Relationship between Expression of Bcl-2 Genes and Growth Factors in Ischemic Acute Renal Failure in the Rat

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Abstract. The promotion of cell survival and regeneration in acute renal failure (ARF) is important for restitution of renal function. This study analyzes the temporal and spatial relationship between expression of pro- and anti-apoptotic members of the Bcl-2 gene family (Bcl-2, Bcl-XL, Bax) and epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), and transforming growth factor-β (TGF-β), growth factors that are thought to be reparative in ARF. A rat model of ischemic ARF involving 30 min of bilateral renal artery occlusion followed by reperfusion for 0 to 14 d was used. Apoptosis and mitosis were quantified and qualitative assessment was made of other cellular damage including necrosis and loss of cellular adhesions. Locality and level of expression of the Bcl-2 and growth factor proteins were determined using immunohistochemistry. Apoptosis peaked between 4 and 14 d postischemia in both proximal and distal tubules. Mitosis peaked at 2 d in proximal tubules and 4 to 14 d in the distal tubules. A spatio-temporal relationship was observed between anti-apoptotic Bcl-2 gene family members and growth factors after ischemia-reperfusion. In control kidneys, expression of Bcl-2, Bcl-XL was low in epithelium of distal tubules, Bax had low-to-moderate expression in the proximal tubule and had low expression in the distal tubule, EGF and IGF-1 had low-to-moderate expression in the distal tubule, and TGF-β had low expression in the proximal tubule. In contrast, within 24 h of reperfusion, distal tubules showed a marked increase in expression of Bcl-2 and a moderate increase in Bcl-XL and Bax. Proximal tubules showed a marked increase in Bax expression and a moderate increase in Bcl-XL. Twenty-four hours after expression of the Bcl-2 proteins was increased, IGF-1 and EGF protein levels were increased in the distal tubule, similar to the Bcl-2 anti-apoptotic proteins, and were also detected in the adjacent proximal tubules, suggestive of paracrine action in these tubules. TGF-β expression was moderately increased in regenerating proximal tubules, but no relationship was seen with the pattern of expression of the Bcl-2 genes. An explanation of these results is that the distal tubule is adaptively resistant to ischemic injury via promotion of survival by anti-apoptotic Bcl-2 genes, and its survival allows expression of growth factors critical not only to the maintenance and regeneration of its own cell population (autocrine action), but also to the adjacent ischemia-sensitive proximal tubular cells (paracrine action).

A major contributor to the development and progression of acute renal failure (ARF) is the loss of functioning tubular epithelial cells by means of various cell deletion or death processes. Although the term “acute tubular necrosis” is still used to describe the pathology of ARF, this is a misnomer because apoptosis (1–4) and cell desquamation (5) also occur. With reference only to the death processes, the relative contributions of necrosis or apoptosis to ischemia-induced ARF probably depend on the severity of the initiating events. After prolonged renal ischemia, necrosis or accidental cell death occurs from the resultant negation of the cells’ energy and protein levels. The initiating ischemia for renal apoptosis is often mild or of short duration (6,7). For the apoptotic process, the cells use their own energy and proteins and thus offer a molecular means of modulating inappropriate levels of apoptosis in disease processes (8).

Susceptibility to necrotic or apoptotic injury varies along the nephron depending on the nature of the injury, hemodynamic changes, ongoing metabolic demands, and local oxygen availability (reviewed in references (9) and (10). Marked sensitivity to hypoxia has been demonstrated in both proximal tubules (11) and the medullary thick ascending limbs (12), although the relative importance of injury to each nephron segment remains controversial. In the medullary thick ascending limb, sensitivity was reduced by the presence of erythrocytes in an isolated perfused rat kidney model (13). Despite a new understanding of the pathology of ARF, however, patient mortality remains at 30 to 50% of ARF cases. Recovery depends not only on the replacement or regeneration of cells deleted by death, the theme of many recent studies, but also on protection of cells from death.

