Angiostatin and matrix metalloprotease expression following ischemic acute renal failure

David P. Basile, Katherine Fredrich, Dorothee Weihrauch, Naoichiro Hattan, and William M. Chilian. Angiostatin and matrix metalloprotease expression following ischemic acute renal failure. Am J Physiol Renal Physiol 286: F893–F902, 2004. First published January 6, 2004; 10.1152/ajprenal.00328.2003.—Ischemic injury to the kidney results in blood vessel loss and predisposition to chronic renal disease. Angiostatin is a proteolytic cleavage product of plasminogen that inhibits angiogenesis, promotes apoptosis of endothelial cells, and disrupts capillary integrity. A combination of lysine-Sepharose enrichment followed by Western blotting was used to study the expression of angiostatin in response to the induction of ischemic renal injury. No angiostatin products were readily detectable in kidneys of sham-operated control rats. In contrast, both 38- and 50-kDa forms of angiostatin were dramatically enhanced in the first 3 days following 45-min ischemia-reperfusion injury. Renal angiostatin levels declined but remained detectable at late time points postrecovery (8–35 days postschemia). Angiostatin-like immunoreactivity was also elevated in the plasma and in urine for up to 35 days following injury. Lysine-Sepharose extracts of either kidney or urine inhibited vascular endothelial cell growth factor-induced proliferation of human aortic endothelial cells in vitro; an effect that was blocked by coinoculation with an angiostatin antibody. RT-PCR verified that mRNA of the parent protein plasminogen was produced in the liver, but it was not present in either sham-operated or postschematic kidney. Matrix metalloproteinase (MMP)-2 and MMP-9, which may mediate angiostatin generation, were enhanced in postschematic kidney tissue and were localized to the renal tubules, interstitial cells, and the tubulo-interstitial space. These data indicate the possible local synthesis of angiostatin following acute renal failure (ARF) and suggest a possible role for MMPs in this activity. Renal angiostatin generation following ARF may modulate renal capillary density postschemia and thereby influence chronic renal function.

renal ischemia/hypoxia; tubulointerstitial space; renal vascular system
METHODS

Animal and surgical procedures. Care of the rats before and during the experimental procedures was conducted in accordance with the policies of the Animal Resource Center, Medical College of Wisconsin, and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All protocols had received prior approval by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Male Sprague-Dawley rats (Harlan, Madison, WI; 250 g) were housed in pairs in standard shoe-box cages with a 12-h light cycle (lights on 0600–1800) and access to water and standard laboratory rat chow (0.8% NaCl, Purina) available ad libitum. Animals were anesthetized with ketamine (100 mg/kg ip) for 10 min following administration of pentobarbital sodium (25–50 mg/kg ip). ARF was induced by performing bilateral renal artery clamping for 45 min followed by reperfusion according to procedures previously described (14). After ischemia, reflow was verified visually and animals were allowed to recover for 1, 2, 3, 8, or 35 days postinjury; sham-operated control animals were treated by exposing the kidneys under identical anesthesia but not clamping. Sham-operated controls were performed in the 3-, 8-, and 35-day groups.

Measurement of renal function and harvesting of tissue. Renal functional parameters were measured at 24 h, 7 days, and 35 days. Tail blood samples (0.5 ml) were collected under light halothane anesthesia into heparinized tubes, and plasma was obtained following centrifugation. Urine collection was for 24 h in metabolic cages (Nalgene). Serum and urine creatinine were determined using standard assays (Sigma creatinine kit 555A). Urine volume was determined gravimetrically.

At the time of death, animals were deeply anesthetized with ketamine/xylazine/acepromazine (2.0/0.6/0.3 mg/kg). The kidneys were removed and cut bilaterally; one-half of the kidney was snap-frozen in liquid nitrogen and stored at −70°C for subsequent biochemical analysis. In some studies, tissue was fixed by immersion in Histochoice (Sigma) for subsequent immunofluorescence.

