EFFECTS OF ANGIOTENSIN II ON THE PROLIFERATION AND FIBRONECTIN PRODUCTION OF GLOMERULAR MESANGIAL CELLS

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Angiotensin II (Ang-II) is a potent vasoactive hormone which plays an important role in the pathogenesis of glomereulosclerosis. Since glomerulosclerosis is characterized by alterations in both cellularities and the extracellular matrix, we investigate the effects of Ang-II on the cellular proliferation and fibronectin production of glomerular mesangial cells. Fibronectin was measured by Western blot analysis and fibronectin mRNA was measured by Northern blot analysis. The results were as follows: (1) Ang-II increased [^{3}H]-thymidine uptake of mesangial cells in a dose-dependent manner. (2) Ang-II increased the number of mesangial cells in a time-dependent manner. (3) Ang-II enhanced fibronectin mRNA expression and its production of mesangial cells in a time-dependent manner. Our results indicate that Ang-II is an active stimulant for the proliferation and fibronectin production of glomerular mesangial cells. (Acta Nephrologica 2004; ***18:*** *57-61)*

Key words: ***angiotensin II, mesangial cell, fibronectin.***

INTRODUCTION

Angiotensin II (Ang-II), the principle effector molecule of the renin-angiotensin system, has long been known to regulate blood pressure, salt and water homeostasis. All components of the renin-angiotensin system, including precursors and enzymes required for the formation and degradation of the biologically active forms of angiotensin, as well as different receptors, have been identified in the kidney.[1] The classical view of Ang-II as a vasoactive agent that participates in local and systemic hemodynamic regulation has been recently enlarged to consider it as a true cytokine with an active role in renal pathology. Recent studies also indicated that Ang-II is an important vasoactive peptide involved in the formation of glomerulosclerosis. One common feature of glomerulosclerosis is the proliferation of resident renal cells, especially the glomerular mesangial cells, and the accumulation of extracellular matrix.[2] Ang-II seems to participate in these phenomena, although its actual role has been poorly defined.

Mesangial cells, as the major intrinsic cells in glomeruli, are one of the targets of Ang-II in various renal diseases. We have previously demonstrated that mesangial cells can be stimulated by several cytokines and can produce many cytokines in response to injury.[3-5] It has also been well established that human mesangial cells are the main cells involved in the development of glomerulosclerosis. One of the major elements of glomeruloeclerosis is fibronectin, since fibronectin is the major component of the extracellular matrix in the glomerular mesangium. We have previously demonstrated that mesangial cells produce fibronectin in both normal and diseased conditions.[3] Accumulation of fibronectin is the hallmark of progressive glomerular diseases. Therefore in present study we measure the effects of Ang-II on the cellular proliferation and fibronectin production of cultured rat mesangial cells.

MATERIAL AND METHODS

Culture of mesangial cells

Mesangial cells were isolated and cultured from rat renal glomeruli according to the methods previous-

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ly described.[3] Briefly, glomeruli were harvested from six to eight male Sprague-Dawley rats (150 to 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 two to three 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 up 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 cells were passaged every four to six days. Cells were starved by incubation in a medium containing 0.5% FCS for 24 hours before adding the reagents.

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. The plates were cultured for 72 hours in a 5% CO_2 humidified atmosphere at 37°C, and then the medium was replaced with RPMI containing 0.2% FCS. After adding Ang-II for three hours, the plates were pulsed with 0.5 μCi/ml of $[^3H]$-labeled thymidine per well for seventeen hours. The cells were then washed with cold PBS, incubated with 10% TCA for two hours, and washed again with 95% alcohol. Cells were finally incubated with 0.2% NaOH 320 μl/well for 20 minutes before counting. The incorporated radioactivity was counted with a -scintillation counter. All assays were performed in triplicate.

Counting of Cell Number

Mesangial cells were cultured in 6-well plates with RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. After a 60-70% confluent, the medium was changed to RPMI1640 containing 0.2% FCS, and cultured for another 3 days. The medium was changed every two days, and the cell number was counted at the 2nd, 4th, and 6th days. The cells were washed twice with PBS and treated with 0.1% trypsin 1 ml/well for counting.

