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Angiotensin II Stimulates the Proliferation and Biosynthesis of Type I Collagen in Cultured Murine Mesangial Cells

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A murine mesangial cell line (MMC) was established from the glomeruli of SJL mice to study the influence of angiotensin II (ANG II) on their growth and function in a serum-free culture. Murine mesangial cells exhibit the phenotypic characteristics of mesangial cells, including staining for desmin, vimentin, Thy 1, and types I and IV collagen by immunofluorescence. The addition of daily doses of 10^{-6} to 10^{-11} mol/l ANG II to MMCs also induced their proliferation in serum-free media. This effect on growth was independent of the presence of insulin in the media, and was receptor mediated, because the specific ANG II-receptor antagonist DuP 753 abolished proliferative growth. Angiotensin II also stimulated mainly the biosynthesis of type I collagen in our MMCs. Transfection of MMCs with chimeric genes containing enhancer/promoter elements for α2(I) and α1(IV) collagens linked to a chloramphenicol acetyltransferase reporter demonstrated that the stimulatory effect of ANG II for type I depends, at least to some extent, on an increase in transcription. These findings indicate collectively that ANG II in serum-free cultures can be a paracrine catalyst for the growth and biosynthesis of type I collagen in mesangial cells. (Am J Pathol 1992, 140:95–107)

Mesangial cells activated by inflammatory events can become important participants in the interactive processes that lead to glomerulonephritis.[1] The proliferation of glomerular mesangial cells, and their production of additional extracellular matrix has been linked, for example, to the development of glomerulosclerosis, proteinuria, and progressive renal failure in a variety of animal models.[2] Mesangial cells appear to be specialized pericytes of considerable biologic complexity.[3,4] These cells can secrete or respond to a wide array of growth factors, hormones, and cytokines.[3–5] *In vitro* studies also have demonstrated convincingly that cytokines, like epidermal growth factor, platelet-derived growth factor, insulinlike growth factor I, insulin, transforming growth factor α, interleukins-1, -4, and -6, endothelin, and prostaglandin $F_{2\alpha}$, all can induce mitogenesis in cultured mesangial cells.[4,5]

The uptake of macromolecules by the mesangium and the proliferation of its resident cells likely contribute to an expansion of mesangial matrix. Both of these processes may be regulated partially by angiotensin II (ANG II).[6,7] Various animal models also provide suggestive evidence that the intrarenal renin-angiotensin system is activated in parallel with circumstances that produce an increase in mesangial matrix.[6,7] Previous studies investigating the direct effects of ANG II on mesangial growth have reported a variety of conflicting results.[8–11] This enigma may be due partly to the presence of serum or other growth factors in each of the various cell culture systems. Most of these studies also have been performed using primary cultures of low-passage rat or human mesangial cells in serum, whereas only a few have examined cells of murine origin. We therefore chose in the present study to analyze the direct effects of ANG II on a transformed mesangial cell line in serum-free culture. Our findings indicate that ANG II can stimulate cell proliferation as well as the transcription and biosynthesis of type I collagen.

Materials and Methods

Isolation and Characterization of Murine Mesangial Cells

Kidneys were harvested from 8- to 10-week-old naive SJL/J (H-2^s) mice. After brief washing in phosphate-

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buffered saline (PBS, pH 7.0), the cortices were separated from the medulla, pooled, minced, and the glomeruli were isolated by differential sieving.[12,13] The resulting preparation contained >95% glomeruli as judged by light microscopy. Glomeruli then were treated with 0.1% collagenase for 30 minutes (type IV from Sigma, St. Louis, MO) and plated in T-25 culture flasks (Nunc, Denmark) in RPMI 1640 containing 15% heat-inactivated fetal calf serum (Gibco-BRL, Gaithersburg, MD), 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml bovine insulin (Sigma). The glomeruli were cultured at 37°C in 5% CO_2, and outgrowths of spindlelike mesangial cells, as well as epithelial cells with a cobblestone appearance, could be observed after 3 to 5 days. After 10 days, the epithelial cells and the remnant glomeruli were no longer visible and mesangial cells were passaged after trypsination. Cells were grown for a further 72 hours in the presence of 50 mmol/l D-valine (Sigma), replacing L-valine in the RPMI 1640, to exclude fibroblasts.[14] Subconfluent mesangial cells grown in T-25 flasks were next transformed with nonreplicating, non–capsid-forming SV-40 virus (strain Rh 911, 108 IU/ml).[15] After integration for a week, these murine mesangial cells (MMCs) were transferred to Dulbecco's modified Eagle's medium (DMEM) containing penicillin/streptomycin, and 5 mmol/l glutamine, where they were cloned, grown, and incubated for prolonged periods (up to 5 days) without the addition of serum. Cells between five to ten passages were used for all studies.

The MMCs were characterized further by immunofluorescence. For this purpose, they were grown in glass slides chambers until they were subconfluent. The cells then were fixed at −20°C in acetone for 10 minutes before their staining. Cells were stained with the following antibodies using indirect immunofluorescence: α-vimentin-Ab, α-desmin-Ab (both from Sigma), α3M-1-Ab (a nephritogenic antigen in proximal tubular cells),[16] a monoclonal αThy 1.1-Ab (Sigma), and with monospecific polyclonal antibodies against type I and IV collagens.[17] Additionally, the MMCs were incubated for 72 hours in the presence of D-valine instead of L-valine, and proliferation was measured as described below. Syngeneic, transformed fibroblasts (TFB) isolated from the renal tubulointerstitium served as a control cell line in these latter experiments.[18]

Cell Proliferation Assays

Murine mesangial cells were transferred to a 96-well microtiter plate, 10^4 cells per well, rested for 48 hours in serum-free DMEM, and then further stimulated for 24 to 48 hours with daily doses (every 24 hours) of 10^{-6} to 10^{-11} mol/l ANG II acetate salt (Sigma) dissolved in media. In selected experiments, cells also were coincubated with 10^{-6} mol/l saralasin (Sar^1-Val^5-Ala^8, Sigma) or 10^{-6} mol/l of the specific, nonpeptide ANG II-receptor antagonist, DuP 753 (2-n-butyl-4-chloro-5-hydroxymethyl-1-[2-(1H-tetrazole-5-yl)biphenyl-4-yl)]methyl) imidazole, gift of Dr. Ronald D. Smith, Dupont Company, Wilmington, DE).[19] The stimulated MMCs were pulsed with 1 μCi ^{3}[H]thymidine (5 Ci/mmol; Amersham, Arlington Heights, IL) during the last 6 hours of culture. Cells were harvested on glass-fiber paper and counted for scintillations.[15,18]

To test the influence of insulin and ANG II on proliferation, cells rested in serum-free DMEM without additives were treated with 1 to 10 μg/ml bovine insulin in the presence or absence of 10^{-8} mol/l ANG II for 48 hours. ^{3}[H]-thymidine incorporation then was measured as described above.

For measurement of cell counts, 5×10^4 MMCs were made quiescent in a 24-well plate using serum-free DMEM. Stimulation with ANG II was carried out as described above. At the end of the stimulation period, cells were scrapped from the plate with 0.5 mol/l ethylenediaminetetra-acetic acid (EDTA) using a rubber policeman. The cells then were counted with an automated cell counter (Coulter Electronics, Hialeah, FL).