Biochemical analysis of angiostatin and MMPs. Protein extracts from frozen kidneys were prepared according to the method described by Rankin et al. (38). Briefly, kidneys were homogenized in 50 mM Tris-HCl, pH 8.0, containing 10% DMSO, 10 mM E64, and 2 mM PMSF followed by centrifugation at 3,000 g for 5 min. The supernatant protein content was determined with the Bio-Rad protein assay kit using the microassay format based on the manufacturer’s recommendation for enhanced sensitivity.

For kidney samples, 20–100 µg of total protein (see figure legends) were combined with standard electrophoresis buffer (10 mM Tris, pH 8.3, containing 10% SDS and 700 mM β-mercaptoethanol). After being heated for 5 min, samples were electrophoresed on a 12.5% (angiostatin) or 10% (MMP) SDS-PAGE gel and transferred to nitrocellulose. To verify efficiency of transfer and equal loading, the membranes were pretained with Porcine S (Sigma). After being blocked, the membranes were incubated for 48 h at 4°C with either of the following antibodies: goat anti-human angiostatin (0.2 µg/ml; R & D Systems) or mouse anti-MMP-2 and -MMP-9 (0.5 µg/ml each; Santa Cruz Biotechnology). Peroxidase-conjugated anti-mouse IgG (Bio-Rad) or anti-goat IgG (Sigma) was used with chemiluminescence (SuperSignal, Pierce) for development. An additional primary antibody used in these studies was a peroxidase-conjugated goat anti-human plasminogen (10 µg/ml; Novus Biologicals), which does not require subsequent incubation in secondary antibody before development.

Purified human angiostatin (Alpha Diagnostic International, San Antonio, TX), human plasminogen (Sigma), and human plasmin (Sigma) were included in several gels as positive controls and were used to identify different molecular mass immunoreactive products.

Lyse-Sepharse enrichment. Washed lysine-Sepharse beads (Pharmacia) equivalent to 5 mg of dry gel were combined with 2 mg of renal protein extract or 15 µl of rat plasma in a volume of 1 ml complete RIPA buffer (10 mM HEPES, pH 7.5, 142.5 mM KCl, 0.2% NP-40 supplemented with protease inhibitor cocktail tablets; Roche). In some studies, urine equivalent to 100 µg creatinine or 500 µg urine protein was treated in the same manner. All samples were incubated overnight at 4°C while being shaken. The beads were subsequently washed by consecutive centrifugation and resuspension twice in complete RIPA and twice in RIPA lacking NP-40. After the final spin, adherent proteins were eluted for Western blot analysis by adding 15 µl of electrophoresis sample buffer and boiling the beads for 5 min.

In other studies, adherent proteins were eluted from lysine-Sephrose beads in 200 µl of 0.2 M e-aminocaproic acid (Sigma); the buffer was changed to 0.1 M phosphate buffer, pH 7.2, using Sephadex G-25 spin columns (Pharmacia), and samples were immediately frozen at −80°C until use in in vitro biological assays.

In vitro endothelial proliferation assay. Proliferation was detected by direct assessment of cell numbers similar to the method described previously (26). Cultures of confluent human aortic endothelial cells (EC) were trypsinized, and the yield of cells was measured using a hemocytometer. Ten thousand cells were seeded in 10-mm wells (24-well plate) and maintained in DMEM/5% FBS overnight. The cells were then serum starved for 72 h (0.5% FBS) to produce growth arrest and synchronize the cell cycle. After 72 h, EC were maintained in 0.5% FBS/DMEM to establish a baseline. Cells were then stimulated with VEGF (50 ng/ml) and coincubated with or without lysine-Sephrose extracts obtained from kidney or urine samples. The extracts added to these cultures corresponded to either 1.5 µg/ml of kidney lysate protein or 0.5 µg/ml of urine protein, as the original starting material before processing with lysine-Sephrose beads. In some wells, a neutralizing angiostatin antibody was also included in the incubation (4 µg/ml; Pharmingen).

