Cell Cycle Regulation: Repair and Regeneration in Acute Renal Failure

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Research into mechanisms of acute renal failure has begun to reveal molecular targets for possible therapeutic intervention. Much useful knowledge into the causes and prevention of this syndrome has been gained by the study of animal models. Most recently, investigation of the effects on acute renal failure of selected gene knock-outs in mice has contributed to our recognition of many previously unappreciated molecular pathways. Particularly, experiments have revealed the protective nature of 2 highly induced genes whose functions are to inhibit and control the cell cycle after acute renal failure. By use of these models we have started to understand the role of increased cell cycle activity after renal stress and the role of proteins induced by these stresses that limit this proliferation.

THE CONSEQUENCES OF nephrotoxic renal injury include segment-specific changes in cell viability and reduced renal function. In experimental models, necrosis of the S3 segment of the proximal tubule is the predominant morphologic injury; apoptosis occurs in a minority of cells, especially those of the distal nephron. Functionally, severe vasoconstriction, principally applied to the afferent arteriole, reduced glomerular filtration rate, and loss of autoregulatory responses characterize the renal microvascular response to injury. The kidney is also unable to generate maximum urinary concentration or to reclaim filtered sodium fully. Reversal of these changes coincides with the reestablishment of the normal renal epithelial barrier with new cells that reline the denuded tubules. We hypothesize that renal injury and recovery are part of the same responses and that these processes depend on proper coordination of the cell cycle machinery. Furthermore, we show that the engagement of the cell cycle not only underlies recovery but also is an important determinant of whether cells survive the injury itself. The process of regeneration and recovery begins shortly after injury, in which both necrotic cells and replicating cells line the injured proximal tubule. The commitment to DNA synthesis is rapid and temporally coincides with the emergence of the morphologic and functional derangements. The rapid appearance of messenger RNA (mRNA) for immediate-early genes (eg, c-fos, c-jun, and egr-1),[1,2] whose expression frequently is associated with the entry of cells from quiescence into the cell cycle, has been observed in acute renal failure arising from several causes. Similarly, proliferating cell nuclear antigen (PCNA) expression increases after acute renal failure.[3-5] PCNA is a nuclear protein that is a subunit of the DNA polymerase responsible for DNA replication.[6,7] Its expression identifies cells that have entered the DNA synthetic phase of the cell cycle.[8] From these parameters it is clear that damage of cells that results in cell death also results in cell replication.

CELL CYCLE PROGRESSION AND ITS REGULATION

Studies with eukaryotic models have elucidated that orderly progression through the cell cycle is regulated by the sequential synthesis, activation, compartmentalization, and degradation of proteins controlling both entry and exit from each phase of the cycle: G1 (gap-1), S (DNA synthesis), G2 (gap-2), and M (mitosis) (Fig 1). These regulations ensure that mitosis cannot begin before DNA synthesis has completed, that DNA synthesis is initiated only after cell division, and that both mitosis and DNA replication cannot proceed with unrepaired DNA damage. One of the major controls on cell cycle progression is the regulation of phosphorylation of different substrates by interacting proteins consisting of a cyclin and a cyclin-dependent kinase (cdk). Cyclins, the regulatory subunit of the heterodimer, originally were found by nature of their cyclic oscillations during the sea urchin cell cycle.[9] (The first described cyclin, now called cyclin B, was synthesized during interphase and degraded during mitosis.) The catalytic subunit of

