′-ATGGATTATTTTCCCGTGAT-3′, and the scramble oligonucleotide had the sequence 5′-ATACACTGGA-AGAATAATCT-3′. Cells were preincubated for 24 hours with the same concentration of ET-1 antisense, sense, and scramble oligonucleotides, and the supernatants were collected after stimulation with 2% FCS for 48 hours.

### *Statistical analysis*

Data were presented as mean ± SEM. One way ANOVA and unpaired *t* test were used to compare the difference between each pair, and $p < 0.05$ was considered to be statistically significant.

## RESULTS

### *Effects of Ang-II on ET-1 production*

The ET-1 production of mesangial cells was significantly enhanced after stimulation with both Ang-II (102.1 ± 3.2 pg/mL, $p < 0.01$) and 2% FCS (110.3 ± 5.8 pg/mL, $p < 0.001$ compared to controls 87.9 ± 2.9 pg/mL) (Figure 1).

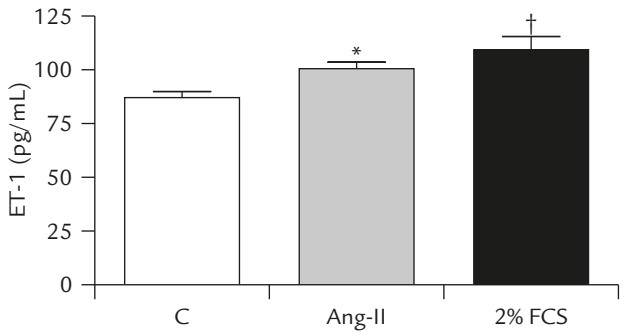


**Figure 1.** *Effects of angiotensin-II (Ang-II, $10^{-7}$ M) and 2% fetal calf serum (FCS) on the endothelin-1 (ET-1) production of mesangial cells. The supernatants were collected after stimulation for 72 hours. Data are presented as the mean ± SEM of all three independent experiments performed in triplicate. *$p < 0.01$ and †$p < 0.001$ compared to control (C) without stimulation.*

### *Effects of ET-1 AON on ET-1 protein production*

ET-1 protein production was significantly suppressed by ET-1 AON at the concentrations of $1 \times 10^{-5}$ M (9.8 ± 0.9 pg/mL, $p < 0.001$), $2 \times 10^{-6}$ M (12.1 ± 1.3 pg/mL, $p < 0.001$) and $4 \times 10^{-7}$ M (10.7 ± 1.8 pg/mL, $p < 0.001$ compared to that stimulated with 2% FCS, 37.5 ± 5.3 pg/mL). The amount of ET-1 production was not affected by either ET-1 sense or scramble oligonucleotides in different concentration (Figure 2).

### *Effects of ET-1 AON on Ang-II-stimulated thymidine uptake*

The [$^3$H]-thymidine uptake of mesangial cells was significantly enhanced by $10^{-7}$ M Ang-II (1,204 ± 75 compared to control 870 ± 52 cpm, $p < 0.001$). The effect was significantly suppressed by ET-1 AON at concentrations of $10^{-8}$ M and $10^{-9}$ M (890 ± 50 and 920 ± 32 cpm, respectively, both $p < 0.01$ compared to the effect of Ang-II). The effect on thymidine uptake was not affected by either ET-1 sense or scramble oligonucleotides at different concentrations (Figure 3).