RT-PCR. Total RNA from the kidney or liver was obtained from frozen tissue using Ultraspace RNA isolation solution (Biotechnology, Houston, TX). The presence of plasminogen mRNA was determined by running reverse-transcription PCR. Two micrograms of RNA were reverse transcribed with Superscript (Life Technologies) and oligo dT primers for 30 min at 39°C according to the manufacturer’s instructions. The resultant RT product was then amplified by PCR using Taq polymerase (Amersham) and the following primers: forward primer 5’-GGATCCATTATGGTGACATATTAGACACCGAGCTGTC-3’; reverse primer 5’-GAATTCATCGACTCATATTAGGACCAAGAGCAGCAG-3’. The underlined sequences refer to the promoter sequence for SP6 and T7 polymerase, respectively, a feature that was not used in the current study. The resultant amplified products were run on a 1% agarose gel and were consistent with the predicted 404-bp base region of the rat plasminogen mRNA. As a positive control, primers for β-actin were included in the reaction mixture as described previously (49).

SDS-PAGE zymography. Gelatinase activity was evaluated by SDS-PAGE zymography as described previously (26). Protein samples (200 µg) from renal extracts were loaded into the wells of a 10% gelatin gel (Bio-Rad) and electrophoresed. The gel was removed and incubated for 1 h at room temperature in 100 ml of renaturing buffer.
(2.5% Triton X-100) on a rotary shaker. The buffer was replaced with 100 ml of development buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM CaCl₂, and 0.02% Brij-35). The gel was incubated at 37°C for 18–24 h. Each gel was stained with 0.5% Coomassie blue G-250 in 30% methanol and 10% acetic acid for 3 h and destained with several changes in 30% methanol/10% acetic acid. The gels were digitized using a CCD camera frame digitizer system and analyzed using NIH Image (density and band area). Positive controls for MMP-2 and MMP-9 were obtained from Calbiochem.

**Immunofluorescence for MMP-2 and MMP-9.** Histochemistry-fixed kidneys were processed by incubation in 10 mM phosphate-buffered saline, pH 7.4, and subsequent daily changes of increasing sucrose (5, 10, and 20%) in the same buffer. Cryostat sections (10 µm) were thaw-mounted and stored desiccated at 4°C until use. The tissues were rehydrated in PBS and blocked in blocking buffer (PBS containing 0.2% Triton X-100, 5% normal sheep serum, and 0.6% bovin serum albumin). Goat anti-MMP-2 or -MMP-9 (10 µg/ml; Santa Cruz Biotechnology) was applied for 18 h at 4°C. Nonimmune goat IgG (Sigma) at the same concentration was used as a control for specificity. After being rinsed, the tissue was incubated with anti-goat-FITC (Pierce) and visualized using a Nikon E600 microscope equipped with Xenon-Arc epifluorescence and a Princeton Instruments Micromax Cooled CCD camera (RS Princeton Instruments, Trenton, NJ). Image acquisition was aided by a Pentium III grade personal computer running MetaFluor imaging software (Universal Imaging version 4.6, Downingtown, PA). Signals were obtained by excitation at 490 nm and an emission filter of 535/540. Tissue autofluorescence was determined using excitation at 405 nm with the same emission filter and exposure time. A FITC-specific signal was then determined by subtracting the signal obtained at 490-nm excitation from the one obtained at 405-nm excitation using MetaFluor imaging software. Both darkfield and brightfield images were then obtained, and these images were overlaid onto the FITC-specific image for contrast.

**RESULTS**

Rats were subjected to 52 min of renal ischemia and allowed to recover for 1, 2, 3, 8, or 35 days postsurgery. Mean serum creatinine values at 24-h postreperfusion were between 3.2 ± 0.3 and 3.7 ± 0.2 mg/dl, indicating evidence of injury (Table 1). Animals that were allowed to recover for 8 days or longer demonstrated a return of serum creatinine to sham-operated control values. In animals surviving for 35 days, daily urinary output remained significantly elevated vs. sham-operated controls, consistent with our previous studies (2, 3) (Table 2).

A standard Western blot approach using an antibody that recognizes kringle 1–3 of angiostatin was used to identify plasminogen/angiostatin in renal protein extracts. The antibody effectively recognized proteins consistent with the size of full-length plasminogen (~93 kDa) and plasmin (~62 kDa; Fig. 1A). The commonly identified 50- and 38-kDa forms of angiostatin, previously identified by us in canine myocardial interstitial fluid (26), were not consistently observed using this standard approach (Fig. 1A).

