Graded ATP depletion can cause necrosis or apoptosis of cultured mouse proximal tubular cells

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Graded ATP depletion can cause necrosis or apoptosis of cultured mouse proximal tubular cells. Am. J. Physiol. 274 (Renal Physiol. 43): F315–F327, 1998.—The mechanisms of cell death induced by ATP depletion were studied in primary cultures of mouse proximal tubular (MPT) cells. Graded ATP depletion, ranging in severity from ~2 to 70% of control levels, was induced by incubating cells with either antimycin or 2-deoxyglucose, with varying concentrations of dextrose. We found that cells subjected to ATP depletion below ~15% of control died uniformly of necrosis. In contrast, cells subjected to ATP depletion between ~25 and 70% of control all died by apoptosis. The rapidity of cell death was proportional to the severity of reduction of cell ATP content and was independent of the mechanism of cell death. Renal growth factors, epidermal growth factor (EGF) and high-dose insulin, did not ameliorate apoptotic cell death induced by ATP depletion. We conclude that ATP depletion can cause either necrosis or apoptosis in MPT cells. Furthermore, we have identified a narrow range of ATP depletion (~15 to 25% of control) representing a threshold that determines whether cells die by necrosis or apoptosis.

Acute renal failure following ischemic injury to the kidney is caused, in large part, by renal tubular epithelial cell injury (2). The proximate cause of tubular cell injury and death following ischemia is a fall in the intracellular content of ATP (31, 32, 37, 38). Studies examining the pathophysiology of ischemic tubular cell injury have focused largely on the factors consequent to ATP depletion that lead to necrotic cell death (38). Acute tubular necrosis, the clinical term used to describe the entity of ischemic or toxic acute renal failure, emphasizes the importance that has traditionally been ascribed to necrosis in the pathophysiology of this disease.

Apoptosis, a form of cell death quite distinct from necrosis (1, 12, 21, 22, 35), has been recognized for many years as an important physiological mechanism for the removal of unwanted cells in many situations, including embryologic development, the normal turnover of gastrointestinal epithelium, and the regulation of the immune system (28). However, accumulating evidence during the past five years has shown that a wide variety of pathological events that are well known to cause necrosis, including ischemia, can also induce apoptosis (5, 7, 16, 33). Most of this evidence has come from studies of cells in culture and has demonstrated that many injurious agents can cause necrosis or apoptosis in the same cell type. In general, the mechanism of cell death appears to be determined by the severity of the injury, with extremely severe insults causing necrosis and milder insults of the same type causing apoptosis (5, 7, 16, 33). Consistent with this concept is our recent study showing that cisplatin, a common cause of acute renal failure, induces necrosis of cultured renal tubular cells at high concentrations (~1 mM) and apoptosis at much lower concentrations (10–100 μM) (21).

Nonetheless, the mechanism of renal tubular cell death induced by ischemia or ATP depletion remains uncertain. Although the factors contributing to renal tubular cell death have been extensively studied over the past few decades, some of these studies examined injurious stimuli that lead exclusively to necrosis, whereas others did not determine the mechanism of cell death at all (1, 6, 38). The purpose of this study was to examine carefully the mechanisms of cell death induced by ATP depletion in renal tubular cells and to establish the role of the severity of ATP depletion in determining whether cells die by apoptosis or necrosis.

We used predominantly morphological criteria to distinguish necrosis from apoptosis, since these remain the most reliable determinants of the mechanism of cell death (8, 15, 18, 22). We also used primary cultures of mouse proximal tubular (MPT) cells, since we have shown that MPT cells are far more susceptible to lethal injury than are immortalized renal cell lines such as Madin-Darby canine kidney, opossum kidney, or LLC-PK1 cells (19, 29, 37). On the basis of these findings, we believe that MPT cells represent a more reliable model than immortalized cell lines for elucidating the in vivo mechanisms of cell death induced by ATP depletion in ischemic proximal tubular cells.

In this study, we show for the first time that there is a narrow range of ATP depletion (~15 to 25% of control), which represents a threshold in determining the mechanism of cell death. Cells exposed to severe ATP depletion (less than ~15% of control) die uniformly by necrosis; cells subjected to milder degrees of ATP depletion (more than ~25% of control) undergo apoptosis. Furthermore, we demonstrate that, independent of the mechanism of cell death, the rapidity with which cells die is proportional to the severity of ATP depletion and that this is true across a wide spectrum of ATP levels, ranging from ~2 to ~70% of control. Finally, we show that renal growth factors do not protect against cell death induced by ATP depletion.

METHODS
Reagents
The vital nuclear dye bisbenzamide (Hoechst 33342 or H-33342) was obtained from Calbiochem (San Diego, CA).
Propidium iodide (PI) was obtained from Molecular Probes (Eugene, OR). All other reagents, including trypan blue, dextrose, antimycin, 2-deoxyglucose (DOG), and dextrose-free Dulbecco's modified Eagle's medium (DMEM), as well as all culture supplies, were obtained from Sigma (St. Louis, MO).

