Relationships between the Mitochondrial Permeability Transition and Oxidative Stress during ara-C Toxicity

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ABSTRACT

The mitochondrial permeability transition and oxidative stress seem to be critical alterations in cellular physiology that take place during programmed cell death. Failure to undergo apoptosis is associated with drug resistance in acute myelogenous leukemia and other cancers. Therefore, it is important to establish causal relationships between the physiological changes that take place in apoptosis, because these are potential targets for novel treatment strategies to overcome this form of drug resistance. We describe the use of multilaser flow cytometry methods to correlate measurements of mitochondrial membrane potential (MMP), the generation of reactive oxygen intermediates, the cellular content of reduced glutathione (GSH), intracellular calcium, and exposure of phosphatidylserine on the cell surface. Using these combined methods, we have mapped a "death sequence" that occurs after treatment of leukemic blasts with clinically relevant concentrations of 1-β-D-arabinofuranosylcytosine (ara-C). Dual labeling of MMP and cellular glutathione content showed that loss of MMP, indicative of the permeability transition, took place in cells that were depleted of glutathione. The loss of MMP coincided with phosphatidylserine exposure and preceded a state of high reactive oxygen generation. Finally, there was an increase in intracellular calcium. These results demonstrate that the mitochondrial permeability transition takes place during ara-C toxicity but suggest that this occurs downstream of the loss of GSH. Thus, oxidative stress after ara-C-induced toxicity seems to be a biphasic phenomenon, with the permeability transition occurring after a depletion of GSH and preceding a state of high reactive oxygen generation.

INTRODUCTION

The lethal effects of ara-C are caused by incorporation of ara-CTP into DNA, where it acts as a chain terminator (1). After initial DNA damage, cells eventually undergo DNA fragmentation that is characteristic of apoptosis (2). We have previously shown that the loss of calcium ion regulation occurs during ara-C toxicity, suggesting that DNA fragmentation is caused by the activation of calcium-dependent endonucleases (3). Using multiparametric flow cytometry with probes for the cellular redox state, we mapped a sequence of events occurring upstream of the increase in intracellular calcium. The earliest detectable change was a modest increase in the rate of reactive oxygen generation, accompanied by an approximately 2-fold increase in the cellular content of GSH. This finding is consistent with previous reports that oxidative stress is an early event in programmed cell death (4, 5); the source of the ROSs is uncertain. The increase in GSH observed is likely to be a cellular response to the increased ROI that protects the normal reducing cellular redox environment. With longer exposure and preceded a state of high reactive oxygen generation. Later, there was an increase in intracellular calcium ([Ca2+]), followed by a loss of surface membrane integrity. Cells that overexpressed the Bcl-2 protein were protected by being held in the high ROI/high GSH reducing state (3). This orderly sequence of events is consistent with the suggestion that oxidative stress is an important mechanism of drug-induced apoptosis and that the protection afforded by Bcl-2 is related to an increased capacity to withstand oxidative stress (6).

Recently, Kroemer and coworkers have shown that mitochondrial changes play a pivotal role during early apoptosis (7–10). The mitochondrial permeability transition results from the opening of large protein pore complexes within the inner mitochondrial membrane (9, 11). An active process, the permeability transition can occur in response to a wide range of factors, including calcium signaling and oxidative stress (11–15). It produces loss of the MMP, thereby uncoupling oxidative phosphorylation. Increases in ROI generation then occur and are likely to be the result of a feedback increase in respiratory chain activity due to the loss of ATP generation (16, 17). The mitochondrial permeability transition could therefore be both a cause and a consequence of oxidative stress.

In addition to mitochondrial membrane changes, there is recent interest in alterations in the surface membrane that take place in early apoptosis. These involve the translocation to the cell surface of phosphatidylserine, an aminophospholipid that is normally confined to the inner leaflet of the lipid bilayer (18–20). This is an active process that allows the recognition of apoptotic cells by phagocytes. It is likely that the various physiological events taking place during apoptosis are interrelated and controlled by genes that determine whether drug treatment results in cell death or survival. These processes are potential targets for novel treatment strategies. Therefore, we developed multilaser flow cytometry methods to map the sequence of changes in cellular physiology after ara-C treatment and identify the critical events associated with drug resistance. Using this technique, we have confirmed that the loss of MMP is an early event that coincides with phosphatidylserine exposure and precedes high ROI generation and the loss of ionized calcium regulation. However, these changes seem to take place downstream of an earlier depletion in the reduced cellular GSH content.

MATERIALS AND METHODS

Cell Culture. The OCI/AML-2 cells used in these experiments are a continuous line of acute myelogenous leukemia blasts originally isolated from a patient at Ontario Cancer Institute/Princess Margaret Hospital. These cells grow independently of added growth factors and were maintained at 37°C in 10% FCS (Sigma, St. Louis, MO) with α-MEM (Life Technologies, Inc., Burlington, Canada) and subcultured routinely every 2–3 days.

ara-C Treatment. ara-C (Upjohn Co., Don Mills, Canada) was dissolved in PBS buffer (pH 6.5) at 10 mM. Cells were treated with 7 μM ara-C for the appropriate length of time, counted, and then resuspended at 1 × 106 cells/ml of medium for flow cytometric analysis. This concentration of ara-C produces an approximately 90% loss of clonogenic survival after 24 h of exposure.