Plasminogen-derived products have high affinity for lysine, and this characteristic has been used to aid in its purification (8, 9, 22). Therefore, renal protein extracts were incubated in the presence of lysine-Sepharose before electrophoresis and Western blot analysis. Ponceau S staining revealed that only a small amount of protein remains following lysine-Sepharose enrichment of up to 2 mg renal protein extract (Fig. 1, D and E). Immunoreactive bands corresponding to plasminogen and plasmin, as well as lower molecular mass species that are consistent with the reported size of angiostatin, were reliably and consistently detectable following lysine-Sepharose enrichment (Fig. 1, B and B'). The relative amount of all plasminogen-derived products, including those corresponding to the 50- and 38-kDa forms of angiostatin, was considerably higher in extracts in the early (days 1–3) posts ischemic period compared with sham-operated controls. The apparent levels of these proteins diminished over time; however, longer exposure of the gels demonstrated that the 50- and 38-kDa immunoreactive products were persistently elevated for as long 8 and 35 days posts ischemia when compared with controls (Fig. 1B').

As further verification of these results, lysine-enriched renal protein extracts were probed with a different antibody termed antiplasminogen, which is conjugated directly to peroxidase. The results using this antibody were qualitatively similar to those obtained with the antiangiostatin antibody (Fig. 1, C and C').

<table>
<thead>
<tr>
<th>Time Point/Group (n)</th>
<th>24-h Postreperfusion</th>
<th>At Death</th>
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<tr>
<td>1 Day</td>
<td></td>
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<tr>
<td>Ischemic (4)</td>
<td>3.7 ± 0.2*</td>
<td>3.7 ± 0.2*</td>
</tr>
<tr>
<td>2 Days</td>
<td></td>
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<tr>
<td>Ischemic (4)</td>
<td>3.5 ± 0.3*</td>
<td>4.2 ± 0.2*</td>
</tr>
<tr>
<td>3-Day Sham</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Ischemic (4)</td>
<td>3.2 ± 0.3*</td>
<td>3.6 ± 0.3*</td>
</tr>
<tr>
<td>8-Day Sham (4)</td>
<td>0.45 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Ischemic (4)</td>
<td>3.6 ± 0.4*</td>
<td>0.5 ± 0.1</td>
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Data are means ± SE. *P < 0.05 vs. sham-operated controls.

**Table 2. Renal function of group following recovery from surgery for 35 days**

<table>
<thead>
<tr>
<th>Creatinine, mg/dl</th>
<th>Sham (n = 4)</th>
<th>Ischemic (n = 4)</th>
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<tbody>
<tr>
<td>24 h</td>
<td>0.4 ± 0.1</td>
<td>3.2 ± 0.3*</td>
</tr>
<tr>
<td>8 Days</td>
<td>0.4 ± 0.1</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>35 Days</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
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<tr>
<td>Urine flow rate, ml/day</td>
<td>17.9 ± 1.9</td>
<td>30.8 ± 8.0*</td>
</tr>
<tr>
<td>8 Days</td>
<td>14.6 ± 2.2</td>
<td>19.3 ± 8.2*</td>
</tr>
<tr>
<td>35 Days</td>
<td>13.8 ± 0.6</td>
<td>20.3 ± 5.6*</td>
</tr>
<tr>
<td>Urine protein excretion, μg/day</td>
<td>N/D</td>
<td>69.1 ± 8.8</td>
</tr>
<tr>
<td>8 Days</td>
<td>61.3 ± 5.3</td>
<td>46.6 ± 2.8</td>
</tr>
<tr>
<td>35 Days</td>
<td>119 ± 9.0</td>
<td>97.2 ± 18.4</td>
</tr>
</tbody>
</table>

Data are means ± SE. Data shown for serum creatinine correspond to animals in which tissue and plasma angiostatin were determined and are n = 4/group. Data from a similarly injured but separate group are shown for urine flow rate and urine protein excretion and correspond to the animals from which angiostatin was analyzed in urine samples (n = 4/group). * P < 0.05 vs. sham-operated controls.
body revealed qualitatively similar results but did not consistently detect the 38-kDa form of angiostatin (Fig. 2, bottom).

