

FORMACION CONTINUADA EN NEFROLOGIA

Apoptotic cell death in renal disease

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Understanding the mechanisms of physiologic cell death (apoptotic or programmed cell death) may provide new diagnostic opportunities and, perhaps, additional therapeutic approaches to the clinical management of renal disease. The availability of advanced techniques in cellular and molecular biology to study the genes that regulate apoptosis has resulted in an exponential growth of information, especially in the fields of immunology, oncology, neurology and development ¹⁻⁴. We will review the concept of apoptosis, the genes involved in its regulation, its role in physiology and its possible participation in renal disease based on the international literature and our own personal experience.

Apoptotic and programmed cell death

Only a limited number of things happen to cells resident in organ tissues. They can remain stationary and support the structure-function relationships of the organ, they can proliferate to reproduce themselves, they can sometimes hypertrophy, or they can die. Complex organisms need a physiologic mode for cell death, so as to keep constant their number of cells and eliminate those damaged or that are no longer necessary. Although the concept of physiologic cell death is quite old, renewed interest in the subject dates to the creation of the term apoptotic and the description of its morphological characteristics by Kerr and colleagues in 1972 ⁵.

The functional concept of programmed cell death implies an active participation of the cell in its own

death (cell suicide) through the activation of a genetic program. In general programmed cell death has the morphologic characteristics of apoptotic, although there are exceptions. In fact, there is functional, morphologic, and genetic evidence of heterogeneity in this process ⁶⁻¹¹, and there are unanswered questions about the physiologic relevance of this diversity. Moreover, there are modes of cell death which may share characteristics of apoptotic and necrosis ¹². With this caveat in mind, and given the scarce knowledge of nonapoptotic modalities of programmed cell death, we use the term apoptosis when the characteristic morphology and pattern of DNA degradation have been demonstrated. However, the pragmatist may define programmed cell death as a process that can be modulated through interference with cell death related genes, independently of the morphology or pattern of DNA degradation.

Apoptotic is an active process which may be prevented or delayed by the administration of growth factors, certain drugs or by gene manipulation. Cells committed to an apoptotic pathway may be rescued by therapeutic maneuvers ¹³. Thus, apoptotic offers the opportunity for a therapeutic intervention and this explains the attempts to discriminate between necrosis and apoptosis as a mode of cell death in renal disease. Necrosis, as a mode of cell death, is a passive process in which the cell swells, loses the integrity of the cell membrane, releases toxic and proinflammatory products, and the DNA is degraded in random manner. Both apoptosis and necrosis, however, can occur at the same time in the same tissue ¹⁴. The occurrence of either may depend on the intensity of the precipitating events. For example, tissue ischemia may kill cells by either necrosis or apoptosis ¹⁴. The proportion of cells dying by each mechanism may vary from individual to individual, and the segment of the cell population predetermined to die by apoptosis might be rescued by interference with the genetic program of apoptotic.

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Characteristics of apoptotic

Apoptosis and necrosis may be differentiated by morphologic and functional criteria (table I)². From a morphologic view, apoptotic cells display decreased cell and nuclear size with chromatin condensation, preservation of organelles, detachment from adjacent cells, membrane cell blebbing, and fragmentation with the formation of membrane bound bodies, the so-called apoptotic bodies. Some of these features may be appreciated by light microscopy, others are more evident with electronic microscopy, or the use of nucleic acid dyes, like propidium iodide and acridine orange. There are several protocols for the identification of apoptotic cells based on changes in cell size or the pattern of nucleic acid staining by cytofluorography¹⁵.

Table I. Characteristics of apoptotic

- Physiologically active.
- Preventable and possibly reversible*.
- Typical morphology.
- Integrity of cell membrane.
- Phagocytosis by adjacent cells.
- Internucleosomal DNA degradation.

* At least as a process in a cell population.

Functionally, apoptotic differs from necrosis in several ways. In apoptosis, the integrity of cell membrane is preserved, and there is no leakage of pro-inflammatory molecules. There is a characteristic pattern of internucleosomal DNA degradation, caused by the activation of a calcium- and magnesium-dependent endonuclease, which results in the formation of multiple DNA fragments with lengths that are integer multiples of 180-200 bp. On gel electrophoresis these fragments are seen as a DNA ladder (figure 1). Other patterns of DNA degradation may occasionally be observed. Apoptotic cells also express new cell membranestructures, like immature glucidic structures, phosphatidyl serines and glycoproteins, which determine the rate of recognition and phagocytosis by adjacent cells, all in the absence of severe inflammation. A tissue transglutaminase is activated, which crosslinks cytoplasmic proteins.

Apoptosis is an active process, which requires energy in the form of ATP, and the expression or suppression of certain genes. The intracellular transduction pathways of apoptosis are poorly understood, and may involve calcium and inositol-phosphates or changes in protein phosphorylation. Oxidative stress also appears to have a causative role¹⁶. The inhibition of mRNA or protein synthesis may cause or prevent apoptosis, depending on the balance between



Figure 1. Internucleosomal DNA fragmentation in renal cells. Tubular epithelial cells were cultured for 24 h in serum free medium in the absence (left lane) or presence (right lane) of 30 ng/mL recombinant murine TNF α . Detached cells were lysed, DNA was isolated, separated in a 7.5% agarose gel and stained with ethidium bromide. Note the characteristic DNA ladder, suggestive of apoptotic, in the TNF α -treated samples.

lethal and protective factors in the cell. From this point of view, three modes of apoptosis can be distinguished²:

- *By induction:* the stimulus which induces apoptosis activates lethal genes, and cycloheximide, an inhibitor of protein synthesis, prevents apoptosis.
- *By release:* the genes promoting apoptosis are already activated, and short lived proteins antagonize them. Macromolecular synthesis inhibitors decrease the levels of protective factors and induce apoptosis.
- *By transduction:* the synthesis of new proteins is not necessary, and the occurrence of apoptosis is not affected by cycloheximide.

Apoptosis usually affects individual cells, its tissue distribution is patchy and asynchronous, and once initiated, progresses rapidly. It has been calculated that the half-life of the apoptotic cell is a few hours, and it may be phagocytosed in less than one hour. A low percentage of apoptotic cells visible in a tissue section (e.g. less than 0.8%), therefore, may be misleading. It has been suggested that such a low percentage may, nevertheless, result in the loss of up to 50% of the non-required cell mass during the development of the central nervous system^{17,18}. It has been demonstrated that apoptosis may also attenuate unneeded metanephric mesenchyme following induction by the ureteric bud¹⁹. The rapid disappearance of the cellular debris and the lack of serious inflammation may make even large scale apoptosis histologically inconspicuous. The lack of easily detectable histological changes is the origin of controversy regarding its normal role in physiology and pathophysiology.

