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Induction of Apoptosis as well as Necrosis by Hypoxia and Predominant Prevention of Apoptosis by Bcl-2 and Bcl-X_L[1]

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ABSTRACT

The molecular mechanism of cell death due to hypoxia has not been elucidated. Our recent observations that overexpression of the anti-apoptotic proto-oncogene *bcl-2* and a *bcl-2*-related gene, *bcl-x*, prevents hypoxic cell death suggest that hypoxia induces apoptosis. Using electron microscopy and confocal and nonconfocal fluorescence microscopy, we show here that hypoxia does, in fact, induce both necrosis and apoptosis, and that the proportion of these two modes is highly dependent on the cell type. Overexpression of Bcl-2 or Bcl-X_L blocks hypoxia-induced apoptosis in a dose-dependent manner.

INTRODUCTION

Apoptosis and necrosis are two fundamental types of cell death with different morphological features (1, 2). Apoptosis is characterized by the generation of fragmented nuclei with highly condensed chromatin, protrusion of cytoplasm, and formation of apoptotic bodies, and necrosis is defined by electron-lucent cytoplasm and mitochondrial swelling with apparent intact nuclei. Apoptosis accounts for the majority of cell death occurrences during development and cell turnover and is induced by various treatments, such as growth factor withdrawal (3, 4). Apoptotic cell death generally occurs in a tightly regulated manner (3), whereas necrosis has been considered a passive degenerative phenomenon induced by direct toxic and physical injuries (5, 6).

Cell death due to hypoxia is a major concern in various clinical entities such as ischemic disease and organ transplantation. However, the mechanisms of hypoxic cell death have not been fully elucidated. Cell death by hypoxia has been generally believed to be represented as necrosis (7, 8), based on various ultrastructural findings in hypoxic cells. In contrast, recent biochemical observations have suggested the possibility of hypoxia-induced apoptosis (9, 10), a hypothesis supported by our recent findings that hypoxic cell death is prevented by the anti-apoptotic proteins, Bcl-2 (11–15) and Bcl-X_L (16), by a reactive oxygen species-independent pathway (17).

Here we studied the morphological features of cell death induced by hypoxia. Using electron microscopy and confocal and nonconfocal fluorescence microscopy, we have found that hypoxia induces apoptosis as well as necrosis. The hypoxia-induced apoptosis was predominantly prevented by overexpression of Bcl-2 or Bcl-X_L in a dose-dependent manner.

MATERIALS AND METHODS

Cell Culture. PC12 cells, a rat pheochromocytoma cell line (18), and 7316A cells, a rat hepatoma cell line (19), were maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM HEPES/Na^+ (pH 7.4), 0.05 mM 2-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. GB11 cells, a mouse early pre-B cell line (20), were maintained in RPMI 1640 containing IL-7, as described previously (20).

Hypoxia was induced with an anerobic device. Briefly, cells plated at low density (5×10^4/cm^2) were transferred to a chamber, and hypoxic conditions were achieved using BBL GasPac Plus (Becton Dickinson, Cockeysville, MD), which catalytically reduces O_2 to undetectable levels (less than 10 ppm) within 90 min, as assessed by oxygen electrode (POG-203-S1; Unique Medical, Osaka, Japan). After the indicated period, cells were harvested and treated as described below using degassed buffer and reagents.

Transfectants. Human *bcl-2* cDNA and chicken *bcl-x_L* cDNA (17) were each inserted in vectors pBC140 (20) and pUC-CAGGS (21) and transfected into PC12 cells. Control clones were prepared similarly using the vector without the inserts. Western blotting was performed using a mouse antihuman Bcl-2 monoclonal antibody (22) or rabbit anti-chicken Bcl-x polyclonal antibody raised against a GST-Bcl-X_L fusion protein and then affinity purified (17).

Electron Microscopy. Cells were harvested and fixed with 2.5% sodium phosphate-buffered glutaraldehyde (pH 7.4) and postfixed for 1 h with 1% veronal acetate-buffered OsO_4. Ultrathin sections were stained in ethanolic uranyl acetate and lead citrate and examined by electron microscopy (H-7000; Hitachi, Tokyo, Japan).

