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Cell survival or death in renal tubular epithelium after ischemia-reperfusion injury

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Cell survival or death in renal tubular epithelium after ischemia-reperfusion injury. A major contributor to the development and progression of ischemia-reperfusion (IR)–induced 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 apoptotic cell death, as well as necrosis, occurs [1, 2] along with desquamation and loss of viable epithelial cells [3]. Apoptosis was first described in renal disease in 1987 in an animal model of hydronephrosis [4]. In ARF, with reference to only the death processes, the relative contribution of necrosis or apoptosis possibly depends on the extent of the initiating events. For example, after prolonged total renal ischemia, necrosis or "accidental cell death" occurs from the resultant negation of the cell's energy and protein levels. In apoptosis, the cells use their own energy processes and proteins to die, and often the initiating ischemia is more mild [5]. Finally, despite prolonged ischemia, within the heterogeneous renal cell populations there are those that are more sensitive to ischemia, such as the proximal straight tubule and to some extent the thick ascending limb (TAL) of the loop of Henle. It may be hypothesized that these cells tend to undergo necrosis in comparison with the less sensitive segments that undergo apoptosis. Because apoptosis is gene driven, its identification is important because of the possibility of its modulation via molecular controls. However, despite these new concepts of ARF, patient death remains high, at approximately 30 to 50% of ARF cases. Recovery from ARF 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. Both processes are dependent on many of the cellular and molecular controls that have evolved in multicellular organisms to manage normal development, differentiation and growth processes, but that then become involved in the pathogenesis and progression of many renal diseases, including ARF.

Much recent understanding of cell survival or death concerns the molecular regulation by the Bcl-2 multigene family, whose members include inhibitors of apoptosis, for example Bcl-2, Bcl-X_L, Mcl-1, or accelerators of apoptosis, for example, Bax, Bcl-X_S, and Bad. Once the death process has been initiated, for example, by hypoxia, the presence of oxygen-derived free radicals or modulation of essential growth factors, the antiapoptotic balance of these genes may act to maintain cell survival [6]. Bcl-2 is an important renal cell survival or development proto-oncogene. For example, grossly abnormal kidney development and augmented metanephric apoptosis occur in Bcl-2–deficient mice. In fetal kidney development, the distribution of apoptotic cells is inversely correlated with expression of Bcl-2 [7].

Several key renal epithelial cell growth factors are known to act in renal regeneration after IR injury. Can their action be mediated via an anti-apoptosis balance of the Bcl-2 gene family cell death or survival genes? The growth factors chosen for our study were epidermal growth factor (EGF), insulin-like growth factor-I (IGF-1), and transforming growth factor-β (TGF-β). In this article regarding IR-induced ARF, we demonstrate a correlation between cell survival promoted by members of the Bcl-2 gene family and regeneration of damaged tubular epithelium by the selected renal growth factors whose expression may be facilitated in cell populations protected by the anti-apoptotic genes.

METHODS

Ethical approval was given by the University of Queensland Animal Experimentation Ethics Committee (AEEC Number PATH/106/96).

Animals and experimental model

Male Sprague-Dawley rats [200 to 250 g, $N = 6$ per IR treated, $N = 4$ per control (sham-treated) group]. The treatment was bilateral renal artery occlusion for 30 minutes, followed by reperfusion. The collection of kidneys was at the end of the ischemic period (T0) or after 1, 2, 4, 7, and 14 days of reperfusion. Body weights were recorded at the start of the experiments and prior to euthanasia of the animal for collection of tissue.

Key words: ischemic renal injury, apoptosis, necrosis, Bcl-2 gene.

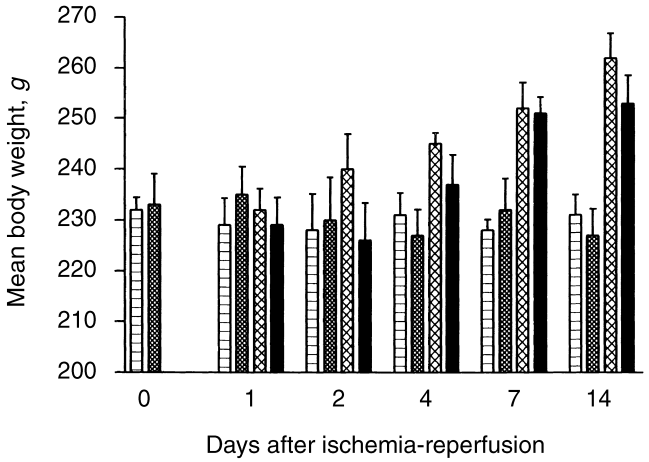


Fig. 1. Comparison of body weights for treated and control animals at each experimental time point. No significant difference was found at each time point.

