Identification of Putative Gene-Based Markers of Renal Toxicity

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This study, designed and conducted as part of the International Life Sciences Institute working group on the Application of Genomics and Proteomics, examined the changes in the expression profile of genes associated with the administration of three different nephrotoxicants—cisplatin, gentamicin, and puromycin—to assess the usefulness of microarrays in the understanding of mechanism(s) of nephrotoxicity. Male Sprague-Dawley rats were treated with daily doses of puromycin (5–20 mg/kg/day for 21 days), gentamicin (2–240 mg/kg/day for 7 days), or a single dose of cisplatin (0.1–5 mg/kg). Groups of rats were sacrificed at various times after administration of these compounds for standard clinical chemistry, urine analysis, and histological evaluation of the kidney. RNA was extracted from the kidney for microarray analysis. Principal component analysis and gene expression–based clustering of compound effects confirmed sample separation based on dose, time, and degree of renal toxicity. In addition, analysis of the profile components revealed some novel changes in the expression of genes that appeared to be associated with injury in specific portions of the nephron and reflected the mechanism of action of these various nephrotoxicants. For example, although puromycin is thought to specifically promote injury of the podocytes in the glomerulus, the changes in gene expression after chronic exposure of this compound suggested a pattern similar to the known proximal tubular nephrotoxicants cisplatin and gentamicin; this prediction was confirmed histologically. We conclude that renal gene expression profiling coupled with analysis of classical end points affords promising opportunities to reveal potential new mechanistic markers of renal toxicity. Key words: biomarkers, cisplatin, gentamicin, microarrays, nephrotoxicity, proximal tubule, puromycin, toxicogenomics. Environ Health Perspect 112:465–479 (2004). doi:10.1289/txg.6683 available via http://dx.doi.org/ (Online 15 January 2004)

Renal toxicity commonly occurs after administration of xenobiotics and a variety of pharmaceutical agents. The process is typically initiated by a toxic injury to tubular epithelial cells in various nephron segments or by injury to specific cell types in the glomerulus. The initial injury is often followed by cellular proliferation and repair that attempts to restore normal renal function (Toback 1992). Changes in the expression of mRNA specifically expressed in the injured kidney cells are some of the earliest events that accompany renal injury. This is accomplished by changes in the expression of other genes that contribute either to cellular repair or recovery of renal function or in those that mediate fibrosis and further pathology of the kidney (Matejka 1998; Norman et al. 1988; Safirstein et al. 1990). For example, elevations in the expression of heme oxygenase 1 (HO-1), kidney injury molecule-1 (KIM-1), claudin-1, thrombin beta 4, osteopontin, and several growth factors have been reported in various models of renal injury (Hammelmann 1998a, 1998b; Huang et al. 2001; Ichimura et al. 1998; Yoshida et al. 2002).

Recent application of microarray-based gene profiling has provided some unique insights into compound-specific and toxicity-related gene expression changes in the liver (Hamadeh et al. 2002a, 2002b; Waring et al. 2001a, 2001b), and several laboratories have applied similar techniques to identify gene expression changes related to nephrotoxicity (Huang et al. 2001; Nagasawa et al. 2001; Yoshida et al. 2002).

This study, conducted as a consortium collaboration with the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) (Kramer et al. 2004; Pennie et al. 2004), compares kidney gene expression profiles induced by cisplatin and gentamicin, compounds that injure the proximal tubule, and puromycin, which produces proteinuria by selectively damaging podocytes, a key component of the glomerular filtration barrier to plasma proteins. Conventional clinical chemistry and histopathological or ultrastructural findings confirmed the induction of tubular or glomerular injury in these studies. Through comparison of the expression profiles derived from kidneys exposed to these three nephrotoxicants at multiple doses and time points (associated with varying types and severity of toxicity), we hypothesized that we could identify patterns of gene expression that reflect different types of nephrotoxicity. An important outcome is the identification of potential gene-based markers of nephrotoxicity and evidence that one can detect region-specific renal toxicity using microarray technology. Additional functional genomics studies may afford the opportunity to validate the proposed novel gene-based markers of nephrotoxicity, which in part may improve current sensitivity to assessing xenobioc-induced nephrotoxicity.

Materials and Methods

General

This study was conducted in accordance with Good Laboratory Practices. All animals were handled and treated in accordance to the NIH Guide for the Care and Use of Laboratory Animals (NIH 1999). Experiments were performed on...
approximately 8- to 9-week-old male (approximately 250–300 g) Sprague-Dawley rats Cr:CD (SD) IGS BR VAF+ (abbreviated SD) purchased from Charles River Breeding Laboratories (Raleigh, NC), Food [Purina Rodent Chow no. 5002 (Purina Milling Inc., St. Louis, MO), or Certified Rodent Diet 18% no. 5LG3, (PMI Feeds, Richmond, IN)], except for study-defined fasting procedures, and water were available ad libitum.

Test Article and Treatment

Cisplatin (CAS no. 15663-27-1; lot 059H3657), gentamicin sulfate (CAS no. 1405410; lot 50K0294), and puromycin dihydrochloride hydrate (CAS no. 58-58-2; Lot 080K4031) were purchased from Sigma Chemical Company (St. Louis, MO). Doses were selected based on previous reports that documented histopathological changes in the kidney by the 7-day time point or later. The rats received a single ip injection of vehicle (0.9% NaCl solution) or cisplatin at a dose of 0.3, 1, or 5 mg/kg (20 per group). Cisplatin- and vehicle-treated rats were sacrificed by inhalation of carbon dioxide 4, 24, 48, and 144 hr after injection of vehicle or cisplatin. This portion of the study was conducted at Pfizer Inc (Groton, CT).

Gentamicin was dissolved in a sterile 0.9% (w/v) NaCl solution and the animals were treated with daily ip injections of vehicle or gentamicin at doses of 2, 20, or 240 mg/kg/day for up to 7 days. A group of rats (three to five per group) were treated at 4, 24, 48, and 144 hr after the initial administration of gentamicin. This portion of the study was conducted at Novartis Pharmaceuticals (East Hanover, NJ) and the Medical College of Wisconsin (Madison, WI).

Puromycin was dissolved in sterile 0.9% (w/v) NaCl solution. The rats received daily ip injections of vehicle or puromycin at doses of 5 or 20 mg/kg/day for up to 21 days. Puromycin-treated and time-matched vehicle control animals were sacrificed at 4, 24, 48, and 144 hr, and 21 days after the first exposure to the compound. This portion of the study was coordinated by staff from the National Institute of Environmental Health Sciences (NIEHS; Research Triangle Park, NC).

Urine Collection

Animals were placed in standard metabolic cages overnight (approximately 12–14 hr) prior to necropsy. Food but not water was removed from the animals during this time. Urine volumes were recorded and the urine samples were frozen and stored at −80°C for subsequent analysis.

Tissue Collection

After CO2 anesthesia, a sample of blood was collected from the vena cava for standard clinical chemistry analysis. The kidneys were collected, weighed, and stored frozen. Half the kidney and the left lobe of the liver were placed in 10% neutral buffered formalin for histology, and the other half was flash-frozen in liquid nitrogen and stored at −80°C for Western blot or immunohistochemical analysis. The right kidney was minced, transferred to 5 mL RLT with betimercaptoethanol (+ BME) buffer (Qiagen, Valencia, CA) in a 50-mL centrifuge tube and homogenized for 60–120 sec. Additional RLT (+ BME) buffer was added to achieve a final volume of 30 mL, which was divided into 15-mL aliquots and stored at −80°C until the RNA was extracted.