Fluorescence Microscopy. Cells were stained for 30 min at 37°C with calcein-AM (10 μM) and propidium iodide (10 μM) and analyzed under a confocal fluorescence microscope (LSM-GB200; Olympus, Tokyo, Japan) with laser beam excitation at 488 nm or stained with Hoechst 33342 (10 μM) and propidium iodide (10 μM) and analyzed under nonconfocal fluorescence microscope (BHS-RFL-LSM; Olympus, Tokyo, Japan) with excitation at 360 nm. Quantitative analysis was performed by counting more than 1000 cells in each examination. Results are expressed as the means ± SD of values obtained from four independent experiments. Statistical evaluation was performed by the paired *t* test or ANOVA. Sheffe's test was used for individual comparisons of groups when a significant change was observed by ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

To assess the type of cell death induced by hypoxia, we used electron microscopy to analyze the morphological features of PC12 (rat pheochromocytoma cells) incubated under hypoxic conditions (undetectable O_2 levels; see "Materials and Methods"). Ultrastructural analysis revealed two modes of cell death with different morphological features. One was characterized as apoptosis by the generation of fragmented nuclei with highly condensed chromatin, protrusion of cytoplasm, and formation of apoptotic bodies (Fig. 1*a*). The other was distinguished by intact chromatin with large cytosolic vacuoles, mitochondrial swelling, and electron-lucent cytoplasm (Fig. 1*b*), characteristic of necrosis (6, 7, 23). These results indicate that hypoxic treatment of PC12 cells induces both necrosis and apoptosis.

To determine the relative frequency of dead cells in each mode, we used fluorescence microscopy in conjunction with cell staining to assess nuclear shapes, cytosolic vacuoles, and membrane integrity of hypoxic PC12 cells. Confocal fluorescence microscopy, using calcein-AM (green), which stains whole cells except vacu-

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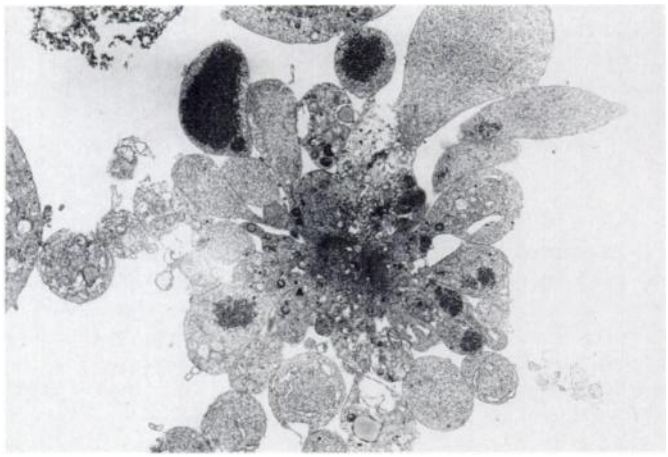