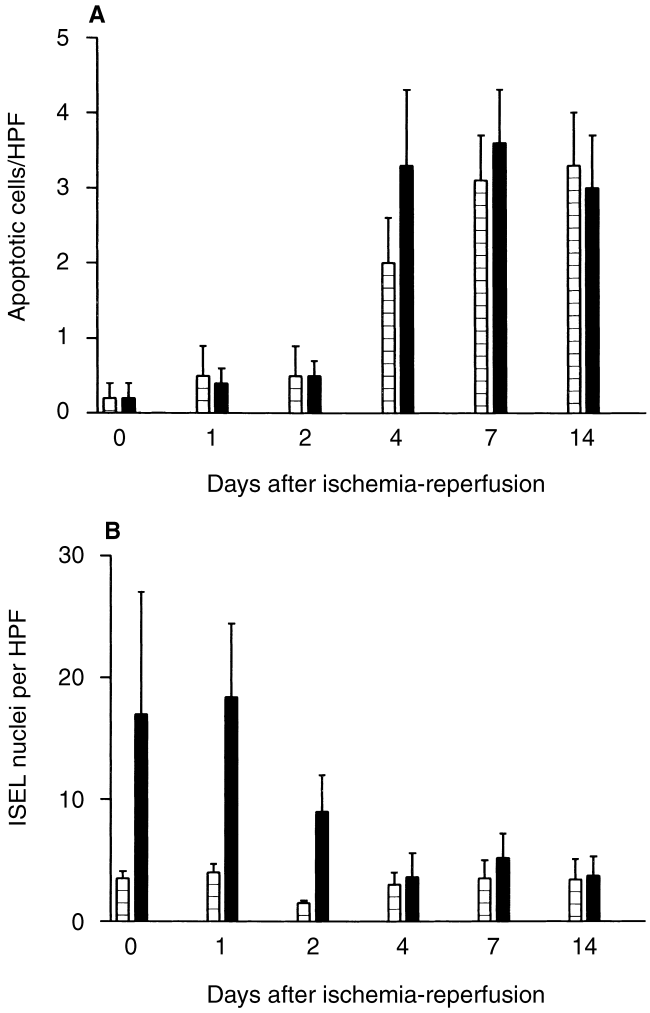


Fig. 2. Comparison of morphological assessment of levels of apoptosis (*A*) and counts for ISEL-positive nuclei (*B*).

Tissue collection

Kidneys were removed, bisected transversely to their length, through the papilla and half of each kidney fixed in either 10% phosphate-buffered formalin or in 4% buffered paraformaldehyde, pH 7.4, at 4°C overnight. Routine preparation was carried out for histology and for immunohistochemistry (paraffin embedding, 4 μm sections cut onto Superfrost Plus slides) and other histological stains such as hematoxylin and eosin (HE), periodic acid-Schiff's reagent (PAS), and Masson's trichrome stain.

Immunohistochemistry

The peroxidase-antiperoxidase method was used. Nonspecific binding of peroxidase or antibodies was blocked with 0.3% H_2O_2 in methanol. Primary antibodies were applied to subserial sections: Bcl-2 (antihuman, 1:50 dilution; Dako, Carpinteria, CA, USA, or Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bax and Bcl-$X_{L/S}$ (1:50; Santa Cruz), EGF (antirat, 1:200; R&D Systems, Minneapolis, MN, USA), IGF-I (antihuman, 1:1000; Gropep, Adelaide, Australia), TGF-β (1:50; Sigma, St. Louis, MO, USA), and proliferating cell nuclear antigen (PCNA, 1:100; Oncogene; CN Biosciences, Alexandria, Australia). Secondary antibody was Vector universal (1 in 400). Chromogen was diaminobenzidine (DAB). Negative controls were prepared without primary antibody or with nonspecific IgG. Positive rat tissue control sections from known unrelated studies were included. Sections were lightly counterstained with hematoxylin.

