Macrophages Promote Prosclerotic Responses in Cultured Rat Mesangial Cells: A Mechanism for the Initiation of Glomerulosclerosis

IZABELLA Z. A. PAWLUCZYK and KEVIN P. G. HARRIS
Department of Nephrology, Leicester General Hospital, Leicester, United Kingdom.

Abstract. Glomerulosclerosis is the final outcome of a number of different causes of glomerular injury, during which the structures of the glomerulus are obliterated by extracellular matrix. Accumulating evidence suggests that infiltrating macrophages play a pivotal role in the progression to glomerulosclerosis. The present study defines the role played by macrophages at both cellular and molecular levels in the initiation of the sclerotic process in cultured rat mesangial cells. Macrophage-conditioned medium (MPCM) generated from thiglycollate-elicited, lipopolysaccharide-stimulated macrophages upregulated mesangial cell fibronectin production in a dose and time-dependent manner, independently of cell proliferation. Immunoprecipitation of metabolically labeled 35S-fibronectin confirmed that the matrix protein was synthesized de novo. The genes for fibronectin and the matrix proteins laminin and collagen IV were also found to be upregulated 2.86 ± 0.24-, 4.94 ± 0.17-, and 3.03 ± 0.31-fold over controls, respectively (P < 0.001). Macrophage modulation of matrix turnover was suggested by an upregulation of both transin and tissue inhibitor of metalloproteinase-1 gene transcription. Transforming growth factor (TGF) β1, platelet-derived growth factor, tumor necrosis factor (TNF) α, or interleukin (IL)-1β could not be detected in the MPCM per se; however, TGFβ1 and platelet-derived growth factor AB were found to be secreted into mesangial cell culture supernatants. Secretion was augmented 1.69 ± 0.16- and 2.28 ± 0.28-fold, respectively (both P < 0.001), in response to MPCM. Northern blot analysis demonstrated that protein secretion had been preceded by upregulation of the genes for these cytokines (2.2 ± 0.4-fold [P < 0.001] and 5.7 ± 1.2-fold [P < 0.004], respectively). Incubation of MPCM with either neutralizing antibody or the growth factor receptor antagonist suramin demonstrated that TGFβ1 played a significant, although minor, role in MPCM-stimulated fibronectin production. In conclusion, this study provides compelling evidence for a direct role of macrophages in the progression to glomerulosclerosis. (J Am Soc Nephrol 8: 1525–1536, 1997)

The histological characteristics of numerous experimental and human glomerular diseases that ultimately progress to glomerulosclerosis are expansion of the glomerular mesangium and deposition of extracellular matrix (1). The etiology of progressive renal scarring is complex and multifactorial, but considerable evidence, largely from animal studies, now suggests that macrophages could play an important pathogenetic role in this process.

For example, the administration of puromycin aminonucleoside (PAN) to rats causes the nephrotic syndrome, which is associated with a glomerular macrophage infiltrate at day 11, coinciding with peak proteinuria (2). Although the nephrotic syndrome resolves, the rats subsequently develop progressive glomerulosclerosis. Maneuvers that abolish the glomerular macrophage infiltrate during the acute nephrotic phase in this model, such as a diet free of essential fatty acids (3) or whole-body X-irradiation (4), are able to prevent the subsequent progression to glomerulosclerosis. A glomerular infiltrate is also an early response to renal ablation, a lesion also known to progress to glomerulosclerosis (5,6). After uninephrectomy, for example, an infiltrate occurs within 1 to 2 wk of surgery, before the observation of histological changes by light microscopy, the detection of microalbuminuria, or the development of renal scarring (5). Glomerular macrophage depletion by X-irradiation has been shown to ameliorate the progression of the glomerular injury in the remnant kidney model (6), further supporting an effector role for these cells in mediating glomerulosclerosis.

Factors elaborated by these infiltrating cells could alter the phenotypic expression of the resident glomerular cells with respect to their proliferative and matrix-producing capacities. Much research has recently been carried out regarding the role of various cytokines and growth factors in the modulation of glomerular cell proliferation and matrix synthesis. In PAN nephrosis, the macrophage infiltrate has been shown to occur in temporal association with increased glomerular transforming growth factor (TGF) β and fibronectin gene expression (7). Furthermore, immunohistochemical labeling studies have demonstrated that the TGFβ protein staining corresponds locally with ED-1-positive macrophages (7). More recently, in the same model, it has been reported that TGFβ mRNA is ex-
pressed by glomerular macrophages, thus identifying these cells as a potential cellular source of the profibrogenic cytokine (8). Okuda et al. have also reported that the level of TGFβ mRNA and the number of cells producing TGFβ protein was higher in glomeruli isolated from Thy-1 nephritics than in glomeruli from normal rats (9). Subsequently, in the same model, it was shown that administration of a neutralizing antibody to TGFβ resulted in a significant suppression of extracellular matrix production with a concomitant reduction in the degree of histological damage (10). Further support for a pathogenic role for TGFβ in renal scarring has been provided by the observation that in vivo transfection of the TGFβ gene increases the production of extracellular matrix deposition in the kidney (11).

These observations provide a strong, although correlative, case for TGFβ mediating the adverse effects of macrophages in the scarring process. However, it seems unlikely that one cytokine acting alone would be solely responsible for mediating mesangial matrix accumulation, given the potential cytokine interactions involving autocrine loops and paracrine pathways that could be involved in this process. Instead, the process of scarring likely requires a number of factors acting in concert.

The present study, therefore, was undertaken to determine the precise role played by macrophages at both cellular and molecular levels in the initiation of the sclerotic process in mesangial cells—functionally the most important cells within the glomerulus.

