Monitoring changes in gene expression in renal ischemia-reperfusion in the rat

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Acute renal failure (ARF), characterized by rapid decline in glomerular filtration rate (GFR), is potentially reversible but is associated with major morbidity and mortality. Despite new insights into the pathogenesis of ARF, neither the incidence nor mortality has declined in decades [1]. During the induction and course of ARF many intrarenal alterations occur, including changes in cell polarity [2–5], levels of signaling molecules [6–11] expression of adhesion molecules [8, 12–15] and patterns of apoptosis and necrosis [16]. Nevertheless, it is probable that additional factors participate in the evolution and repair of ARF but are yet to be appreciated. Discovering such factors may lead to improved strategies for preventing ARF and treating this serious disorder.

Certain changes seen in the course of ARF involve infiltrating cells and adhesion molecules. Neutrophil infiltration is induced by adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin via the mediation of tumor necrosis factor-α (TNFα) and nuclear factor-κB (NF-κB). Infiltrating neutrophils induce adhesion molecules such as integrins [12], E-selectin [13], P-selectin [14], intercellular adhesion molecule-1 [8], and neural cell adhesion molecule [15]. Inhibition of NF-κB using a decoy oligonucleotide has been reported to suppress the inflammatory response that follows ischemia-reperfusion injury [17]. Animals that express aberrant adhesion molecules may be protected from renal injury, as noted in ICAM-1–deficient mice that are resistant to ischemic renal injury [18].

Depolarization of epithelial cells occurs in the course of renal injury and repair, in part secondary to changes in adhesion molecules. For example, in ARF induction of transforming growth factor-β (TGF-β), deactivation of integrin, and increased expression of adhesion molecules is associated with disruption of the cytoskeleton in proximal tubular cells [19]. In this setting the actin microfilament network becomes redistributed into large
cytoplasmic aggregates [19], which leads to depolarization and exfoliation of epithelial cells. Newly expressed integrin appears to restore cell polarization, accompanied by increased expression of laminin and fibronectin [19].

Ischemic injury results in apoptosis as well as necrosis. Apoptosis is mediated through several signaling cascades, one of which is the mitogen-activated protein (MAP) kinase cascade. Members of this family, such as p38MAPK [10], JNK and ERK [11] have been previously reported to play a role in ARF. Park, Chen and Bonventre suggested that activation of JNK or p38 is involved in protection against ischemic injury in the kidney [11]. TNF-α and integrin also have been reported to mediate apoptosis [20, 21].

DNA microarray technology offers the advantage of analyzing thousands of genes simultaneously, with the potential to uncover new mechanisms of pathogenesis. Thus, microarray analysis coupled with bioinformation could detect changes in genes previously unknown to participate in renal injury and repair. Such an approach could lead, potentially, to a new paradigm through which ARF may be better understood. In the present study, we examined changes in gene expression induced by ischemia-reperfusion injury. A total of 2,100 mouse cDNAs spotted onto a glass slide were used for profiling gene expression. As expected, we found that many of the changes in expression occur in genes that encode proteins previously implicated in ischemia-reperfusion injury. However, and importantly, ADAM2 (a disintegrin domain and metalloproteinase), cytochrome p450 IID6, peroxisome proliferator-activated receptor gamma (PPARγ) were newly identified to participate in ARF.

METHODS

Animals and RNA extraction

Male Wistar rats (200 to 250 g, N = 16 ischemia-reperfusion, N = 20 sham) were housed in a 12-hour light/dark cycle, and allowed free access to food and water. Control and treated animals were weight- and age-matched at the time of initiation of bilateral ischemic injury, and body weights were recorded at initiation and completion of experiments. The animals were allowed free access to standard rodent chow and water until overnight prior to surgery when food (but not water) was removed. Animals were anesthetized intraperitoneally with pentobarbital sodium, 65 mg/kg. Both renal arteries were exposed using a midline incision and then clamped for 30 minutes followed by reperfusion. Sham animals had an incision plus 30 minutes of waiting time without clamping. After ischemia or sham treatment, muscle layer incisions were sutured, and skin incisions closed with Michel clips. We gave a total of 6 mL of 0.9% saline solution IP to the rats during and after surgery to prevent dehydration. In the present study, we chose to analyze RNA from animals that showed more prominent creatinine changes. Animals were then followed for 1, 2, 3, or 4 days (at least N = 3 per group per time point).