Radioimmunoassay for Secreted Collagens and Biolabeling with ^{3}[H]Proline

After stimulation for 48 hours with the different mediators, secreted type I and IV collagens were measured in the supernatant using monospecific, polyclonal rabbit anti-types I and IV collagen antibodies.[20–22] Radioimmunoassay for collagens was performed on 10^4 rested MMCs in serum-free DMEM supplemented with 50 μg/ml each of L-ascorbic acid and β-aminopropionitrile.[23] Data were expressed as nanograms per milliliter culture supernatant after normalization for cell number.[23] Measurement of the biosynthesis of collagens also was performed by standard technique.[22] In T-75 flasks, 10^6 rested MMCs were incubated for 48 hours with daily doses of 10^{-8} mol/l ANG II in the presences of 10 μCi ^{3}[H]proline (L-(2,3,4,5)-^{3}[H]proline, 130 Ci/mmol, Amersham), L-ascorbic acid, and β-aminopropionitrile. At the end of the incubation period, the supernatant and the cells were harvested separately in a cocktail of protease inhibitors (15 mmol/l N-ethylmaleimide, 20 mmol/l EDTA, 1 mmol/l phenylmethylsulfonylfluoride, and 10 U aprotinin). Murine mesangial cells were lysed in 5% acetic acid on ice, centrifuged briefly, and the supernatant then was transferred to a new tube. The proteins from the supernatants and lysates were precipitated by adding absolute ethanol (final concentration, 33%, vol/vol) at 4°C overnight. The

samples were centrifuged at 4°C, the pellets were dissolved in Laemmli buffer containing 5% 2-mercaptoethanol, and the total protein concentration was determined for each sample.[23] Some samples were resuspended in 0.1 mol/l TRIS (hydroxymethyl)aminomethane (TRIS-HCl, pH 7.4), 15 mmol/l $CaCl_2$, and treated with 20 U high purity collagenase VII (Sigma) for 60 minutes at 37°C. Equivalent concentrations of proteins were separated under denaturing conditions on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel. After staining, the gel was treated with Amplify™ (Amersham) and fluorographed at − 70°C for 24 hours (supernatants) or 7 days (cell lysates) using intensifying screens. Collagen standards for types I and IV and purified fibronectin were run in addition to the routine size markers.

Trichloracetic acid (TCA)-precipitable total ^{3}[H]-proline incorporation also was measured in some experiments. For these studies, 5×10^4 cells were plated in 24-well plates and rested for 24 hours in serum-free media. The MMCs were stimulated for 48 hours either with control media, daily doses of 10^{-8} mol/l ANG II, 10^{-6} mol/l DuP 753, or 10^{-8} mol/l ANG + 10^{-6} mol/l DuP 753. For the last 12 hours, 1 μCi ^{3}[H]proline was included in each well. The cells were washed two times in ice-cold PBS, and then were precipitated twice with 10% TCA, redissolved in 0.5 mol/l NaOH with 0.1% Triton X-100, and counted for β-emissions. Results were expressed as counts/well normalized for cell number.

Chimeric Chloramphenicol Acetyltransferase (CAT) Reporter Genes

Rested MMCs, 2×10^5 in a culture petri dish, were transfected with 15 μg of plasmid DNA using calcium phosphate.[18,21] The following plasmids were used for transfections: pA_oCAT containing only the CAT gene was used as a negative control; pSV_2CAT containing the CAT gene linked to the SV40 promoter and enhancer served as positive control for transfection efficiency; pR41 containing the CAT linked to the α2(I) collagen promoter and enhancer[24]; and p184 containing α1(IV) collagen promoter and enhancer.[21,25] After completing the transfection, ANG II, DuP 753, or saralasin were added to the media and the cells were incubated for another 48 hours. These MMCs were then washed in PBS at the end of the incubation period, lysed in 0.25 mol/l TRIS-HCl (pH 7.0), and the lysate was spun at 4°C. The protein concentration was determined in an aliquot of the supernatant,[23] and the CAT assay was performed with equal protein concentrations.[18,21,26] ^{14}[C]chloramphenicol products were extracted with ethyl acetate and separated by thin-layer chromatography using chloroform-methanol (95:5, vol:vol) as a tracer.[26] Plates were autoradiographed and the appropriate bands for acetylated products were cut out and counted for scintillations. Enzyme activities are reported as relative CAT-activities with $pA_o = 1$ (CAT-RV).

Statistical Analysis

All data are presented as a mean ± standard error of the mean (SEM). Statistical significance between more than two groups was first tested with the nonparametric Kruskal-Wallis test for multiple comparisons. If this test was significant, individual groups were compared using the Wilcoxon-Mann-Whitney test. A P value of < 0.05 was considered significant.

Results

Characterization of Murine Mesangial Cells

One of the objectives of the present study was to generate a cell line of MMCs that could function for prolonged periods in serum-free media. The cells can be grown in serum-free culture conditions for at least 5 days, although the thymidine incorporation slightly decreases over time (data not shown). These MMCs grew normally in this media in a typical stellate or spindle-form fashion with extensive cytoplasmatic projections. Figure 1 demonstrates the immunofluorescent staining pattern of these MMCs after labeling with antibodies specific for a variety of phenotypic traits. These cells, for example, stained positive with antibodies against the cytoskeletal filaments desmin and vimentin (Figures 1E, F), suggesting a mesenchymal origin.[12,13,27] They also express the Thy 1.1 antigen (Figure 1D), but failed to bind antibody recognizing the proximal tubular 3M-1 antigen (Figure 1C). Staining MMCs with antibody recognizing murine collagen type I demonstrated a typical intracellular staining pattern (Figure 1B), whereas the basement-membrane–associated collagen type IV was localized more homogeneously over the cell surface (Figure 1A).

Although we have never observed glomerular outgrowths of fibroblasts from 8- to 10-week-old mouse kidneys, we nevertheless incubated MMCs for 72 hours in the presence of 50 mmol/l D-valine instead of L-valine. Syngeneic, transformed interstitial fibroblasts (TFBs), harvested originally from the tubulointerstitial microenvironment, served as a specificity control.[18,28] Table 1 demonstrates that MMCs grew equally well in RPMI supplemented either with 0.5% FCS and D-valine or with 0.5% FCS and L-valine, as measured by ^{3}[H]thymidine incorporation. The syngeneic fibroblasts, in contrast, did not grow well in the presence of D-valine or in 0% FCS (data not shown).

