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Extracellular Matrix Modulates Mesangial Cell Apoptosis and mRNA Expression of Cathepsin-B and Tissue Transglutaminase

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Abstract Mesangial matrix is a dynamic structure which modulates mesangial cell function. Since accumulation of matrix precedes the development of focal glomerulosclerosis, we studied the effect of different matrices on mesangial cell (MC) apoptosis. Suspended mesangial cells became apoptotic in a time dependent manner. Collagen type III did not modulate MC apoptosis when compared to cells grown on plastic. MCs grown on Matrigel, collagen type I and IV showed an increased number of apoptotic cells when compared to MCs grown on plastic. DNA end-labeling further confirmed these observations. MCs grown on Matrigel showed enhanced ($P < 0.05$) mRNA expression for tissue transglutaminase (TTG) and cathepsin-B. Mesangial cells grown on Matrigel also showed enhanced expression of superoxide dismutase (SOD). We conclude that mesangial cells require attachment to the matrix for their survival and alteration of the quality of matrix modulates mesangial cell apoptosis. J. Cell. Biochem. 68:22–30, 1998.

Key words: cathepsin-B; tissue transglutaminase; mesangial cell apoptosis; mRNA expression

Although extracellular matrix (ECM) was thought merely to provide an inert support for adjoining cells, it is now considered to be essential for the survival of many cell types [Ernsberger et al., 1987; Kalcheim et al., 1987; Talhouk et al., 1992; Meredith et al., 1993]. Gut epithelial cells and ureteral epithelial cells have been demonstrated to develop apoptosis when in suspension [Meredith et al., 1993]. In addition, neurons are also dependent on extracellular matrix for survival [Ernsberger et al., 1987; Kalcheim et al., 1987]. In contrast, mesothelial cells and fibroblasts have been reported to survive in suspension [Meredith et al., 1993]. The role of ECM in the survival of cultured glomerular mesangial cells is not clear. We previously reported that mesangial cells grown on a polyhema substrate did not spread and remained rounded [Singhal and Hays, 1988]. We have also recently shown that lack of adhesion to substrate results in mesangial cell apoptosis by a mechanism that appears to be dependent upon impaired tyrosine phosphorylation [Mattana et al., 1996]. Other investigators studied the effect of ECM on mesangial cell morphology, proliferation, and protein synthesis [Hay, 1984; Foellmer et al., 1987; Person et al., 1988; Yaoita, 1989]. These effects of ECM on MC are receptor mediated; MC express receptors for fibronectin, laminin, and collagen type I, III, and IV [Cosio et al, 1990].

Focal glomerulosclerosis (FGS) has been demonstrated to be a common pathway for the progression of renal injury in a variety of human as well as animal experimental renal diseases [Floege et al., 1992]. Expansion of the mesangium has been shown to be a precursor of FGS [Pesce et al., 1991; Floege et al., 1992]. In animal experimental models of FGS, expansion of mesangium occurs as a result of mesangial cell proliferation and accumulation of matrix [Pesce et al., 1991; Floege et al., 1992]. In the natural course of the disease, mesangial cell

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hypercellularity gradually declines and accumulation of matrix continues. The mechanism of matrix accumulation has been studied extensively [Oomura et al., 1989; Sraer et al., 1993]. However, the mechanism of cell death in this glomerular lesion is not clear [Sraer et al., 1993]. Recently, glomerular cell apoptosis has been demonstrated to play a role in the reduction of glomerular hypercellularity [Harrison, 1988; Savill, 1994; Sugiyama et al., 1996a]. What triggers the occurrence of glomerular cell apoptosis is not clear. It has been suggested that the quality of accumulated matrix may determine the cellularity of the mesangium. Recently, Mooney et al. [1996] and Sugiyama et al. [1996a] studied the effect of matrices on the suppression of apoptosis of mesangial cells. However, these investigators did not study occurrence of MC apoptosis under physiological conditions.

The glomerulus is composed of mesangial, endothelial and epithelial cells. Because the morphology of an apoptotic cell is similar to other cells it may be difficult to identify the cell type on the basis of morphology in in vivo studies. In addition, apoptotic cells are rapidly phagocytosed and thus may not be able to maintain distinctive markers of cell origin. Therefore, we planned in vitro studies to evaluate the effect of ECM on occurrence of mesangial cell apoptosis. Since the occurrence of apoptosis is programmed genetically, we also studied the effect of different matrices on the expression of genes which are associated with active cell death.