Apoptosis

Morphology [8] and *in situ* end labeling (ISEL) were quantitated in 10 to 20 light microscope fields per section at ×400 magnification. Morphological characteristics included cellular rounding and shrinkage, eosinophilic cytoplasm, nuclear chromatin compaction, especially along the nuclear envelope in a crescentic manner, membrane-bound cellular blebbing, the formation of apoptotic bodies, which may appear in the tubular lumina or be phagocytosed by intrinsic renal cells or invading macrophages.

Necrosis

Morphological characteristics included cellular swelling and lysis, cytoplasmic eosinophilia, membrane rupture, densities in swollen mitochondria, pyknotic or irregularly clumped nuclear chromatin, and an associated leukocytic infiltrate that is absent in apoptosis [8]. Semiquantitative assessment was carried out.

Cell proliferation

Proliferating cell nuclear antigen (PCNA) nuclear labeling, which correlates well with DNA synthesis, as measured by ^{3}H-thymidine uptake in the kidney, was used. Labeled nuclei were quantitated as for ISEL. This

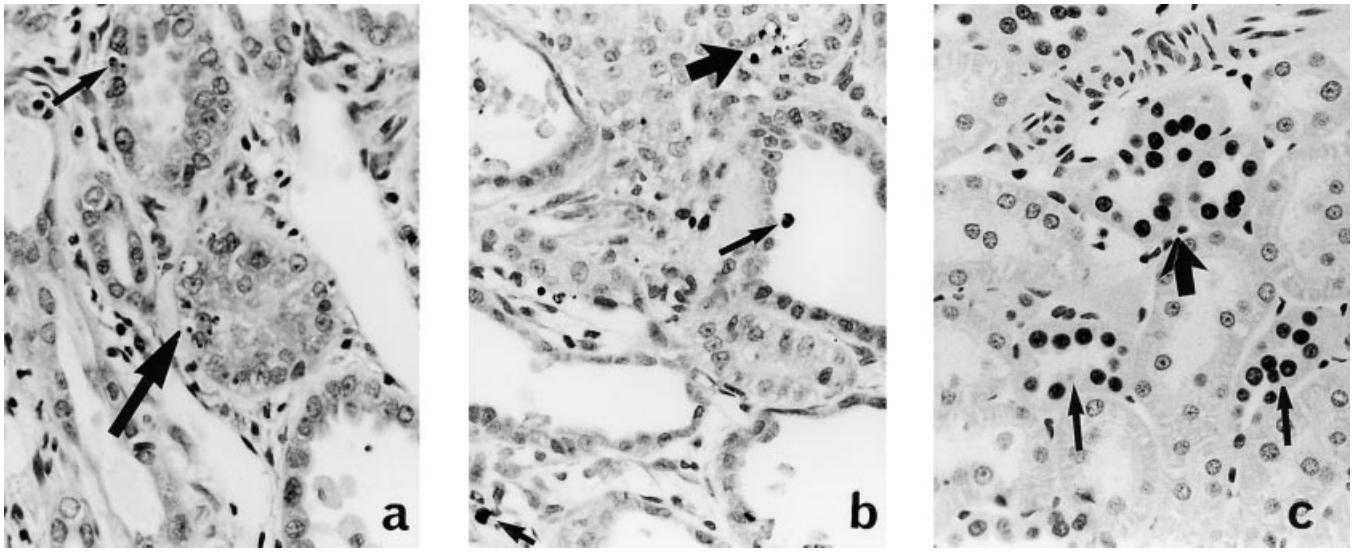


Fig. 3. Apoptosis. Examples of (*a*) apoptotic cells in distal and proximal tubular epithelium are arrowed. (*b* and *c*) ISEL associated with apoptosis (examples arrowed in b) is compared with high, apparently nonapoptotic ISEL in the distal tubule at 24-hour post-IR (c).

method gives a profile similar to assessing mitosis by morphology.