Lysine-Sepharose enrichment was also used to analyze plasminogen/angiostatin content in the urine of postischemic rats. Samples for Western blots were normalized to either creatinine content (Fig. 3, top) or protein content (Fig. 3, bottom). Regardless of the method of normalization, there was little evidence of immunoreactive products in urine from sham-operated control rats. Postischemic animals demonstrated largely elevated urinary plasminogen/angiostatin immunoreactivity, although the pattern of detectable immunoreactive products was inconsistent. However, postischemic urine samples at 2, 8, and 35 days postsurgery tended to contain detectable levels of the 50- and 38-kDa angiostatin isoforms.

As an additional verification of presence of angiostatin in kidney and urine, lysine-Sepharose-enriched fractions were analyzed for their ability to inhibit VEGF-induced angiogenesis in vitro. VEGF stimulation (50 ng/ml) resulted in a ~90% increase in EC number; lysine-Sepharose extracts completely inhibited the VEGF-induced stimulation of EC number (Fig. 4). Under the conditions of the assay used, the inhibition of VEGF-induced stimulation was maximal and identical in extracts derived from either sham-operated or postischemic animals. Data in Fig. 4 represent pooled data from both sham-operated and postischemic kidney and urine extracts. Coincubation of lysine-Sepharose extracts with a neutralizing angiostatin antibody restored VEGF-induced cell proliferation (Fig. 4). These data suggest that the lysine-Sepharose-enriched fraction from urine and kidney homogenates contain biologically active angiostatin.

Plasminogen is synthesized primarily in the liver, although extrahepatic synthesis of plasminogen has been reported (46). RT-PCR was used to determine if plasminogen synthesis might occur in the kidney following injury. Figure 5 demonstrates that the predicted amplicon corresponding to plasminogen mRNA was generated following RT-PCR of total liver RNA. This product was not detectable by RT-PCR of total RNA from kidney of either sham-operated or postischemic animals. These
data suggest that the source of plasminogen is not renal and is likely hepatic.

It is likely that increased angiostatin expression is the result of increased local conversion of plasminogen within the kidney following I/R injury. Therefore, we sought to determine whether MMP-2 and MMP-9, two enzymes reported to convert plasminogen to angiostatin, were altered in the setting of renal ischemic injury. Western blot analysis (Fig. 6A) and zymography (Fig. 6B) demonstrate the increase in MMP-2 and MMP-9 protein and activity, respectively, in extracts obtained from rat kidney 1, 2, and 3 days postischemia. Densitometric analyses of Western blots revealed significant increases ranging from two- to threefold in both MMP-2 and MMP-9 compared with sham-operated controls (P < 0.05, n = 4/group/time point). Similarly, there were significant increases postischemia in the activities of both MMP-2 and MMP-9. Densitometric values from zymography ranged from two- to fourfold between days 1 and 3 for MMP-2 (P < 0.05 for each time point). Values for MMP-9 activity were not different on day 1, but two- to threefold higher on days 2 and 3 (P < 0.05).

Immunofluorescence was used to identify the site of increased MMP-2 and -9 expression in tissues of rats 3 days following ischemic injury. In sham-operated controls, fluorescent signal corresponding to MMP-2-like immunoreactivity could be distinguished near the basolateral aspect of tubular structures in the cortex (arrow, Fig. 7A) and this pattern was more prominent in the outer medulla (arrow, Fig. 7B). In contrast, no signal was obtained in sham sections using an antibody for MMP-9 (Fig. 7A and B) or a nonimmune antibody (not shown). Immunofluorescent signal for MMP-2 was increased moderately in the cortex (Fig. 7C) and more dramatically in the outer medulla (Fig. 7D) 3 days following ischemic injury. Higher-magnification images illustrate that MMP-2 immunoreactivity was primarily localized to the basolateral aspect of the tubule and in the tubulointerstitial area (Fig. 7E). When visualized relative to an image obtained from brightfield microscopy, MMP-2 signal can be seen over the basement membrane (arrow, Fig. 7F) and outside the tubule in the interstitial space.