Histology

The tissues were fixed for a maximum of 48 hr and then transferred to 70% ethanol for up to 10 days before being placed in paraffin. The tissues were embedded in paraffin blocks, cut into 5-µm sections, and stained with hematoxylin and eosin (H&E). Ultrastructural analysis using electron microscopy was performed on kidney tissue obtained from animals treated with gentamicin. In this case the kidney tissue was fixed with paraformaldehyde rather than formalin.

Clinical Chemistry and Urinalysis

At scheduled necropsies, blood (fasted, vena cava bleeding; approximately 2–3 mL) was collected into serum separation collection tubes and allowed to clot. Serum was collected into serum separation collection tubes and stored frozen at −70°C. Clinical Chemistry and Urinalysis

Tissue Collection

Gene expression analysis was conducted on the same RNA samples using several platforms including Affymetrix (Santa Clara, CA) and NIEHS custom cDNA microarrays. The analysis of samples using the Affymetrix platform was performed according to the manufacturer details (Affymetrix 2003; Lockhart et al. 1996) and is detailed in the accompanying paper (Thompson et al. 2004).

The analysis using the custom cDNA microarray manufactured at NIEHS is as follows: Sequence-verified rat cDNA clones that covered the 5’ end of the gene and ranged in size from 500 to 2000 bp (Research Genetics, Huntsville, AL) were printed on glass slides. M13 primers were used to amplify insert cDNAs from purified plasmid DNA in a 100-µL polymerase chain reaction (PCR) reaction mixture. A sample of the PCR products (10 µL) was separated on 2% agarose gels to ensure quality of the amplifications. The remaining PCR products were purified by ethanol precipitation, resuspended in ArrayIt buffer (TeleChem Intl. Inc., Sunnyvale, CA), and spotted onto poly-l-lysine-coated glass slides using a modified robotic DNA arrayer (Beecher Instruments, Bethesda MD) with TeleChem pins to produce the NIEHS rat chip containing > 7,000 clones. A list of clones on this chip is available on}

RNA Extraction

Homogenized kidney samples (one of two 15-mL samples per animal) were processed using a Qiagen RNeasy Maxi kit (Qiagen) according to the manufacturer instructions. Final RNA product was quantified from the ultraviolet absorbance at 260 nm (Beckman DU5200UV/Vis Spectrophotometer; Beckman Coulter Inc., Fullerton, CA). The quality of the RNA was evaluated by measuring the 260-/280-nm ratios and confirmed by formaldehyde agarose gels or analysis of the sample on a Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA was deemed of a suitable quality for microarray analysis if the 260/280 ratio was between 1.6 and 2.0 and the gel and Bioanalyzer traces showed no visible degradation products lower than the 18S ribosomal band. An equal amount of RNA extracted from the kidney of all rats in a treatment group was pooled into single samples. In select instances RNA from kidney of individual animals was also analyzed by microarray and/or by RT–PCR. Samples were stored at −80°C until hybridization.

cDNA Microarray Manufacturing

Gene expression analysis was conducted on the same RNA samples using several platforms including Affymetrix (Santa Clara, CA) and NIEHS custom cDNA microarrays. The analysis of samples using the Affymetrix platform was performed according to the manufacturer details (Affymetrix 2003; Lockhart et al. 1996) and is detailed in the accompanying paper (Thompson et al. 2004).

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the NIEHS web site (NIEHS Microarray Group 2003a). Methods were adapted from DeRisi et al. (1996) and are available on the NIEHS web site (NIEHS Microarray Group 2003b).

cDNA Microarray Hybridization and Analysis

For microarray hybridizations, each total RNA sample (35–75 µg) was labeled with Cyanine 3 (Cy3)- or Cyanine 5 (Cy5)-conjugated dUTP (Amersham, Piscataway, NJ) by a reverse transcription reaction using the reverse transcriptase SuperScript (Invitrogen, Carlsbad, CA) and the primer Oligo dT (Amersham). Control samples were labeled with Cy3, whereas samples derived from chemically exposed animals were labeled with Cy5. The fluorescently labeled cDNAs were mixed and hybridized simultaneously to the cDNA microarray chip. Each RNA pair was labeled and hybridized independently in duplicate or quadruplicate to a total of two to four arrays. The cDNA chips were scanned with an Axon Scanner (Axon Instruments, Foster City, CA) using independent laser excitation of the two fluoros at 532 and 635 nm wavelengths for the Cy3 and Cy5 labels, respectively. Image analysis, background subtraction, normalization, and determination of significant gene changes were conducted using the ArraySuite, version 2.0 extensions of the IPLab image processing software package (Scanalytics, Fairfax, VA) (Chen et al. 1997) as previously described (Bushel et al. 2001, 2002; Hamadeh et al. 2002a, 2002b, 2002c).

Hierarchical cluster analysis was carried out with the Cluster, version 2.12, and TreeView, version 1.6, package (Eisen et al. 1998). The cDNA data set is available from NIEHS (NIEHS Microarray Group 2003c). The complete data set is currently being submitted to ArrayExpress (EMBL-European Bioinformatics Institute, Hinxton, U.K.; http://www.ebi.ac.uk/arrayexpress) and will be available for public download by the second quarter of 2004. Accession numbers referencing this data set will be available on the HESI web site (http://hesi.ilsi.org/index.cfm?pubenti=120). GeneSpring, version 4.2 (Silicon Genetics, Redwood City, CA), Cluster, and TreeView software (Eisen et al. 1998) were used to perform the self-organizing maps (SOM) procedure and/or visualize sets of genes constituting the union of all significant gene changes from each dose and time point. SOM is a neural network architecture that categorized similar trends in the expression pattern of genes across multiple doses and time points. In addition, Partek Pro, version 5.02 (Partek Inc., St. Charles, MO), was used to further elucidate discriminate gene sets using principal component analysis (PCA).

Real-Time Quantitative PCR

RNA samples representing single animals treated with cisplatin or gentamicin were also used to validate the expression profile of several genes identified using cDNA microarray data including osteopontin, inositol polyphosphate multikinase, l-arginine glycine amidinotransferase, prosaposin, lipocalin, synaptogyrin 2, kallikrein, KIM-1, and an expressed sequence tag (EST) (GenBank accession no. AA957270). The primers for these genes were designed using Primer Express (Applied Biosystems, Foster City, CA) and custom made (Bioserve Biotechnologies, Laurel, MD) except for renal kallikrein primers and probe that was purchased from Applied Biosystems assay by design service.

Real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) according to the manufacturer instructions. Primer and probe sequences are listed in later section. Primer and probe concentrations were optimized such that the amplification efficiency of the endogenous reference was similar to that of the target gene. The endogenous reference used for samples was actin RNA. For the cisplatin samples the SYBR Green I labeling kit (Applied Biosystems) was used according to the manufacturer instructions to detect double-stranded DNA generated during PCR amplification. Reverse transcription and PCR reactions were performed at the same time in a 50-µL reaction containing 4 mM magnesium chloride, 0.8 mM of each dNTP, 100 ng total RNA, 0.4 µM reverse primer, 0.4 µM forward primer, 0.4 U/µL RNasin, 0.025 U/µL AmpliTaq Gold DNA polymerase (Roche), and 0.25 U/µL MuIV reverse transcriptase (Roche). Amplification reactions were carried out using the following temperature profiles: 48°C, 30 min; 95°C, 10 min; 95°C, 15 sec; 60°C, 1 min) for 40 cycles. Fluorescence emission was detected for each PCR cycle and the threshold cycle (CT) values were determined. The CT value was defined as the actual PCR cycle when the fluorescence signal increased above the background threshold. Induction or repression of a gene in a treated sample relative to control was calculated as follows: fold increase/ decrease = 2[CT (exposed) − CT (control)]. Values are reported as an average of triplicate analyses. For the gentamicin samples, reverse transcription and PCR were carried out in a 25-µL reaction volume using 10 ng total RNA in triplicate per reaction, 5 nM forward and reverse primer, and 25 nM FAM-MGB–labeled probe. Taqman one-step PCR master mix reagent kit (Applied Biosystems) was used for all reactions according to manufacturer’s specifications. All reactions were carried out using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Transcript levels were measured using the comparative Ct methods described in User Bulletin No. 2 (Applied Biosystems 1997).