Assessment of immunolocalization

Light microscopic evaluation was performed at a magnification of ×400. Bcl-2, Bcl-X, Bax, IGF-1, EGF, and TGF-β protein expression was assessed in code-labeled sections. Immunohistochemical localization (numbers of labeled tubules) and intensity in paraffin sections were graded at four levels of positivity for either proximal or distal tubules.

Statistical analyses

Data were analyzed using standard statistical methods using Student's *t*-test for unpaired samples.

RESULTS

Body weights

No significant difference was found among body weights (Fig. 1) when comparisons were made between treated and control groups at each set experimental time.

Apoptosis or necrosis

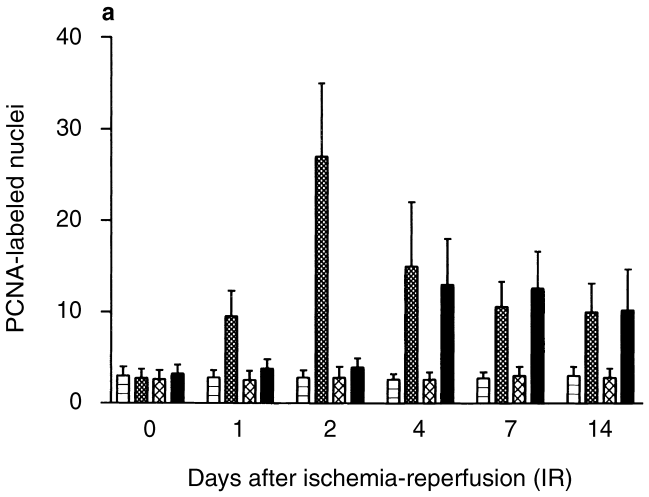
The kidneys of control animals had negligible levels of apoptosis and no necrosis. Increased levels of apoptosis occurred in both the proximal and distal nephron segments post-IR compared with control sections. Morphological assessment of apoptosis (Fig. 2a) in distal or proximal tubular epithelium showed peaks of apoptosis to be in the latter stages of the study (4-, 7-, and 14-days post-IR). Proximal tubular necrosis and desquamation were evident at one- to four-days post-IR. High levels of "ISEL" (Fig. 2b) were recorded early (0 to 2 days) in the study, mainly in the distal tubule. This labeling does not appear to eventuate in apoptosis, but is more likely single- or even double-stranded DNA damage that is reparable and may be under the influence of the cell survival gene Bcl-2. Figure 3 demonstrates the morphological evidence of apoptosis (Fig. 3a), ISEL associated with apoptotic cells (Fig. 3b) and at high nonapoptotic levels in the distal tubule (Fig. 3c).

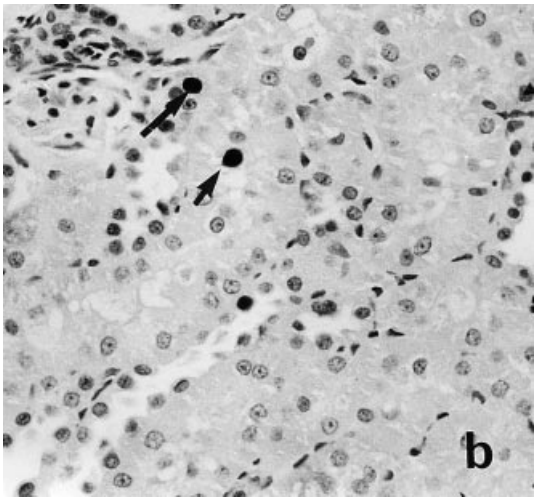
Cell proliferation/regeneration

Figure 4a gives a graphical comparison of the relative changes in cell proliferation after IR. Low levels of PCNA nuclear labeling were identified in sections from control animals. Proximal tubular epithelial regeneration peaked at two to four days and at four to seven days in the distal tubule, reflecting the levels of cell deletion in each nephron segment. Desquamation of the entire necrotic epithelium had sometimes occurred, but where the basement membrane was preserved, there was evidence of regeneration of dedifferentiated tubular epithelium. Figure 4 b and c compare control PCNA labeling (Fig. 4b) with regenerating renal tubular epithelium after IR (Fig. 4c).