Primary Culture of MPT Cells

Cells were cultured from collagenase-digested fragments of proximal tubules obtained from the cortices of kidneys of C57Bl/6 mice by a modification of previously described methods (29). Cortical tubules were plated in serum-free defined culture medium (1:1 mixture of DMEM and Ham's F-12, containing 2 mM glutamine, 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 5 mg/ml transferrin, 5 mg/ml insulin, 50 mM hydrocortisone, 500 U/ml penicillin, and 50 mg/ml streptomycin). MPT cells grew to confluence from tubules over 4–5 days and were studied between the 5th and 10th days of culture. MPT cell monolayers were previously shown to be of proximal tubular origin by a combination of morphological, biochemical, and transport characteristics (29).

ATP Depletion of MPT Cells Using Chemical Anoxia

Cells were incubated in dextrose-free DMEM at 37°C in a humidified atmosphere containing 5% CO₂-95% air. For control monolayers not subjected to ATP depletion, dextrose (10 mM) was added to the DMEM. ATP depletion of varying severity was induced by adding antimycin (2 µM, a mitochondrial inhibitor) or DOG (5 mM, an inhibitor of glycolysis), and antimycin ± 10 mM dextrose in the absence of metabolic inhibitors. Baseline ATP values are expressed as nanomolar ATP per milligram cell protein. ATP levels following incubation with metabolic inhibitors are expressed as a percent of the control value.

Assay of Cell Viability

Cell viability was quantitated by counting the number of viable cells, defined as cells that remained adherent to the culture dish and excluded trypan blue. Although trypan blue positivity and loss of cell adherence cannot be used to determine the mechanism of cell death, the combined use of these criteria is a well-established technique for determining whether any intervention alters overall “cell survival” in the setting of either necrosis (29) or apoptosis (21). The exact mechanism of cell death, whether necrosis or apoptosis, must be established by other methods (described below).

Nonadherent cells were removed by two washes with ice-cold phosphate-buffered saline (PBS). Adherent cells were harvested by incubation with 0.05% trypsin-0.53 M EDTA-4 Na for 10 min at 37°C. Trypsin was neutralized by addition of DMEM containing 10% calf serum. Cells were centrifuged for 5 min at 1,000 revolutions/min and resuspended in DMEM. Trypan blue (0.04 g/dl) was added for 10 min, and the number of viable cells excluding trypan blue was counted in a hemocytometer. Cell viability was expressed as the percentage of viable cells in experimental wells compared with that in control wells in which cells were incubated in the presence of 10 mM dextrose and in the absence of metabolic inhibitors.

Cell ATP Content

Cell ATP content was measured using the luciferase assay, as previously described (29). ATP levels were measured at 10, 20, and 60 min after incubation of cells for each of the six metabolic conditions described above. The ATP levels at each of these three time points were not different from one another for any of the six experimental conditions. We therefore averaged the three ATP values for each experimental condition and expressed the ATP as a single value. For each experiment, baseline ATP levels were measured in uninjured monolayers incubated in DMEM containing 10 mM dextrose in the absence of metabolic inhibitors. Baseline ATP values are expressed as nanomolar ATP per milligram cell protein. ATP levels following incubation with metabolic inhibitors are expressed as a percent of the control value.

Agarose Gel Electrophoresis of DNA

MPT cells were depleted of ATP, and supernatant and detached cells were pooled before a single fraction of DNA per sample was extracted for electrophoresis. For cells treated with antimycin alone, antimycin + 0.25 mM dextrose, and antimycin + 1.0 mM dextrose, DNA was extracted after 6 h. For cells treated with antimycin + 10 mM dextrose, DOG alone, or DOG + 10 mM dextrose, DNA was extracted after 24 h of ATP depletion.

Cells were lysed in 0.5% Triton X-100, 5 mM tris(hydroxymethyl)aminomethane hydrochloride, pH 7.4, and 20 mM EDTA for 30 min at 4°C. After centrifugation at 15,000 g for 20 min, the supernatants were extracted with phenol-chloroform. Sodium acetate (3.0 M, pH 5.2; 1/10th volume) and MgCl₂ (1.0 M, 1/30th volume) were added, and the DNA was precipitated in ethanol. Samples were separated by electrophoresis on a 1.2% agarose gel containing ethidium bromide.

Immunofluorescent Studies of Cells Stained with H-33342 and PI

We used fluorescence microscopy to distinguish apoptosis and necrosis, using previously described techniques (21). Plasma membrane integrity was assessed by staining cells with the membrane-permeant DNA dye PI (excitation wavelength, 500 nm; emission wavelength, 640 nm). Nuclear morphology was assessed by staining with the supravital DNA dye H-33342 (excitation wavelength, 497 nm). H-33342 enters live cells and therefore stains the nuclei of viable cells, as well as those that have died by apoptosis or necrosis. Apoptotic cells can be distinguished from viable and necrotic cells on the basis of nuclear condensation and fragmentation, as well as by increased fluorescent intensity of nuclei stained with H-33342.