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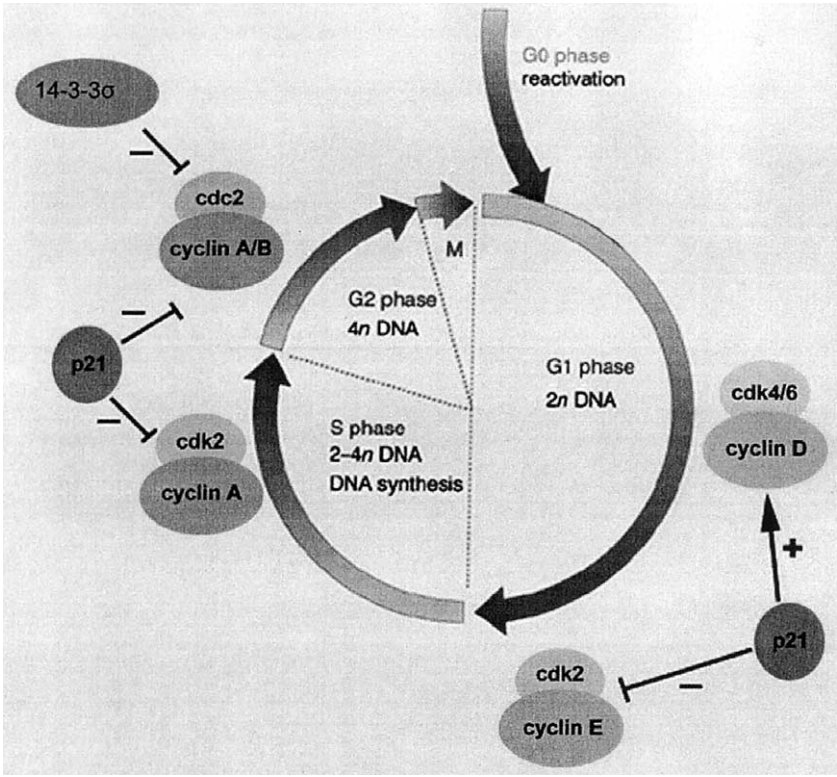

Fig 1. The cell cycle and some of its controls.

the complex is a serine/threonine protein kinase, cdk,[10] which is inactive unless associated with a cyclin. The binding of the cyclin to its cdk induces several conformational changes in the active site of the cdk,[11] conferring basal kinase activity,[12] and full activity is dependent on threonine phosphorylation of the cdk by the heterotrimeric cdk-activating kinase.[13] In vertebrates, several different cyclins and cdk partners are sequentially active throughout the cell cycle. In lower eukaryotes (eg, budding and fission yeast), different cyclins associate with the same cdk subunit.

Because cells enter the cycle in G1, usually requiring transmission of extracellular signals by growth factor receptors and integrin-derived adhesion signals,[14] cyclin D is synthesized and activates the kinase activity of cdk 4/6.[15] These kinases phosphorylate the carboxyl-terminal domain of the retinoblastoma protein (Rb), a transcriptional repressor, displacing the binding of histone deacetylase,[16] and blocking active repression by Rb. The partial inactivation of Rb is correlated with increased expression of cyclin E and cyclin E–cdk2 kinase activity. This kinase causes hyperphosphorylation of Rb, resulting in the release of Rb-bound E2F transcription factor, and activating a cascade of responsive genes, primarily those involved in DNA synthesis.[17] Cyclin E–cdk2 activity peaks at the G1/S transition, but shortly after entry into S, cyclin E begins to degrade, cyclin A starts to be synthesized, and cyclin A–cdk2 activity starts to increase. Peaks of cdk2 activity occur during S phase and just before mitosis.[18] During late G2, cyclin B accumulates in the cytoplasm. At the beginning of mitosis, cyclin B translocates to the nucleus, its associated kinase is activated, and cyclin B–cdc2 kinase now controls entry into M phase. During anaphase and telophase of mitosis, cyclin B is degraded by ubiquitin- and proteosome-dependent proteolysis,[19] causing cdc2 inactivation, and the divided cells reenter G1 to begin another cycle.

Examination of cell cycle mutants revealed that most mutations result in arrest at specific stages of the cycle. This led to the concept of cell cycle surveillance mechanisms (checkpoints) that detect

defects in DNA synthesis and chromosome segregation to block cycle progression.[20] These checkpoints also ensure that each phase of the cycle is irreversible, that each phase is completed before another is initiated, and that each phase follows the other in a sequential fashion. One of the major regulatory checkpoints in the cell cycle occurs at the G1 to S transition, when the cell either commits to genomic DNA replication or to quiescence and/or differentiation. It is also a major regulatory intersection for cells that have sustained genomic damage to undergo repair before entering the DNA synthetic phase. In early G1, the level of a 21-kd protein (p21) usually increases naturally, which acts to prevent further cell cycle progression because p21 is a potent inhibitor of cdk2 activity. This increase in p21 protein can occur for other reasons. The mRNA can be induced by the p53 transcription factor after DNA damage,[21] or by p53-independent mechanisms, as we have reported after renal injury.[22] As cyclin D–cdk4/6 increases, it titrates the level of p21 by sequestering it as part of a quaternary protein complex also containing proliferating cell nuclear antigen (PCNA, the DNA polymerase processivity factor). The titration of excess p21 by cyclin D allows cyclin E–cdk2 to become activated, which is necessary for cell cycle progression through G1 and into S. During late G1, both cyclin D and p21 are degraded. The mechanism of p21 degradation has not been characterized fully, but it can be degraded by the proteasome independently of ubiquitination[23] and also by caspase-3[24,25] in cells in which an apoptotic cascade has been activated.