Measurement of Fibronectin

The mesangial cells were grown in 24-well tissue culture plates. The medium of subconfluent cultures was switched to the Ang-II stimulation. At the end of each experiment, the fibronectin content of the mesangial conditioned tissue culture supernatant was measured. Cells were then exposed to 0.05% trypsin in 0.53 mM EDTA and counted in a hemocytometer. The primary antibody was a polyclonal rabbit anti-rat fibronectin antiserum (Calbiochem, CA, USA), and the secondary antibody was the peroxidase conjugated goat anti-IgG (Calbiochem, CA, USA). After incubation overnight at 4°C, the plates were washed with TTBS (1M Tris, pH 7.6; 5M NaCl; 0.1% Tween-20), stained with ECL system (Amersham, Buckinghamshire, England), and autoradiographed with Kodak X-OMAT-AR film.

Measurement of Fibronectin mRNA Expression

Total cellular RNA was isolated according to the acid guanidinium thiocyanate-phenol-chloroform method as previously described.[6] Briefly, RNA (20 μg/lane) was separated by 1% agarose/formaldehyde gel electrophoresis and transferred to nylon filters. The filters were hybridized with a random-primed ^{32}P-labeled rat fibronectin cDNA probe. Then the filters were washed in 30 mM NaCl, 3 mM sodium citrate and 0.1% sodium dodecyl sulfate at 65°C. The filters were autoradiographed with Kodak X-OMAT-AR film for 24 to 72 hours at -70°C. To correct for difference in RNA loading, we subsequently rehybridized the filters with a -actin cDNA probe. Each lane of the autoradiographs was measured in a densitometer (Molecular Dynamics, model 100S).

Statistics

One-way ANOVA and unpaired t test were used to compare the difference between each pair.

RESULTS

Dose-Response for $[^3H]$-Thymidine Uptake (Fig. 1)

The $[H^3]$-thymidine uptake of mesangial cells increased progressively after stimulation with Ang-II at a concentration from 10^{-10} to 10^{-7}M. From this result, we chose a concentration of 10^{-7}M for study.

Time-Response for Cell Number (Fig. 2)

The number of mesangial cells after stimulation with Ang-II (10^{-7}M) had not increased at the 2nd day, however, the cell number had increased significantly at the 4th day and 6th days after stimulation. The control group without Ang-II stimulation did not show an increase in cell number.

Fibronectin mRNA Expression (Fig. 3)

The fibronectin mRNA expression of mesangial cells increased after 18 hours of Ang-II (10^{-7}M) stimulation.

The expression was also significantly increased at 24 hours and decreased thereafter.

Fibronectin Production (Fig. 4)

The fibronectin production of mesangial cells increased significantly at 18 hours after Ang-II (10^{-7}M) stimulation (P<0.001). The amount remained higher than the control at 24 hours (P<0.01), and decreased thereafter.

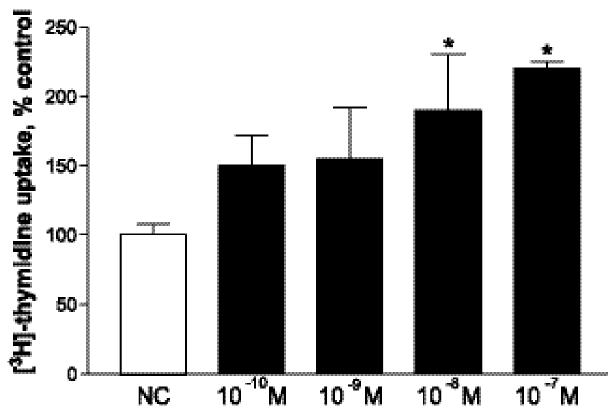

Fig. 1. Effects of different concentrations of angiotensin II on the [^{3}H]-thymidine uptake of cultured rat glomerular mesangial cells. The cells were preincubated with angiotensin II for 3 hours and then pulsed with [^{3}H]-thymidine for 17 hours. Data are presented with the mean±SEM of all three independent experiments performed in quadriplicate. *: P<0.05 compared to normal controls (NC).

