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Glomerular matrix accumulation is linked to inhibition of the plasmin protease system

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Glomerular matrix accumulation is linked to inhibition of the plasmin protease system. TGF-β plays a pivotal role in the pathological accumulation of extracellular matrix in experimental glomerulonephritis. Increased TGF-β expression leads to increased synthesis and deposition of extracellular matrix components while administration of antiserum to TGF-β suppresses the major manifestations of the disease. We hypothesized that TGF-β might also enhance matrix accumulation by decreasing matrix turnover via effects on protease/protease inhibitor balance. Plasmin is a potent protease capable of degrading a variety of matrix molecules. Plasmin generation from plasminogen is regulated by plasminogen activator(s) (PA) and plasminogen activator inhibitor(s) (PAI). In this study PA activity was markedly reduced and PAI-1 synthesis dramatically increased when TGF-β was added to normal glomeruli. Diseased glomeruli also showed decreased PA activity, increased PAI-1 synthesis and increased PAI-1 deposition into matrix. Administration of anti-TGF-β serum to glomerulonephritic rats blocked the expected increase in glomerular PAI-1 deposition. Thus changes in the PA/PAI balance favoring accumulation of matrix are induced by TGF-β in normal glomeruli and are present in nephritic glomeruli when endogenous TGF-β production is high. Our findings implicate the plasmin protease system in tissue repair following acute glomerular injury and suggest another mechanism by which TGF-β enhances the matrix accumulation characteristic of many glomerular diseases.

A central feature of progressive glomerular disease, whether due to glomerulonephritis or diabetes, is accumulation of extracellular matrix leading to the development of glomerulosclerosis [1, 2]. In acute mesangial proliferative glomerulonephritis induced in rats by the administration of anti-thymocyte serum (ATS), we have shown that immunological injury induces elevated glomerular expression of transforming growth factor-β (TGF-β) mRNA and TGF-β protein. The expression of TGF-β in the glomerulus is followed closely by induction of synthesis of two chondroitin/dermatan sulfate proteoglycans, decorin and biglycan, which are considered markers of TGF-β activity [3], and a major matrix glycoprotein, fibronectin. Further demonstration of the causal role of TGF-β in this model is that administration of a neutralizing antibody to TGF-β suppresses glomerular accumulation of extracellular matrix [4]. Other work in an experimental model of rapidly progressive glomerulonephritis induced by administration of anti-glomerular basement membrane antibody, has also shown a close correlation between expression of TGF-β and the development of renal fibrosis [5].

TGF-β is a growth factor unique in its widespread effects on extracellular matrix [6]. From *in vitro* experiments using a number of cell lines, TGF-β has been shown to: (1) increase the production of matrix glycoproteins and proteoglycans [7–13]; (2) modulate the expression of some integrin-type receptors on cells in a manner that might lead to increased cell adhesion to matrix [14, 15]; (3) decrease the production of matrix degrading proteases [16–18]; and (4) increase the level of protease inhibitors [15–19]. Modulation of the balance between proteases and their inhibitors is one potentially important mechanism by which TGF-β enhances accumulation of extracellular matrix following tissue injury.

One of the protease cascades strongly influenced by TGF-β is plasminogen activator (PA)/plasmin system. Plasmin is a potent serine protease best known for its activity against fibrin, but plasmin is also capable of degrading extracellular matrix and probably plays an important role in the proteolysis that accompanies tissue repair [20, 21]. Plasmin generation from plasminogen is regulated by the interaction of activators and inhibitors. There are two distinct types of plasminogen activators (PA) that convert the zymogen plasminogen to plasmin, tissue-type (t-PA) and urokinase-type (u-PA). u-PA has been implicated in a variety of extravascular processes including tissue remodeling and cell migration. The PAs are counterbalanced by inhibitors of which plasminogen activator inhibitor-1 (PAI-1) is one of the most important.

In our previous work using the ATS model of glomerulonephritis we showed a close correlation between expression of TGF-β and mesangial matrix expansion. Other investigators have shown a role for PDGF in mesangial cell proliferation in this model of glomerulonephritis [22]. Both of these cytokines induce high levels of PAI-1 in many cell lines [16, 17, 23–25]. A recent study showed dramatic accumulation of PAI-1 mRNA in mouse kidney three hours after injection of purified human recombinant TGF-β1 [26]. Although these cytokines induce large changes in PAI-1 synthesis, the *in vivo* significance of these actions is unknown. In addition to the strong induction of matrix synthesis by TGF-β and the mesangial cell proliferation brought about by PDGF, we questioned whether effects on protease/protease inhibitor balance might contribute to the accumulation of pathologic matrix. In the current study, we report that TGF-β markedly stimulates active PAI-1 synthesis by normal glomeruli. Furthermore, nephritic glomeruli show

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decreased PA activity and increased PAI-1 synthesis which precede the accumulation of extracellular matrix in the nephritic glomeruli. Returns to normal PAI-1 synthesis and PA activity precede resolution of accumulated matrix during recovery from disease, results consistent with the hypothesis that the PA/PAI-1 protease system plays a role in matrix accumulation during disease and possibly matrix degradation during recovery. Finally, a significantly increased deposition of PAI-1 in nephritic glomeruli is largely prevented by *in vivo* administration of neutralizing antibodies to TGF-β, indicating a causal role for TGF-β in the increased glomerular deposition of PAI-1 in disease.