Adherent MPT cells and cells that had detached spontaneously from the monolayer were processed separately. Cells were washed in PBS and then stained with H-33342 (10 mg/ml) and PI (1 mg/ml) for 10 min at 37°C. Cells were then freshly washed in PBS. Adherent cells were fixed with 3.7% paraformaldehyde, while spontaneously detached cells were examined under epifluorescence microscopy as a wet preparation. Each field of cells was photographed twice (magnification, ×400), using appropriate filters to examine and compare H-33342 and PI fluorescence staining in the same cells.

Electron Microscopy

MPT cells that had detached from the culture dish were centrifuged at 500 g for 15 min, fixed with 2.5% glutaraldehyde in PBS for 1 h at 4°C, and washed three times in Sabatini’s solution (PBS with 6.8% sucrose). Samples were postfixed with 1% osmium tetroxide (1 h), washed three times in Sabatini’s solution, passed through a graded series of alcohols (30, 50, 70, 90, and 100% for 15 min each), and
treated with propylene oxide (15 min), a 1:1 Epon-propylene oxide mix (1 h), and three changes in pure Epon (twice for 3 h and overnight). Polymerization occurred overnight at 64°C. Ultrathin sections (~50 nm) were cut with a MT2 Sorvall ultramicrotome, stained with lead citrate and uranyl citrate, and examined with a JEOL 100CX transmission electron microscope at 60 kV, using a 20-mm objective aperture.

Statistics
Duplicate wells were examined in each experiment, and the results of duplicate wells were averaged. All data are expressed as means ± SE. Comparisons between the multiple different groups were made using a Student's t-test. When more than one comparison was necessary, the Bonferroni correction was used.

RESULTS

Effect of Metabolic Inhibitors on Cellular ATP Content

Figure 1 shows the effect on cellular ATP content of antimycin or DOG in combination with varying concentrations of dextrose. The mitochondrial inhibitor, antimycin, in the absence of dextrose, produced the most severe depletion of ATP levels to 1.5 ± 0.4% of control levels. The addition of dextrose (0.25, 1.0, and 10 mM) to antimycin resulted in progressively less severe reductions in cellular ATP (4.1 ± 0.3, 14.5 ± 0.2, and 49.4 ± 1.3% of control levels, respectively). The glycolytic inhibitor, DOG, in the absence of dextrose, reduced cellular ATP to 23.7 ± 1.9% of control, whereas the addition of 4 mM dextrose to DOG reduced cellular ATP to 46.6 ± 1.6% of control. DOG + 10 mM dextrose produced the mildest depletion of cellular ATP (70.0 ± 1.6% of control levels). Thus, by using antimycin or DOG in combination with various concentrations of dextrose, we were able to produce a graded severity of ATP depletion, ranging from ~2 to ~70% of control. Antimycin + 10 mM dextrose and DOG + 4 mM dextrose therefore resulted in a comparable degree of ATP depletion (49.4 ± 1.3 and 46.6 ± 1.6% of control, respectively).

There was no difference in baseline levels of ATP obtained for each of the seven metabolic conditions. The average value of cellular ATP for all control ATP measurements (n = 34) was 19.1 ± 1.5 nM ATP/mg protein.

Effect of ATP Depletion on Cell Survival

MPT cell survival correlated directly with cellular ATP levels (Figs. 2 and 3). Cells incubated with antimycin in the absence of dextrose (~2% of control ATP levels) died rapidly, with 2.5 ± 1.2% cells remaining viable after 6 h (n = 8) (Fig. 2). The addition of dextrose (0.25 and 1.0 mM) to antimycin improved survival at 6 h to 19.9 ± 2.7 (n = 7) and 42 ± 4.2% (n = 8), respectively (Fig. 2). This increase in viability is in accord with the increased cellular ATP content produced by addition of dextrose (~4 and ~15%, respectively). No cells remained viable after 24 h under any of these conditions (data not shown).

With further increases in cellular ATP content, cell death occurred less rapidly, and loss of cell viability became apparent only after 6 h. Thus, for cells incubated with DOG alone (n = 10), antimycin + 10 mM dextrose (n = 8), DOG + 4 mM dextrose (n = 5), or DOG + 10 mM dextrose (n = 8), cellular ATP was >85% (Fig. 1), and viability at 6 h in each case was >85% of control (Fig. 3). By 24 h, however, viability had markedly decreased, with 23.4 ± 1.2% of cells remaining viable for DOG alone, 61.1 ± 3.2% for antimycin + 10 mM dextrose, 60.2 ± 6.1% for DOG + 4 mM dextrose, and 87.8 ± 2.5% for DOG + 10 mM dextrose (Fig. 3). Viability at 48 h was further reduced to 0, 50.4 ± 5.5, 47.6 ± 6, and 74.7 ± 1.1%, respectively (Fig. 3). Survival correlated directly with increasing cellular
The pattern of DNA fragmentation is not completely reliable in determining the mechanism of cell death (see discussion). Therefore, we examined the mechanism of cell death induced by each degree of ATP depletion using three independent morphological methods: 1) cell morphology, examined under light (phase-contrast) microscopy; 2) nuclear morphology of cells stained with H-33342 and PI, examined under epifluorescence microscopy; and 3) electron microscopy.