A second major cell cycle checkpoint occurs at the G2 to M transition when the cell commits to beginning cell division, having completed DNA replication. Transport of cyclin B to the nucleus is possibly dependent on phosphorylation,[26] whereas its associated cdk (cdc2) is both activated and repressed by phosphorylation. Phosphorylation of cdc2 at threonine[161] by cyclin H-cdk7 is essential for activity,[27] whereas phosphorylation at threonine[14] and tyrosine[15] by the kinases Wee1[28] and Myt1[29] causes inactivation. The phosphorylation at these latter 2 sites is regulated by cdc25C phosphatase, which dephosphorylates these sites before mitosis[30] when cyclin B–cdc2 moves into the nucleus. Activated cdc2 complexes phosphorylate several substrates, including lamins, condensins, and Golgi matrix components—events important for centrosome separation, breakdown of the nuclear envelope, spindle assembly, chromosome condensation, and Golgi fragmentation.[31] DNA damage and incomplete replication inhibit this process by stimulating synthesis of ATM and ATR protein kinases. These kinases in turn activate chk-1 and -2 kinases. Together these kinases activate transcription factors such as p53 and cause phosphorylation and subsequent cytoplasmic compartmentalization of both cdc25C and cdc2,[28,32-34] whose nuclear localization is crucial for G2 to M transition. The compartmentalization of phosphorylated cdc25C and cdc2 is primarily through binding by 14-3-3 proteins, induced by p53-dependent[35] and, as we have shown,[36] p53-independent mechanisms. Similarly, both p53-dependent and -independent mechanisms activate p21, which directly inactivates the cdc2 kinase to cause G2 arrest.

CELL CYCLE REGULATION BY CYCLIN KINASE INHIBITORS

Two families of proteins interact with and inhibit cyclin-dependent kinases. One family specifically inhibits cdk4/6, the Ink4 (inhibitor of cdk4) proteins.[37] These are small molecular weight proteins, ranging from 14 to 19 kd,[38-42] each containing ankyrin repeats. They bind the kinase subunit, preventing formation of an active cyclin-cdk complex. As inhibitors of cdk4/6 kinase they prevent Rb phosphorylation, arrest the cell cycle in G1 phase, and require a functional Rb to arrest the cycle.[40,43] Their role in normal cell cycle progression is to act as checks on the assembly and activity of cyclin D–cdk4/6. Members of this family have been associated with terminal differentiation and senescence, and their mutations or deletions have been associated with cancer. The second family, of which p21 is a member, also contains $p27^{Kip1}$,[44] and $p57^{Kip2}$,[45,46] the Kip (cdk inhibitory protein) proteins. Xiong et al[47] found that p21 could inhibit the activity of each member of the cyclin-cdk cascade and that p21 overexpression inhibited the proliferation of mammalian cells. Similarly, p21 also can inhibit PCNA[47-49] and interfere with its role in DNA replication. These 2 binding moieties of p21 are located on separate domains of the protein.[50,51] Although p57 seems to be expressed in only a limited number of tissues, both p21 and p27 are expressed in most cells, and p21 mRNA is induced by stress

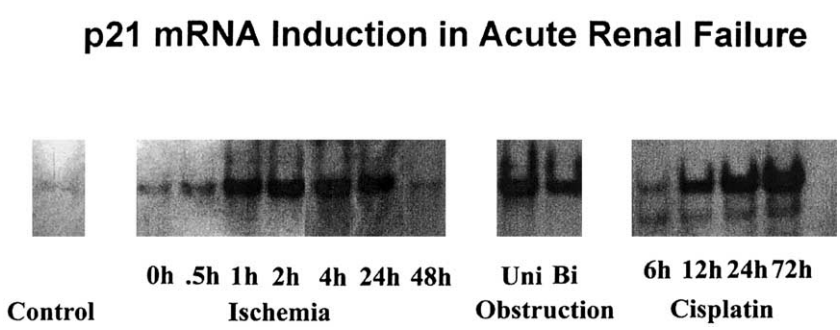

Fig 2. Northern blot analysis of p21 mRNA transcripts in rat kidney cells.

in p53-dependent and p53-independent pathways. The p27 inhibitor is highly expressed in quiescent cells, but as cells enter G1, nuclear p27 is transported into the cytoplasm and degraded after ubiquitination. The p21 protein is expressed maximally during G1 in which it negatively regulates cdk2 activity. As cyclin D–cdk4/6 levels increase during G1, p21 is titrated, releasing the cdk2 inhibition.

