

## Cell death induced by acute renal injury: a perspective on the contributions of apoptosis and necrosis

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**Padanilam, Babu J.** Cell death induced by acute renal injury: a perspective on the contributions of apoptosis and necrosis. *Am J Physiol Renal Physiol* 284: F608–F627, 2003; 10.1152/ajprenal.00284.2002.—In humans and experimental models of renal ischemia, tubular cells in various nephron segments undergo necrotic and/or apoptotic cell death. Various factors, including nucleotide depletion, electrolyte imbalance, reactive oxygen species, endonucleases, disruption of mitochondrial integrity, and activation of various components of the apoptotic machinery, have been implicated in renal cell vulnerability. Several approaches to limit the injury and augment the regeneration process, including nucleotide repletion, administration of growth factors, reactive oxygen species scavengers, and inhibition of inducers and executioners of cell death, proved to be effective in animal models. Nevertheless, an effective approach to limit or prevent ischemic renal injury in humans remains elusive, primarily because of an incomplete understanding of the mechanisms of cellular injury. Elucidation of cell death pathways in animal models in the setting of renal injury and extrapolation of the findings to humans will aid in the design of potential therapeutic strategies. This review evaluates our understanding of the molecular signaling events in apoptotic and necrotic cell death and the contribution of various molecular components of these pathways to renal injury.

renal ischemia; molecular components; signal transduction; renal tubular epithelial cells; therapeutic approaches

CELLULAR DEATH AND RESORPTION are critical biological processes that are crucial not only to normal histogenesis and organelle turnover but also to the pathogenesis of tissue injury and diseases (67, 73, 99, 141, 188, 199). Normal physiological cell death or programmed cell death (PCD) occurs continuously in proliferating tissues and counterbalances excessive cell proliferation during mitosis. PCD is an integral part of maintaining the normal functioning of the immune system and in sculpting the early embryo (67, 188, 199). Nonsynchronized apoptosis can result in pathophysiological outcome and is implicated in causing a variety of human diseases. Excessive PCD can lead to impaired growth and development, neurodegenerative diseases, and acquired immunodeficiency syndrome (14, 193). On the other hand, indiscrete suppression of apoptosis can result in autoimmune diseases and cancer (62, 101). Tissue injury resulting from hypoxic-ischemic, toxic, and thermal insults can result in both pathological cell death (necrosis) and PCD (apoptosis). Unlike apoptosis that occurs in normal and disease states, necrosis is

induced only when cells or tissues are exposed to severe and acute injury (60, 97, 149). The molecular pathways leading to the different modes of cell death in various human diseases and clinical conditions and their regulation have been under intense scrutiny during the past two decades. Dissecting and discriminating the roles of key molecules in various cell death programs are crucial as they are attractive targets for therapeutic intervention.

### MODES OF CELL DEATH

A distinction between the morphology of cells undergoing physiological cell death and pathological cell death was observed more than 50 years ago (67). However, it was not until the seminal report by Kerr et al. (101) in 1972 that cytologists and pathologists adopted the term “apoptosis” for a morphologically distinct form of cell death. The perpetual flow of information in the past two decades defining the effector and regulatory pathways that result in apoptosis has raised further interest in the role of this mode of cell death in various diseases and pathological conditions, including ischemia-reperfusion injury (IRI) of the heart, brain, and the kidney.

Apoptosis is highly coordinated and is generally thought to be mediated by active intrinsic mechanisms,

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although extrinsic factors can contribute (16, 30, 205). Apoptosis is genetically controlled and is defined by cytoplasmic and nuclear shrinkage, chromatin margination and fragmentation, and breakdown of the cell into multiple spherical bodies that retain membrane integrity (25, 101, 138, 246). The factors contributing to necrosis are mostly extrinsic in nature, such as osmotic, thermal, toxic, hypoxic-ischemic, and traumatic insults (25, 60). Necrosis is characterized by progressive loss of cytoplasmic membrane integrity, rapid influx of  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ , and water, resulting in cytoplasmic swelling and nuclear pyknosis (9, 18, 200, 244). The latter feature leads to cellular fragmentation and release of lysosomal and granular contents into the surrounding extracellular space, with subsequent inflammation (138). A recent report indicates that the release of the high-mobility group 1 protein by necrotic cells is involved in promoting the inflammation. Cells deficient for high-mobility group 1 protein showed greatly reduced ability to promote inflammation (201).

Apoptosis and necrosis often occur simultaneously in a wide variety of pathological conditions as well as in cultured cells exposed to physiological activators, physical trauma, or toxins and chemicals (141). The same type of insult can induce either apoptosis or necrosis, but whether one mode of cell death is preferred over the other usually depends on the severity of the insult and the idiosyncrasy of the target cell (9, 25, 128, 141). The perception that apoptosis and necrosis are functionally opposed forms of cell death is fading, and a consensus has developed that both forms of cell death constitute two extremes of a continuum.

### MORPHOLOGICAL, BIOCHEMICAL, AND MOLECULAR MARKERS OF APOPTOSIS AND NECROSIS

In settings of acute injury such as IRI to the kidney where apoptosis and necrosis coexist (204, 205), discriminating between the two modes of cell death is essential. Despite the recent progress in elucidating the molecular determinants of cell death, a precise histological or biochemical marker to differentiate apoptosis from necrosis has not been identified. Detection of structural alterations using electron microscopy as described originally by Kerr et. al (101) still remains as the reliable criterion to distinguish between the two modes of cell death. It is now agreed upon that a rational combination of at least two techniques should be utilized, one to visualize morphological changes and the second to determine biochemical changes, when the two modes of cell death are asserted.

### MORPHOLOGY OF APOPTOSIS

In apoptosis, the earliest characteristic change occurs in the nucleus with chromatin condensation, pyknosis, and karyorrhexis (101, 246). The condensed chromatin appears as crescents along the periphery of the nuclear membrane or as spherical bodies within the nucleus. The cytoplasmic condensation instigates the cell to shrink and form numerous vacuoles within the cytoplasm (99, 246). Subsequently, the nuclear and plasma membranes become convoluted, and small masses of condensed chromatin undergo fragmentation along with condensed cytoplasm to form "apoptotic bodies." Apoptotic bodies are plasma membrane bound and often contain functional mitochondria and other organelles (246). The stereotypical morphological

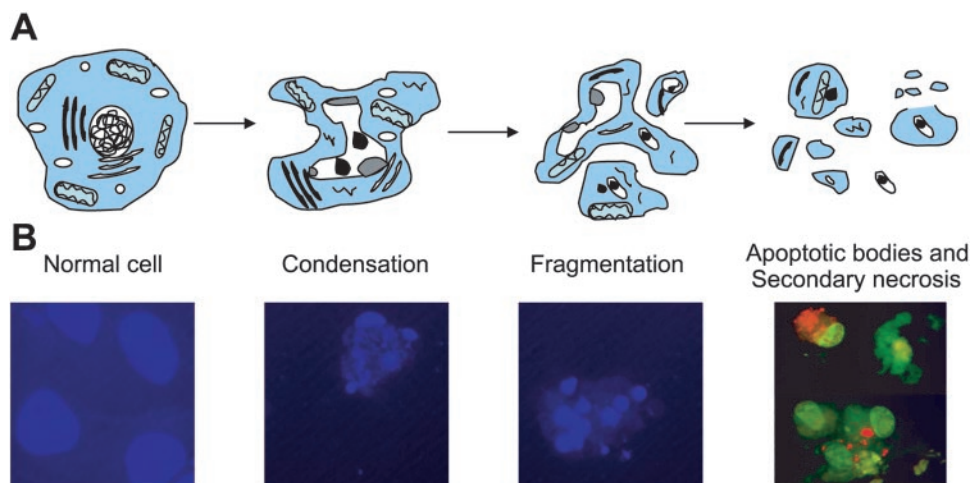


Fig. 1. Illustration of the various stages of apoptotic cell death. *A*: depiction of the stereotypical changes including condensation, changes in nuclear structure, and fragmentation of the cell into small apoptotic bodies. In vivo, the apoptotic bodies are phagocytosed by neighboring cells, whereas in vitro they undergo swelling and eventual lysis (secondary necrosis). *B*: photographs of LLC-PK<sub>1</sub> cells undergoing apoptosis at the corresponding stages as shown in *A*. Apoptosis was induced by overnight exposure of the cells to 50  $\mu\text{M}$  cisplatin. The cells in the first 3 photographs were stained with Hoechst dye, and the cells in the last photograph were stained with acridine orange and ethidium bromide. In the last photograph, viable cells appear green, whereas the apoptotic cells with intact plasma membrane appear green with yellowish dots representing condensed chromatin; apoptotic cells and bodies that are undergoing secondary necrosis appear bright orange or red due to the plasma membrane damage and entry of ethidium bromide.

changes associated with apoptotic cell death are depicted in Fig. 1. The phosphatidyl serine residues that are normally localized to the inner membrane are re-located to the outside of the cell membrane before its fragmentation. The phosphatidyl serine residues on the apoptotic bodies serve as a signal to the neighboring healthy cells to phagocytose and clear the cellular debris, thus avoiding an inflammatory response (211). In a recent report, Brown et. al (24) provide evidence that homophilic binding between the adhesion receptor CD31 (platelet-endothelial cell adhesion molecule-1) present on leukocytes and macrophages plays a key role in phagocytosis. Both viable cells and dying cells attach to macrophages through these receptors, but the viable cells get detached through an unknown repulsive mechanism. The repulsive signaling is impaired in the dying cells, and they are engulfed (24). It is not known at present whether analogous systems exist in cells other than leukocytes. In vitro, in the absence of phagocytosis, apoptotic bodies ultimately will swell and lyse, and this terminal process of cell death has been termed "secondary necrosis." Secondary necrosis may occur in vivo in autoimmune disorders associated with impaired clearance of apoptotic cells (243).