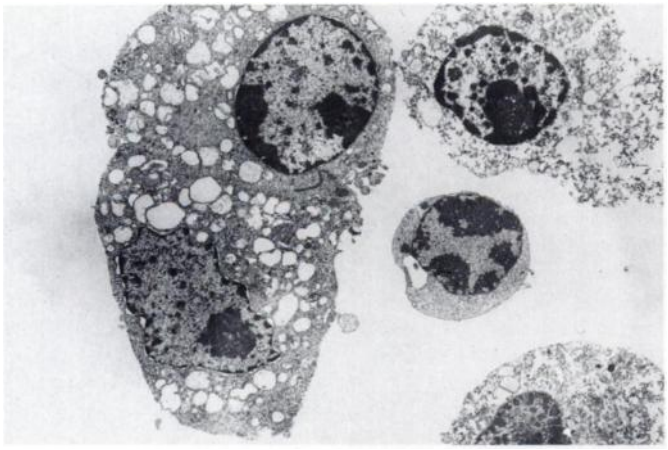

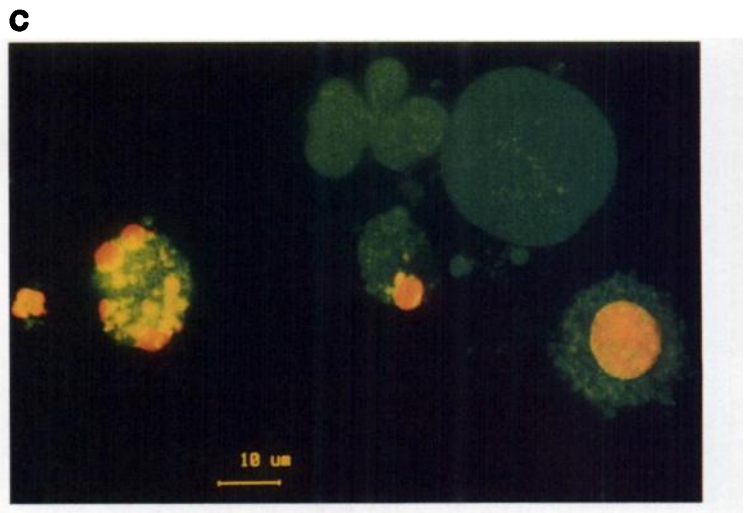

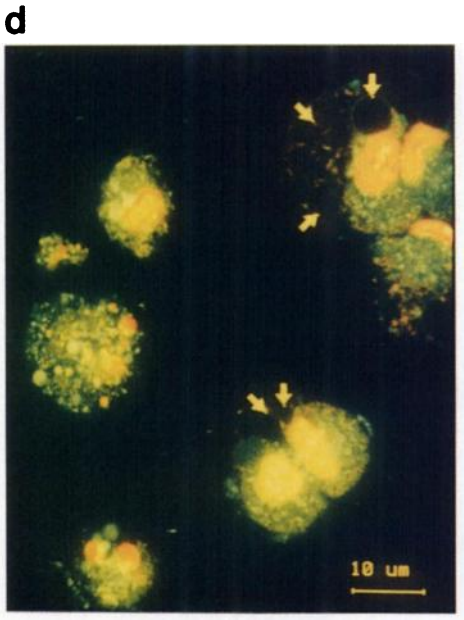

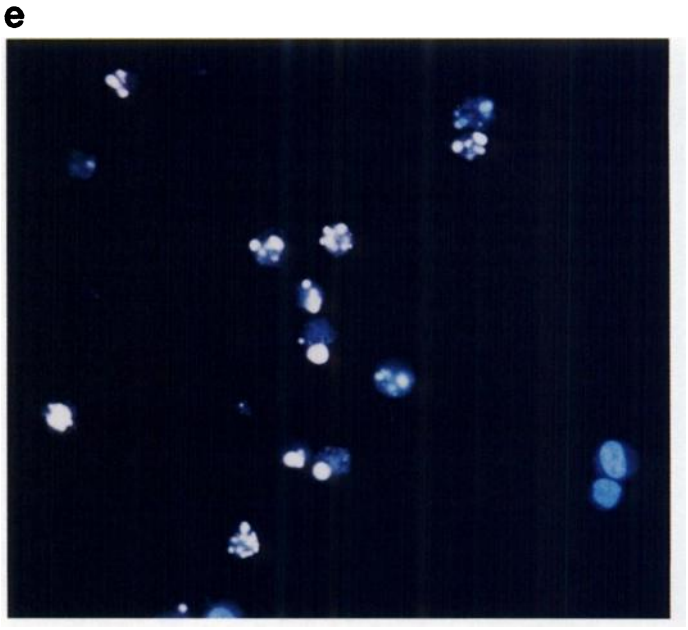

Fig. 1. Representative morphology of hypoxic PC12 cells. Ultrastructural analysis of PC12 after 24 h hypoxia showed two different morphological features: *a*, apoptosis (×5500); and *b*, necrosis (×5200). *c* and *d*, confocal fluorescence microscopic analysis of PC12 cells treated with 48 h hypoxia after being stained with calcein-AM (green) and PI (red). Necrotic cells have red round nuclei and large vacuoles (*arrows*), and "terminal" apoptotic cells have red fragmented nuclei. Round cells and fragmented cells without PI staining are viable and "early" apoptotic cells, respectively. *e*, nonconfocal fluorescence microscopic analysis of PC12 cells treated with 48 h hypoxia after being stained with PI (pink) and Hoechst 33342 (blue). Shown are cells with blue round nuclei, red round nuclei, blue fragmented nuclei, and red fragmented nuclei, which correspond to viable, necrotic, "early" apoptotic, and "terminal" apoptotic cells, respectively.