Methods

Animals

Four- to six-week-old Sprague-Dawley rats from Sasco, Inc. (Omaha, Nebraska, USA) and four-month-old New Zealand white rabbits from Ellis and Prichard (American Fork, Utah, USA) were maintained in specific pathogen-free conditions and used in all experiments.

Growth factors, antibodies and reagents

Porcine platelet TGF-β1 was from R&D Systems (Minneapolis, Minnesota, USA). PDGF-BB was from Boehringer Mannheim (Indianapolis, Indiana, USA). Rabbit anti-rat PAI-1 IgG was obtained from American Diagnostica (New York, New York, USA). Biotin was from Calbiochem (San Diego, California, USA); avidin-FITC and biotin blocking reagents were from Pierce (Rockford, Illinois, USA).

Sieves for glomerular isolation and tissue culture plastic ware were from Baxter Scientific (Salt Lake City, Utah, USA). Tissue culture supplies included RPMI 1640 culture medium and HEPES buffer solution from Irvine Scientific (Irvine, California, USA), methionine- and glutamine-free RPMI, penicillin and streptomycin from ICN Flow Laboratories (Costa Mesa, California, USA) and fetal calf serum (FCS) from Hyclone (Logan, Utah, USA). Reagents to measure PA activity, including plasminogen, low molecular weight human u-PA and H-D-Val-Leu-Lys-p-nitroanilide (S-2251) were from KabiVitrum (Franklin, Ohio, USA). Electrophoresis supplies were from Bio-Rad (Richmond, California, USA). Precast polyacrylamide gels (4 to 12% gradient) were obtained from NOVEX (Encinitas, California, USA).

^{35}S-methionine (EXPRE^{35}S^{35}S-protein labeling mix) and ENLIGHTNING came from New England Nuclear Dupont (Boston, Massachusetts, USA). Ketamine-HCl from Aveco (Fort Dodge, Iowa, USA) and xylazine from Miles Laboratories (Shawnee, Kansas, USA) were used for anesthesia. X-ray films were from Eastman Kodak (Rochester, New York, USA). Staphylococcal protein-A-Sepharose CL4B and all other reagents not specifically mentioned were from Sigma Chemical Company (St. Louis, Missouri, USA).

Induction of experimental glomerulonephritis

Anti-thymocyte serum (ATS) was produced in New Zealand white rabbits and glomerulonephritis was induced in Sprague-Dawley rats by intravenous administration of 1 ml of ATS as previously described [3]. The day of injection was designated as day 0. Rats were killed 3, 7, and 14 days after ATS administration for histologic examination of kidney tissue and isolation of glomeruli for culture.

Glomerular culture

Rats were anesthetized intramuscularly with ketamine HCl, and xylazine (10 mg, and 0.5 mg per 100 g body wt, respectively). The kidneys were perfused *in situ* via the aorta with PBS pH 7.4 and excised. The capsules were removed and the cortical tissue was dissected out and minced with a razor blade. Glomeruli were isolated using the graded sieving technique [3]. Isolated glomeruli were washed three times in PBS and resuspended at 1.5×10^3 glomeruli per ml in methionine-, serum- and antibiotic-free RPMI-1640 in six well plates. After two hours of incubation with various concentrations of TGF-β the cultures were biosynthetically labeled by addition of 50 μCi/ml ^{35}S-methionine for an additional 24 hours. Phenylmethyl-sulfonyl fluoride (PMSF) was added to a final concentration of 1 mM. Samples were centrifuged to remove glomeruli and culture supernatants were frozen at −20°C until use. Where samples were to be assayed for PA activity, radiolabel and PMSF were omitted.

Electrophoretic technique

Samples for SDS-PAGE were mixed with sample buffer [27] containing 3% sodium dodecyl sulfate (SDS) and 10% 2-mercaptoethanol, and were heated for five minutes at 100°C. Twenty microliter aliquots were applied to 4 to 12% gradient gels, and gels were run for three hours at constant power. For autofluorography, gels were incubated in ENLIGHTENING for one hour, dried and exposed to x-ray film, typically for a one to two day exposure. Autofluorograms were scanned with an Ultrascan XL Enhanced Laser Densitometer (Pharmacia, Uppsala, Sweden) to compare and quantitate the relative intensities of the protein bands. Autofluorograms exposed for different time periods were scanned to check the linearity of densitometer readings.

Plasminogen activator (PA) assay

PA activity was assayed by measuring the hydrolysis of synthetic substrate by formed plasmin in the presence of plasminogen [28]. Assays were performed in polyvinyl chloride microtiter plates. The total volume of 125 μl was comprised of the following: sample, H-D-Val-Leu-Lys-*p*-nitroanilide (0.01 μM), and plasminogen (0.03 μM) in 0.5% Triton X-100, 0.1 M Tris, pH 8.0. The amount of *p*-nitroaniline released was measured at 410 nm with a Dynatech MR700 microplate reader (Dynatech Laboratories, Alexandria, Virginia, USA). A standard curve was generated with each assay using low molecular weight human u-PA. Each sample was also assayed without plasminogen to establish the plasminogen-dependence of the enzyme activity. The PA activity in culture supernatant or cell lysate was expressed as IU/1000 glomeruli. Where indicated amiloride (1 mM), an inhibitor of u-PA but not t-PA [29], was included in the assay mixture.