At sacrifice the kidneys were removed and snap-frozen in liquid nitrogen. The samples were stored at −70°C until further study. The serum creatinine level over time is shown in Figure 1, and documents successful induction of ARF. Total RNA was isolated from kidney by Trizol (Gibco, Rockville, MD, USA) extraction.

High-density DNA microarray preparation

High-density cDNA microarrays were generated by standard methods [22]: Mouse expressed sequence tags (ESTs; Research Genetics, Huntsville, AL, USA) were amplified by the polymerase chain reaction (PCR), according to the manufacturer’s recommendations. Each sample was run in a 1% agarose gel for quality verification (data not shown). The sizes of PCR products ranged from approximately 300 bp to 1.2 kb. DNA microarrays, containing 2,100 distinct ESTs, were printed onto glass slides using a pin-style arrayer (BioRobotics, Cambridge, UK). The identities of all 18 genes were confirmed by DNA sequencing using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA) performed by the Genome Center staff at Brigham & Women’s Hospital. The sequence was confirmed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

To make microarrays, sialylated glass slides (CEL Associates Inc., Houston, TX, USA) were cleaned for two hours in a solution of 2 N NaOH. After rinsing in distilled water, the slides were treated with a 1:5 dilution of poly-t-lysine adhesive solution (Sigma, St. Louis, MO, USA) for one hour, and then dried for five minutes at 80°C in a vacuum oven. DNA samples from 100-μL PCR reactions were precipitated with ethanol in 96-well microtiter plates. The resulting precipitates were resuspended in 20 μL 3 × standard sodium citrate (SSC; 20 ×
were resuspended in 20 mM H9262 Fibronectin.

Quantitative real time PCR

Quantitation with real-time, one-step reverse-transcription polymerase chain reaction (RT-PCR) was performed with SYBR Green PCR Reagents and an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Reactions were performed using 1.0 μL of RNA at a concentration of 100 ng/μL in a reaction volume of 50 μL. An RT reaction was performed at 48°C for 30 minutes, followed by PCR consisting of AmpliTaq activation for 10 minutes at 95°C, then 40 cycles with heating to 95°C for 15 seconds, and cooling to 60°C for one min. mRNA levels were normalized to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We elected to confirm changes in five genes using real-time one-step quantitative RT-PCR. These five genes and PCR primers are as follows: Fibronectin.

Forward primer, 5'-GTGCGTGGGCTTCAACTTCTC-3'
Reverse primer, 5'-GTGGTTGCAAACCTTCAAT-3'

HO-1.

Forward primer, 5'-TCTATCGTGGCATCGAAG-3'
Reverse primer, 5'-CAGCTCCTCAACAGCCTAA-3'

ADAM2.

Forward primer, 5'-GGGATCTGTTGGCAGCTGAT-3'
Reverse primer, 5'-ATTAGGTCGCAGTCTTGT-3'

PPARγ.

Forward primer, 5'-TTGACCTCTCCTGTGATGGA-3'
Reverse primer, 5'-CATTGGGTCAGCTTTGTGA-3'

Cytochrome P450 IIId6.

Forward primer, 5'-AGCTTCAACACCAGCTATGG-3'
Reverse primer, 5'-CAGCAGTGTCCTCTCCATG-3'

GAPDH.