ROI Generation. Generation of ROI was measured using DHR-123 (Molecular Probes, Eugene, OR). This is weakly fluorescent but is oxidized by ROI to the highly fluorescent rhodamine 123. DHR-123 was made up as a 1 mM stock solution in 100% ethanol and stored in 1-ml aliquots under nitrogen.

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2 The abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine; GSH, reduced glutathione; ROI, reactive oxygen intermediate; MMP, mitochondrial membrane potential; MBBr, monobromobimane; DHR-123, dihydrodorhamidine 123; [Ca2+]i, intracellular ionized cytosolic calcium.
GSH. The nonfluorescent sulfhydryl probe MBBr (Molecular Probes) was used as described previously (21). This compound binds avidly to -SH groups, producing brightly fluorescent conjugates. Under the staining conditions used, fluorescence is highly correlated with the cellular content of GSH (22). MBBr was made up as a 4 mM stock solution in 100% ethanol.

MMP. This was measured using the cyanine dye DiIIC(6), obtained from Molecular Probes. This is a structural analogue of the widely used MMP probe DiOC(6) but is capable of excitation using a 633 nm HeNe laser. Because of their cationic and amphiphatic nature, these cyanine dyes are concentrated in energized mitochondria. Dual staining of cells undergoing apoptosis using DiOC(6) and DiIC(6) gave identical patterns, confirming that DiIC(6) is a suitable red light-excited MMP probe. DiIC(6) was made up as a 40 µM working dilution in 100% ethanol.

Phosphatidylserine Expression. This was measured using a fluorescein conjugate of the natural ligand annexin V, obtained from R&D Systems (Minneapolis, MN). Cells were labeled with a final concentration of 5 µg/ml annexin V-FITC for 5 min at room temperature.

Ionized Calcium. This was done using the fluorescent calcium indicator indo-1, loaded into cells as the membrane-permeant ester indo-1 AM (Molecular Probes).

Surface Membrane Integrity. The membrane-impermeant DNA stain propidium iodide (10 µg/ml) was added before running the samples. Cells that had lost surface membrane integrity were identified by their bright nuclear fluorescence.

Flow Cytometry. Flow cytometry was done using an Epics Elite cell sorter (Coulter Corp., Miami, FL) equipped with argon, HeCd, and HeNe lasers emitting at 488, 325, and 633 nm, respectively. The argon laser was used as the reference beam, with the HeCd and HeNe running collinearly with a 40-ms time delay. The signals were processed using a gated amplifier. The following excitation lasers and bandpass filters were used. Annexin V-FITC and DHR-123 were excited by the argon laser with fluorescence collected at 525 nm, and propidium iodide was excited using the HeCd laser with fluorescence collected at 640 nm. The HeCd laser was used to excite MBBr, and fluorescence was collected at 450 nm. Indo-1 was also excited using the HeCd laser, and [Ca²⁺], was obtained from electronic ratio measurements of the calcium-bound (405 nm) and calcium-free (525 nm) emissions. The 633 nm HeNe laser was used to excite DiIIC(6), and emission was collected at 675 nm.

Combined Labeling Methods. To identify relationships between various changes in cell function that take place during apoptosis, two quadruple-labeling methods were used, each using triple-laser excitation. Method 1, designed to monitor changes in cellular redox state, involved staining with 1 µM DHR-123 and 40 nM DiIIC(6) for 25 min at 37°C, after which cells were resuspended in fresh medium containing 40 µM MBBr and 10 µg/ml propidium iodide and incubated for an additional 5 min at 37°C. Method 2, which examines events downstream of the loss of GSH, consisted of 3 µM indo-1 AM and 40 nM DiIIC(6) for 25 min at 37°C for 25 min, followed by resuspension in fresh medium containing annexin V-FITC and propidium iodide for 5 min at room temperature.

RESULTS

Relationships between Cellular Redox State and MMP. Dead cells that had lost the capacity to exclude propidium iodide showed low levels of MBBr and DHR-123 fluorescence. These represented <20% of the total cell population and were gated out from further analyses. As previously found (3), there was striking heterogeneity in ROI generation and GSH content after ara-C treatment. A subpopulation appeared with an increased rate of ROI production, whereas the distribution of individual cell GSH content became much broader, with the appearance of cells with greater and lower values than those seen in untreated control populations. Correlated two-parameter plots of GSH content versus reactive oxygen show that the low GSH cells correspond to the high ROI population (Fig. 1). Using DiIIC(6) to measure MMP, a distinct population of low fluorescent cells was seen after ara-C treatment. The data are shown in Fig. 2 as correlated two-parameter contour plots of glutathione content (top) and reactive oxygen generation (bottom) plotted against MMP. The low MMP cells appearing after drug exposure showed decreased GSH (top right panel), and no cells were seen that had high levels of GSH and depolarized mitochondria. The subpopulation of low MMP cells showing high rates of ROI generation (bottom right panel) mapped to the low MMP/flow GSH cluster (top right panel).