CYCLIN KINASE INHIBITOR EXPRESSION DURING ONTOGENY

The expression of p21 has been reported in mouse embryo,[52,53] especially in differentiating myoblasts. We have found[54] that in 12-day-old mouse embryo, p21 expression is high in the comma- and S-shaped bodies throughout the kidney. In day 18 embryos, expression is still high in the S-shaped bodies of the outer cortex; however, there is little if any expression in the differentiated tubules and within the inner cortex and medulla. Expression of p21 is minimal in adult kidney. Similarly, p21 expression has been observed using in vitro differentiation systems.[55-60] The other proteins of the p21 family also have been associated with differentiation in vitro and embryonic development in vivo.[46,61-64] However, gene ablation experiments have shown that both p21 and p27 knock-out mice develop somewhat normally, and that only p57 expression is necessary for embryonic development.[65-70]

CELL CYCLE ACTIVATION AND RENAL FAILURE

Shortly after acute renal failure, many normally quiescent kidney cells enter the cell cycle. There are increases in nuclear PCNA levels, as well as ^{3}H-thymidine and bromodeoxyuridine (BrdU) incorporation into nuclear DNA. However, coincident with this increased activity, we have shown that the $p21^{WAF1/CIP1/SDI1}$ gene is activated in murine kidney cells.[22] The Northern blot in Fig 2 shows the relative amounts of hybridization found in RNAs isolated from rat kidney before and after different models of acute renal failure, probed with p21-specific radiolabeled complementary DNA. No p21 mRNA could be detected at this exposure time in kidney from the untreated rat, but there was a marked induction of p21 mRNA in all experimental models of acute renal failure. In the ischemia model, there was a slight increase of p21 mRNA even before release of the clamp (0 hours); the major increase started 1 hour after reflow and persisted thereafter with maximum expression at 4 hours. There was a marked induction after 24 hours of unilateral or bilateral ureteral obstruction, and the highest level was detected in kidney isolated after cisplatin treatment.

The sites of p21 mRNA overexpression were localized by in situ hybridization using an antisense digoxigenin-labeled RNA probe. Highest amounts of p21 mRNA were found in the outer stripe of the outer medulla, in the cells of the thick ascending limbs. The distal convoluted tubule cells in the cortex also were stained. The localization of p21 mRNA in all types of acute renal failure is similar. A more sensitive localization for p21 protein using immunohistochemistry showed the protein to be present in nuclei of both distal and proximal tubule cells.

THE INFLUENCE OF p21 ON ACUTE RENAL FAILURE

The effect(s) of p21 induction in acute renal failure was studied by comparing wild-type *p21(+/+)* mice with mice homozygous for a *p21*

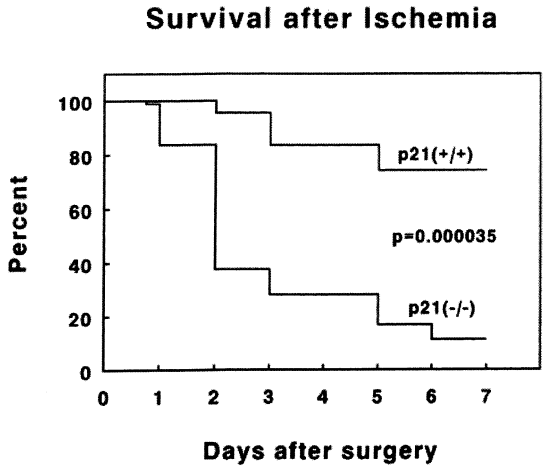

Fig 3. Kaplan-Meier survival curve. Comparison of the survival of p21(+/+) and p21(−/−) mice after 30 and 50 minutes of renal ischemia.

