Correlation of oxidant-induced acute ATP depletion with delayed cell death in human neuroblastoma cells

HENRIKKA AITO, T. KRISTIINA AALTO, AND KARI O. RAIVIO
Hospital for Children and Adolescents, FIN-00029 Helsinki, Finland

Correlation of oxidant-induced acute ATP depletion with delayed cell death in human neuroblastoma cells. Am. J. Physiol. 277 (Cell Physiol. 46): C878–C883, 1999.—We correlated the adenine nucleotide (AN) levels and energy charge (EC) at the end of a transient oxidative exposure to the delayed death of neuronal cells. When wild-type (WT) or Bcl-2-overexpressing (BCL-2) human neuroblastoma cells (Paju) were exposed to 250 µM H2O2 for 60 min, the EC of WT cells was unchanged, but that of BCL-2 cells decreased from 0.91 ± 0.03 to 0.67 ± 0.02. Depletion of ANs was significantly greater in BCL-2 (66.7 ± 2%) than in WT (38.8 ± 2%) cells. Proliferation of both lines decreased, averaging 63 ± 17% of control by 48 h. Exposure to 5 mM H2O2 caused no further change in ANs in BCL-2 cells but in WT cells decreased the EC to 0.45 ± 0.08 and depleted ANs to 41 ± 9% of control; after 24 h, WT cells became pyknotic and showed DNA fragmentation but no chromatin condensation, whereas BCL-2 cells died by delayed necrosis. After 10 mM H2O2, EC dropped to 0.15 ± 0.1, and both lines were acutely killed. The EC after an oxidative insult correlated well with further growth of both cell lines. A significant decline in EC led to delayed death. Bcl-2 did not protect against the fall in EC or AN depletion, but, although survival was not improved, the mechanism of death appeared to be different.

adenine nucleotides; Bcl-2; energy charge; delayed neuronal death; hydrogen peroxide; adenosine 5'-triphosphate

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Cell viability. Cell number and viability were determined with a hemocytometer after staining cells, which were resuspended in PBS, with 0.04% trypan blue.

Oxidant Exposure

The H$_2$O$_2$ exposures were started using cell cultures in four-well dishes (Nunc) at a controlled density averaging 1,000 cells/mm$^2$. The medium was replaced with serum-free RPMI, and H$_2$O$_2$ was added to a final concentration of 100 µM, 250 µM, 5 mM, or 10 mM H$_2$O$_2$ (Merck, Darmstadt, Germany). After 60 min of exposure, the cells were either immediately analyzed or the exposure medium was replaced by regular culture medium, and the cells were further incubated for 24, 48, or 72 h and then analyzed. The H$_2$O$_2$ concentrations were verified immediately before each exposure by spectrophotometric analysis at 240 nm, using the molar extinction coefficient of 44 for H$_2$O$_2$.

Analysis

AN depletion assay. Before starting exposure experiments, Paju cells were prelabeled for 6 h with [1$^4$C]adenine (final concentration 100 mM, specific activity 51–55 mCi/mmol; Amersham, Amersham, UK) in normal culture medium. The labeled medium was then removed, and cells were washed three times with RPMI 1640 and exposed as appropriate. Directly after exposure, the medium was removed, and cellular ANs (ATP, ADP, and AMP) were extracted on ice with perchloric acid and quantified (1). Radioactivities were measured with a liquid scintillation counter (Rackbeta 1209; LKB Wallac, Turku, Finland). The adenylate EC of ([ATP] + $\frac{1}{2}$[ADP]) / ([ATP] + [ADP] + [AMP]), where brackets denote concentration, was calculated from the radioactivities in the ANs (5), which have been shown to correspond to their intracellular concentrations (1).

Gel electrophoresis. DNA from the exposed cells was extracted with phenol-chloroform, end-labeled with digoxigenin-UTP (Boehringer Mannheim, Pentzberg, Germany), electrophoresed (50 V, 3.5 h) on 2% standard low-melting agarose gel, and analyzed by autoradiography as described (33). Paju WT cells were treated for 30 min by potassium cyanide and deoxylglucose to induce apoptosis in 12 h (our unpublished results) were used as a positive control for DNA fragmentation.

Assessment of nuclear morphology. Aliquots of the exposed cells were fixed in 4% phosphate-buffered formaldehyde, stained overnight with the fluorescent DNA-binding probe Hoechst (Molecular Probes Europe, Leiden, Netherlands) at a concentration of 0.4 mg/ml, resuspended in 50% glycerol in PBS, and visualized under fluorescent microscopy using a UV-A filter with an excitation wavelength of 340–380 nm (Leica, Germany).