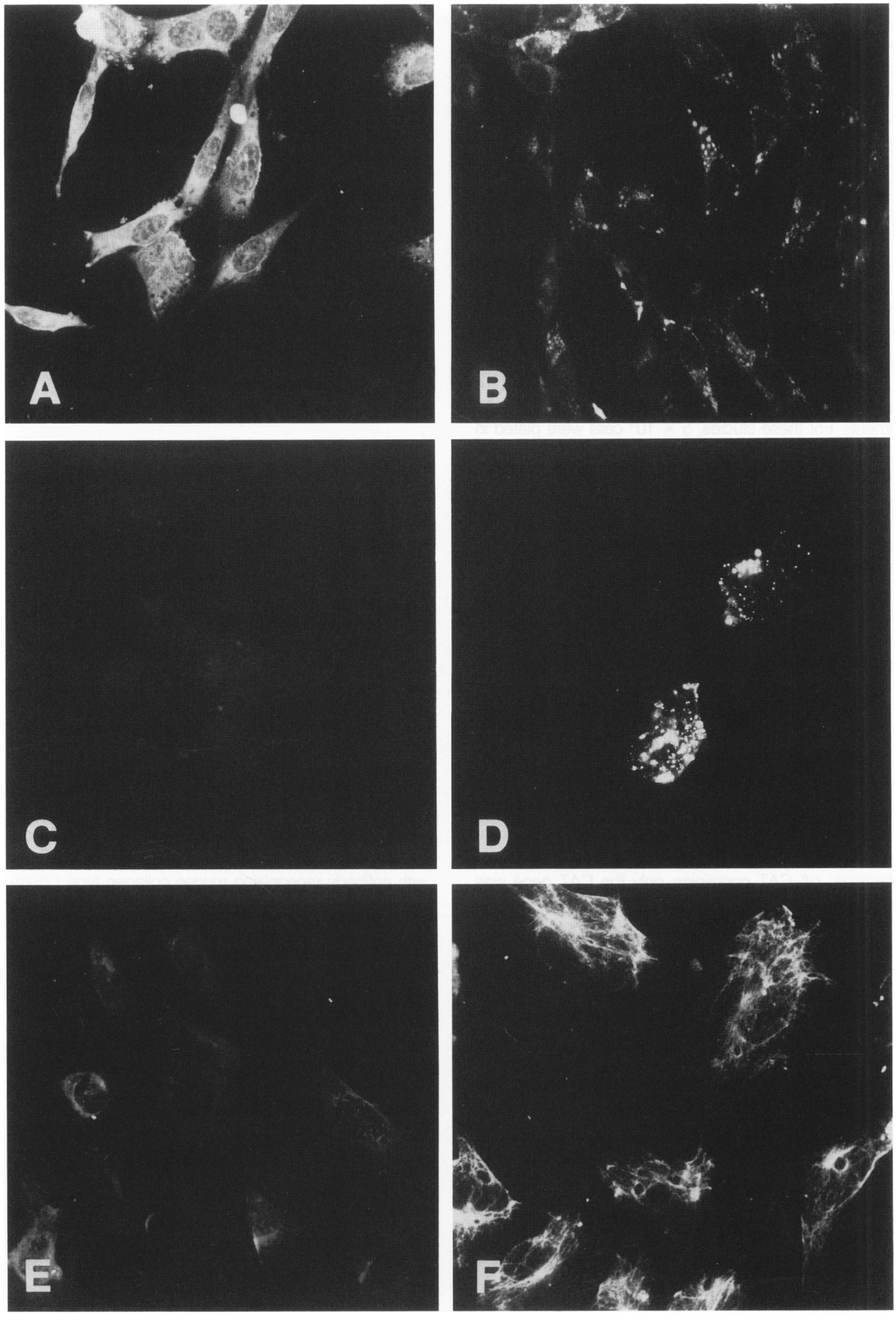
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Table 1. *Influence of D- and L-valine Containing Media on the Incorporation of ^{3}[H]thymidine in Mesangial Cells (MMCs) and Tubulointerstitial Fibroblasts (TFBs)*

	cpms × 10^3/well	
Cells	L-valine	D-valine
MMCs	36.5 ± 3.5	29.1 ± 9.5
TFBs	66.9 ± 7.1	5.8 ± 1.1*

* $P < 0.005$ vs. cells grown in the presence of L-valine, n = 10. ^{3}[H]thymidine incorporation was measured after 72 hours, as described in Material and Methods.

Effect of Angiotensin II on the Cellular Proliferation of Mesangial Cells

To determine whether ANG II had any effects on the proliferation of MMCs, quiescent cells were treated for either 24 or 48 hours with various concentrations of ANG II. In Figure 2, a single dose of 10^{-5} to 10^{-7} mol/l ANG II increased significantly their thymidine incorporation after 24 hours. Cells cultured for 48 hours with daily doses of ANG II (10^{-6} to 10^{-10} mol/l given twice every 24 hours) also demonstrated a stimulation of proliferation. The increase in thymidine incorporation was blocked by coincubation with the specific, nonpeptide ANG II-receptor antagonist DuP 753 (10^{-6} mol/l DuP 753 + 10^{-8} mol/l ANG II for 48 hours: 41.4 ± 3.6 × 10^3 cpm/well, $P < 0.02$ *versus* ANG II alone, n = 10). Dup 753 alone was without significant effect on proliferation (10^{-6} mol/l DuP 753 for 48 hours: 40.9 ± 5.5 × 10^3 cpm/well, P = not significant *versus* controls, n = 9). Similarly, the ANG II-induced increase in thymidine incorporation also was prevented by treatment with saralasin (10^{-6} mol/l saralasin + 10^{-8} mol/l ANG II for 48 hours: 30.5 ± 2.1 × 10^3 cpm/well, $P < 0.02$ *versus* cells treated with ANG II alone, n = 10). Saralasin by itself did not stimulate significantly any thymidine incorporation (10^{-6} mol/l saralasin for 48 hours: 48.9 ± 2.0 × 10^3 cpm/well, P = not significant *versus* controls, n = 9).

The increase in thymidine incorporation after stimulation by ANG II also was reflected in an increase in cell number. Figure 3 demonstrates a significant increase in numbers of MMCs 48 hours after stimulation with 10^{-7} to 10^{-9} mol/l ANG II. This effect was blocked by DuP 753, or by saralasin (10^{-6} Dup 753 + 10^{-8} mol/l ANG II for 48 hours: 526 ± 38 × 10^3 cells/well, $P < 0.05$ *versus* ANG II alone, n = 6; 10^{-6} saralasin + 10^{-8} mol/l ANG II for 48 hours: 445 ± 47 × 10^3 cells/well, $P < 0.05$ *versus* ANG II alone, n = 6). Both blockers alone did not influence cell number significantly (10^{-6} mol/l DuP 753 for 48 hours: 419 ± 41 × 10^3 cells/well, 10^{-6} mol/l saralasin for 48 hours: 497 ± 70 × 10^3 cells/well, n = 6 for both). Angiotensin II, 10^{-8} mol/l for 48 hours, was also without significant effect.

Because it has been reported that the contractile properties of ANG II on mesangial cells require the presence of insulin,[29] we next tested whether insulin was necessary for ANG II–induced proliferation. Insulin alone, in a pharmacologic dose range of 10 μg/ml for 24 hours, induced a statistically significant increase in thymidine incorporation (Figure 4). The effect of ANG II on MMC proliferation, however, was independent of insulin, and higher concentrations of insulin had no additional effect on ANG II–induced proliferation (Figure 4).