gene deletion, *p21(−/−)*. After either cisplatin administration or after 30 or 50 minutes of ischemia, *p21(−/−)* mice displayed a more rapid onset of the physiologic signs of acute renal failure, developed more severe morphologic damage, and had a higher mortality than their *p21(+/+)* littermates.[4,5] Blood urea nitrogen values in untreated animals were nearly identical, and 1 day after cisplatin injection the values in the wild-type mice population were still within the untreated range. However, at this time, the values in the *p21(−/−)* population were elevated. Two and 3 days after cisplatin injection, the blood urea nitrogen level of the wild-type mice was increased, but never to the extent of the *p21(−/−)* mice. Similar findings were observed after ischemia. A marked difference in mortality also was observed (Fig 3). After either cisplatin-induced or ischemic acute renal failure, morphologic damage in kidneys of the *p21(−/−)* mice was evident throughout the cortex, whereas in the p21(+/+) kidneys it was restricted primarily to

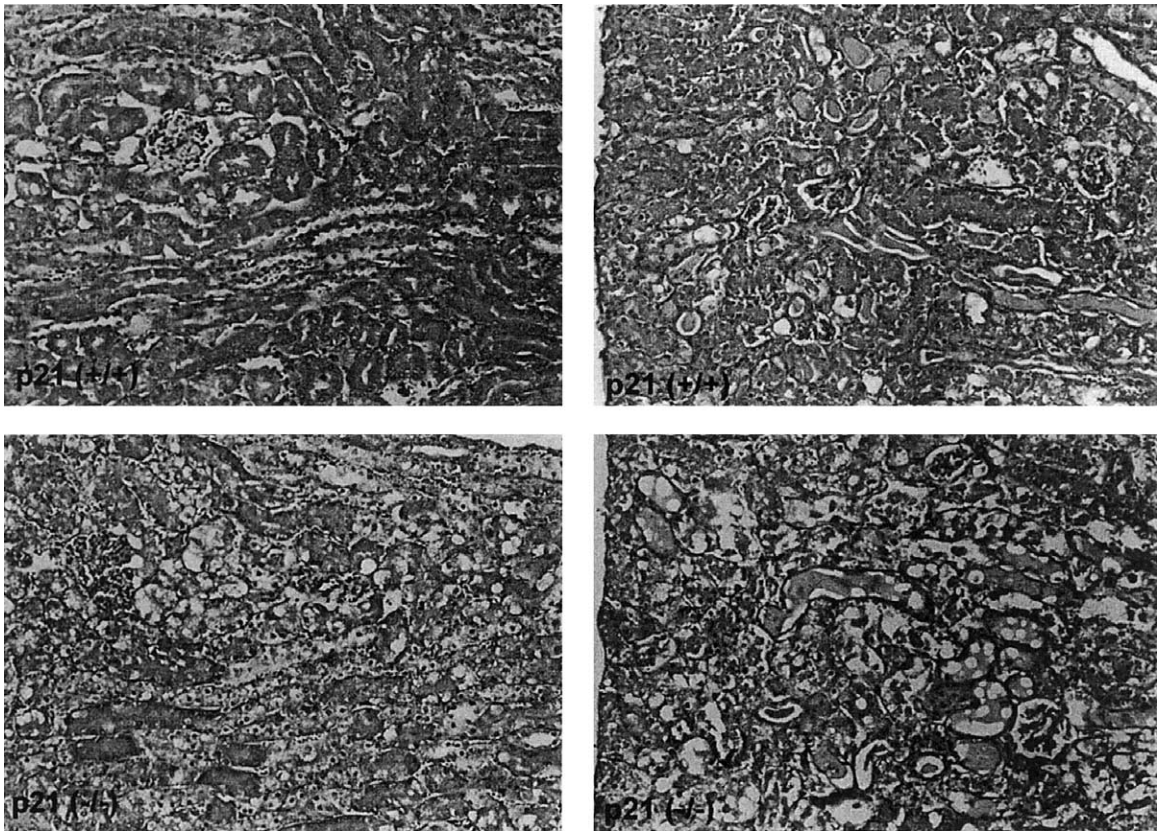

Fig 4. Histology of kidney after cisplatin injection. Representative sections from either 1 day (left panels) or 3 days (right panels) after injection of wild-type or p21(−/−). Magnification ×390.