#### MORPHOLOGY OF NECROSIS

The morphology of a necrotic cell is very distinct from that of a cell undergoing classic apoptosis, with ultrastructural changes occurring in both the cytoplasm and the nucleus. The main characteristic features are chromatin flocculation, swelling and degeneration of the entire cytoplasm and the mitochondrial matrix, blebbing of the plasma membrane, and eventual shedding of the cytoplasmic contents into the extracellular space (100). Unlike in apoptosis, the chromatin is not packed into discrete membrane-bound particles, but it forms many unevenly textured and irregularly shaped clumps, a feature that is being used for differentiating between the two modes of cell death (224). The mitochondria undergo inner membrane swelling, cristolysis, and disintegration (112). Polyribosomes are dissociated and dispersed throughout the cytoplasm, giving the cytoplasmic matrix a dense and granular appearance. Dilation and fragmentation of the cisterns of rough endoplasmic reticulum and Golgi apparatus are frequently observed (225).

#### ENDONUCLEASES AND DNA FRAGMENTATION IN APOPTOSIS AND NECROSIS

Analysis of DNA fragmentation by agarose gel electrophoresis is one of the most widely used biochemical markers for cell death. The detection of internucleosomal DNA cleavage (DNA laddering) is considered to be an indicator of apoptosis, whereas the random digestion of DNA resulting in a smear pattern is a marker of necrotic cell death (4, 8, 245). However, recent data from several studies indicate that discriminating between apoptosis and necrosis based on DNA fragmentation pattern is questionable, because both modes of

cell death can occur in the absence or presence of DNA fragmentation (49, 192, 229).

The cleavage of double-strand DNA in apoptotic DNA degradation is believed to occur by the activation of endogenous  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonucleases that specifically cleave between nucleosomes to produce DNA fragments that are multiples of  $\sim 180$  base pairs (35, 229). DNA fragmentation represents a point of no return from the path to cell death, because no more new cellular protein synthesis for cell survival can occur. Although several endonucleases that are involved in apoptosis have been identified and characterized in the past several years, the indispensability of these enzymes for the apoptotic process has been lacking. Two newly identified apoptotic endonucleases [caspase-activated DNase (CAD) and endonuclease G (Endo G)] with different enzymatic properties appear to be involved in cellular disassembly and cell-autonomous apoptosis (123, 131, 180). The biochemical pathways and the candidate endonucleases involved in DNA degradation are shown in Fig. 2.

#### CAD

CAD is a magnesium-dependent endonuclease that normally resides in the nuclei bound to its chaperone and inhibitor of CAD (ICAD). On an apoptotic stimulus, caspases-3 or -7 or granzyme B cleave ICAD, dissociating it from CAD, which results in activation of CAD and apoptotic internucleosomal DNA degradation (57, 198, 256). ICAD is essential for proper folding of CAD and its activation (161). Targeted deletion of ICAD led to absent apoptotic DNA cleavage in thymocytes and splenocytes treated with staurosporine (254). However, DNA fragmentation did occur in certain tis-

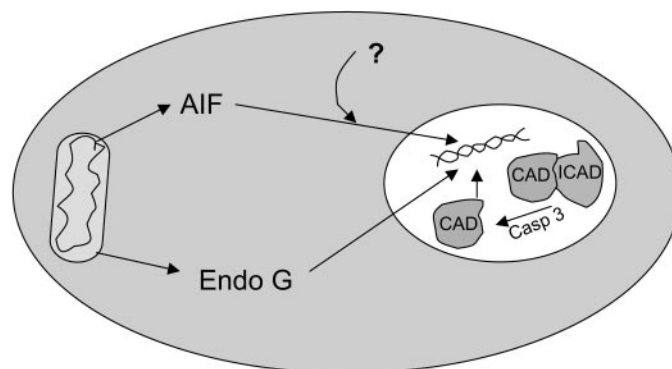


Fig. 2. Pathways leading to DNA degradation. Mitochondrial derived endonuclease G (Endo G) and nuclear-derived, caspase-activated DNase (CAD) are 2 known apoptotic endonucleases involved in nuclear DNA degradation. After an apoptotic stimulus, caspase-3 cleaves the inhibitor of CAD (ICAD)/CAD complex and activates CAD to elicit DNA fragmentation in dying cells. Endo G is released from mitochondria in response to various apoptotic stimuli and translocates to nucleus, where it is involved in DNA degradation. The apoptosis-inducing factor (AIF) derived from mitochondria is not a self-nuclease but may participate in DNA degradation, possibly by recruiting other endonucleases. Unlike CAD, activation of Endo G and AIF is independent of caspase activation. It is possible that Endo G and AIF may participate in nuclear DNA degradation when caspase activation is limited.

sues from ICAD-deficient mice, suggesting that endonucleases other than CAD may also participate in the apoptotic process.

### *Endo G*

A search for the endonucleases that are responsible for CAD-independent DNA fragmentation using biochemical and genetic methods identified mitochondrial Endo G as a second apoptotic endonuclease. Endo G is released from the mitochondrial intermembrane space and translocates to the nucleus to elicit DNA fragmentation (123, 180). Endo G activity is stimulated by DNA breaks, and its specificity for DNA degradation is more toward single-strand DNA than duplex DNA. Thus, unlike CAD, Endo G is a mitochondria-derived endonuclease whose activity is independent of caspase activation.

Apoptosis-inducing factor (AIF) is another molecule involved in chromatin degradation independent of caspase activation. AIF, on its release from mitochondrial intermembrane space, migrates to the nucleus, interacts with DNA (248), and induces partial chromatin condensation and large-fragment (50-kb) DNA fragmentation. However, AIF has no intrinsic endonuclease activity, and AIF-mediated DNA degradation does not require caspase activation (93, 216). It is yet to be determined whether Endo G, CAD, or other endonucleases participate in AIF-mediated DNA degradation (255).

## MOLECULAR MECHANISMS OF APOPTOSIS

Studies by Horvitz and colleagues (85) first elucidated the existence of a genetically controlled cell death program in which at least three gene products, CED-3, CED-4, and CED-9, participate to cause selective PCD during *Caenorhabditis elegans* development. Subsequent studies in other organisms revealed that several cysteine proteases that share homology to CED-3 are present in mammalian cells. Fourteen such cysteine proteases have been identified so far, and they are identified as caspase-1 to caspase-14 (220, 221).

### *Molecular Executioners of Apoptosis*

Caspases are the molecular executioners of apoptosis because they bring about most of the morphological and biochemical characteristics of apoptotic cell death. They are a family of constitutively expressed proenzymes that undergo proteolytic processing to generate its activated form (212, 220, 221). Functionally, the caspase family can be divided into two major subfamilies. Caspases-1, -4, and -5 are involved in the maturation of cytokines such as interleukin-1 $\beta$  and interleukin-18 and promote proinflammatory functions. The other members of the family function as part of the apoptotic pathway, and they are subdivided into initiator (caspases-2, -8, -9, and -10) and effector caspases (caspases-3, -6, and -7) (212, 220). The initiator caspases are activated by adapter-facilitated self-cleavage in response to apoptotic stimuli. The effector caspases are activated through cleavage by initiator

caspases (78). During apoptosis, the effector caspases cleave numerous proteins located in the cell membrane, nucleus, and cytoplasm, and the significance of this proteolysis in the apoptotic process is incompletely elucidated. The activation of CAD (see above) to facilitate DNA degradation (57), cleavage of nuclear lamins to facilitate nuclear shrinkage and budding (189), and activation of p21-activated kinase 2 to cause active blebbing in apoptotic cells (195) are a few of the important functions mediated by caspases in the apoptotic process.