The following primers, listed in the 5’ to 3’ direction, were used for reverse transcription-PCR (RT–PCR) verification of gentamicin-induced gene expression changes: kallikrein (GenBank accession no. M19647) forward = GCACCGGCTTT GTCAGTCAA, probe = CCCTCACC CTGACTAC, reverse = TCGGGTGTTG GTTCCCTCATG; prosaposin (GenBank accession no. NM_013013) forward = AAGAGCCCAACGAGCTCGCAT, probe = CGCCCCATGTCGCT, reverse = TGT TCCAATAGTGACACCGCTT CT; osteopontin (GenBank accession no. M99252) forward = CCAGCACACAAAG CAGAGTTT, probe = CAGTCTGATGT CCTGAC, reverse = CAGTCCGTAA GCAAGCTATCA; KIM-1 (AF35963) forward = CGCAGAGAAACCGCAG TAAG, reverse = CAAAGCTCAGAGAG CCCCATC; glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (GenBank accession no. M17701) forward = AGATGG TGAAGGTCGGTGT, reverse = ACTG TGTTCTAGCT GCTTCCT; cyclin B1 (GenBank accession no. J01435) forward = CGTCACTGCCCCA TGCAATTCG, reverse = CTGGTCTACCC TGCTACAGCT.

The following primers, listed in the 5’ to 3’ direction, were used for RT–PCR verification of cisplatin-induced gene expression changes: Mimpk (GenBank accession no. NM_134417) forward = TGCCCTGTTCCA CACTACATACA GATG, reverse = CGGGATTTGGAAT CGGTCTT; l-arginine glycine amidinotransferase (GenBank accession no. U07971) forward = CGAGGCCGGTGC ACTATAC, reverse = GCAGCAGGAATT TTTGGGAGGA; lipocalin/EST (GenBank accession no. NM_130741) forward = GCAGTTGGCCTAGTGTTTCA, reverse = GCAAGGAAATT TGCGAG GAA; synaptogyrin 2 (GenBank accession no. AF058493) forward = TGCTCG GCTCCCAACTT, reverse = CTGGGACG AGGTTGGCACGTA; prosaposin = GenBank accession no. M19936) forward = ACTGCTGCCCCATGCAAT, reverse = AAGTAGGGCACTTCTGCAA GT.
Western Blots
Samples of renal tissue were homogenized in 10 mM potassium homogenization buffer containing 250 mM sucrose, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, adjusted to pH 7.6. Homogenates were centrifuged at 3,000 × g for 5 min and 9,000 × g for 15 min. The renal homogenates (50 µg) were separated by electrophoresis on an 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and transferred to a nitrocellulose membrane in a transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) at 100 volts at 4°C for 2 h. The membranes were blocked in a TBST-20 buffer (10 mM Tris, 150 mM NaCl, and 10% nonfat dry milk) overnight. The membranes were washed with TBST-20 and then developed with enhanced chemiluminescence (Pierce Hospital, Boston, MA) for 2 hr. The membranes were blocked with bovine serum albumin. The sections were incubated with a 1:200 dilution of a horseradish peroxidase–coupled secondary antibody. The membrane was washed with TBST-20 and then developed using a avidin-biotin-peroxidase complex, counterstained with H&E, and viewed using a 60x oil objective.

Immunohistochemistry
Paraffin sections 3 µm thin were prepared and mounted on glass slides. The sections were deparaffinized, rehydrated, and permeabilized with hyaluronidase (1 mg/mL) in a sodium acetate buffer. The sections were placed in an antigen-retrieval solution at 95°C for 45 min (Dako Corp., Carpentaria, CA) and nonspecific binding was blocked with bovine serum albumin. The sections were incubated with a 1:200 dilution of a KIM-1 primary antibody (provided by J. Bonventre) or vimentin antibody, washed with Tris-buffered saline (provided by J. Bonventre) or vimentin antibody, washed with Tris-buffered saline and exposed to a biotinylated secondary antibody for 1 hr. The sections were developed using an avidin-biotin-peroxidase complex, counterstained with H&E, and viewed using a 60x oil objective.

### Table 1.
| Dose (mg/kg) | Body weight (g) | Kwt (g) | Cr (mg/dL) | BUN (mg/dL) | Glucose/Cr | Prot/Cr | Ca/Cr | Protein excretion (mg/day) |
|-------------|-----------------|--------|------------|-------------|------------|--------|------|---------------------------|
| Vehicle     | 279 ± 7         | 2.5 ± 0.1 | 0.30 ± 0.01 | 15 ± 1      | 0.23 ± 0.01 | 1.0 ± 0.1 | 0.07 ± 0.01 | 39 ± 3                    |
| 0.3         | 288 ± 8         | 2.7 ± 0.1 | 0.24 ± 0.02 | 14 ± 1      | 0.17 ± 0.06 | 1.1 ± 0.1 | 0.11 ± 0.02 | 28 ± 3                    |
| 1           | 289 ± 10        | 2.7 ± 0.1 | 0.22 ± 0.04 | 11 ± 1      | 0.15 ± 0.04 | 1.5 ± 0.2 | 0.12 ± 0.03 | 39 ± 4                    |
| 5           | 252 ± 3*        | 2.7 ± 0.3 | 1.4 ± 0.4*  | 63 ± 20*    | 6.1 ± 2.0*  | 1.8 ± 0.3* | 0.23 ± 0.08* | 66 ± 10                   |

### Table 2.
| Dose (mg/kg) | Body weight (g) | Kwt (g) | Cr (mg/dL) | BUN (mg/dL) | Glucose/Cr | Prot/Cr | Ca/Cr | Protein excretion (mg/day) |
|-------------|-----------------|--------|------------|-------------|------------|--------|------|---------------------------|
| Vehicle     | 269 ± 7         | 2.0 ± 0.1 | 0.19 ± 0.02 | 16 ± 1      | 0.19 ± 0.02 | 0.5 ± 0.1 | 0.07 ± 0.01 | 5 ± 1                     |
| 2           | 278 ± 8         | 2.1 ± 0.1 | 0.15 ± 0.02 | 13 ± 1      | 0.16 ± 0.02 | 0.4 ± 0.1 | 0.06 ± 0.01 | 4 ± 1                     |
| 10          | 283 ± 9         | 2.1 ± 0.1 | 0.16 ± 0.03 | 14 ± 1      | 0.20 ± 0.02 | 0.5 ± 0.1 | 0.09 ± 0.02 | 28 ± 2                    |
| 80          | 264 ± 7         | 2.2 ± 0.2 | 0.16 ± 0.03 | 15 ± 2      | 0.23 ± 0.02 | 1.3 ± 0.1* | 0.14 ± 0.07* | 14 ± 1*                   |
| 240         | 252 ± 8         | 3.2 ± 0.2* | 1.9 ± 0.1*  | 127 ± 41    | 0.49 ± 0.01* | 4.9 ± 0.3* | 0.26 ± 0.07* | 142 ± 31*                 |

### Table 3.
| Dose (mg/kg) | Body weight (g) | Kwt (g) | Cr (mg/dL) | BUN (mg/dL) | Glucose/Cr | Prot/Cr | Ca/Cr | Protein excretion (mg/day) |
|-------------|-----------------|--------|------------|-------------|------------|--------|------|---------------------------|
| Vehicle     | 269 ± 7         | 2.0 ± 0.1 | 0.19 ± 0.02 | 16 ± 1      | 0.19 ± 0.02 | 0.5 ± 0.1 | 0.07 ± 0.01 | 91.7 ± 17                 |
| 5           | 278 ± 8         | 2.1 ± 0.1 | 0.15 ± 0.02 | 13 ± 1      | 0.16 ± 0.02 | 0.4 ± 0.1 | 0.06 ± 0.01 | 39.5 ± 44                 |
| 10          | 280 ± 6         | 2.1 ± 0.1 | 0.16 ± 0.03 | 13 ± 1      | 0.20 ± 0.02 | 0.5 ± 0.1 | 0.09 ± 0.02 | 1991 ± 1728               |

Abbreviations: Ca, calcium; Cr, creatinine; Kwt, total kidney weight in grams; Prot, protein.