Materials and Methods

Unless stated otherwise, chemicals and reagents were obtained from Sigma Chemical Co. (Poole, United Kingdom).

Culture of Rat Mesangial Cells

Mesangial cells were cultured from the glomerular explants of adult Wistar rat kidneys, using standard techniques (12). The cells were cultured in RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum (FCS), 100 μg/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), 5 μg/ml bovine insulin, and 2 mM glutamine (Life Technologies). Cultured cells were characterized by their typical stellate fusiform morphology, their positive staining for the Thy-1 antigen, and their resistance to the toxic effects of o-valine.

Mesangial cells of passages 2 through 10 were cultured in 24-well plates (ICN Flow, Oxfordshire, United Kingdom) or 25-cm² flasks (Corning, High Wycombe, United Kingdom), allowed to grow to confluence, and then made quiescent in medium containing 0.5% FCS for 72 h before use.

Isolation of Macrophages and Preparation of Macrophage-Conditioned Media

Macrophages were obtained from adult Wistar rats by injecting 10 ml of 3% thioglycollate broth into the peritoneal cavity. After 5 d, the peritoneal cavity was lavaged with 20 ml of cold Hanks' balanced salt solution (HBSS). The majority (~90%) of the exudate cells obtained in this way were macrophages, as judged by positive immunohistochemical staining for the rat monocyte/macrophage marker ED-1. Macrophage-conditioned medium (MPCM) was prepared using a modified method of Kohan and Schreiner (13). The exudate cells were purified by temporarily plating them at a cell density of 5 × 10⁶ cell/ml in 25-cm² tissue culture flasks. After 2 h of incubation at 37°C in a humidified 5% CO₂/95% air atmosphere, nonadherent cells were removed by washing with HBSS buffered with 20 mM Hepes. The macrophages were then stimulated with lipopolysaccharide (LPS) (from Escherichia coli 026 B6) at a final concentration of 1 μg/ml for 16 h, and then washed three times and cultured for an additional 48 h in serum-free RPMI. The MPCM was harvested and centrifuged for 10 min at 2000 rpm, and then frozen at −20°C until needed.

Culture of Mesangial Cells in the Presence of Macrophages or MPCM

Confluent quiescent mesangial cells were exposed to thioglycolate-elicited peritoneal macrophages at final (macrophage) cell densities of 0.3, 3.13 × 10⁵, 6.25 × 10⁵, 12.5 × 10⁵, and 25 × 10⁵ cells/ml per well. In parallel, macrophages at the same cell densities were cultured directly on the 24-well plates as controls.

In additional experiments, confluent quiescent mesangial cells were exposed to a 50% solution of MPCM. The final concentration of FCS remained at 0.5%. The cultures were maintained in this medium for up to 7 d. The tissue culture supernatants were harvested and centrifuged for 30 s at 11,600 × g to remove cell debris and were then stored at −20°C for subsequent analysis of soluble fibronectin or the cytokines TGFβ, platelet-derived growth factor (PDGF), tumor necrosis factor (TNF) α, or interleukin (IL)-1β.

Preparation of Cell Lysates

After removal of tissue culture supernatants, cell monolayers were washed with PBS, scraped into 1% Nonidet P40 in wash buffer (PBS containing 0.3 M NaCl and 1% Tween 20), and then incubated at room temperature for approximately 30 min. The cell scrapings were then transferred to 2-ml tubes, sonicated for 5 s, and centrifuged for 30 s at 11,600 × g. Sonication and centrifugation were repeated, and the lysate supernatants were assayed for fibronectin and total cell protein.

Effect of Suramin on MPCM

To assess the contribution of TGFβ and PDGF to MPCM-mediated fibronectin production, the antihelmintic, polyanionic drug suramin was used. Suramin has been shown to antagonize the binding of growth factors such as TGFβ and PDGF to their receptors (14,15). Confluent, quiescent mesangial cells were exposed to MPCM, in combination with 150 μg/ml suramin, for 24 h. Supernatants and cell lysates were assayed for fibronectin.

TGFβ and PDGF Cell-Binding Assays

Confluent, quiescent mesangial cells in 24-well plates were fixed with 200 μl/well 0.25% glutaraldehyde for 10 min at room temperature. The plate was washed twice with 2 ml of HBSS/well. The fixed mesangial cells were then treated with 200 μl/well of either 10 ng/ml TGFβ (or PDGF) ±150 μg/ml suramin or medium ±150 μg/ml suramin for 1 h at room temperature. After incubation, the plate was washed three times with enzyme-linked immunosorbent assay (ELISA) wash buffer and blocked with 2% bovine serum albumin (BSA) (in wash buffer) for 30 min. A total of 200 μl of rabbit anti-TGFβ (or goat anti-human PDGF) was added to each well and incubated for 1 h at room temperature. After three washes, 200 μl/well of goat anti-rabbit Ig-horseradish peroxidase (HRP)-conjugated antibody (Dako, High Wycombe, United Kingdom) at 1:1000 dilution (or rabbit anti-goat Ig-HRP [Dako] at 1:5000 dilution) was
added to each well and incubated for an additional hour at room temperature. After three more washes, 200 µl/well 1,2-phenylenediamine dihydrochloride substrate solution was added (see section, Fibronectin ELISA, below). The reaction was stopped with 200 µl of 1 M H₂SO₄. Duplicate 200-µl aliquots from each of the 24 wells were transferred to the wells of a 96-well plate, and the optical density was determined.