### *Mechanisms of Caspase Activation*

Two converging molecular signaling pathways can lead to the activation of caspases, and the choice between the pathways that the cell adapts for its final demise is profoundly influenced by the initial apoptotic stimulus. The first is the receptor-mediated death-signaling pathway that is triggered mostly by extrinsic signals exemplified by the binding of a TNF ligand to its receptor (e.g., Fas to the Fas receptor). The second apoptotic pathway is mediated by mitochondria and is triggered mostly by intrinsic stress signals and developmental instructions (82, 92, 94, 252). An overview of the two death-signaling pathways is presented in Fig. 3.

### *Signaling Through Death Receptors*

Caspase activation through cell death receptors is mediated by a subset of the TNF receptor superfamily, which includes Fas/CD95, TNF receptor (TNFR)-1, and death receptor-3. Binding of the ligand trimerizes the receptors and recruits death domain-containing molecules such as FADD/MORT, TRADD, and RAIDD to their cytoplasmic regions of the receptors to form a death-inducing signaling complex (92, 94, 238). The interactions between death effector domains (DED) present in both FADD and pro-caspase-8 lead to the recruitment of several pro-caspase-8 molecules to the complex. The key initiator caspase that instigates the downstream caspase cascade in the death receptor pathway is caspase-8 (6). The molecular mechanism that mediates the initiator caspase activation is still unclear, but it is thought to be regulated by protein-protein interactions. It is suggested that the close proximity of these molecules will activate the low intrinsic protease activity in them (159). Pro-caspase-8 undergoes autoproteolytic activation and initiates a caspase cascade to activate the effector caspases.

c-FLIP is a naturally occurring dominant negative antagonist of death receptor-mediated caspase-8 activation and contains two DEDs and a defective caspase-like domain. c-FLIP can associate with DEDs of FADD and caspase-8, thus interfering with the recruitment of caspase-8 to FADD (88, 226). Mouse embryonic fibroblasts derived from caspase-8-deficient mice are resistant to Fas, TNF-R1, or DR3 stimulation but susceptible to agents that utilize the mitochondrial death pathway (232). This further demonstrates the crucial

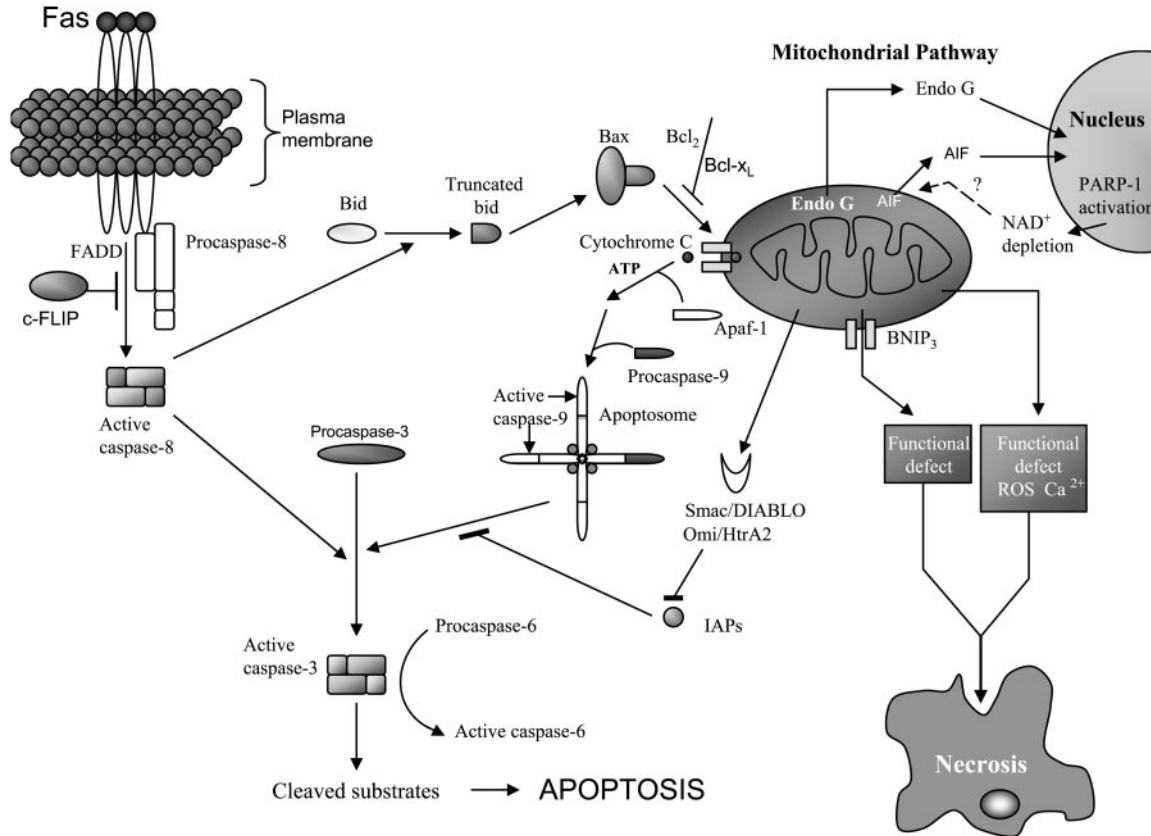


Fig. 3. Overview of death-signaling pathways in mammalian cells: The death receptor pathway (*left*) is initiated by the binding of a ligand (Eg: FasL) to its receptor Fas, which results in the sequential recruitment of FADD and pro-caspase-8. c-FLIP can block the recruitment of pro-caspase-8 to the complex. The proximity of several pro-caspase-8 molecules results in its activation. Caspase-8 can proteolytically activate caspase-3, or it can cleave Bid to its truncated form t-Bid, which binds to Bax and gets integrated into the mitochondrial membrane to release cytochrome *c*. In response to various cellular stress-induced apoptotic stimuli, the intrinsic mitochondrial pathway is activated. This pathway involves the translocation of proapoptotic molecules such as Bax from the cytosol to the mitochondrial membrane. Bax can release cytochrome *c* from the mitochondria into the cytosol. Cytochrome *c* associates with Apaf-1 and caspase-9 to form the apoptosome and subsequent activation of caspase-3. Mitochondria also release AIF and Endo G, which may exert their effects on the nuclei. Mitochondria released Smac/Diablo and Omi/HtrA2 sequesters inhibitors of apoptosis (IAPs) to prevent them from inhibiting caspase-3. BNIP3 is a Bcl2 family member that is translocated and integrated into the mitochondria. Unlike other Bcl2 family members, BNIP3 can induce necrotic cell death in response to death stimuli. Activation of poly (ADP-ribose) polymerase (PARP) leads to NAD<sup>+</sup> depletion and may induce mitochondrial depolarization to release AIF. ROS, reactive oxygen species.

role played by caspase-8 in transducing signals downstream of the death receptors.

#### Mitochondria and Bcl2 Family as Regulators of Cell Death

The apoptotic signal transduction pathways that are undertaken by the cell in response to an intrinsic signal such as DNA damage, glucocorticoids, perturbations in redox balance, and ceramide and growth factor deprivation involve mitochondrial release of proapoptotic molecules (92, 108, 215). Bcl2 family members control the permeability of the mitochondrial outer membrane to release various proapoptotic proteins (92).

The ever-growing mammalian Bcl-2 family of apoptotic regulators shares homology with the *C. elegans* antiapoptotic molecule CED-9 (32, 106). Based on their structure and functional similarities, Bcl2 family mem-

bers are divided into the proapoptotic (Bax, Bak, and Bok) and antiapoptotic (Bcl2, BclX<sub>L</sub>, Bcl-w, Mcl-1, and A1) groups (92). A third class of death effector molecules sharing homology only to the Bcl-2 homology-3 (BH3) domain can activate proapoptotic Bcl2 family members or inactivate antiapoptotic members (86, 213). The family of BH3-only proteins include Bin, Bid, Bad, Bik, BNIP3, Noxa, Puma, and Hrk (86, 162, 213). Pro- and antiapoptotic members of the Bcl-2 family can homodimerize or heterodimerize, thus forming a large number of combinations within a cell. Heterodimerization between a proapoptotic member and an antiapoptotic member can nullify the functions of each (191). The outcome of a cell that received an apoptotic stimulus is thought to depend partly on the ratio of the death promoter to the death suppressor (1, 2). The precise mechanisms by which the Bcl-2 family members modulate apoptosis are still not completely eluci-

dated, but their key functions revolve around the release of proapoptotic factors, especially cytochrome *c* from the mitochondrial intermembrane compartment into the cytosol (168, 217).

Several models have been proposed to explain how Bcl-2 family members can cause the exit of large molecules such as cytochrome *c* from the mitochondria. It is suggested that 1) Bcl-2 proteins may insert themselves into the outer mitochondrial membrane, where they could form channels that allow the passage of molecules (191); 2) Bcl-2 family members may interact with other mitochondrial membrane proteins (e.g., the voltage-dependent ion channel or VDAC) to form large-pore channels (191, 209, 227, 228); and 3) Bcl-2 family members may alter mitochondrial membrane permeability, causing mitochondrial swelling and eventual rupture of the outer membrane, thus releasing intermembrane proteins into the cytosol (82). Although evidence to support all of the above models is presented by various investigators, further studies are needed to resolve this crucial issue.

