Characterization of cell clones isolated from hypoxia-selected renal proximal tubular cells

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We recently selected death-resistant cells via repeated episodes of hypoxia. In the present study, 80 clones were isolated from the selected cells and their response to apoptotic injury was characterized. Compared with the wild-type cells, the isolated clones showed a general resistance to apoptosis: 13 were extremely resistant to azide-induced apoptosis, 10 to staurosporine, and 9 to cisplatin. The cell clones that most consistently demonstrated resistance or sensitivity to injury were further studied for their response to azide treatment. Azide induced comparable ATP depletion in these clones and wild-type cells. Hypoxia inducible factor-1 (HIF-1) was upregulated in several clones, but the upregulation did not correlate with cell death resistance. The selected clones maintained an epithelial phenotype, showing typical epithelial morphology, forming “domes” at high density, and expressing E-cadherin. Azide-induced Bax translocation and cytochrome c release, two critical mitochondrial events of apoptosis, were abrogated in death-resistant clones. In addition, cell lysates isolated from these clones showed lower caspase activation on addition of exogenous cytochrome c. Bax, Bak, and Bid expression in these clones was similar to that in wild-type cells, whereas Bcl-2 expression was higher in all the selected clones and, interestingly, Bcl-XL was markedly upregulated in the most death-resistant clones. The results suggest that apoptotic resistance of the selected clones is not determined by a single factor or molecule but, rather, by various alterations at the core apoptotic pathway.

Compared with other organs, oxygen supply in the kidneys is somewhat unique. The parallel arrangement of arterial and venous vessels increases direct oxygen diffusion across the arterioles into the postcapillary venous system. As a consequence, renal cells, particularly those in the medulla, are constantly exposed to an environment of low-oxygen mild, yet chronic, hypoxia (11). On the other hand, renal cells, in general, are highly active in terms of oxygen consumption because of their energy-demanding activities of filtration and reabsorption. The unique feature of oxygen supply and demand makes the kidneys particularly susceptible to ischemic or hypoxic injury. Consistently, hypoxia has been implicated in the development of acute, as well as chronic, renal diseases (4, 7, 11, 23, 24, 29). Investigation of the response of kidney cells to hypoxia and related stresses may enhance our understanding of renal pathophysiology.

To examine the factors that determine the cellular sensitivity to hypoxic injury, we recently subjected renal proximal tubular cells to repeated episodes of hypoxia and selected a population of cells that are resistant to apoptosis (9). Interestingly, these cells upregulated Bcl-XL, a known antiapoptotic protein. Nevertheless, the selected cells are expected to be heterogeneous. Thus, for further analysis of the apoptotic resistance, clones were isolated from the cell population and their response to different types of injury were characterized.

MATERIALS AND METHODS

Materials. The immortalized rat kidney proximal tubular cell (RPTC) line was originally obtained from Dr. U. Hopfer (Case Western Reserve University, Cleveland, OH) and maintained for experimentation as previously described (9, 28). Antibodies were purchased from the following sources: monoclonal anti-cytochrome c from BD Biosciences (San Diego, CA), monoclonal anti-Bax and anti-Bcl-XL from NeoMarkers (Fremont, CA), monoclonal anti-Bcl-2 and polyclonal anti-E-cadherin from Santa Cruz Biotechnology (Santa Cruz, CA), polyclonal anti-Bak from Upstate Biotechnology (Lake Placid, NY), monoclonal anti-hypoxia inducible factor (HIF)-1α from Novus Biologicals (Littleton, CO), polyclonal anti-Bid from Dr. X. Yin (University of Pittsburgh, Pittsburgh, PA), and secondary antimouse and anti-rabbit horseradish peroxidase-conjugated antibodies from Pierce Biotechnology (Rockford, IL). Other reagents were purchased from Sigma (St. Louis, MO).

Cloning of hypoxia-selected cells. The hypoxia-selected cells were obtained through seven cycles of severe hypoxia injury followed by recovery (9). The cells were stored in liquid nitrogen before the present study. For isolation of individual clones, the cells were
revitalized from liquid nitrogen and grown in full culture medium for a few days. The cells were then digested into individual cells with trypsin and plated at a density of 500–1,000 cells per 100-mm cell culture dish. Growth of the cell colonies was monitored by light microscopy. When the individual colonies reached ~100–200 cells, their positions were marked and the colonies were collected into sterile cloning cylinders. To ensure the purity of the clones, the collected colonies were expanded and cloned once more for experimental testing.