Substrate gel electrophoresis (zymography)

The method of Granelli-Piperno and Reich [30] was modified as follows: plasminogen (0.03 μM) and α-casein (0.2 mg/ml) were added to a 12.5% polyacrylamide gel mixture prior to casting. Samples were not boiled or reduced prior to the

analysis. After electrophoresis the gel was soaked for one hour in 2.5% Triton X-100 and then overnight in 0.1 M glycine buffer, pH 8.0 at 37°C. Clear α-casein zones of lysis were localized by staining the gel with Coomassie Blue. The samples were also run on gels without plasminogen to establish that the zones of lysis were attributable to PA.

Immunoprecipitation

Samples (1 ml) were preabsorbed with 50 μl rabbit serum and 100 μl 50% (vol/vol) Protein-A Sepharose CL-4B at 4°C for two hours. After centrifugation the media were collected and 5 μg of anti-PAI-1 IgG in 100 μl distilled water was added together with 50 μl protein-A Sepharose. The samples were then incubated in a rotary shaker at 4°C for 16 hours. The antigen-antibody complexes bound to Protein-A Sepharose were collected by centrifugation, washed three times with 10 mM Tris-HCl buffer, pH 7.5, containing 0.5% Triton X, 0.1% SDS, and 0.5% sodium deoxycholate, and once with PBS. The precipitated proteins were dissolved in Laemmli's sample buffer, boiled for five minutes and analyzed by SDS-PAGE as described above. When appropriate decreasing quantities of media were used to ensure that immunoprecipitation was quantitative.

Immunofluorescent staining

Kidneys were perfused via the aorta with cold PBS, isolated and minced. The mince was snap-frozen in 2-methylbutane that had been cooled in liquid nitrogen. Three to five micrometer sections were obtained using a cryostat (Tissue-Tek-II, Miles Laboratories, Elkhart, Indiana, USA). The tissue was air dried and infixed. After blocking with biotin and avidin, sections were stained with 2.5 μg anti-PAI-1 IgG which had been biotinylated by the method of Berman and Basch [31]. Avidin-FITC was then applied and immunofluorescence was examined using an epifluorescence microscope (Zeiss, Germany).

Effect of in vivo administration of TGF-β antiserum and immunohistological staining

Paraffin fixed tissues were examined from an earlier study where anti-TGF-β1 was given at the time of induction of glomerular disease and was shown to suppress the increased production and accumulation of extracellular matrix [4]. Briefly, anti-TGF-β serum or one of two control rabbit sera were injected for six consecutive days starting with the day anti-thymocyte serum was given. Normal control and disease control animals were injected with normal rabbit serum (NRS) instead of anti-TGF-β serum. On days 7 and 14 after induction of disease, kidneys were isolated, fixed in 10% neutralized formalin and paraffin embedded. One micrometer sections were mounted on poly-L-lysine coated slides, deparaffinized and exposed to 0.3% hydrogen peroxide for 30 minutes. Immunohistochemical staining with rabbit anti-rat PAI-1 was detected with biotinylated goat anti-rabbit IgG, followed by avidin-peroxidase (ABC kit, Vector Laboratories). Each incubation was followed with a five minute wash with PBS. Staining was visualized with 0.05% 3,3'-diaminobenzedene/0.03% hydrogen peroxide in PBS. Immunoperoxidase staining was quantitated in a blinded fashion by scoring distribution and intensity of glomerular staining on a scale of 1 to 4 in at least 20 glomeruli from three rats in each group. All slides were stained simultaneously and photographed using the same light intensity and exposure time.

Statistical comparisons were done on data from three replicate experiments using *t*-tests.

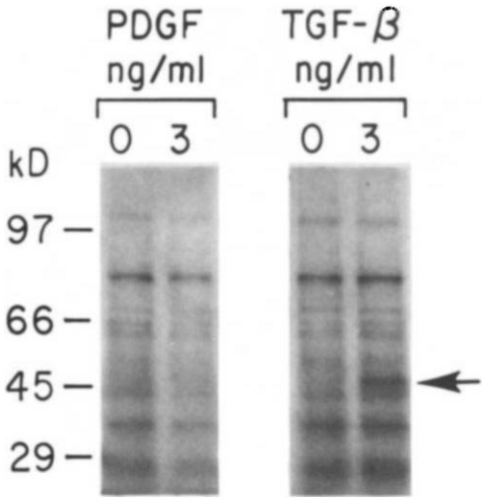

Fig. 1. *Effects of PDGF and TGF-β on normal glomeruli.* Equal numbers of normal rat glomeruli were exposed to either PDGF-BB or TGF-β1 for 24 hours in the presence of ^{35}S-methionine. The fluorogram shown is from SDS-PAGE of 20 μl of each culture supernatant. The position of PAI-1 is indicated by an arrow.