**Effect of Neutralizing Anticytokine Antibodies on MPCM**

Mesangial cells were exposed to: (1) MPCM, in combination with either 10 µg/ml rabbit pan-specific anti-TGFβ, goat anti-human PDGF, goat anti-murine TNFα, or goat anti-murine IL-1β; (2) MPCM, in combination with a “cocktail” of neutralizing antibodies (10 µg/ml each of αTGFβ, αPDGF, αTNFα, and αIL-1β); and (3) cytokines (natural human TGFβ, natural human PDGF, recombinant murine TNFα, and recombinant murine IL-1β; 2.5 ng/ml each), in combination with the “cocktail” of neutralizing anticytokine antibodies. Incubations were carried out for 7 d, after which cell lysates, supernatants, or both, were assayed for fibronectin. All cytokines and anticytokine antibodies were purchased from R&D Systems, Inc. (Minneapolis, MN).

**Fibronectin ELISA**

Culture supernatants or cell lysates were assayed for fibronectin, using an inhibition ELISA. A total of 60 µl of rat plasma fibronectin standard (19 to 5000 ng/ml) (Calbiochem, Nottingham, United Kingdom), supernatant sample, or cell lysate was incubated with an equal volume of rabbit anti-fibronectin diluted 1:2000 in wash buffer at 4°C overnight. Fifty microliters of this reaction mixture was then transferred to each well of a 96-well microtiter plate (Nunc Immunoplate, Roskilde, Denmark) that had been precoated at 4°C overnight with rat plasma fibronectin (1 µg/ml in 0.05 M carbonate buffer, pH 9.6) and blocked with 2% BSA in wash buffer for 1 h at room temperature. The plate was incubated with the reaction mixture at room temperature for 2 h. After washing each well four times, 50 µl of goat anti-rabbit IgG conjugated to HRP (Dako) was added to the well and incubated at room temperature for 1 h. After four more washes, 50 µl of 0.67 mg/ml 1,2 phenylenediamine dihydrochloride was added in 0.03 M citrate buffer, pH 5.0, containing 0.012% H₂SO₄, and absorbance was read at 492 nm on a Tittertek Multiskan Plus microtiter plate reader (Flow Laboratories, Oxfordshire, United Kingdom).

**Cytokine ELISA**

Cytokine measurements were carried out on culture supernatants, using commercially available ELISA assays for the following cytokines: human TGFβ (Predicia, Genzyme Diagnostics, R&D Systems), human PDGF-AB (Biotrak, Amersham), rat TNFα (Factor-Test X, Genzyme Diagnostics), and IL-1β (Intertest-1β-X, Genzyme Diagnostics). Assays were carried out according to the manufacturer’s instructions.

**DNA Assay**

Cell monolayers were washed four times with ice-cold 0.9% wt/vol NaCl and then scraped into 10% ice-cold perchloric acid (PCA). The wells were washed with further aliquots of 10% PCA, after which the washings were pooled, vortexed, and spun at 3000 × g for 10 min at 4°C. After discarding the supernatants, 500 µl of fresh 10% PCA was added, and the solution was hydrolyzed by heating at 70°C in a water bath for 20 min. After hydrolysis, the tubes were chilled on ice to precipitate as much protein as possible. The tubes were then centrifuged for 10 min at 3000 × g at 4°C to spin down the protein precipitate, which was subsequently analyzed for protein concentration. The supernatants containing the DNA were transferred to plastic test tubes. A total of 250 µl of diphenylamine reagent (1 g of diphenylamine/25 ml of glacial acetic acid) followed by 50 µl of acetaldehyde solution (1.6 mg/ml in water) was added to the 250-µl cell hydrolysate or calf thymus DNA standard (0 to 200 µg/ml). The mixture was vortexed and incubated at 25 to 30°C for 16 to 20 h. The absorbance of the reaction solution was read at 595 and 710 nm, and the difference between the absorbances at the two wavelengths was calculated. (Light scattering at 710 nm was measured to eliminate any effects arising from the slight turbidity of the samples.) A standard curve of change in absorbance versus DNA concentration was constructed, from which the DNA concentration was determined.

**Protein Determination**

For cell monolayers that had been precipitated with PCA for DNA determinations, the cell protein content was measured by the method of Lowry et al. (16), using BSA standards. The protein content of cell lysates dissolved in Nonidet P40 was determined using a commercial BioRad DC protein assay, using BSA standards.

**[3H]Thymidine Incorporation Assays**

Mesangial cell proliferation was measured by [3H]thymidine incorporation. Confluent, quiescent mesangial cells were exposed to MPCM or medium alone for 3 d. Then, 1 µCi/well [3H]thymidine (Amersham) was added to directly to each well. The cells were incubated for an additional 24 h, after which the culture media were discarded and the wells rinsed once with PBS. One milliliter of 0.1 mM cold thymidine in RPMI + 0.5% FCS was then added to each well and incubated at 37°C for 20 min. The wells were washed once with ice-cold PBS, twice with 10% trichloroacetic acid, and then once with PBS again. A total of 250 µl of 0.5 M NaOH was added to each well, and the plate was incubated at 60 to 70°C for 30 min to dissolve the cell monolayer. Two hundred microliters of cell lysate from each well was added to 4 ml of Ecoscint A scintillation fluid (National Diagnostics, Mansville, NJ), followed by 20 µl of concentrated HCl. 3H activity was counted on an LKB 1219 liquid scintillation counter (LKB Instruments, Bromma, Sweden).

