Extracellular Matrix Degradation by Cultured Mesangial Cells: Mediators and Modulators

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Decreased degradation of the glomerular extracellular matrix (ECM) is thought to contribute to the accumulation of glomerular ECM that occurs in diabetic nephropathy and other chronic renal diseases. Several lines of evidence indicate a key role for the plasminogen activator/plasminogen/plasmin system in glomerular ECM degradation. However, which of the two plasminogen activators (PAs) present in renal tissue, tissue plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA), is responsible for plasmin generation and those factors that modulate the activity of this system remain unclear. This study utilized mesangial cells isolated from mice with gene deletions for tPA, uPA, and plasminogen activator inhibitor 1 (PAI-1) to further delineate the role of the PA/plasminogen/plasmin system in ECM accumulation. ECM degradation by uPA-null mesangial cells was not significantly different from controls (92% ± 1%, $n = 12$). In contrast, ECM degradation by tPA-null mesangial cells was markedly reduced (–78 ± 1%, $n = 12$, $P < 0.05$) compared with controls, whereas tPA/uPA double-null mesangial cells degraded virtually no ECM. Previous studies from this laboratory have established that transforming growth factor-β1 (TGFβ1) inhibits ECM degradation by cultured mesangial cells by increasing the production of PAI-1, the major physiological PA inhibitor. In keeping with this observation, TGFβ1 (1 ng/ml) had no effect on ECM degradation by PAI-1-null MC. High glucose levels (30 m*M*) in the presence or absence of insulin (0.1 m*M*) caused a moderate increase in ECM degradation by normal human mesangial cells. In contrast, glycated albumin, whose concentration is known to increase in diabetes, produced a dose-dependent (0.2–0.5 mg/ml) inhibition of ECM degradation by normal human mesangial cells. Taken together, these results document the importance of tPA versus uPA in renal plasmin production and indicate that in contrast to elevated glucose, glycated albumin may contribute to ECM accumulation in diabetic nephropathy. Exp Biol Med 228:1018–1022, 2003

Key words: tPA; ECM degradation; plasmin; mesangial cells; glycated albumin

Accumulation of the glomerular mesangial extracellular matrix (ECM) leading to glomerulosclerosis is a common finding in diabetic nephropathy and other chronic renal diseases (1–3). Several lines of evidence indicate that ECM accumulation in such chronic renal diseases results from both increased synthesis and degreased degradation of ECM components (4). It is widely accepted that ECM degradation in glomeruli and glomerular cells is mediated by a plasminogen activator-plasmin-matrix metalloproteinase-2 (MMP)-2 cascade. For example, we have shown that ECM degradation by human (5, 6), rat (7), and mouse (this paper) mesangial cells is dependent on the production of plasmin and, to a lesser extent, active MMP-2. In addition, a variety of studies have reported decreased plasminogen activator (PA) activity, decreased plasmin activity, or increased levels of PA inhibitor 1 (PAI-1, the major PA inhibitor) in glomeruli obtained from animals with experimentally induced glomerular injuries known to result in mesangial matrix accumulation (8–11). Interestingly, Haraguchi *et al.* (12) have recently reported that IV administration of recombinant tissue plasminogen activator (tPA) prevents renal ECM accumulation associated with experimentally induced anti-Thy 1.1 glomerulonephritis.

Although the central role of the PA/plasminogen/plasmin system in renal ECM degradation is widely accepted, the specific PA responsible for plasmin production (tPA, urokinase-type plasminogen activator [uPA], or both) is unknown. In addition, with the exception of transforming growth factor-β (TGFβ), a well-documented inhibitor of renal ECM degradation (13–15), the factors that regulate the activity of this system are also unknown. In the present study, we have utilized mesangial cells isolated from the kidneys of mice with gene deletions for tPA, uPA, tPA/uPA, and PAI-1 to delineate the role of tPA versus uPA in renal ECM degradation. In addition we have examined the effects

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of elevated glucose, insulin, and glycated serum albumin (three compounds whose levels are known to be abnormal in diabetics) on ECM degradation by cultured human mesangial cells.