Phase-contrast microscopy. MPT cells incubated with dextrose in the absence of any metabolic inhibitors demonstrated the typical cobblestone appearance of a normal epithelial cell monolayer. All cells excluded trypan blue (Fig. 5A). MPT cells subjected to a reduction of ATP to ~15% of control by treatment with antimycin in the presence of 1.0 mM dextrose for 6 h are shown in Fig. 5B. A number of these cells are swollen, enlarged, and trypan blue positive, all features typical of necrosis. Cells dying from more severe ATP depletion, induced either by antimycin alone or by antimycin + 0.25 mM dextrose, show the same necrotic features (not shown). Eventually, the dead cells detached in sheets of interconnected cells (not shown).

In contrast, when MPT cells were subjected to less severe ATP depletion (~25% of control) by treatment with DOG alone, dying cells showed features quite distinct from those of necrosis under phase-contrast microscopy (Fig. 5C). Cells became progressively smaller and more rounded and detached individually from the monolayer. Furthermore, all cells still adherent to the monolayer were trypan blue negative, indicating maintenance of an intact cell membrane. Also, unlike necrotic cells, cells dying from apoptosis detached individually from the monolayer. These morphological features

DNA Electrophoresis

DNA electrophoresis of MPT cells subjected to ATP depletion below 15% of control (antimycin alone, antimycin + 0.25 mM dextrose, or antimycin + 1.0 mM dextrose) demonstrated degradation of DNA into small fragments of random size (Fig. 4, lanes 2-4). This “smear” pattern of DNA fragmentation is consistent with necrotic cell death. In contrast, electrophoresis of DNA from MPT cells in which ATP levels were reduced to 25–70% of control levels (DOG alone, antimycin + 10 mM dextrose, or DOG + 10 mM dextrose), resulted in a “ladder” pattern, indicative of internucleosomal cleavage of DNA (Fig. 4, lanes 5-7). A “ladder” pattern of DNA fragmentation has been reported in most cells undergoing apoptosis and is considered one of the characteristic biochemical features of apoptosis. It should also be noted that the ladder is most intense in lane 5 and progressively less intense in lanes 6 and 7 (Fig. 4). Thus the degree of DNA fragmentation is roughly proportional to the number of dead cells, as determined by the viability assays (Fig. 3).

Morphological Determination of the Mechanism of Cell Death Induced by Graded ATP Depletion

The pattern of DNA fragmentation is not completely reliable in determining the mechanism of cell death...
on phase contrast are typical of cells dying by apoptosis. Less severe reductions of cell ATP (~49, 47, and 70% of control), induced by treatment with either antimycin + 10 mM dextrose, DOG + 4 mM dextrose, or DOG + 10 mM dextrose, respectively, showed the same morphological features of apoptosis under phase-contrast microscopy (not shown).

Thus, in these phase-contrast studies, depletion of cell ATP to a level between 15–25% of control seems to represent a threshold dividing apoptotic from necrotic cell death.

Fluorescence microscopy of MPT cells. Adherent cells. Control MPT cell monolayers stained with H-33342 demonstrated the faint delicate chromatin pattern of normal nuclei (Fig. 6A). As expected, these normal cells excluded PI (Fig. 6B). After ATP depletion to ~15% of control (antimycin + 1.0 mM dextrose) for 6 h, some cells are dead, as evidenced by positive nuclear staining with PI (Fig. 6D). The nuclei of other cells are PI negative, indicating that they are still potentially viable (Fig. 6D). These findings are comparable to the trypan blue stain in Fig. 5C and are typical of necrotic cell death. It should be noted, however, that, irrespective of whether these cells are PI positive or negative, the nuclear morphology and intensity of nuclear fluorescence of all cells subjected to severe ATP depletion (~15% control levels) were similar to those of normal cells on H-33342 staining (compare Fig. 6, A and C). Hence, H-33342 nuclear staining alone cannot be used to distinguish necrotic from viable cells. Necrotic cells can be identified under epifluorescence microscopy by the combination of positive PI staining and a morphologically normal nuclear pattern with H-33342. Cells subjected to more severe ATP depletion (<15% control) by treatment with antimycin alone or antimycin + 0.25 mM dextrose showed the same features (not shown).

Unlike normal or necrotic cells, the nuclei of adherent MPT cells subjected to milder ATP depletion (~25% control) by exposure to DOG alone for 24 h had strikingly varied chromatin morphology on H-33342 staining (Fig. 6E). Although some nuclei appeared normal, many nuclei had abnormal morphological features typical of apoptosis. The nuclei of apoptotic cells appear as very brightly staining homogeneous bodies of condensed chromatin (Fig. 6E). The condensed nuclei of apoptotic cells then fragment into pieces of varying size (Fig. 6E). Some cells, in earlier phases of apoptosis, maintain a normal overall nuclear morphology but stain more intensely with H-33342 and are slightly smaller than normal nuclei (Fig. 6E). Thus H-33342 staining alone permits the visualization of two charac-