**Identification of Newly Synthesized Fibronectin**

**Biosynthetic Labeling with 35S-Methionine.** Confluent, quiescent mesangial cells were exposed to MPCM or medium alone diluted 1:1 in methionine-free RPMI (Sigma) for 3 d. Eighteen hours before termination of the experiment, each well was pulsed with 50 µCi of 35S-methionine (Amersham). The culture media were retained, and the cell monolayers were washed twice in PBS. Cell lysates were prepared as described above.

**Immunoprecipitation.** To immunoprecipitate the newly synthesized fibronectin, 20 µl of goat anti-fibronectin (Calbiochem) was added to 500 µl of culture medium or 200 µl of cell lysate. Normal goat serum was used as control. The samples were incubated overnight at 4°C. Fifty microliters of insoluble protein A cell suspension (10% wet vol/vol of nonviable S. aureus [Cowan strain] cells in 0.04 M sodium phosphate buffer, pH 7.2, 0.15 M NaCl, and 0.05% sodium azide) was then added to precipitate out the antigen:antibody complexes and then incubated for 4 h at 4°C. The samples were centrifuged for 10 min at 3000 × g, and the pellets were washed three times with ice-cold immunoprecipitation buffer (PBS containing 0.5 M NaCl, 0.1% sodium dodecyl sulfate [SDS], and 1% Triton X-100, pH
7.4), vortexing the pellet thoroughly between each wash. Finally, the pellets were washed with ice-cold PBS and dissolved in 70 μl of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer containing 3% SDS and 10% 2-mercaptoethanol and heated for 7 min in a boiling water bath. The dissolved pellets were centrifuged in a microfuge for 30 s.

The radiolabeled proteins were resolved by SDS-PAGE on 5% polyacrylamide gels. Newly synthesized proteins were detected by autoradiography of dried gels. Bands were quantified by scanning densitometry on an LKB Ultrascan densitometer (LKB Instruments).

**Northern Blotting**

Confluent, quiescent mesangial cells were exposed to MPCM or medium alone for 24 h. Total RNA was extracted using TRIzol reagent (Life Technologies), according to the manufacturer’s instructions. Aliquots (30 μg) of RNA were electrophoresed on a 1% agarose gel containing 1.9% formaldehyde in MOPS (3-(N-N-morpholino)propanesulfonic acid). The resolved RNA was transferred onto Hybond-N nylon membranes (Amer sham) by capillary action using 20X SSC (1X SSC = 15 mM trisodium citrate, 150 mM sodium chloride). The membranes were prehybridized for 4 h at 37 or 42°C (depending on probe) with 200 μg/ml denatured salmon sperm DNA in 50% formamide, 1% SDS, 5X Denhardt’s, and 5X saline-sodium phosphate ethylenediaminetra-acetic acid (SSPE) (1X SSPE = 11.5 mM sodium phosphate, 150 mM sodium chloride, 1 mM EDTA)). The membranes were then hybridized overnight with a [32P]dCTP cDNA probe that had been Klenow DNA polymerase-labeled, using a random primer labeling system (Prime-a-Gene, Promega) in fresh buffer (same composition as prehybridization buffer). After hybridization, the membranes were washed twice with 1% SDS, 2X SSPE at room temperature, twice with 0.2% SDS, 0.2% SSPE at 65°C, then exposed to X-Omat LS film (Kodak) with intensifier screens at -70°C. Membranes were subsequently stripped in boiling 5% SDS, 0.5X SSPE before reprobing. Densitometric analysis of the transcripts was carried out on a BioRad GS 700 imaging scanner. RNA loading was normalized using a cDNA probe for cyclophilin. Cyclophilin expression was not affected by treatment with MPCM (data not shown).

**Probes**

All of the cDNA probes were generous gifts. cDNA for rat fibronectin was from Dr. R. O. Hynes (Massachusetts Institute of Technology) (17); murine α1 (IV) collagen and laminin B1 chain cDNA were from Dr. Kurkinen (Department of Medicine and Dentistry of New Jersey-Rutgers Medical School, Piscataway, NJ) (18,19); murine TGFβ1 was from Dr. R. Akhurst (Department of Medical Genetics, Glasgow University, Glasgow, Scotland) (20); murine PDGF B chain was from Dr. C. D. Stiles (Dana Farber Cancer Institute, Harvard Medical School, Boston, MA) (21); murine tissue inhibitor of metalloproteinase (TIMP)-1 cDNA was from Dr. D. T. Denhardt (Department of Biochemistry, Rutgers University, Piscataway, NJ); rat transin was from Professor R. Breathnach (Laboratoire de Recherche, Nantes, France) (22); and human cyclophilin was from SmithKline Beecham Pharmaceuticals.

**Statistical Analyses**

Data were expressed as means ± SEM. For comparison of means between two groups, an unpaired t test was used. To compare values between multiple groups, ANOVA with a Bonferroni correction was applied. Statistical significance was defined as P < 0.05.

**Results**

**Effect of Macrophage/Mesangial Cell Coculture on Fibronectin Production**

Macrophages cultured in the presence of 0.5% FCS for 3 d generated small amounts of secreted fibronectin dose dependently (Figure 1). Mesangial cells also constitutively secreted fibronectin. However, direct coculture of macrophages with mesangial cells resulted in a synergistic increase in fibronectin production proportional to the number of added macrophages (Figure 1).

**Effect of MPCM on Mesangial Cell Fibronectin Production**

To verify that the macrophage effect on mesangial cell fibronectin production was due to secreted factors and could occur independently of cell:cell contact, mesangial cells were exposed to MPCM.

Although macrophages cultured for 3 d in 0.5% FCS secreted fibronectin, the matrix protein could not be detected in MPCM per se. Cultured mesangial cells constitutively secreted low levels of fibronectin into the culture medium (Table 1). Addition of MPCM significantly enhanced this production.