Materials and Methods

Reagents. TGFβ1 was obtained from Boehringer (Petersburg, VA). Glycated serum albumin (A8624, prepared by the reaction of glucose with albumin in sterile phosphate buffer, pH 7.4) and insulin (I-4011) were obtained from Sigma-Aldrich Chemical (St. Louis, MO). All other chemicals were reagent grade or higher and obtained from commercial sources as described in our previous papers (5–7). Exogenously added agents (e.g., plasminogen, TGFβ, glucose, insulin, glycated albumin) were prepared immediately before use and dissolved in RPMI-1640 containing 0.2% lactalbumin hydrolyzate (RPMI-LH) at the concentrations indicated.

Knockout Mice and Mesangial Cell Culture. Mice with gene deletions for tPA, uPA, tPA and uPA, and PAI-1 (knockout mice) were obtained from Dr. Thomas Bugge (National Institute of Dental and Craniofacial Research, Bethesda, MD). Lack of each specific gene (tPA, uPA, tPA/uPA, and PAI-1) was documented in each mouse used for the preparation of mesangial cells by polymerase chain reaction on DNA isolated from tail clippings. Mesangial cells from C57Bl/6 (controls) and null mice were isolated from glomeruli prepared by sieving and centrifugation essentially as described in our previous paper (16). Mouse mesangial cells were stellate or spindle shaped with irregular cytoplasmic projections. The cells did not exhibit contact inhibition and became multilayered if allowed to grow beyond confluency. Mouse mesangial cells exhibited positive immunoperoxidase staining for Thy 1.1 and actin and negative staining for von Willebrand factor. The preparation and characterization of human mesangial cells, a gift from Dr. Hanna Abboud (University of Texas Health Sciences Center, San Antonio, TX), is described in our previous papers (5, 6).

Measurement of ECM Degradation by Cultured Mesangial Cells. ECM degradation by cultured mouse and human mesangial cells was measured as described in detail in our previous papers (5, 6) and summarized briefly here. Thin films of [^{125}I]labeled radiolabeled Matrigel (BD Biosciences, San Jose, CA) were produced in 24-well culture plates as described previously (5, 6). Wells containing dried Matrigel films (25 μg of protein, 20–25,000 cpm) were washed three times with 1.0 ml of serum-free RPMI-1640 medium immediately before addition of mesangial cells (25,000 cells per well) in Waymouth medium containing 15% fetal calf serum. Plates were then incubated for 48 hr (37°C, 5.0% CO_2) to allow the mesangial cells to attach to the Matrigel films and to recover from the plating procedures. After 48 hr, the medium was carefully removed and the cells were washed three times with 1.0 ml of serum-free RPMI-1640 and then incubated as above for 72 hr in 500 μl of serum-free RPMI-1640 (without phenol red) containing 0.2% RPMI-LH and any modulator (i.e., protease inhibitors, glucose, insulin, glycated albumin, or TGFβ) as previously described. ECM degradation by cultured mesangial cells was measured by the release of radioactivity into the cell-free culture medium. At the end of the incubation period (72 hr), the medium from each well was carefully removed and counted (gamma counter). ECM degradation is expressed as mean ± SEM μg Matrigel degraded for the number of determinations (each carried out in triplicate) indicated in each figure. Matrigel degradation was calculated from the decay-corrected specific radioactivity of the Matrigel after correction for the appropriate controls (Matrigel incubated with cells only; Matrigel incubated with medium only; and Matrigel incubated with plasminogen only) included on each 24-well plate.

Statistical Analysis. Results are expressed as the mean ± SEM for the number of replicates indicated. Significance of difference between means was compared by the Student's t test with $P < 0.05$ considered significantly different.

Results

ECM Degradation by Cultured Mouse Mesangial Cells. As shown in Figure 1A, ECM degradation by cultured mouse mesangial cells was dependent on the presence of plasminogen in the medium. ECM degradation was not significantly affected by E-64 (10^{-5} M, a specific cysteine proteinase inhibitor) or pepstatin (5.0 μg/ml, a specific aspartic proteinase inhibitor). Degradation was completely inhibited by aprotinin (216 KIU/ml, a plasmin inhibitor). Orthophenanthroline (100 μM), a metal chelator that inhibits metalloproteinases, reduced ECM degradation by about 35% (Fig. 1B). ECM degradation by mouse mesangial cells was dependent on cell number (10–100,000 cells/well) and length of incubation (1–3 days). Zymography documented that the cells produced both latent and active MMP-2 and that the latter was decreased in the presence of aprotinin (data not shown). Taken together, these data document that ECM degradation by cultured mouse mesangial cells is virtually identical to that by cultured human and rat mesangial cells and dependent on the presence of a functioning PA/plasminogen/plasmin system.