The release of cytochrome *c* by mitochondria is almost a universal feature found in response to various intracellular stimuli, including DNA damage, glucocorticoids, oxidative injury, and growth factor deprivation, although they may not play a significant role in receptor-mediated apoptosis (3, 109). Cytosolic cytochrome *c* triggers the formation of the mitochondrial apoptosome, which consists of cytochrome *c*, apaf-1, and caspase-9 (92, 94). Cytochrome *c* binds and oligomerizes the adapter protein apaf-1, which recruits procaspase-9. This causes procaspase-9 to autocatalytically activate to form its active form caspase-9 and proteolytically activate caspase-3. As expected, cytochrome *c*-deficient embryonic stem cells were resistant to UV light and staurosporine-induced apoptosis and partially resistant to apoptotic stimuli from serum deprivation. However, cytochrome *c*-deficient cells readily underwent apoptosis in response to receptor-mediated apoptotic stimuli (92, 94).

In addition to cytochrome *c*, mitochondria release a large number of other polypeptides, including AIF (135), Endo G (see above), second mitochondrial activator of caspases (Smac/DIABLO) (52), HtrA2/Omi (124), and pro-caspases-2, -3, and -9 from the intermembrane space (134). Smac/DIABLO and HtrA2/Omi promote apoptotic cell death by inhibiting a set of proteins termed inhibitors of apoptosis (IAPs) (52, 124). IAPs (e.g., c-IAP1, c-IAP2, X-linked IAP, Survivin) can bind to caspases and block cell death induced by a variety of stimuli (207).

AIF is a flavoprotein that translocates from mitochondria to nuclei on apoptotic stimulation and induces caspase-independent chromatin condensation and large-size (50-kb) DNA fragmentation (216). AIF can also induce mitochondrial release of cytochrome *c* and thus augment the cell death process through the apoptosome pathway (216). Recent studies indicate that heat shock protein 70, which is upregulated in cellular stress (231), exerts its cytoprotective function by binding and inhibiting AIF activity (190).

Thus mitochondria participate in the apoptotic pathways through at least two independent and redundant pathways, one involving the activation of caspases and the other mediated by AIF. Studies utilizing knockout mice for cytochrome *c*, apaf-1, caspase-9, and AIF together with caspase inhibition studies suggested that AIF-mediated apoptosis is dependent on the initial stimulus (92). AIF-deficient embryonic stem cells, when cotreated with caspase inhibitors, were protected from apoptosis induced by the oxidative stress inducer menadione, whereas it was only partially protected from apoptosis in response to serum withdrawal. The hierarchical nature and the interactions between AIF and the apoptosome in cell death pathways remain largely unknown (92).

Although the extrinsic and intrinsic signals are considered to take two distinct pathways to execute cell death, receptor-initiated cell death can involve the mitochondrial pathway through the BH3-only protein Bid. In hepatocytes, activation of caspase-8 by Fas leads to cleavage of Bid to its active form t-Bid. t-Bid translocates to mitochondria and associates with Bcl-2-like proteins to disrupt mitochondrial integrity (70, 240). It should be noted that the cross talk between the two pathways is minimal under most conditions. A recent report indicates that cytotoxic stress induced mitochondrial permeability, and release of various apoptogenic factors is mediated by caspase-2 in human fibroblasts transfected with adenoviral oncogene E1A. Small interfering RNA (SiRNA)-mediated silencing of caspase-2 expression prevented cisplatin, etoposide, and UV light-induced apoptosis in these cells. It is argued that in this setting, mitochondria are amplifiers of caspase activity rather than initiators of caspase activation (114).

Mitochondria can also initiate a necrosis-like PCD independently of caspase activation. On stimulation by TNF, mitochondria are shown to produce reactive oxygen species (ROS) that can induce necrotic cell death, and ROS scavengers attenuated the injury (203, 233). An impairment in the cytochrome *c* or AIF pathway can switch the cell death mode from apoptosis to necrosis. Inhibition of caspases can also switch apoptosis to necrosis once mitochondria are induced. Thus mitochondria play a central role in executing different modes of cell death (116, 118, 119). In addition to mitochondria, several other cellular organelles may participate in apoptotic cell death, and the details are reviewed elsewhere (63).

#### APOPTOSIS-BASED THERAPEUTIC AGENTS

The participation of an ever-growing number of molecules in the apoptotic machinery offers a plethora of opportunities for apoptosis modulation (81). Various strategies that are being developed include the use of antisense oligonucleotides, recombinant proteins, small molecules to disrupt protein-protein interactions, and caspase inhibitors (163, 194).

An antisense oligonucleotide (G-3139) that targets the first six codons of the Bcl-2 open reading frame is

shown to downregulate its mRNA. Continuous infusion of G-3139 markedly reduced tumor growth of Merkel cell carcinomas that were xenografted into SCID mice. G-3139 is presently in clinical trials for malignant melanoma and other forms of human cancers. Anti-sense strategies are also being attempted to modulate the expression of Bcl-XL, c-FLIP, and survivin (163).

Caspase inhibition using active site mimetic peptide ketones, such as fmk [benzyloxycarbonyl (z)-VAD-fluoromethylketone], cmk (z-YVAD-fmk/chloromethylketone), z-DEVD-fmk/cmk, and z-D-cmk, have provided valuable information regarding their use as apoptotic inhibitors (66). Caspase inhibition has shown remarkable efficacy in inhibiting apoptotic cell death in different models of IRI, including cerebral, cardiac, and kidney (33, 43, 58). Cell-permeable specific caspase inhibitors are being developed by various pharmaceutical companies and will undoubtedly be more viable in treating acute injuries such as IRI, transplantation, and cerebral stroke (163).

### MOLECULAR MECHANISMS OF NECROSIS

Necrosis is the prominent mode of cell death that occurs in various neurodegenerative conditions and as a consequence to ischemic injury in various organs including the brain and heart. Even though great progress has been made in the last decade in understanding the molecular mechanisms of apoptosis, the biochemical pathways leading to necrotic cell death remain poorly understood (141). Necrosis is long thought to be a "passive" process occurring as a consequence of acute ATP depletion. Several ATP-dependent ion channels become ineffective, leading to ion dyshomeostasis, disruption of the actin cytoskeleton, cell swelling, membrane blebbing, and eventual collapse of the cell (9, 137, 169). Recent reports suggest that in addition to the passive mechanisms, "active" mechanisms may also participate in the necrotic process.

#### *Na<sup>+</sup> Overloading*

In ischemia or hypoxic injury, energy depletion occurs by defective ATP production combined with the rapid consumption of ATP by ion pumps and through hydrolysis and leakage. The necrotic volume increase associated with necrotic cell death is initiated by an influx of Na<sup>+</sup> and release of ATP due to membrane leakage (171). The increased Na<sup>+</sup> level in the cytosol activates Na<sup>+</sup>-K<sup>+</sup>-ATPase, resulting in dissipation of ATP. In the beginning stages of the injury, a simultaneous efflux of K<sup>+</sup> maintains ion homeostasis. Severe depletion of ATP leads to failure of the pump-leak balance mechanism, leading to an influx of Na<sup>+</sup> and water that results in swelling and collapse of the cell. Thus the overload of Na<sup>+</sup> concomitant with severe ATP depletion seems to be the major determinant of a necrotic outcome (27). The mechanism by which the rapid influx of Na<sup>+</sup> takes place is still not elucidated. A role for several of the ion channels such as the Na<sup>+</sup>/K<sup>+</sup> pump, Na<sup>+</sup>/H<sup>+</sup> exchanger, Na<sup>+</sup>/Ca<sup>2+</sup> exchanger, and Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransporter has been reported in var-

ious cell lines (27, 28, 120). However, a universal role for any of these ion channels in augmenting necrotic cell death is yet to be established.

#### *Ca<sup>2+</sup> Accumulation*

Cytosolic Ca<sup>2+</sup> plays a role in linking ATP depletion and necrosis in some cell types (9), but several other cell types including hepatocytes and renal tubules can undergo necrotic cell death in its absence (90). The ROS-mediated necrotic volume increase and Na<sup>+</sup> influx are suggested to be initiated by the binding of the free radicals to ion channels including nonselective Ca<sup>2+</sup> channels (10, 83, 84, 105). The increased levels of Na<sup>+</sup> activates Na<sup>+</sup>-K<sup>+</sup>-ATPase and consumes ATP, which further activates nonselective Ca<sup>2+</sup> channels, resulting in massive cytosolic Ca<sup>2+</sup> accumulation. High levels of Ca<sup>2+</sup> can participate in ATP depletion by activating Ca<sup>2+</sup>-ATPase and mitochondrial depolarization. The increased levels of Ca<sup>2+</sup> activate endonucleases to degrade DNA and activate cellular proteases such as calpain to degrade several structural and signaling proteins (239). The role of Ca<sup>2+</sup> in oxidative stress is reviewed in detail by Ermak and Davies (59).