### Clinical Chemistry and Urinalysis

**Cisplatin.** A summary of the clinical chemistry data for all three nephrotoxicants is presented in Table 1. The low dose (0.3 and 1 mg/kg) of cisplatin produced only minor changes in the histologic appearance of the kidney or clinical chemistry at any time point during the experiment. The high dose (5 mg/kg) of cisplatin had minimal effect on urine or serum chemistry relative to the values seen in the time-matched control animals injected with vehicle at 4, 24, and 48 hr (data not shown).

**Gentamicin.** A summary of the clinical chemistry data for all three nephrotoxicants is presented in Table 1. The low dose (0.3 and 1 mg/kg) of cisplatin produced only minor changes in the histologic appearance of the kidney or clinical chemistry at any time point during the experiment. The high dose (5 mg/kg) of cisplatin had minimal effect on urine or serum chemistry relative to the values seen in the time-matched control animals injected with vehicle at 4, 24, and 48 hr (data not shown).

**Puromycin.** A summary of the clinical chemistry data for all three nephrotoxicants is presented in Table 1. The low dose (0.3 and 1 mg/kg) of cisplatin produced only minor changes in the histologic appearance of the kidney or clinical chemistry at any time point during the experiment. The high dose (5 mg/kg) of cisplatin had minimal effect on urine or serum chemistry relative to the values seen in the time-matched control animals injected with vehicle at 4, 24, and 48 hr (data not shown).
shown). However, 7 days after injection of the 5 mg/kg dose of cisplatin, plasma creatinine and serum BUN concentration were elevated by approximately 5-fold. Rats treated with the high dose of cisplatin exhibited glucosuria (elevated glucose/creatinine concentration ratio) indicative of proximal tubular injury. They also exhibited a 2-fold increase in urine protein and calcium concentration.

**Gentamicin.** Daily treatment of the rats with 2 and 10 mg/kg doses of gentamicin produced very little change in serum or urine clinical chemistry compared with the values seen in time-matched vehicle control animals at day 7 (Table 1). Urinary total protein was significantly increased \( (p < 0.001) \) in rats dosed at 80 mg/kg/day at 4 hr and at 7 days. They also exhibited an increase in urinary \( \text{Ca}^{2+} \) concentration at 4 hr \( (p < 0.01) \) and 2, 3, and 7 days \( (p < 0.01) \) after administration of gentamicin (data not shown). Because the degree of renal injury was minimal in the strain of rat SD-ICS (Charles River Labs) used in the present studies, we repeated the study in three additional rats using a higher dose of gentamicin (240 mg/kg/day). This dose produced the expected severe renal injury as reflected by the marked increase in BUN and creatinine. These rats also exhibited a pronounced proteinuria and elevated levels of glucose and \( \text{Ca}^{2+} \) in the urine (Table 1).

**Puromycin.** Rats treated with puromycin (20 mg/kg/day) for 21 days exhibited marked proteinuria (Table 1) and an elevation in urinary \( \text{Ca}^{2+} \). The chronic loss of protein in the urine was reflected by a dramatic fall in serum protein and albumin levels at day 21 in rats chronically exposed to 20 mg/kg/day puromycin. Despite the marked proteinuria, BUN and creatinine were not significantly elevated at any of the time points in the study, indicating that the glomerular filtration rate (GFR) remained relatively normal in these rats.

### Renal Histopathology

**Cisplatin.** The low dose (0.3 mg/kg) of cisplatin had very little effect on the histology of the kidney. Four of the five animals treated with the 1 mg/kg dose of cisplatin exhibited some degree of single-cell tubular necrosis and some mild tubular degeneration at 48 and 144 hr after administration of the compound. Rats treated with the high dose (5 mg/kg) of cisplatin exhibited severe proximal tubular necrosis and the formation of protein casts, especially in the pars recta of proximal tubules (Table 2; Figure 1A, B). There was also evidence of mild interstitial inflammation and edema in both the renal cortex and outer medulla, and some glomeruli exhibited thickening of the basement membrane.

**Gentamicin.** Rats treated with gentamicin at 2 and 10 mg/kg/day exhibited no discernable renal pathology. Even the rats treated with the higher dose of gentamicin (80 mg/kg/day) exhibited only subtle compound-related changes in the histology of the kidney after 3 and 7 days of exposure (Table 2; Figure 1C, D). These changes included minimal degeneration of some proximal convoluted tubules, apoptosis, tubular basophilia, tubular casts, increased mitotic figures, and mononuclear cellular infiltrates. Ultrastructural analysis revealed that one control animal had essentially normal proximal tubular epithelium, whereas another had hyaline droplets of different densities (Figure 1E). The proximal tubular epithelium of two treated animals exhibited large lysosomes of varying density that accumulated lamellar material forming typical myeloid bodies (Figure 1F). In addition, all of the animals treated with

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**Figure 1.** Histopathological evidence of cisplatin- and gentamicin-induced proximal tubular toxicity. Representative H&E-stained sections histological sections from kidney of (A) vehicle-treated (magnification 20×), (B) cisplatin-treated rats (5 mg/kg, 6 days) (magnification 20×), (C) vehicle-treated rats (magnification 200×), and (D) gentamicin-treated rats (80 mg/kg/day, 7 days) (magnification 200×). Representative electron micrographs from (E) vehicle-treated rats (magnification 3,000×) and (F) gentamicin-treated rats (80 mg/kg/day, day 7) (magnification 4,500×). Increased apoptosis and cellular infiltrates and myelin figures were observed in rats treated with gentamicin (80 mg/kg/day, day 7).
240 mg/kg/day gentamicin for 7 days exhibited severe proximal tubular necrosis and formation of protein casts in the proximal tubule.

**Puromycin.** There were minimal background findings in the control and treated groups, defined as less than three foci of the following: interstitial lymphocytic inflammatory infiltrates, interstitial fibrosis, tubular mineralization, and tubular cysts in the cortex or medulla. There was a dose-related minimal increase in severity of one or more of the following lesions: interstitial lymphocytic cellular infiltrate, glomerular sclerosis, lymphocytic perivascular cellular infiltrate in the renal capsule, and tubular regeneration in one of five animals from each time point (Figure 2; Table 2). The most significant findings were dilation of renal tubules, protein casts, expansion of the mesangial matrix material in the glomerulus, and focal segmental glomerulosclerosis in the rats treated with puromycin (20 mg/kg/day) for 21 days. In two of five rats there were multiple areas of involvement in the renal cortex, which occupied approximately 25–35% of the kidney. Sloughed tubular epithelial cells were sometimes present in the tubular lumen. However, the majority of the glomeruli appeared normal at the light microscopic level.