One of the major multigene families involved in the molecular controls of cell survival or death is the Bcl-2 gene family (14,15). Its members include inhibitors of apoptosis (e.g., Bcl-2, Bcl-XL, Bcl-w, and Mcl-1) or accelerators of apoptosis (e.g., Bax, Bcl-XS, Bak, Bik, and Bad). To date, approximately
15 members have been identified, all distinguished by highly conserved domains referred to as the Bcl-2 homology (BH) domains, of which five subtypes are known. Translated products of the Bcl-2 genes form homodimers or heterodimers to become functionally active (16–18). Heterodimerization between anti-apoptotic Bcl-2 and pro-apoptotic Bax may negate the function of either protein. After heterodimerization between, and neutralization of anti- and pro-apoptotic proteins, it is homodimerization of the excess protein that appears to determine the fate of the cell through its then active function.

The Bcl-2 genes are important in renal cell survival, development, and repair, although the exact functions of some of the lesser-known family members are not yet well defined. In fetal kidneys, the distribution of apoptotic cells is inversely correlated with expression of Bcl-2 (19,20). Bcl-2 deficiency causes increased metanephrine apoptosis, development of polycystic kidneys, and evidence of exfoliated renal epithelial cells in the convoluted tubules (21–23). Deregulated expression of Bax and Bcl-XL were also found to impair normal renal development, and repair, although the exact functions of some of the lesser-known family members are not yet well defined. In fetal kidneys, the distribution of apoptotic cells is inversely correlated with expression of Bcl-2 (19,20). Bcl-2 deficiency causes increased metanephrine apoptosis, development of polycystic kidneys, and evidence of exfoliated renal epithelial cells in the convoluted tubules (21–23). Deregulated expression of Bax and Bcl-XL were also found to impair normal renal developmental processes (24–26). The exact patterns of expression of members of this multigene family throughout the normal and diseased adult kidney are less clear. Low levels of Bcl-2 are expressed in the normal distal tubule, with higher levels of expression in the collecting tubule in the inner medulla (20,27). Augmented expression of several of the anti-apoptotic members of the Bcl-2 gene family, including Bcl-2 and Bcl-XL, has been found in biopsies of human renal disease states (27) and in experimental models of ischemic ARF (4). Bax expression is reported to be downregulated after some instances of ischemic renal damage, and is related to tubular regeneration (4).

The inter-relationship between altered expression of the Bcl-2 genes and expression of reparative growth factors has not been studied. Several key growth factors involved in renal tissue injury and repair, including in ARF, have been identified (reviewed in references (28) and (29)). These include epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), and transforming growth factor-β (TGF-β). Growth factors bind to specific receptors on the surface of cells, they may act locally via autocrine or paracrine mechanisms, and they may be increased or decreased in tissue injury and repair. Apoptosis, mitosis, and cellular differentiation can each be modulated by alterations in growth factor expression, which is regulated by serum depletion, reactive oxygen species, nitric oxide, or other stresses associated with ischemic injury (30–33).

In the present study of the molecular pathology of ischemic ARF using a rodent model, we have analyzed the relationship between temporal and spatial expression of several pro- and anti-apoptotic Bcl-2 genes (Bcl-2, Bcl-XL, and Bax) and selected key renal growth factors (EGF, IGF-1, and TGF-β). Functional parameters were compared with histopathology.

### Materials and Methods

Experiments followed the guidelines for care and use of animals in experimental research issued by the National Health and Medical Research Council of Australia. Ethical clearance number for the work was AECC PATH/106 (University of Queensland).

### Animals and Experimental Model

Mature male Sprague Dawley rats (200 to 250 g, n = 6 per ischemia treatment, n = 4 per control group) were housed at 21°C, 12-h light/dark cycle, and allowed free access to food and water. Under Nembutal anesthesia (pentobarbitone sodium, 60 mg/kg, intraperitoneally), both renal arteries were exposed using flank incisions, and clamped for 30 min. Control animals received sham treatment. After ischemia or sham treatment, clamps were removed, muscle layer incisions sutured, and skin incisions closed with Michel clips. For one group of animals, the kidneys were removed at the end of the ischemic period (T₀). For other groups, kidneys were reperfused for 1, 2, 4, 7, and 14 d when the relevant group of treated or control animals was given a lethal dose of Nembutal and kidneys were collected. Control and treated animals were weight- and age-matched at time of initiation of bilateral ischemic injury, and body weights were recorded at initiation and completion of experiments. The 30-min period for ischemia gave 100% survival in treated rats over the 2-wk study period.