ECM Degradation by Mesangial Cells Isolated From tPA-, uPA-, and tPA/uPA-Null Mice. Mesangial cells produce both tPA and uPA (5). To determine which of these PAs is involved in the generation of plasmin for renal ECM degradation, we examined ECM degradation by mesangial cells obtained from mice with gene deletions for tPA, uPA, and both tPA and uPA. As shown in Figure 2A, deletion of uPA had little effect on ECM degradation (–8.0%). In contrast, in the absence of tPA, ECM degradation was reduced nearly 80% whereas in the absence of both tPA and uPA there was virtually no degradation of the ECM. We also measured plasmin activity in medium obtained from control and knockout cells. As shown in Figure 2B, plasmin activity correlates well with ECM degradation (Fig. 2A).

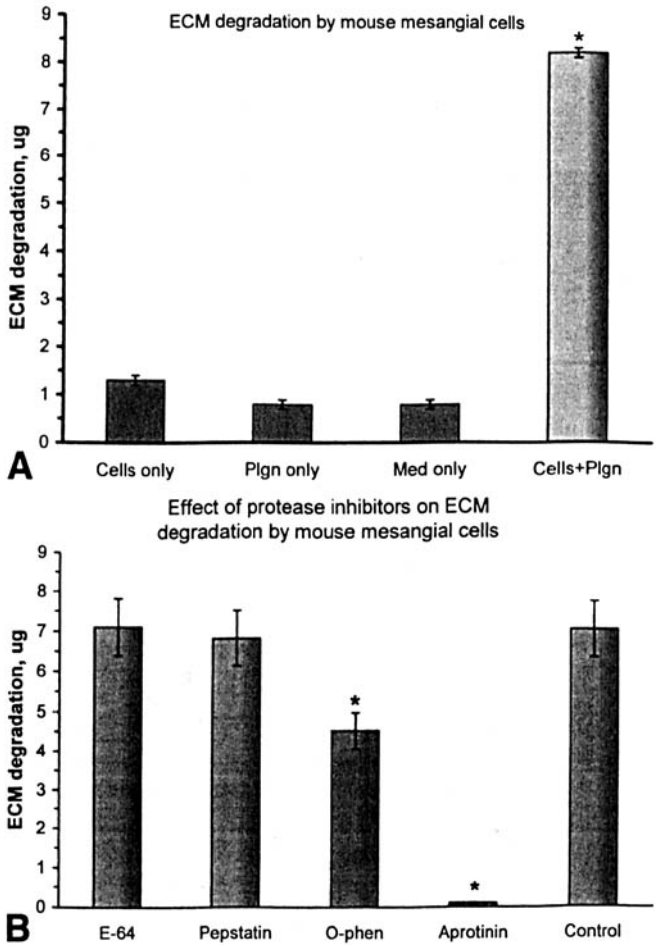

Figure 1. Characterization of ECM degradation by cultured mouse mesangial cells. Mesangial cells were incubated for 72 hr in the presence of plasminogen (Plgn) (A) or the inhibitors indicated (B). Results are presented as the mean ± SEM of a single experiment carried out in triplicate. $^*P < 0.05$.

These data confirm our previous work (5, 7) indicating that tPA is the major PA involved in the production of plasmin by cultured mesangial cells and that ECM degradation is proportional to the plasmin activity.

Effect of TGFβ on ECM Degradation by PAI-1-Null Mesangial Cells. Previous work from this laboratory has documented that TGFβ is a potent inhibitor of ECM degradation by cultured human mesangial cells and indicated that this effect is mediated by increased expression of PAI-1, the major physiologic inhibitor of both tPA and uPA (6). As shown in Figure 3, TGFβ had no effect on ECM degradation by PAI-1-null mesangial cells, documenting that TGFβ acts by increasing the expression of PAI-1.

