Identification of persistently altered gene expression in the kidney after functional recovery from ischemic acute renal failure


Identification of persistently altered gene expression in the kidney after functional recovery from ischemic acute renal failure. Am J Physiol Renal Physiol 288: F953–F963, 2005. First published January 4, 2005; doi:10.1152/ajprenal.00329.2004.—Recovery from ischemic acute renal failure (ARF) involves a well-described regenerative process; however, recovery from ARF also results in a predisposition to a progressive renal disease that is not well understood. This study sought to identify alterations in renal gene expression in postischemic, recovered animals that might play important roles in this progressive disorder. RNA isolated from sham-operated control rats or rats 35 days after recovery from bilateral ischemia-reperfusion (I/R) injury was compared using a cDNA microarray containing ~2,000 known rat genes. A reference hybridization strategy was utilized to define a 99.9% interval and to identify 16 genes that were persistently altered after recovery from I/R injury (12 were upregulated and 4 were downregulated). Real-time PCR verified the altered expression of six of eight genes that had been positively identified. Several genes that were identified had not previously been evaluated within the context of ARF. S100A4, a specific marker of fibroblasts, was identified in a population of interstitial cells that were present postischemic injury. S100A4-positive cells were also identified in tubular cells at earlier time points postischemia. Genes associated with calcification, including osteopontin and matrix Gla protein, were also enhanced postischemic injury. Several proinflammatory genes were identified, including complement C4, were enhanced in postischemic tissues. Conversely, renal kallikrein expression was specifically reduced in the postischemic kidney. In summary, genes with known inflammatory, remodeling, and vasoactive activities were identified in rat kidneys after recovery from ARF, some of which may play a role in altering long-term renal function after recovery from ARF.

Ischemia–reperfusion (I/R) in rats is widely utilized to study ARF. This model is characterized by a rapid increase in vascular resistance, inflammation, and tubular epithelial cell damage (11, 29). Morphologically, the injury is most obvious in pars recta but may also be present in cortical proximal tubule and distal nephron segments. ARF in rats is reversible, and the recovery response is characterized by the restoration of glomerular filtration rate (GFR) and remodeling of the renal tubular system (21, 29).

However, there are persistent alterations in renal function postischemic injury. Postischemic animals have a permanent compromise in urinary concentrating ability associated with a reduction in renal medullary tonicity (5). In terms of renal structure, we have reported that there is a permanent reduction in renal microvessels after recovery from ARF at all time points analyzed, demonstrating that the renal vascular system has little regenerative capacity (5). Moreover, despite the renal regenerative response, postischemic animals gradually develop manifestations of secondary chronic renal disease such as proteinuria and interstitial fibrosis (5, 34), which become apparent at later time points after the initial recovery response. A secondary loss of GFR is observed after recovery from ARF with a solitary kidney (3, 9, 34). Thus between 4 and 8 wk post-I/R, kidneys have largely normal structure and function but are prone to develop secondary disease at later time points.

The development of secondary chronic renal failure (CRF) after recovery from ARF is largely unexplored. Among the potential factors contributing to CRF may be persistent renal hypoxia after recovery from ARF, due in part to the alterations in vascular structure (3). It has also been suggested that the genesis of fibrosis occurs secondarily to atrophy in a subpopulation of damaged nephrons (34), is enhanced by renal angiotensin II activity (33), and may occur after the establishment of a fibrotic cell type and/or persistent inflammation (9, 17).

The purpose of this study was to characterize a molecular basis for susceptibility of CRF after ARF. The aim was to identify alterations in renal mRNA expression profiles between normal (i.e., sham-operated control rats) and rats after recovery from ischemic ARF but before the subsequent development of secondary disease. Utilizing a customized cDNA microarray containing ~2,000 known rat genes, we employed conservative analysis strategies and an experimental design to minimize

RENAL ISCHEMIA-HYPOXIA IS a leading cause of acute renal failure (ARF), a clinical condition associated with rapid loss of renal function. Mortality rates from ARF are reported to range from 30 to 80%. Despite these high rates, ARF is largely reversible, and complete restoration of renal function is often observed in surviving patients. A portion of patients demonstrate incomplete recovery and/or a secondary decline of renal function (1, 7, 8). Moreover, ischemic injury in the setting of renal transplant results in delayed graft function, which predisposes the development of allograft nephropathy and posttransplant hypertension (15, 16, 19, 28, 31).