Recently, two novel Ca<sup>2+</sup>-permeable cation channels belonging to the long transient receptor potential (TRP) channel family, LTRPC2 and LTRPC7, are found to be activated by a disruption of the redox (oxidation-reduction) status (79, 160, 182). LTRPC7 is an intracellular ligand-gated ion channel that can permeate both Ca<sup>2+</sup> and Mg<sup>2+</sup> and is suppressed by higher Mg<sup>2+</sup>-ATP concentration. In ischemic conditions, the levels of ATP-bound Mg<sup>2+</sup> fall, which may activate inward and outward currents through LTRPC7 (160). LTRPC2 is a NSCC that is permeant to both Na<sup>+</sup> and Ca<sup>2+</sup>. It is activated by binding of ADP-ribose and increased concentration of arachidonic acid and Ca<sup>2+</sup> but suppressed by high Na<sup>+</sup> levels. LTRPC2 is activated by micromolar levels of H<sub>2</sub>O<sub>2</sub> and agents that produce ROS or reactive nitrogen species, thus representing an intrinsic mechanism that mediates Ca<sup>2+</sup> and Na<sup>+</sup> overload in response to changes in redox status (79, 182).

Mild oxidative stress such as that induced by moderate levels of H<sub>2</sub>O<sub>2</sub> can increase cytoplasmic Ca<sup>2+</sup> levels by its release from internal stores such as the endoplasmic reticulum (ER). This process is mediated through protein kinase C and inositol triphosphate (InsP<sub>3</sub>). A recent study demonstrated that in *C. elegans*, the Ca<sup>2+</sup>-binding proteins calreticulin and calnexin are essential for stimulating necrosis by stresses not involving Ca<sup>2+</sup> influx across the plasma membrane (247).

#### *The Mitochondrial Connection*

It is well known that mitochondria participate in necrotic and apoptotic cell death by opening the mitochondrial permeability transition pore. Several second messengers and proapoptotic proteins including Bcl2 family members can induce the permeabilization of the mitochondrial permeability transition pore (38, 108).

BNIP3 is a member of the Bcl2 family that is loosely associated with mitochondria in the normal state but gets fully integrated into the mitochondrial outer membrane after a death stimulus. BNIP3-transfected cells are found to undergo cell death independently of Apaf-1, caspase activation, cytochrome *c* release, and nuclear translocation of AIF. The cells exhibited morphology typical of the necrotic form of cell death with plasma membrane permeability, mitochondrial damage, extensive cytoplasmic vacuolation, and mitochondrial autophagy. It is proposed that BNIP3 can mediate necrosis-like cell death through mitochondrial permeability transition pore opening and mitochondrial dysfunction (230). The expression of BNIP3 is shown to be induced in several cell lines in response to hypoxic injury. Overexpression of the hypoxia-inducible factor-1 $\alpha$  also induced the expression of BNIP3, resulting in a necrotic form of cell death (71).

One of the major factors that determine whether a cell undergoes apoptosis or necrosis is the level of intracellular ATP. Poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme that adds ADP-ribose polymers to various proteins and to itself when activated by DNA strand breaks (143). PARP activation is thought to exacerbate ATP depletion and induce necrotic cell death (72, 184). Overactivation of PARP after a cellular injury can result in consumption of its substrate  $\beta$ -NAD<sup>+</sup>. In an effort to resynthesize NAD<sup>+</sup>, ATP is massively depleted and the cells die from lack of energy (72, 184). PARP inhibition protected cells from necrotic cell death in neuronal ischemia, myocardial ischemia, and renal ischemia (113, 133, 140). PARP-deficient mice are protected against neuronal and myocardial ischemic injury and diabetic pancreatic damage (56, 183, 257), but they are susceptible to apoptotic cell death elicited by TNF- $\alpha$  and Fas in hepatocytes (117) and  $\gamma$ -irradiation. Fibroblasts from PARP  $-/-$  mice are protected from ATP depletion and necrotic cell death but not from apoptotic cell death (72). A recent study in L929 fibrosarcoma cells showed that ligation of CD95 induces apoptosis, whereas TNF elicits necrosis in the cells. Further investigation into the mechanism of this discrepancy showed that TNF induces PARP activation, leading to ATP depletion and necrosis. In contrast, CD95 ligation cause PARP cleavage, thereby maintaining ATP levels, and the cells die by apoptosis (136).

A recent report by Yu et. al (249), however, indicates that NAD<sup>+</sup> depletion and subsequent energy depletion may not be the cause of cell death after PARP activation. Instead, NAD<sup>+</sup> decrement may act only as a signal for AIF translocation from mitochondria to cytosol and to nucleus. AIF initiates nuclear condensation and subsequent chromatin fragmentation, culminating in cellular demise.

#### CELL DEATH IN ISCHEMIC ACUTE RENAL FAILURE

A reduction in the glomerular filtration rate (GFR) is the primary change in renal function caused by ARF in humans and experimental animal models (53). Patho-

physiological mechanisms involved in the decline in GFR can be attributed to persistent vasoconstriction due to an imbalance between vasoconstrictive and vasodilatory mediators (36, 37); vascular obstruction caused by endothelial-leukocyte interactions (31, 187); tubuloglomerular feedback in response to increased solute delivery to the macula densa (104, 142); tubular obstruction caused by detachment of tubular epithelial cells from the basement membrane and back-leak of glomerular filtrate as a consequence of disruption of the epithelial cell layer (5, 20, 21, 65).

#### *Vascular Dysfunction in Renal Ischemia*

After an ischemic insult, total renal blood flow returns toward normal, but marked, regional alterations occur. The outer medullary region is marginally oxygenated under normal conditions and has high energy demands. The blood flow to outer medullary or corticomedullary junction region remains  $\sim$ 10% of normal during reperfusion (234, 235). The microvasculature in this region becomes congested due to interstitial edema, red blood cell trapping, leukocyte adherence, and extravasation (22). It is still unclear whether the medullary vascular congestion or the preglomerular vasoconstriction is the primary reason for causing the impaired GFR (206). The reduced blood flow and hypoxic conditions that occur in renal ischemia lead to deprivation of vital nutrients and loss of ATP in the vascular cells and in the nephron segments of the outer medullary region. In the vascular smooth muscle cells and endothelial cells, disorganization of F-actin is observed. The endothelial cells undergo swelling, leakage, cell activation, and dysfunction (111, 158). Injection of endothelial cells expressing endothelial nitric oxide synthase into rats subjected to renal ischemia resulted in the implantation of these cells in the renal microvasculature and functional protection of ischemic kidneys (23). A recent study demonstrated that permanent damage to peritubular capillaries occurred in rats that underwent renal ischemia and may partly account for the pathogenesis of chronic renal failure in this setting (11).

#### *Tubular Dysfunction in Renal Ischemia*

At the tubular level, the S3 segment of the proximal tubule that traverses the outermedullary segment is extremely susceptible to ischemic injury. This is due to their low glycolytic capacity to generate ATP in the setting of rapid ATP depletion resulting from impaired oxidative phosphorylation. The acute, severe ATP depletion leads to necrotic cell death. The medullary thick ascending limbs, although situated in the same region, do not undergo the same level of injury because they have greater glycolytic capacity to generate ATP under ischemic conditions (20). However, the cells in this nephron segment and other distal tubule cells undergo sublethal changes and produce various chemokines and cytokines that may have autocrine and/or paracrine effects on the injury and regeneration process of the kidney postischemia (125, 127). The avail-



ability of ATP in the distal tubule cells makes them less vulnerable to injury and to promote apoptotic cell death pathways under severe stress conditions.

Irreversible cell death induced by ischemic, toxic, and obstructive ARF was long considered to be of the necrotic type, but recent data from several laboratories indicate that both apoptosis and necrosis can occur simultaneously in these forms of ARF in humans and experimental animal models (46). The relative contribution of the two mechanisms to the initial cell loss depends on the severity of the injury and the cell type (127).

#### MECHANISMS OF NECROSIS IN RENAL ISCHEMIA

The molecular pathways adopted by the renal tissue that lead to necrotic cell death after an ischemic episode are not fully understood and are now obscured even more by attempts to explore the contribution of apoptosis to renal injury. Hypoxia resulting from decreased blood flow leads to a variety of secondary effects, including a breakdown in cellular energy metabolism, endothelial and epithelial cell dysfunction, cell swelling, generation of ROS, increase in free cytosolic  $\text{Ca}^{2+}$ , and activation of phospholipases, proteases, and endonucleases (20, 197, 229). Recent evidence indicates that active mechanisms such as activation of PARP play important roles in necrotic cell death after renal ischemia (140). A hypothetical scheme of biochemical events that may engage in positive-feedback loops to accelerate cellular disintegration culminating in necrotic cell death is shown in Fig. 4.