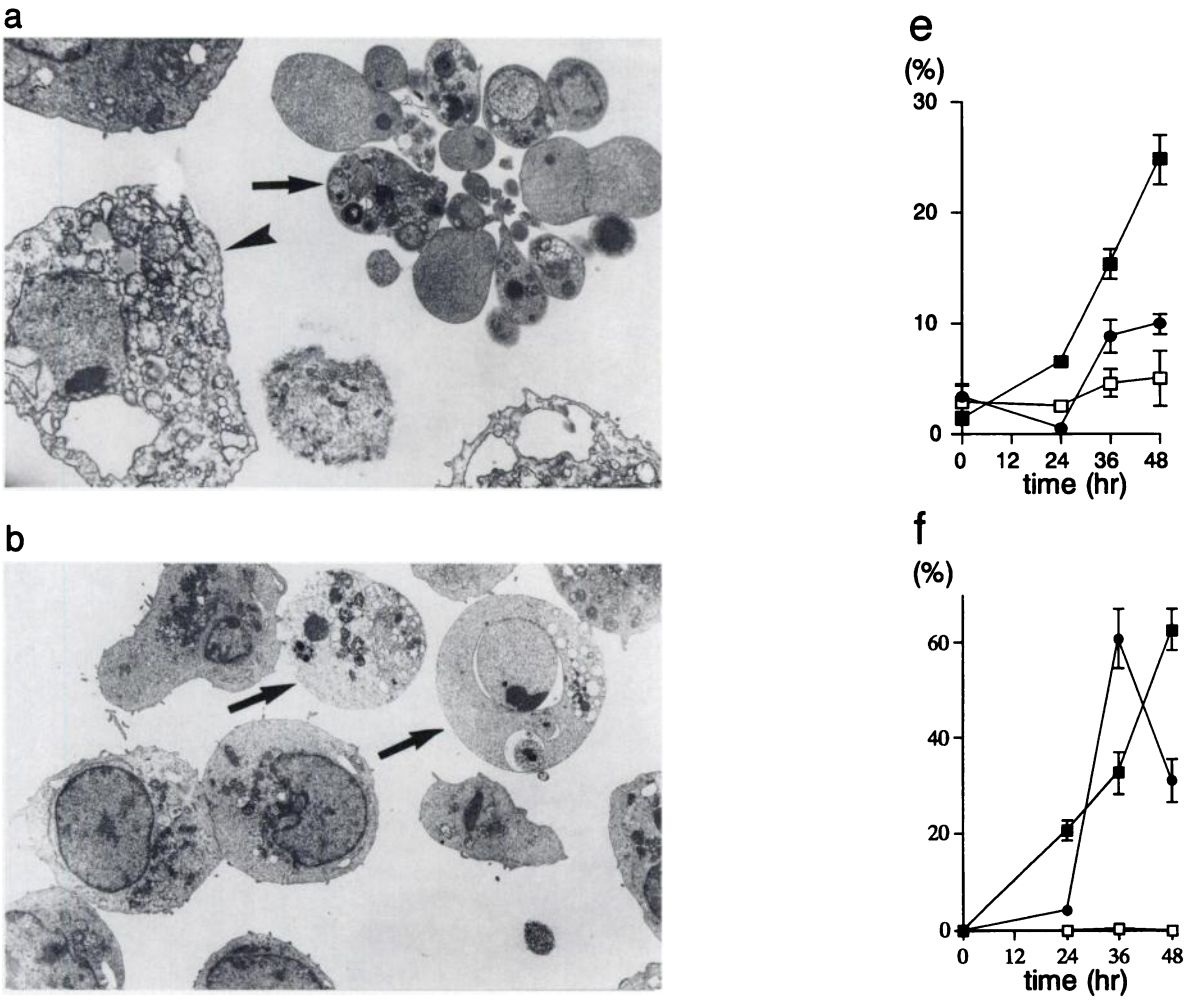

Fig. 2. Representative morphology of hypoxic 7316A and GB11 cells. Ultrastructural analysis (*a* and *b*) and nonconfocal fluorescence microscopic analysis (*c* and *d*) of 7316A (*a* and *c*) and GB11 (*b* and *d*) cells were carried out after 24 h hypoxia (*a*, ×5200; *b*, ×4500). *Arrow*, apoptotic cell; *arrowhead*, necrotic cells. *e* and *f*, time course of cell death of 7316A *e* and GB11 *f*, respectively. Values are the percentage of each morphological mode among total cells (□, necrosis; ●, early apoptosis; ■, terminal apoptosis). Results are expressed as the means of values obtained from four independent experiments; *bars*, SD.