Induction of apoptosis. Apoptosis was induced by azide, staurosporine (STS), or cisplatin. Azide blocks cellular respiration and, in the absence of glucose, induces ATP depletion of the cells (39). ATP depletion activates Bax and releases mitochondrial cytochrome c. When ATP-depleted cells are returned to glucose-containing medium, the cells develop typical apoptotic morphology (28, 36, 37). In the present study, the cells were incubated with 10 mM azide for 3 h in glucose-free Krebs-Ringer bicarbonate solution and then returned to full culture medium for 2 h for examination of apoptosis, as described in our recent work (5). STS, a broad-spectrum inhibitor of protein kinases, induces apoptosis in a variety of cells, including the RPTC line (9, 36). In the present study, the cells were incubated with 1 μM STS for 5 h to examine apoptosis. Cisplatin, a cancer therapy drug, induces renal cell injury, including apoptosis. In the present study, the cells were incubated with 10 mM cisplatin for 3 h in an atmosphere of 1% O2/99% N2 to examine apoptosis, as described in our recent work (16, 17).

Morphological examination of apoptosis. Typical apoptotic morphology, indicated by cell shrinkage and formation of apoptotic bodies or blebs, was examined by light microscopy. Nuclear condensation and fragmentation were examined by fluorescence microscopy after staining with 5 μg/ml Hoechst 33342. For semiquantification, the percentage of apoptosis was assessed by examination of four fields in each dish with ~200 cells per field.

Measurement of caspase activity. Caspase activity was measured by an enzymatic assay, as described previously (9, 36). Cells were extracted with 1% Triton X-100. The lysate of 25 μg protein was added to an enzymatic reaction containing 50 μM DEVD-7-amido-4-trifluoromethylcoumarin (DEVD-AFC), a fluorogenic substrate of caspase-3, caspase-6, and caspase-7 (carbobenzoxy-Asp-Glu-Val-Asp-AFC; Enzyme Systems Products, Dublin, CA). After 1 h of reaction at 37°C, fluorescence was measured at 360-nm excitation and 530-nm emission with a GENios plate reader. For each measurement, free AFC was used to construct a standard curve. The fluorescence reading of the samples was converted to nanomolar liberated AFC using the standard curve. In each experiment, the caspase activity of the parental RPTC line was arbitrarily set to 1, and the data from various cell clones were normalized accordingly.

Subcellular fractionation. For analysis of the distributions of Bax and cytochrome c during apoptosis, fractionation of cells into cytosolic and membrane-bound organelar fractions was carried out using low concentrations of digitonin, which selectively permeabilizes the plasma membrane. This fractionation method, which has been used previously, has been confirmed by other fractionation approaches and by immunofluorescence (10, 22, 27, 28). Briefly, cells were incubated with 0.05% digitonin in an isotonic sucrose buffer for collection of cell cytosol. The remaining digitonin-insoluble part was dissolved with 2% SDS for collection of the membrane-bound fraction containing mitochondria.

Immunoblot analysis. Electrophoresis and immunoblot analysis of proteins were performed in a NuPAGE gel system. After the samples were blocked with 2% BSA or 5% milk, the blots were incubated overnight with specific primary antibody at 4°C and then exposed to horseradish peroxidase-conjugated secondary antibody. The antigens were revealed by exposure to an enhanced chemiluminescence kit (Pierce Biotechnology).

Immunofluorescence. Cells grown to confluence on collagen-coated glass coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.1% SDS, and incubated with 5% normal goat serum for blocking. The cells were subsequently exposed to anti-E-cadherin (1:100 dilution) for 1 h, washed extensively, and exposed to Cy3-labeled goat anti-rabbit IgG. Fluorescence was examined by fluorescence microscopy.

In vitro reconstitution of caspase activation. Reconstitution of caspase activation by addition of exogenous cytochrome c was performed as described previously (8). Briefly, cell cytosol was collected and concentrated to 4–5 mg protein/ml with 10-kDa cutoff microcentrators. Concentrated cytosol of 25 μg protein was added to a reaction mixture containing 1 μl of 0.5 mg/ml cytochrome c, 1 μl of 10 mM dATP, and 0.5 μl of 200 mM MgCl2. The reaction mixture was incubated for 1 h at 30°C and then transferred to caspase assay buffer containing DEVD-AFC for determination of caspase activity.