Address for reprint requests and other correspondence: D. P. Basile, Dept. of Cellular and Integrative Physiology, Indiana University School of Medicine, 635 Barnhill Dr., Indianapolis, IN 46202 (E-mail: dpbasile@iupui.edu).

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the effect of gene expression associated with early injury or repair processes of this model. Using this approach, we anticipated that several genes would be identified with the potential to influence hemodynamic, inflammatory, and fibrotic processes in the subsequent development of renal disease. The identification of such genes might prove useful in developing future studies aimed toward understanding CRF progression after ARF.

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 guidelines 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 shoebox cages with a 12:12-h light-dark cycle (lights on 0600–1800) and access to water and standard laboratory rat chow (8.8% NaCl, Purina) available ad libitum. Animals were anesthetized with ketamine (100 mg/kg ip) for 10 min, followed by administration of pentobarbital sodium (25–50 mg/kg ip). ARF was induced by performing bilateral renal artery clamping for 52 min, followed by reperfusion according to procedures previously described (5, 6). After ischemia, reflow was verified visually, and the animals were allowed to recover for various periods of time according to the experimental protocol, as described below.

Study group I. This group comprised animals that were allowed to recover for 35 days posts ischemic or sham surgery. This group was used for the isolation of the total RNA that was applied to microarrays in this study.

Study group II. This group comprised animals that were treated identically to group I but were allowed to recover for 3, 8, or 35 days postsurgery. This group was utilized for additional verification of gene expression patterns and immunohistochemical analyses.

Measurement of Renal Function and Harvesting of Tissue

Renal functional parameters were measured at 24 h and 35 days. Tail blood samples (0.5 ml) were collected under light halothane anesthesia into heparinized tubes, and plasma was obtained after centrifugation. Urine collection was done for 24 h in metabolic cages and stored at −70°C for subsequent biochemical analysis. In

Experimental Design and Data Analysis

Rats were paired for experimental analysis according to the schema shown in Fig. 1. A total of six posts ischemic animals were paired with six sham-operated control animals. Comparisons were made by using dye switching for each pair, for a total of 12 hybridizations. In addition, six within-group comparisons were utilized from the sham-operated control group for determination of the reference distribution. Dye switching was not necessary for within-group comparisons, since two rats of each pair were considered identical.

Fig. 1. Schema for the design of microarray analysis to identify persistently altered genes after renal ischemia-reperfusion (IR) injury. The diagram illustrates the nature of the 18 hybridizations that were utilized in this study: 12 hybridizations compared 6 sets of posts ischemic [experimental (E)] and sham-operated [control (C)] animals. Individual Ln(ratios) were determined for each gene for each animal pair (n = 6) after the averaging of results from dye-switched replicate samples. Six additional hybridizations were utilized to obtain the distribution of Ln(ratios) within the control group and were utilized for determination for the threshold of differentially expressed genes. ARF, acute renal failure.
Raw values of fluorescent intensity in each spot were obtained from microarray images using Imagene 4.01 software (BioDiscovery, Los Angeles, CA). These raw data were categorized, selected, and adjusted to yield log-transformed, normalized ratios following the systematic method that was described previously (26). This data analysis method quantitatively identifies and systematically excludes spots with low intensities or high local background to avoid the generation of disproportionate and misleading ratios. Of 1,871 genes, 834 passed the data selection process in at least 4 of the 6 “between-group” comparisons. Differentially expressed genes were identified relative to a reference distribution obtained in sham vs. sham comparisons, based on the method previously described (25) and elaborated in RESULTS.

Raw data from Imagene 4.01 from the 18 hybridizations were uploaded to the Gene Expression Omnibus (GEO) website http://www.ncbi.nlm.nih.gov/geo/; the series accession number is GSE1714.

Real-Time PCR

FAM-labeled primers (Lux primers, Invitrogen, Carlsbad, CA) specific for each of the genes of interest are shown in Table 1. Real-time PCR reactions were carried out on an ABI Prism 7900HT (ABI, Applied Biosystems, Foster City, CA) using a TaqMan One-Step RT-PCR Master Mix Reagents Kit (ABI) according to the manufacturer’s recommendation. Real-time reactions were carried out simultaneously for 18S RNA in parallel wells. Standard curves (0.15–80 ng total RNA) were generated using pooled RNA samples from control rats for both 18S RNA and the mRNA for the genes of interest to determine the relative fold-change between sham-operated and postischemic kidney.

Verification of Clone Identity

Sequence verification of clones of interest was carried out by PCR amplification and subsequent sequencing using BDT chemistry (Applied Biosystems). Retrieved sequences were then subjected to BLAST searches for verification of their identity.