Effect of ANG II on the Biosynthesis of Collagen

Because MMCs are capable potentially of producing several collagens, we next wanted to determine whether stimulation by ANG II had any influence on collagen synthesis in our cell line. As an initial approach, we measured total ^{3}[H]-proline incorporation into TCA-precipitable proteins. Figure 5 shows that 10^{-8} mol/l ANG II for 48 hours significantly stimulated proline incorporation into *de novo* synthesized proteins, probably collagens preferentially. Data were normalized for cell number, excluding that the observed effects were solely caused by the ANG II–induced proliferation. The stimulatory effect of ANG II was blocked by 10^{-6} mol/l DuP 753.

To gain a better insight as to which collagens are synthesized, cells were labeled with ^{3}[H]-proline in the presence of L-ascorbate and cross-linking inhibitors during stimulation with ANG II for 48 hours. Equal protein concentrations of the supernatant, harvested from a serum-free culture, were run on denaturing SDS-polyacrylamide gels in parallel with cell lysates. The size of the bands after autoradiography were compared with standard collagens run in parallel. As shown in Figure 7A, ANG II stimulates mainly the production and secretion of α1(I) and α2(I) collagens, whereas the secretion of type IV collagens seems to be only marginally influenced by ANG II. A syngeneic proximal tubular cell line (MCT cells), which synthesize only small amounts of type I collagen,[15,20,21,23] served as an additional control. The autoradiogram of this gel indicates that most of these collagens are secreted into the culture supernatant, because only relatively small amounts of collagen were observed in the cell lysates. Furthermore the stimulatory effects of ANG II on collagen synthesis were blocked by coincu-

Figure 1. *Immunofluorescence staining of MMCs grown in chambers on glass slides for 48 hours in serumfree medium. The cytoskeleton of the MMCs is stained by α-desmin* **(E)** *and α-vimentin* **(F)** *antibodies. An antibody specific for Thy 1 binds to MMCs* **(D)**, *but MMCs did not stain with an antibody against 3M-1, a major nephritogenic antigen from proximal tubular cells* **(C)**. *Collagen type I was mainly localized intracellularly* **(B)**, *whereas an antibody against type IV collagen stained MMCs more homologeous including the cell membrane* **(A)**, *(original magnification ×200).*

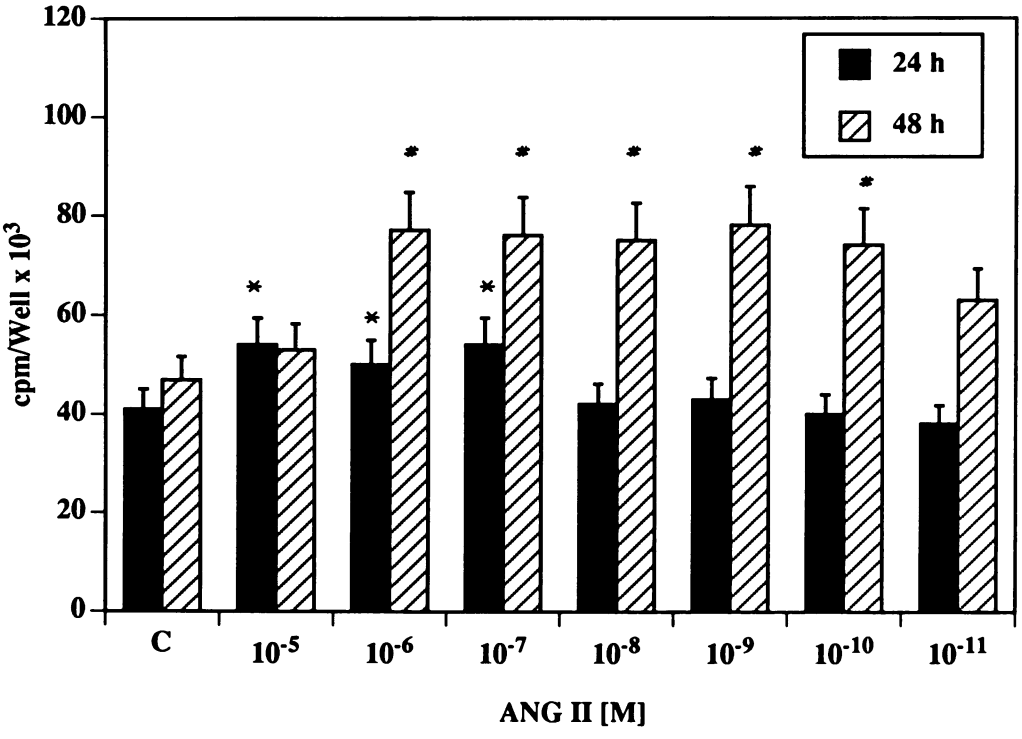


Figure 2. *^{3}[H]thymidine incorporation into rested MMCs 24–48 hours after daily administration of ANG II. A single dose of 10^{-5}–10^{-7} M ANG II for 24 hours increased significantly thymidine incorporation although the effect was more pronounced after 48 hours when ANG II had been added twice. Cells were pulsed for the last 6 hours with 1 μCi/well ^{3}[H]thymidine. *P < 0.05 versus controls after 24 hours, #P < 0.01 versus controls after 48 hours, n = 10.*

bation with 10^{-6} mol/l DuP 753 (Figure 7B). Pretreatment of samples with collagenase and subsequent electrophoretic separation showed that the bands represented collagenous proteins (data not shown). We also measured the secretion of types I and IV collagens with a sensitive radioimmunoassay established previously in our laboratory.[15,18,21,23] Table 2 demonstrates that the treatment of quiescent MMCs with ANG II stimulates significantly the secretion of type I collagen but had no significant effect on type IV collagen.

To determine whether this increase in the synthesis of type I collagen might be due to enhanced gene transcription, a chimeric reporter gene construct (pR41) containing type I enhancer/promoter elements linked to the CAT was transfected transiently into MMCs. As shown in Figure 8A, the presence of 10^{-6} to 10^{-8} mol/l ANG II for 48 hours increased significantly the CAT activity of this plasmid construct. The activity of the control construct, pAo, which contains a promoterless CAT gene, is arbitrarily assigned a relative value of 1.0. The transfection of MMCs with pR41 followed by incubation in control media without ANG II showed a relative CAT-activity of 1.1. Angiotensin II, 10^{-8} mol/l, increased the relative activity of pR41 to 4.9. This increase after ANG II was reduced to a relative activity level of 1.5 by 10^{-6} mol/l DuP 753 (DuP 753 + ANG II). Although saralasin exhibited some intrinsic stimulatory activity (3.2), the relative CAT-activity of saralasin and ANG II (3.6) was also less than with ANG II alone, suggesting an effect of partial receptor blockade. The CAT-activity of the type IV construct p184 was not significant changed by ANG II in MMCs (Figure 8B), although the cells could be transfected successfully with the positive control pSV_2.