Measurement of cell ATP. ATP was determined with an ATP bioluminescence assay kit (Sigma) (28). Briefly, cells were extracted with perchloric acid. After extraction, the acid was neutralized with K2CO3. The neutralized sample was mixed at a 1:1 ratio with the bioluminescence reagent, and luminescence was measured immediately on a luminometer (Turner Designs, Sunnyvale, CA). For each measurement, a standard curve was constructed using serial dilutions of ATP stock solution. The luminescence reading was converted to nanomolar amounts of ATP according to the standard curve.

Statistical analysis. Values are means ± SD (n ≥ 3). Statistical differences were determined by Dunnett’s multiple comparison post test following ANOVA using GraphPad Prism software; P < 0.05 was considered significantly different.

RESULTS

Isolation of cell clones from hypoxia-selected cells. Our recent work selected a population of death-resistant cells through repeated episodes of hypoxia (9). The selected cell population was expected to be heterogeneous, preventing further characterization. Therefore, in this study, we first isolated >200 clones from the hypoxia-selected cells and then tested 80 clones for their response to cell injury.

Cell injury was induced by the ATP-depleting chemical azide, the broad-spectrum protein kinase inhibitor STS, and the DNA-damaging agent cisplatin. All three treatments induced apoptosis. The cells showed a typical apoptotic morphology, with cell shrinkage, formation of apoptotic bodies, and nuclear condensation and fragmentation. Necrosis was minimal in these experiments, as shown by the absence of propidium iodide staining (not shown). Thus we initially analyzed apoptosis by cell morphology to indicate cell injury. According to the apoptosis rate, the cell clones were categorized into four types (Fig. 1): very resistant (<15% apoptosis), resistant (15–35% apoptosis), nonresistant (35–50% apoptosis), and sensitive (>50% apoptosis). Of the 80 cell clones tested, 13 were very resistant and 42 were resistant to azide-induced apoptosis. Seventeen clones showed 40–50% apoptosis after azide incubation, comparable to parental wild-type cells. Eight clones were more sensitive to azide injury, showing >50% apoptosis (Fig. 1A). Similar results are shown for cell injury induced by cisplatin (Fig. 1B) and STS (Fig. 1C). Analysis of injury response of individual clones showed clearly that the majority (68 clones) were resistant to at least one type of injury, with 21 clones resistant to all three (Fig. 1D). Interestingly, the sensitivity of a small number (total 12) of clones to cell injury was similar to or more sensitive than that of the parental wild-type cells.

Apoptosis and caspase activity induced by azide, STS, and cisplatin in selected cell clones. On the basis of the results of the initial screening, 15 clones were selected for further anal-
ysis. Of these clones, 6 were very resistant, 4 were moderately resistant, and 5 were sensitive to cell injury (Fig. 1). Their responses to azide-, STS-, and cisplatin-induced cell injury were first confirmed by analysis of apoptosis using morphological criteria. As shown in Fig. 2A, clones 7, 10, 19, 24, 41, and 79 were very resistant to apoptosis, whereas clones 21, 65, 66, and 70 were moderately resistant. On the contrary, clones 8, 32, 40, 67, and 78 were sensitive. To confirm the morphological results, we measured caspase activity (Fig. 2B). Overall, caspase activity showed a positive correlation with the morphological examination, with lower activity in the resistant clones and higher activity in the sensitive clones (Fig. 2). Fig. 3 shows cell morphology of wild-type cells and clone 24, which was very resistant to apoptotic injury. Clearly, azide and azide-, cisplatin-, and staurosporine (STS)-induced apoptosis. A: isolated cell clones were incubated for 3 h with 10 mM azide in glucose-free buffer and allowed to recover for 2 h in full culture medium. B: isolated cell clones were incubated with 1 μM STS in full culture medium for 5 h. C: isolated cell clones were incubated with 20 μM cisplatin for 16 h in full culture medium. D: summary of apoptosis-resistant cell clones.
cisplatin induced typical apoptotic morphology in wild-type cells, showing cell shrinkage, formation of apoptotic blebs, and nuclear condensation and fragmentation. The apoptosis rate was noticeably lower in clone 24. We selected six cell clones for further characterization using the injury model of azide treatment. These clones most consistently demonstrated resistance or sensitivity to cell injury and represented the three main categories: very resistant (clones 24 and 79), moderately resistant (clones 65 and 66), and sensitive (clones 8 and 32).