![Figure 1. Effect of macrophage/mesangial cell coculture on fibronectin production. Increasing numbers of macrophages were cultured alone or in combination with confluent, quiescent mesangial cells for 3 d in medium containing 0.5% fetal calf serum (FCS). Tissue culture supernatants were assayed for fibronectin. Results are means ± SEM of eight wells.](image-url)
Table 1. Time course of fibronectin secretion in response to MPCM

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<th>Day</th>
<th>Fibronectin (µg/ml)</th>
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<td>Medium</td>
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<td>0</td>
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<tr>
<td>3</td>
<td>1.21 ± 0.13</td>
<td>4.48 ± 0.46</td>
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<tr>
<td>5</td>
<td>1.83 ± 0.18</td>
<td>5.92 ± 0.46</td>
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<tr>
<td>7</td>
<td>2.34 ± 0.37</td>
<td>9.40 ± 1.15</td>
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* Mesangial cells were incubated with MPCM for 3, 5, and 7 d. Supernatants were assayed for fibronectin. Values are means ± SEM (n = 3) each carried out in quadruplicate. MPCM, macrophage-conditioned medium.

with time (Table 1). The effect of MPCM demonstrated a clear dose dependency (Figure 2A), which was maintained when fibronectin production was expressed as a function of total cell DNA (Figure 2B) or protein (Figure 2C), indicating that the effect was not secondary to an increase in cell number.

MPCM also caused an increase in cell-associated fibronectin. Of the total amount of fibronectin produced by mesangial cells, approximately 80% was secreted into the supernatant, whereas 20% was cell-associated (427.5 ± 59 ng/µg cell protein in supernatant versus 80.1 ± 16.1 ng/µg cell protein in cell lysate). Values are means ± SEM (n = 4).

The increase in fibronectin was not a result of residual or contaminating endotoxin in the MPCM activating the mesangial cells, because addition of LPS to a final concentration of 1 µg/ml directly to mesangial cells did not result in an increase in constitutive fibronectin levels in either the secreted or cell-associated forms (data not shown).

**Effect of MPCM on Mesangial Cell Proliferation**

[^3]HThymidine incorporation assays were used to assess mesangial cell proliferation in response to MPCM. MPCM suppressed mesangial cell proliferation dose dependently: the greater the concentration of MPCM, the lower the incorporation of [%[^3]H]thymidine (Figure 3).

**Effect of MPCM on De Novo Fibronectin Synthesis**

To assess whether the observed increase in fibronectin levels was due to an increase in protein synthesis, biosynthetic labeling of fibronectin was carried out. Autoradiographs of the immunoprecipitated, biosynthetically labeled fibronectin showed that exposure of mesangial cells to MPCM resulted in an increased fibronectin synthesis over that of medium alone (Figure 4, A and B). Densitometric analysis of the 220-kD fibronectin band from three independent experiments showed 5.9- and 4.4-fold increases over control in the secreted and cell-associated forms of MPCM-induced fibronectin, respectively (0.31 ± 0.06 versus 0.053 ± 0.007, P < 0.001, and 0.31 ± 0.07 versus 0.073 ± 0.016, P < 0.005 arbitrary densitometric units).

*Figure 2. Effect of macrophage-conditioned medium (MPCM) on mesangial cell fibronectin production. Confluent, quiescent mesangial cells were incubated with 0, 1, 10, 20, 50, and 100% MPCM for 7 d. Culture supernatants were assayed for fibronectin. Cell lysates were assayed for total cell DNA and total cell protein. Results are expressed as micrograms of fibronectin secretion per milliliter (A), per microgram of cell DNA (B), and per microgram of cell protein (C). A representative experiment from three experiments carried out in quadruplicate is shown.*
Effect of MPCM on Mesangial Cell Extracellular Matrix Gene Transcription

Figure 5 illustrates that there was an increase in mesangial cell fibronectin mRNA in quiescent rat mesangial cells exposed to MPCM for 24 h. Analysis of the 8-kb fibronectin band by scanning densitometry showed that fibronectin mRNA was increased 2.9 ± 0.24-fold (P < 0.001, n = 5) over control cells exposed to medium alone (normalizing for RNA loading with cyclophilin). A second, larger transcript (>9.49 kb) was also seen, but was not included for analysis in this study. As well as message for fibronectin, the genes for the matrix proteins collagen IV (α1 (IV) collagen) and laminin (B1 chain) were also upregulated 3.1 ± 0.3-fold (P < 0.001, n = 4) and 4.9 ± 0.2-fold (P < 0.001, n = 4), respectively (Figure 5).

Effect of MPCM on Mesangial Cell Transin and TIMP-1 Gene Transcription

Stromelysin or rat transin is a matrix metalloproteinase (MMP) that is able to degrade various matrix components, including fibronectin (23). Northern blot analysis demonstrated that MPCM induced de novo transin expression in mesangial cells, whereas TIMP-1 mRNA was upregulated 15.2 ± 2.5-fold (P < 0.001, n = 6) over control levels (Figure 6).

Detection of Growth Factors in MPCM and Tissue Culture Supernatants

TGFβ, PDGF, TNFα, and IL-1β are cytokines whose presence in the glomerulus has been associated with the progression to glomerulosclerosis. None of the cytokines could be detected in MPCM using the commercially available ELISA. After concentration of MPCM 20× using Amicon Centriprep 3 concentrators (Amicon), TGFβ, PDGF, and IL-1β remained undetectable, although TNFα was detected at a concentration of 90 pg/ml. However, TGFβ and PDGF-AB were detected in mesangial cell culture supernatants (2.56 ± 0.69 and 1.18 ± 0.22 ng/ml, respectively), suggesting that these cytokines were autocrinally secreted by mesangial cells. Furthermore, this autocrinal secretion was upregulated 1.69 ± 0.16- and 2.29 ± 0.28-fold, respectively (P < 0.001, n = 8), in response to MPCM. The secreted TGFβ was in the latent, or inactive, form, since it could not be detected in the assay unless the samples had been activated by acidification.