Immunohistochemistry and Western Blotting

Immunohistochemistry and Western blot analysis were utilized to further characterize the expression of some of the genes of interest. Primary antibodies utilized in this study were rabbit anti-S100A4 (Dako), goat anti-C4 (Dako), and rabbit anti-matrix Gla protein (MGp; two different antibodies referred to as COV-2 and MGP-1225; a generous gift from Dr. Gerard Karsenty, Baylor University, Dallas, TX). The osteopontin (OPN) antibody (MII1B10) was developed by Dr. Michael Solursch and Ahnders Franzen and was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences. Immunohistochemical localization was carried out on formalin-fixed, paraffin-embedded tissues using a standard indirect avidin-biotin-horseradish peroxidase method (Zymed, South San Francisco, CA); an antigen retrieval step of microwaving slides in 0.1 M citrate buffer, pH 6.0, for 10 min was utilized for S100A4. Either AEC or DAB was utilized as the chromogens for horseradish peroxidase localizations. The procedure and antibody utilized for the immunohistochemical localization of kallikrein were described previously (38). To quantify kallikrein, images (×40 magnification) of the cortex from each section (Sham and ARF) were acquired using a Nikon Eclipse-600 microscope and Nikon DXM1200 digital camera. The kallikrein-immunoreactive area in each image was determined by image analysis using Simple PCI software (Compix). The values corresponding to total immunostained (brown) cells were averaged and expressed as the mean absolute values and the mean percentage of stained cells area per field (0.064 μm²) with a modification of a previously described method (44, 48).

RESULTS

Identification of Differentially Expressed Genes After Recovery from ARF in Postischemic Rat Kidneys

Rats were subjected to I/R injury or sham surgery and allowed to recover for 35 days. This recovery period was chosen because it is associated with functional and morphological recovery from injury but precedes the manifestations of secondary renal disease such as overt fibrosis, proteinuria, or elevated arterial blood pressure (3, 5). Functional data are shown in Table 2. Plasma creatinine values were significantly elevated by 24 h postischemia but returned to values seen in sham-operated controls within 1 wk. Postischemic animals at 5 wk of recovery manifested a significant diuresis, which is consistent with our previous reports using this model (3, 5) (Table 2).

A ~2,000-gene rat cDNA microarray was used to compare the relative mRNA expression in total kidney of postischemic recovered rats vs. sham-operated control rats as shown in Fig. 1. Individual samples from the sham-operated group were hybridized and compared with each other to generate a reference distribution of ratios and determine the variance of the biological and experimental platform (25). The Ln(ratio) values obtained from the six microarrays from the sham vs. sham comparisons were averaged for each gene; averaged ratios were included if detectable and of good quality in a minimum of four of the six comparisons. Ratios were obtained from 811 genes in these hybridizations; the averaged Ln(ratio) values followed a normal distribution with a SD of 0.138 (Fig. 2).

Comparisons were then made in the between-group ARF vs. sham hybridizations; 834 genes were detectable in at least four of six comparisons; these comparisons also followed a normal distribution (Fig. 2) with a SD of 0.148. Utilizing 3 × SD of the reference distribution, the 99.9% interval was set at ±0.414. At this threshold, a total of 16 genes were differentially expressed after recovery from ARF (4) were downregulated, 12 were
unregulated); these genes with their clone ID and Ln(ratio) values are presented in Table 3.

All identified genes were verified by sequencing of the cDNA clones. A BLAST search of the sequenced clones revealed that 15 of 16 genes were accurately identified, whereas one single clone could not be positively identified (Table 3). The differential expression of a subset of eight sequence-verified genes was further evaluated using real-time PCR; the differential expression of six of these eight genes was verified using this approach (Table 3). In a previous study, we demonstrated that there were two other genes in Table 3 that were differentially expressed in ARF vs. sham comparisons is illustrated in the open bars. The line refers to the 3 SD of this distribution of Ln(ratio) values of gene expression in Table 3 [i.e., collagen IV (α1) and tissue inhibitor of metalloproteïnase-1] had prolonged enhanced expression after ARF (6).

Real-time PCR was also used to measure the expression of an additional gene in Table 3 (i.e., sgk), the single clone in this experiment that could not be sequence verified. Real-time PCR analysis did not detect differences in sgk. In addition, three other genes that did not reveal differential expression by microarray (VEGF, p27kip, and Glb) were not differentially expressed as indicated by real-time PCR analysis (data not shown).