**Azide-induced ATP depletion in the selected cell clones.** Azide is an inhibitor of mitochondrial respiration at complex IV. In the absence of the glycolytic substrate glucose, azide induces ATP depletion, which is a key to the induction of apoptosis. Accordingly, the observed cellular sensitivity or resistance to azide injury (Figs. 1–3) might be caused by the differences in ATP depletion in these cell clones. To test the possibility, we determined cell ATP during azide incubation. Wild-type cells and the cell clones were incubated in 10 mM azide in a glucose-free buffer. Before azide treatment, the control levels of cell ATP varied to some extent among the cell clones (Fig. 4). On azide incubation, ATP declined drastically in wild-type cells as well as in all selected cell clones. The degrees of ATP depletion were comparable in these cells, reaching a maximum at the end of 2 h. Importantly, no correlations were shown between the sensitivity of the cells to injury and ATP depletion in the cell clones. For example, clones 24 and 79 were most resistant to azide-induced apoptosis; however, ATP depletion in these two clones was complete after 1 h of azide incubation. Clone 8 was sensitive to azide injury, but ATP depletion was slower for clone 8 than for clones 24 and 79 (Fig. 4). The results suggest that the sensitivity or resistance to injury of the selected cell clones is not due to variations in ATP depletion during azide treatment.

**HIF-1α expression in selected cell clones.** The cell clones used in this study were isolated from a population of renal tubular cells that were selected by repeated episodes of hypoxia (9). In response to hypoxia, mammalian cells may activate robust adaptive mechanisms, of which HIFs play an important regulatory role (21, 30). HIFs include HIF-1 and HIF-2. In renal tubular cells, HIF-1, but not HIF-2, is induced by hypoxia and relevant conditions (26). Consistently, HIF-2 was not detected in the renal tubular cells that were used in the present study (not shown). Thus our study was focused on HIF-1.

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**Fig. 3. Morphology of wild-type (WT) and clone 24 cells after incubation with azide and cisplatin.** Wild-type and clone 24 cells were incubated with 10 mM azide for 3 h and allowed to recover for 2 h or incubated with 20 μM cisplatin for 24 h. Control groups were not treated with azide or cisplatin. After incubation, cells were stained with Hoechst 33342. Cell morphology was recorded by phase contrast microscopy, and nuclear morphology, shown as Hoechst staining, was recorded by fluorescence microscopy. Azide and cisplatin induced development of typical apoptosis. Clone 24 was highly resistant to apoptotic injury induced by azide and cisplatin.

**Fig. 4. Azide-induced ATP depletion in selected cell clones.** Cells of wild-type and selected clones were incubated with 10 mM azide in glucose-free buffer for 0, 1, 2, or 3 h and then extracted with perchloric acid for ATP measurement. Protein was determined in parallel dishes. Results are from 3 separate experiments with duplicate dishes for each clone in every experiment; error bars (SD) are omitted for clarity. Azide induced drastic ATP depletion in wild-type cells as well as the selected clones.
Specifically, we analyzed the expression of HIF-1α, the subunit that regulates HIF-1 activity. Figure 5 shows low, yet detectable, HIF-1α expression in wild-type cells (lane 1). Cobalt, a known pharmacological activator of HIF-1, induced HIF-1α in these cells (lane 2). In the selected cell clones, high levels of HIF-1α were shown in clones 8, 66, and 79. Clone 65 and wild-type cells expressed similar amounts of HIF-1α, whereas clones 24 and 32 expressed lower HIF-1α. Apparently, there were no correlations between the levels of HIF-1α expression and resistance or sensitivity to cell injury. For instance, clones 8 and 79 showed high expression of HIF-1α, yet clone 8 was sensitive and clone 79 was resistant to injury. For clones 24 and 32, HIF-1α expression was low, but their sensitivity to injury was strikingly different. The results suggest that HIF-1 may not be the key to the cellular response to injury in these clones.