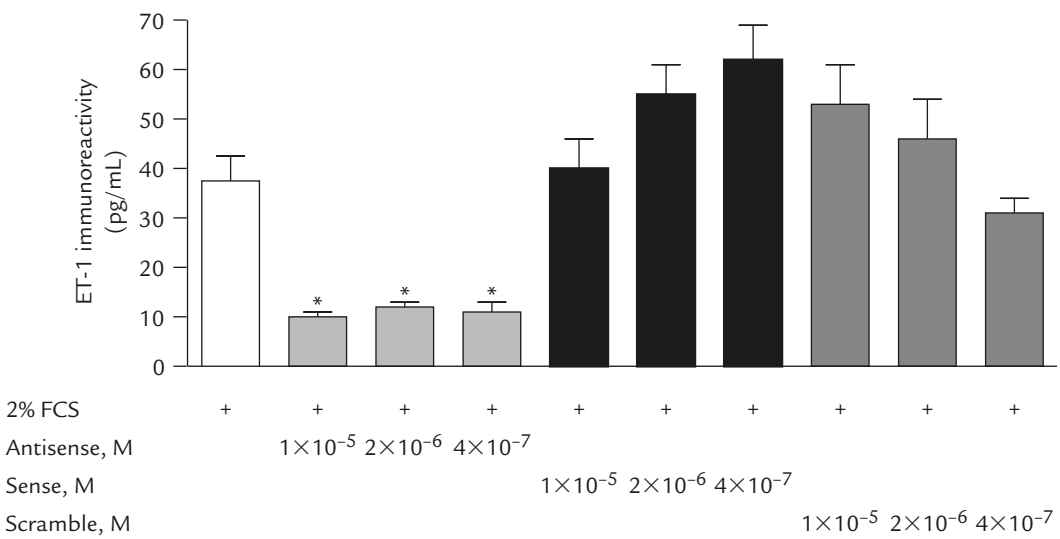


**Figure 2.** *Effects of endothelin-1 (ET-1) antisense oligonucleotide (AON) on ET-1 production of glomerular mesangial cells. Cells were preincubated for 24 hours with the same concentrations of ET-1 antisense, sense and scramble oligonucleotides, and the supernatants were collected after stimulation with 2% fetal calf serum (FCS) for 48 hours. *$p < 0.001$ compared to the effect of 2% FCS only. Data are presented as the mean ± SEM of three independent experiments performed in triplicate.*

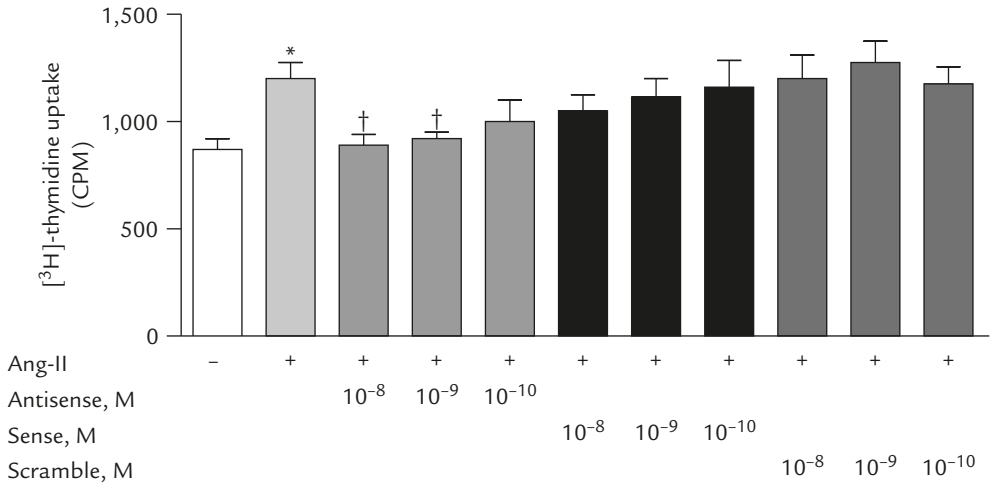


**Figure 3.** *Effects of endothelin-1 (ET-1) antisense oligonucleotide (AON) on [$^3$H]-thymidine uptake of glomerular mesangial cells stimulated with angiotensin-II (Ang-II). Cells were preincubated for 24 hours with the same concentrations of ET-1 antisense, sense and scramble oligonucleotides, and then stimulated with Ang-II ($10^{-7}$ M) before adding [$^3$H]-thymidine. *$p<0.001$ compared to control; †$p<0.01$ compared to the effect of Ang-II only. Data are presented as the mean ± SEM of three independent experiments performed in quadriplicate.*