Activation of a genetic program for cell death

The development of *Caenorhabditis elegans*, a nematode, requires the formation of 1090 cells while 131 undergo programmed cell death. The study of the genes involved in apoptosis in this model has originated concepts that have been later applied to amniotes. Two lethal genes have been identified in *C. elegans*, *ced-3* and *ced-4*, which need to be expressed for cell death to occur, and a survival gene, *ced-9*, which has to be suppressed ^{6,20}.

Recent studies have also identified genes that are involved in apoptosis in amniotes (table II). Molecular studies in tumors have uncovered genes, such as *bcl-2* and *p53* ^{21,22}, whose alterations result in excessive or defective function that promote tumor growth by decreasing the likelihood of apoptosis. Others have been discovered by chance, like *Fas/APO-1*, first identified with a cytotoxic monoclonal antibody ^{23,24}. Finally, a systematic approach, comparing the content of cDNA libraries or antigen expression between apoptotic and nonapoptotic cells has yielded a number of genes or proteins, such as *tcl-30*, the function of which remains unclear ²⁵. We will limit our discussion to genes which have been shown to be expressed by renal cells, drawing on some data from our own experience (figure 2).

Table II. Apoptosis-related genes

- Prevent apoptosis: *bcl-2*, *bcl-xL*.
- Promote apoptosis:
 - Receptors: *Fas*, *55pTNFr*, *75pNGFr*.
 - Transcription factors: *p53*, *c-myc*, *c-fos*, *c-jun*.
 - Intracellular proteins: *bax*, *bcl-xS*, *IL-1β*-convertase.
- Role in apoptosis not well characterized: *tcl30*, *clusterin*, *c-fes*, *WT-1*.

Most of the genes under discussion have been well studied in neurons, lymphohemopoietic, and tumor cells. It remains to be demonstrated that their role in apoptosis is similar in renal cells. There is, however, evidence that the basic mechanisms of apoptosis are well preserved among species, and probably among different organs of the same individual. For instance, the human *bcl2* gene protects *C. elegans* cells from apoptosis ²⁶.

Protective genes: *bcl-2* and *bcl-X*

The activation of *bcl-2* and the *bcl-xL* isoform of the *bcl-x* gene prevents or retards apoptosis.

***bcl-2*.** *bcl-2* encodes a membrane-bound protein expressed on mitochondria and other intracellular

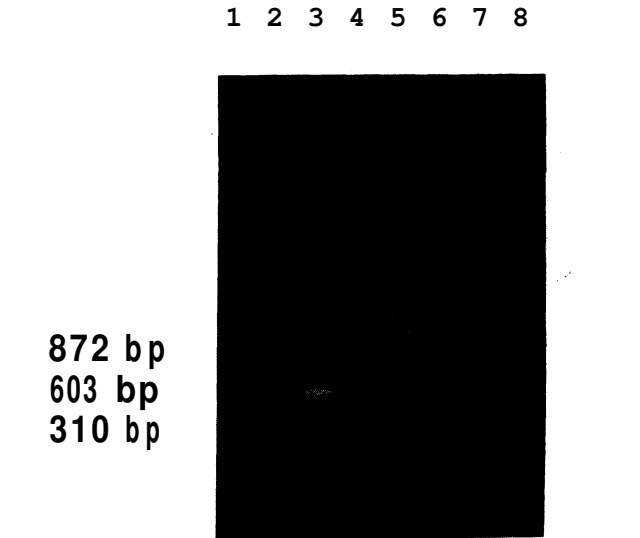


Figure 2.-RT-PCR of apoptosis related genes in renal cells. cDNA WAS made by reverse transcription of murine tubular epithelial cell mRNA. PCR was performed employing primers derived from the published gene sequences. The PCR products (shown in the figure) were cloned into the PCR II vector and sequenced to confirm their identities. Northern analysis of RNA from renal cells and kidney revealed the presence of adequate sized transcripts. Lane 1, *bcl-2*; 2, *bcl-x*; 3, *bax*; 4, *p53*; 5, molecular weight markers; 6, *Fas*; 7, *tcl30*; 8, *clusterin*.

mem branes ^{27,28}. It belongs to a recently identified family of interactive apoptosis-related proteins which includes *bcl-x*, *bax* and *MCL 1* ²⁹. *bcl-2* appears to function by blocking lipid peroxidation and/or inhibiting the production of reactive oxygen species ^{30,31}.

The *bcl-2* gene was identified in the locus of a t(14;18) (q32;21) translocation present in 85% of human follicular lymphomas ²¹. This translocation results in the uncontrolled expression of a chimeric protein which retains the survival function of *bcl-2* ²¹. It is thought that this protein may confer a vital advantage to oncogenic clones, and in conjunction to other mutations, could lead to lymphoma. Excessive expression of *bcl-2* has also been described in epithelial neoplasia ³². *bcl-2* overexpression confers protection against apoptosis, and interference with *bcl-2* function predisposes to apoptosis in several *in vitro* and *in vivo* systems ^{6, 8-10, 33-39}. Specifically, *bcl-2* protects from *c-myc*-induced apoptosis ³³ glucocorticoid-induced apoptosis in lymphocytes ⁹, *Fas/TNFα* induced cell death ^{34,35} and some, but not all, models of growth factor deprivation-induced apoptosis ^{8, 36-39}. *bcl-2* also protects against death induced by oxidants ³⁰ and physical factors such as heat shock and irradiation ⁶. However, it offers no protection against cytotoxic T cell killing ⁴⁰. The over-

expression of *bcl-2* in B-lymphocytes of transgenic mice results in the abnormal survival of autoreactive clones as well as the development of a proliferative glomerulonephritis⁴¹. Interestingly, *bcl-2* mRNA levels in circulating lymphocytes have been reported to be elevated in patients with systemic lupus erythematosus⁴², although this may just represent the state of activation of those lymphocytes⁴³. *bcl-2* may also play a physiological role, as suggested by its elevated expression during development^{44,45}, as well as in adult, long-lived cells such as neurons and memory lymphocytes⁴⁶. *bcl-2* has a further potential role in renal development, as mice carrying a targeted mutation in *bcl-2* develop polycystic renal disease that progresses to renal failure⁴⁷.

We have recently demonstrated that different murine renal cells, including mesangial cells, tubular epithelium, fibroblasts and metanephric stem cells, express *bcl-2* mRNA and the protein product is detectable by indirect immunofluorescence⁴⁸. *bcl-2* mRNA is also present in blood-free adult mouse kidney. As has been previously described in other organs, renal cells and kidneys express *bcl-2* mRNA transcripts of several sizes (7.5, 4.1 and 2.4 kb), the 7.5 kb transcript being the most abundant.

bcl-x. Bcl-x has recently been described as a new member of the *Bcl-2* family of proteins⁴³. In humans different alternatively spliced isoforms of *bcl-x* protect from (*bcl-xL*) or predispose to (*bcl-xS*) apoptotic cell death in growth factor-deprived cells. The apoptotic promoting action of *bcl-xS* seems to be related to the inhibition of *bcl-2* action. We have demonstrated that murine kidneys and renal cells in culture express two *bcl-x* mRNA transcripts, detectable by northern hybridization, the smaller one having a shorter half life. Both murine transcripts hybridize to a human *bcl-xL* specific probe, although their function remains undefined.