METHODS

Mesangial Cell (MC) Culture

Mouse MC transformed with nonreplicating, noncapsid forming SV-40 virus (strain RH 911) were kindly provided by Dr. E. Neilson, University of Pennsylvania, Philadelphia. These cells have previously been characterized [Wolf et al., 1992]. MC were grown in Dulbecco's Modified Eagle medium (DMEM; Sigma Chemical Company, St. Louis, MO) containing 10% fetal calf serum (FCS, GIBCO, Grand Island, NY), 50 U/ml of penicillin and 50 microgram/ml of streptomycin (GIBCO).

The following experimental agents were used. Matrigel^{R} (Collaborative Biomedical Products, Becton-Dickinson, Bedford, MA), a murine extracellular matrix derived from Engelbreth-Holm-Swarm (EHS) tumor, was diluted in serum-free, phenol red free RPMI 1640 at 4°C, and this solution was added to Petri dishes and incubated at room temperature to produce an adherent coating of extracellular matrix; additional plastic dishes were coated with collagen type I, III, and IV (Southern Biotechnology Assoc. Inc., Birmingham, AL). Collagen stock solutions of 500 mg/ml were diluted 1:100 with PBS and plates were coated. The plates were incubated overnight at 4°C. Unbound collagen was aspirated and plates were gently rinsed with normal saline just before use.

H-33342 and Propidium Iodide Staining

H-33342 stains the nuclei of live cells and identifies apoptotic cells by increased fluorescence; whereas propidium iodide costains the dead cells. Double staining by these two agents provides the percentage of live, apoptotic, and necrosed cells under control and experimental conditions. To determine the effect of different matrices on MCs, equal numbers of mesangial cells (10,000 cells/well) were plated in 24-well plates coated either with buffer (plastic), collagen I, III, IV, or Matrigel and incubated in media containing FCS for 48 h (10% FCS for initial 4 h and subsequently changed to 1% FCS for the remaining 44 h). At the end of the scheduled incubation periods, cells were incubated with H-33342 (1 microgram/ml) for 7 min at 37°C. Subsequently, cells (without a wash) were placed on ice and propidium iodide (final concentration, 1 microgram/ml) was added to each well. Cells were incubated with the dyes for 10 min on ice, protected from light, and examined under ultraviolet light. Percentage of live, apoptotic (bright fluorescence by condensed or fragmented nuclei), and necrosed (stained pink with propidium iodide) cells were recorded in eight random fields. Four sets of experiments were carried out. The total number of cells examined varied from 857 to 1,260 cells in different groups (plastic, 1,260 cells; collagen I, 1,130 cells; collagen III, 1,211 cells; collagen IV, 1,170 cells; Matrigel, 850 cells).

To determine the fate of suspended cells, equal numbers of mesangial cells (10,000 cells) were seeded either in charged or noncharged plastic Petri dishes in media containing DMEM + 5% FCS. for variable periods (6, 18, 24, and 48 h). At the end of the scheduled period, the cells were stained with H-33342 and propidium iodide. Four sets of experiments were carried out.

Detection of Mesangial Cell Apoptosis by DNA End-Labeling

This is a sensitive method for detection of apoptosis. Briefly, equal numbers of mesangial cells were seeded on 100 mm Petri dishes coated either with buffer (plastic), Matrigel, collagen types I, III, or IV for 48 h. Subsequently, the cells were lysed and incubated with 5 microliter/ml (μl/ml) proteinase K (Promega, Madison, WI) overnight at 37°C. The DNA was extracted and precipitated overnight at −20°C in a 0.3 mM final concentration of Na acetate, 8 ml of 1 M $MgSO_4.7H_2O$ and 2.5 volumes of absolute ethanol. The samples were centrifuged and resuspended in TE buffer (10 mM Tris and 1 mM EDTA) and DNA concentrations were determined. Five mg of isolated DNA from the cells grown on different matrices were end-labeled with 5U Klenow polymerase (Promega) in the presence of 10 mM tris (pH 7.5), 5 mM $MgSO_4.7H_2O$, and 0.5 ml of ^{32}P dCTP (DuPont NEN, Boston, MA) for 10 min at room temperature. The end-labeled DNA was electrophoresed on a 1.8% agarose gel at five volts per cm in 0.5X TE buffer containing 10 mg/ml ethidium bromide. After drying the gel on a slab gel dryer for 45 min at 40°C, radiolabeled fragments were visualized by exposure to Kodak X-ray film at −70°C for 30 min to 3 h.