Epithelial phenotype of selected cells. Hypoxic incubation may induce transdifferentiation and phenotypic changes of epithelial cells. Manotham et al. (20) showed that cultured renal tubular cells underwent epithelial-mesenchymal transition (EMT) during chronic hypoxia. To determine whether phenotypic changes occurred in our selected clones, we first examined the expression of E-cadherin, a hallmark of epithelium. E-cadherin was expressed in wild-type clones (Fig. 6A), regardless of their apoptotic sensitivity. As a negative control, E-cadherin was not detected in mouse embryonic fibroblasts. Immunofluorescence showed localization...
of E-cadherin mainly in the expected intercellular junction area (Fig. 6B). The selected cells also maintained a cuboidal shape, as shown by phase contrast microscopy (Fig. 6C). Noticeably, when grown at high confluence, these cells formed a “dome”-like structure, indicating fluid accumulation at the basolateral side and the presence of a functional epithelial tight junction (Fig. 6). Together, these results suggest that the selected cell clones did not have EMT and have maintained an epithelial phenotype.

Azide-induced cytochrome c release and Bax translocation in selected cell clones. To further characterize the selected cell clones, we examined the apoptotic pathway. Previous work suggested that apoptosis following ATP depletion involves critical apoptotic events at the mitochondrial level (see Ref. 29 for review). Noticeably, Bax, a proapoptotic Bcl-2 family protein, translocates from cytosol into mitochondria, leading to permeabilization of the outer membrane and release of cytochrome c. To examine azide-induced cytochrome c and Bax redistributions in the selected clones, we fractionated the cells into cytosolic and membrane-bound organellar fractions for immunoblot analysis (Fig. 7). As expected, cytochrome c was detected in the membrane fraction containing mitochondria in control cells, which were not incubated with azide (Fig. 7A, lane 1). After 3 h of azide treatment, significant amounts of cytochrome c were released into the cytosolic fraction in the wild-type cells (Fig. 7A, lane 4). Similar release was shown in clones 8 and 32 (Fig. 7A), which were very sensitive to azide injury (Fig. 2). In sharp contrast, clones 24 and 79 showed less cytochrome c release, consistent with their resistance to azide-induced apoptosis (Fig. 2). Interestingly, clone 65 released cytochrome c (Fig. 7A, lane 4), although it was resistant to azide-induced apoptosis (Fig. 2). On the other hand, clone 66 showed limited cytochrome c release (Fig. 7A, lane 4) but was somewhat sensitive to azide-induced apoptosis (Fig. 2).

The release of cytochrome c was generally corroborated by Bax accumulation in mitochondria in the selected clones. Bax was detected mainly in the cytosolic fraction under control condition in wild-type cells as well as the selected cell clones (Fig. 7B, lane 6). During azide incubation, Bax translocated into the membrane fraction in wild-type cells (Fig. 7B, lane 7). Similar Bax translocation was shown in clones 8 and 65, but not in clones 24, 79, and 66 (Fig. 7B). Interestingly, clone 32 did not show Bax translocation (Fig. 7B, lane 7), although cytochrome c was released in this clone (Fig. 7A), suggesting that other factors, including other Bcl-2 family proteins, may induce mitochondrial injury in this cell clone. The immunoblot results were substantiated by semiquantification via densitometry of blots from separate experiments (Fig. 7, C and D). Together, the results further support a critical role for the mitochondrial pathway in tubular cell apoptosis under conditions of ATP depletion. Importantly, it is suggested that the cellular sensitivity or resistance to azide injury in the selected clones might be related to changes at the mitochondrial level.

Reconstitution of caspase activation by addition of exogenous cytochrome c to cytosolic extracts from selected cell clones. As shown above, there was a general correlation between azide-induced cytochrome c/Bax redistribution and apoptosis in the selected cell clones. However, there were exceptions. Notably, clone 65 showed a massive cytochrome c release during azide treatment (Fig. 7A) but did not develop apoptosis (Fig. 2). On the other hand, clone 66 released limited amounts of cytochrome c (Fig. 7A) yet was sensitive to azide-induced apoptosis (Fig. 2). These results are interesting, because they suggest differences in the apoptotic cascade down-
stream of cytochrome c release between these clones. To test this possibility, we conducted an in vitro reconstitution experiment. In this experiment, cytotoxic extracts were collected from wild-type cells and the selected clones. Equal amounts of recombinant cytochrome c were then added to the cytotoxic extracts. Caspase activity stimulated by the exogenous cytochrome c was monitored to indicate the cytotoxic capacity for caspase activation (Fig. 8). Clearly, cytotoxic preparations of clones 24, 79, and 65 were resistant to cytochrome c stimulation, whereas clones 8, 32, and 66 were sensitive to caspase activation by cytochrome c. The results suggest that, in addition to mitochondria, cytotoxic factors also play a role in the determination of cellular sensitivity or resistance to apoptotic injury. It is possible that clone 65 releases cytochrome c into the cytosol during azide treatment (Fig. 7A), but its cytotoxic activity is not the result of less apoptosis occurs.