Fig. 5. Control and ATP-depleted MPT cells stained with trypan blue and examined under phase-contrast microscopy. A: MPT cells incubated with dextrose in the absence of metabolic inhibitors demonstrate cobblestone appearance typical of a normal epithelial monolayer. B: Many of the cells subjected to severe ATP depletion (~15% of control) by exposure to antimycin + 1.0 mM dextrose for 6 h are swollen, enlarged, and trypan blue positive. These features are consistent with necrosis. C: MPT cells subjected to less severe ATP depletion (~25% of control) by exposure to DOG alone for 24 h are all trypan blue negative. However, normal cobblestone appearance is lost because most of the cells have become rounded and smaller and have lost contact with surrounding cells. Gaps in the monolayer represent areas in which cells have spontaneously detached from the monolayer. These features are all indicative of apoptosis.
Fig. 6. Epifluorescence microscopy of MPT cells still attached to the monolayer and stained with H-33342 and propidium iodide (PI). A and B: MPT cells incubated with dextrose in the absence of metabolic inhibitors have normal nuclear morphology on H-33342 staining. Nuclei stain faintly, and delicate pattern of normal chromatin is clearly seen (A). All these nuclei, as expected, exclude PI (B). C and D: nuclei of cells subjected to severe ATP depletion (−15% of control) with antimycin + 1.0 mM dextrose for 6 h that are still adherent to the monolayer appear relatively normal on H-33342 staining (C). However, although some cells are viable and exclude PI, many are necrotic as evidenced by bright staining with PI (D). E and F: cells exposed to moderate ATP depletion (−25% of control) with DOG alone for 24 h that are still adherent to the monolayer show varied morphology on H-33342 staining (E). Although some nuclei appear normal, others appear as brightly staining homogeneous masses of chromatin (arrows, E). This appearance is due to condensation of nuclear chromatin. Condensed bodies of chromatin are also of varying size because of nuclear fragmentation into multiple pieces (arrows, E). Nuclear condensation and fragmentation are the most reliable features of apoptosis and do not occur in necrotic cells. Some nuclei show an intermediate morphology between normal and completely condensed nuclei. These nuclei are somewhat smaller and more brightly staining than normal nuclei (curved arrow, E). All nuclei, whether normal, condensed, or fragmented, exclude PI (F), indicating preservation of plasma membrane.
teristic features of apoptosis not seen in necrosis, namely, chromatin condensation and nuclear fragmentation. It is also important to note that the nuclei of adherent apoptotic cells, whether normal or condensed, were all negative for PI (Fig. 6F). This is consistent with the observation that apoptotic cells maintain plasma membrane integrity long after nuclear condensation and fragmentation have occurred (15, 18, 22). Cells subjected to less severe ATP depletion (>25% control) by exposure to antimycin + 10 mM dextrose, DOG + 4 mM dextrose, or DOG + 10 mM dextrose showed the same morphology as cells subjected to DOG alone (not shown).

DETACHED CELLS. Normal viable cells do not detach spontaneously from the monolayer. In contrast, both necrotic and apoptotic cells eventually lose adherence and float into the incubation medium. Necrotic cells tend to detach from the monolayer in sheets of intercon-nectected cells, whereas apoptotic cells detach individually from the monolayer.

Spontaneously detached cells from monolayers subjected to severe ATP depletion (antimycin + 1.0 mM dextrose) show a relatively normal chromatin pattern on H-33342 staining (Fig. 7A), whereas their nuclei are uniformly PI positive (Fig. 7B), indicating that all these detached cells have undergone necrotic cell death. The nuclei of cells that have spontaneously detached after less severe ATP depletion (DOG in the absence of dextrose) show the classic apoptotic features of condensation and fragmentation (Fig. 7C), comparable to those seen in apoptotic cells still adherent to the monolayer (Fig. 6E). Although most of these cells have an intact plasma membrane and exclude PI, some of these cells have become PI positive (Fig. 7D). The loss of plasma membrane integrity in apoptotic cells is a late feature of apoptosis and is seen predominantly in

Fig. 7. Epifluorescence microscopy of MPT cells stained with H-33342 and PI after spontaneously detaching from monolayer following moderate and severe ATP depletion. A and B: nuclei of cells subjected to severe ATP depletion (antimycin + 1.0 mM dextrose) that have detached from the monolayer appear normal on H-33342 staining (A) but are revealed as necrotic by PI staining (B). C and D: in contrast, nuclei of spontaneously detached cells subjected to moderate ATP depletion (DOG alone) all show condensation and fragmentation, features characteristic of apoptosis. However, unlike adherent cells subjected to same degree of ATP depletion, some detached cells have lost plasma membrane integrity and are PI positive (arrowheads). Despite PI positivity, nuclei of these cells are condensed and fragmented, demonstrating that apoptosis was primary cause of cell death.
cells in culture. Cell dying from apoptosis in vivo are rapidly phagocytosed, generally before the plasma membrane breaks down (28). This process of plasma membrane degeneration in apoptotic cells has been called “secondary necrosis” or, more accurately, “post-apoptosis” (9). Nonetheless, Fig. 7, C and D, demonstrate clearly that, despite nuclear PI positivity, the nuclei of the PI-positive cells are condensed and fragmented, indicating that the primary cause of cell death in these cells was apoptosis and not necrosis (see DISCUSSION).