Initial Characterization of Genes Differentially Expressed After I/R

Four genes that have not previously been examined in the setting of ARF were selected for further characterization on the basis of their potential involvement in progressive fibrosis, inflammation, and/or vascular reactivity. Real-time PCR analysis was carried out on RNA from tissues at a time point early in the injury process, i.e., 3 days posts ischemia. There were directionally consistent alterations in the expression of these genes at this early postischemic time point compared with sham-operated controls [Ln(ratio) complement C4, 0.5; S100A4, 2.1; kallikrein, −0.9; and MGP 1.9; P < 0.05, by Student’s t-test vs. corresponding sham-operated control].

Further characterization of these genes was pursued by immunohistochemical analysis. One of these genes, S100A4, has also been referred to as FSP-1 or mts-1 and is thought to be a specific marker of tissue fibroblasts (22). S100A4-like immunoreactivity was generally localized in an isolated few interstitial cells in the kidneys of sham-operated control rats but was expressed more prominently in interstitial cells of postischemic recovered kidneys. At 3 days postinjury, several S100A4+ cells can be appreciated surrounding renal tubules (Fig. 3B). There is significant resolution of tubular structure at 8 days postinjury; at this time point, occasional S100A4-positive cells could be appreciated in the tubular epithelia (Fig. 3C, arrow). At 35 days post-I/R injury, S100A4-positive cells were persistent in the interstitial area and appeared as either isolated interstitial cells (Fig. 3D, small arrow) or circumventrally around small blood vessels (Fig. 3D, large arrow). In a study conducted in parallel with the current study, we demonstrated that there was a two- to threefold increase in S100A4-positive cells in the outer and inner medulla after 35 days of recovery from ARF (42).

Complement C4 showed distinctive immunohistochemical localization in the kidney, being localized primarily in the cortical distal tubule (Fig. 4A, large arrow) and collecting duct (thin arrow). However, there was no apparent alteration in the intensity or distribution of complement C4 immunoreactivity in kidneys after recovery from ARF (Fig. 4B). Despite the prominent effects of ARF on the mRNA expression of C4 that was verified by real-time PCR (Table 3), Western blot analysis failed to reveal any strong effect of ARF on C4 protein levels (Fig. 4C). The primary immunoreactive product in rat kidney was at ~60 kDa, and this was not affected by ARF. The ~60-kDa band is consistent with the reported size for iC4b, an inactive intermediate form of C4 (46). However, other immunoreactive bands present at much lower levels were also identified and showed modest regulation by ARF. For example,
Table 3. Differentially expressed genes in the postischemic recovered kidney