### Functional Studies

Bladder urine and aortic blood were collected under anesthesia, just before euthanasia and collection of kidneys. Serum creatinine and urea, and urine creatinine, y-glutamyltranspeptidase (GGT) (an indicator of proximal tubular brush border damage), lactate dehydrogenase (LDH) (a cytoplasmic enzyme), and the lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAG) were used to estimate renal function or tubular and cellular integrity. Urine samples were centrifuged to remove insoluble salts and debris. Aliquots were stored at −20°C for the estimation of NAG after gel filtration on Sephadex G-25 (34,35). Urine samples for LDH and GGT were assayed on fresh unfrozen samples. Serum urea and creatinine were measured using an automated analyzer.

### Tissue Collection

At indicated times, both kidneys were rapidly removed, bisected transversely to their length, fixed in 4% buffered paraformaldehyde (pH 7.4) at 4°C overnight, then transferred to 70% buffered ethanol before preparation for normal histology and for immunohistochemistry. All antibodies that were used for the study responded well to tissue fixed in this way. Tissue was blocked routinely in paraffin, and 4-μm sections were cut onto Superfrost Plus slides for staining. Histologic stains included hematoxylin and eosin, periodic acid-Schiff reagent for protein localization, and Masson’s trichrome stain for collagen. Several extra animals (7 d reperfusion) were perfusion-fixed with physiologic saline followed by 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), and the kidneys were prepared routinely for electron microscopy for ultrastructural verification of apoptosis.

### Immunohistochemistry

Subserial sections were cut, numbered, deparaffinized, and rehydrated before staining with the peroxidase-anti-peroxidase method. Primary antibodies were: Bcl-2 (1:50 dilution; anti-human, Dako M887 mouse monoclonal and Santa Cruz polyclonal), Bax and Bcl-Xl/S (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA); EGF (1:200 dilution; anti-rat, R&D Systems, Minneapolis, MN), IGF-1 (1:1000 dilution; anti-rat, Groep, Adelaide, South Australia), TGF-β1 (1:50 dilution; Sigma, St. Louis, MO). The appropriate secondary antibody was applied at a dilution of 1 in 400 in Tris buffer, followed by the ABC-peroxidase reaction for 30 min. The chromogen was diaminobenzidine in 0.01% H₂O₂, applied for 2 to 5 min. Non-
specific binding of peroxidase or antibodies was blocked with 0.3% hydrogen peroxide (H₂O₂) in methanol followed by incubation in diluted normal swine serum. Negative controls were prepared without primary antibody, or with nonspecific serum. Positive tissue control sections from unrelated studies were included (Bel-2 against lymphoma and known positive rat parotid tissue, Bax and Bel-Xₐ against rat gut tissue Paneth cells, EGF against normal adult rat renal papillary collecting tubule cells, IGF-1 against known highly expressing rat renal distal tubular cells, TGF-β against rat renal vessel walls). Batch staining was done per antibody type. Sections were lightly counterstained with hematoxylin and then dehydrated to xylene and mounted in Depex.

In Situ End Labeling for DNA Fragmentation

Following the methods of Ansari and colleagues (36), sections were dewaxed, rehydrated, digested in 0.5% pepsin in 0.1 M HCl for 15 min, washed, and then labeled using the Klenow fragment of DNA polymerase I (Pharmacia, Rydalmere, NSW, Australia) and biotin-labeled dUTP (Boehringer-Manheim, Castle Hill, NSW, Australia). The reaction was terminated by washing in water, endogenous peroxidase activity was blocked with 0.1% H₂O₂, and biotin-labeled nuclei were visualized with horseradish peroxidase-conjugated avidin developed in diaminobenzidine and counterstained with hematoxylin. Negative controls had no Klenow polymerase, and positive controls were rat renal and hepatic sections from other experiments in which high levels of in situ end labeling (ISEL) were known to correlate with high levels of apoptosis assessed morphologically. (The ISEL method was counterchecked using another enzymatic technique called terminal deoxynucleotidyl transferase-mediated nick end labeling [TUNEL]) (37). No differences in levels of labeling were found between TUNEL and ISEL.