Evaluation of the Effect of Matrices on Mesangial Cell mRNA Expression of Genes Associated With Active Cell Death

Occurrence of apoptosis has been demonstrated to be associated with the expression of active cell death genes in a variety of cell lines [Fesus et al., 1987; Michel et al., 1992; Guenette et al., 1994; Picksley and Lane, 1994]. We studied the effect of matrices on the expression of genes associated with active cell death such as cathepsin-B and tissue transglutaminase. In brief, mesangial cells were seeded (5 X 10^5 cells/dish) on Petri dishes coated either with buffer, collagen type I, III, IV, or Matrigel and incubated in media containing DMEM and 1% FCS for 48 h. Three sets of experiments were performed. At the end of the incubation period, cells were harvested and total RNA was extracted by the method of Chomczynski and Sacchi [1987]. Northern blots were generated and probed with cDNA probes specific for cathepsin-B (a gift from Martin Tenniswood, Lake Placid, NY) and tissue transglutaminase (a gift from Prof. Peter J. Davis, University of Texas, Houston, TX). Membranes were stripped and reprobed for GAPDH to evaluate whether equal amounts of RNA were added.

Recently, superoxide dismutase (SOD) has been shown to be expressed by glomerular cells exposed to reactive oxygen species [Gwinner et al., 1995]. Since reactive oxygen species are considered to play a role in mesangial cell apoptosis [Sugiyama et al., 1994], we studied the effect of matrices on mesangial cell expression of SOD.

Evaluation of the Effect of Matrices on Mesangial Cell mRNA Expression of SOD

Equal numbers of mesangial cells (5 X 10^5 cells/dish) were plated on Petri dishes coated either with buffer or Matrigel and incubated in media containing DMEM and 1% FCS for 48 h. At the end of the incubation period, total RNA was extracted. Northern blots were prepared and probed with a probe specific for Cu/Zn-SOD (a gift from Dr. Michael Haas, University of Miami, FL). The membranes were stripped and reprobed for GAPDH to evaluate whether equal amounts of RNA were added. Two sets of experiments were carried out.

Autoradiographic Densitometry Instrumentation and Quantitation of Northern Blots

The Digital Imaging System IS-1000 (Alpha Innotech Corp., San Leandro, CA) was used to quantitate densitometry of bands from autoradiographs. Each defined peak was then integrated and density displayed in terms of total area under the peak. This area was then divided by the area of the corresponding GAPDH probed band, and the ratio was expressed.

Statistical Analysis

For comparison of mean values between two groups, the unpaired *t*-test was used. To compare values between multiple groups, analysis of variance was applied and a Newman-Keuls multiple range test was used to calculate a q value. All values are mean ± SE except where otherwise indicated. Statistical significance was defined as $P < 0.05$.

RESULTS

Effect of Matrix on Mesangial Cell Apoptosis

The effect of matrices on mesangial cell apoptosis is shown in Table I. Collagen type III did

TABLE I. Effect of Matrix on Mesangial Cell Apoptosis‡

Plastic	Collagen I	Collagen III	Collagen IV	Matrigel
1.9 ± 0.1	6.7 ± 0.1*	2.2 ± 0.2**	4.8 ± 0.9	5.8 ± 1.7***

‡Equal numbers of mesangial cells (10,000 cells/well) were plated in 24-well plates coated either with buffer (plastic), collagen I, III, IV, or Matrigel and incubated in media containing 1% FCS for 48 h. At the end of the scheduled incubation periods, cells were stained with H-33342. Percentage of live and apoptotic cells were recorded in eight random fields. Results (means ± SEM) are from four sets of experiments.
*$P < 0.01$ compared with plastic.
**$P < 0.05$ compared with collagen I.
***$P < 0.05$ compared with plastic and collagen III.