**Identification of Tubular Toxicity Based on Gene Expression Changes**

For each treatment (compound, dose, and time), genes with statistically significant expression changes were identified using the approach described by Bushel et al. (2001). Hierarchical clustering was used to aid in visualization and biological interpretation of this extensive data set, and in particular, to identify correlated expression patterns that reflect potential biological/toxicological processes occurring in the renal tissues of the animals treated with cisplatin, gentamicin, or puromycin at varying doses and time points. Hierarchical clustering, described by Eisen et al. (1998), was applied across the various treatment groups, doses, and time points, using a combined list of genes identified to be altered statistically in at least one of the samples studied relative to control (Figure 3A, B). Using this approach, it was possible to extract correlated patterns and natural classes present in the data set that could be correlated with biological processes relevant to nephron segment-specific toxicity as well as gene expression alterations reflecting histopathological changes.

Hierarchical clustering revealed two distinct separations in experimental groups that correlated with the severity of renal toxicity. As shown in Figure 3A, the hierarchical order of the clustering tree indicated several correlations and allowed sample separation based on severity, region, and type of toxicity. For instance, the hierarchical ordering of gene expression changes by cisplatin (5 mg/kg/day, 24 and 144 hr), gentamicin (2, 10, 80, and 240 mg/kg/day, day 7), and puromycin (5 mg/kg/day, day 7; 20 mg/kg/day, days 1 and 7) fall within one node and are much more closely correlated compared with the remaining lower-dose, earlier time point samples. This separation in the expression patterns appeared to be correlated with the severity of the renal injury (based on changes in serum biochemistry and/or urinalysis changes or histopathology) and are linked to the treatment, dose, time, and severity of each of these compounds. The second major node separating the remaining treatment groups consists of samples associated with less prominent renal toxicity.

**Figure 2.** Representative H&E-stained histological sections from kidney of (A, B) vehicle-treated and (C, D) puromycin-treated rats. (A, B) Intact renal tubules and glomeruli and uniform nuclei are observed in controls. In addition, uniform tubules with single layer of epithelium lining are observed in renal cortex of control rats. (C) Increased protein casts and necrosis of renal tubular cells observed in rats treated with puromycin (20 mg/kg/day, day 21). (D) Thickened glomerular membranes and affected tubules observed in rats treated with puromycin (20 mg/kg/day, day 21). Magnification 40×.
Figure 3. Hierarchical clustering, as described by Eisen et al. (1998), was applied across the various treatment groups, doses, and time points, using a combined list of genes identified to be altered statistically significantly in at least one of the samples studied relative to control. Gent, gentamicin. (A) Separation of experimental groups. The A and B notation behind the high-dose cisplatin indicates two biological replicates. Gene expression changes were determined using 2–4 replicate hybridizations from pooled kidney samples and include fluor-reversal. (B) Node containing several putative markers of renal toxicity. Green indicates genes that are downregulated; red indicates genes that are upregulated in treated animals relative to control animals. Accession numbers are from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/). (C) PCA of cisplatin-induced (red), gentamicin-induced (green), and puromycin-induced (yellow) gene expression changes at varying doses and time points. The labels of severity and region of toxicity were overlaid from the clinical pathology data.
Clustering based on gene expression profiling of samples obtained from independent replicate experiments performed using the same compounds and protocols can reveal if there are variations in gene expression changes across studies. In Figure 3A, cisplatin 5 mg/kg, 144 hr (A) and cisplatin 5 mg/kg, 144 hr (B) represent renal gene expression changes observed based on two independent experiments performed with cisplatin in different groups of animals (A and B denote the first and second biological experiments, respectively). The gene expression changes observed in these two different experiments performed with cisplatin at 5 mg/kg/day were highly correlated, indicating minimal overall variability in gene expression changes across two independent *in vivo* experiments, and hence the profiles clustered together.

Multidimensional visualization by PCA using all of the statistically altered renal gene expression changes was used to observe the spatial distribution of the treatment groups in multidimensional space (Figure 3C). This approach offers an opportunity to visualize expression patterns that can reflect similarities in biological responses. The visualization of high-dimensional data in two- or three-dimensional principal components reveals unsupervised clusters within the data. Samples can be scored using the results of a PCA on known samples, and these scores may place the unknown into one of the previously identified clusters where gene expression changes are clearly linked to biological/pathological events. As shown in Figure 3C, three major separations of the various treatment groups were evident. The first includes treatment groups in which severe renal proximal tubular toxicity was observed (cisplatin 5 mg/kg, 24 and 144 hr). The data from these groups were clearly separated from early time points after exposure to cisplatin and from the low-dose samples where no histologic evidence of tubular injury were observed. In addition, the second principal component shows separation of the high-dose cisplatin data with tubular injury from the puromycin data that exhibited marked proteinuria and glomerular toxicity.

The samples analyzed by microarrays in these experiments consisted of pooled samples. However, we further characterized the response of individual animals at one dose/time point for cisplatin (Thompson et al. 2004) and for gentamicin (day 7) and compared the response of these animals to the animals from a related, biologically replicated experiment. In the gentamicin replicate experiment, the results from animals dosed with 240 mg/kg of gentamicin for 7 days (to produce severe injury) were compared with the results obtained using the 80-mg/kg dose for 7 days in the initial experiment. The higher dose (240 mg/kg) resulted in the expected severe proximal tubular necrosis and proteinuria. Although not identical, the overall changes in renal gene expression observed after treatment with 240 mg/kg compared with 80 mg/kg gentamicin for 7 days were still closely correlated and hence clustered together despite the less severe changes in renal histology and threshold changes in the urinary excretion of protein, glucose, and Ca²⁺ observed after treatment with 80 mg/kg gentamicin (Figure 4).

The power of microarray analysis is that it allows elucidation of groups of genes that correlate with certain expression patterns that accompany particular types of biological effects. Analysis of groups or clusters of genes that contribute to similar pathways/functions may be more robust.
than focusing on a single gene. In addition, gene clusters may be used to identify known and novel putative gene-based markers of renal toxicity and can potentially direct future research to validate these putative gene-based markers in urine/serum using animal and clinical models.

Figure 5 shows a group of genes strongly downregulated in samples that exhibited proximal tubular necrosis. Many of these genes are functionally localized to the proximal tubules, further strengthening the hypothesis that regional-specific toxicity may be discerned through analysis of gene expression patterns (Table 3). Interestingly, the puromycin samples showed similar downregulation of some of these genes, indicating that tubular toxicity may have occurred from this treatment. Indeed, upon further histopathological evaluation, the less prominent tubular toxicity was observed in the rats receiving the higher dose of puromycin for 21 days (Figure 4).

**Elucidation and Verification of Putative Biomarkers**

One of the intended outcomes of these studies was the elucidation of putative new sensitive biomarkers of nephrotoxicity. We examined the significantly changed genes for those that displayed induction in a dose- and time-dependent manner for further verification in this study. Figure 6 shows a grouping of genes after high-dose cisplatin and gentamicin treatment that appear to be upregulated in a dose- and time-dependent fashion. Overlap of these genes on clusters derived from both the NIEHS and Affymetrix microarrays indicate several potential biomarkers of renal toxicity/repair, including KIM-1, osteopontin, vimentin, and several ESTs (Figure 6).