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Clone ID</th>
<th>Sequence Verified?</th>
<th>Microarray Ratio</th>
<th>Real-Time PCR Ratio</th>
<th>Previous Identification in ARF?</th>
<th>Related Pathway/Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat retinol-binding protein (RBP)</td>
<td>UI-R-A0-aj-c-09-0-UI</td>
<td>Yes</td>
<td>−0.78</td>
<td>−0.21</td>
<td></td>
<td>Vitamin A homeostasis/tissue remodeling</td>
</tr>
<tr>
<td>gene</td>
<td>UI-R-A0-ar-b-02-0-UI</td>
<td>Yes</td>
<td>−0.60</td>
<td>−0.60*</td>
<td>41</td>
<td>Stress response</td>
</tr>
<tr>
<td>Rat α-crystallin B chain mRNA,</td>
<td>UI-R-E1-ft-h-01-0-UI</td>
<td>Yes</td>
<td>−0.55</td>
<td>ND</td>
<td></td>
<td>Cell cycle regulation</td>
</tr>
<tr>
<td>complete cds</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rattus norvegicus CCND1 mRNA for</td>
<td>UI-R-A1-ee-h-08-0-UI</td>
<td>Yes</td>
<td>−0.43</td>
<td>−1.0*</td>
<td></td>
<td>Vasodilation/sodium excretion</td>
</tr>
<tr>
<td>cyclin D1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Rat submaxillary gland PS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>kallikrein mRNA</td>
<td></td>
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<tr>
<td><strong>Genes downregulated by ischemic</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>injury</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rattus norvegicus C4 complement</strong></td>
<td>UI-R-C0-hc-f-02-0-UI</td>
<td>Yes</td>
<td>1.06</td>
<td>1.02*</td>
<td></td>
<td>Inflammation/association with hypertension</td>
</tr>
<tr>
<td>protein mRNA</td>
<td>UI-R-A0-ap-e-12-0-UI</td>
<td>Yes</td>
<td>0.92</td>
<td>ND</td>
<td></td>
<td>Inflammation</td>
</tr>
<tr>
<td>Protein 9-kDa calcium-binding</td>
<td>UI-R-C0-hy-g-02-0-UI</td>
<td>Yes</td>
<td>0.70</td>
<td>0.5*</td>
<td></td>
<td>Fibrosis/EMT</td>
</tr>
<tr>
<td>protein (S100A4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Matrix Gla protein</td>
<td>UI-R-E0-cg-h-08-0-UI</td>
<td>Yes</td>
<td>0.54</td>
<td>1.1*</td>
<td></td>
<td>Calcification, cell migration and division</td>
</tr>
<tr>
<td>Rattus norvegicus mRNAs for</td>
<td>UI-R-A1-ed-f-03-0-UI</td>
<td>Yes</td>
<td>0.54</td>
<td>ND</td>
<td></td>
<td>Extracellular matrix</td>
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<tr>
<td>collagen α1 type 1</td>
<td>UI-R-C0-id-a-09-0-UI</td>
<td>Yes</td>
<td>0.51</td>
<td>ND</td>
<td></td>
<td>TGF-β regulation/fibrosis</td>
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<tr>
<td>ESTs, similar to TGF-β binding</td>
<td>UI-R-E0-bu-g-11-0-UI</td>
<td>Yes</td>
<td>0.49</td>
<td>ND</td>
<td></td>
<td>Inflammation-related</td>
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<tr>
<td>protein:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Similar to procollagen α1 IV</td>
<td>UI-R-A1-dy-e-06-0-UI</td>
<td>Yes</td>
<td>0.46</td>
<td>ND</td>
<td>6</td>
<td>ECM</td>
</tr>
<tr>
<td>Thymosin β-10 mRNA</td>
<td>UI-R-E1-dq-g-05-0-UI</td>
<td>No</td>
<td>0.43</td>
<td>−0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum and glucocorticoid-regulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>kinase (sgk)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue inhibitor of metalloprotease</td>
<td>ATCC no.: 1005922</td>
<td>Yes</td>
<td>0.43</td>
<td>0.53*</td>
<td>23, 33</td>
<td>Renal fibrosis, inflammation, and ECM homeostasis,</td>
</tr>
<tr>
<td>(TIMP1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>tissue calcification</td>
</tr>
<tr>
<td>Osteopontin</td>
<td></td>
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</tbody>
</table>

All numbers shown are Ln(ratio) values. Therefore, a positive value indicates an upregulation in the postischemic group vs. the sham-operated control group, and a negative value indicates a downregulation. TGF, transforming growth factor; EMT, epithelial-mesenchymal transdifferentiation; ND, not done. *Real-time PCR gene expression, P < 0.05 vs. sham-operated controls by Student’s t-test. Microarray ratio and real-time PCR ratio sequences were verified as originally identified; however, a BLAST search reveals similar homology to a number of other sequences identified as Ig-like light chain variable domain sequences.

an ~200-kDa fragment showed a modest enhancement after ARF, and this band is consistent with the size reported for native complement C4 (46). In addition, a band of ~30 kDa that likely corresponds to the γ-chain of C4 was reduced in postischemic kidneys compared with sham-operated kidneys (Fig. 4, bottom).

The expression of MGP investigated by immunohistochemistry revealed only faint immunoreactivity in kidneys of sham-operated control animals, using the COV-2 antibody (Fig. 5A). In contrast, MGP-like immunoreactivity was present in the cytosol but was more distinctly present within nuclei. A different MGP antibody (referred to as MGP-1225) also showed a similar nuclear localization pattern in tubular epithelial cells in postischemic tissue (data not shown).