Genes that promote cell death: *p53*, *c-myc*, *c-fos*/*c-jun*, *bax* and *IL-1 β* -converting enzyme

The *p53* gene is required for certain modes of apoptosis. Excessive *c-myc* expression results in dependence of external factors to prevent cell death. *c-fos* and *c-jun* have a role in growth factor deprivation-induced apoptosis. *bax* is an endogenous antagonist of *bcl-2*. *IL-1 β* -converting enzyme is the mammalian homologue of the death gene *ced-3*.

p53. *p53* is a nuclear phosphoprotein with characteristics of a transcription factor, whose action is mediated by binding to DNA and other proteins^{22,49}. *p53* is the most frequently mutated or deleted gene in solid neoplasia, and is inactivated by several oncoviral pro-

teins²². There has been a misunderstanding of the physiological role of *p53* because the original functional studies were performed with mutated *p53*. It is now clear that wild-type *p53* is able to induce apoptosis⁷. *p53* is required for apoptosis induced by radiation and DNA damaging drugs, but not for dexamethasone-induced apoptosis in thymocytes^{50,51}. Moreover, apoptosis induced by *p53* in leukemic cells, is partially blocked by growth factors such as IL-6⁵². The adenovirus E1/B protein, which blocks *p53* function and inhibits *p53*-mediated apoptosis⁵³, also protected against Fas and TNF α -mediated cell killing⁵⁴, suggesting a role for *p53* in receptor-initiated apoptosis. *p53* is highly expressed in the developing kidney, and is also present in most adult tissues⁵⁵. However, the possible role of *p53* in renal cell death is unclear. Studies in other organs suggest that *p53* may play a role in non-neoplastic processes. *p53* immunoreactivity is increased in soft tissue and cervix inflammatory lesions^{56,57}, as well as in relation to neuronal cell death during experimental cerebral ischemia⁵⁸.

c-myc. *c-myc* is a proto-oncogene which functions as a transcription factor⁵⁹. *c-myc* activity depends on the formation of a heterodimer by binding to another intracellular protein, Max. Max is always found in excess relative to *c-myc*, and *c-myc* levels determine the formation and activity of the heterodimer⁵⁹. *c-myc* had been traditionally considered a stimulatory factor for cellular proliferation. In fact, *c-myc* transgenic mice develop cystic kidney disease and hyperplasia in glomerular and tubular epithelium⁶⁰. Recent studies have modified this concept. In fibroblasts the uncontrolled expression of *c-myc* increases cellular proliferation in the presence of growth factors, but in cells deprived of them, it induces apoptosis⁶¹. Growth factor deprivation in IL-3-dependent cells is associated with decreased *c-myc* expression, and if *c-myc* is overexpressed, growth factor deprivation-induced apoptosis is accelerated⁶². Moreover, *c-myc* antisense oligonucleotides prevent anti-CD3-mediated activation-induced apoptosis in T cell hybridomas⁶³. It is quite possible that *c-myc* activates a common genetic program that may determine both cell division and cell death. The presence or absence of additional signals may determine the cell fate. This may be related to the activity of *bcl-2*, which inhibits *c-myc*-mediated apoptosis³³. The relationship between *c-myc*-induced cell death and its capacity to activate the transcription of *p53* is not clear⁶⁴. *c-myc* may also protect from cell death under certain conditions. During steroid-induced killing of T cells, *c-myc* transcripts are down-regulated, and transient expression of *c-myc* protects cells from death⁶⁵.

c-fos* and *c-jun. *fos* and *jun* homo or heterodimerize to form the AP-1 transcription factor. The expres-

lpr/lpr mice (manuscript in preparation). The fact that endotoxin increases the expression of a receptor capable of inducing cell death may provide new insights into the pathogenesis of organ dysfunction in septic shock. Specifically, the mechanisms of renal failure in sepsis are poorly understood. Although **TNF α** has been considered a primary mediator of endotoxin-induced organ damage, mice lacking the 55 Mr TNF receptor are still susceptible to endotoxin, but not to **TNF α /IL1 β -mediated** death ⁹⁶. In this regard, Fas, a receptor similar to the 55 Mr TNF receptor, is an excellent candidate to explain why mice carrying targeted mutations in the TNF receptor are not completely protected from the deleterious effects of LPS.

Fas has also been shown to be expressed in metanephric stem cells under the regulation of **TNF α** (unpublished data). These data seem to fit with the recently proposed general role of macrophages, possibly through engagement of Fas or similar receptors, in tissue remodeling during organogenesis ⁹⁷.

Other apoptosis related genes

tcl30. tcl30 encodes a 2.4 kb transcript discovered during a comparison of cDNA libraries from normal and apoptotic thymocytes ⁹⁸. Apoptotic thymocytes express high levels of *tcl30*, which in vivo was detected only in the thymus. The possible role of tcl30 in apoptosis is unknown. However, it is interesting that it encodes a membrane protein with a vitronectin-like domain, and that the vitronectin receptor has been implicated in the phagocytosis of apoptotic debris by mesangial cells and macrophages ⁹⁹. We have recently shown by RT-PCR and northern blot that tubular epithelial cells express tcl30 mRNA.

Clusterin (complement lysis inhibitory protein).

Clusterin is a multifunctional glycoprotein, which has received a variety of names (table III) ¹⁰⁰. Clusterin is associated with apoptosis, although its role in this process, if any, is unknown. Together with *myc*, clusterin is one of the few apoptosis-related genes which has been extensively studied in the kidney. Clusterin expression is increased in several models of acute renal failure, and in chronic models, such as subtotal nephrectomy and polycystic diseases ¹⁰¹⁻¹⁰⁶, some of which have been associated with the occurrence of apoptosis. Clusterin is also found deposited in the glomeruli in immune glomerulonephritis ¹⁰⁷⁻¹⁰⁹, and one of its best characterized functions is the inhibition of the activity of the terminal complement complex (C5 b-C9) ¹¹⁰. We recently observed increased levels of clusterin mRNA in the kidneys of mice with anti-CBM disease and in MRL-lpr/lpr lupus mi-

ce, as well as in the glomeruli of LPS-treated mice ¹¹¹. Thus, clusterin deposited in the glomeruli may be of local origin. We also demonstrated that mesangial cells in culture express clusterin mRNA ¹¹¹. However, in these cells, clusterin expression was decreased by conditions that induced cell death compatible with apoptosis, such as **TNF α** and serum deprivation, as well as by **γIFN**, which did not kill the cells. This indicates that clusterin expression may be regulated in glomeruli by cytokines, independently of apoptosis. Other authors have raised questions about the relationship of clusterin and apoptosis in other experimental systems and have proposed a possible role for clusterin in cell differentiation ¹¹². These authors found abundant clusterin mRNA in newly polarized cells of comma and S-shaped bodies, but not in uninduced or non-polarized metanephric mesenchyma. In keeping with these findings, our recently characterized metanephric stem cell line MMR ¹¹³ did not express clusterin mRNA, unlike renal fibroblasts, mesangial and tubular cells ¹¹¹.