Northern blot analysis demonstrated that the observed increase in secreted cytokines in response to MPCM had been preceded by an upregulation of message for these cytokines (Figure 7). TGFβ mRNA transcription was upregulated 2.2 ± 0.4-fold (P < 0.001, n = 4) and PDGF B chain mRNA 5.7 ± 1.2-fold (P = 0.004, n = 5) over control levels (Figure 7). Neither TNFα nor IL-1β could be reliably detected in tissue culture supernatants from mesangial cells exposed to MPCM.

Effect of Suramin on MPCM-Mediated Mesangial Cell Fibronectin Production

To ascertain the contribution of autocrinally secreted TGFβ and PDGF to the observed increase in fibronectin levels, the growth factor receptor antagonist suramin was used. Binding of exogenously added TGFβ and PDGF to fixed mesangial cells could readily be detected under control conditions, as evidenced by an increase in A492 (Table 2). Addition of suramin significantly decreased detectable, bound TGFβ and PDGF. Indeed, addition of suramin produced A492 readings that were similar to those observed in medium without exogenously added cytokines (i.e., background), confirming that suramin could effectively abolish TGFβ and PDGF binding to mesangial cells by interfering with cytokine receptor binding.

Exposure of mesangial cells to MPCM, in combination with 150 µg/ml suramin, resulted in a reduction of secreted fibronectin to 83.8 ± 4.4% (P = 0.02, n = 3) and a reduction of cell-associated fibronectin to 59.5 ± 3.0% (P = 0.005, n = 3) of control levels. Suramin had no statistically significant effect on basal fibronectin production by mesangial cells.

Effect of Neutralizing Antibodies on MPCM-Mediated Fibronectin Production

In an alternative approach to assessing the contribution of known cytokines to the observed effects of MPCM, mesangial cells were exposed to a panel of neutralizing antibodies. Anti-TGFβ significantly reduced MPCM-mediated supernatant fibronectin production to 72.9 ± 3.7% of untreated MPCM (P < 0.001, n = 5) and cell-associated fibronectin production to 66.3 ± 6.8% (P = 0.003) (Table 3). Antibodies to PDGF, TNFα, and IL-1β had no significant effect, suggesting that these cytokines, at least individually, played no role in MPCM-mediated fibronectin production. (Antibody concentrations of up to 50 µg/ml did not produce any greater reduction in
Figure 4. Effect of MPCM on de novo fibronectin synthesis. Autoradiograph of immunoprecipitated supernatant (A) and cell-associated 35S-fibronectin (B). A representative autoradiograph from three experiments is shown.

fibronectin production by mesangial cells [data not shown]. A "cocktail" of neutralizing antibodies to the cytokines TGFβ, PDGF, TNFα, and IL-1β was effectively able to abolish fibronectin secretion stimulated by exogenously added cytokines (6.7 ± 5.7, P < 0.001) (Figure 8A). However, the same cocktail was only able to reduce MPCM-stimulated fibronectin secretion to 77.9 ± 7.4% of untreated MPCM (P = 0.04, n = 3) (Figure 8B), a degree of reduction that was no greater than that observed with anti-TGFβ alone.

Discussion

The present study demonstrates that macrophage-derived factors can initiate renal scarring directly, by production of matrix proteins, and also indirectly, via the production of profibrogenic cytokines and modulation of matrix turnover.

In the current study, MPCM caused cultured mesangial cells to secrete fibronectin in a time- and dose-dependent manner. The fibronectin was derived from mesangial cells since the matrix protein was not detected in the MPCM per se, although fibronectin was secreted by macrophages cultured for 3 d in medium containing 0.5% FCS. This is in agreement with observations by other investigators who demonstrated that human and mouse macrophages also secrete fibronectin (24,25). The function of macrophage fibronectin may be to aid binding of macrophages to mesangial cells, because Dubois et al. (26) have shown that, in vitro, rat peritoneal macrophages preferentially bind to rat mesangial cells via interactions with fibronectin expressed on the macrophage cell surface. However, our data indicate that macrophages can induce mesangial cell fibronectin production independently of macrophage:mesangial cell contact. This increase in fibronectin secretion could not be accounted for by endotoxin contamination of MPCM, because addition of LPS directly to mesangial cells had no effect on basal fibronectin production.

Northern blot analysis, taken together with the biosynthetic

Figure 5. Effect of MPCM on mesangial cell extracellular matrix gene transcription. Northern blot analysis was carried out on RNA extracted from mesangial cells incubated with MPCM or medium alone for 24 h. RNA was probed for fibronectin, α1(IV) collagen IV, and laminin B1 chain. Representative blots from four to five experiments are presented.
Transin 1.9kb

TIMP-1 1.0kb

Cyclophilin 1.8kb

Medium MPCM

Figure 6. Effect of MPCM on mesangial cell transin and tissue inhibitor of metalloproteinase-1 (TIMP-1) gene transcription. Northern blot analysis of mesangial cell RNA after treatment with MPCM or medium alone. Representative blots from five and six experiments, respectively, are shown.