Results

Response of normal glomeruli to PDGF and TGF-β1

Normal glomeruli were isolated and incubated with and without increasing concentrations of PDGF and TGF-β1 in the presence of ^{35}S-methionine for 24 hours. Aliquots of culture supernatant were run on PAGE. Fluorograms obtained from a representative gel are shown in Figure 1. In the presence of TGF-β1 a clear increase in a band at 50 kD is seen, consistent with the molecular weight of PAI-1. This increase is absent from glomeruli treated with PDGF-BB. Concentrations of PDGF-BB as high as 30 ng/ml showed no increase in this 50 kD band (data not shown). These data suggest that TGF-β1 but not PDGF-BB stimulate PAI-1 synthesis by normal glomeruli.

The effect of TGF-β1 was examined further by measuring the PA activity in cultures of normal glomeruli using two methods, chromogenic substrate assays (Fig. 2A) and substrate gel electrophoresis (Fig. 2B). Both methods showed the presence of PA activity in normal glomerular cultures and a marked decrease in PA activity in response to the lowest dose of TGF-β1 tested (1 ng/ml). Substrate gel electrophoresis showed a 50 kD plasminogen-dependent zone of lysis consistent with u-PA. Gels without added plasminogen showed no 50 kD band in the absence of TGF-β1 (data not shown). That the PA activity was due to u-PA and not t-PA was suggested by the nearly complete inhibition of the activity by amiloride (data not shown) [29].

To examine further PAI-1 expression in this system, normal glomeruli were metabolically labeled in the presence of varying concentrations of TGF-β1. After 24 hours, PAI-1 was immunoprecipitated from culture supernatants. Aliquots of the same samples precipitated with normal rabbit serum (1:100 dilution) showed no labeled proteins, confirming the specificity of the

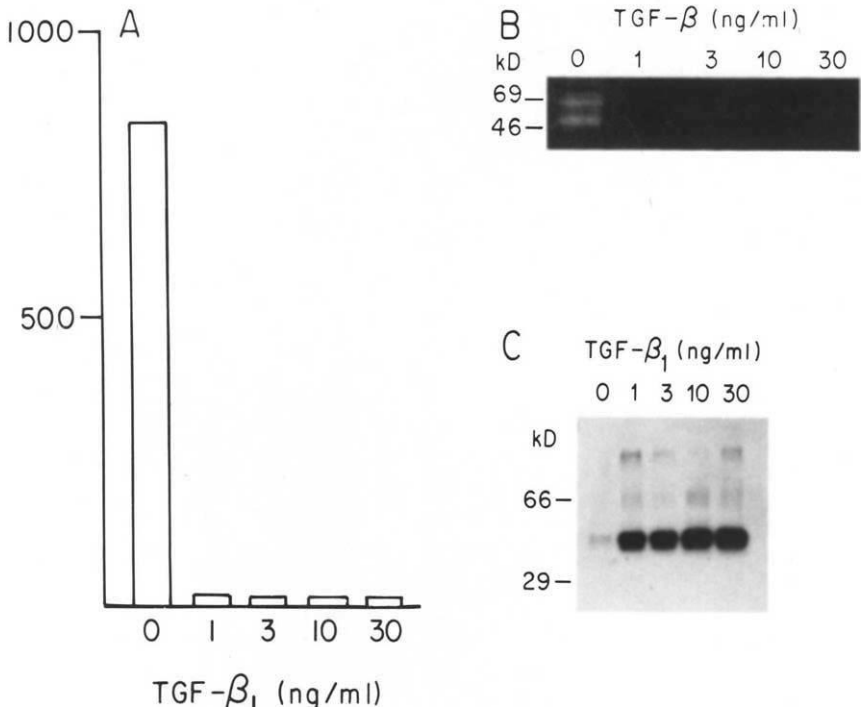

Fig. 2. *Effect of TGF-β1 on PA and PAI-1 production by isolated normal glomeruli.* Equal numbers (1.5×10^5) of normal rat glomeruli were exposed to increasing concentrations of TGF-β1 for 24 hours. **(A)** PA activity in the medium was analyzed by chromogenic substrate assay and expressed per 1000 glomeruli. **(B)** PA activity analyzed by substrate gel electrophoresis. With plasminogen in the gel, conditioned medium produced a clear lysis zone at 50 kD, consistent with u-PA activity. In the presence of TGF-β, this band was not seen. This band was absent when plasminogen was omitted from the gel (data not shown). A 70 kD plasminogen-independent zone of lysis was also decreased by TGF-β. **(C)** Glomeruli were metabolically labeled with ^{35}S-methionine at the time of addition of TGF-β. PAI-1 was immunoprecipitated from 1 ml of labeled medium and analyzed by SDS-PAGE and fluorography.

immunoprecipitation (data not shown). The results show that normal glomeruli spontaneously produce small amounts of PAI-1. With the addition of low concentrations of TGF-β1 striking increases in PAI-1 synthesis are seen (Fig. 2C). Densitometry indicates that the lowest concentration (1 ng/ml) of TGF-β1 induces an 8.5 ± 0.70-fold increase over control in newly synthesized PAI-1 in culture supernatant. Higher concentrations of added TGF-β1 produce further stimulation of PAI-1 synthesis resulting in a 15.2 ± 1.2-fold increase over control at 30 ng/ml TGF-β1/ml (Fig. 2C). Compared to normal control glomeruli, PAI-1 immunoprecipitated from culture supernatant was significantly ($P < 0.01$) greater for all concentrations of TGF-β1.