MMP-9 immunoreactivity was also apparent in the cortex and medulla of postischemic animals, although the pattern of

![Fig. 2. Angiostatin/plasminogen detection in plasma following I/R injury. Top: 15 μl of rat plasma were subjected to lysine-Sepharose enrichment and probed using either the angiostatin antibody or antiplasminogen HRP antibody (bottom). Shown are samples obtained from 4 sham-operated rats (8 days) and from 4 postischemic rats at 8 and 35 days post-I/R.](image1)

![Fig. 3. Angiostatin/plasminogen detection in urine following I/R injury. Urine samples were collected for 24 h in metabolic cages from sham-operated and postischemic animals. Aliquots of either 100 μg creatinine (top) or 500 μg of urinary protein (bottom) were subjected to lysine-Sepharose enrichment and Western blot analysis. Samples were collected from postischemic animals and sham-operated animals at the indicated times; urinary levels from sham-operated controls are shown from 35-day samples. However, these did not differ as a function of time following sham surgery (data not shown). Results shown are those using the R and D antibody. Positive controls shown in standard (std) lane in this blot correspond to purified human plasminogen, plasmin, and angiostatin, as marked.](image2)
postischemic kidney would result in the loss of renal vessels and/or the inhibition of an endogenous repair response, resulting in a net decrease in total vessel number. There are many potential angiogenic and antiangiogenic factors that may be influenced in the setting of ischemic ARF. Although the current study focused on angiotatin, the characterization of several factors with the potential to modulate vessel density in the setting of ischemic injury may likely be warranted in future studies.

Plasminogen produced in the liver has long been recognized as the parent protein of plasmin, by either tissue-type or urokinase-type plasminogen activator. Plasmin degrades plasma fibrin and ECM components either directly or indirectly through the activation of MMPs (39). Plasminogen is also processed by a number of different enzymes, including, but limited to MMP-2, and MMP-9, to generate angiotatin, a molecule with antiangiogenic activity that comprises the kringle 1–4 domains (K1–4) of the parent molecule (25, 33–35). The K1–4 angiotatin was originally identified as a 38-kDa peptide (33); however, angiotatin has also been identified as a 45- to 50-kDa form. In a previous study, we identified bands of ~50 and 38 kDa in canine myocardial interstitial fluid (26). In this study, bands of ~38 and 50 kDa were also identified in postischemic rat kidneys. The possibility that these bands correspond to angiotatin is supported by their physicochemical lysine affinity and the use of two different antibodies against angiotatin/plasminogen. Moreover, lysine-Sepharose extracts suppressed VEGF-induced proliferation of EC cells; an effect that was blocked using a third unique antibody against angiotatin. Although not the focus of this study, it is worth noting that there was a substantial increase in immunoreactivity of two bands ~65 kDa, which likely corresponds to plasmin.

Renal gene expression is altered dramatically postischemia. RT-PCR results argue against the possibility that plasminogen is produced locally by injured kidney and suggested that local conversion of angiotatin occurs. The precise localization of angiotatin could not be determined because the antibodies that were used to identify angiotatin also recognize other plasminogen-derived products. However, the analysis of MMP-2 and -9, which are two of many possible enzymes that may mediate cleavage of plasminogen to angiotatin, allows speculation on the possible site of production of angiotatin. Thus it is of interest that the majority of MMP-2 signal appears to be

expression was different. Although some interstitial staining could be observed, MMP-9 immunoreactivity was present to a significant degree within cells of the tubular epithelium (arrowhead, Fig. 8, C–E) and in cells residing in the tubulointerstitial area (arrow, 8, C–E). Figure 8F illustrates the lack of signal obtained by a nonimmune goat IgG in the outer medulla of a postischemic rat.