Temporal and spatial immunolocalization of Bcl-2 proteins and growth factors

Table 1 compares the grades of expression. In renal sections from control animals, Bcl-2 and Bcl-X_L expression was minimal in tubular epithelial cells. Bax was moderately expressed in the proximal tubule, while EGF,





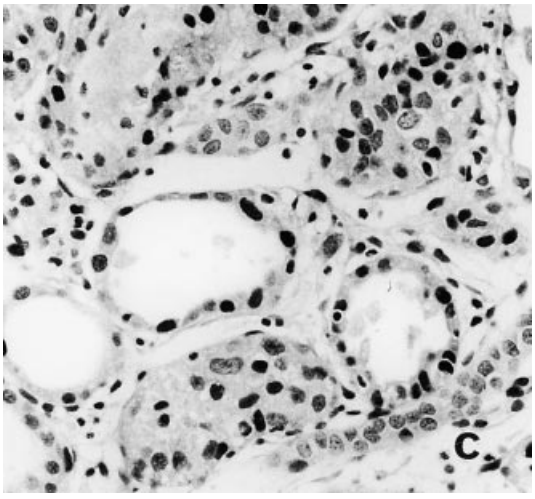


Fig. 4. Cell proliferation. Numbers of PCNA-positive nuclei are compared after IR (*a*). Photomicrographs (*b* and *c*) demonstrate PCNA positive nuclei in controls (b; examples arrowed) and at 2 days post IR (c), where many darkly stained positive nuclei may be seen.

IGF-1, and TGF-β1 were expressed at low to moderate intensity in the distal nephron, and TGF-β1 was also expressed at low levels in the proximal convoluted tubule. In comparison, IR kidneys at the acute phases (0 to 2 days) showed markedly increased Bcl-2 expression of the distal but not the proximal tubular segments. This expression was associated with increased survival of both the distal (Bcl-2 expressing) and adjacent proximal segment, which is suggestive of "cell-to-cell cross-talk" that the promoted survival of both segments. Bax and Bcl-X_L expression were found in the proximal tubule and were similar to Bcl-2 expression in the distal tubule in experimental animals, probably verifying the importance of proportions of Bax and Bcl-2 in the "dueling dimers" concept of cell death or survival. Of the growth factors studied, EGF was down-regulated in necrotic/damaged nephron segments, but interestingly had moderate expression in distal and proximal segments in areas where Bcl-2 had promoted distal tubular survival. IGF-1 had expression that closely reflected Bcl-2 expression. There was moderate expression in the control animal distal tubules, which intensified in the surviving distal tubular segments after IR and was up-regulated in proximal tubular segments abutting the surviving distal tubular segments. It was also up-regulated in the regenerating proximal tubular segments after necrotic damage. TGF-β1 expression was not correlated with Bcl-2 expression but was up-regulated in regenerating proximal proximal convoluted tubule (PCT) and proximal straight tubule (PST) epithelium during repair of necrotic damage.

DISCUSSION

"Cell-to-cell cross-talk" is the current descriptive jargon for interactive processes between cells. This idea is particularly relevant in the kidney, where a heterogeneous population of cells with a different structure and function sit side by side. Although the structure of the renal nephron is usually discussed with an emphasis on the maintenance of normal renal function or physiology,

Table 1. Grades of expression showing temporal and spatial immunolocalization of Bcl-2 proteins and growth factors

Protein and experimental timepoint *day*		PCT (cortex)	DCT (cortex)	PST (medulla)	TAL (medulla)	CD
Bcl-2	C	0	0−+	0	0−+	0−+
	0	0−+	+−++	0−+	+−++	++
	1	0−+	++−+++	0−+	+−+++	++
	2	+−++	++−+++	0−++	+−+++	++
	4	+−++	++	0−++	0−++	++
	7	0−+	+	0−+	0−+	++
	14	0−+	+	0−+	0−+	0−+
Bax	C	0−++	+−++	0−++	0−++	+−++
	0	++	+−++	0−++	+−++	++
	1	+++	+++	++	+−+++	+++
	2	++−+++	++	+++	++	++
	4	+++	++	++	++	++
	7	++	++	++	++	++
	14	++	++	++	++	++
Bcl-X_L	C	0	0−+	0−+	0−+	+−++
	0	+	++	+	++	++
	1	+−+++	++−++	+	++−+++	++
	2	+++	+++	++	+++	++
	4	+	+++	+	+++	++
	7	+	++	+	++	++
	14	+	++	+	++	++
EGF	C	0	++	0	++	+−++
	0	0−+	+−++	0−+	0−++	0−++
	1	+	++	+	++	++
	2	++	+++	++	++	++
	4	++	+++	++	++	++
	7	0−+	++	+	++	++
	14	0−+	++	0−+	++	++
IGF-I	C	0	+	0	++	+
	0	0−+	+	0−+	++	+
	1	+−++	++−+++	0−+	++−+++	+−++
	2	+−++	++−+++	++	+++	+−++
	4	+−++	+++	++	++	++
	7	+−++	++	++	++	++
	14	+	++	+	++	++
TGF-β	C	+	0	+	0−+	+
	0	++	+	+	+	+
	1	++	++	++	+	+
	2	++	++	++	++	+
	4	+	++	+	++	+
	7	+	+	+	++	++
	14	+	+	+	++	++