To study the time course of PAI-1 synthesis induced by TGF-β1, normal glomeruli were plated into multiple tissue culture wells containing TGF-β (10 ng/ml), serum-free medium and ^{35}S-methionine. TGF-β1 was omitted from the control wells. Culture supernatant was removed from the wells at 6, 12, 24 and 48 hour and immunoprecipitated with antiserum to rat PAI-1. In the presence of 10 ng/ml TGF-β1, PAI-1 synthesis and secretion is considerably greater than without exogenous TGF-β1 (Fig. 3 A and B). A plot of these data reveals that without exogenous TGF-β1 PAI-1 synthesis and secretion proceeds at a linear rate for 24 hours of culture. In the presence of TGF-β1 (10 ng/ml) this rate of appearance of newly synthesized PAI-1 in the supernatant is 4.1 and 5 times greater than in the absence of added TGF-β1 in replicate experiments (Fig. 3C).

Nephritic glomeruli—PA activity

Intact glomeruli were isolated from normal and glomerulonephritic rats 3, 7 and 14 days after injection of ATS. After 24 hours in culture, supernatants were collected and PA activity was measured by the chromogenic substrate assay. Figure 4A shows that normal glomeruli secrete plasminogen-dependent proteolytic activity consistent with active PA. Inhibition by amiloride suggests this activity is mostly u-PA (Fig. 4A). On day 3 of the disease this activity is significantly decreased to 15 ± 3% of control activity ($P < 0.001$). It returns to control values (101 ± 7%) by day 7 and shows a significant increase over controls to 142 ± 12% at day 14 of disease ($P < 0.005$).

Substrate gel electrophoresis run on culture supernatants from isolated nephritic glomeruli showed a 50 kD plasminogen-dependent zone of lysis consistent with u-PA (Fig. 4B). No 50 kD band was seen when plasminogen was omitted from the gel (data not shown). Changes during disease in the 50 kD zone of lysis (Fig. 4B) parallel the chromogenic substrate assay data (Fig. 4A).

The second zone of lysis at approximately 70 kD was also observed in substrate gels without plasminogen. This protease, potentially from the serine, cysteine or metalloproteinase family, has not been characterized to date.

Nephritic glomeruli—Synthesis of PAI-1

Nephritic and normal glomeruli were isolated and placed in culture for 24 hours with ^{35}S-methionine. Culture supernatants were then immunoprecipitated with PAI-1 antiserum. Nephritic glomeruli synthesize and secrete large quantities of PAI-1 (Fig. 4C), similar to the PAI-1 synthesis seen when normal glomeruli were exposed to TGF-β (Fig. 2C). Densitometry reveals significant increases of 4.8 ± 0.6-fold and 5.4 ± 0.4-fold in PAI-1 synthesis at days 3 and 7 of disease, respectively ($P < 0.005$). By day 14 of disease a nonsignificant increase to 1.6 ± 0.4 times control values is seen.