Protein expression of kallikrein, the bradykinin-generating enzyme, showed prominent immunoreactivity in tubular epithelial cells from sham-operated control animals (Fig. 6A). Kallikrein was expressed primarily in the connecting tubule (large arrow) in close apposition to renal arteries (small arrow). The staining pattern was consistent with its localization in connecting tubule cells and not in intercalated cells and was consistently reduced in these structures 35 days after recovery (Fig. 6B). Morphometric analysis revealed that the kallikrein immunostaining per field (64,000 μm²) or as the percentage of stained area (sham 2,443 ± 323 μm²/field vs. ARF 860 ± 167 μm²/field) or as the percentage of stained area (sham 3.79 ± 0.66% vs. ARF 1.34 ± 0.26%). Finally, we further characterized the immunohistochemical localization of OPN after 35 days of recovery from ARF, because this protein has also been implicated as a central component in progressive renal fibrosis. OPN-like immunoreactivity was difficult to detect in the cortex and outer medulla kidneys of sham-operated rats compared with ARF rats (P < 0.005) expressed as either absolute area stained (sham = 2,443 ± 323 μm²/field vs. ARF = 860 ± 167 μm²/field) or as the percentage of stained area (sham = 3.79 ± 0.66% vs. ARF = 1.34 ± 0.26%).
tively stained tubules could be distinguished into two groups. In one group, OPN+/H11001 tubules could be identified with largely normal tubular morphology but in close apposition to areas enriched with interstitial cells (Fig. 7B). Indeed, some tubules demonstrated OPN-positive cells on the side close to interstitial cells (Fig. 7B, small arrow), whereas the side of the tubule with no interstitial cells was OPN negative. Additional OPN staining could be observed in dilated tubules, which are occasionally

Fig. 4. Immunohistochemistry and Western blot analysis of complement C4. Shown are kidneys of sham-operated control rat (A) and of a rat after 35 days recovery from I/R injury (B). Immunohistochemical localization of C4 was evident in the tubular epithelial cells of the distal tubule (thick arrow) and the collecting duct (thin arrow). Representative of at least 5 animals/group is shown. Magnification is shown. C: Western blot analysis for complement C4 was analyzed in protein extracts (100 µg) obtained from sham-operated control kidneys or from kidneys 35 days after induction of I/R (ARF). The migration of molecular mass markers is shown as is the putative identification of C4-immunoreactive fragments based on previous reports (46).
present in 35-day postischemic kidneys and may represent incompletely regenerated tubules (Fig. 7C). In addition to tubular staining, OPN-like immunoreactivity was observed to a lesser degree in small blood vessels in the renal medulla (Fig. 7D).

**DISCUSSION**

The microarray approach utilized in the current study incorporated a ~2,000-gene array and a study design based on some of our previous reports, which depend on biological replication, duplicate hybridizations, and dye-switching techniques; these have been shown to increase the reliability of cDNA microarray studies (25, 26). In addition, we incorporated a reference distribution technique in which the threshold for differential expression is determined experimentally. The distribution of ratios obtained from the reference hybridizations represents the random variation contained within the biological and experimental platform used in this study. The ratios follow a normal distribution, and the use of a 99.9% interval derived from the reference hybridization would not be expected to identify more than 1 gene (of the ~1,000 detectable genes) as differentially expressed. Clearly, genes that are differentially expressed may be missed in this procedure if their change in expression is smaller than the threshold cutoff determined experimentally. We chose to utilize the 99.9% threshold value to provide a minimal number of false positives at the expense of missing false negatives with moderate alterations in gene expression.

The general schema resulted in the identification of 16 genes modified by I/R injury. All but one of these clones were sequence verified, and the differential expression was verified in six of eight genes tested by real-time PCR. In addition, two other identified genes had been previously

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**Fig. 5.** Immunohistochemical localization of Matrix gla protein in kidney of sham-operated and post-I/R animals. Shown are 5-μm cross sections stained using the COV-2 antibody. Kidneys in A illustrate a lack of immunoreactivity in sham-operated control animals, whereas MGP-positive staining was present throughout the renal cortex and medulla of postischemic animals within 3 days of I/R. Higher magnification of post-I/R animals at 8 and 35 days postsurgery (C and D) reveals prominent cytoplasmic staining as well as heavy nuclear localization. An equal amount of nonimmune rabbit serum did not result in detectable signal in tissue 35 day posts ischemia (E). Representative of at least 5 animals/group is shown. Magnification is shown in A for A and B. Magnification for C–E is shown in E.

**Fig. 6.** Immunohistochemical localization of renal kallikrein protein in sham-operated and postischemic animals. Shown are 5-μm paraffin sections through rat renal cortex of a sham-operated rat (A) and a postischemic rat 35 days postrecovery (B). Localization of kallikrein is detected within the connecting tubule (large arrow) in close apposition to renal arterioles (small arrow). Kallikrein immunohistochemical localization was reduced in intensity as well as the number of stained cells within in kidneys of postischemic animals at 35 days of recovery (B). G, glomerulus. Representative of a minimum of 5 animals/group is shown. Bar = 50 μm.
reported to be differentially expressed in this model (6). In previous studies using these arrays, we demonstrated a significant correlation between microarray and Northern blotting in 62 genes, highlighting the reliability of the array format utilized (25, 26).