Expression of Bcl-2 family proteins in the selected cell clones. To gain further insights into injury sensitivity or resistance of the selected cell clones, we analyzed the expression of several Bcl-2 family proteins. Bcl-2 family proteins are important regulators of apoptosis, particularly the critical events governing the integrity of mitochondria (1, 6). The expression of Bax and Bak, two proapoptotic multi-Bcl-2 homology (BH) domain proteins, was comparable in wild-type and selected clones (Fig. 9). The cells also expressed similar levels of Bid, a BH3-only proapoptotic protein. On the contrary, Bel-2 was upregulated in all the selected clones compared with the wild-type cells. Bel-2 expression was highest in clones 24, 65, and 79, which were resistant to azide-induced apoptosis (Fig. 2). Bel-2 expression was relatively low in clones 8 and 66, which were sensitive to apoptosis (Fig. 2). Interestingly, clone 32 expressed high levels of Bel-2, although it was sensitive to apoptosis (Fig. 2). Bcl-xL, another well-recognized antiapoptotic protein, was significantly upregulated in clones 24 and 79, but not in clones 8 and 32 (Fig. 9C), suggesting a correlation of Bcl-xL expression with resistance or sensitivity to apoptosis in these cell clones. Bcl-xL was moderately upregulated in clones 65 and 66 (Fig. 9C). However, although clone 65 was resistant to azide-induced apoptosis, clone 66 was sensitive (Fig. 2), suggesting the involvement of other factors in determination of apoptotic sensitivity of these two clones.

DISCUSSION

In this study, we have isolated cell clones from hypoxia-selected renal tubular cells and characterized their responses to apoptotic injury induced by azide, STS, and cisplatin. The results support three conclusions. 1) There is a general apoptotic resistance in the selected cell clones. 2) The selected clones have maintained their epithelial phenotype and did not undergo EMT after repeated episodes of hypoxia. 3) Apoptotic resistance of the selected clones is not determined by a single factor or molecule but, rather, by molecular alterations at the core apoptotic pathway.

The majority of the clones show resistance to at least one type of injury, and some clones are resistant to all three. Azide, in the absence of glucose, induces cell injury via ATP depletion. STS induces apoptosis presumably by inhibiting protein kinases. On the other hand, cisplatin is a known DNA-damaging agent. Thus the initiating signals triggered by these treatments are vastly different. Nevertheless, the different signals may eventually activate relevant or overlapping apoptotic pathways (14). Mitochondria appear to be critically involved in tubular cell apoptosis induced by azide, STS, and cisplatin (9, 16, 25, 36). Thus the cell clones that show resistance to a specific treatment in our experiments may be protected at the initiating signaling level, whereas those resistant to all three types of injury may have acquired resistance at the core apoptotic machinery. This scenario is supported by the observation that the most resistant clones (e.g., clones 24 and 79) consistently demonstrate lower Bax accumulation in mitochondria and less cytochrome c release from the organelles. In addition, the cytotoxic isolated from these clones is resistant to cytochrome c-stimulated caspase activation. Collectively, the results suggest that the selected clones have alterations at the core cell death machinery, which make them “tough” to endure subsequent or other types of insults.

The selected clones may acquire apoptotic resistance as a result of adaptation to hypoxic stress, or they may have inherent resistance. Cellular adaptation is a common phenomenon in response to stress, which may account for various cytoprotective effects of “preconditioning.” However, our results suggest that the apoptotic resistance of the selected clones is less likely to be a result of temporary adaptation or preconditioning. These clones maintained their resistance throughout the ~6 mo of apoptosis testing. During the testing, all cell clones were grown under normal oxygen, and no clones reversed their apoptotic responses. The time line suggests that the apoptotic resistance is an inherent property of the selected clones that perpetuates. Conceivably, the parental cells before hypoxic selection represented a heterogeneous population containing apoptosis-sensitive and -resistant cells. Repeated episodes of hypoxia killed most of the sensitive cells, leading to the enrichment of cell clones with inherent apoptotic resistance.