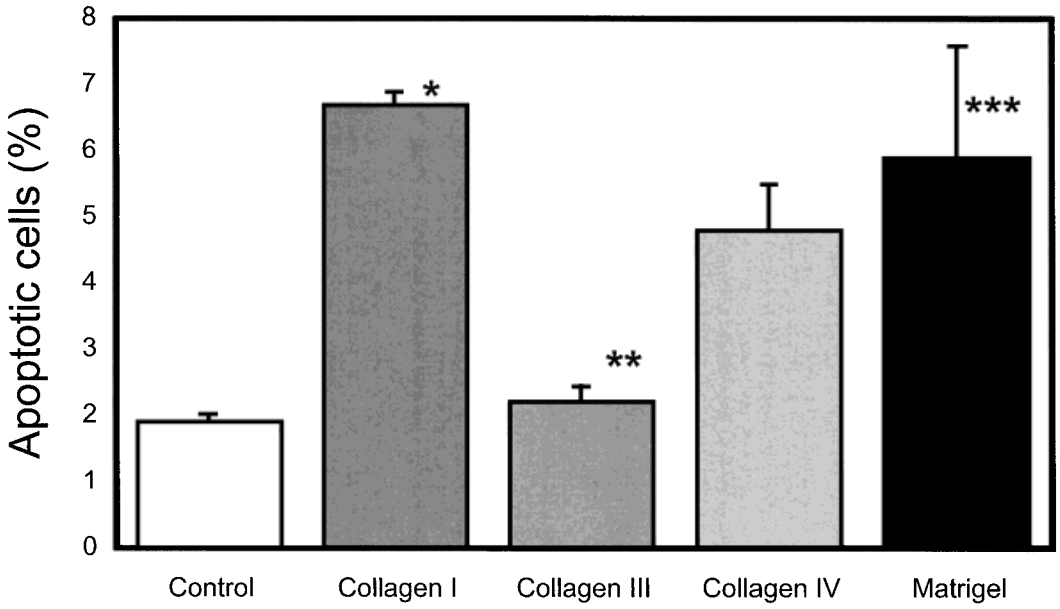

Fig. 1. Effect of matrices on mesangial cell apoptosis. Equal numbers of mesangial cells were plated in 24-well plates coated either with buffer (plastic), collagen I, III, IV, or Matrigel and incubated in media containing 1% FCS for 48 h. At the end of the incubation period, cells were stained with H-33342. Percentage of live and apoptotic cells were recorded in eight random fields. Results (means ± SEM) are from four sets of experiments. *$P < 0.01$ compared with plastic, **$P < 0.05$ compared with collagen I, ***$P < 0.05$ compared with plastic (control) and collagen III.

not alter MC apoptosis when compared to cells grown on plastic. MCs grown on Matrigel and collagen types I and IV showed an increased number of apoptotic cells (plastic, 1.9 ± 0.1%, collagen I, 6.7 ± 0.1%; collagen IV, 4.8 ± 0.9%; Matrigel 5.8 ± 1.7% apoptotic cells/field, Fig. 1). Representative photographs of cells growing on plastic, collagen I and Matrigel are shown in Figure 2. A few cells grown on collagen I and Matrigel showed fragmented nuclei with bright fluorescence.

Fate of Suspended (Unattached) Mesangial Cells

The fate of suspended (unattached) mesangial cells is shown in Tables II and III. Suspended mesangial cells showed an increase in apoptosis in a time dependent manner. A decrease in the percentage of apoptosis of mesangial cells at 48 h may be related to secondary necrosis of previously apoptotic cells (Table III). These results suggest that mesangial cells require attachment to survive.

DNA End-Labeling of Cells Grown on Different Matrices

The cells grown on Matrigel, collagen I, and IV showed a classic ladder pattern (integer multiples of 180 base pairs, Fig. 3). The cells grown on plastic and collagen type III showed a minimal amount of DNA fragmentation.