Table 2. Effect of suramin on the binding of TGFβ and PDGF to mesangial cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Control A₄₉₂</th>
<th>+ Suramin A₄₉₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>0.327 ± 0.007</td>
<td>0.326 ± 0.003</td>
</tr>
<tr>
<td>TGFβ</td>
<td>0.456 ± 0.008</td>
<td>0.370 ± 0.008⁻ᵇ</td>
</tr>
<tr>
<td>PDGF</td>
<td>0.438 ± 0.007</td>
<td>0.346 ± 0.007⁻ᵇ</td>
</tr>
</tbody>
</table>

*Glutaraldehyde-fixed mesangial cells were incubated with 10 ng/ml of either TGFβ or PDGF in the presence or absence of 150 µg/ml suramin. Bound cytokine was detected using anti-TGFβ or anti-PDGF antibodies followed by the appropriate horseradish peroxidase-labeled second antibody and colorimetric analysis (expressed as A₄₉₂). Each value represents means ± SEM of six wells (one of three representative experiments). A, absorbance; TGF, transforming growth factor; PDGF, platelet-derived growth factor.

⁻ᵇ P < 0.001 versus corresponding suramin-untreated cells.

Table 3. Effect of neutralizing antibodies on MPCM-mediated fibronectin production

<table>
<thead>
<tr>
<th>Group</th>
<th>Supennatant Fibronectin (%)</th>
<th>Cell Lysate Fibronectin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPCM</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>αTGFβ</td>
<td>72.9 ± 3.7⁻ᵇ</td>
<td>66.3 ± 6.8⁻ᶜ</td>
</tr>
<tr>
<td>αIL-1β</td>
<td>107.0 ± 4.8</td>
<td>110.5 ± 7.5</td>
</tr>
<tr>
<td>αPDGF</td>
<td>102.5 ± 5.3</td>
<td>95.0 ± 7.1</td>
</tr>
<tr>
<td>αTNFα</td>
<td>100.1 ± 0.6</td>
<td>99.0 ± 7.2</td>
</tr>
</tbody>
</table>

*Mesangial cells were exposed to MPCM in combination with antibodies (α) to TGFβ, IL-1β, PDGF, or TNFα. Supernatants and cell lysates were assayed for fibronectin. Values are means ± SEM (n = 3 to 5), each carried out in quadruplicate. IL, interleukin; TNF, tumor necrosis factor.

⁻ᵇ P < 0.001.

⁻ᶜ P = 0.003 versus untreated MPCM.

Cyclophilin 1.8kb

Medium MPCM

Figure 7. Effect of MPCM on TGFβ and PDGF B chain gene transcription. Northern blot analysis of mesangial cell RNA after treatment with MPCM or medium alone. One representative blot from five experiments is shown.

labeling studies, suggests that the MPCM-mediated upregulation of mesangial cell fibronectin production occurs, at least in part, as a result of increased mRNA transcription and protein synthesis. However, MPCM was also found to induce the expression of mRNA for the MMP transin and to upregulate mRNA for TIMP-1. Although upregulation of message does not always suggest translation of protein, a number of reports have suggested that TIMP-1 mRNA expression and protein secretion are tightly coupled (27). These observations suggest that macrophage-derived products may also modulate matrix turnover, and they raise the possibility that fibronectin accumulation could also result from a net decrease in degradation rate, depending on the balance between MMP and TIMP-1 production. However, the fivefold accumulation in fibronectin protein as assessed by both the ELISA and immunoprecipitation studies confirms that the net effect of macrophage-derived products is the accumulation of matrix proteins.

The observed increase in fibronectin levels was not secondary to a stimulation of cell proliferation. When fibronectin secretion was expressed per unit cell DNA or cell protein, the
effects of MPCM were still observed. [3H]Thymidine incorporation assays showed that MPCM actually suppressed mesangial cell proliferation, or at least [3H]thymidine uptake. The suppressive effect of MPCM is in agreement with the findings of Ooi et al. (28), who demonstrated suppression of mesangial cell proliferation by murine MPCM derived from endotoxin-treated mice. These observations contrast with those of other researchers who have found that MPCM can stimulate mesangial cell proliferation (29–32). The differences between these studies may be a function of the concentration of mitogenic factors in MPCM, because Mattana and Singhal (33) found that macrophage supernatants had both stimulatory and suppressive effects on mesangial cell proliferation, depending on the concentration of the supernatant used. Alternatively, the disparity may be due to the particular experimental conditions used.

At present, it is not clear whether the factor(s) suppressing proliferation and inducing fibronectin production are identical. Many in vivo studies have reported that cell proliferation precedes matrix accumulation, which has led to the formulation of the hypothesis that mesangial cell proliferation and accumulation of extracellular matrix are inextricably linked (34). Eng et al. (35), however, were able to demonstrate that the two processes could be partially dissociated. They demonstrated that administration of interferon-γ to anti-Thy-1 nephritic rats significantly reduced cell proliferation while increased levels of matrix deposition were maintained. These in vivo experiments thus concur with the findings reported in the study presented here, i.e., that proliferation and matrix synthesis can be dissociated. This is further supported by the findings of Groggel and Hughes, who demonstrated that heparan sulfate could stimulate rat mesangial cell matrix production while decreasing cell number (36), and those of Zhu et al. (37), who demonstrated that light chains isolated from patients with biopsy-proven light-chain deposition disease were able to increase mesangial cell fibronectin, laminin, and collagen IV in the absence of cell proliferation.