Table III. Clusterin

Synonyms:

- **SGP2; sulfated glycoprotein 2 (rat).**
- **TRPM: testosterone repressed protein messenger (rat).**
- **GP80: glycoprotein 80 (canine MDCK cells).**
- **SP40,40: serum protein 40,40 (human membranous nephropathy).**
- **APO j (human).**
- **Glycoprotein III (bovine).**

Functions:

- **Binding and inactivation of C5b-C7 complement complexes.**
 - **Increased in relation to apoptosis *in vitro* and *in vivo*.**
 - **Apolipoprotein.**
 - **Neuroendocrine cell granule component.**
-

Other genes. Reports on the gene regulation of apoptosis are frequent. Recent papers have reported that antisense oligonucleotides complementary to the *c-fes* oncogene induces apoptosis in HL60 cells ¹¹⁴. Activated T24-ras and v-abl have also been shown to protect from apoptosis, at least under certain conditions ²⁹.

Apoptosis in pathophysiology

The apoptosis is a normal biologic process in most embryonic and adult tissues. Together with mitosis it regulates the number of cells in each tissue at each stage of development (figure 3). In fact, cell proliferation and cell death seem intimately related with examples suggestive of a common activation pathway that may result in one or the other ^{61,63}, depen-

sion of *c-fos* and *c-jun* is rapidly and transiently induced upon growth factor deprivation in IL-2 and IL-6-dependent cell lines, preceding apoptosis⁶⁶. Moreover, antisense inhibition of either of them protected against death⁶⁶. An association between *c-fos* expression and apoptosis in transitory embryonic structures has also been noted⁶⁷. *fos* and *jun* may also be involved in apoptosis regulation through the formation of the transcription complex NF-AT. This complex requires a nuclear formation, which includes *c-fos* and *c-jun*⁶⁸, and a component that has to be translocated from the cytoplasm. Cyclosporin A, which blocks activation-induced apoptosis in T cells⁶⁹, also blocks the translocation of the cytoplasmic component of NF-AT, probably by inhibition of calcineurin activity⁷⁰.

bax. *Bax* was described in August 1993 as a member of the Bcl-2-like family of proteins⁷¹. *Bax* forms heterodimers with Bel-2. In cells transfected with both *bcl-2* and *bax*, the *bcl-2/bax* ratio appears more important in determining the susceptibility of the cell to growth factor deprivation-induced apoptosis than either of the two alone: excessive *bax* predisposes cells to apoptosis under these conditions, but not in the presence of growth factors. The original report of this finding suggested that the murine kidney expresses two *bax* transcripts of 1.5 and 1 kb abundantly. In our own laboratory we have found the 1 kb *bax* transcript to be preferentially expressed by mesangial cells, proximal tubular cells and metanephric stem cells as well as by whole kidney.

IL-1 β -converting enzyme. CED-3 is required for cell death to occur in *C. elegans*. Mammalian IL-1 β -converting enzyme is highly homologous to CED-3⁷². In fact, overexpression of IL-1 β -converting enzyme has been shown to induce cell death in rat fibroblasts⁷³. This effect was prevented by mutating the gene, by the IL-1 β -convertase antagonist *crmA* gene, and by *bel-2*. It had already been suggested that this enzyme might have other roles besides cleaving IL-1 β , as it is expressed by cells that lack the cytokine, and it is activated in macrophages undergoing apoptosis, but not in those undergoing necrosis⁷⁴. Other *ced-3* related genes such as *Nedd-2*, may also have a role in apoptosis.

Receptors that induce apoptosis: Fas and the TNF receptors family of proteins

Fas (CD95) is a cell membrane protein, and a member of a family of receptors that includes both TNF receptors^{75,76}, the low affinity NGF receptor⁷⁷, CD40⁷⁸, OX40⁷⁹, CD27⁸⁰, and CD30⁸¹. This protein

family is defined by similarities in their extracellular domains that are absent in the intracellular portion of the molecule. Several of these proteins modulate the occurrence of apoptosis. Both the activation of Fas and the 55 Mr TNF receptor induce cell death with features of apoptosis through a relatively conserved intracellular domain^{82,83}. However the 75 Mr NGF receptor lacks that domain, but still induces apoptosis when not bound to NGF: unlike Fas and the TNF receptor, it is NGF's nonoccupancy of its receptor which induces apoptosis⁸⁴.

Fas was initially described by two independent groups which developed monoclonal antibodies (APO-1 and Fas) that were cytotoxic to human cells^{23,24}. A recently described anti-murine monoclonal antibody also induces apoptosis *in vitro* and *in vivo*⁸⁵. Fas is another example, along with *bcl-2*, of apoptosis-related genes whose alteration leads to an immune-mediated glomerulonephritis^{41,86}. The genetic defect of Fas in MRL-lpr/lpr lupus mice results in the survival of autoreactive T cell clones and the appearance of a generalized autoimmune disease. This defect consists in the insertion of an endotransposon in the second intron of the Fas gene, leading to abnormal splicing of the gene, with nearly total absence of normal sized transcripts and the expression of low levels of abnormally sized messages in the thymus and liver⁸⁷⁻⁹⁰. The abnormal transcripts contain a 168 bp endotransposon fragment within the coding region of the gene. Interestingly, the normal splicing pattern can apparently be restored in the thymus of TCR β transgenic MRL-lpr/lpr mice⁸⁹. This lead us to hypothesize that certain, as yet unknown stimuli, may induce the expression of normal transcript in the kidney of these animals.