Discussion

The effects of ANG II on the proliferation of mesangial cells in culture is not assiduously resolved by the current published literature. Some investigators have reported that ANG II can induce proliferation of mesangial cells,[9,10] whereas others have found little or no effect.[8,11] A few experiments have even suggested alternatively that ANG II in culture may induce cellular hypertrophy, rather than proliferation in mesangial cells.[11]

All of these reports have used either human or rat mesangial cells, often in the presence of serum. Serum contains several proteases that can easily cleave ANG II, and serum itself is a highly complex mixture of factors with either growth stimulatory or inhibitory properties. Mesangial cells also express intrinsic proteases capable of peptide degradation.[4] What concentrations of ANG II are

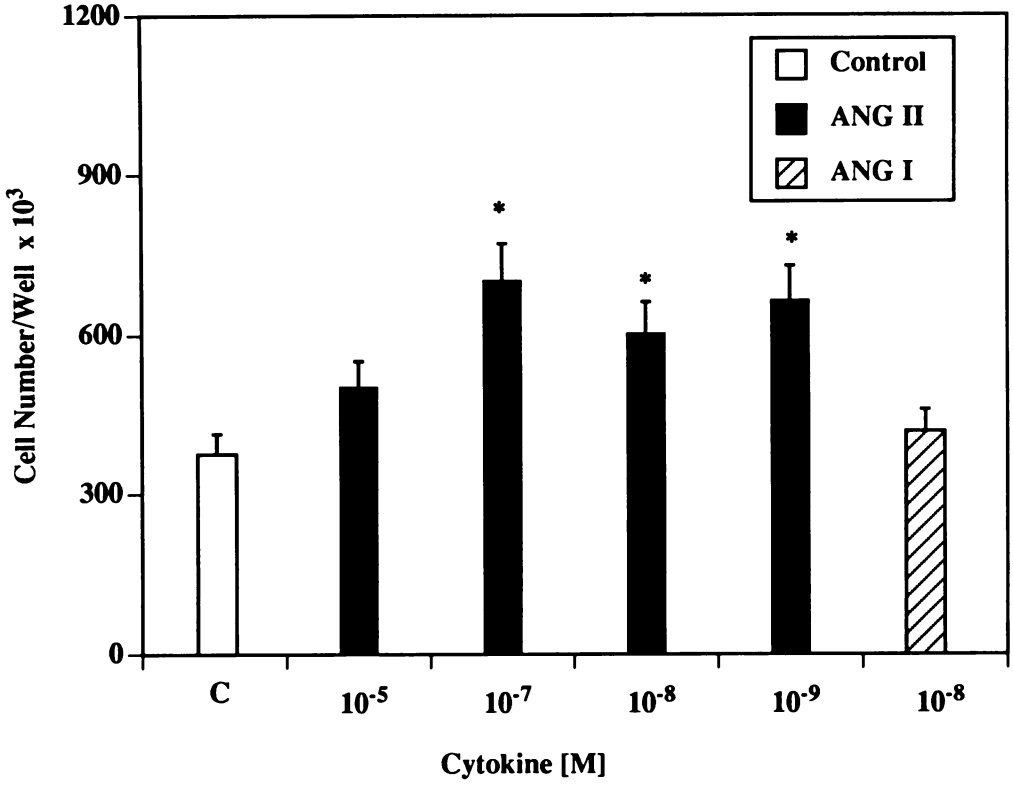


Figure 3. *Total cell number/well 48 hours after daily ANG II stimulation. 10^{-7}–10^{-9} M ANG II increased significantly the cell number whereas 10^{-8} M angiotensin I (ANG I) was without major effect. 5 × 10^4 cells were plated originally per well.* *P < 0.02 *versus controls, n = 6.*

actually seen by these cells over prolonged periods in culture is still contentious. It also has been reported that some primary cultures of mesangial cells may no longer respond to ANG II after multiple passages, probably as a result of a drop in numbers of expressed receptors.[30]

We established a cultured line of transformed MMCs that can be grown for prolonged periods without serum to evaluate some of these issues. Our MMCs have the phenotypic characteristics of primary mesangial cells in culture, including a typical shape, and a positive staining for vimentin, desmin, and Thy 1.[12,13,27,31] These MMCs, furthermore, did not express proximal tubular antigens,[16] but did grow in the presence of D-valine, unlike fibroblasts.[14] Angiotensin II, as a single factor added daily to serum-free media, easily induced proliferation in these MMCs. Although proliferation could be detected 24 hours after a single dose of ANG II (Figure 2), mitogenesis was more pronounced after 48 hours, when ANG II had been given twice. The proliferative effect of ANG II was blocked by incubation with saralasin or DuP 753, indicating signal-transduction through specific ANG II-receptors, preferentially of the AT-1 type.[32] It is possible, however, that mesangial cells may have other subtypes of ANG II-receptors besides AT-1. Recent micropuncture studies in normal rats also have reported that ANG II concentrations in the glomerular filtrate can be in the nanomolar range.[33] This is a concentration that was mitogenic clearly in our cell culture system.

It has been observed that insulin also may be required for the expression of a contractile response by mesangial cells to ANG II in culture[29] and for a proliferative effect in cultured fetal human mesangial cells.[10] Insulin alone in some mesangial culture systems also has been observed to increase ^{3}[H]-thymidine incorporation into DNA.[34] Our studies confirmed that insulin, alone, induced proliferation in MMCs. Insulin in a variety of doses, however, was neither necessary in our system to demonstrate a proliferative effect of ANG II in MMCs, nor did ANG II in combination with insulin have an effect on proliferation that was different from what we observed with ANG II alone. The basis for this discrepancy is unclear presently. Besides the obvious species differences, the glucose concentration in the media, the presence or absence of other supplementary factors in the serum, as well as the fact that our cells are transformed, may account for all or some of these findings.

We have not compared thoroughly the phenotypic characteristics of primary cultured mesangial cells with our MMCs in this study. Initial studies did show, however, that transformed MMCs proliferate approximately 10%