#### ATP Depletion

Ischemic and toxic renal injury leads to a rapid decrease in the level of the adenine nucleotide pool (ATP, ADP, and AMP) (241). In the absence of reper-

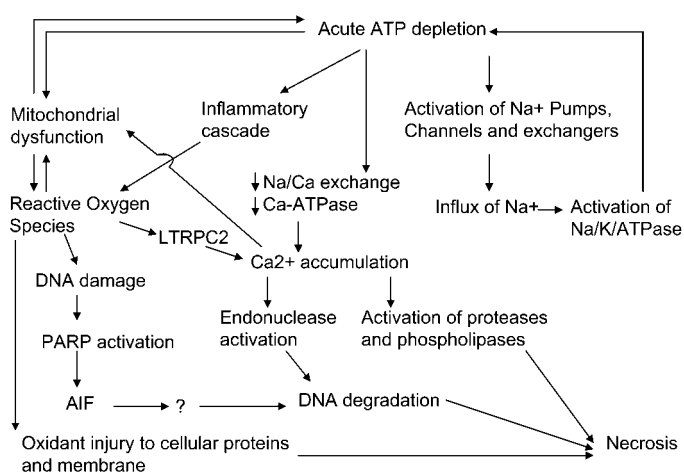


Fig. 4. Hypothetical sequence of various biochemical events eliciting necrotic cell death. The acute depletion of ATP associated with ischemic-hypoxic injury is exacerbated by mitochondrial dysfunction and activation of various ATP-dependent ion channels, which further depletes ATP by a feedback mechanism. An increase in cytosolic  $\text{Ca}^{2+}$  and ROS triggers various converging biochemical pathways, leading to activation of cellular degradative enzymes and ultimate cellular disintegration.

fusion, the adenine nucleotides are degraded to the purine nucleosides adenosine and inosine and to the purine base hypoxanthine. The purine metabolites and the base are membrane permeable. Prolonged ischemia will lead to the continued loss of these precursor nucleosides, and the cell is dependent on the endogenous precursor compounds that become available during the reperfusion for ATP resynthesis (7, 214). Prolonged ischemia also leads to mitochondrial dysfunction and impaired oxidative phosphorylation. Thus the rate of repletion of ATP is dependent on the severity and duration of the injury (125, 129).

#### $\text{Na}^+$ Influx and "Oncosis"

ATP depletion leads to disruption in the microvillous actin, the cytoskeletal meshwork, and the cortical actin of the proximal tubule epithelial cell (154, 156). Disruption of the cortical actin cytoskeleton leads to redistribution of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  to the apical membrane of the proximal tubule epithelial cell, and this will alter the  $\text{Na}^+$  handling of the proximal tubule (155, 157). A high fraction of filtered  $\text{Na}^+$  will reach the macula densa and result in increased vasoconstriction via the tubuloglomerular feedback mechanism (153). Prolonged ischemia can compromise  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity, leading to influx and accumulation of  $\text{Na}^+$ ,  $\text{Cl}^-$ , and water in the cytosol. Severe depletion of ATP leads to failure of the pump-leak balance mechanism, resulting in cell swelling or oncosis, a typical feature of necrosis (241).

#### Increased Cytosolic $\text{Ca}^{2+}$ Concentration

Renal proximal tubular cells undergo a significant increase in free cytosolic ionized  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) from 170 to 390 nM during a 5-min exposure to hypoxic injury. The increase in  $\text{Ca}^{2+}$  preceded hypoxic membrane damage and was reversible if reoxygenated before the cell undergoes lethal injury (53). A role for  $\text{Ca}^{2+}$  in mediating the injury is further substantiated by the findings that removal of extracellular  $\text{Ca}^{2+}$ , and chelation of intracellular  $\text{Ca}^{2+}$ , protected the cells from hypoxic injury (53). Furthermore,  $\text{Ca}^{2+}$  channel blockers are found to ameliorate ischemic renal injury in experimental animal models and in humans. Overloading of  $\text{Ca}^{2+}$  in mitochondria results in uncoupling of oxidative phosphorylation and subsequent reduction in ATP synthesis and increased production of superoxides. The mechanisms by which a rise in  $\text{Ca}^{2+}$  level contributes to the pathophysiology of ARF may include activation of  $\text{Ca}^{2+}$ -dependent proteases, phospholipases, and endonucleases (20, 53).

#### Proteases

Prolonged increases in  $[\text{Ca}^{2+}]_i$  levels activates the  $\text{Ca}^{2+}$ -dependent cysteine protease calpain. Renal proximal tubules constitutively express calpains and are activated in response to toxic and hypoxic injuries. Inhibition of calpain activity using various pharmacological agents ameliorated necrotic cell death in prox-

imal tubule cells (PTC) subjected to hypoxic injury or ATP depletion (80, 130, 202, 208).

Meprin (metallopeptidase from renal tissue) is a zinc-dependent metalloendopeptidase that is present in the brush-border membrane of renal proximal tubular epithelial cells, accounting for ~5% of the total tubular protein (223). Meprin is localized to the apical brush border (29, 237). After renal tubular epithelial cell injury, it translocates to the basement membrane and cleaves one of the basement membrane components, nidogen. Mouse strains expressing lower levels of meprin are less susceptible to ischemic injury compared with those expressing normal levels (223). Exogenously added meprin is cytotoxic to renal PTC, and inhibition of meprin provided both histological and functional renal protection after ischemic injury (29, 237).

### *Phospholipases*

Ischemic cell injury is associated with phospholipolysis and activation of various phospholipases. PLA<sub>2</sub> is one of the acyl hydrolases with various isoforms that are dependent on or independent of Ca<sup>2+</sup> for their activation (19). PLA<sub>2</sub> activation causes membrane phospholipid breakdown during ischemic injury in various tissues including the kidney (185, 186). The mechanism by which PLA<sub>2</sub> activation appends the injury is not clear. It is possible that the breakdown in cell membrane integrity, generation of inflammatory mediators, and the cytotoxicity resulting from the accumulation of lysophospholipids and free fatty acids accentuate cellular injury (20, 144). The role of free fatty acids in mediating the injury, however, is controversial because exposure of proximal tubules to unsaturated free fatty acids protected against hypoxic injury (250, 251).

### *Endonucleases*

Fragmentation of DNA has been demonstrated after IRI in animal models and hypoxia/reoxygenation injury in isolated PTC culture models (15, 49, 89, 204). DNA ladder formation and DNA strand breaks demonstrated in postischemic kidneys and isolated proximal tubular cells were characteristic of endonuclease activation. A 15-kDa nuclear endonuclease and a 30-kDa cytosolic DNase/endonuclease that are induced and activated postischemic renal injury have been described (13). Inhibition of the activities of these endonucleases protected renal cells from hypoxia-mediated necrotic cell death. The mechanisms by which these enzymes are activated are not elucidated. It is proposed that the DNA strand breaks induced by oxidative damage may induce and activate the endonucleases. IRI leads to massive DNA damage shortly after injury in renal epithelial cells (229). The degree of DNA damage that renders cell death irreversible and whether inhibition of the activity of the endonucleases at the reversible stage ameliorate cell death in renal ischemia are not known.

### *ROS*

IRI in the kidney is associated with generation of ROS in the neutrophils and in parenchymal cells such as proximal tubules and endothelium. The various sources of production of ROS include the impaired mitochondrial electron transport chain, cyclooxygenases, lipoxygenases, and xanthine oxidase (219). The superoxides can react with nitric oxide (NO) and form peroxynitrate (53, 69). ROS has been implicated in mediating ischemic renal injury, and treatment with antioxidants or free radical scavengers ameliorated renal injury (23, 167, 181). The mechanisms by which ROS induce damage to the cells include peroxidation of lipid membranes, protein denaturation, and DNA strand breaks (34). The massive DNA damage associated with renal ischemia leads to excessive activation of the DNA repair enzyme PARP (64, 140) and subsequent ATP depletion (72, 253).

### *PARP*

Inhibition of PARP protected renal PTC from oxidant injury and necrotic cell death. Prolonged incubation of renal PTC in the presence of PARP inhibitors, however, induced apoptotic cell death. The mechanism of induction of the apoptotic pathway in the absence of PARP is yet to be determined. Administration of PARP inhibitors to rats post-ischemic renal injury prevented the decline of ATP in renal tissues. The levels of serum creatinine and BUN values returned to normal levels at a faster rate in PARP-inhibited animals compared with that in vehicle-treated animals (140).