Laminin and collagen are matrix proteins more commonly associated with the basement membrane, although they are present in the mesangium in small amounts. Under pathophysiological conditions, increased amounts of these matrix proteins are found in the mesangium. This study demonstrates that MPCM produces an increase in mesangial cell production of these three matrix proteins, consistent with the notion that macrophage-derived products play a role in the development of glomerulosclerosis. The combined expression of fibronectin, laminin, and collagen IV in mesangial cells has been reported previously in a number of other experimental systems, including: (1) exposure to high glucose conditions (38); (2) after stimulation with thromboxane (39); and (3) after treatment with morphine-stimulated macrophages (30). Differential expression of the three matrix proteins has also been described (40). The extracellular matrix in sclerosed mesangial areas generally contains these matrix proteins, although the exact composition varies among diseases. Whether the production of the three matrix proteins occurs in a coordinated manner in response to one factor via the activation of a common tran-
scriptional element or whether each matrix protein is independently regulated by several different factors is yet to be elucidated. TGFβ, for example, has been shown to directly stimulate the activity of α2(I) collagen promoter (41) and the fibronectin promoter (42) in human cell lines, and it has been speculated that matrix genes, often coexpressed after stimulation with TGFβ, might be activated by a common transcriptional factor such as nuclear factor 1 (43).

TGFβ has been shown to be expressed in many conditions, leading to glomerulosclerosis. Ding et al., in experiments on PAN-nephrotic rats, have tentatively identified glomerular macrophages as a potential source of TGFβ (8). In the current study, TGFβ was not detected in MPCM per se, although mesangial cells were shown to constitutively secrete TGFβ (and PDGF-AB) into their culture media, and this autocrine secretion was further upregulated in response to MPCM. The secreted TGFβ was found to be in the latent or inactive form, in accord with the findings of other investigators (44).

The experiments with the neutralizing antibodies suggest that, with the exception of TGFβ, the other cytokines, individually, play no role in MPCM-mediated fibronectin production. The antibodies used in this study were neutralizing for either mouse or human cytokines and were able to completely block the activity of exogenously added human or mouse cytokines at the concentration used; whether they would also neutralize endogenous rat cytokines is difficult to ascertain with the current, limited availability of rat cytokines. However, it is noteworthy that higher concentrations of antibody (50 μg/ml) demonstrated no additional effect. Moreover, suramin, whose action to inhibit growth factor binding would be species-independent, demonstrated a comparable (and no greater) reduction in fibronectin accumulation to those seen with the anti-TGFβ neutralizing antibody. Taken together, the data suggest that TGFβ plays a small but significant role in MPCM-mediated fibronectin production. However, the majority of the fibronectin-stimulating activity in MPCM cannot be attributed to this growth factor. The identity of the factor(s) responsible for the majority of the stimulatory activity remains to be elucidated. Although TGFβ has been implicated in the pathogenesis of glomerulosclerosis (45), it is of note that Border and associates have reported that in rat mesangial cells expression of fibronectin, laminin, and collagen IV is not substantially affected by TGFβ, as demonstrated by immunoprecipitation of metabolically labeled culture supernatants (46).

Whether the macrophage model used in this study, i.e., thioglycollate-elicited, LPS-stimulated peritoneal macrophages, represents or reflects the effects of infiltrating macrophages found in conditions of renal disease is unclear. To date, the precise activation state of infiltrating macrophages has not been fully described and no doubt varies according to a number of criteria, including cell origin, maturity, and environmental and immunological factors (47,48). However, certain comparisons can be made between the present in vitro system and the pathophysiologial in vivo conditions. Infiltrating macrophages are, in a broad sense, elicited into the glomerulus by the actions of chemokines, etc. Macrophage adherence to plastic surface has been shown to mimic the spreading of these cells to vascular surfaces (49). In vitro, the process of adhesion itself has been shown to upregulate the gene transcription of certain cytokines, which are generally not translated until they have received a second signal from agents such as LPS (50). In addition, it has been shown previously that peritoneal macrophages behave in a qualitatively similar way to glomerular macrophages. For example, peritoneal and glomerular macrophages from diet-induced hypercholesterolemic and PAN-nephrotic rats both exhibit an upregulation of TGFβ gene expression (10). The current studies clearly demonstrate that macrophage-derived products can induce potent profibrogenic characteristics in mesangial cells. The use of MPCM more closely mimics the likely events occurring in vivo than those studies that used heterologous, purified recombinant proteins (traditionally associated with macrophages) added in "industrial," or at least nonphysiological, concentrations to achieve similar effects.

In conclusion, this study provides direct evidence of a role for macrophages in the development of glomerulosclerosis; macrophage secretory products comprise all of the elements required for the initiation of the sclerotic process, as evidenced by the upregulation of the expression of proteins, genes, or both, for matrix proteins and profibrogenic growth factors and the ability to modulate matrix turnover by augmenting the mRNA expression of both the MMP transins and the inhibitor of matrix degradation TIMP-1.

Although TGFβ plays an important role in the fibrogenic process, other factors, some of which are yet to be identified, are also involved.

Acknowledgments

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References

2. Schreiner GF, Cotran RS, Unanue ER: Modulation of IgA and leukocyte common antigen expression in rat glomeruli during the course of glomerulonephritis and aminonucleoside nephrosis. Lab Invest 51: 524–533, 1984
33. Mattana J, Singhal PC: Macrophage supernatants have both stimulatory and suppressive effects on mesangial cell proliferation. J Cell Physiol 154: 289–293, 1993
42. Dean DC, Newby RF, Bourgeois S: Regulation of fibronec tin biosynthesis by dexamethasone, transforming growth factor β and camp in human cell lines. J Cell Biol 106: 2159–2170, 1988
43. Roberts AB, Flanders KC, Kondaiah P, Thompson NL, Ob-