The endogenous ligand for Fas has recently been characterized and shown to induce apoptosis⁹¹. The Fas ligand is thought to be encoded by the gene defective in *gld* lupus mice⁹². Studies based on this assumption suggest a role for *Fas* in T cell-mediated cytotoxicity⁹³. Cytotoxic T cells can play a pathogenic role in experimental and human renal diseases⁹⁴, and *Fas* may participate in this process. We have recently shown that murine mesangial cells and tubular cells express *Fas* mRNA under the regulation of cytokines and endotoxin⁹⁵. Cytokines thought to play a pathogenic role in kidney damage, such as TNF α , IL1 β and γ IFN, increased levels of *Fas* mRNA and expression of the Fas receptor in renal cells, and levels of *Fas* mRNA, for example, are increased in anti-GBM disease⁹⁵. Endotoxin is also a potent inducer of new *Fas* transcripts by renal cells in culture and by the kidney, liver and lung *in vivo*⁹⁵. Moreover, endotoxin resulted in expression of near normal levels of normal sized *Fas* message in the kidneys and liver from MRL-

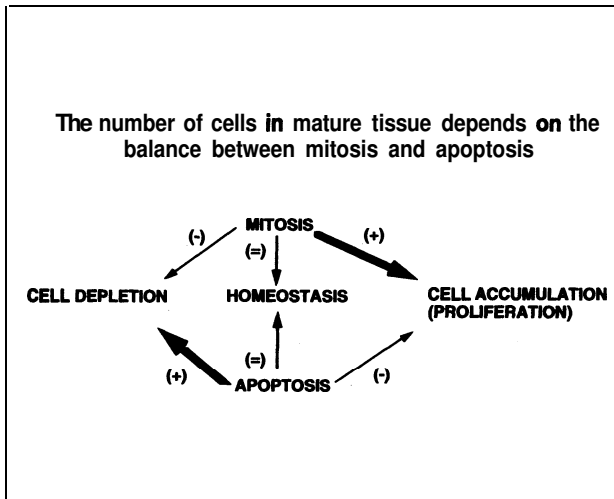


Figure 3.-The homeostasis of cell number depends on the balance between cell division (mitosis) and cell death (apoptosis). An excess of cell mitosis, especially if associated to a decreased rate of cell death, may result in disease characterized by excessive cell accumulation, such as proliferative glomerulonephritis. An increased rate of cell death, especially if not compensated by increased cell division, may result in cell depletion, such as chronic renal atrophy.

ding on the instructions of endogenous or exogenous factors. Inhibition of apoptosis, for example, may be viewed as cell proliferation in *in vitro* studies.

Table IV summarizes some of the physiological processes in which a role for apoptosis has been suggested. The rapid tissue turnover and modification that characterizes embryonic development would not be possible without apoptosis. The role of apoptosis in the elimination of unwanted cells during development may be more fundamental than the previously thought. A recent study has suggested that prevention of apoptosis, per se, in the absence of differentiating factors, may be enough for differentiation to proceed¹¹⁵. In some parts of the central nervous system, for example, up to 60% of the neurons that are formed die by apoptosis¹⁷.

There is limited information on apoptosis and kidney development. Developing kidneys are known to contain apoptotic cells^{19,116} and to express high levels of several of the apoptosis-related genes, like bcl-2 and p53^{44, 45, 55}. In fact, mice carrying targeted mutations of bcl-2 have neonatal polycystic kidney disease with persistence of immature cells⁴⁷. Our own data indicate that bax and bcl-x are expressed by embryonic kidney and metanephric stem cells in culture. The relative levels of the two murine bcl-x transcripts in the kidney change during development with lower amounts of the larger transcript found in the adult kidney (unpublished observation). Uninduced mesenchyme dies by apoptosis¹¹⁶, and WT-1 is required for the prevention of apoptosis even in the

presence of adequate inducers: mice carrying targeted mutations of WT-1 suffer from renal agenesis as a result of massive apoptosis of the metanephric blastema¹¹⁷. Growth factors such as NGF and EGF can rescue metanephric cells from apoptosis^{19, 116}, and anti-sense oligonucleotide experiments suggest that the 75 Mr NGF receptor participates in kidney development¹¹⁸. Embryonic human kidneys also express high levels of the T cell and monocyte chemoattractant RANTES (A. Krensky, personal communication). Although its role during development is as yet unclear, this fits with the recently proposed essential role of macrophages during embryonic apoptosis. Moreover, metanephric stem cells can be induced in culture by macrophage products, such as TNF α , to express transcripts that encode for an apoptosis transducing receptor, Fas (unpublished observation).

Table IV. Apoptosis in physiopathology

Partial list of physiological processes characterized by the occurrence of apoptosis.

- Embryonic development.
- Tissue turnover:
 - Keratinocytes.
 - Intestinal epithelium.
- Degeneration of hormone-dependent tissues after removal of trophic hormones:
 - Thyroid, adrenals.
 - Prostate, breast, endometrium.
- Regulation of the immune response:
 - Negative selection.
 - Control of peripheral immune response (activation induced apoptosis).
 - CD4 and CD8 J cell cytotoxicity.
- Hematopoiesis:
 - Regulation of circulating blood cell number.
 - Generation de erythrocytes by normoblast apoptosis.

Alterations in the frequency of apoptosis can cause disease characterized by insufficient or excessive number of cells. The loss of function of genes that induce apoptosis or the increased expression of genes that prevent apoptosis can result in autoimmunity, excess of autoreactive cells (bcl-2, Fas), or neoplasia (bcl-2, p53)^{21,22, 41,86}. An excess of apoptosis has been implicated in the neuronal loss of Alzheimer's and Parkinson's diseases, and in the disappearance of T lymphocytes following HIV infection^{6 119, 120}. Altered regulation of apoptosis may also result in persistent inflammation or fibrosis, if the numbers of leukocytes and fibroblasts in a site of injury are not adequately control led¹²¹.

Apoptotic and renal disease

Studies on apoptotic and renal disease are recent and more abundant in abstracts presented at meetings than in the published literature. Published studies have demonstrated the existence of apoptotic morphology and the typical pattern of DNA degradation in several renal diseases, although there are numerous unanswered questions regarding the factors that induce and prevent apoptosis, its gene regulation, its relative contribution to renal damage, and the possible therapeutic value of the modulation of apoptosis. Table V summarizes renal diseases in which apoptosis may play a role.

Table V. Possible participation of apoptotic in renal diseases

- **Metanephric development.**
- **Acute renal failure: toxic, ischemic, obstructive.**
- **Chronic renal failure: renal atrophy and interstitial fibrosis.**
- **Inflammation: loss of parenchymal cells, resolution of inflammation and of cell proliferation (hyperplasia).**
- **Autoimmunity and transplantation: regulation of the immune response.**
- **Polycystic renal disease.**
- **Nephrotic syndrome.**
- **Diabetic nephropathy.**
- **Tumors.**

There are four general aspects to the relationship of apoptosis to renal disease:

Apoptotic as a mechanism of depletion of intrinsic renal cells

Apoptotic induced by a reduced supply of growth factors, or the presence of soluble or membrane bound products of macrophages or T cells may play a role in the loss of parenchymal cells observed in ischemic, toxic and inflammatory processes.