Effects of Elevated Glucose, Insulin, and Glycated Albumin on ECM Degradation by Cultured Human Mesangial Cells. Decreased levels of insulin (absolute or relative) and hyperglycemia are the hallmarks of diabetes mellitus. As shown in Figure 4, elevated glucose (30 m*M*), in the presence or absence of insulin (0.1 m*M*), caused a moderate increase in ECM degradation. In addition to direct effects, elevated glucose levels lead to glycation (nonenzymatic, covalent addition of glucose) of the ECM and serum proteins, both of which have been implicated in the complications of diabetes mellitus (17). Thus we examined the effects of glycated albumin on ECM degradation by cultured human mesangial cells. As shown in Figure 5, glycated albumin resulted in a dose-dependent decrease in ECM degradation, reaching 80% inhibition at a concentration of 0.5 mg/ml. In contrast, sorbitol-treated albumin (0.5 mg/ml) had no effect.

Discussion

Accumulation of the renal ECM and thickening of the glomerular basement membrane are well-documented complications of diabetic nephropathy (1–3). Both increased synthesis and decreased degradation of the glomerular ECM are thought to contribute to this accumulation (4–6). Mesangial cells are known to be responsible for the synthesis and turnover of the mesangial ECM. Previous studies from

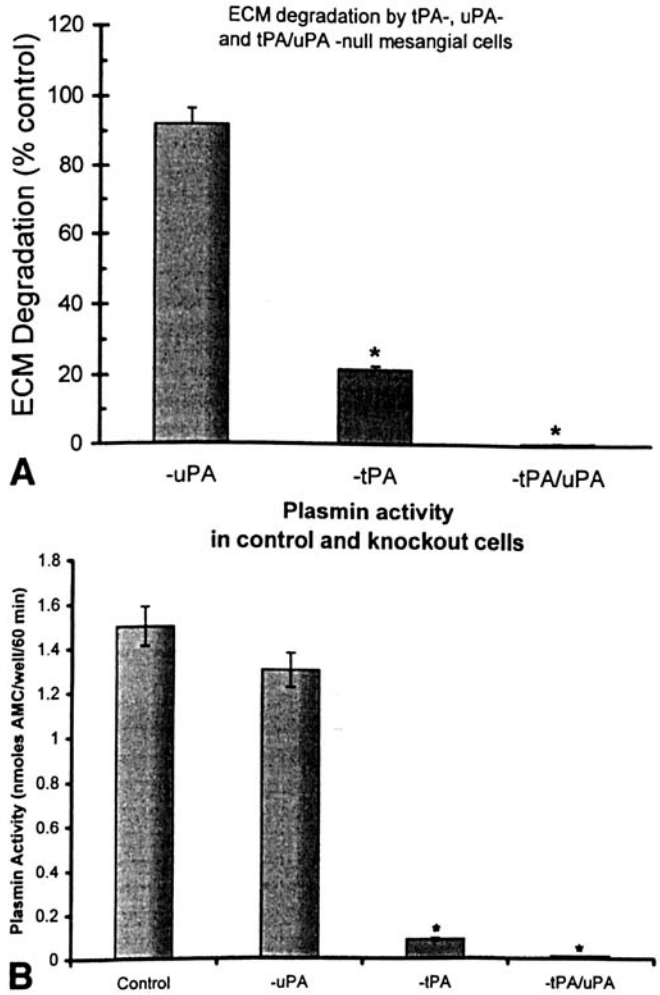

Figure 2. (A) ECM degradation by cultured mouse mesangial cells with gene deletions for tPA and uPA. tPA-null, uPA-null, or tPA/uPA-null mesangial cells were incubated for 72 hr. Results are presented as the mean ± SEM of 4 or more determinations (carried out in triplicate). (B) Plasmin activity in medium obtained from control and knockout cells was measured as described by Wong *et al.* (7). Results are presented as the mean ± SEM for 4 determinations carried out in triplicate. $^*P < 0.05$.