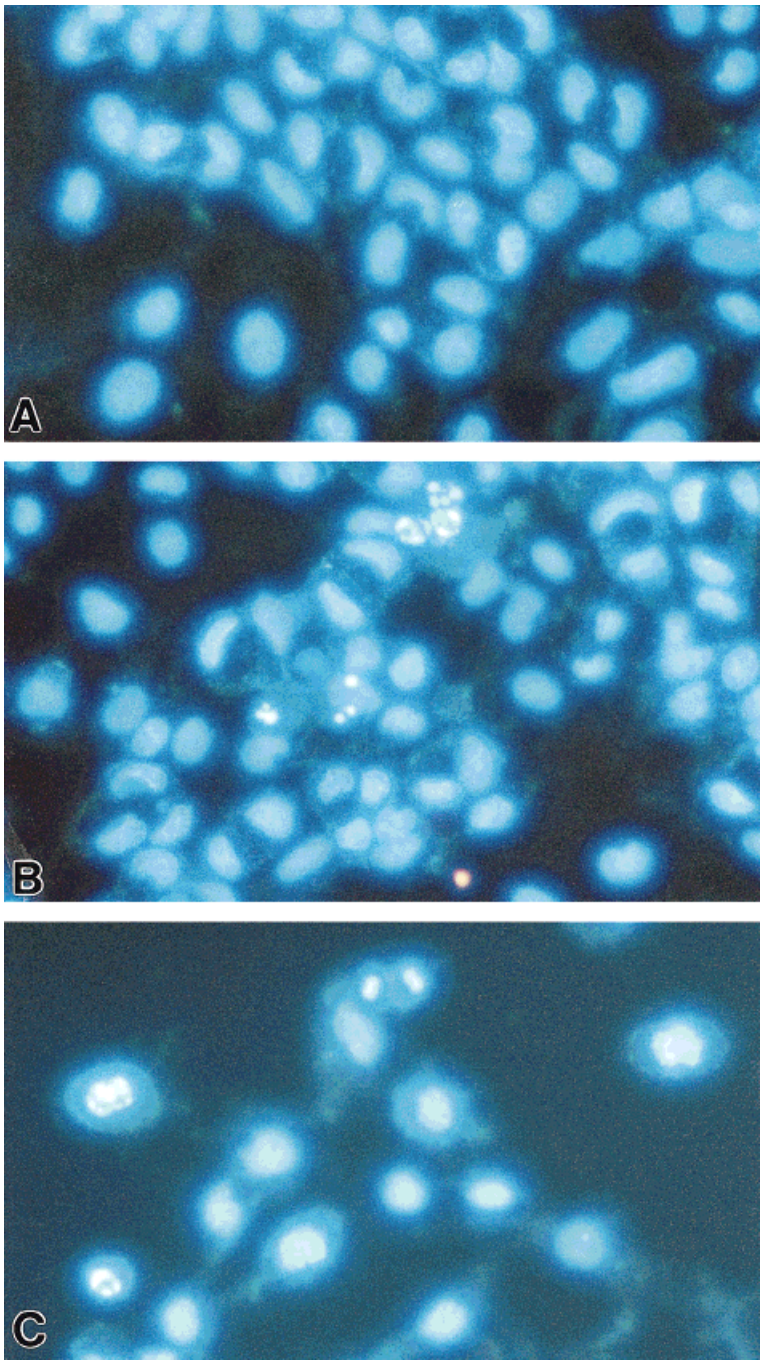

Fig. 2. Equal numbers of mesangial cells were plated in 24-well plates coated either with buffer (plastic), collagen, I, or Matrigel and incubated in media containing 1% FCS for 48 h. At the end of the incubation period, cells were stained with H-33342. **A**: Mesangial cells grown on plastic. **B**: Mesangial cells grown on collagen I. **C**: Mesangial cells grown on Matrigel.

TABLE II. Fate of Suspended Mesangial Cells (% of Apoptotic Cells/Field)‡

6 h		18 h		24 h		48 h	
ATC	UNATC	ATC	UNATC	ATC	UNATC	ATC	UNATC
2.3 ± 0.5	3.7 ± 0.1	3.3 ± 0.3	22.0 ± 0.7*	3.7 ± 0.3	40.3 ± 2.5*	4.4 ± 0.4	17.09 ± 2.8*

‡Equal numbers of mesangial cells were seeded either in charged (ATC) or noncharged (UNATC) plastic Petri dishes in media containing DMEM + 5% FCS for variable time periods (6, 18, 24, and 48). At the end of the scheduled periods, the cells were stained with H-33342 and propidium iodide at 12, 24, and 48 h. Percentage of apoptotic cells were counted in eight random fields. Results (means ± SEM% of apoptotic cells [bright fluorescence with condensed or fragmented nuclei]/field) are from four sets of experiments. *$P < 0.001$ compared to respective ATCs.