Proliferating Cell Nuclear Antigen Labeling for Cell Proliferation

Using methods described previously for immunohistochemistry, the proliferating cell nuclear antigen (PCNA) monoclonal antibody (1:50 dilution; PC10, Oncogene Sciences, Uniondale, NY) was applied at 4°C overnight, then processed routinely. Negative controls had nimmune serum, and positive controls were rat tissue with a known high proliferative rate that had also been assessed morphologically.

Assessment of Morphologic and Immunohistochemical Characteristics

Sections were labeled only with an identifying number and scored without knowledge of reperfusion time. Control sections had no pathologic changes. Light microscopic evaluation was performed at a magnification of ×400 (high-power microscope field, HPF) in 10 to 20 adjacent fields, and different factors were estimated in proximal and distal tubules. The term “proximal tubule” included both the proximal convoluted and straight segments. The term “distal tubule” included the thick ascending limb, the distal convoluted tubule, and the collecting duct. Proximal and distal tubules were distinguished using morphologic characteristics. Also used was periodic acid-Schiff positivity of the brush border or the luminal surface of the proximal tubule in damaged tubules where brush border vestiges were found.

Apoptosis. Previously defined morphologic criteria were used to count apoptotic cells (38,39). These characteristics included cellular rounding and shrinkage, eosinophilic cytoplasm, nuclear chromatin compaction especially along the nuclear envelope in a crescentic manner, membrane-bound cellular blebbing, and formation of apoptotic bodies that may appear in the tubular lumina or be phagocytosed by intrinsic renal cells or invading macrophages. Presence of apoptosis assessed in this way was verified in selected samples as described in Materials and Methods, using electron microscopy and ultrastructural characteristics of apoptosis.

DNA Fragmentation. ISEL nuclear positivity was counted in distal and proximal tubules. ISEL is an indication of DNA fragmentation and is often equated with apoptosis. A description of the need for caution with this method is found in the Discussion section. Care was taken in the current study to exclude labeled nuclei that had the cellular appearance of necrosis or mitosis.

Necrosis. This was assessed morphologically by cellular swelling and lysis, cytoplasmic eosinophilia, membrane rupture, densities in swollen mitochondria, pyknotic or irregularly clumped nuclear chromatin, and an associated leukocytic infiltrate that was absent in apoptosis (38,39). Other evidence of tissue damage, such as brush border loss, cytoplasmic blebbing, luminal cast formation, loss of cellular adhesion, and abnormal but non-necrotic nuclear swelling, was also recorded.

Cell Proliferation. PCNA-positive nuclei were counted. Mitotic nuclei were also assessed in comparison with PCNA labeling.

Bel-2, Bel-Xₐ, Bax, IGF-1, EGF, and TGF-β Expression.

Expression levels were determined by immunohistochemical localization and intensity in paraffin sections. An immunohistochemical index of five grades was recorded for tubular sections, for either proximal or distal tubules, according to published methods (20,40): 0 = no positivity; 1 to 4 based on arbitrarily designating the staining intensity of positive cells in comparison with control positive sections, which were designated for each batch as 4.

Statistical Analyses

Data were analyzed using standard statistical methods: ANOVA, t test for unpaired samples, or the Mann–Whitney U test. Most data are presented in graph form as the mean ± SEM. Significance was assessed at P < 0.05.

Results

Functional Studies

Serum creatinine levels increased at days 1 and 2, but this did not reach statistical significance (P > 0.05) (Figure 1A). Serum urea was significantly elevated 24 and 48 h after bilateral renal ischemia (P < 0.05) (Figure 1B), and returned to baseline values by day 4. The data for urinary enzymes for the control animals are shown as dashed lines in Figure 2. The brush border enzyme GGT showed a slight increase immediately after ischemia (Figure 2A), which corresponds with the loss of proximal tubular brush border in early ischemic injury. The cytoplasmic and lysosomal enzymes LDH and NAG, respectively, were elevated for several days but returned to baseline by day 14 (Figure 2, B and C). The patterns of excretion of urinary LDH and NAG reflect the ongoing tubular injury as described from the histologic sections.