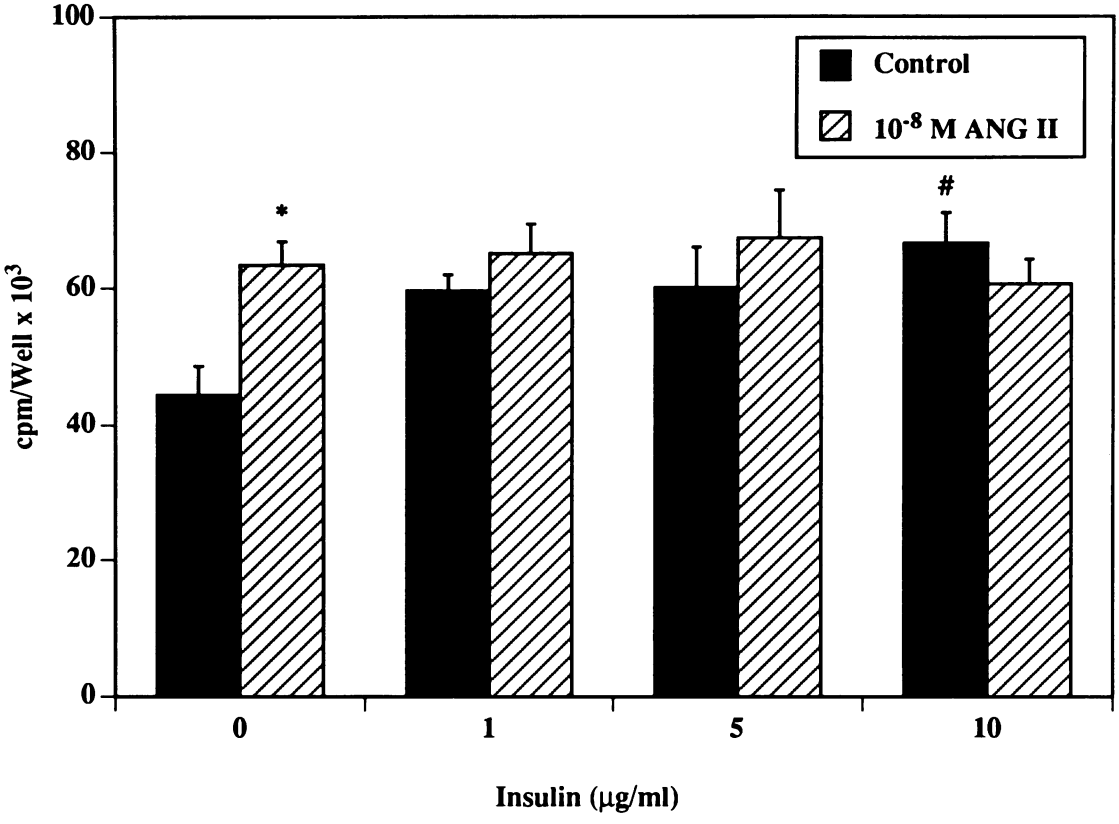


Figure 4. *Influence of insulin on ANG II-induced ^{3}[H]thymidine incorporation. MMCs were stimulated for 24 hours with 10^{-8} M ANG II in the presence or absence of 1–10 μg/ml bovine insulin. Additional cells were incubated with insulin alone. 10 μg/ml of insulin stimulated ^{3}[H]thymidine incorporation significantly in the absence of ANG II. ANG II-induced proliferation was insulin independent. #P < 0.05 versus controls without insulin. *P < 0.01 versus ANG II treated cells in the absence of insulin, n = 10.*

better in serum compared with nontransformed parental cells. Furthermore, in contrast to nontransformed mesangial cells, the MMCs can be incubated in serum-free media for at least up to 5 days. We are aware, of course, that transformed MMCs may have distinct phenotypic properties different from those of nontransformed cells. The phenotypical and functional characteristics studied so far, however, showed that our MMCs, besides their ability to grow in serum-free medium, do not seem to behave differently from other reports on nontransformed mesangial cells. A recent report using transformed MMCs in an antigen-presenting capacity also found no substantial differences in their phenotypic characteristics from primary cultures.[35] We also have established several syngeneic cell lines from different segments along the nephron, including proximal and distal tubular cells as well as tubulointerstitial fibroblasts,[15,18,28] and these groups of cells respond distinctively to ANG II and exhibit functional characteristics according to their origin. Angiotensin II induces cellular hypertrophy in syngeneic proximal tubular cells (MCTs), for example, rather than the proliferative response we observed with MMCs.[23] Such differences may be due to distinct signaling pathways for ANG II in MCTs *versus* MMCs. The ANG II–induced hypertrophy in proximal tubular cells was transduced by a decrease in intracellular cyclic adenosine monophosphate.[21] In contrast, ANG II tends to stimulate phospholipase C in mesangial cells, resulting in increases of inositol 1,4,5-triphosphate and release of diacylglycerol.[4,36] Apparently the same signal transduction pathway is used by vasopressin, which is mitogenic for mesangial cells in culture.[4,37] Interestingly it has also been reported in other cell culture systems that ANG II, through a protein kinase C-dependent mechanism, phosphorylates nuclear laminins.[38] A phosphorylation of laminins has been traditionally considered necessary for the disassembly of the nuclear envelope inducing progression from the G_2-phase of the cell cycle toward mitosis.[39]

It has been recently demonstrated that ANG II enhances the tyrosine phosphorylation of five proteins of molecular masses between 70 and 225 kd in rat mesangial cells.[40] This tyrosine phosphorylation was partly independent from activation of protein kinase C, and a similar pattern of phosphoproteins was induced by endothelin and vasopressin. These interesting findings suggest that the tyrosine phosphorylation of a distinct group of

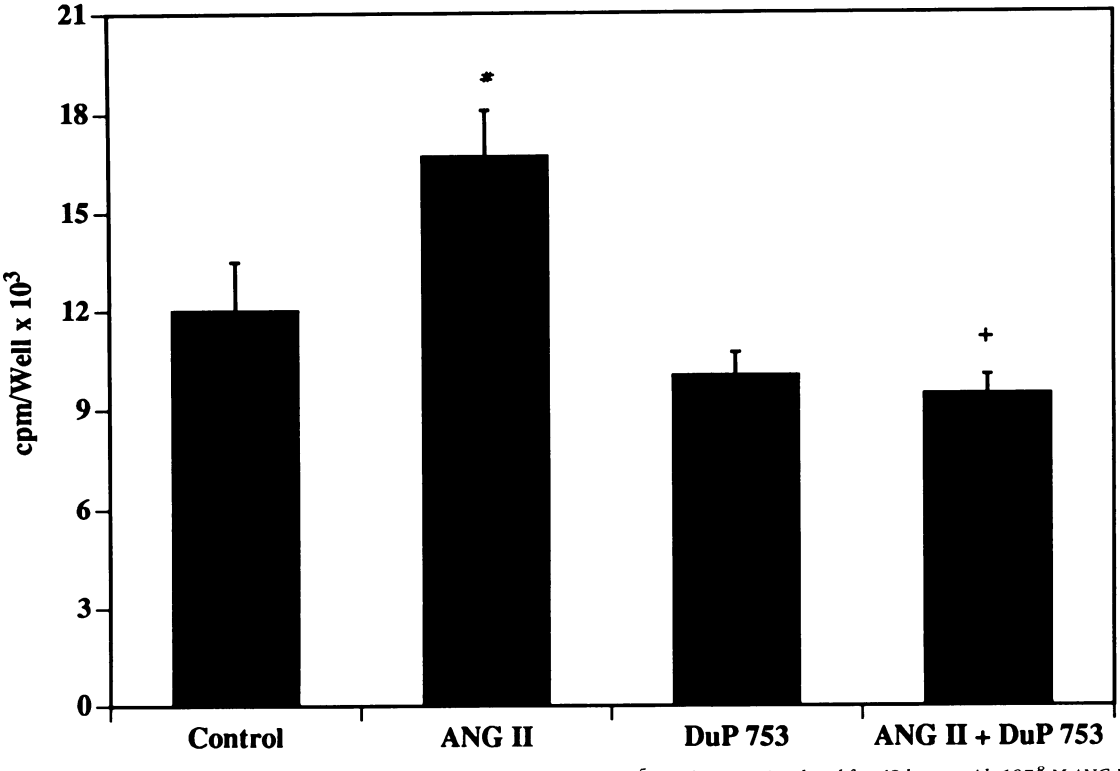


Figure 5. *³[H]proline incorporation into* de novo *synthesized proteins. 5 × 10⁵ MMCs were stimulated for 48 hours with 10⁻⁸ M ANG II in the presence or absence of 10⁻⁶ M of the specific ANG II-receptor antagonist DuP 753. Some cells were incubated with DuP 753 alone (10⁻⁶ M). During the last 12 hours, MMCs were pulsed with 5 μCi ³[H]proline per well. After repeated washings, the cellular proteins were precipitated twice in 10% trichloroacetic acid, redissolved in 0.5 N NaOH and counted for radioactivity. Data are reported as cpms/well, normalized for cell number. ANG II stimulated significantly ³[H]proline incorporation into precipitable proteins. This effect was blocked by DuP 753 co-incubation. DuP 753 alone had no significant effect. #P < 0.02 versus controls without ANG II, †P < 0.005 versus MMCs treated with ANG II in the absence of DuP 753, n = 6.*

membrane and cytosolic proteins by vasoactive peptides might play a critical role in the mitogenic response to these factors.[40]