Data analysis. Results are expressed as means ± SD of 4–12 parallel independent experiments performed at least in duplicate. The statistical analysis was done using the nonparametric Mann-Whitney U-test, and the association between EC and AN concentrations and cell viability was assessed by Pearson correlation analysis after logarithmic transformation of the values (SPSS 7.5 software for Windows). P < 0.05 was considered significant.

RESULTS

Acute Nucleotide Depletion

The uptake of [1$^4$C]adenine and its distribution between the ANs was similar in WT and BCL-2 cells (data not shown). Exposure to 100 µM H$_2$O$_2$ for 1 h did not cause a significant depletion of ANs in either cell line. In BCL-2 cells, ANs were maximally depleted at 250 µM H$_2$O$_2$ exposure, with no further effect at 5 or 10 mM H$_2$O$_2$. In WT cells, 250 µM H$_2$O$_2$ had a significantly smaller effect on AN depletion (P = 0.001 compared with BCL-2 cells). At higher concentrations, AN depletion was dose dependent, and, at 10 mM H$_2$O$_2$, AN depletion was more severe than in BCL-2 cells (Fig. 1).

Energy Charge

The EC of the control cultures (0.90 ± 0.04, n = 12) did not differ between the cell lines. Exposure to 100 µM H$_2$O$_2$ for 60 min did not affect the EC in either cell line. In WT cells, exposure to 250 µM H$_2$O$_2$ for 60 min did not affect cellular EC (0.88 ± 0.02) but caused a significant decrease to 0.67 ± 0.02 (P = 0.001) in BCL-2 cells. At higher concentrations, the EC decreased similarly in both cell lines (Fig. 2).

Cell Survival

Acute toxicity. After exposure to all H$_2$O$_2$ concentrations, the WT and BCL-2 cells remaining attached to
the culture dish (i.e., cells that were collected for the AN depletion assay) all excluded trypan blue. However, there was a dose-dependent loss of viable cells at the end of exposure, with no difference between the WT and BCL-2 lines (Fig. 3, time 60 min).

Delayed Cell Death

Cell numbers of the unexposed WT and BCL-2 cultures did not differ when followed for 48 h (Fig. 3). Exposure to 100 µM H$_2$O$_2$ had no effect on the growth properties of either cell line (data not shown). Exposure to 250 µM H$_2$O$_2$ for 60 min caused an inhibition on the proliferation of both cell lines during the follow-up period, but there were no microscopic indications of increased cell death (Fig. 3). At 48 h, the respective counts of viable WT and BCL-2 cells were $107 \pm 17$ and $127 \pm 11\%$ (not significant) of initial values (Fig. 3).

After the 60-min exposure to 5 mM H$_2$O$_2$, there were no significant differences between WT and BCL-2 cultures in the number of cells surviving for 24 or 48 h (Fig. 3). However, both were significantly lower than cell counts at the end of the 60-min exposure and when compared with unexposed control cultures. In WT cells, the decline in viability progressed to 72 h (10 ± 5%), and there was a significant difference ($P = 0.05$) in the number of viable cells between 48 and 72 h, whereas the counts of BCL-2 cells showed no significant change between 24 and 72 h. After exposure to 10 mM H$_2$O$_2$, all cells of both lines had died by 24 h.

Correlation of the Acute EC and AN Depletion With Delayed Cell Death

The EC at the end of 60 min of H$_2$O$_2$ exposure correlated well ($r^2 = 0.99, P < 0.01$ and $r^2 = 0.99, P = 0.05$, for WT and BCL-2 cells, respectively) with viability at 24 h (Fig. 4, A and B). The correlation of the initial AN depletion with viability at 24 h was strong in WT cells ($r^2 = 0.99, P < 0.05$) but nonsignificant in BCL-2 cells ($r^2 = 0.32$).

DNA Fragmentation

At the end of a 60-min exposure to 250 µM or 5 mM H$_2$O$_2$, electrophoresis of the extracted DNA showed no fragmentation or degradation in either cell line (for 5 mM H$_2$O$_2$, Fig. 5, lanes 2 and 3). Exposure to 10 mM H$_2$O$_2$ for 60 min resulted, in both lines, in necrotic DNA degradation detected as a smear (not shown). When studied 12 h after a 60-min exposure to 5 mM H$_2$O$_2$, electrophoresis of WT cells showed DNA fragmentation suggestive of apoptosis (Fig. 5, lane 4), whereas BCL-2 cells showed a uniform smear (Fig. 5, lane 5). After 24 h...
and 48 h, nonspecific DNA fragmentation was observed in both cell lines (not shown).