Multiple approaches were used to robustly determine and confirm the expression changes observed in the present microarray studies. First, the genes that were differentially modulated were defined using the approach of Chen et al. (1997) that employs a specified confidence level (95% in the present study) for determining differentially expressed genes. Second, to reduce false positives, replicate hybridizations (typically n = 4, with reverse labeling) were performed using the pooled samples and/or biological replicates, and a binomial distribution was used to model the results of the analyses at given confidence levels. Third, dye reversal was used to avoid false positives due to biases in Cy-dye-specific
Table 3. Downregulated gene expression changes correlated with regional specific damage.\(^a\)

| Glomerulus | Renal distribution and enzyme | Distribution\(^b\) | Cisplatin | Gentamicin |
|------------|------------------------------|------------------|-----------|------------|
| Adenosine-deaminase\(^c\) | S1 > S2 > S3 | √ | | |
| Proximal tubule | Glucose-6-phosphatase\(^d\) | S1 > S2 > S3 | | |
| | Fructose-1,6-bisphosphatase\(^d\) | S1 < S2 < S3 | | |
| | Phosphoenolpyruvate carboxykinase\(^d\) | S1 > S2 > S3 | | |
| | Fructokinase\(^a\) | S1 = S2 < S3 | | |
| | Fructose-1 phosphate aldolase\(^d\) | S1 = S2 > S3 | | |
| | Glycerokinase\(^a\) | S1 = S2 > S3 | | |
| | Glycerol 3-phosphate dehydrogenase\(^d\) | S1 = S2 = S3 | | |
| | Glutamine synthetase | S3 | | |
| | Glutathione S-transferase\(^d\) | S1 = S2 = S3 | | |
| | Alanine aminotransferase | S1 < S2 < S3 | | |
| | GABA transaminase | S1 < S2 > S3 | | |
| | Gamma glutamyltransferase | S1 < S2 < S3 | | |
| | Glycerokinase | S1 < S2 < S3 | | |
| | Fructokinase | S1 < S2 < S3 | | |
| | Phosphoenolpyruvate carboxykinase | S1 < S2 < S3 | | |
| | Isocitrate dehydrogenase | S1 < S2 < S3 | | |
| | Lactic acid dehydrogenase | S1 < S2 < S3 | | |
| | L-Hydroxyacid oxidase | S1 < S2 < S3 | | |
| | Gamma glutamyl cysteine synthetase | S3 | | |
| | Glucose-6-phosphatase | S3 | | |
| | Fructokinase | S3 | | |
| | Glutamine synthetase | S3 | | |
| | L-arginine-glycine amidinotransferase | 0.3 0.5 – – – – | | |
| | Inositol polyphosphate kinase | 0.5 0.7 – – – – | | |
| | Osteopontin | 5.2 4.1 0.9 1.1 1.4 3.9 | | |
| | Kallikrein | – – 1.01 1.0 0.5 0.3 | | |
| | Synaptogyrin 2 | 0.5 1.8 – – – – | | |
| | Prosaposin | 1.07 1.05 1.0 1.0 0.9 0.9 | | |
| | Aminopeptidases | 1.07 1.05 1.0 1.0 0.9 0.9 | | |
| | Lipocalin | 2 7.7 – c –– – – | | |

Abbreviations: \(\sqrt{\text{√}}\), change in gene expression observed; \(\sqrt{\text{√√}}\), change in gene expression observed in more than 1 gene in this family. \(^*\)Information modified from WHO (1991). \(^\text{S1, S2, and S3 denote renal tubular subregions.}\)

In addition, several of the observed gene expression changes identified in the present study were confirmed using alternate approaches, including RT–PCR, in situ hybridization, immunohistochemistry, and/or Western blot analysis. As shown in Table 4, RT–PCR analysis was performed using kidney RNA isolated from rats treated with cisplatin for 24 hr or 144 hr or gentamicin exposed for 7 days. Genes changed with microarray were generally in qualitative agreement when determined using RT–PCR. The expression of genes known to be implicated in the mechanism(s) of renal toxicity that were confirmed include kallikrein, heme oxygenase 1, clusterin, osteopontin, and KIM-1 (Table 4; Figure 7A). In addition, several

Figure 6. Putative gene-based markers of nephrotoxicity. Graph represents level of change relevant to control animals. The level of expression is shown as the log2 of the ratio of the expression in treated samples versus vehicle-treated samples. The values for the expression in cisplatin-treated animals is shown in closed bars, and the open bars represent the expression in gentamicin-exposed animals.

Table 4. Comparison of cDNA microarray and RT–PCR measurements.

| GenBank accession no.\(^a\) | Gene | RT–PCR (5 mg/kg) | cDNA microarray (5 mg/kg) | Day 2 RT–PCR | Day 7 RT–PCR |
|-----------------------------|------|-----------------|--------------------------|--------------|--------------|
| AA86431                     | Osteopontin | 5.2 | 4.1 | 0.9 | 1.1 | 1.4 | 3.9 |
| AA80117                     | Inositol polyphosphate kinase | 0.5 | 0.7 | – | – | – | – |
| AA820297                    | L-Arginine-glycine amidinotransferase | 0.3 | 0.5 | – | – | – | – |
| AA858514                    | Prosaposin | 1.07 | 1.05 | 1.0 | 1.0 | 0.9 | 0.9 |
| AI058493                    | Synaptogyrin 2 | 0.5 | 1.8 | – | – | – | – |
| AA957270                    | EST | 3.5 | 2.9 | – | – | – | – |
| M19647                      | Kallikrein | – | – | 1.01 | 1.0 | 0.5 | 0.3 |
| AA495030                    | Lipocalin 2 | 7.7 | – | – | – | – | – |

\(^a\)From GenBank (http://www.ncbi.nlm.nih.gov/GenBank/). \(^b\)Sequence not present on cDNA microarray. Observed based on similarity to KIM-1 expression on Affymetrix platform (data not shown).
genes for which there is limited understanding of a role in renal toxicity were validated. These include inositol polyphosphate multikinase and t-arginine-glycine amidinotransferase. In addition to RT–PCR verification, heme oxygenase and KIM-1 protein expression in rat kidney after cisplatin treatment was confirmed by Western blot analysis (Figure 7A–D). Using in situ hybridization, expression of KIM-1 was confirmed to be increased in the proximal tubule after exposure to cisplatin (Figure 8B). Increased vimentin expression was also confirmed by immunohistochemistry (Figure 8D).

RT–PCR verification of two ESTs identified to be induced in rat kidney after exposure to 5 mg/kg cisplatin for 144 hr was also performed. Lipocalin 2 (LCN2) (GenBank accession no. AA946503) expression was initially identified in cisplatin gene expression data generated with the same RNA on an Affymetrix platform (data not shown). In fact, the expression of LCN2 was similar to that observed for KIM-1. Induction of KIM-1 expression after cisplatin treatment was confirmed by in situ hybridization, RT–PCR, and Western blot analysis (Figure 7). The increased expression of another EST (GenBank accession no. AA957270; now known as a tumor necrosis factor (TNF) receptor superfamily, member 12a, or Tweak receptor) was verified using RT–PCR Table 4). The role of Tweak receptor in mediating cisplatin renal toxicity remains to be determined.

Discussion

In this study, we used cDNA microarrays to examine temporal changes in gene expression patterns in the kidney after treatment of rats with cisplatin, gentamicin, and puromycin, three nephrotoxins that primarily injure the proximal tubule (cisplatin and gentamicin) or the glomerulus (puromycin) via different mechanisms of action. The results of our microarray study are consistent with numerous reports of the modulation in expression of various mRNA after renal injury induced by cisplatin, gentamicin, or puromycin (Girton et al. 2002; Huang et al. 2001). For instance, using data from cisplatin treatment, it was possible for us to functionally annotate gene expression changes to various previously published and novel categories. These categories include biochemical pathways related to creatinine biosynthesis, osmoregulation, kinase signaling, cell cycle–related genes, renal transporters, renal injury, and regenerative responses, as well as gene expression changes related to drug metabolism, detoxification, and drug resistance.