An increase in the accumulation of collagen in glomeruli-producing fibrosis has been demonstrated in a variety of inflammatory states associated with mesangial proliferation.[2,41–44] The relationship between the proliferation of mesangial cells and glomerulosclerosis, however, is still uncertain.[2] Most of the newly deposited intraglomerular matrix seems to contain type IV collagen[41,42] and laminin,[42,43] although increased mesangial staining for type I collagen has been reported in one model of proliferative glomerulonephritis.[43] mRNA encoding glomerular α1(I) was also more abundant, and more elevated persistently than mRNA encoding type IV mRNA.[44] Mesangial cells in culture synthesize mainly type I collagen.[2,45–48] Biolabeling of our MMCs with ³[H]-proline followed by SDS-polyacrylamide gel electrophoresis (PAGE) demonstrated a pattern of collagen expression in accordance with the findings of earlier investigators.[23,47] In the presence of cross-linking inhibitors, most of the collagen chains were secreted into the culture supernatant. It has been demonstrated in rat mesangial culture that collagen type I, in contrast to other collagens, is not incorporated into the underlying matrix,[48] and it was produced continually even by cells that had reached confluence.[48] Angiotensin II treatment of the MMCs stimulated ³[H]proline incorporation into newly synthesized proteins, apparently through an ANG II-receptor–mediated mechanism. The SDS-PAGE showed that mainly type I biosynthesis was stimulated by ANG II. This finding was independently confirmed using a radioimmunoassay with monospecific, polyclonal antibody against type I collagen. The increase in the synthesis of type I collagen by ANG II does not reflect simply the induction of proliferation, because the data were normalized for cell number. In contrast, collagen type IV secretion as measured by the radioimmunoassay was, after normalization for cell number, not significantly influenced by ANG II. This finding is consistent with a preliminary report that ANG II may stimulate the incorporation of proline into collagenase-digestible proteins.[11] We can not exclude the possibility, however, that ANG II might have some mild stimulatory effects on the synthesis of other collagens.

Transfection of our MMCs with chimeric gene constructs containing enhancer/promoter elements from the

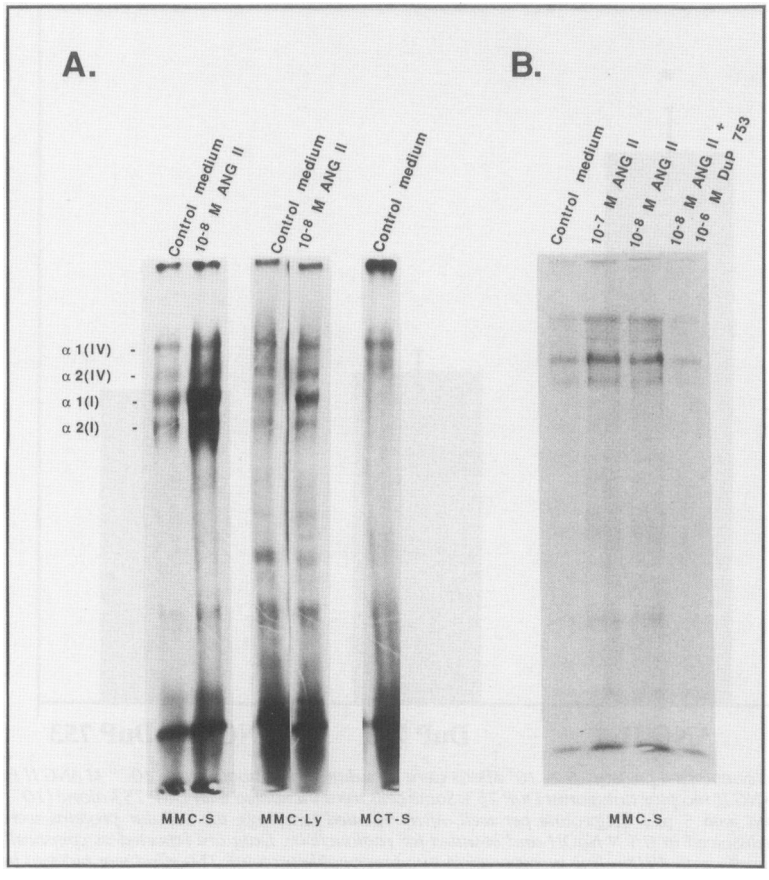
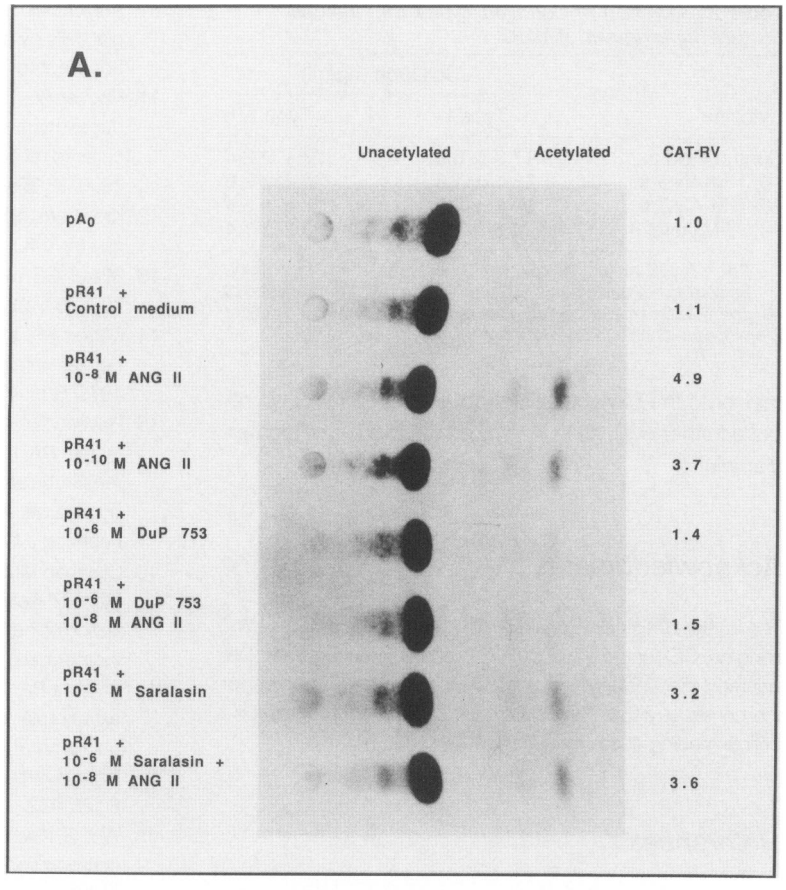