Apoptotic and renal failure. There is evidence for the involvement of apoptosis in both the acute and chronic loss of renal parenchyma^{14, 122, 123}. Internucleosomal DNA degradation and the presence of apoptotic bodies have been reported to occur in both ischemic acute renal failure and chronic ischemic atrophy^{14, 123}. In fact, after brief (<45 min) ischemia and reperfusion, apoptotic figures were detected in rat kidneys at 12 hours and the classic internucleosomal DNA fragmentation was also evident¹²³. In chronic ischemia apoptotic figures could be observed up to 28 days¹⁴. In these conditions there is also ongoing necrosis¹⁴, and the relative contribution of the two mechanisms of death to cell loss may depend on

the balance of regulatory events. For obscure reasons, apoptosis seems to be specially frequent in post-transplant acute renal failure¹²⁴. Evidence of apoptosis has also been found in toxic and obstructive acute renal failure^{48, 122, 125}, cortical atrophy post-papillary necrosis¹²⁶ and in the chronic renal disease caused by subtotal nephrectomy¹²⁷. We and others have demonstrated that a sensitive southern blot technique permits the identification of internucleosomal DNA degradation even in the normal kidney¹²⁸. However, the short in vivo half life of the apoptotic cell makes it a challenge to study its participation in disease. Apoptotic indices below 1.5% result in the loss of 50% of the cells during central nervous system development¹⁷. Moreover, in renal injury, detachment is an early event in apoptosis, and apoptotic cells can be shed into the tubular lumen. While the meaning of the presence of viable tubular cells in the urine during acute renal failure is not yet fully understood¹²⁹, we have hypothesized that cells destined to undergo apoptosis are rescued from cell death by in vitro culture in the presence of growth factors¹³.

Little is known about the precise contribution of apoptosis to the damage of acute renal failure. Apoptotic cell death may be a direct consequence of exposure to noxious stimuli. Apoptosis may also occur in cells proliferating in a compensatory fashion after renal injury. These cells may be specially sensitive to absolute or relative deficits in exposure to growth factors. In this setting apoptosis might contribute to the persistence or delayed recovery from acute renal failure. Apoptosis may also be a physiologic balance to check or even reverse an exaggerated compensatory proliferative response. Shimizu et al observed, after 60 minutes of ischemia, an early peak of necrosis and apoptosis in the first 48-72 h of acute renal failure, and a second, bigger peak of apoptosis after 7-14 days, when the necrotic tubules had been completely relined by hyperplastic epithelium¹²⁹. Elevated apoptotic indexes persisted for up to 4 months¹²⁹. Apoptosis has also been proposed to participate in the regression of renal hyperplasia induced by lead and gentamicin^{130, 131}. An issue that has not been addressed in the experimental setting, but that may be relevant in clinical practice, is the effect of repeated low level insults to the kidney, such as recurrent episodes of hypoperfusion/hypotension, or infection, on the ability of cells to withstand apoptotic cell death. In theory, such insults may select for apoptosis, rather than necrosis, because of the mildness of the insult or its repetitive nature, which would deprive cells of growth factors during compensatory cell replacement.

Apoptotic of parenchymal cell in inflammation. Little is known about the role of apoptosis in renal cell

death induced by inflammatory cells. However, as reviewed below, several cytokines and inflammatory mediators released by macrophages induce apoptosis in other cell types. Moreover, both CD4+ and CD8+ cytotoxic T lymphocytes may induce apoptosis in target cells^{132,133}, and donor cell death in the setting of untreated acute rejection in experimental liver transplantation shows histologic features of apoptosis¹³⁴.

A possible role for apoptotic in nephrotic syndrome. It is generally accepted that idiopathic nephrotic syndrome is the consequence of podocyte damage. However, there is little information regarding the nature of this damage. Denudation of basement membrane and podocyte detachment and death have been reported in several forms of human nephrotic syndrome, including minimal change disease, focal and segmental glomerulosclerosis, glomerulonephritis and diabetic nephropathy¹³⁵. While the morphological features were originally interpreted as those of necrosis, it is possible that coexistent apoptosis was missed due to the already commented difficulties in identifying apoptosis *in vivo*. Further studies, applying newly developed techniques, such as *in situ* detection of DNA fragmentation¹³⁶, should be performed. In the experimental setting, the observed cytotoxicity of adriamycin and puromycin aminonucleoside on cultured glomerular epithelial cells¹³⁷ might be related to their ability to induce apoptosis^{138,139}. Although it is unclear why podocytes should be especially sensitive to their toxic effects *in vivo*, it is interesting that apoptosis induced by adriamycin depends on the activation of p53¹³⁹. Glomerular podocytes are the only renal cells that express WT-1 in the adult kidney¹⁴⁰, and the interaction between p53 and WT-1 increases the transcriptional activity of p53¹⁴¹.

A reduced rate of apoptotic may result in abnormal accumulations of somatic cells

Some nephropathies are defined by an abnormal accumulation of renal cells. Examples may be mesangial hypercellularity in proliferative glomerulonephritis, the increased number of interstitial fibroblasts observed in interstitial fibrosis of advanced renal diseases, and the epithelial hyperplasia of renal cystic disease. An increased mitotic rate may explain some of the histologic alterations in these nephropathies. However, the lack of a compensatory increase in cell death may lead to the accumulation of cells. Although this possibility has not been directly studied, there are indications that it might be so. Fibroblasts obtained from fibrotic kidneys accumu-

te more rapidly in culture and survive longer than those obtained from healthy kidneys¹⁴². Similar observations have been made with mesangial cells obtained from animals with proliferative glomerulonephritis. Shimizu et al. have recently reported that the resolution of the glomerular proliferation characteristic of anti-Thy-1 nephritis depends on apoptosis of excessive numbers of glomerular cells¹⁴³.

Both the occurrence of apoptotic¹⁴⁴, and abnormalities of apoptosis-related genes have been reported in polycystic kidneys. Transgenic mice overexpressing c-myc suffer from polycystic kidneys⁶⁰, and over-expression of c-myc has been detected in the kidneys of cpk mice¹⁴⁵. Interestingly, bcl-2 protects from c-myc-induced apoptosis³³. Although the mechanism is still unclear, the lack of functional Bel-2 results in neonatal polycystic renal disease in mice⁴⁷. These mice displayed renal cysts, proliferative glomerular and tubulointerstitial lesions, the presence of immature cells and mitotic and apoptotic figures, suggesting that an increase in the rate of cell death leads to a "compensatory" proliferation⁴⁷. Alternatively, premature cell death may compromise differentiation¹¹⁵. It is theoretically possible that overexpression of *bax*, the endogenous antagonist of *bcl-2*, may result in the development of renal cysts. However, we have not observed abnormal levels of *bax* mRNA in kidneys or tubular cells from cpk/cpk mice (unpublished observation). A decreased apoptotic rate may also participate in the development of renal neoplasias, and altered p53 genes have been found in some renal carcinomas.