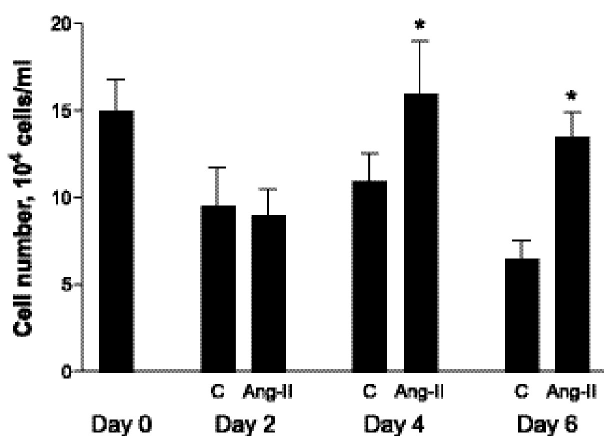

Fig. 2. Effect of angiotensin II (Ang-II, 10^{-7}M) on the growth of glomerular mesangial cells. Data are presented with the mean±SEM of all three independent experiments performed in triplicate. *: P<0.01 compared to control (C) cell number on the same day.

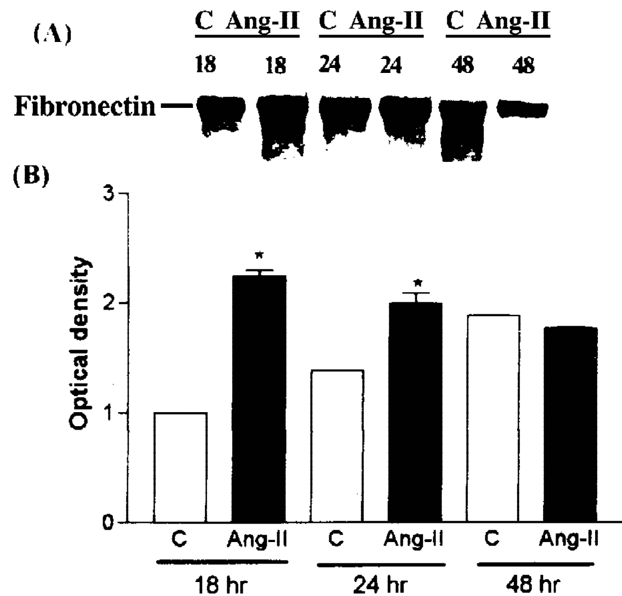

Fig. 3. Effect of angiotensin II (Ang-II, 10^{-7}M) on the fibronectin mRNA expression of glomerular mesangial cells. Cells were collected at 18, 24 and 48 hours, respectively. Upper (A) is a representative of autoradiography, and lower (B) is the mean±SEM calculated by densitometer of three independent experiments. *: P<0.001 compared to control (C) value at 18 hours.

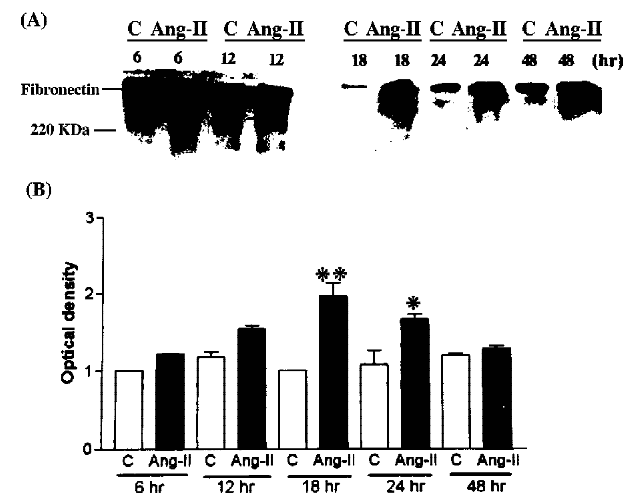

Fig. 4. Effect of angiotensin II (Ang-II, 10^{-7}M) on the fibronectin protein production of glomerular mesangial cells. Cells were collected 6, 12, 18 and 24 hours, respectively. Upper (A) is a representative of autoradiography, and lower (B) is the mean±SEM calculated by densitometer of three independent experiments. *: P<0.01, **: P <0.001 compared to control (C) value at 18 hours.