Thus, in these fluorescence studies, we again demonstrate that there is a threshold for reduction in cell ATP, between 15 and 25% of control, that determines the mechanism of cell death.

Electron microscopy. Cells that have undergone severe ATP depletion (antimycin + 1.0 mM dextrose for 6 h) demonstrate features of necrosis (Fig. 8, A and B), including loss of plasma membrane integrity, swelling of mitochondrial cristae, and degeneration of chromatin (karyolysis) in the absence of chromatin condensation. In contrast, cells that have been subjected to more modest ATP depletion (DOG without dextrose) demonstrate all the classic features of apoptosis (Fig. 9, A–C). In contrast to necrotic cells, the plasma membrane of apoptotic cells remains intact, the mitochondria remain relatively normal, and cytoplasmic volume is dramatically reduced. Finally, the morphological hallmark of apoptosis, i.e., condensation and fragmentation of nuclear chromatin, is clearly evident (Fig. 9). Because the nuclei of apoptotic cells condense while the cytoplasmic volume decreases, the overall size of apoptotic cells markedly decreases (Fig. 9).

Effect of Growth Factors on Cell Survival After ATP Depletion

We have previously shown that the renal growth factor EGF (10 nM), as well as activation of the insulin-like growth factor I (IGF-I) receptor [using high-dose insulin (5 µg/ml)], inhibits apoptosis induced by growth factor deprivation (W. Lieberthal, V. Triaca, S.S. Koh, and J.S. Levine, unpublished observations). We therefore examined the effect of these same growth factors on the survival of cells exposed to two levels of ATP depletion, both of which induce apoptosis. In the absence of growth factors, the survival of cells after treatment with DOG alone for 48 h was 15 ± 4% of control. Neither EGF nor high-dose insulin improved survival [21 ± 1 and 20 ± 1% of control, respectively; P = not significant (NS) (n = 5)]. For cells exposed to antimycin + 10 mM dextrose for 48 h, cell survival was 48 ± 1% of control in the absence of growth factors, 55 ± 6% in the presence of EGF, and 58 ± 7% in the presence of high-dose insulin (P = NS) (n = 5). Thus neither EGF nor high-dose insulin afforded protection against apoptosis induced by DOG or antimycin + 10 mM dextrose.

DISCUSSION

The role of necrotic cell death in the pathophysiology of acute ischemic injury to the brain, heart, and kidney has long been well recognized. The presence of necrosis is easily identified in histological sections because necrotic cells swell and ultimately rupture, spilling injurious cytosolic contents, thereby inducing an inflammatory response that is easily seen morphologically (22, 28). In marked contrast, the presence of apoptotic cells is often difficult to demonstrate in tissue sections, even when apoptosis is responsible for rapid and extensive cell loss (15, 22). The difficulty in identifying apoptotic cell death is attributable to two factors. First, apoptotic cells are very rapidly and efficiently cleared via phagocytic uptake by macrophages and neighboring parenchymal cells (15). Second, unlike necrotic cells, apoptotic cells maintain an intact plasma membrane until relatively late, permitting their clearance before release of intracellular material, so that extensive apoptosis can occur without any signs of a surrounding inflammatory response (15, 22, 28). This difficulty in identifying apoptosis in tissue sections accounts in large part for the fact that the pathophysiological importance of apoptosis in many pathological states has gone unrecognized for so many years (21).

Evidence that cells can die by apoptosis or necrosis in response to many injurious stimuli (21, 28, 33) has led to attempts to identify apoptotic cells in organs subjected to ischemic injury. Despite the difficulties in identifying apoptosis in vivo, there is evidence implicating apoptosis as a cause of cell death following acute ischemia-reperfusion injury to the brain (11, 17, 23). In these studies, cells within the central zone of ischemia have been shown to die rapidly by necrosis, whereas cells at the periphery of the ischemic zone die more slowly by a process morphologically resembling apoptosis (11, 17, 23). These observations are consistent with the concept that severe ATP depletion causes rapid metabolic collapse and necrosis, whereas more modest degrees of ATP depletion induce apoptosis.

Although a role for apoptosis in acute cerebral ischemia is gaining acceptance, the pathophysiological importance of apoptosis in acute ischemic injury to the kidney remains controversial. Although some investigators have reported the presence of apoptotic cells in kidneys subjected to ischemic injury (27, 30), others have failed to find any evidence of apoptosis (13, 27, 30). The extent to which apoptosis contributes to loss of cells in ischemic renal injury awaits the development of more sensitive methods to detect apoptosis in vivo.

The purpose of this study was to test the hypothesis that renal tubular cells in culture subjected to graded ATP depletion will die by necrosis or apoptosis, depending on the severity of ATP depletion, and to determine, if possible, whether a level of ATP depletion can be identified which clearly divides these two forms of cell death.