Apoptotic in the regulation of renal inflammation

Recent data suggest that leukocytes in the inflammatory site are eliminated by apoptosis if there is not an adequate microenvironment of cytokines and cell-preservation factors¹⁴⁶⁻¹⁴⁸. The clearance of inflammatory cells by apoptosis might be a mechanism regulating resolution of glomerular and interstitial inflammation¹²¹, and failure of this clearance may contribute to persistence of the inflammatory process. There is evidence in support of this hypothesis such as the capacity of mesangial cells to engulf apoptotic neutrophils¹⁴⁹ as well as the visualization of apoptotic debris inside mesangial cells during recovery from acute glomerulonephritis¹⁵⁰. Identification of the survival signals for leukocytes may help understand the persistence of certain renal inflammatory events. Moreover, if the survival factors for leukocytes differ from those of renal intrinsic cells the door would be open to the manipulation of inflammation through modulation of leukocyte apoptosis without damaging

the renal parenchyma. In fact, certain cytokines may have a dual action on the regulation of apoptosis: **TNF α** , for example, prevents apoptosis in monocytes, while killing renal cells *in vitro* ^{48, 148}.

It is relevant at this point to comment on the harbored notion that apoptosis itself does not generate inflammation. It is conceivable that apoptosis may generate inflammation through several mechanism. If massive apoptosis occurs in an organ not physiologically prepared for such an event, some of the dying cells may not be phagocytised by adjacent cells, especially if the latter are dysfunctional because they have been exposed to the same pernicious toxins. This may result in the disintegration of apoptotic bodies and the release of non-specific proinflammatory factors. Cell lysis has been observed *in vivo* when massive apoptosis of the liver takes place upon injection of anti-Fas antibody ⁸⁵. It is also theoretically possible that the genetic programs activated during apoptosis may provide specific chemotactic substances for more phagocyte recruitment, as it does provide new surface determinants for recognition and phagocytosis. There is some, scattered evidence for this notion. Macrophages undergoing apoptosis process IL-1 β (74). We have recently observed that peak transcription of a luciferase construct under the control of the T cell and monocyte chemotactic chemokine Scya 5 (RANTES) promoter is induced by **TNF α** in doses that induce apoptosis ^{48, 151, 152}. Kidney development, in which widespread apoptosis of unwanted cells takes place, is associated with elevated levels of RANTES expression (A. Krensky, personal communication). The monocyte chemoattractant JE expression is elevated as early as 4 hours after renal ischemia, peaks at 24-48 h and remains elevated for as long as 168 h ¹⁵³. In fact, invading macrophages were detected in the kidneys during renal cortical atrophy, a process characterized by apoptosis in the absence of necrosis and other tissue injury ¹²⁶. Although previous studies have not made a clear connection between inflammatory infiltrates and apoptosis, recent data from transgenic mice suggest that macrophages are indispensable in eliciting cell death by apoptosis during development ⁹⁷. The exact role of macrophages, as primary determinants of the occurrence of apoptosis through engagement of receptors such as Fas, or as a complement to signals derived from the apoptotic cells, as well as the means of macrophage recruitment are poorly understood.

Apoptotic as a modulator of the immune response

There is a growing body of evidence that apoptosis plays a fundamental role in the control of the immu-

ne response in the thymus and the periphery (reviewed in 2). While the details of this involvement are beyond the scope of this review, it should be noted that alterations in apoptosis-related genes such as bcl-2 and Fas result in autoimmune diseases with renal damage. Moreover the mechanism of action of drugs used for the therapy of immune-mediated diseases and transplant rejection may involve the regulation of lymphocyte apoptosis ^{69, 154, 155}.

Regulation of apoptotic in renal cells

There is little information on the factors that regulate apoptotic in the kidney. In general, apoptosis may be the consequence of withdrawal of growth factors and/or the exposure to apoptosis inducing factors. The idea that cell viability depends on a balance of external signals has increasingly been accepted ²⁰. The growth factor requirement may vary with cell type, functional status of the cell, or the additional presence of pernicious toxins. The requirement for external survival factors has been well characterized for some neuron and hemopoietic cells. Much less is known about the needs of renal cells. We and others have shown that serum deprivation results in apoptosis of mesangial and tubular cells ^{48, 156}. The specific growth factors that account for the survival promoting activity of serum in renal cells are unknown. However, there is information suggesting the involvement of several cytokines. Both EGF and IGF-I are decreased during acute renal failure ¹⁵⁷⁻¹⁵⁹ and their administration improves the evolution of experimental acute renal failure ^{125, 160, 161}, and even decreases internucleosomal DNA degradation ¹²⁵. Both factors have also been shown to protect against apoptosis in other cell culture systems ^{162, 163}. In fact, there is evidence that EGF prevents apoptosis in the metaphoric kidney and in proximal tubular cells ¹⁹. PDGF is the main element responsible for the mitogenic activity of serum ¹⁶⁴. Its increased expression has been related to the accumulation of cells in proliferative glomerulonephritis ¹⁶⁵, and it is temporally associated with the recovery of a normal appearance by tubular cells following toxic injury ¹⁵⁸. PDGF might also protect from cell death and account for part of the survival activity in serum. In fact, PDGF prevents apoptosis in developing oligodendrocytes ¹⁸. A better understanding of the survival factors in renal tissues may have therapeutic interest in clinical practice, as several recombinant cytokines are available, and, at least *in vitro*, their addition up to 24 hours after withdrawal may rescue cells from apoptosis that otherwise were destined to die ¹³.

Several cytokines and inflammatory mediators may

also induce apoptosis (table VI) ¹⁶⁶⁻¹⁷⁰. Studies in renal cells are not abundant. It has been reported that H₂O₂ induces internucleosomal DNA degradation in tubular renal cells ¹⁷⁰. Our group had previously reported that TNF α is cytotoxic for glomerular epithelial and mesangial cells in culture ¹³⁷. Other authors have reported that TNF α cytotoxicity is related to the occurrence of apoptosis ^{168, 171}. We have recently observed that TNF α induces cell death and internucleosomal DNA fragmentation in mesangial and tubular epithelial cells ⁴⁸. It has been suggested that the ability of TNF α to induce apoptosis may be mediated by lipid moieties such as ceramide ¹⁶⁹, or by oxygen radical species ¹⁷². There is also evidence that platelet-activating factor (PAF) may participate in TNF α -induced cytotoxicity of renal cells ¹³⁷. We are not aware, however, of reports on a possible role for PAF in cytokine-induced apoptosis.