### *Inducible Nitric Oxide Synthase and Osteopontin*

NO produced in the renal proximal tubules in response to ischemic injury is mediated by the inducible form of nitric oxide synthase (iNOS) (181). Mice deficient for iNOS are protected against ischemic renal injury (69). Specific inhibition of iNOS using antisense oligonucleotides before inducement of ischemic renal injury also resulted in a dramatic functional protection of kidneys from acute ischemia in rats (69). There is some evidence that expression of iNOS post-renal injury may be modulated by osteopontin. Recombinant osteopontin can inhibit iNOS expression and production of NO in renal epithelial cells (87). Osteopontin and its receptor CD44 are highly induced postinjury in distal tubules, and administration of renoprotective agents such as IGF-1 further enhanced its expression (121, 177). Osteopontin-deficient mice are more susceptible to injury and showed increased levels of iNOS expression and prevalence of nitrosylated proteins (166).

### *Protein Kinases*

The PKC family of serine-threonine kinases encompasses 12 different isozymes. PKC can be activated by Ca<sup>2+</sup>, phosphatidyl serines, and diacylglycerol. The PKC family transduces a myriad of signals

by activating G protein-coupled receptors, tyrosine kinase receptors, and nonreceptor tyrosine kinases (164). The increased levels of intracellular  $\text{Ca}^{2+}$  and the phospholipid hydrolysis products that exist in renal tubular cells postischemia provide a suitable environment for PKC activation. PKC isozymes and the receptor for activated C kinase are induced and activated post-ischemic injury in various tissues including the kidney (173, 174). PKC- $\alpha$ , - $\beta$ II, and - $\zeta$  are induced and translocated to subcellular components postischemia in rat kidneys (173). Exposure of LLC-PK<sub>1</sub> cells to oxidant injury induced expression of PKC isozymes. Activation of PKC is shown to protect the cells from oxidant injury and subsequent necrotic cell death (175). The mechanisms by which PKC activation ameliorates necrotic cell death are yet to be addressed. Recent reports indicate a role for PKC in ischemic preconditioning and heat shock-induced renal protection (115, 147).

SAPK/JNK is a member of the MAPK family. SAPK transduces signals to the nucleus in response to cellular stresses such as inflammatory cytokines, ischemia, reversible ATP depletion, heat shock, and genotoxic stress. SAPK activity is markedly increased in the proximal and distal tubules after IRI (110, 178). Inhibition of SAPK activity during ischemia ameliorates renal failure (48). ERKs are another class of the MAPK family involved in mitogenic response and cellular differentiation. ERK is also activated post-renal injury, but its expression is localized to thick ascending limbs in the inner stripe. Studies in several cellular systems have suggested that JNK activation can be modulated by the coexpression of ERKs. It is suggested that the activation of the ERKs in distal tubules during ischemic insult may protect the cells from the injurious effects of JNK activation (47).

#### MOLECULAR MECHANISMS OF APOPTOSIS IN RENAL ISCHEMIA

Apoptotic cell death has been documented in experimental animal models and humans post-renal ischemia, and inhibition of apoptotic cell death is shown to ameliorate the injury and inflammation (42, 43). Several factors that can induce necrotic cell death, including growth factor deprivation, loss of cell-cell and cell-matrix interactions, cytotoxic stimuli, and cell death receptor activation, are also suggested to trigger apoptotic cell death in renal ischemia (126). The severity of the injury caused by these factors and the degree of cellular ATP and/or GTP depletion play crucial roles in determining the mode of cell death (126). A severe depletion of ATP favors necrotic cell death whereas GTP depletion is shown to promote apoptotic cell death (98, 126). The elucidation of various apoptotic pathways and the identification of the vast array of molecules that regulate apoptosis provide new opportunities for investigating the mechanisms by which apoptotic cell death occurs in renal ischemia.

#### DEPLETION OF GTP

GTP and GTP-binding proteins play major roles in signal transduction pathways, leading to cell growth, receptor activation, and cellular homeostasis. Selective depletion of intracellular guanylates results in inhibition of proliferation and induces apoptosis in pancreatic  $\beta$  cells (122). It is speculated that the cell death induced by GTP depletion might be modulated or mediated by GTP-binding proteins.

Among the various factors contributing to ischemic injury, ATP depletion has always been assumed to be the main culprit (128). The role of other cellular nucleotide pools in ischemic conditions was not investigated. Recent reports indicate that depletion of GTP occurs concurrently with depletion of ATP in in vitro and in vivo models of ischemic renal injury (44). Selective depletion of GTP induced apoptosis in renal tubular cells. Supplementation of guanosine to renal tubular cells subjected to chemical anoxia selectively enhanced GTP levels close to normal values and significantly reduced apoptotic cell death (44). Administration of guanosine before inducement of ischemic renal injury improved renal functions and significantly reduced the number of cells undergoing apoptotic cell death (98). The novel finding that manipulation of guanine nucleotide levels could modulate apoptotic cell death in renal ischemia offers new avenues for therapeutic intervention.

#### *TNF Receptor Family-Mediated Apoptosis*

Members of the TNF family of receptors including CD95 (Fas), TNFR-1, and CD27 have been implicated in the pathogenesis of IRI (50, 51, 61, 165, 176). TNF is a mediator of inflammation, which exerts its biological effects through its interaction with TNFR-1 or -2. Induced expression of TNF occurs post-IRI in various organs including the kidney. Simulated ischemia in LLC-PK<sub>1</sub> cells induced TNF- $\alpha$  mRNA expression and bioactivity. The production of TNF post-renal injury is triggered by the locally produced ROS, which activates the transcription factor NF- $\kappa$ B through p38 MAP kinase (51). Inhibition of p38 MAP kinase using specific inhibitors suppressed the expression of interleukin-1 $\beta$  and TNF- $\alpha$  and reduced apoptotic cell death in mice and dogs that underwent ischemic renal injury. Neutralization of TNF- $\alpha$  bioactivity using antibodies to TNF- $\alpha$  or inhibition of p38-MAP kinase (146) or NF- $\kappa$ B activity (145) prevented apoptosis in LLC-PK<sub>1</sub> cells subjected to simulated ischemia.

Fas is the best characterized TNF receptor that can trigger apoptosis in various cells including renal tubular epithelial cells (103, 172). Renal proximal tubular cells subjected to ATP depletion or exposed to LPS underwent apoptosis and was accompanied by increased Fas protein expression (91, 102). That mice deficient for Fas are protected from ischemic renal injury suggests an involvement of Fas-FasL system in renal ischemia (165).

Another member of the TNFR family implicated in inducing apoptosis post-ischemic renal injury is CD27. CD27 and its death domain-containing binding partner Siva are induced at sites of apoptosis in tubular cells post-renal injury (176). Hypoxic injury in LLC-PK<sub>1</sub> cells induced the expression of both CD27 and Siva. Transient transfection of Siva in LLC-PK<sub>1</sub> cells induced 100% apoptotic cell death in transfected cells. The role of CD27-mediated cell death post-renal injury is under investigation.

### Caspases

Several studies have documented caspase activation after IRI in the kidney and post-hypoxic injury in renal PTC. The expression of caspases-1, -2, -3, -6, -7, -8, and -9 has been characterized in rat kidneys at the mRNA level (95). The expression and activity of caspases-1, -2, and -6 are altered in kidneys post-IRI (96). LLC-PK<sub>1</sub> and Madin-Darby canine kidney (MDCK) cells subjected to chemical hypoxia underwent apoptosis with a marked increase in activation of caspases-3 and -8 (55). The activation of caspase-3 is accompanied by Bax translocation from cytosol to mitochondria and cytochrome *c* release from mitochondria (196, 197). Inhibition of caspase activity protected the cells from undergoing apoptotic cell death.

Inhibition of caspases using a pancaspase inhibitor is shown to protect kidneys from ischemic injury. The pancaspase inhibitor protects against ischemic ARF in mice by inhibition of apoptosis and subsequent inflammation (43). The results from these studies clearly demonstrate a role for caspases in IRI. However, the role of individual caspases contributing to the injury and inflammation post-renal injury cannot be discriminated from these studies because nonspecific caspase inhibitors were utilized. The availability of specific inhibitors of individual caspases will provide better clues as to the functions of individual caspases in renal IRI. Induction of IRI in mice deficient for caspase-1 by two different groups furnished contrasting results. Results from one study indicated that caspase-deficient mice underwent less severe injury than their wild-type counterparts and that this was due to impaired interleukin-18 activation (148), whereas a second group found no changes in the severity of the injury between control groups and caspase-1-deficient mice (41).

### Mitochondria and Bcl-2 Family Members

Mitochondria participate in inducing apoptosis after IRI through multiple changes, including generation of oxygen free radicals, calcium translocations, altered permeability transitions, and release of cytochrome *c*, apoptogenic factors, and Bcl2 family members. Renal IRI in rats can induce mitochondrial swelling, rupture of inner and outer membranes, and release of Bcl2 postinjury (17). A recent study investigated the proximate events that lead to mitochondrial permeability transition and release of cytochrome *c* after hypoxia/reoxygenation injury in kidney proximal tubular cells (242). A persistent respiratory defect occurs in complex

I-dependent substrates during reoxygenation after hypoxia, and this defect is associated with condensed mitochondrial configuration and incomplete recovery of mitochondrial membrane potential. Amelioration of impaired substrate flux through complex I and ATP generation by  $\alpha$ -ketoglutarate plus greatly improved mitochondrial function and cellular recovery (242). The identification of these upstream pathways of anaerobic metabolism and the possibility of metabolic manipulations to improve mitochondrial functions at an early stage may help to prevent irreversible mitochondrial damage in renal ischemia.