Whereas previous gene-profiling studies have focused on early time points after I/R (45, 50), the current study utilized a 5-wk time point after recovery from a bilateral I/R in the rat. The rationale for this approach is that renal regeneration is largely complete at this time point, being characterized by a resolution in GFR and proximal tubular structure. Renal proximal tubules demonstrate a modest hypercellularity (21, 39). There is a diminished urinary concentrating ability, a reduction in the number of renal microvessels, and exacerbated hypoxia (3, 5). In addition, postischemic animals demonstrate enhanced presor activity to ANG II (5). Importantly, however, this time point precedes the development of obvious interstitial scarring, proteinuria, or a secondary reduction in GFR (3, 5, 21). Whereas some identified genes may represent residual expression due to the early injury or the active deposition of renal interstitial scars, we posit that some of the identified genes may underlie alterations in the physiology of postischemic kidneys and contribute to the predisposition of progressive CRF. Therefore, it appears significant that a number of these genes were identified with the potential to modulate hemodynamics, inflammation, and ECM remodeling.

Several of these genes have been previously investigated in the setting of ARF (6, 40). Two of these, collagen IVα1 and tissue inhibitor of metalloprotease-1, are profibrotic genes associated with the deposition of ECM. In a previous study, we demonstrated that the mRNA expression of these genes is dramatically enhanced within 1–3 days of I/R, peaks at 1–2 wk, and returns toward baseline by 4 wk (6). The expressions of these genes are at least partially influenced by transforming growth factor (TGF)-β activity (6); however, the development of interstitial fibrosis is not prominent when the expression of these genes is maximal. Therefore, it is unclear whether the expression of these ECM genes at 5 wk is associated with the progressive development of interstitial fibrosis or whether they represent residual activity from the early recovery period.

It is of particular interest that OPN was identified in this study, which is well known to promote inflammatory processes associated with progressive fibrosis. OPN is enhanced rapidly in the postischemic kidney (23, 32) and may represent an adaptive response, because the disruption of the OPN gene in null mice results in more severe injury after I/R (30). In the current study, immunohistochemical staining of OPN was most apparent in tubules in close apposition to interstitial cells; however, we are unable to discern whether OPN expression in the tubules promotes the deposition of interstitial cells or whether the deposition of interstitial cells results in OPN expression. However, the persistent expression of OPN may be relevant toward the overall long-term pathology of the postischemic kidney. This possibility is further suggested by the report of Persy et al. (36) demonstrating that OPN null mice were resistant to macrophage infiltration at 5 and 7 days postischemic injury.

Besides its described role in inflammation, OPN promotes tissue calcification. Tissue calcification has been described in patients after ARF as well as in animal models (12, 13, 18, 20). MGP is a 14-kDa vitamin K-dependent protein found most abundantly in the bone and cartilage (13, 18). MGP is a binding protein for bone morphogenetic protein-2 and acts as a calcification inhibitor in vivo (13, 18). MGP null mice develop extensive arterial calcification of the tunica media and cartilaginous tissue and perish within ~6 wk of age. It has been
suggested that upregulation of MGP at the site of calcification is in an attempt to clear calcium from the vessel wall (13, 18). Hence, the dramatic enhancement in the expression of MGP in the setting of ARF described in this study may represent a compensatory mechanism to limit the extent of tissue calcification, perhaps influenced by OPN.

The gene encoding the 9-kDa S100A4 calcium-binding protein was prominently increased in postischemic tissue. This protein has also been referred to as fibroblast-specific protein or FSP-1 and is a marker of interstitial fibroblasts or myofibroblasts that are critical in the development of interstitial scarring (22). The tubular expression of S100A4 has been suggested to promote epithelial-mesenchymal transdifferentiation (EMT), a process by which epithelial cells become myofibroblasts and move into the interstitial space. In the current study, immunohistochemistry localized S100A4-positive cells almost exclusively in the tubulointerstitial space. S100A4 cells were clearly resolved in interstitial fibroblasts or myofibroblasts between tubules or in association with small renal blood vessels (Fig. 3). However, occasional S100A4-positive cells were noted in the tubular epithelium at earlier time points postischemia. These data raise the possibility that EMT occurs during tubular regeneration after I/R, which may account for the expansion of the interstitial myofibroblastic cell type and contribute to the development of interstitial scarring.