Table VI. Factors involved in the development of apoptosis

- *Deprivation of survival factors:*
 - Hormones: testosterone and prostate.
 - Cytokines: TNF α and neurons or neutrophils. IL-3 and hemopoietic cells. Renal cells and serum, EGF. Others: IGF-1, IL-2, IL-3, IL-4, IL-6, CNTF, PDGF.
 - Drugs.
- *Factors that induce apoptosis:*
 - Hormones: glucocorticoids and T cells.
 - Cytokines: TNF α , TGF β 1, Fas ligand.
 - Other mediators of inflammation: NO, H₂O₂ and oxygen radicals, ceramide, thromboxane B₂.
 - CD4+ and CD8+ cytotoxic T cells.
 - Lymphocyte activation.
 - Drugs: cancer chemotherapy, colchicine.
 - Physical factors: heat shock, radiation.

Occurrence of apoptosis or necrosis may depend on the intensity of the stimulus or on the presence of additional stimuli.

A balance and interplay of survival and lethal factors. There is evidence that cell fate may depend on the interaction of survival factors and apoptosis-inducing factors. In oligodendrocytes, ciliary neurotrophic factor (CNTF) not only prevented growth factor deprivation-induced apoptosis, but also protected from TNF α -induced cell death ¹⁷³. Several interleukins can also rescue lymphocytes from glucocorticoid-induced cell death ¹⁵⁵. Our recent data from experiments on renal cell death support the hypothesis that renal cell fate is also determined by an interplay of survival and lethal factors. These factors seem to modulate the expression of apoptosis-related genes (figure 4). Growth factor deprivation of tubular cells resulted in predictable cell death associated with increased levels of bax mRNA and decreased bcl-2/bax ratios.

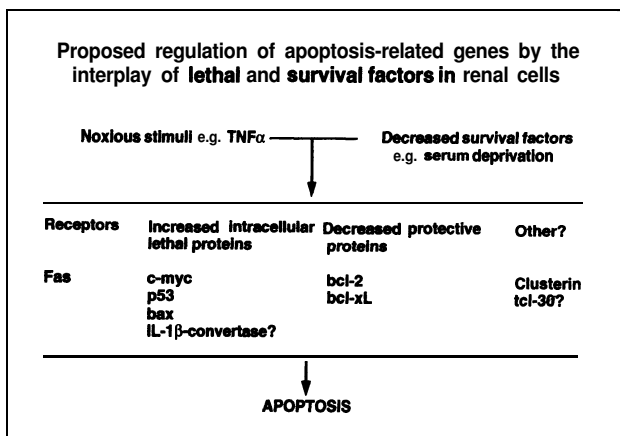


Figure 4.-Proposed regulation of apoptosis-related genes by the interplay of lethal and survival factors in renal cells. Data from our laboratory suggest that the lethal effect of TNF α on serum-deprived renal cells in culture may be related to its capacity to modulate the expression of genes that regulate the occurrence of apoptosis. TNF α increased the expression of the apoptosis-inducer receptor fas, the transcription factors c-myc and p53, and the intracellular antagonist of bel-2, bax. Moreover the levels of bel-2 and bel-x, which protect against the development of apoptosis, were reduced. TNF α also decreased the expression of clusterin, which binds to the terminal complement attack complex and prevents complement-induced cell lysis.

The addition of TNF α resulted in more prominent cell death with features of apoptosis. Cell death induced by TNF α was not as intense in the presence of growth factors. In our experimental system, TNF α resulted in increased c-myc mRNA levels in serum-deprived tubular cells. Increased levels in c-myc were the earliest of the changes in apoptosis-related genes induced by TNF α . This may have a role in the increased susceptibility of these cells to TNF α -induced cell death when growth factors are absent as increased c-myc expression confers a dependability on external growth factors to prevent cell death ⁶¹. TNF α cytotoxicity had previously been associated with increased c-myc expression ¹⁷⁴. Furthermore, TNF α decreased bcl-2 expression, leading to a marked diminution of the bcl-2/bax ratio, upregulated p53 mRNA levels and increased Fas mRNA expression, facilitating the action of other apoptotic inducing pathways ^{48,95}. Thus, TNF α -induced cell death may be related to the modulation of apoptosis-related genes, perhaps tilting the balance towards the occurrence of cell death. This might also be true for other receptors of the same family, like Fas.

In concordance with these findings in TNF α and serum-deprivation-induced death in cultured tubular cells, decreased bcl-2, and increased levels of c-myc, bax, bcl-x and Fas mRNA are associated with the de-

velopment of cell death and internucleosomal DNA fragmentation in experimental acute renal failure^{48,95}. It is interesting that increased renal expression of c-myc and c-fos has been reported in the early stages of several models of acute renal failure, including ischemia¹⁷⁵⁻¹⁷⁷. c-fos has been shown to participate in growth factor deprivation-induced cell death in vitro⁶⁶. If c-myc confers a dependence on external growth factors in renal cells in vivo, as it does to fibroblasts *in vitro*⁶¹, an inadequate supply of growth factors, perhaps compounded by an excess of apoptosis-inducing factors, may create a cytokine microenvironment favoring cell death in the kidney during acute renal failure, and may increase its severity, or even delay recovery. Evidence for such adverse microenvironment has been suggested by several investigations. For example, renal levels of pre-pro-EGF and IGF-1 mRNA have been reported to be reduced^{157,158}, and systemic TNF α and local TGF β increased in models of acute renal failure^{159,178}. Moreover, as predicted from this work, the exogenous administration of EGF or IGF-I can improve the evolution of acute renal failure^{125,160}, and decrease internucleosomal DNA degradation¹²⁵.

Apoptotic-related genes and elevated ambient glucose. The molecular basis for the development of diabetic nephropathy and for the susceptibility of the diabetic kidney to acute renal failure has not been determined. We have recently noted that culturing tubular cells in a high glucose medium decreases the expression of bcl-2 mRNA and the bcl-2/bax ratio. Decreased bcl-2 levels may predispose cells to apoptotic cell death in the presence of an adverse microenvironment. However, high glucose also decreased p53. While the physiological relevance of this is unclear, high ambient glucose decreases the accumulation of tubular cells *in vitro*, while increasing the accumulation of mesangial cells¹⁷⁹. The modulation of apoptosis-related genes may underlie some of these observations.

Summary

There is increasing evidence that apoptosis is an integral part of the normal functioning of the kidney and other organs. Derangements in its regulation, as a hypothesis, may result in renal disease. The apoptotic rate of renal cells might be abnormally increased in nephropathies characterized by cell death or cell depletion such as acute tubular necrosis, acute rejection, necrotizing glomerulonephritis or renal atrophy, and decreased whenever there is an abnormal accumulation of cells, such as during proliferative glomerulonephritis, polycystic renal disease, renal fibrosis and neoplastic.

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