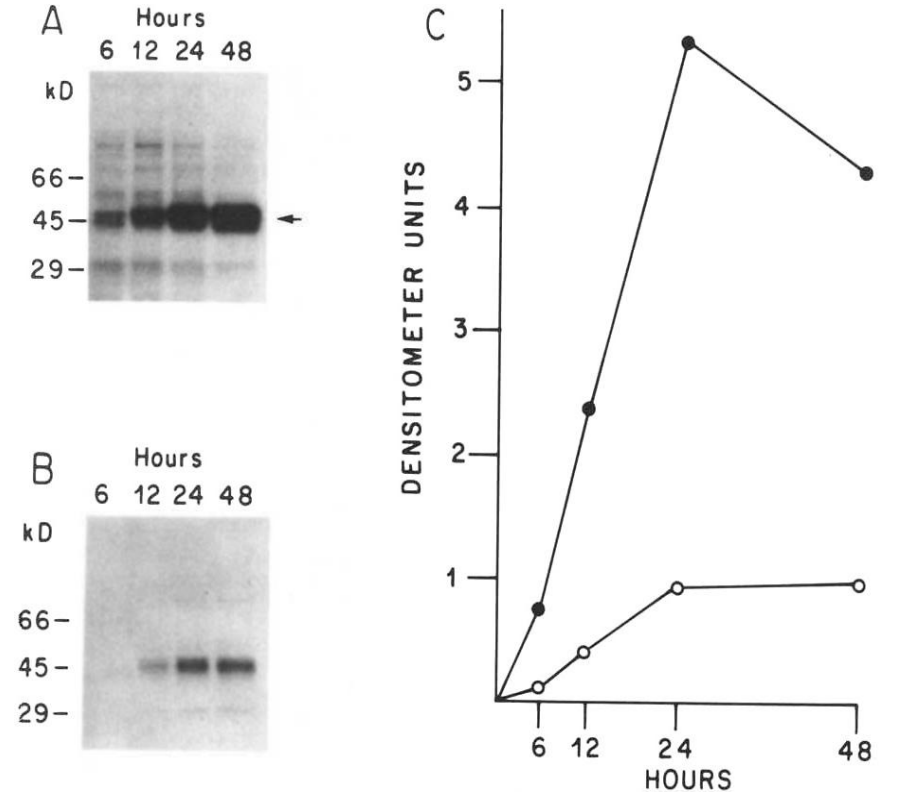

Fig. 3. *Time course of PAI-1 immunoprecipitated from medium conditioned by normal glomeruli for 6, 12, 24 and 48 hours with (A) and without (B) 10 ng/ml TGF-β1.* Densitometry of these gels yielded the data plotted in C. The arrow indicates the PAI-1 band.

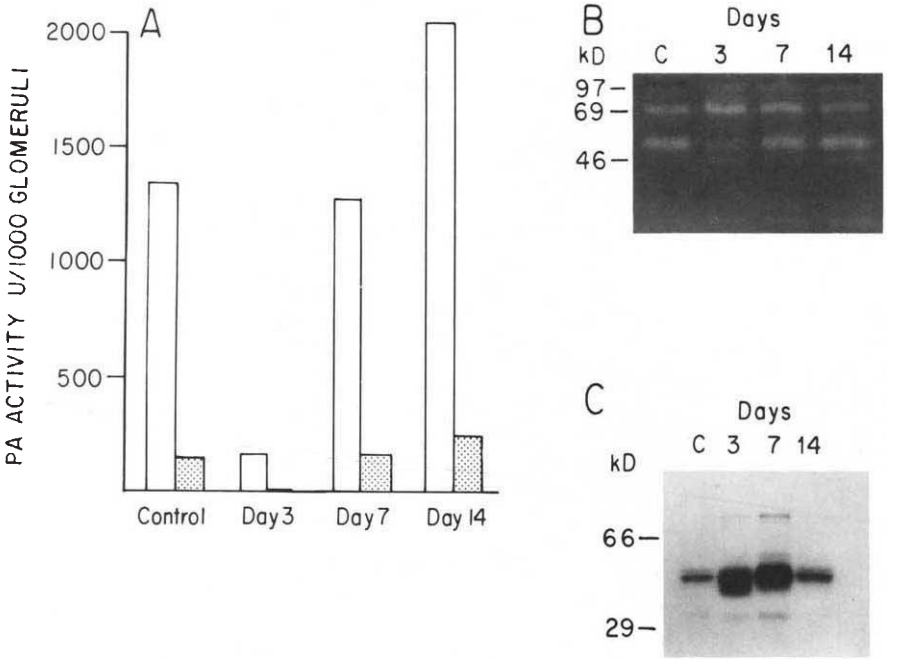

Fig. 4. *PA and PAI-1 production by isolated nephritic glomeruli.* Equal numbers of normal control or nephritic glomeruli isolated on days 3, 7, or 14 of ATS-induced glomerulonephritis were placed in culture for 24 hours. **(A)** PA activity was analyzed and expressed as in Figure 1. Open bars represent total PA activity and dotted bars show PA activity measured after amiloride treatment. **(B)** Substrate gel electrophoresis of glomerular culture supernatant from nephritic glomeruli. The 50 kD zone of lysis was present in normal glomeruli, decreased at day 3 of disease and increased at day 7 and 14. Gels prepared without plasminogen showed the 70 kD but not the 50 kD band of lysis (data not shown). **(C)** PAI-1 was immunoprecipitated as described in Figure 2C from medium conditioned by the nephritic glomeruli.

Immunofluorescent staining for PAI-1

In Figures 5A, B and C are shown representative fluorescence micrographs of PAI-1 staining in glomeruli from control, day 7 and day 14 of disease, respectively. Using this method, staining appears to be primarily in the mesangial matrix and is reminiscent of the pattern of fibronectin seen in this disease model [3, 32]. Compared to control (Fig. 5A), there is a clear increase in PAI-1 staining on day 7 of disease (Fig. 5B) and further increase on Day 14 (Fig. 5C). The apparent increase in tubular staining seen in Figure 5C was seen in all samples and therefore likely represents a true increase in PAI-1 at day 14 of disease. These data suggest that the increase in glomerular