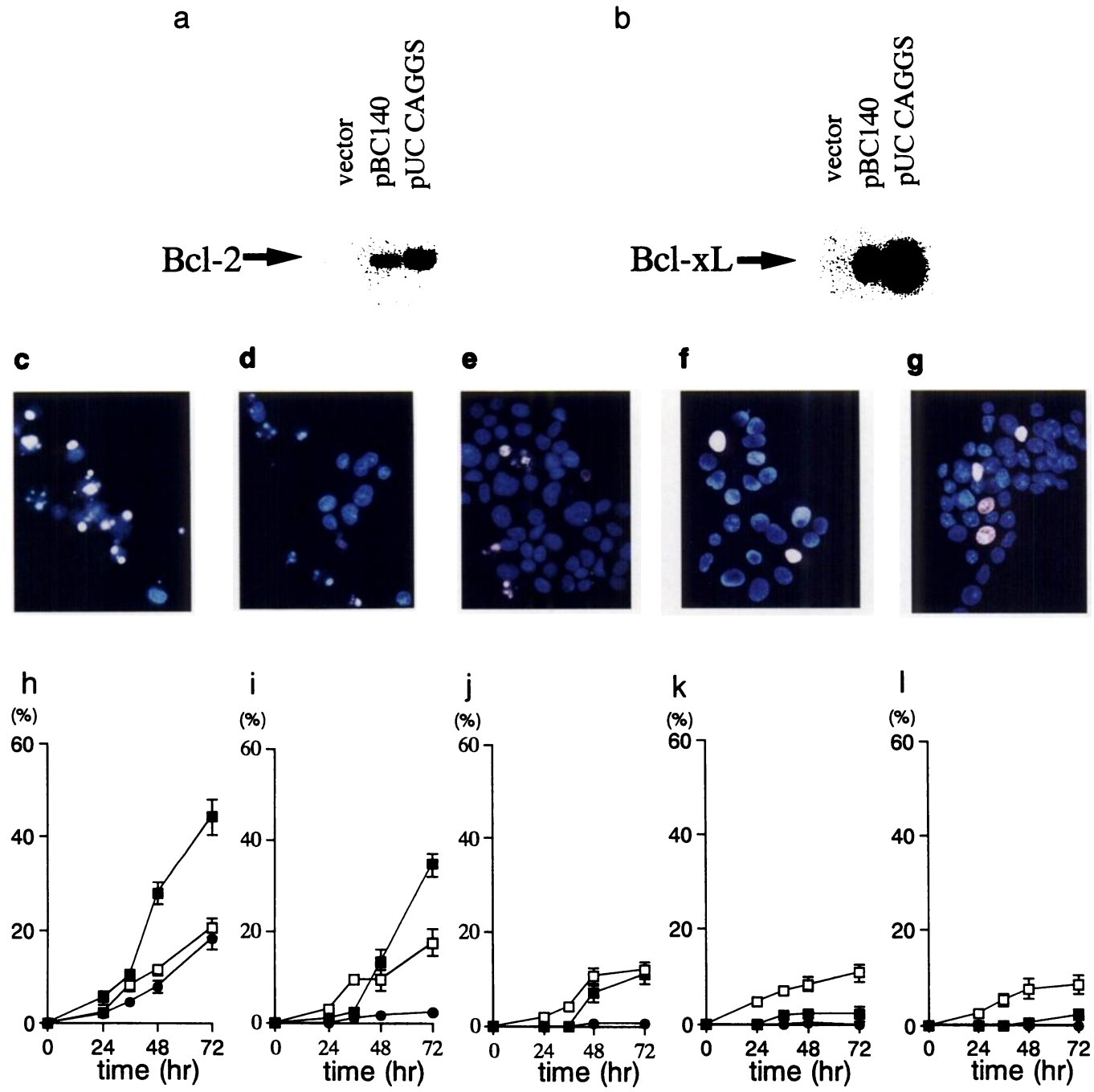

Fig. 3. Effects of Bcl-2 and Bcl-X_L on modes of hypoxic cell death. Expression of Bcl-2 *a* and Bcl-X_L *b*. Representative clones were analyzed by Western blotting. More than two bands were observed for Bcl-X_L, probably due to some posttranslational modification. *c–l*, representative cell morphology *c–g* of 48 h hypoxic cell death of PC12 transfectants and their quantitative analysis *h–l*. Representative data of vector control *c* and *h*, pBC140-hbcl-2 *d* and *i*, pBC140-cbcl-x_L *e–j*, pUC-CAGGS-hbcl-2 *f* and *k*, and pUC-CAGGS-cbcl-x_L *g* and *l* are shown. Values are the percentage of cells in each morphological mode among total cells (□, necrosis; ●, early apoptosis; ■, terminal apoptosis). At least 1000 transfected cells were counted for each experiment. Results are expressed as the means from values obtained from four independent experiments; *bars*, SD.

oles irrespective of membrane integrity, and PI[3] (red), which stains only nuclei in cells with disrupted membrane integrity (Fig. 1, *c* and *d*), delineated four groups based on nuclear shape, *i.e.*, round or fragmented, and cell membrane integrity (Table 1). Almost all cells with PI-stained round nuclei had large vacuoles and low cytosolic density (Fig. 1, *c* and *d*), corresponding to necrosis defined by electron microscopy. In contrast, apoptotic cells with PI-positive fragmented nuclei had no large vacuoles. Cells that were not stained with PI had no large vacuoles and were either round or fragmented, probably corresponding to viable cells and apoptotic cells