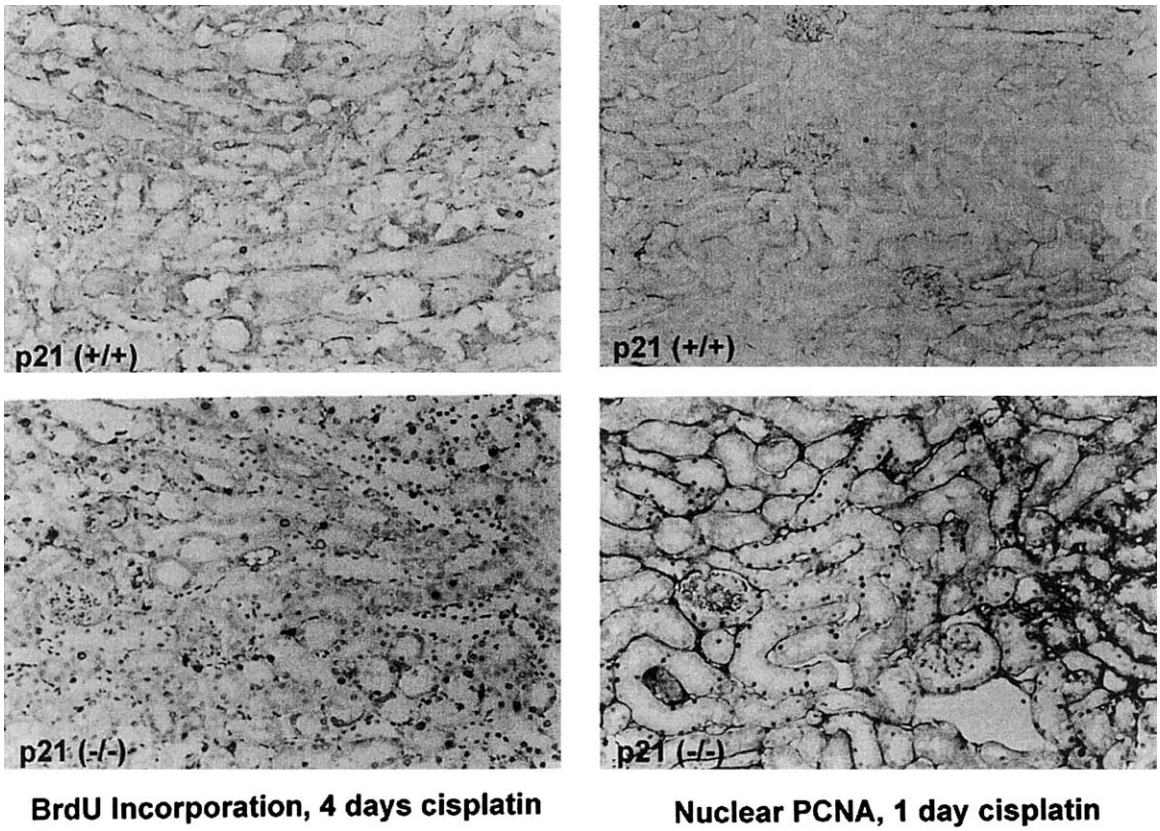

Fig 5. Cell-cycle analysis in kidney after cisplatin injection. Immunodetection of nuclear BrdU incorporation 4 days and of nuclear PCNA localization 1 day after injection. Sections were from wild-type and p21(−/−) mice. Magnification ×200.

the S3 segment of the proximal tubules. Representative photomicrographs of kidney sections from days 1 and 3 after cisplatin administration are shown (Fig 4).

In addition to necrosis, apoptosis also was more widespread in the *p21(−/−)* mice after cisplatin treatment. In the wild-type mice, most of the apoptotic cells were located in the distal nephron, whereas in the *p21(−/−)* mice, both distal nephron and proximal tubules contained apoptotic cells. Apoptosis was not found to be a major reaction in the first several days after ischemia either in *p21(+/+)* or *p21(−/−)* mice.

As would be expected from the role of p21 as a cell cycle inhibitory protein, parameters such as BrdU incorporation into nuclear DNA and increases of PCNA content were much higher and more widespread after acute renal failure in *p21(−/−)* mice, compared with *p21(+/+)* mice. Representative photomicrographs of kidney sections from 1 day after cisplatin injection stained for PCNA and from 4 days after cisplatin stained for BrdU are shown (Fig 5). Similarly, in another model of acute renal failure, ureteral obstruction, Hughes et al[71] found that p21 expression limited kidney cell proliferation.

MECHANISM OF p21 PROTECTION?