Forward primer, 5'-ACTCCCCATCTTCACCTTCTTT-3'
Reverse primer, 5'-TACTCTTGGAGGCCATG-3'

All primer sets were designed according to manufacturer's recommendations, and when feasible, to span one or more introns. All amplicons ranged between 142 and 148 base pairs. Electrophoretic analysis of expected product sizes was performed for all primer sets prior to one step, real time RT-PCR, to confirm the fidelity of the reaction.

cDNA probe labeling

cDNA probes were synthesized as previously reported [23] to improve signal detection on high-density microarrays using a fluorescent oligonucleotide dendrimeric signal amplification system. Briefly, 10 μg of total RNA was primed with 1.0 picomole of modified reverse transcription (RT) primer with capture sequence for Cy3 or Cy5 (Genisphere, Montvale, NJ, USA). The RT reaction was performed according to standard protocols (SuperScript RT II; Gibco), followed by the addition of 3.5 μL of 0.5 mol/L NaOH/50 mmol/L ethylenediaminetetraacetic acid (EDTA), and heating at 65°C for ten minutes to denature the DNA/RNA hybrids. This reaction was then neutralized with 5 μL of 1 mol/L Tris, pH 7.5. For double-labeled detection, RT reactions were mixed and precipitated using 3 mol/L sodium acetate and 100% ethanol, then washed once with 70% ethanol. The pellets were resuspended in 20 μL distilled H2O.

Hybridization

cDNA microarrays were prehybridized with 500 μL of preheated (65°C) ExpressHyb (Clontech, Palo Alto, CA, USA) for an hour in a microarray hybridization chamber (Grace Bio-Labs, Bend, OR, USA) in a rotisserie hybridization oven. Probe was put into the hybridization chamber and incubated overnight in a rotisserie hybridization oven. The next day the microarrays were washed for five minutes in 2 × SSC/0.2% sodium dodecyl sulfate (SDS) at 65°C, then in 2 × SSC and 0.2 × SSC for two minutes each. For dendrimer detection, 1.0 μL of the Cy3- and Cy5-labeled dendrimers (Genisphere) were suspended in 15 μL of 4 × SSC/2% SDS and layered on the array. The arrays were covered with a coverslip and then incubated at 65°C for eight hours in a microarray hybridization cassette (Telechem International, Sunnyvale, CA, USA). The chips were then washed as above.

Data collection, normalization, and analysis

Each time point was analyzed twice with reverse color labeling. The mean of the two measurements was used to determine the fold changes. As was stated, dye reversal was used. This analysis uses a log-normal error model. The cut off was based on at least twofold changes for the genes of interest. The chips were scanned in a ScanArray 5000 confocal laser scanner (GSI Lumonics, Marina Del Rey, CA, USA). The signal and background signals were then quantified with Imagen 4.0 quantification software (BioDiscovery, Billerica, MA, USA). Signals were measured as the mean pixel intensity within each circumscribed spot and average background was measured using the mean pixel intensities of blank spots in the array (without cDNA) and as the mean pixel intensity outside the circumscribed spot diameter for calculating specific signals.
RESULTS
cDNA microarray analysis

Representative differential expression patterns are shown for sham (Fig. 2A) and ischemia-reperfusion samples (Fig. 2B) at day 1 on one glass slide. Ischemia-reperfusion (IR) samples were labeled with Cy3 and sham with Cy5. This figure demonstrates clear signals with background and several instances of clear differential gene expression. Every spot is well rounded and has virtually the same diameter. Figure 2C shows an overlay picture of sham and IR such that green spots indicate up-regulation and red spots indicate down-regulation, and yellow spots indicate no change, respectively. The numbers 1, 2, 3, 4, and 5 indicate clusterin, ADAM2, HO-1, cyp2d6, PPARγ, respectively. Of these, clusterin, ADAM2, and HO-1 are increased and cyp2d6 and PPARγ are decreased in IR.

As shown in Figure 3A, a scatter plot of sham (Cy3) versus sham (Cy5) fell on a line of identity. When a scatter plot of sham (Cy3) versus IR (Cy3) was performed, some changes in individual genes were seen (Fig. 3B). Red spots, identified as Adam2, HO-1, and clusterin signify up-regulated genes in IR and green spots (identified as cyp2d6 and PPARγ) signify down-regulated genes. To confirm these data, reverse labeling was performed. Instead of labeling IR with Cy3 and sham with Cy5, IR was labeled with Cy5 and sham with Cy3. In Figure 3C, a scatter plot of this reverse labeling is shown; now green spots (identified as Adam2, HO-1, and clusterin) signify genes decreased in IR and red spots (identified as cyp2d6 and PPARγ) signify transcripts increased in IR. Thus, these reverse labeling experiments demonstrated consistent results. Furthermore, all 18 transcripts altered were sequence-verified to confirm their identities.