TABLE III. Time Course Effect of Charged and Noncharged Petri Dishes on Necrosis (% of Necrotic Cells/Field) of Mesangial Cells*

6 h		18 h		24 h		48 h	
ATC	UNATC	ATC	UNATC	ATC	UNATC	ATC	UNATC
1.5 ± 0.1	1.6 ± 0.1	1.8 ± 0.2	7.2 ± 1.4	1.5 ± 0.3	12.1 ± 2.0	4.0 ± 1.2	37.6 ± 6.5

*Equal numbers of MMCs were plated either in charged (ATC) or noncharged (UNATC) plastic Petri dishes in media containing DMEM + 5% FCS for variable time periods (6, 18, 24, and 48). At the end of the scheduled periods, the cells were stained with H-33342 and propidium iodide. Percentage of necrotic cells were counted in eight random fields. Results (means ± SEM% of necrotic cells [stained pink]/field) are from four sets of experiments.

Effect of Matrices on Mesangial Cell mRNA Expression for Cathepsin-B and Tissue Transglutaminase

The effect of different matrices on mesangial cell mRNA expression for cathepsin-B and tissue transglutaminase is shown in Figures 4 and 5. Mesangial cells grown on Matrigel showed enhanced ($P < 0.05$) mRNA expression for tissue transglutaminase as well as for cathepsin-B (Figs. 4 and 5).

Effect of Matrices on Mesangial Cell mRNA Expression of SOD

The effects of matrices on mesangial cell mRNA expression of SOD is shown in Figure 6. Mesangial cells grown on Matrigel showed a higher mean ratio between SOD and GAPDH vs. cells grown on plastic (plastic, 0.43 ± 0.05 vs. Matrigel, 0.93 ± 0.34).

DISCUSSION

The present study demonstrates that an altered matrix modulates the occurrence of mesangial cell apoptosis. Mesangial cells are destined to be apoptotic in the absence of attachment to the matrix. Mesangial cells grown on Matrigel showed an increased number of apoptotic cells when compared to cells grown

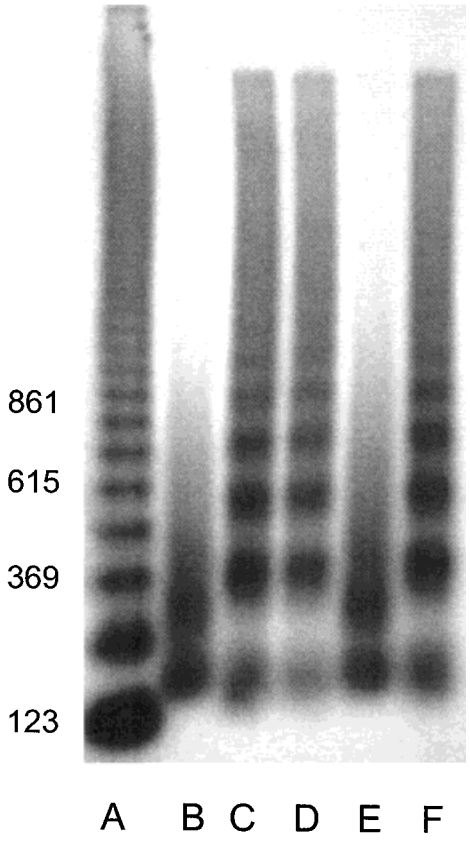

Fig. 3. DNA end-labeled from cells grown on different matrices. **A**: Marker. **B**: Cells grown on plastic. **C**: Cells grown on matrigel. **D**: Cells grown on collagen I. **E**: Cells grown on collagen III. **F**: Cells grown on collagen IV.