After cisplatin injection or renal ischemia in vivo, we found that in kidneys of *p21(−/−)* mice, a more widespread cell death was associated with an increased cell cycle activity and increased nuclear size.[36] In considering possible causes for this increased size, we investigated whether these cells contained greater than normal amounts of nuclear DNA. Figure 6 is an in situ hybridization for chromosome 15 in nuclei isolated from mice after acute renal failure. Characteristic of this analysis, 2 spots of hybridization can be seen in interphase nuclei having a normal 2N DNA content (Fig 6A). How-

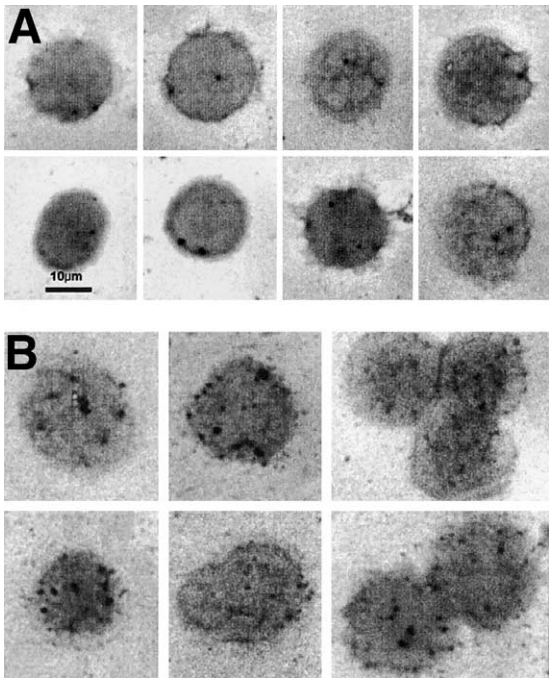

Fig 6. In situ hybridization analysis of kidney nuclei for ploidy determination. Representative nuclei isolated from kidney of mice before cisplatin injection, or from (A) wild-type mice after cisplatin injection, or from (B) p21(−/−) mice 4 days after cisplatin.

ever, several areas of hybridization can be seen in kidney nuclei isolated from *p21−/−* mice after acute renal failure (Fig 6B), showing polyploid DNA content, resulting from an uncoordinated cell cycle.

We also found the induction of a protein, 14-3-3σ, a regulator of G2 to M transition, after both cisplatin and ischemia-induced acute renal failure in vivo.[36] Recently, it was shown that this protein may influence cell fate after injury.[33,35,72] In the absence of p21 induction, overexpression of the 14-3-3σ gene in growing cells caused an uncoordinated cell cycle in which cells did not divide synchronously after G2, but rather entered another DNA synthetic phase. This increased DNA content in the cells, which in turn led to cell death. However, expression of both p21 and 14-3-3σ led to cell cycle inhibition rather than to cell death. To explore the roles of p21 and 14-3-3σ in relevant in vitro models of renal cell injury, we determined the effect of either cisplatin or hydrogen peroxide exposure on cells in which one or both of these genes were deleted.[36] Our results showed that as compared with wild-type cells, cells with the gene deletions had much decreased viability, both in dose-response experiments and in survival times after cisplatin or hydrogen peroxide exposure.

These studies are compatible with the idea that cell stress induces pathways that compete between cell death and cell cycle arrest (Fig 7). In wild-type cells, stress results in induction of cell cycle inhibitors that lead to arrest, whereas in *p21*- and/or *14-3-3σ*–deleted cells, similar stress causes cell death pathways to predominate. Our results indicate that coordinated cell cycle control, initially manifested as cell cycle inhibition, is necessary for optimum recovery from acute renal failure. Be-

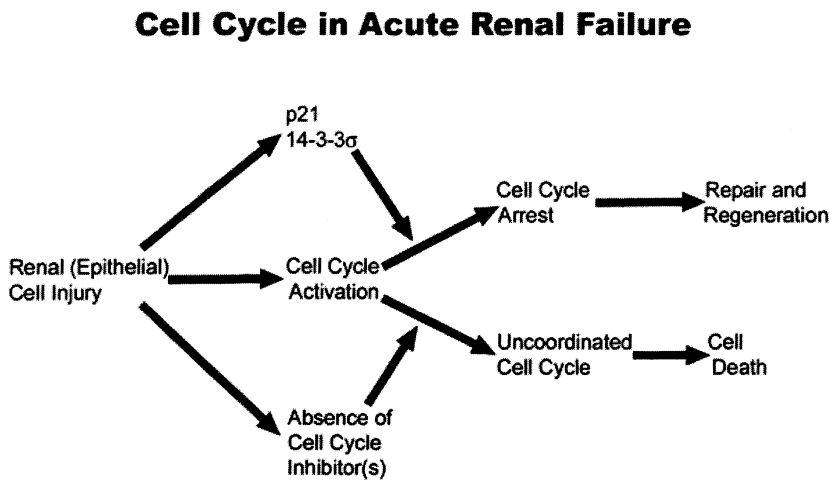

Fig 7. Proposed mechanism for the interaction of cell cycle inhibitors with the course of acute renal failure.