Body Weights

Mean ± SEM of initial body weights, at time of assignment of animals to control or treated groups, and final body weights at time of euthanasia and collection of tissue, are shown in Figure 3. Mean body weights of ischemia-treated animals were...
Apoptosis or Necrosis

The kidneys of control animals had negligible levels of apoptosis assessed morphology, negligible ISEL, and no necrosis. Increased levels of apoptosis occurred in both the proximal and distal nephron segments postischemia compared with control sections. Morphologic assessment of apoptosis (Figure 4A) in distal or proximal tubular epithelium showed peaks of apoptosis to be in the later stages of the study (4, 7, and 14 d posttreatment). Proximal tubular necrosis and desquamation were evident, and maximal at 1 to 4 d postischemia (not shown in graph). In Figure 4B, counts of ISEL-positive nuclei peaked earlier (0 to 2 d) than the peak in apoptosis seen using morphology. The labeling is an indication of DNA fragmentation and is often equated with apoptosis. In the early times after reperfusion (up to 2 d), the amount of labeling did not indicate morphologically assessed apoptosis, or necrotic nuclear changes. An explanation for this is that there may have been reperfusion (free radical)-induced single- or double-strand DNA breaks that are reparable. Such repair processes have been described in earlier literature (41–43). These cells would label as positive soon after reperfusion, but most would repair rather than proceed to apoptosis. Photomicrography of ISEL, with and without morphologic evidence of apoptosis, is presented in Figure 4, C and D. Figure 4C demonstrates the high level of ISEL at 24 h posts ischemia, compared with ISEL

Figure 1. Serum creatinine and urea. (A) Serum creatinine in control rats (dashed line) and in treated rats (solid line). (B) Serum urea in control rats (dashed line) and in treated rats (solid line). Results are given as mean ± SEM. *P < 0.05, significant difference from control group (unpaired t test).

Figure 2. Urinary enzymes. Urinary γ-glutamyltranspeptidase (GGT) (A), lactate dehydrogenase (LDH) (B), and N-acetyl-β-D-glucosaminidase (NAG) (C) in control rats (dashed line) and in treated rats (solid line). Results are given as mean ± SEM.
Figure 3. Mean body weights ± SEM (initial and at end of experimental time point). No significant difference was found among body weights (P > 0.05) when comparisons were made between treated and control groups at each experimental time. At T0, animals have only one set of data because the initial body weights coincided with control groups at each experimental time.

Figure 5, A through F, demonstrates the pathology of the present model of ARF, using low- and high-power photomicroscopy to demonstrate specific features. Figure 5A demonstrates necrotic tubular epithelium adjacent to a tubule in which apoptotic cell death has occurred, at 24 h postischemia. In Figure 5B, also at 24 h postischemia, a proximal tubule has many apoptotic cells and the distal tubule below it has minimal damage. Cells indicated in the lumen in Figure 5C either have an apoptotic nuclear appearance (small arrows) or a more normal nucleus (large arrow), the latter being supportive of literature that reports desquamation of normal cells. Regeneration of the tubular epithelium at 2 d postischemia is demonstrated in Figure 5D. High-power light microscopy and ultrastructure provide verification of the presence of tubular epithelial cell apoptosis in Figure 5, E and F, at 4 and 7 d postischemia, respectively.

Cell Proliferation/Regeneration

The relative changes in cell proliferation after ischemia, compared with control levels, are shown in Figure 6A. Low levels of PCNA nuclear labeling were identified in sections from control animals. Proximal tubular epithelial regeneration peaked at 2 to 4 d and distal tubular regeneration at 4 to 14 d. Where desquamation of necrotic epithelium of the proximal tubule epithelium had occurred but basement membrane was preserved, complete regeneration of the tubular epithelium appeared to occur by 7 to 14 d with high levels of PCNA positivity detected. The results confirm the work of Witzgall and colleagues (44), who recorded high levels of PCNA labeling, particularly in the S3 segment, during regeneration after ischemic injury. Figure 6, B and C, demonstrates low levels of PCNA nuclear labeling in control renal sections (B) compared with the greatly increased labeling seen in regenerating renal tubular epithelium, illustrated here at 4 d postischemia (C). Figure 5D gives further morphologic evidence of tubular epithelial mitosis.