**DISCUSSION**

The potential that the kidney may generate molecules with antiangiogenic activity has, to date, received little attention and was the major focus of this study. It is now well-recognized that renal vessel loss is a common feature of several models characterized by tubulointerstitial disease and a predisposition to hypertension (10, 19–21, 30, 40). Similarly, we showed that there is a permanent reduction in the density of renal blood vessels following the apparent recovery from I/R injury (3). In addition, evidence of peritubular capillary loss has been suggested from analysis of biopsies of patients with tubulointerstitial disease of many etiologies (11). Hence, it would appear that a complete understanding of the factors involved in maintaining vessel integrity might be useful to our understanding of the pathogenesis of renal diseases.

We suggest that in the setting of I/R injury, there is a shift in the balance between trophic, angiogenic factors and antiangiogenic factors. Such alterations in the humoral milieu of the postischemic kidney would result in the loss of renal vessels and/or the inhibition of an endogenous repair response, resulting in a net decrease in total vessel number. There are many potential angiogenic and antiangiogenic factors that may be influenced in the setting of ischemic ARF. Although the current study focused on angiotatin, the characterization of several factors with the potential to modulate vessel density in the setting of ischemic injury may likely be warranted in future studies.

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<tr>
<th>Cell number (% of 0.5% FBS control)</th>
<th>0.5% FBS</th>
<th>VEGF</th>
<th>Kidney Extract</th>
<th>Urine Extract</th>
<th>Antiangiotatin</th>
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Fig. 4. Inhibition of endothelial cells (EC) proliferation by lysine-Sepharose-enriched kidney and urine extracts. Human aortic EC were cultured as described in METHODS and stimulated with vascular endothelial cell growth factor (VEGF), with or without lysine-Sepharose-enriched fractions from kidney and urine as shown. Data are means ± SD and are expressed as percent increase in cell number relative to 0.5% FBS control. Positive control (VEGF) is based on n = 8 and VEGF + antibody, n = 4. Data obtained from tissue and urine extracts correspond to the mean values obtained from 4 different animals. As extracts from sham-operated and postischemic animals were both maximally inhibitory, the data were pooled (i.e., 2 sham and 2 postischemic; each extract tested in quadruplicate). *P < 0.05 vs. 0.5% FBS control. †P < 0.05 vs. VEGF stimulated. ‡P < 0.05 vs. corresponding antibody-treated samples.

Fig. 5. Plasminogen mRNA is not detectable in kidney by RT-PCR. Primers were designed to amplify mRNA of plasminogen or β-actin; 1 μg of total RNA of whole kidney or liver (as labeled) was subjected to RT-PCR as described in METHODS. A minimum of 4 sham and postischemic kidneys (3 days) were tested and failed to demonstrate an amplified product corresponding to plasminogen mRNA in kidney.
either near the tubular basement membrane or the interstitial space, while MMP-9 is present in tubular cells as well as interstitial cells. The identity of these interstitial cells is not known, but they likely represent an inflammatory cell type. In one previous study, Ziswiler et al. (51) reported that there was no change in the MMP-2 or -9 activities in renal cortex at either 12- or 24-h postischemia in rats. The apparent disparity may relate to the fact that we used whole kidney homogenates containing the outer medullary region. Our immunofluorescence studies suggest the strongest intensities of the MMP-2 and -9 signals were in the outer medulla (Figs. 7 and 8).

The localization of MMPs within the tubulointerstitial area is of significant interest with regard to their potential role in angiostatin generation because this region is the location of peritubular capillaries. Thus the potential local activation of angiostatin by MMPs in close proximity to blood vessels may

![Fig. 6. Expression of matrix metalloproteinase (MMP)-2 and MMP-9 in kidney in response to I/R injury. A: DMSO protein extracts (20 μg) from kidneys of sham-operated (sh) rats or rats 1, 2, or 3 days postischemia were subjected to PAGE, transferred to nylon membranes, and probed with antibodies against MMP-2 (~65 kDa; top) or MMP-9 (~90 kDa). B: gelatin zymography of DMSO protein extracts (50 μg) from kidneys of sham-operated rats or rats 1, 2, or 3 days postischemia. Human MMP-2 (M-2) and MMP-9 (M-9) standards are shown in lanes 1 and 2.](image)