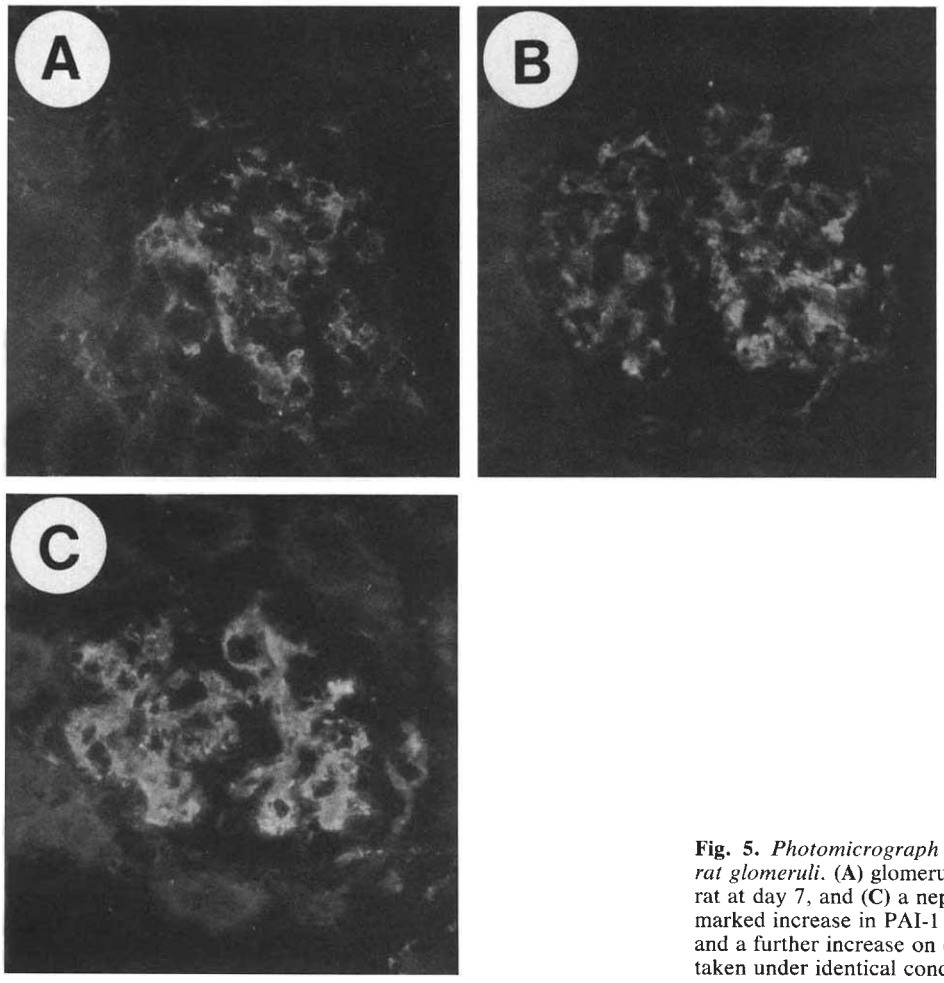

Fig. 5. *Photomicrograph of immunofluorescence staining of PAI-1 in rat glomeruli.* **(A)** glomerulus of a normal control rat, **(B)** a nephritic rat at day 7, and **(C)** a nephritic rat at day 14 of disease. There is a marked increase in PAI-1 deposition in glomerular matrix on day 7 and a further increase on day 14 of disease. The micrographs were taken under identical conditions. Magnification ×500.

PAI-1 synthesis leads to an increase in PAI-1 deposition into extracellular matrix. Note that the PAI-1 staining is greatest on day 14 while glomerular synthesis of PAI-1 peaks at day 7 and is close to normal by day 14.

Immunoperoxidase staining of PAI-1 and effect of in vivo administration of TGF-β antiserum

The results of immunoperoxidase staining of PAI-1 in nephritic glomeruli from normal animals, diseased animals and diseased animals treated with neutralizing antiserum to TGF-β are shown quantitatively in Figure 6. A significant increase in staining was seen in diseased animals compared to controls at both days 7 and 14 ($P < 0.001$), consistent with the immunofluorescence data. When diseased animals were injected with antibodies to TGF-β for six successive days, glomerular staining was significantly decreased compared to disease control animals on day 7 ($P < 0.001$) and on day 14 ($P < 0.005$; Fig. 6). These data indicate that TGF-β plays a causal role in the increased glomerular deposition of PAI-1 in this disease model.

Discussion

The current study demonstrates that TGF-β induces changes in the PA/PAI-1 protease system within glomeruli which potentially reduce or inhibit the involvement of plasmin in matrix degradation. Small quantities of TGF-β markedly decrease PA activity and increase PAI-1 synthesis when added to normal glomeruli in culture. The increase in PAI-1 synthesis is detectable as early as six hours after exposure to TGF-β and continues so that by 24 hours PAI-1 is a dominant protein being secreted by glomeruli exposed to TGF-β.