**Nuclear Morphology**

The nuclear morphology of unexposed WT or BCL-2 cells remained unchanged over at least 48 h in culture (Fig. 6A). Assessment of nuclear morphology directly after exposure to 250 µM or 5 mM H₂O₂ did not reveal differences between the two cell lines or in comparison with unexposed cells (data not shown). After 24 h, WT cells exposed to 5 mM H₂O₂ for 60 min were morphologically unchanged (data not shown), but by 48 h the nuclei had become pyknotic (Fig. 6B). Condensation of chromatin typical of apoptosis was not detected. In BCL-2 cells exposed to 5 mM H₂O₂ for 60 min, nuclear enlargement and fragmentation were observed after 24 h (data not shown), and these changes progressed by 48 h (Fig. 6C). No morphological features of apoptosis were detected.

**DISCUSSION**

Transient exposure to oxidants leads to an impairment in cellular energy metabolism (1, 2). Disturbances in mitochondrial function and ATP synthesis have been associated with the onset of apoptosis (19), but ATP appears to be required for downstream events (10). Therefore, a permanent failure may cause necrosis (34), whereas cells retaining the potential to produce high-energy phosphates have been shown to pass on to apoptosis more readily (4, 7). The possibility of a mechanism other than apoptosis or necrosis has also been implicated in oxidant-induced delayed neuronal damage (11, 22, 23).

We developed this cell model to evaluate whether the severity of the acute disturbance in energy metabolism caused by an oxidative insult predicts the fate of the cells in vitro. In some cell models, ATP depletion has been shown to induce apoptosis (13), and many apoptotic stimuli have been shown to impair ATP production. To clarify the role of Bcl-2 protein in preventing the acute and delayed events of oxidant-induced cell death, we used a cell line overexpressing this protein.
We found that the acute disturbance of cellular energy status, as reflected in EC, correlated well with the prolonged survival of the cells after an oxidative insult that did not yet affect acute viability. The degree of the acute AN depletion was also dependent on the intensity of the exposure but predicted cell survival only in WT cells.

As in freshly isolated cortical cell cultures (7), our continuously growing cell lines showed a dose-dependent response to oxidant exposure, ranging from no demonstrable effect at 100 μM H₂O₂ to acute necrosis at 10 mM H₂O₂. Intermediate concentrations triggered delayed cell death. A significant decline in EC, which did not yet affect acute viability, was correlated with the process of delayed cell death. In WT cells, this process had some apoptotic characteristics, such as DNA laddering detectable at 12 h and pyknosis of the nuclei by 48 h, although we failed to detect clear nuclear condensation in these cells. Overexpression of Bcl-2 protein conferred no protection against the initial AN depletion or the decline in EC caused by H₂O₂ exposure, and it did not save the cells from delayed death in this setting. These results are partly in agreement with previous data (24) that showed that Bcl-2 overexpression did not protect hematopoietic cells from ATP depletion induced by mitochondrial inhibitors, although it rescued these cells from apoptosis. It has been suggested that protection by Bcl-2 overexpression against oxidants, at least H₂O₂, may be concentration dependent or limited to the apoptotic mode of death (12, 20). Because all of the criteria were not fulfilled, we cannot ascribe the death of our WT cells to apoptosis. The low-level constitutive expression of Bcl-2 of the WT cells may also modify their mode of death. The delayed death of the BCL-2 cells in our model showed no apoptotic features.

Cultured malignant neuroblastoma cells are not an ideal model to evaluate the process of oxidant-induced injury in neural cells in vivo. However, these human cells offer a useful tool to assess the processes of oxidative injury, since they are rather resistant to the acute toxicity of oxidants and can thus be reliably analyzed and followed up. Also, because the differentiation stage of these cells can be modified in vitro, they can be used as a model to assess the relation of oxidant resistance to the stage of differentiation.

We conclude that, in this cell model, the EC directly after a transient oxidative exposure correlates well with prolonged survival. In WT cells, an exposure that causes a significant decline in EC but does not yet affect immediate viability triggers a process of delayed cell death with some apoptotic features. Neither the initial drop in EC, AN depletion, nor delayed cell death is inhibited by Bcl-2 overexpression. On the other hand, the characteristics of the ensuing cell death are different. Our data are in contrast with previous findings that showed increased capacity of BCL-2 cells to maintain mitochondrial proton gradient and ATP levels (29). However, the more sustained oxidative insult used in our model suggests that this capacity is limited.

This study was supported by the Foundation for Pediatric Research (H. Aito), Sigrid Juselius Foundation, and the Research Foundation of Helsinki University Central Hospital, Finland (T. K. Aalto).

Address for reprint requests and other correspondence: H. Aito, Hospital for Children and Adolescents, PO Box 280, FIN-00029 Helsinki, Finland (E-mail: henrikka.aito@huch.fi).

Received 20 October 1998; accepted in final form 29 June 1999.

REFERENCES