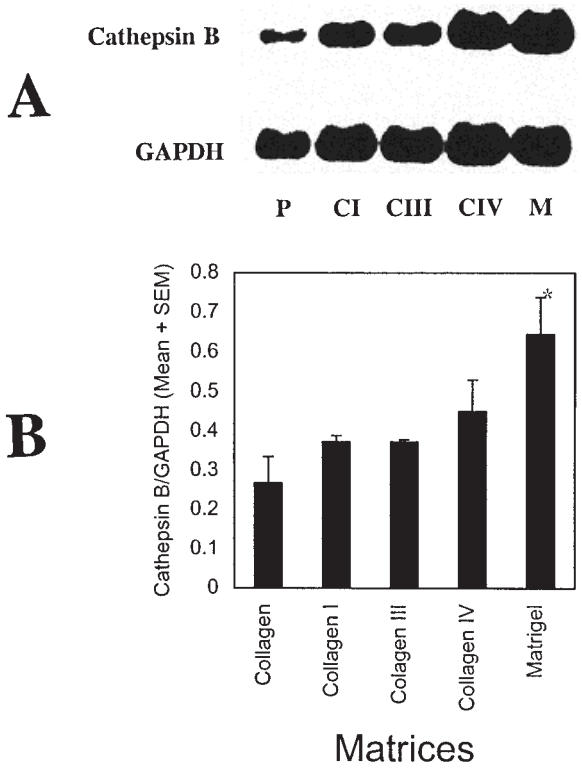

Fig. 4. Effect of matrices on mesangial cell expression of cathepsin-B. **A**: A representative gel. The upper panel shows mRNA expression of cathepsin-B and the lower panel shows expression of GAPDH by mesangial cells grown on plastic (P), collagen types I (I), III (III), IV (IV), or Matrigel (M). **B**: Results (mean ± SEM) are from three sets of experiments. *$P < 0.05$ compared with control.

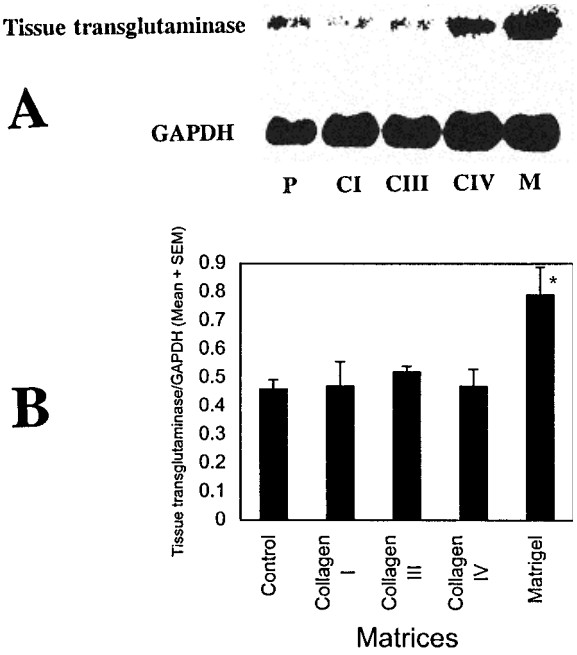

Fig. 5. Effect of matrices on mesangial cell tissue transglutaminase expression. **A**: A representative gel. The upper panel shows mRNA expression of tissue transglutaminase and the lower panel shows expression of GAPDH by mesangial cells grown on plastic (P), collagen types I (CI) , III (CIII), IV (CIV), or Matrigel (M). **B**: Results (mean ± SEM) are from three sets of experiments. *$P < 0.05$ compared with control.

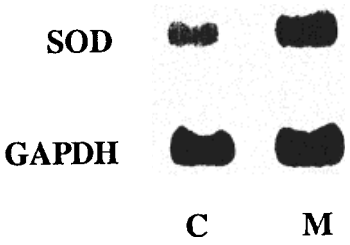

Fig. 6. Effect of matrices on mesangial cell mRNA expression of SOD. The upper panel shows mRNA expression of SOD by mesangial cells grown on plastic and Matrigel. The lower panel shows mRNA expression of GAPDH by mesangial cell grown on plastic (C) and Matrigel (M).

either on plastic or plastic coated with collagen type III. MCs grown on Matrigel also showed enhanced ($P < 0.05$) mRNA expression for tissue transglutaminase and cathepsin-B.