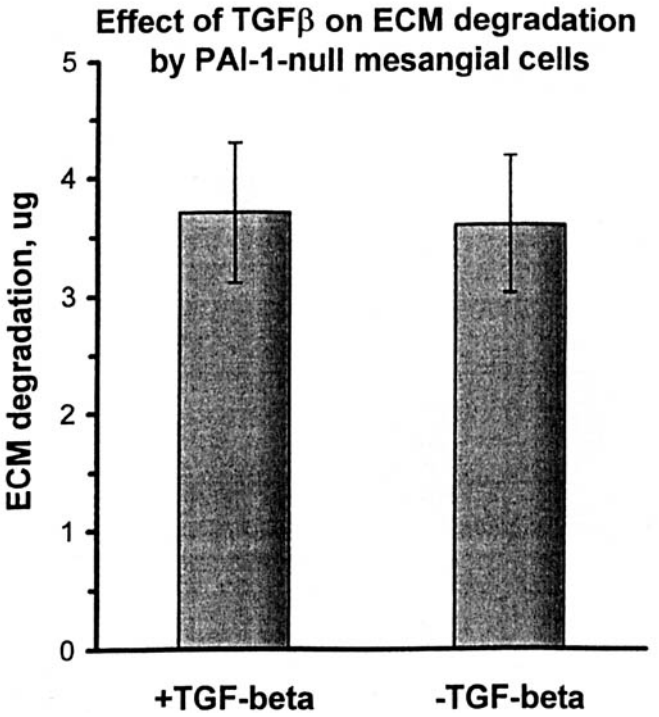

Figure 3. Effect of TGFβ1 on ECM degradation by PAI-1-null mouse mesangial cells. PAI-1-null mesangial cells were incubated for 72 hr in the absence or presence of 1 ng/ml TGFβ1. ECM degradation is expressed as the mean ± SEM for 3 (–TGFβ1) or 6 (+TGFβ1) determinations carried out in triplicate.

our laboratory have established that plasmin, produced by the action of PAs on plasminogen, is the key proteinase involved in ECM degradation by mesangial cells (5–7). In addition to direct degradation of several ECM components, plasmin is thought to activate, directly or indirectly, latent MMP-2 that also contributes to ECM degradation (5, 6). Mesangial cells produce both tPA and uPA (5). However,

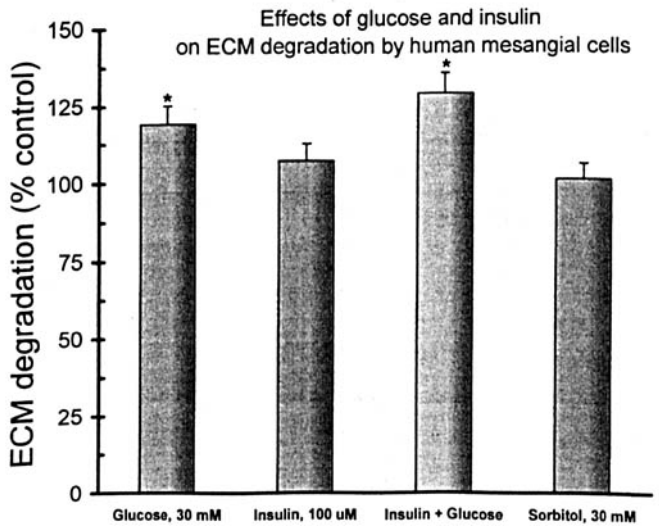

Figure 4. Effect of glucose and insulin on ECM degradation by cultured human mesangial cells. Mesangial cells were incubated for 72 hr in the presence of glucose (30 m*M*) or insulin (0.1 m*M*). ECM degradation is expressed as the mean ± SEM for 3 or more determinations carried out in triplicate. $^*P < 0.05$.

despite the clear role of plasmin in ECM degradation, the specific PA(s) responsible for plasmin production in the mesangial matrix was unclear. The availability of mice with gene deletions (knockouts) for uPA and tPA allowed us to resolve this issue. Although it was desirable to measure tPA and uPA expression in cells obtained from knockout mice, antibodies specific for mouse tPA or uPA are not available. Our results, presented using mesangial cells obtained from mice with gene deletions for tPA and uPA (Fig. 2A), clearly establish that tPA is the major PA involved in plasmin production in this system. In the absence of tPA, ECM degradation was reduced approximately 80% whereas in the absence of uPA the decrease in ECM degradation was less than 10%. The level of ECM degradation correlates well with the plasmin activity in medium obtained from control and knockout cells, as shown in Figure 2B.