Abbreviations are: PCT, proximal convoluted tubule; DCT, distal convoluted tubule: PST, proximal straight tubule; TAL, thick ascending limb; CD, collecting duct; EGF, endothelial growth factor; IGF-I, insulin-like growth factor-I; TGF-β, transforming growth factor-beta.

the identification of altered gene or protein expression in abutting nephron structures can also explain pathological and regenerative processes. Renal cell "cross-talk" has been proposed for some time [9]. Proving that it actually occurs probably requires *in vitro* experimentation or the use of mice null for certain genes. Neither model is perfect, because in both cases the cells no longer have the normal ocean of cytokines, growth factors, or other essential proteins that they may need to interact for normal renal function of one gene. *In vivo*, results such as those presented here can give some pointers for *in vitro* study, and in the following paragraphs, we discuss the possible interactions between growth factors and Bcl-2 gene family members, as indicated from our results.

We chose to concentrate on growth factors for which some indication of success in human and experimental model ARF treatment is available [10]. IGF-1 promotes tubular remodeling and repair after ischemic insult. The EGF solution delivered daily by subcutaneous injection was shown to suppress apoptosis, and it also acts on cell proliferation in proximal tubular cells. Although TGF-β is best known for its role in renal fibrosis, its early expression is also thought to aid in renal regeneration.

Figure 5 summarizes a possible Bcl-2–mediated mechanism of cell survival and regeneration that may exist in ARF, and one that may be modulated for improving cell death or tissue damage associated with ARF, using molecular techniques. Our current hypothesis, devel-

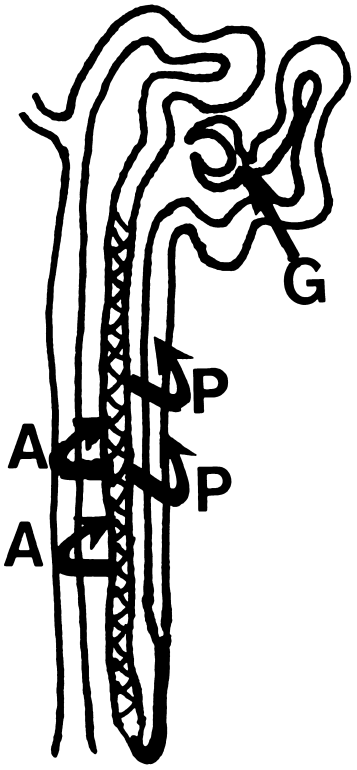


Fig. 5. Schematic representation of a possible mechanism of cell death and survival in IR-induced ARF. Arrows indicate autocrine (A) or paracrine (P) movement of growth factors from the TAL (hatched) after protection by Bcl-2. The glomerulus (G) is indicated.

oped from these results, is that, in some instances, the distal tubular/thick ascending limb (DT/TAL) epithelial cells are adaptively resistant to IR-induced injury via Bcl-2 (and/or Bcl-X_L) up-regulation and, possibly, Bax down-regulation. The maintenance of DT cell survival via this mechanism allows any single-stranded or possibly double-stranded DNA fragmentation to be repaired, as demonstrated by ISEL results. The DT cells can also function as a reservoir for the production of growth factors critical to the maintenance and/or regeneration of their own cell population (autocrine action) and also the ischemia-sensitive proximal tubular cells that abut them (paracrine action).

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