![Fig. 7. Immuno- fluorescence localization of MMP-2 in normal and postischemic rat kidneys. Shown are representative frozen sections (10 μm) through the renal cortex (A, C) and outer medulla (B, D, E, F) of sham-operated rats (A, B) or of rats 3 days postischemia (D-F). Sections were stained with antibodies against MMP-2 and appropriate FITC-labeled secondary antibody. FITC-specific fluorescence (see METHODS) was labeled green and is shown relative to the image obtained from darkfield to visualize structure (shown in blue). There is modest MMP-2-immunoreactivity staining in the basolateral aspect of tubules from sham-operated rats (A, B, arrows). MMP-2-like immunoreactivity is enhanced slightly in cortex (C) and more appreciably in the renal medulla (D) 3 days following renal injury. E: higher magnification of D, illustrating the localization of MMP-2 in the interstitial space (arrow). F: FITC signal from E is shown relative to image obtained from brightfield microscopy (blue); the basement membrane can be observed as a thin line (arrow). FITC signal can be appreciated over the basement membrane as well as outside the basement membrane area. Bar in A refers to A-D, and bar in E refers to E and F.](image)
result in a negative influence on capillary stability. It should also be emphasized that a number of other MMPs can lead to angiostatin generation such as MMP-3 (stomelysin-1), MMP-12 (matrix metalloelastase), and MMP-7 (34–35). These have not been addressed in these studies. Moreover, plasminogen activator inhibitor-1 and transforming growth factor-β are also capable of affecting angiostatin generation (32), and these are both enhanced in the setting of ischemic ARF in rats (4).

The localization of MMPs in the tubulointerstitial area suggests that it may also influence the regulation of ECM homeostasis in the tubular basement membrane following I/R injury. To our knowledge, no studies evaluated the ramifications of MMP activity on the long-term structure of the tubular basement membrane following ischemic injury. Clearly, further studies will be required to determine whether MMPs influence ECM control and/or angiostatin generation in this setting.

The results from these studies raise the possibility that angiostatin, and potentially other antiangiogenic factors, predisposes vessel dropout and/or inhibits compensatory angiogenic activity post-I/R. With regard to the latter possibility, angiostatin-like proteins were identified in extracts, albeit at reduced levels, for up to 35 days postinjury. It is of interest that elevated angiostatin levels were also identified in plasma.

Recent studies showing decreased barrier function of peritubular capillaries following I/R (45) suggest the possibility that plasminogen can leak from the vasculature into the interstitial space where it may be acted on by MMPs. Such a breakdown in barrier function also opens the possibility of backleak into the vascular system, which may account for presence of angiostatin in the plasma. In addition, administration of radiolabeled angiostatin is found in kidney and urine, suggesting that it is cleared by the kidney (28). Therefore, reduced clearance of angiostatin by postischemic kidneys may also account for elevated plasma angiostatin levels. However, determination of these putative mechanisms would require further careful investigation. Whether plasma angiostatin affects other tissues following I/R is unknown.

It is also of interest that some angiostatin was detectable in the urine of postischemic animals, although the pattern of immunoreactive products in urine was not consistent. We suggest that heterogeneity in the reestablishment of the tubular epithelial barrier as well as the level of microalbuminuria may create a very complicated set of parameters that affect the detection of angiostatin in the urine. Nevertheless, it is possible that urinary angiostatin may represent a potentially interesting marker of renal injury in the setting of ARF or delayed graft function.
In this model of reversible ARF, there is a net reduction in the total number of renal vessels, suggesting a shift in the balance between antiangiogenic factors and proangiogenic factors. In the current study, we identified the generation and sustained expression of angiostatin, a well-known antiangiogenic factor, following renal I/R injury. Further studies will be required to determine whether this antiangiogenic factor participates in blood vessel homeostasis and whether angiostatin may also be present in kidney of other progressive disorders characterized by a decrease in blood vessel loss.

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REFERENCES