DISCUSSION

Ang-II is known as a critical factor for the progression of chronic renal diseases.[7] We have demonstrated that Ang-II enhances proliferation of glomerular mesangial cells. The proliferating cells would, in turn, activate renal cells in an autocrine fashion, through the release of a wide range of growth factors, including Ang-II, and therefore contribute to the perpetuation of kidney damage. We have also shown that Ang-II enhanced fibronectin production of mesangial cells. These data show that Ang-II could contribute to the glomerulosclerotic process and the consequent kidney damage.

Ang-II affects cellular behavior by binding to its cell surface receptor,[8] and the Ang-II type A receptor has been found to ameliorate the glomerulosclerosis/ interstitial fibrosis and retard the progression of chronic renal diseases.[9] The downstream signal transducers of Ang-II in the signalling pathway may involve arachidonic acid, Rac1, Nox4, reactive oxygen species and ERK1/ERK2.[10] Recent data also provide in vivo evidence that injured glomeruli are sensitive to local tissue actions of Ang-II, which promote proliferation and matrix accumulation within the glomerulus.[11] JNK also mediates the proliferative effect of Ang-II in cultured human mesangial cells and thus represents a novel therapeutic target for treatment of chronic renal diseases.[12] Ang-II also promotes apoptosis of mesangial cells. This effect of Ang-II is mediated through downstream signaling involving TGF-beta, phospholipase D, and calcium, contributing to the activation of NADPH oxidase and generation of reactive oxygen species.[13]

The synthesis of extracellular protein in mesangial cells plays an important role in mesangial expansion and pathogenesis of glomerulosclerosis.[14] Fibronectin is one of the major components of the mesangial matrix. Fibronectin is a high-molecular mass adhesive glyocoprotein implicated in a wide variety of cellular properties, including cell adhesion, differentiation, proliferation, migration, and apoptosis.[15] Fibronectin regulates many cellular functions after directly binding to integrins.[16] It has been reported that the expression of fibronectin could be regulated by many molecules, such as TGF- , cAMP, epidermal growth factor (EGF), PDGF, and interferon- (IFN-). However, the mechanism responsible for production and accumulation of fibronectin remains poorly understood. AngRem104 is a novel human gene recently found to be potentially involved in the regulation of fibronectin induced by Ang-II in human mesangial cells, which may provide new insights into the mechanisms of glomerular sclerosis associated with Ang-II.[17] This study shows that Ang-II can be one of the major stimulants for fibronectin production of mesangial cells. Another study demonstrated that Ang-II-induced fibronectin production was suppressed by an RNA interference technique.[18] It is interesting that mesangial cells slowly underwent apoptosis after infection with a retrovirus that continuously suppressed fibronectin synthesis. These studies, which explored a new method for simultaneously inhibiting the proliferation of mesangial cells and the accumulation of extracellular matrix, may represent a novel therapeutic approach to glomerulosclerosis.[18] Ang-ll has also been shown to induce a phenotypic alteration in mesangial cells, enhance the mesangial expression of nonmuscle myosin heavy chain (SMemb) and stimulate the fibronectin synthesis.[19] These results suggest that the mesangial expression of SMemb is related to glomerular matrix accumulation.

In conclusion, these results indicating that Ang-II enhance cellular proliferation and fibronectin production of glomerular mesangial cells provide important evidence for the role of mesangial cells in the process of glomerulosclerosis.

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