Temporal and Spatial Immunolocalization of Bcl-2 Proteins and Growth Factors

Figure 5 is a graphical summary of localization and expression levels of the Bcl-2 and growth factor proteins. Figure 8, A through F, and Figure 9, C through F, demonstrate the immunohistochemistry of the Bcl-2 proteins and growth factors, respectively. Localization of the proteins was generally cytoplasmic. Bcl-2 proteins were diffuse in the control sections but tended to be punctate in the sections from treated animals, indicating a relocation from the cytosol to cytoplasmic organelles such as mitochondria and endoplasmic reticulum. Immunoelectron microscopy would be needed to confirm the relocalization.

Control Animals. Expression of Bcl-2 and Bcl-XL expression was low in distal tubular epithelial cells (Bcl-2 is demonstrated in Figure 8A). Bax (Figure 8B) had low-to-moderate expression in distal and proximal tubules. EGF and IGFl were expressed at low intensity in the distal nephron (Figure 9, C and E, respectively), and TGF-β was expressed at low levels in the proximal convoluted tubule, the collecting duct, and vessel walls (presented graphically in Figure 7; histology not demonstrated).

Treated Animals and Bcl-2 Genes. During the acute phase (0 to 2 d), markedly increased Bcl-2 and moderately increased Bcl-XL expression levels were seen in distal tubule (Figure 8, E through F). Low levels of expression of Bcl-2 were occasionally seen in the proximal tubule, with moderate levels of Bcl-XL found. Expression of Bax was moderate in the distal tubule and markedly increased in the proximal tubules, with a trend toward higher expression in the proximal tubule by 2 d of treatment (Figure 8F). The temporal profile of expression in the proximal tubule was similar to that seen for Bcl-XL.

Where anti-apoptotic protein expression appeared to protect most cells of the distal tubule epithelium, adjacent proximal segments tended to have sporadic apoptotic, and not necrotic, cell death (Figure 8D).

Treated Animals and Growth Factors. EGF expression was decreased distally in the first 24 h after reperfusion, then increased to moderate-to-high levels (Figure 9, C and D). Moderate-to-high levels were detected in the proximal tubule from 2 to 7 d. The increase in EGF expression both in the distal and in the proximal tubules trailed the anti-apoptotic Bcl-2 proteins by 24 to 48 h. Figure 9D indicates the pattern of expression at 4 d postischemia. IGFl had a distal tubular expression pattern similar to, but trailing by 24 h, the pattern of
expression for Bcl-2. By 2 d, expression of IGF-1 was also detectable in the proximal tubules. Protein levels were then maintained in the proximal tubule until 14 d (Figure 9F demonstrates expression at 7 d). It was not apparent that the increased growth factor protein levels were anti-apoptotic, but their presence was often associated with increased levels of mitosis. TGF-β expression, both cytoplasmic and nuclear, was increased transiently (24 to 48 h) in the regenerating proximal tubule after damage, and there was a moderate increase in distal tubular expression detected at 7 and 14 d (not demonstrated in photomicrography). There was no apparent temporal or spatial association between Bcl-2 proteins and TGF-β.

Discussion

Several key apoptosis-regulating genes have been identified (reviewed in references (45) and (46). Although the caspase and Bcl-2 multigene families predominate, the one that appears to be particularly relevant to apoptosis in renal disease is the Bcl-2 family. Several key renal epithelial cell growth factors are known to participate in renal regeneration in ischemic ARF injury. Their action may be mediated via an anti-apoptosis balance of the Bcl-2 gene family cell death or survival genes.

Apoptosis has been recorded previously in both the proximal and distal tubular cell populations in ischemic renal injury (1–4,7,8). In the present study, we have verified a role for...
apoptosis in ischemia-induced ARF. However, the high levels of ISEL, described early (0 to 2 d) after reperfusion, need to be explained in comparison with the moderate increase in apoptosis identified at the same time points using morphologic means.