Ischemic renal injury is associated with a marked increase in the expression of the antiapoptotic Bcl2 family of proteins, Bcl2, Bcl-X<sub>L</sub>, and the apoptotic protein Bax, in distal tubules and moderate increases in the proximal tubules (12, 40). The marked upregulation of the antiapoptotic proteins in the distal tubules may tip the balance in favor of cell survival, and this imbalance may be involved in its adaptive resistance to ischemic injury. It is suggested that this survival mechanism may allow the cells to produce growth factors that may aid in the protection and/or regeneration of the distal tubules by an autocrine mechanism and of the more vulnerable proximal tubules by a paracrine mechanism (68).

The relative expression of the Bcl2 family of proteins in distal and proximal tubules subjected to oxidant injury *in vitro* is similar to that seen *in vivo*. However, the expression of BclX<sub>L</sub> is decreased in PTC and a translocation of BclX<sub>L</sub> from the cytosol to the mitochondria is observed in the surviving distal tubule cells. No change in the subcellular distribution of Bax was observed in the surviving distal tubule cells, and it remained widely distributed in the cytosol. The expression of Bcl2 or Bax was also unchanged in PTC post-oxidant injury. It is unclear if the translocation of BclX<sub>L</sub> plays a role in its protection from the oxidative injury (40). In a separate study, proximal tubules subjected to ATP depletion induced by hypoxic injury or impaired oxidative phosphorylation are shown to translocate Bax from the cytosol to the mitochondria. It is suggested that Bax may form pores in the mitochondrial outer membrane causing the release of cytochrome *c* from the mitochondrial intermembrane space and may activate apoptotic pathways (196).

### Growth Factors

Administration of growth factors pre- or postinjury to animal models of renal ischemia is shown to ameliorate the injury and enhance renal regeneration. The beneficial effects may be attributed to their antiapoptotic, proliferative, and proangiogenic influences (74–77). In addition, they are shown to enhance GFR postinjury possibly by enhancing NO production, increased local blood flow (39, 54), and vasodilatation of renal microvessels in juxtamedullary nephrons (222).

Among the various growth factors, hepatocyte growth factor (HGF) is one of the most potent renoprotective growth factors (76, 150, 151, 236). After acute

renal injury, the expression of the HGF receptor c-met is exclusively induced in kidneys (132). HGF, when administered in its peptide form, is rapidly removed from blood circulation by the liver and has a very short half-life. Recently, it was shown that intravenous administration of a naked plasmid encoding the HGF gene results in its expression for up to 6 days in the kidney (45). Intravenous administration of a single dose of HGF plasmid DNA protected tubular epithelial cells from both apoptotic and necrotic forms of cell death after folic acid-induced renal injury. This novel gene-delivery system may help to circumvent the difficulty of maintaining sustainable levels of the growth factor in the blood circulation and offers a novel therapeutic strategy (45).

### *The Inflammatory Cascade in Renal IRI*

The inflammatory cascade of renal injury is initiated by ATP depletion and is further exacerbated during reperfusion (170). Several candidate systems that originate from endothelial, epithelial, and infiltrating inflammatory cells are shown to mediate the inflammatory process (206). On stimulation by IRI, vascular endothelial cells express various adhesion molecules such as ICAM-1, P- and E-selectins, and  $\beta$ 2-integrins (26). ICAM-1 binds to lymphocyte function-antigen (LFA-1; CD11 $\alpha$ /CD18) and Mac-1 (CD11 $\beta$ /CD18) receptors on neutrophils, resulting in their recruitment and activation (187). The endothelial cells also produce leukotriene B<sub>4</sub> and platelet-activating factor, which further stimulate inflammatory cells. On adherence, neutrophils release ROS, proteases, elastases, myeloperoxidase, and other protein- and matrix-degrading enzymes (219). Furthermore, the endothelial cell dysfunction and activation of leukocytes contribute to the inflammatory process with the coordinated release of several cytokines that include IL-1, IL-6, IL-8, TNF- $\alpha$ , chemokines, and monocyte chemoattractant protein-1 (MCP-1) (218). The molecular mechanisms by which the inflammatory reactions ensue necrotic and/or apoptotic cell death in renal epithelial cells are incompletely characterized. The role of other inflammatory cells such as macrophages and T and B lymphocytes in mediating inflammatory responses in renal ischemia remains to be established (107, 179, 187).

### **DETECTION OF APOPTOSIS IN ISCHEMIC RENAL TISSUES**

Detection and quantitation of apoptosis in renal tissues that underwent ischemic injury are challenging due to the nonspecificity of the methods that are presently available. The use of a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay has been criticized for its lack of sensitivity and specificity in discriminating among apoptotic, necrotic, and sublethally injured cells (139). Although electron microscopic analysis still remains as the reliable criterion to distinguish among the different forms of cell death, it provides a qualitative rather than a quantitative evaluation of apoptotic cell death,

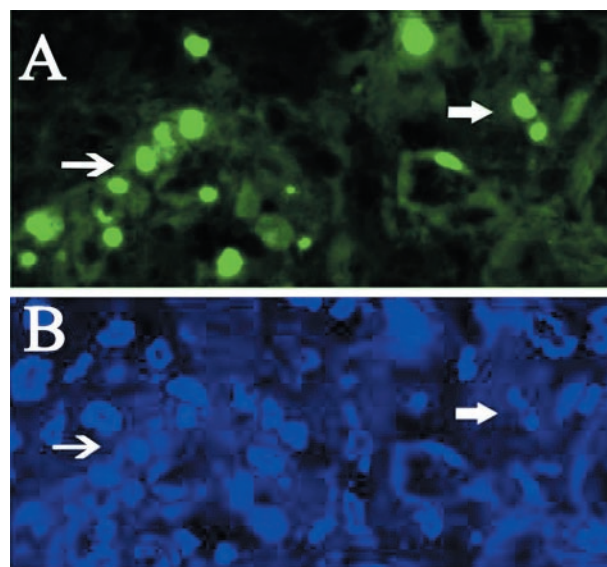


Fig. 5. Double staining of ischemic renal tissue with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) and Hoechst 33342. A TUNEL assay was performed on formalin-fixed ischemic mouse kidney sections as previously described (176). The tissue sections were counterstained with Hoechst 33342 and mounted. Apoptotic cells were visualized under a fluorescent microscope, and the different fluorescent labels were detected by interchanging the fluorescent filter. The nuclear morphology of TUNEL-positive cells detected in A is altered, as shown by arrows in B.

especially in tissue sections. The online website <http://www.cyto.purdue.edu/flowcyt/research/cytotech/apopto/data/> provides electron microscopic photographs of cells undergoing apoptosis and necrosis.

Another reliable approach that can be easily carried out is to use a combination of a biochemical assay and a morphological assay on the same tissue. Double staining of kidney sections using the TUNEL technique in conjunction with a nuclear stain such as Hoechst 33342 has been successful, as shown in Fig. 5. The morphological changes in nuclear chromatin structure are visualized histologically and can be used to confirm the apoptotic nature of the TUNEL-positive cells in an ischemic renal tissue. The advantages and disadvantages of various methodologies used to detect apoptosis in tissue sections are reviewed elsewhere (152, 210).

### **CONCLUDING REMARKS**

Apoptotic and necrotic forms of cell death coexist in ischemic renal tissues. The relative contribution of the two modes of cell death after an insult depends on the severity of the injury, level of ATP and/or GTP depletion, and the idiosyncrasy of the cell. Necrotic cell death can be mediated by passive as well as active mechanisms. Several molecules that are involved in the active mechanisms are just beginning to be identified. Tremendous progress has been made in elucidating the biochemical and molecular complexity of the apoptotic pathway. The vast array of molecules involved in both the apoptotic and necrotic cell death

pathways offers several potential targets for therapeutic intervention. Inhibition of apoptotic and necrotic pathways ameliorated ischemic renal injury in animal models. Several pharmacological agents are presently under development to modulate apoptosis. The successful progression of a few of these agents into various phases of clinical trials suggests a transition for the apoptosis field from benchside to bedside. However, a few theoretical questions that need to be addressed before attempting apoptosis based therapy remain unanswered. If apoptosis can be selectively modulated in a specific tissue or cell type in a timely fashion without affecting other key systems and if the cells that are salvaged from apoptosis will be functional, especially in settings such as acute renal ischemia, remain to be established.

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