Figure 6. *Biolabelling of MMCs with ^{3}[H[proline grown in serum-free media in the presence or absence of daily doses of 10^{-8} M ANG II. After 48 hours similar amounts of protein were harvested from the culture supernatants and cell lysates, and were then run under denaturing conditions on a 12% SDS-polyacrylamide gel. The migration of collagen markers are noted at the left. Autoradiography of the lanes with supernatant (S) was performed for 24 hours whereas the lanes with the cell lysates (Ly) were autoradiographed for 7 days.* (A) *The majority of collagen proteins are secreted into the supernatant in the presence of crosslinking inhibitors. ANG II stimulates mainly the synthesis of type I collagens in MMC-S and MMC-Ly. Cell supernatants from MCT cells (MCT-S), a syngeneic proximal tubular cell line (15) grown in serum free media for 48 hours, produce less collagen type I, and is shown as an additional control.* (B) *The ANG II-induced increase in collagen synthesis in MMC-S is abolished by co-incubation with 10^{-6} M DuP 753.*

murine α2(I) gene linked to CAT revealed that ANG II-induced type I collagen synthesis, and that the increase was, at least to some extent, mediated by a change in transcription. Mature type I collagen from mesangial cells is comprised of a mixture of either α1(I)/α2(I) heterodimers or α1(I) trimers.[24,45] The ANG II–mediated increase in transcription seems to be receptor-mediated, because DuP 753 and saralasin both blocked the observed effects. In contrast to DuP 753, the antagonistic effects of saralasin were not complete, and the substance alone seemed to exhibit some stimulatory actions. This is in agreement with earlier observations that saralasin has some agonistic activity.[23] In contrast to type I, ANG II had no stimulatory activity on the transcription of the type IV construct. How ANG II stimulates the transcription of collagen type I is unclear. Some of the above-mentioned second messenger pathways might be involved in such a process.[4,36] Angiotensin II is internalized with its receptor after binding in vascular smooth muscle cells.[49] It also has been suggested in older literature that ANG II binds to nuclei and increases RNA transcription in hepatocytes.[50,51] Certainly further experiments are necessary to evaluate which signal-transduction pathways are involved in the ANG II–stimulated transcription of type I collagen in MMCs.

The local concentration of ANG II in the glomerular microenvironment is determined not only by the generation of ANG II, but also by its degradation from specific proteases.[52] We have shown previously that the activity of aminopeptidase A (ATA) in glomeruli, which cleaves ANG II, is elevated with the activation of the intrarenal renin–ANG II system.[52] Intriguingly, ATA activity was not elevated significantly 14 weeks after 1⅓ nephrectomy, a setting in which the structure of the glomerular tuft is, after an initial proliferation of mesangial cells, disturbed by early glomerulosclerosis and proteinuria. A similar redistribution of glomerular ATA activity has been found in the kidneys of patients with proliferative glomerulonephritis.[53] These findings suggest that a decrease of glomerular ATA might lead to enhanced local levels of ANG II in pathophysiologic situations associated with mesangial proliferation.

It is intriguing and appealing to consider that ANG II might influence the mitogenic activity of mesangial cells *in vivo*. The serum-free culture system we have used, however, might not be analogous totally to the situation *in vivo*, where mesangial cells are exposed to plasma. The coordination and functional relationship of ANG II to the effects of hemodynamic alterations, mesangial uptake of macromolecules, stimulation of mesangial cell prolifera-



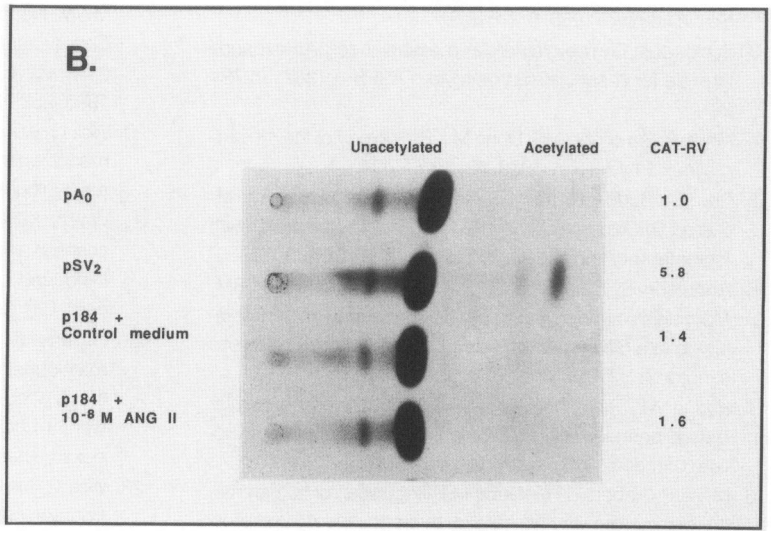


Figure 7. *Representative autoradiograms of thin-layer chromatography filters containing separated CAT products after an acetylation reaction on transfected MMCs. An increase in acetylated product reflects stimulation of gene transcription.* (A) *There is little transcriptional activity of the transfected type I construct pR41 in control media. 10^{-8} M ANG II, however, increased significantly this CAT activity. This increase was blocked by DuP 753. Similarly, 10^{-6} M saralasin partly blocked ANG II-induced transcription. However saralasin alone, in contrast to DuP 753, had some stimulatory effect on CAT-activity. This finding is in accordance with the well-known agonistic activity of saralasin.* (B) *In contrast, the CAT activity of the type IV construct p184 is not stimulated significantly by ANG II. Transfection of MMCs with the pSV_2 containing SV 40 enhancer/promoter elements demonstrates that the cells can be transfected. The shown autoradiograms are representative for three independent transfection and CAT-assay experiments. (CAT-RV = relative CAT activity calculated after assigning pA_0 a relative value of 1.0).*

Table 2. *Secretion of Collagen Type I into the Cell Culture Supernatant of MMCs*

Cytokine	Collagen (ng/ml)	
	Type I	Type IV
Control media	17.3 ± 0.8	27.1 ± 2.4
10^{-6} M ANG II	18.1 ± 1.3	28.9 ± 2.0
10^{-8} M ANG II	32.0 ± 4.9**	28.6 ± 3.4
10^{-10} M ANG II	26.6 ± 2.8**	23.2 ± 1.4

** $P < 0.005$ versus control, n = 10 for type I, n = 7 for type IV. Stimulation of quiescent MMCs for 48 hours in the presence of 50 μg/ml each of L-ascorbic acid and the crosslinking inhibitor β-aminopropionitrile. Values are normalized for cell number.

tion, or to the biosynthesis of collagens, certainly will require further evaluation in established models of glomerular injury.

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