Figure 5. Selected morphologic changes associated with acute renal failure (ARF). Necrotic tubular epithelium (thick arrows) is found adjacent to a tubule in which apoptotic cell death predominates (thin arrows) at 24 h postischemia (A). In Panel B, also at 24 h postischemia, a proximal tubule has many apoptotic cells (thin arrows). The distal tubule below it has minimal damage. Panel C demonstrates desquamation of an apparently normal cell (thick arrow), as well as cells with an apoptotic nuclear appearance (thin arrows). Panel D shows regeneration of the tubular epithelium at 2 d postischemia (mitotic cell indicated by an arrow). Verification of the presence of apoptosis was provided by high-power photomicroscopy (Panel E, 4 d postischemia, apoptotic cell indicated by an arrow) and electron microscopy (Panel F, 7 d postischemia). In Panel F, the apoptotic cell (arrow) shows the typical condensation and margination of the nuclear chromatin as described for apoptosis in references (38) and (39), and has been phagocytosed by an adjacent epithelial cell. Magnification: ×450 in A and B; ×800 in C through E; ×7400 in F.

Several previous publications have shown that the enzymatic method of labeling DNA fragmentation, often associated only with apoptosis, will label necrotic (nonspecific DNA degradation) and sometimes mitotic (transient DNA strand breaks) as
well as apoptotic cells (3,41,47–49). In the current study, care was taken to exclude labeled nuclei that had the cellular appearance of necrosis or mitosis. However, there are other important considerations in this ARF model that used a period of ischemia followed by reperfusion. Free radical damage to DNA may occur after ischemia-reperfusion and may manifest as single- and double-strand breaks, both detectable by enzymatic labeling. Both forms of DNA strand breaks are repairable (40,42,50). Cell protection via Bcl-2 upregulation may allow time for DNA strand repair to occur. The cells in which DNA repair is not completed continue to apoptosis. Involvement of Bcl-2, and in some cases Bcl-XL, in protecting against cell death induced by oxidative stress is proven in other instances (51–53). Another important consideration for quantification of apoptosis using only enzymatic means is the influx of leukocytes into the tubulointerstitial area during the repair processes of ARF. These cells are known to infiltrate the tubular epithelium, may undergo apoptosis, and also may be expelled into the tubular lumen as apoptotic bodies. Because they are apoptotic, the enzymatic label will also be positive for these cells. The current model was designed to minimize necrosis, the form of death most likely to initiate a noticeable increase in leukocytic infiltrate, and in this scenario the labeling of apoptotic leukocytes would be minimal. Nonetheless, the current results confirm the need for caution in interpretation of enzymatic apoptotic labeling and emphasize the complexity of use of enzymatic label alone for analysis of modes and locality of cell death.

Members of the Bcl-2 gene family can form homo- or heterodimers, and the relative proportions of anti-apoptotic members such as Bcl-2 or Bcl-XL and pro-apoptotic members such as Bax are now thought to govern whether the cell lives or dies after damage. This “dueling dimers” theory (16) can be used in the current study to explain how the anti-apoptotic balance of these proteins promoted cell survival in the distal tubule, even though all three gene family members had altered expression in this nephron segment. The proportionally high Bax expression in the proximal tubule suggests a more vulnerable state, yet in many instances the proximal tubule was also viable. A positive relationship between anti-apoptotic Bcl-2 genes and growth factors is one explanation for proximal as well as distal tubular protection, and this is discussed in the following paragraphs.

EGF functions in the recovery processes of ischemia-induced renal injury (54). EGF has moderate distal synthesis and expression in normal kidneys (55), and its receptor is present on the basolateral and, to some extent, on the apical surfaces of the proximal and distal tubules (33,56). EGF synthesis and expression are transiently decreased after ischemia-reperfusion.
injury (57), but then the protein functions as a potent mitogen to reestablish normal tubular epithelium (56,58). The function of IGF-1 in the aftermath of renal disease is also mitogenesis and repair (59), and its role in renal ischemia-induced injury may complement EGF (60). IGF-1 is moderately synthesized and expressed distally (medullary thick ascending limb and collecting ducts) in normal kidneys (55), and the proximal tubular cells, while having minimal IGF-1 expression normally, are known to have abundant receptors (61) that are activated after renal injury (62). In our model, the profile of expression of these growth factors distally was similar to expression of Bcl-2, but the peak expression time trailed that of Bcl-2. The results suggest a joint role for Bcl-2 and these growth factors. Withdrawal of growth factors during ischemia may induce Bcl-2 expression in the distal nephron population. The Bcl-2-protected cells are able to upregulate EGF and IGF-1 protein, or activate expression from existing preproteins via proteolysis. The increased levels of EGF and IGF-1 protein then not only help to re-epithelialize any deleted home base cells, but also to act, via paracrine action, to maintain and/or re-epithelialize the adjacent ischemia-sensitive proximal tubule. Without the protection of Bcl-2, neither may be possible, and extensive cell death of apoptotic (distal tubular) or necrotic (proximal tubular) types may result.