Genes up-regulated by ischemia-reperfusion injury

Figure 4 shows the nine genes increased in IR. Of these, three increased acutely. These include heme oxygenase-1, thymosin β4, and uncoupling protein (UCP)-2 (Fig. 4A). Heme oxygenase-1 (HO-1) increased at day 1 followed by normalization, a finding previously reported in a rat model of ARF [24]. Thymosin β4 (Tb4) increased approximately twofold. Uncoupling protein (UCP)-2 mRNA level increased by 2.8-fold at day 1 and recovered to baseline at day 4.

Six genes were elevated throughout the four-day time course of IR (Fig. 4B). These included ADAM2, clusterin, FK506 binding protein 1a (Fkbp1a), fibronectin, vanin1, and heat-responsive protein 12 (hrsp12). The expression of clusterin (also known as a TRPM-2 or sulfated glycoprotein 2) was increased approximately fivefold and maintained at that level until day 4. Fibronectin increased by about 2.5 fold and remained so for four days, consistent with other reports [12, 25]. ADAM2 was up-regulated by 3.3 fold and gradually normalized.

Genes down-regulated by ischemia-reperfusion injury

Figure 5 shows nine genes that were decreased in IR. Of these, four genes decreased acutely, including alpha-albumin protein, cytochrome P450 IID6 (cyp2d6), cytochrome P450 IIId9 (cyp2d9), and glutamine synthetase (Fig. 5A). Cyp2d6 was markedly decreased, to approximately 20% of the sham value.

Five genes were chronically down-regulated, including alcohol dehydrogenase-B2, cytochrome P450 IVa14 (cyp4a14), PPARγ, Xist gene, and uromodulin (Fig. 5B). Uromodulin was decreased to approximately 20 to 25% of the sham value.

Confirmation of change in expression

Microarray data of five genes of interest were confirmed by real-time quantitative PCR (Fig. 6); others were not tested. Fibronectin was chosen because of its well-known increase in ischemic injury [12]. HO-1 was selected as increased on gene array and previously described to be increased in ARF [24, 26–28]. ADAM2 was chosen as a gene that was increased on gene array, and a previously undescribed finding in ARF. PPARγ and Cyp 2d6 were chosen as they were decreased on gene array, previously undescribed findings in ARF. Using real-time PCR, HO-1 and ADAM 2 were confirmed to be transitional increased whereas cytochrome p450 IId6 and PPARγ were decreased in ischemia-reperfusion. Thus, there was excellent concordance between microarray data and real time PCR, though the magnitude of changes were more pronounced than those seen with microarray analysis.

DISCUSSION

In the present study, we demonstrated that multiple gene transcripts were increased or decreased by ischemia-reperfusion, and we identified several genes previously unknown to be involved in renal injury and repair. For example, it has been previously reported that clusterin [16] and fibronectin [12, 29] are up-regulated by renal ischemia-reperfusion injury, and that HO-1 is up-regulated during ARF [24, 30, 31]. Uromodulin has previously been shown to decrease after renal ischemia-reperfusion injury [32].

The transcript with the largest observed change was clusterin, known as a marker for cell injury in the tissues. In the present studies clusterin was increased from day 1 to day 4 after ischemic injury. Clusterin mRNA is overexpressed in apoptosis-resistant cells [33] and is required for survival of cells treated with TNFα or subjected to oxidative stress [34–36]. Thus, we infer from these find-
Fig. 2. Representative differential expression by cDNA array. Representative differential expression is shown by microarray of sham (A, Cy3) and ischemia-reperfusion (B, Cy5) at day 1 on one glass slide. (C) Overlay picture of sham and IR microarray is shown. In this picture, each red spot indicates higher signal in sham and each green spot indicates higher in IR. Numbers denote: (1) clusterin, (2) ADAM2, (3) HO-1, (4) cyp2d6, (5) PPARγ. Note that the signal of spots numbered 1, 2 and 3 are higher on the right compared to the left, indicating an increase. In contrast, the spots numbered 4 and 5 indicate a decrease in expression.