Using chemical anoxia, we were able to induce a wide range of reductions in cellular ATP ranging from ~2 to 70% of control. Inhibition of both glycolysis and mitochondrial respiration (with antimycin in the absence of dextrose) reduced cell ATP to <2% of control (Fig. 1). Addition of the antimycin of increasing concentrations of dextrose (0.25, 1.0, and 10 mM) increased cell ATP levels to ~4, 15, and 49% of control, respectively (Fig. 1).
DOG, which enters cells via the dextrose transport system, reduces cell ATP in two ways. It decreases ATP generation by acting as a competitive inhibitor of glycolysis, and it depletes existing ATP stores by undergoing extensive phosphorylation (4). Thus DOG can induce ATP depletion by a mechanism independent of the glycolytic pathway and therefore can reduce cell ATP stores in the presence or absence of dextrose.
Fig. 9. Electron microscopy of MPT cells subjected to moderate ATP depletion. MPT cells subjected to moderate ATP depletion (~25% of control) by incubation with DOG alone for 24 h demonstrate typical features of apoptosis. A: low-power magnification (×3,300) shows many small cells that contain multiple pieces of condensed chromatin. Condensed pieces of chromatin vary widely in size, because many of the nuclei have undergone fragmentation. B and C: under high power (×8,200), cells have an intact plasma membrane, normal mitochondrial cristae, and a markedly reduced cytoplasmic volume, all features typical of apoptosis. These low- and high-power views also show condensation of nuclear chromatin, a feature characteristic of apoptosis that does not occur in necrosis. Early in apoptosis, chromatin condenses against the nuclear membrane, producing the crescentic pattern (arrowheads). Later, chromatin condenses into solid, rounded masses that undergo fragmentation (A).
In incubation of cells with DOG alone, in the absence of dextrose, reduced cell ATP to ~25% of control, a level intermediate between those achieved by antimycin + 1.0 mM and antimycin + 10 mM dextrose (Fig. 1). The effect of cell ATP levels induced by DOG + 4 mM dextrose was comparable to that of antimycin + 10 mM dextrose. Incubation of cells with DOG + 10 mM dextrose resulted in reduction of cell ATP to ~75% of control, the least severe degree of cellular ATP depletion of all metabolic interventions used.

The rapidity of cell death correlated roughly with the degree of ATP depletion. Cells exposed to severe reductions in ATP to ~2, 4, and 15% of control (Fig. 1) died over a 2- to 6-h period (Fig. 2). In marked contrast, there was little or no loss in cell viability at 6 h for cells subjected to moderate reductions in ATP levels (~25, 50, and 70% of control (Fig. 3)). By 48 h, none of the cells subjected to a reduction in ATP to ~25% of control (DOG without dextrose) remained viable (Fig. 3). For cells subjected to ATP depletion to ~50% (antimycin + 10 mM, as well as DOG + 4 mM dextrose), viability was comparable (50.4 ± 5.5 and 47.6 ± 6%, respectively). A decrease in ATP levels to ~70% reduced viability to 74.7 ± 1.1% of control (Fig. 3).

We also found that the mechanism of cell death depended on a clear threshold of ATP depletion, which ranged between 15 and 25% of control levels. MPT cells with ATP levels <15% of control died uniformly by necrosis, whereas those with ATP levels of ≥25% of control died uniformly by apoptosis.

We determined the mechanism of cell death by many approaches, including DNA electrophoresis, phase-contrast microscopy, fluorescence microscopy, and electron microscopy. In all three conditions for which ATP was severely depleted (<15% of control), a smear pattern of DNA degradation, typical of necrosis, was found on DNA electrophoresis, without any laddering, to suggest apoptosis of even a proportion of the cells (Fig. 4, lanes 2–4). Phase-contrast micrographs of cells subjected to the same degree of ATP depletion demonstrated cell swelling and trypan blue positivity, both features long recognized as typical of necrosis. Staining of nuclei of these cells with H-33342 revealed that the nuclei of cells subjected to severe ATP depletion demonstrated normal chromatin morphology (Figs. 6C and 7A). However, in contrast to viable cells which excluded PI (Fig. 6B), most of the cells subjected to severe ATP depletion still adherent to the monolayer (Fig. 6D) and all cells that spontaneously detached from the monolayer (Fig. 7B) were PI positive. Finally, electron microscopy of cells subjected to severe ATP depletion for 6 h showed the classic features of necrosis (Fig. 8).