Nevertheless, some clones are still sensitive to cell injury after selection. Of the 80 clones we have characterized, 12 are

Fig. 8. Caspase activation after cytochrome c stimulation in cell cytosol isolated from selected clones. Cytotoxic extracts were collected from the wild-type and selected cell clones, and equal amounts of cytochrome c were added to cytotoxic extracts. Caspase activity after cytochrome c stimulation was measured by enzymatic assays containing DEVD-7-amido-4-trifluoromethylcoumarin. Activity of wild-type cell cytosol was arbitrarily set as 1 in each experiment, and activity of selected clones was normalized accordingly. Values are means ± SD (n = 4). *Significantly different from WT.
as sensitive as or even more sensitive than the parental wild-type cells. In general, these clones are sensitive to all three types of injury. Selective analysis of clones 8 and 32 has shown that these clones are particularly sensitive to mitochondrial membrane permeabilization and cytochrome c release. Moreover, their cytosols are quite competent for caspase activation on exogenous cytochrome c stimulation. These observations indicate that the cells of these clones contain a highly sensitive apoptotic machinery. It is surprising that, after seven cycles of vigorous hypoxic selection, we still found a low percentage of injury-sensitive cell clones. One possibility is that these clones are resistant to hypoxic injury but are sensitive to azide-, STS-, and cisplatin-induced injury. This possibility is not supported by the fact that the same signaling pathway is activated by hypoxia and azide to induce apoptosis. Hypoxia and azide block cellular respiration and, in the absence of glucose, induce ATP depletion, which is the key to Bax activation, cytochrome c release, and the induction of apoptosis under the pathological conditions (29). Alternatively, these cell clones were originally death resistant but have lost their resistance during cloning and subsequent testing. Loss of apoptotic resistance during the cloning period is possible, because, after the cells were revascularized from liquid nitrogen, they were grown under normal oxygen tension for ~3 mo for cloning. The loss of resistance during the cloning period would suggest that some cells had a temporary adaptive response during hypoxic cycles. After cloning, the selected cells, however, maintained their apoptotic responses throughout the ~6-mo testing period, suggesting that their sensitivity or resistance is stable and inherent.

Using the azide injury model, we have selectively analyzed six clones to gain insights into their sensitivity or resistance to apoptosis. Azide treatment induces ATP depletion in all the cell clones (Fig. 4). ATP depletion in the selected clones was similar to or even more severe than that in the wild-type cells. However, they differ in subsequent apoptosis (Fig. 2). On the basis of these results, it is concluded that the different responses of these clones to azide-induced apoptosis are not due to their ability of energy maintenance; rather, downstream factors may be more important. We have also analyzed HIF in these cell clones (Fig. 5). HIFs include HIF-1 and HIF-2. Although HIF-1 is ubiquitously expressed, HIF-2 is expressed in specific cells such as endothelium (21, 30). In renal tubular cells, HIF-1, but not HIF-2, is induced by hypoxia and relevant conditions (26). Consistently, HIF-2 was not detected in the RPTC line that was used in the present study (not shown). To examine HIF-1, we specifically focused on HIF-1α, the regulatory subunit. Our results show a variation of HIF-1α expression in these clones (Fig. 5). Nevertheless, HIF-1α expression does not correlate with death resistance of the clones. For example, HIF-1α is highly expressed in clones 8 and 79; yet, although clone 79 is injury resistant, clone 8 is not. Among low HIF-1α clones, clone 24 is resistant to injury. Thus it is suggested HIF may not be the key determinant of sensitivity to cell injury in the selected clones. Consistently, recent work indicates that HIF regulation of cell injury may relate to its ability to modulate glucose utilization (3). In the presence of high glucose, HIF deficiency does not sensitize the cells to injury, whereas in the presence of low glucose, HIF may improve glucose uptake and utilization to help the cells survive. In our experiments, azide injury was induced in the absence of glucose; thus the regulation of cellular sensitivity by HIF is expected to be minimal. Collectively, it is suggested that HIF may not be directly involved in the determination of cellular sensitivity to injury.