that retain membrane integrity, respectively. These four groups were also easily distinguished and quantified by non-confocal fluorescence microscopic analysis of hypoxic PC12 cells stained with PI and Hoechst 33342 (blue), which stains all nuclei (Table 1; Fig. 1*e*). Cells with fragmented nuclei were classified based on PI staining as "early" apoptotic with membrane integrity and "terminal" apoptotic without membrane integrity.

To determine whether induction of both apoptosis and necrosis by

[3] The abbreviation used is: PI, propidium iodide.

Table 1 *Cell death modes defined by fluorescence staining*

	Confocal		Nonconfocal	
	Calcein-AM		PI	Ho342
	Cell shape	Vacuoles	Nuclei	Nuclei
Viable	Round	−	Not stained	Round
Necrosis	Round	+	Round	Round
Early apoptosis	Fragmented	−	Not stained	Fragmented
Terminal apoptosis	Fragmented	−	Fragmented	Fragmented

hypoxia is a general phenomenon, we examined a rat hepatoma cell line, 7316A, and a mouse early pre-B cell line, GB11. Although the results obtained with hypoxic 7316A cells were virtually identical to those obtained for PC12 cells by electron and fluorescence microscopy (Fig. 2,[5] *a* and *c*), nearly all hypoxic GB11 cells appeared apoptotic, and necrotic cells were rarely observed (Fig. 2, *b* and *d*). Numbers of 7316A cells exhibiting necrosis, "early" and "terminal" apoptosis among total cells, increased as a function of time (Fig. 2*e*), whereas necrotic GB11 cells were rarely detected at any time point (Fig. 2*f*). Thus, the proportion of necrosis and apoptosis induced by hypoxia appears to vary among cell lines.