Fig. 3. Scatter plot graph of microarray data. (A) Sham labeled by Cy3 versus sham labeled by Cy5. All data points are on a 45° angle, confirming the accuracy of this technique. (B) Scatter plot of sham labeled by Cy5 versus ischemia-reperfusion (IR) labeled by Cy3 at day 1 was shown. Red spots, (typed as ADAM2, HO-1, and clusterin) signify genes that are up-regulated and green spots, (typed as cyp2d6 and PPARγ) signify genes that are down-regulated. (C) Scatter plot of IR labeled by Cy5 vs sham labeled by Cy3 as shown on day 1, as a reverse labeling of scatter plot B. Red spots, (typed as ADAM2, HO-1, and clusterin) signify up-regulated and green spots, (typed as cyp2d6 and PPARγ), down-regulated. Thus, reverse labeling confirmed the data. Abbreviations are: ADAM2, a disintegrin domain and metalloproteinase; Clu, clusterin; HO1, heme oxygenase-1; Cyp2d6, cytochrome P450 2d6; PPARγ, peroxisome proliferator activated receptor-gamma.
ings that clusterin may play role in protection from ischemia-induced apoptosis in kidney.

In the present study, HO-1 increased at day 1, decreased gradually at day 2 and normalized by day 4. HO-1 has already been shown to be increased in the kidney in various models of ARF [24, 26–28]. Shimizu et al reported that HO-1 conferred a protective effect in ischemic acute renal failure, in that when HO activity was blocked by tin mesoporphyrin, increases in microsomal heme concentration, and serum creatinine concentration were observed, along with extensive tubular epithelial cell injury [31].

Uromodulin (Tamm-Horsfall protein) was greatly decreased in our model. This protein, one of the most abundant in the renal tubule, is exclusively localized to the thick ascending limb of Henle’s loop [37]. Safirstein et al and Bachmann et al reported that uromodulin mRNA declined to undetectable levels by 24 hours after renal ischemia-reperfusion [32, 37]. We confirm their observation on day 1 after ischemia injury. In addition, our data show that uromodulin gene expression was continuously inhibited up to day 4 post-injury.

The microarray data showed that ADAM2 was increased on day 1 after IR injury and gradually returned to normal on day 4. The real-time PCR confirmed that there is an increase of ADAM2 on day 1. However, the PCR results further showed no changes or decrease after day 2. We do not know the reasons for this discrepancy. Nevertheless, the PCR results did confirm that ADAM2 was transiently up-regulated on day 1. Novel observations included increased ADAM2 in ARF. This gene, also known as fertilin beta, is a well-characterized mole-
cule that mediates cell-cell communication in sperm-egg interactions. ADAM2 has not previously been known to be involved in ARF. Interestingly, this protein increases in the central nervous system in cases of Alzheimer's disease, along with alteration in cell-matrix contact in the brain [38]. We thus infer that this molecule plays a role in cell-matrix interactions within the kidney. Several members of the ADAM family have the ability to cleave transmembrane proteins, such as growth factors, as well as their receptors, cell adhesion molecules, and cytokines, resulting in soluble forms of those substances, a phenomenon that is termed ectodomain shedding [39]. For example, ADAM-17 (TNFα converting enzyme, TACE), a member of this family, cleaves transmembrane pro-TNFα to produce mature form of TNFα. Tissue inhibitor of metalloproteases-3 (TIMP-3) inhibits these proteins that are involved in ectodomain shedding [40–43]. It may be that ADAM2 plays a role as a shedder, during the regeneration needed during tissue repair after renal damage. Thus, we speculate that ADAM2 plays an important role in repair after tissue injury with in the kidney.