In contrast to the effects of severe ATP depletion, MPT cells subjected to more moderate reductions in cell ATP (25–70% of control) (Fig. 1) died by apoptosis. MPT cells dying by apoptosis demonstrated DNA fragmentation with “laddering” on DNA electrophoresis (Fig. 4, lanes 5–7). Furthermore, MPT cells dying by apoptosis were easily distinguished from those dying by necrosis by strict morphological criteria. Phase-contrast microscopy of cells dying by apoptosis showed a decrease in cell size, as opposed to the cell swelling associated with necrosis (Fig. 5C). Also, apoptotic cells became rounded and eventually detached individually from surrounding cells (Fig. 5C). This is in sharp contrast to necrotic cells, which were enlarged and detached from the monolayer in interconnected sheets (Fig. 5B). Fluorescent microscopy with H-33342 of MPT cells subjected to severe ATP depletion also demonstrated typical features of apoptosis. Some of the cells still adherent to these surfaces showed normal appearing nuclei (Fig. 6E). However, the majority of nuclei showed two morphological features specific for apoptosis, namely, condensation and fragmentation of nuclear chromatin. Condensed nuclei appear as brightly staining and featureless masses (Fig. 6E) that are quite distinct from the nuclei of normal (Fig. 6A) and necrotic cells (Fig. 6C). Nuclear condensation and fragmentation are the most specific morphological features of apoptosis and do not occur in necrotic cells. Furthermore, the plasma membrane of apoptotic cells remained intact until relatively late, so that the cells still adhere to the monolayer, whether viable or apoptotic, all excluded PI (Fig. 6F).

Although apoptotic cells still adherent to the monolayer uniformly excluded PI, some of the apoptotic cells that had detached into the supernatant were PI positive. This is indicative of a loss of plasma membrane integrity in cells that have escaped phagocytosis and have undergone secondary degenerative changes while in the supernatant culture medium. These changes have been called secondary necrosis but more recently have been more accurately referred to as post-apoptosis (9). Two important points need to be made about the changes that occur in postapoptotic cells. First, these changes, although a common phenomenon in vitro, occur rarely in vivo, where apoptotic cells are rapidly cleared by phagocytosis before loss of plasma membrane integrity can occur (28). Second, with cells in culture, the primary mechanism of cell death of PI-positive cells, whether necrosis or apoptosis, can be determined by examining the chromatin morphology with H-33342 staining. For PI-positive cells subjected to severe ATP depletion (Fig. 6, C and D, and Figs. 7, A and B), nuclear staining with H-33342 shows no condensation or fragmentation, indicating that necrosis was the primary cause of cell death. In contrast, for PI-positive cells subjected to moderate ATP depletion, the presence of nuclear condensation and fragmentation indicates that apoptosis was the primary cause of death, despite loss of plasma membrane integrity (Fig. 7, C and D).

Electron microscopy confirmed the specificity of nuclear staining using H-33342 and PI for determining the mechanism of cell death. All cells subjected to severe ATP depletion are necrotic under electron microscopy (Fig. 8), whereas all cells subjected to modest ATP depletion have undergone apoptosis (Fig. 9).

Two pieces of evidence suggest that the mechanism of cell death correlates with the severity of ATP depletion and is not related to the type of metabolic inhibitor. First, depending on the accompanying concentration of dextrose, antimycin is capable of inducing either necro-
sis or apoptosis. Second, the same degree of ATP depletion, whether induced by antimycin + 10 mM dextrose or DOG + 4 mM dextrose results in the same type of cell death (apoptosis).

Although the results of DNA electrophoresis were consistent with our morphological studies in demonstrating internucleosomal cleavage of DNA (“laddering”) in apoptotic cells and random fragmentation of DNA in necrotic cells, there is substantial evidence that the pattern on DNA electrophoresis is not completely specific in determining the mechanism of cell death. Ueda et al. (36), in a careful study of hypoxic proximal tubular fragments, demonstrated DNA laddering in cells from isolated proximal tubules that, when examined by light and electron microscopy, were necrotic and showed no evidence of apoptosis. Furthermore, there are now several reports demonstrating that apoptosis, defined by morphological criteria, can occur in the absence of DNA laddering (3, 21–24–26). Thus DNA laddering cannot be used alone, in the absence of morphology, to determine the cause of cell death.

Renal growth factors, such as EGF, IGF-I, and hepatocyte growth factor, have all been shown to accelerate the recovery of acute experimental renal failure (10, 14, 34). The extent to which this effect of growth factors is due to inhibition of apoptosis remains uncertain. We have demonstrated that EGF and high-dose insulin (which stimulates IGF-I receptors) inhibit apoptosis triggered by the withdrawal of growth factors (W. Lieberthal, V. Triaca, J. S. Koh, and J. S. Levine, unpublished observations). In contrast, in this study, neither EGF nor high-dose insulin ameliorated apoptosis caused by ATP depletion induced by either DOG alone or antimycin + 10 mM dextrose.

In summary, we have demonstrated that renal tubular cells undergo necrosis when their cellular ATP stores are severely depleted to a level incompatible with maintenance of basal metabolism and activity of membrane transport pumps (38). Because apoptosis is an active energy-requiring process, it would seem logical that apoptosis could occur in ATP-depleted cells only if sufficient amounts of energy are available to permit activation of the pathways that induce and mediate apoptotic cell death (28). More modest ATP depletion, if sustained, can also be lethal but leads instead to apoptosis. In these studies, we have found that the degree of reduction in the level of ATP that represents the threshold between necrotic and apoptotic cell death occurred between 15 and 25% of control levels. Our findings are consistent with the hypothesis that apoptosis and necrosis both contribute to cell death following ischemic renal injury and that the severity and duration of ATP depletion within each individual renal tubular cell likely determine the mechanism of death in lethally injured cells (21).

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