To examine the effects of Bcl-2 and Bcl-X_L on the various modes of cell death, these proteins were overexpressed in PC12 cells using different constructs made with two vectors: pBC140 with a cytomegalovirus promoter-enhancer; and pUC-CAGGS with a β-actin promoter and cytomegalovirus enhancer. Western blot analysis revealed that the amounts of Bcl-2 and Bcl-X_L expressed using the pUC-CAGGS system were much higher than those using the pBC140 system (Fig. 3, *a* and *b*). Vector transfectants were used as control cells. After hypoxic treatment of these derivatives of PC12 cells, the proportion of necrotic and early and terminal apoptotic cells was analyzed by fluorescence microscopy. In vector transfectants, all fractions increased in a time-dependent manner, and 21% of cells appeared necrotic, 18% appeared early apoptotic, and 44% appeared terminal apoptotic after 72 h of hypoxia (Fig. 3, *c* and *h*). However, in PC12 cells transfected with pBC140-Bcl-2, which overexpressed lower amounts of Bcl-2, apoptosis was partially blocked (Fig. 3, *d* and *i*), and in pBC140-Bcl-X_L transfectants, which overexpressed lower amounts of Bcl-X_L, such blocking was even more extensive (Fig. 3, *e* and *j*). On the other hand, PC12 cells with highly overexpressed Bcl-2 (Fig. 3, *f* and *k*) or Bcl-X_L (Fig. 3, *g* and *l*) using the pUC-CAGGS system showed essentially no "early" or "terminal" apoptosis. Necrosis was significantly blocked by pBC140-Bcl-X_L, pUC-CAGGS-Bcl-2, and pUC-CAGGS-Bcl-X_L ($P < 0.05$) but not by pBC140-Bcl-2. These results indicate that Bcl-2 and Bcl-X_L act in a dose-dependent manner and that sufficient amounts of either proteins can provide complete block of apoptosis and partial prevention of necrosis; the latter are almost consistent with the observations that Bcl-2 inhibits the necrosis induced by glutathione depletion (24) and by sodium azide (25). Similar protective effects were also observed in hypoxia-induced apoptosis of 7316A and GB11 cells (data not shown). Complete blocking of hypoxia-induced apoptosis by overexpression of Bcl-2 and Bcl-X_L was confirmed by electron microscopy (data not shown). Thus, both Bcl-2 and Bcl-X_L act predominantly to prevent apoptosis.

Under hypoxic conditions, oxidative phosphorylation is blocked, and the resulting decrease in ATP levels is thought to cause sufficient elevation of cytosolic Ca^{2+} concentrations (26) to activate Ca^{2+}-dependent proteases (27), leading to the disruption of the cytoskeleton and membrane (28), and ultimately to necrosis. This scenario is generally accepted as the mechanism of necrosis induced by hypoxia. Our data show that hypoxia activates the apoptotic pathway concomitant with the necrotic pathway, although it remains unclear what determines the modes of cell death. In the present study, hypoxic conditions were achieved in the presence of glucose, which rather mimics pathological hypoxia *in vivo* where glucose is always present, so that ATP might be supplied from glycolysis, although oxidative phosphorylation should be completely blocked in the presence of undetectable amounts of oxygen molecules. Since "chemical hypoxia" induced with the cyanide and iodoacetate (29), which completely abolishes ATP production, resulted in almost necrosis,[4] incomplete or relatively slow ATP depletion might underlie hypoxia-induced apoptosis, or alternatively, other events caused by oxygen depletion might lead to apoptosis. The fact that the hypoxic treatment induces apoptosis as well as necrosis and complete prevention of apoptosis by Bcl-2 and Bcl-X_L raises the possibility of reducing the tissue damage caused by low oxygen concentrations by exploiting molecules such as Bcl-2 and Bcl-X_L with potent anti-apoptotic activity.

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[4] Unpublished results.

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