For example, we detected a reduction in the expression of t-arginine-glycine amidinotransferase and guanidinoacetate methyltransferase at days 1 and 6, respectively, after treatment with 5 mg/kg cisplatin, a dose that induced renal lesions. Mapping of these gene products to a biochemical pathway points to a role for them in the formation of creatinine from t-arginine (see map in Kramer et al. 2004). Creatinine is formed from creatine and excreted by the kidneys. Traditionally, measurements of increased serum urea and creatinine are used clinically as indices of changes in glomerular filtration rate. However, they are relatively insensitive markers of glomerular injury, as typically up to 75% of nephrons have to be nonfunctional before there are significant elevations in serum levels of BUN or creatinine. Although ours is the first study to demonstrate the modulation in the expression of these genes in rat kidney after cisplatin treatment, Lee et al. (1998) demonstrated that cisplatin alters the expression of t-arginine-glycine amidinotransferase and guanidinoacetate methyltransferase in the male rat reproductive tract. Using Western blot analysis, Lee et al. (1998) demonstrated that t-arginine-glycine amidinotransferase is
In contrast, treatment with 5 mg/kg cisplatin that resulted in mild pathological changes, treated with 1 mg/kg, a dose of cisplatin arginine-glycine amidinotransferase in rats of toxicity and biochemical feedback mechanism, however, it may be indicative of progression of mesangial cells. In addition, NOS is present in the renal tubular segments and the juxtaglomerular apparatus and may enhance the renal damage incurred after nephrotoxicant administration.

The expression of kallikrein decreased in the kidney after gentamicin (80 mg/kg/day, 7 days) (Table 4). Kallikrein is a serine protease that cleaves kininogen to produce kinin, a vasoactive and natriuretic peptide (Clements 1989; Schmaier 2003). Kallikrein is produced and secreted in distal nephron segments and has been implicated in the control of sodium and water excretion and the long-term control of arterial pressure. More recently, kallikrein has also been implicated in determining the pathogenesis of renal injury after administration of nephrotoxicants. For example, Murakami et al. (1998) reported that systemic transfection of the kidney with an adenovirus expressing kallikrein attenuates the development of gentamicin-induced nephrotoxicity in rats. Murakami et al. (1998) suggest that the renoprotective effect of kallikrein gene delivery may be related to the ability of kinins to activate phospholipase A and prevent phospholipidosis, which is characteristic of gentamicin toxicity. In addition, recent studies have revealed that urinary kallikrein levels are reduced and there are polymorphisms in the kallikrein gene in patients with renal disease (Yu et al. 2000, 2002). These results suggest the potential utility of gene expression profiling to identify genes and pathways that may play a role in determining the genetic susceptibility to nephrotoxicity (tocogenomics).

Puromycin treatment, in the present studies, was associated primarily with glomerular toxicity, in addition to the less prominent proximal tubular toxicity. Numerous genes were identified as upregulated or downregulated at various doses and time points after puromycin treatment. Examples of genes upregulated primarily at 24 hr after treatment with 5 mg/kg puromycin include serum amyloid p-component (GenBank accession no. AA819830), discussed earlier), and cytosolic epoxide hydrolase (GenBank accession no. AA111911), cathepsin H (GenBank accession no. AA819336), cathepsin B (GenBank accession no. AA963225), alcohol dehydrogenase (GenBank accession no. AA875140), solute carrier family 4, member 4 (SLC4A4; GenBank accession no. A1144995), macrophage inflammatory protein 1-alpha (Mip-1-alpha; GenBank accession no. AA924105), interferon, alpha inducible protein 27-like (GenBank accession no. AA955996), retinol binding protein (GenBank accession no. AA860061), and glucose-6-phosphatase (GenBank accession no. AA964628). An increase in expression of transporter genes may function to facilitate reuptake of solutes and small molecules passing through the damaged glomerulus. Solute carrier family 4, for example, is involved in the coupled movement of sodium and bicarbonate; the expression of this gene may be among the renal responses for maintaining solute homeostasis during renal injury. Cathepsins are primarily cysteine-dependent lysosomal proteases and play an important role in protein degradation and turnover. The authors speculate that the upregulation of cathepsin genes may be a compensatory response to allow for protein degradation as the nephron compensates for proteinuria by increasing protein reuptake associated with glomerular toxicity. In addition to the genes described above, retinol-binding protein was upregulated in the present study and retinol-binding protein mRNA is known to be expressed in rat renal tissue. It is likely that the induction of retinol-binding protein may be in response to increased urinary loss associated with glomerular and/or tubular toxicity. Recent studies demonstrate that urinary retinol-binding protein has been investigated as a prognostic marker of proximal tubular dysfunction in patients with glomerulopathies (Kirschzajn et al. 2002).

In contrast to the observed upregulation of dentin sialophosphoprotein at 24 hr after treatment with 5 mg/kg/day puromycin, dentin sialophosphoprotein was among some of the more downregulated genes at 24 hr after treatment with 20 mg/kg puromycin. Acidic nuclear phosphoprotein 32 (GenBank accession no. A1070967), l-glycine arginine amidinotransferase (GenBank accession no. AA900287, discussed earlier), and cytosolic epoxide hydrolase (GenBank accession no. AA819330) were downregulated at 24 hr after treatment with 20 mg/kg puromycin. Although the role of these genes in the molecular responses to glomerular toxicity is unclear, the downregulation of some of these genes (i.e., cytosolic epoxide hydrolase, acidic nuclear phosphoprotein 32) was observed at both 24 hr and after 7 days of treatment with 20 mg/kg/day of puromycin. Hydrolases have been identified in rat renal tissue. Mesangial cells and have been implicated in glomerulonephritis (Nakao et al. 1999).
Renal lesions induced by cisplatin, gentamicin, and puromycin generally occur in discrete anatomical regions (e.g., tubular or glomerular components), although as observed in this study, injury can diffuse to additional regions of the nephron when toxicity is severe. Discrete toxicity to an organ with site-selective functional capacity suggests that observed changes of the biochemical properties of the affected renal region can provide pertinent information regarding the molecular outcome of the lesion. To take advantage of this in facilitating the interpretation of mRNA expression changes in our study, we used the published literature related to enzymology of kidney tissue to compile information regarding the expression of specific genes within specific regions of the nephron, particularly within various tubular segments (Table 3) (Mattenheimer 1968; WHO 1991). As shown in Figure 4, decreased expression of several mRNAs was detected by microarray profiling, particularly in kidneys of rats treated with cisplatin (5 mg/kg, day 6) and to a lesser extent after gentamicin treatment (80 mg/kg/day, day 7). One explanation could be that the loss of expression of these RNAs could, in part, reflect massive injury and death of proximal tubular cells. Although necrosis of tubular segments was confirmed histologically after treatment with each of the three compounds, it varied in severity (cisplatin > gentamicin > puromycin). One important point to highlight is that the large grouping of genes that are downregulated and grouped together in the clustering diagram (Figure 5) could, as a group, serve as a sensitive diagnostic indicator of proximal tubular damage. We confirmed this by using this set of genes to correctly predict puromycin-induced proximal tubular injury before histopathological analysis was conducted. Further work will be needed to validate the regulation of the expression of these genes during regenerative/repair processes.