Figure 7. Graphical comparison of expression of the Bcl-2 genes and growth factors. Expression levels were determined by immunohistochemical localization and intensity in paraffin sections using an immunohistochemical index of five grades: 0 = no positivity; 1 to 4 based on arbitrarily designating the staining intensity of positive cells in comparison with control positive sections, which were designated for each batch as 4. Temporal and spatial expression patterns are discussed in text of Results section.
The roles of EGF and IGF-1 are more easily explained than that of TGF-β. In control sections, TGF-β1 protein was seen in the proximal tubule and collecting duct, with only a moderate increase in renal expression in the ischemia-treated animals. Recent publications have shown that at low levels, TGF-β1 expression may aid repair of injured renal tubular epithelium after ischemic injury (63). In contrast, maintenance of upregulated TGF-β expression after repair of renal epithelium is injurious, associated with activation of fibroblasts and macrophages in the renal interstitium, deposition of collagen, and renal fibrosis (64). In our model, there was little relationship with the Bcl-2 gene and it is doubtful that an autocrine-paracrine mechanism exists for this protein under the influence of anti-apoptotic Bcl-2 genes.

Our theory of a Bcl-2/Bcl-X<sub>L</sub>-mediated cell survival and regeneration in ischemic ARF is demonstrated in schematic

**Figure 8.** Immunohistochemistry of Bcl-2 genes. Panel A is an example of Bcl-2 expression in control kidneys and shows low levels of expression in the distal tubule epithelium. Bcl-X<sub>L</sub> had a similar pattern of expression and is not demonstrated for control sections. Panel B shows level of Bax expression in controls and its localization in the cytoplasm of the proximal and distal tubules. Panels C and D indicate the marked increase in distal tubular epithelial cell expression of Bcl-2 at 1 and 2 d postischemia, respectively. The expression was cytoplasmic and punctate, indicating relocalization of the protein to cellular organelles. In Panel D, some proximal tubular expression is seen, but the intensity is much less than that of the distal tubule. In the same figure, apoptotic bodies are indicated (thin arrows) mostly in the proximal tubule, whereas the distal tubules had little apoptosis (thick arrow). Panels E and F are examples from the same animal at 4 d postischemia. Bcl-X<sub>L</sub> (Panel E) has increased expression in the distal and proximal tubules but has stronger expression in the distal tubule. Bax (Panel F) has increased expression in both distal and proximal tubules, but expression is greater in proximal tubules. Expression of both proteins was cytoplasmic and punctate or diffuse.
In response to ischemia, the proximal tubule (PT1) undergoes necrosis and/or apoptosis, loss of viable cells, and regeneration. The less ischemia-sensitive distal tubule undergoes mainly apoptosis. Based on the present results, in some instances the distal tubular epithelial cells are adaptively resistant to ischemia-induced injury via Bcl-2/Bcl-X\textsubscript{L} upregulation, and only minimal cell death by apoptosis occurs. Maintenance of distal tubular viability allows time for reperfusion-induced single- or double-strand DNA damage to be repaired. The Bcl-2-protected distal tubule also functions as a reservoir for the production of growth factors critical to its own maintenance and/or regeneration and also to preservation of the proximal tubule (PT2) that abuts them, with only minimal apoptosis occurring.

Although this theory is unproven, we believe it is worth consideration. “Cross talk” describes interactive processes between cells, particularly those involving growth factors. It is particularly relevant in the kidney, where heterogenous populations of cells with different structure and function sit side by side, and the identification of altered gene or protein expres-
Bcl-2 and Growth Factors in Renal Failure


60. Lin J, Cybulsky AV, Goodyer PR, Fine RN, Kaskel FJ:


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