Using the ATS model of glomerulonephritis, we have shown previously that expression of TGF-β mRNA and TGF-β protein, as well as synthesis of proteoglycan and fibronectin by nephritic glomeruli, are increased by day 4 of disease. These events were followed by mesangial matrix expansion by day 7 which peaked by day 14. We now provide evidence that prior to matrix accumulation, nephritic glomeruli also show striking changes in the PA/PAI-1 system which would favor matrix accumulation. These changes are similar to those seen when normal glomeruli are exposed to exogenous TGF-β1. PA activity dramatically decreases and PAI-1 synthesis increases by day 3 of disease and returns toward control levels by day 7. The increased synthetic rate of PAI-1 is reflected in significantly elevated PAI-1 deposition into glomerular extracellular matrix on days 7 and 14 of disease. While there is considerable evidence that secreted PAI-1 is rapidly converted to a latent conformation, binding of PAI-1 to matrix appears to stabilize the active conformation [33]. These data indicate that the

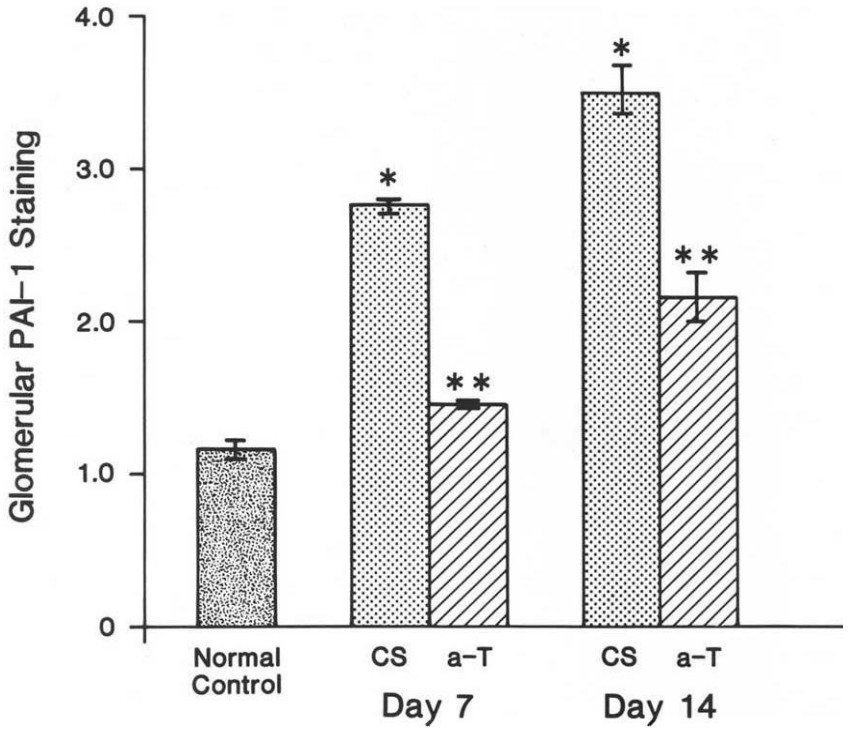

Fig. 6. *Quantitation of immunoperoxidase staining with rabbit anti-rat-PAI-1.* Anti-TGF-β significantly blocked PAI-1 deposition in the injured glomeruli on both days 7 and 14 of disease. Mean and standard error of values from three rats in each group are shown. *$P < 0.005$, diseased rats given control serum (CS) compared to normal rats; **$P < 0.0001$, diseased rats given control serum (CS) compared to diseased animals given anti-TGF-β serum (a-T).

components necessary for blocking matrix degradation by plasmin are in place at an early time point in the disease, prior to histologic evidence of matrix accumulation. A causal role for TGF-β in induction of these changes in the PAI-1/PA protease system was confirmed by the demonstration that *in vivo* administration of anti-TGF-β largely blocks the disease-induced increase in glomerular PAI-1 deposition.

In glomerulonephritis induced by ATS there is evidence that platelets rapidly aggregate in areas of mesangial injury. This could result in release of active TGF-β1 and PDGF. TGF-β1 is capable of inducing its own expression as well as that of PDGF [34]. PDGF is known to play a role in mesangial cell proliferation in this disease model. PDGF along with IL-1 and TNF-α have been shown to induce PAI-1 expression in other cell lines [25]. The data presented in Figure 1 suggest that PDGF does not increase PAI-1 synthesis in normal glomeruli whereas TGF-β1 does. Although we cannot be sure that TGF-β alone is responsible for the changes we see with disease in the activity and expression of PAI-1, several lines of evidence suggest that TGF-β is a dominant factor: (1) TGF-β1, when added to normal glomeruli produces changes in the PA/PAI-1 balance similar to those seen in nephritic glomeruli that are secreting increased levels of active TGF-β. (2) PDGF had no visible effect on total protein synthesis or the 50 kD band when added to normal glomeruli. (3) Anti-TGF-β largely blocks deposition of PAI-1 into matrix and, as we have shown previously, suppresses proteoglycan synthesis and matrix accumulation in this model.

An interesting question is how matrix accumulation is reversed during resolution of experimental glomerulonephritis. Our finding that PA activity at day 14 of disease rebounds to 1.4 times normal, when PAI-1 synthesis is not significantly different from normal, is intriguing. This activity is likely to represent newly synthesized PA released into the medium and there is evidence suggesting that PAI-1 can be removed from matrix by binding to PA [33]. Furthermore, recent evidence indicates that binding of PAI-1 to cell surface receptor-bound u-PA provides a signal for internalization and lysosomal degradation of the u-PA-PAI-1 complex [35]. Thus increased production of PA may lead to increased plasmin generation directly and may also be part of a mechanism for removal and degradation of PAI-1 from the extracellular matrix. The effect would be a reversal of the PA-PAI-1 balance toward matrix degradation.

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