cause these proteins are highly expressed in terminally differentiated cells only after injury, we propose that cell cycle coordination by induction of these proteins could be a general model of tissue recovery from stress and injury. Our model of cell cycle regulation after injury is that after acute renal failure, in which epithelial cells are damaged, normally quiescent cells enter the cell cycle. In kidneys of wild-type animals, cell cycle inhibitors (p21 and 14-3-3σ) also are induced, and their combined activities check the cell cycle at G1 and G2. As extrapolated from the in vitro results, the presence of both p21 and 14-3-3σ is necessary to coordinate the cell cycle, and the absence of either of these factors will result in increased cell death and increased mortality from acute renal failure. In this model, cell cycle arrest is a prerequisite for renal cell repair and/or regeneration after injury and the inhibition of the cell cycle allows the repair of cellular damage to occur before cell replication.

The elevation of p21 expression has been associated with suppression of apoptosis caused by a variety of stimuli and in a variety of cells. Human colorectal cancer cells (HCT116 or RKO) could be protected from either p53- or prostaglandin-induced apoptosis by p21-induced growth arrest.[73,74] Using RKO cells, other agents, such as serum withdrawal and the growth factor receptor antagonist suramin, causing growth arrest in the absence of p21 elevation, did not prevent prostaglandin-induced apoptosis, whereas mimosine, which caused growth arrest and p21 elevation, could protect against prostaglandin-induced apoptosis.[74] Apoptosis was promoted in MCF7 breast cancer cells after prostaglandin treatment by use of antisense RNA to lower induced p21 mRNA.[75] Other transformed cells, such as carcinomas,[76] melanomas,[77] leukemias,[24] hepatomas,[18,78] myoblasts,[79] and neuroblastomas[80] have been found to be susceptible to p21 growth arrest and protection from apoptosis. The suppression of p21 induction by the Myc oncogene/transcription factor was correlated with increased p53-induced apoptosis in HCT116 cells.[81] Several nontransformed cell types also have been used to correlate p21 induction with protection from cell death. Murine hematopoeitic cells undergo apoptosis after interleukin 3 withdrawal and exposure to ionizing radiation, which is correlated with decreased p21 expression.[82] Apoptosis in umbilical vein endothelial cells caused by growth factor deprivation is correlated with p21 cleavage and its exit from the nucleus.[83] Prevention of p21 cleavage by appropriate caspase-resistant p21 mutants suppressed apoptosis in the endothelial cells.

Inguaggiato et al[84] have proposed that resistance to cell death in kidney by heme oxygenase-1 overexpression is by p21 up-regulation, and Miyaji et al[85] have speculated that p21 induction contributes to acquired resistance to cisplatin-induced acute renal failure. We have shown that p21(−/−) mice are more sensitive to renal ischemia and cisplatin administration than p21(+/+) mice,[3,4] that p21(−/−) cultured cells have more cell death than p21(+/+) cells when exposed to hydrogen peroxide and cisplatin,[36] and that primary cultures of proximal tubule cells isolated from p21(−/−) mice are more sensitive to cisplatin than those from p21(+/+) mice.[86]

FUTURE STUDIES

Confronted with a hostile environment, the kidney mounts a response that is initiated by signaling molecules that engage multiple pathways including those that regulate the cell cycle. The cell undergoing these changes may decide to check the progression of the cycle and repair damage before proceeding or entering a pathway destined to cell death. This decision point is regulated carefully and cyclin-dependent kinase inhibitors, especially p21, are important in this decision. The interface between these pathways and the cell death pathways are first emerging but phosphorylation events critical to cell function reside in the cyclin-dependent kinases and the kinases, phosphatases, inhibitors, and activators that regulate their activities. The identification of the precise pathways engaged in this process is an area of active research not only in acute renal failure but also in the field of cell biology in general.

Manipulation of cell cycle inhibitors, either by up-regulation of these genes or by the use of cdk inhibitory drugs, could ameliorate the effect of renal stresses such as cisplatin administration or ischemia reperfusion. Similarly, timely down-regulation of cyclin kinase inhibition could be a strategy for accelerating cellular regeneration after injury.

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