Although the role of the PA/plasminogen/plasmin system in renal ECM degradation is well accepted, factors that regulate the activity of this ECM-degrading system are less clear. Our previous studies (6) have documented that TGFβ is a potent inhibitor of ECM degradation and that this effect is mediated by increased expression of PAI-1, the major physiological inhibitor of PAs. The inability of TGFβ to decrease ECM degradation by PAI-1 null mesangial cells (Fig. 3) confirms this observation. Hoffman *et al.* (18) have reported that elevated glucose levels increase TGFβ production by cultured mouse mesangial cells. Such an effect would be expected to decrease ECM degradation, given our observations that even low levels of TGFβ inhibit ECM degradation (6). Surprisingly, we observed a moderate increase in ECM degradation in the presence of elevated glucose levels, in either the presence or absence of insulin (Fig. 4). The reasons for this discrepancy are unknown but may be related species differences or the fact that their studies included serum in the cell culture medium.

The nonenzymatic addition of glucose (glycation) to free amino groups on proteins is proportional to the glucose concentration. The resultant covalent adducts, including serum proteins, hemoglobin, and ECM components, are known to increase in diabetics and could have important metabolic consequences. Indeed, as shown in Figure 5, glycated albumin caused a dose-dependent decrease in ECM degradation. Thus elevated glucose levels appear to impact ECM degradation in two ways: a moderate increase in ECM degradation (possibly *via* direct effects on mesangial cell metabolism) and a much larger negative effect mediated by glycated albumin. Interestingly, our preliminary data indicate that glycated ECM is resistant to degradation by cultured mesangial cells, further exacerbating the effects of elevated glucose levels.

The identification of tPA as the major PA involved in the production of plasmin for renal ECM degradation, and glycated albumin as an potent inhibitor of renal ECM degradation, extends our understanding of the potential biochemical mechanisms leading to renal ECM accumulation in diabetic nephropathy and may provide additional

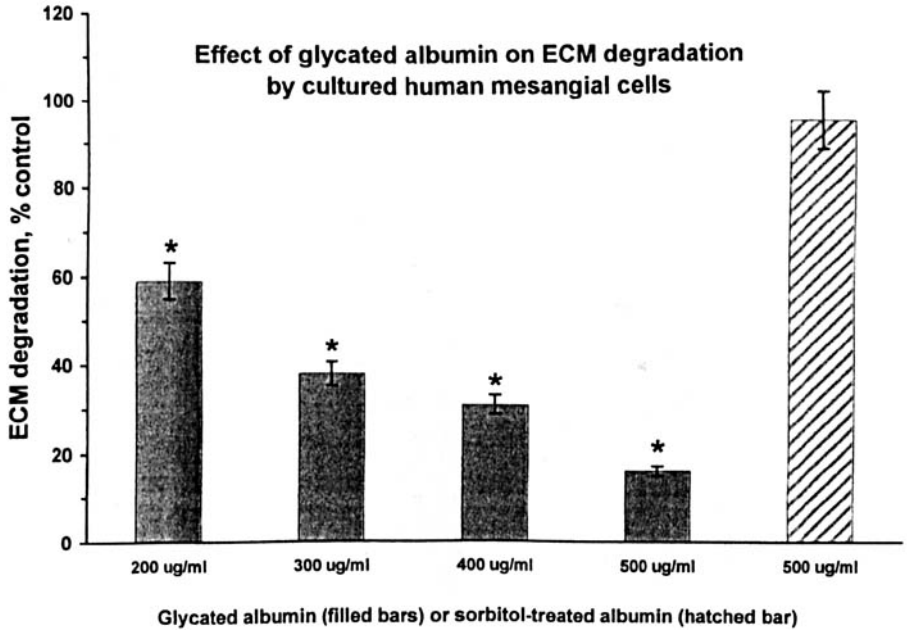

Figure 5. Effect of glycated albumin on ECM degradation by cultured human mesangial cells. Mesangial cells were incubated for 72 hr in the presence of glycated albumin or sorbitol-treated albumin (control) in the concentrations indicated. ECM degradation is expressed as the mean ± SEM for 3 or more determinations carried out in triplicate. $^*P < 0.05$.

targets for the design and application of therapeutic strategies to reduce or eliminate a major complication of diabetes mellitus.

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