This work further highlights the application of microarray in identifying putative biomarkers of injury. For practical considerations in detection assays, it may be most useful to consider those genes that have low expression in control tissue but are induced/upregulated with damage. In this article we have identified several potential biomarkers that appear to be upregulated in a dose- and time-dependent manner upon injury. These include KIM-1, osteopontin, clusterin, and several ESTs, including two that were recently defined, LCN2 and TNF receptor superfamily, member 12a (Tweak receptor). Although our study confirmed that the expression levels of these genes did increase in the kidney, further work will be needed to truly validate these as biomarkers of nephrotoxicity. Studies to investigate reversibility, specificity, and sensitivity will be needed as part of this validation (Kramer et al. 2004). One of these gene products, KIM-1, a membrane-spanning protein cleaved on the extracellular surface after tubular injury (Bailly et al. 2002). In addition, antibodies to KIM-1 are now being used to measure protein levels in human urine after renal ischemic injury (Han et al. 2002). This study indicates that KIM-1 may indeed be a specific marker of tubular injury, which could provide a more sensitive indication of damage compared with traditional clinical measurements (Han et al. 2002). As mentioned earlier, RT–PCR verification of two ESTs induced after exposure to 5 mg/kg cisplatin for 144 hr was performed. Tweak receptor, or TNF receptor superfamily, member 12 (GenBank accession no. AA957270), belongs to the TNF receptor superfamily. Death ligands and receptors such as TNF participate in apoptosis regulation in the course of renal injury (Ortiz et al. 2001), and its induction is likely involved in apoptotic signaling and regulatory mechanisms contributing to cisplatin-induced renal injury. LCN2 (GenBank accession no. AA946503) was identified as a putative biomarker of kidney injury based on the similarity of its expression pattern to KIM-1.

The human homolog of LCN2 is neutrophil-associated lipocalin (NGAL) and the mouse homolog is 24P3. LCN2 has been implicated in the regulation of cell homeostasis and immune response and can function as a carrier protein for the general clearance of endogenous and exogenous compounds (Flower 1996; Flower et al. 2000; Yang et al. 2003). Lipocalin products have been found in neutrophilic granules and may play a role in apoptosis (Devededy et al. 2001; Yang et al. 2003). NGAL/24P3 expression has also been detected in inflamed epithelia (Nielsen et al. 1996), perhaps explaining that the appearance of this transcript may be related to the damage occurring in the renal tubules and the accompanying infiltrates observed histopathologically. Relevant to the present studies is the role implicated for lipocalin superfamily members (i.e., NGAL/24P3) in inducing the formation of kidney epithelia (Yang et al. 2003). However, the underlying molecular signaling mechanism(s) are not fully known. NGAL protein has been identified as a complex with matrix metalloproteinases in urine of patients with cancer (Yang et al. 2001). Lipocalin-type prostaglandin D synthase (also known as L-PGDS or β-trace) is another member of the lipocalin family (Hoffmann et al. 1993; Nagata et al. 1991), and elevated serum and urinary levels of β-trace (L-PGDS) have been observed in patients with renal failure (Hoffmann et al. 1997; Melegos et al. 1999). L-PGDS is a secretary glycoprotein that catalyzes the isomerization of a precursor of prostanooids, prostaglandin (PG) H2, to produce prostaglandin D2 (Urade and Hayashi 2000). Oda et al. (2002) developed and evaluated an enzyme-linked immunosorbent assay (ELISA) method to screen for human urinary L-PGDS. Using this method, Oda et al. determined that L-PGDS excretion was significantly increased in patients with renal disease, even in the absence of increases in serum creatinine. Therefore, its ability to be monitored in urine of patients (Yan et al. 2001) and, in particular, the ability to monitor L-PGDS (a member of the lipocalin family) in urine of patients with epithelial renal disease warrants its further evaluation in models of renal injury. This premise is confirmed in these studies. In addition to the ESTs described above, the expression of several other ESTs (e.g., GenBank accession nos. AA819209, AA899472, and AA899737) were commonly modulated by cisplatin and gentamicin at doses that caused proximal tubular toxicity. In most instances, the expression of these ESTs correlated with severity of proximal tubular injury, and hence, expression was more robust in cisplatin-treated compared with gentamicin-treated rats with tubular toxicity. For instance, one of the ESTs (GenBank accession no. AA899472) appears to have weak similarity to dentin phosphophrotein precursor/dentin sialoprotein (DSP), whose role in response to renal tubular injury has not been previously studied. DSP is a 53-kDa protein that has an overall composition similar to that of the sialoprotein osteopontin (Ritchie et al. 1994). An increase in level of osteopontin mRNA expression in the present studies was observed with cisplatin and gentamicin treatment (Figure 3B) [for RT–PCR validation data, refer to Thompson et al. (2004)]. Although little is known about the function/role of DSP in response to renal injury, an increase in the expression of osteopontin in proximal tubular epithelium has been demonstrated in human and animal models of renal injury and can be associated with monocye or macrophage infiltration (Hudkins et al. 2001; Magil et al. 1997; Pichler et al. 1995). Khan et al. (2002) reported concomitant renal expression and urinary excretion of osteopontin in a rat model of ethylene glycol–induced calcium oxalate nephrothiliasis. Expression of osteopontin protein and mRNA in proximal tubular epithelium has been reported in...
the absence of prominent macrophage influx in both pretransplant donor biopsies and biopsies with cyclosporine toxicity (Hudkins et al. 1999, 2001).

Induction of clusterin expression in these studies was observed after cisplatin- and gentamicin-induced renal injury and has great potential to be used as a biomarker of nephrotoxicity. Clusterin is a secreted heterodimeric glycoprotein that circulates in blood at concentrations of 50–300 µg/mL (Rosenberg and Silkensen 1995a, 1995b). Clusterin has been induced in a variety of models of renal tubular injury and/or remodeling after treatment with cisplatin (Huang et al. 2001; Silkensen et al. 1997) as well as in tubular epithelial cells, in response to proteinuria, in kidneys of nephrotic rats (Correa-Rotter et al. 1998) after puromycin treatment. Depletion of clusterin enhances immune glomerular injury in the isolated perfused kidney (Saunders et al. 1994), and mice deficient in clusterin are more susceptible to injury due to immunocomplexes (Rosenberg et al. 2002). Urinary and serum clusterin levels have been studied in various models of renal injury (Aulitzky et al. 1992) and may play a role in early detection of renal injury. Clearly, the potential use of clusterin as a biomarker of nephrotoxicity has to be confirmed.

Numerous efforts are ongoing to identify and validate markers of renal function and injury in rodent and human models. Muramatsu et al. (2002) demonstrated that cysteine-rich protein 61, a secreted growth factor–inducible immediate early gene, is induced in proximal straight tubules of rodents within 2 h of renal ischemia and can be detected in rat urine as early as 3–6 h after renal injury. Cystatin C is a 13-kDa plasma protein that inhibits cysteine proteases and is freely filtered at the glomerulus. Cystatin C appears to have improved diagnostic accuracy compared with creatinine measurements as a serum marker of GFR (Christensson et al. 2003; Laterza et al. 2002; Newman 2002). Oda et al. (2002) have demonstrated the utility of monitoring lipocalin-type urinary β-trace in patients with renal disease. Star et al. (2002) noted that the approach described in the article by Oda et al. (2002) for developing and evaluating an ELISA-based approach for lipocalin-type urinary β-trace provides valuable insight into efforts that need to be undertaken to translate a novel marker into a valuable laboratory assay.

In conclusion, this study indicates that gene expression arrays may be used to discern regional specific damage of nephrotoxicity. Additionally, this study provides a foundation for opportunities that exist to apply gene expression profiling to begin identifying novel markers of renal toxicity. Further expansion of these analyses may lead to the identification of groups of genes that could ultimately result in the detection of sensitive markers of renal damage. Traditional measures of renal damage such as proteinuria, creatinine clearance, and elevated BUN, usually occur after significant kidney damage has occurred. Elucidation and validation of new sensitive markers could help clinicians more efficiently monitor patients who are administered potentially nephrotoxic drugs.

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