Our results show that the selected cell clones maintained an epithelial phenotype and did not undergo EMT. This conclu-
sion is supported by morphological examination and by E-cadherin expression (Fig. 6). Manotham et al. (20) showed that cultured renal tubular cells transdifferentiate into a fibroblastic- like phenotype via EMT during chronic hypoxia. However, the hypoxic condition in the study of Manotham et al. was quite different from that in the present study: we selected cells subjected to seven cycles of hypoxia (6 h per cycle), whereas Manotham et al. subjected cells to continuous hypoxia for 3–15 days, and significant EMT were shown after 6 days of chronic hypoxia. The results indicate that EMT can be induced in renal tubular cells by chronic hypoxia, but not by acute hypoxia, even with repeated episodes. Thus it is suggested that the apoptotic resistance of selected cell clones in our study is not due to phenotypic changes of the cells.

Our results demonstrate a correlation between Bcl-2 family protein expression and cellular response to injury in the selected cell clones. Bcl-2 family proteins, characterized by the presence of Bcl-2 homology domain(s), play an important role in the regulation of apoptosis and, perhaps, cell death in general (1, 6). Although these proteins may regulate multiple intracellular targets, mitochondria seem to be critical. Functionally, Bcl-2 family proteins can be pro- or antiapoptotic. The interaction and balance between the pro- and antiapoptotic proteins may determine viability of the cells. In this study, the expression of proapoptotic Bcl-2 proteins, including Bax, Bak, and Bid, was comparable to that of wild-type cells and appeared to be constant among the selected clones. On the contrary, there were noticeable differences in the expression of Bcl-2 and Bcl-xL, two well-recognized antiapoptotic proteins (Fig. 9). Interestingly, all the tested clones showed higher Bcl-2 expression than the wild-type cells. This was surprising, because in our earlier work, Bcl-2 expression was not particularly high in hypoxia-selected cells (9). The cause of the discrepancy between these two studies regarding Bcl-2 expression remains unclear. Bcl-2 expression was higher in clones 24, 32, 65, and 79, than in clones 8 and 66 (Fig. 9). However, although clones 24 and 79 were resistant to azide-induced cytochrome c release, clones 32 and 65 were not (Fig. 7). With lower Bcl-2, clone 66 was resistant to cytochrome c release (Fig. 6). Thus the levels of Bcl-2 expression in these clones did not correlate well with their responses to azide-induced mitochondrial injury. These results do not imply that Bcl-2 is unimportant in the determination of mitochondrial integrity in the selected clones; rather, they suggest that Bcl-2 is not the single determining factor. In support of this idea, we show that Bcl-xL may have a role. Bcl-xL expression was highest in clones 24 and 79, two of the most injury-resistant clones. Conversely, Bcl-xL expression was low in clones 8 and 32, two of the most injury-sensitive clones (Fig. 9). The results suggest a good correlation of Bcl-xL expression with apoptotic resistance in these four clones. The level of Bcl-xL expression was moderate in clones 65 and 66. Although clone 65 was resistant to azide-induced apoptosis, clone 66 was sensitive (Fig. 2). The results suggest that Bcl-xL is not the key determinant of apoptotic sensitivity in these two cell clones. The resistance of clone 65 to azide-induced apoptosis may be related to high Bcl-2 expression (Fig. 9). In addition, cytosol from this clone is less susceptible to cytochrome c stimulation for caspase activation (Fig. 8). Together, the results suggest that although Bcl-2 family proteins are involved in the determination of apoptotic sensitivity, no single molecule or factor plays a determining role in all the selected clones. Hypoxic stress likely selects cells with resistance at various levels of the core apoptotic pathway.

The results in the present study may have implications in pathological conditions involving hypoxic selection. For example, because of the malformation and malfunction of vasculature, hypoxic regions persist in solid tumors. This provides a selection pressure, eliminating the death-sensitive cells and accumulating death-resistant cells. The selected cancerous cells become resistant not only to hypoxic injury but also to radio- and chemotherapies (15, 31, 35). Notably, Bcl-xL expression has been demonstrated in malignant solid tumors, including colorectal adenocarcinomas, Kaposi’s sarcoma, and multiple myeloma (12, 18, 33). In the kidneys, hypoxic injury during renal ischemia-reperfusion leads to tubular cell death. Under this condition, some cells die, but others survive. Depending on the severity of the insult, the surviving cells may dedifferentiate and proliferate to reestablish the tubular structure (4). Interestingly, regulation of Bcl-2 and Bcl-xL has been demonstrated during renal ischemia-reperfusion: these molecules may participate in tissue repair and remodeling following the injurious insult (2, 13). Together with these in vivo observations, our data suggest that molecular alterations in the apoptotic machinery may have a pathophysiological role in the determination of life or death of the cells.

GRANTS

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