However, it has also been suggested that fibroblasts may derive from blood vessels in a process termed endothelial-mesenchymal transdifferentiation. Therefore, it is also reasonable to speculate that this process may occur in the setting of I/R. Indeed, our immunohistochemical localization demonstrates a large number of S100A4-positive cells in the early postischemic time period residing in the interstitial space where peritubular capillaries reside. In a recent study, we demonstrated increased interstitial S100A4 fibroblasts after 5 wk of recovery. Moreover, immunoneutralization of TGF-β during recovery attenuated the increase in fibroblasts and preserved the reduction in peritubular capillary density typically observed in this model (42); the possibility that these two observations can be attributable to an effect of TGF-β on the conversion of endothelial cells to fibroblasts is an important consideration that deserves further investigation.

The expression of OPN in the current study, as well as the identification of several other genes including Igk light chain, IgE binding protein, and complement C4, suggests that the postischemic recovered kidney remains in a proinflammatory state. We suggest that the inability to suppress the inflammatory response completely after I/R is an important factor in progressive disease posts ischemic injury.

Indeed, complement C4 was the most consistently upregulated gene in this study. Whether complement C4 expression might impinge on the late manifestations of ARF is a possibility worthy of further consideration. It is important to note that complement C4 mRNA was also identified as a prominently upregulated gene in a model of chronic hepatic iron overload in rats using a subtraction hybridization cloning strategy (14). It was determined that C4 expression was associated with activation of stellate cells associated with increased smooth muscle actin deposition and liver fibrogenesis.

Despite the consistent enhanced expression of complement C4 mRNA in the model of renal I/R injury, it is difficult to speculate on a prominent role for complement C4 in the pathogenesis of secondary disease in this model. First, whereas complement C4 has been identified in a number of glomerular diseases, examination of C4 in different models (e.g., lupus nephritis, Heymann nephritis) suggests that it may either promote or inhibit progressive disease (10, 35, 51). Second, whereas deposition of the C4d cleavage product in peritubular capillaries is seen in humoral rejection (37), we failed to detect alterations in the immunohistochemical staining of C4d in posts ischemic animals (data not shown). Finally, whereas mRNA expression is clearly enhanced posts ischemia, there is no clear evidence for an increase in complement C4 protein expression (Fig. 4). Most of the immunoreactivity was found to be present at ~60 kDa, which is consistent with the reported migration of the α chain of iC4b, an inactive product, and was unchanged by I/R (46). Moreover, we detected only a modest expression of a ~200-kDa band postischemia that may correspond to a native complement C4 protein.

With regard to the consideration that renal I/R might alter gene expression regulating systemic blood pressure, the most notable alteration in our study was the substantial downregulation of renal kallikrein. Kallikrein is produced in the cortical connecting tubule whereas its substrate kininogen is synthesized downstream in the collecting tubules. These act to generate bradykinin, an NO-dependent vasodilatory factor (2, 49).

There is abundant evidence that indicates that the KKS plays an important role in blood pressure regulation, salt sensitivity, and electrolyte excretion (27). Kallikrein administration to hypertensive patients reduces blood pressure, and this is more effective in salt-sensitive vs. salt-resistant individuals (2). The long-term infusion of a subdepressor dose (700 ng/day iv) of purified rat urinary kallikrein attenuates renal injury in salt-induced hypertension in Dahl S rats (47). In addition, a selective breeding scheme has been utilized to generate a kallikrein-deficient rat strain; this strain manifests polydipsia and hypertension in response to elevated Na intake as well as progressive renal scarring (27).

In consideration of the reports, we have recently determined that posts ischemic animals manifest a Na-dependent hypertension and accelerated secondary renal damage (Ref. 41 and Basile D, unpublished observations). It is worth noting that a reduction in kallikrein expression was recently reported by Suga et al. (43) in a model of hypokalemia-induced renal injury. The model of hypokalemia-induced injury is analogous to the model of recovery from I/R in other ways, such as a reduction in peritubular capillaries and exacerbated medullary renal hypoxia (43). The interrelationship among the reduction in kallikrein, renal hypoxia, and capillary rarefaction in the progression of renal disease and hypertension post-IR remains unclear.

In conclusion, the current study has identified a subset of genes that remains persistently expressed in the kidney after ARF. These genes provide potential insight into the altered physiological state of the posts ischemic kidney. Further investigation into some of these genes may illuminate the nature of the predisposition of CRF in the setting of acute reversible injuries.

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