Cells can die as a result of necrosis or apoptosis. Necrosis is a violent death which results from severe ATP depletion causing the collapse of cellular homeostasis [Majno and Joris, 1995]; whereas apoptosis is a highly regulated and genetically directed process [Majno and Joris, 1995; Hockenberry, 1995]. Morphology is considered to be a reliable method for distinguishing between apoptosis and necrosis [Majno and Joris, 1995; Hockenberry, 1995]. Necrotic cells along with their organelles are swollen, show the loss of plasma membrane integrity and leak their cellular contents, triggering inflammation [Martin et al., 1994; Majno and Joris, 1995; Hockenberry, 1995]. In contrast, apoptotic cells are smaller as a result of a reduction of cytosolic volume and condensation of nuclear chromatin [Martin et al., 1994; Majno and Joris, 1995; Hockenberry, 1995]. The plasma membrane remains intact and does not release cytosolic contents to the outside. Apoptotic cells also undergo budding which leads to the fragmentation of the cell into multiple apoptotic bodies. The latter are rapidly phagocytosed by macrophages and neighboring cells [Majno and Joris, 1995]. Cells undergoing apoptosis actively degrade their DNA and form extensive cross-links [Khar, 1994; Bortner et al., 1995; Kroemer et al., 1995]. DNA degradation requires activation of an endogenous deoxyribonuclease. In some cells this

deoxyribonuclease is specific for internucleosomal DNA, and the degraded DNA will form a 200 bp ladder pattern [Khar, 1994; Bortner et al., 1995; Kroemer et al., 1995]. Protein crosslinking appears to be dependent on the activation of an endogenous transglutaminase required for the formation of apoptotic envelopes [Fesus et al., 1987]. In the present study mesangial cells grown on Matrigel showed enhanced expression of tissue transglutaminase confirming that the interaction of Matrigel with MC programs the MC to the path of apoptosis.

Recently, collagen IV and laminin have been shown to suppress mesangial cell apoptosis through an integrin mediated pathway under apoptotic stimulus [Mooney et al., 1996]. Similarly, basement membrane matrix was linked to suppression of mesangial cell apoptosis under apoptotic stimulus [Sugiyama et al., 1996a]. These investigators have used a serum deprivation technique to stimulate apoptosis of mesangial cells. The deprivation of serum to mesangial cells is likely to shut off the message for cell proliferation. However, the use of basement membrane matrix in those conditions may have provided some growth factors (for an example Matrigel contains TGF-b and other growth factors) to serum deprived cells which may have modulated the effect of the apoptotic stimulus. Serum deprivation is an artificial condition which does not occur in vivo. In vivo, mesangial cells are continuously perfused with plasma. In the present study, mesangial cells were not deprived of serum; therefore, results may not be comparable to those studies. The entire process of apoptosis, from the first structural changes to phagocytosis takes approximately 2 h [Raff, 1992]. Therefore, it may often be difficult to detect apoptosis in routine histology sections [Wyllie, 1994]. However, clearance of apoptotic cells is significantly delayed in cell culture and thus it becomes much easier to detect the occurrence of apoptosis in cultured cells. Delay in the clearance of apoptotic cells leads to the degeneration of apoptotic cells that results in loss of plasma membrane integrity and may produce some features associated with cell necrosis [Lieberthal et al., 1994]. However, cells undergoing this type of secondary necrosis still demonstrate the nuclear morphological changes of apoptosis such as chromatin condensation and fragmentation; these alterations are not present in cells dying primarily as a result of necrosis. Secondary necrosis is a common occurrence in cell culture. In the present study, many necrotic cells showed condensation of chromatin as well as fragmentation suggesting secondary necrosis of apoptotic cells. Reactive oxygen species (ROS) have been reported to induce apoptosis in cultured mouse mesangial cells [Sugiyama et al, 1994b]. In the present study too, mesangial cells grown on Matrigel showed enhanced mRNA expression of SOD which is likely a feedback mechanism to combat ROS-induced injury. Therefore, it is possible that Matrigel-induced MC apoptosis may partly be mediated through the generation of reactive oxygen species. Since cells grown on collagen IV showed a lower percentage of apoptosis when compared to cells grown on Matrigel the other constituents (laminin, TGF-b, etc.) of Matrigel may be contributing to Matrigel-induced mesangial cell apoptosis.

We conclude that matrix modulates mesangial cell apoptosis and expression of genes associated with active cell death. This effect of ECM may be contributing to the development of mesangial cell hypoplasia in focal glomerulosclerosis.

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