We also noted that thymosin β4 (Tb4) was increased in ischemia-reperfusion injury. The beta-thymosins constitute a family of actin monomer-sequestering proteins widely distributed among vertebrates, and Tb4 is most abundant protein in this family. Tb4 has been reported to bind to actin monomers and to enhance wound healing [44–46]. Additionally, it is up-regulated following focal brain ischemia [47]. Our findings lead us to hypothesize that Tb4 has a general role in repair of cytoskeleton and in re-polarization of renal tubular epithelial cells after IR injury.

UCP-2 was increased by IR injury, a previously unreported finding. This gene is involved in mitochondrial hydrogen peroxide generation [48]. UCP-2 mRNA has been reported to be up-regulated approximately three-fold in fatty liver, during which hydrogen peroxide production by mitochondria was increased. It has been speculated that this increase may result from chronic apoptotic stress and may play a role in adaptation to such stress [49]. Li et al reported that UCP-2 is involved in cellular defense against oxidative stress (hydrogen peroxide 200 mmol/L) in clonal beta-cells that over-express UCP-2 [50], further supporting this theory. We speculate that UCP-2 plays an analogous role in renal injury.

Vanin-1 increased by about 2.7-fold and remained elevated throughout the study period. Vanin-1, also called pantetheinase, hydrolyzes pantethein to cysteamine (a powerful antioxidant) and pantothentic acid [51]. Therefore, we hypothesize that vanin-1 acts as an endogenous anti-oxidant in renal repair after ischemia-reperfusion injury [52].

We noted PPARγ to be down-regulated in ischemia-reperfusion injury, a novel finding. PPARs (peroxisome proliferator-activated receptors), a family of transcription factors, have multiple roles that are of great interest. Although etomoxir, a PPARγ agonist, was reported to protect the kidney in ischemia-reperfusion injury [53], to our knowledge the present work is the first to find changes in PPARγ in this model. PPARγ is a nuclear receptor that serves as a transcription factor and thus regulates several physiologic pathways, particularly those involved in lipid metabolism [54]. Fahmi et al reported that PPARγ inhibited interleukin-1 induced matrix metalloproteinase 13 [54]. Furthermore, Yee et al reported that the stromelysin-1 (MMP-3) gene, which is involved in tissue remodeling, contains a PPAR element in its promoter [55]. Therefore, it seems likely that PPARγ plays a role in tissue repair, especially in control of MMP.

Nakajima et al observed that heterozygous PPARγ-deficient mice subjected to intestinal ischemia suffered more pronounced injury than control mice [56]. Furthermore, when such mice were treated with a PPARγ-activating ligand, BRL-49653, they were partially reconstituted and suffered less damage. This report supports our hypothesis that PPARγ may act through one of its pathways related to apoptosis and/or tissue repair after ischemic injury.

Cytochrome p450 IId6, IId9, and IVa14, all of which are involved with oxidation, were suppressed. When these enzymes are active, they lead to consumption of oxygen. Hence, when tissue is subjected to severe ischemic injury, these enzymes are likely suppressed in an attempt to minimize ischemic injury. We speculate that these enzymes are suppressed during ischemic renal injury in an
attempt to minimize oxidation injury. Suppression of these cytochrome p450s may occur during the ischemic state to slow down oxidative metabolism, which would lead to cytoprotection.

The present analysis of the global gene expression profile in ischemia-reperfusion used whole kidney. Thus, the expression pattern within various intrarenal structures is not distinguishable from the whole. It also is possible that certain of the observations could be the result of inflammatory cell infiltration or injury to the microvasculature. Nonetheless, given the early stage and high cost of array technology, we chose not to focus on dissected cell types (such as, glomerulus, proximal tubule, thick ascending loop of the limb of Henle, distal tubule, collecting duct, etc.) in an initial study of renal ischemia-reperfusion. Once routine amplification technology is established, pursuing such a goal will be more feasible.

In conclusion, using DNA microarray we have identified 18 genes that in mRNA expression lead in the course of ischemia-reperfusion injury. The identities of these 18 genes were confirmed by DNA sequencing. Changes in five genes (fibronectin, ADAM2, cytochrome p450 IIId6, HO-1 and PPARy) were further confirmed by quantitative real-time PCR. Several genes that have not previously been identified in ischemic ARF may have important implications concerning the evolution of this disease.

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