## Discussion

In this study, we have demonstrated that ET-1 AON effectively suppressed the ET-1 production and the Ang-II-stimulated [$^3$H]-thymidine uptake of mesangial cells. The use of AON as therapeutic agents has become a popular topic in both the research and the clinical fields [11–13]. The principle of antisense technology is the sequence-specific binding of an AON to target mRNA, resulting in the prevention of gene translation and the modulation of gene expression involved in the pathogenesis of diseases. There has been a rapid increase in the number of antisense molecules progressing past Phase I, II and III clinical trials in recent years [14].

In studies with AON, researchers must differentiate between the desired sequence-specific inhibition of the targeted mRNA from the undesired sequence-related and non-sequence-related effects, in order to choose and design appropriate therapeutic oligonucleotides. Negative controls serve to rule out the possibility that the effects of AON are caused by non-sequence-specific mechanisms, while positive controls provide additional evidence that a true antisense effect is the reason for the biologic effects. Usually, a combination of at least two different controls is recommended. In this study, we used sense control which has a sequence complementary to that of the AON, and we also used scrambled control which is generated by mixing up the AON bases in a randomized manner. Similar effects of ET-1 AON was not seen in sense or scramble oligonucleotides.

Our study demonstrated that Ang-II is an active stimulant for the proliferation of glomerular mesangial cells. Ang-II is known as a critical factor for the progression of chronic renal diseases [15]. Ang-II contributes to the inflammatory process in glomerular disorders, facilitating the migration of mononuclear cells to the glomeruli and ultimately participates in the fibrotic process. We have also demonstrated that the effects of Ang-II on mesangial cells were partly mediated by ET-1, another vasoactive peptide involved in glomerular diseases [3]. ET-1 had been found to induce the proliferation of mesangial cells and the production of extracellular matrix [4], both are similar to the action of Ang-II and therefore may explain the possible mechanisms for the effect of Ang-II on mesangial cells. We have also demonstrated that ET-1 AON suppresses ET-1 production, while sense and scramble do not have similar effects. Therefore, ET-1 AON may also be used in the treatment of related disorders.

In conclusion, we have demonstrated that ET-1 AON effectively suppressed the ET-1 production and the Ang-II-stimulated proliferation of mesangial cells. Therefore, ET-1 AON may be used in the treatment of ET-1 and Ang-II-related glomerular disorders in the future.

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# 內皮素-1 反譯寡核苷酸抑制第二型血管收縮素所誘發之腎絲球間質細胞增生

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反譯寡核苷酸 (以下簡稱 AON) 最近被報告可能做為臨床治療用途。我們曾報告第二型血管收縮素 (以下簡稱 Ang-II)，會刺激另一個更強的細胞激素，內皮素-1 (以下簡稱 ET-1) 之產生，因此本研究之目的即探討 ET-1 AON 對 Ang-II 所誘發之腎絲球間質細胞增生之影響。ET-1 係以放射免疫分析法測定。研究結果 (1) Ang-II 可誘發腎絲球間質細胞產製較多量 ET-1 (2) ET-1 AON 可抑制腎絲球間質細胞 ET-1 之產量，而 ET-1 sense 及 scramble 寡核苷酸則沒有類似作用 (3) Ang-II 可刺激腎絲球間質細胞增加 thymidine 攝取量，此增加可受 ET-1 AON 部分抑制，但 ET-1 sense 及 scramble 寡核苷酸則沒有類似作用。本研究結果顯示 ET-1 AON 可明顯抑制腎絲球間質細胞之 ET-1 產量及 Ang-II 所誘發之細胞增生。

**關鍵詞**：第二型血管收縮素，反譯寡核苷酸，內皮素-1，腎絲球間質細胞

(高雄醫誌 2007;23:170－5)

收文日期：95 年 5 月 30 日

接受刊載：95 年 9 月 29 日

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