Relationships between MMP, [Ca²⁺], and Phosphatidylserine Exposure. Using annexin V-FITC labeling, a subpopulation of cells that expressed phosphatidylserine but excluded propidium iodide could be clearly identified after ara-C treatment. As shown in Fig. 3, annexin V positivity was strongly correlated with low MMP, and very few cells were seen in the transition from high MMP/annexin V negative to low MMP/annexin V positive. These findings indicate that the loss of MMP and the exposure of phosphatidylserine on the cell surface take place rapidly and within minutes of each other. Fig. 4 shows correlated two-parameter dot plots of ionized calcium versus MMP (top) and annexin V binding (bottom). After ara-C treatment, a population of cells appeared with very high [Ca²⁺] values. All of these high [Ca²⁺] cells showed reduced MMP and were annexin V positive, whereas approximately 50% of the low MMP/annexin V-positive cells showed normal calcium values, indicating that the increase in [Ca²⁺], occurred later than the mitochondrial and surface membrane changes.

Time Course of ara-C Treatment. The single 24-h time point experiments suggested that a sequence of events was occurring during ara-C toxicity. This was further investigated by making flow cytometry measurements at 2-h intervals during continuous ara-C treatment for 26 h. The findings are summarized in Fig. 5, which for clarity

**Fig. 1.** Correlated contour maps of reactive oxygen generation (log scale) versus cellular glutathione content (linear scale), showing increased heterogeneity after 24 h of treatment with 7 µM ara-C (right panel).
Fig. 2. Correlated contour maps of MMP versus glutathione content (top) and reactive oxygen generation (bottom). All cells with loss of MMP after ara-C treatment show low glutathione, and a proportion of these show high reactive oxygen generation (right panels).

shows only the 16-, 20-, and 24-h time points. The percentage of cells showing loss of MMP increased with time of ara-C exposure. The lower panels in Fig. 5 show GSH content, annexin V binding, and [Ca^{2+}], plotted against MMP. Note that the basic patterns, such as the loss of MMP in low GSH cells and the strong correlation between MMP and annexin V labeling, were maintained throughout the time course. Thus, the heterogeneity observed at 24 h using the live cell function assays is explained on the basis of the cells going through a sequence of discrete stages in an asynchronous manner. This asynchrony is probably due to the fact that ara-C incorporation into DNA only takes place during S-phase of the cell cycle.

DISCUSSION

Considerable interest has been generated recently by the proposal that the mitochondrial permeability transition plays a key role in apoptosis after a wide range of physiological and nonphysiological initiating factors (7–10, 12, 23, 24). It is an active process that involves the opening of complex pore structures in the mitochondrial inner membrane, resulting in the loss of MMP and the uncoupling of oxidative phosphorylation (17). High levels of ROI generation may then occur, as found in the present paper, and are probably due to the loss of feedback inhibition of the respiratory chain after depletion of cellular ATP. Although these events are by themselves injurious to cells, there is evidence that the nuclear changes associated with apoptosis are due to the release of a death effector from the permeabilized mitochondria (8, 9). The identity of this effector is uncertain, although it is apparently not the interleukin 1β converting enzyme-like protease CPP-32 that has recently been implicated in ara-C-induced apoptosis (25).

The detailed composition of the permeability transition pore com-
plex is unknown. However, there is evidence that it is associated with other inner membrane proteins such as the ADP/ATP translocase and a peripheral benzodiazepine receptor (24). The Bcl-2 protein might also be physically linked and might inhibit apoptosis by a direct action on the pore complex (9). Although not fully characterized, the permeability transition seems to be ubiquitous in mammalian cells. It can be initiated by cell signaling (12), glucocorticoids (10), and also by oxidative processes such as disulfide bonding or the generation of lipid peroxidation breakdown products within the matrix space (13—15, 23). Thus, the permeability transition may cause oxidative stress due to increased respiratory chain activity and may also be the consequence of oxidative stress.

Using multiparametric flow cytometry, we have mapped out a sequence of events occurring during ara-C toxicity that is defined in physiological terms. As previously reported (3), the earliest events detected were an increase in the cellular content of glutathione and a modest increase in ROI generation. These early changes are probably due to an as-yet-uncharacterized oxidative stress that occurs after DNA damage by ara-C. Oxidative stress has been extensively documented after a wide variety of cellular insults, such as hyperthermia (26), UV irradiation (27), glucocorticoid treatment (6), or transient hypoxia (27, 28). An increase in GSH commonly occurs in response to oxidative stress, probably as an antioxidant defense mechanism (28, 29). With increasing time of ara-C treatment, we later observed a decrease in cellular GSH that coincided with the loss of MMP. No cells with high GSH values showed loss of MMP, excluding the possibility that the permeability transition is the cause of the GSH depletion. More likely, the permeability transition is due to failure of antioxidant mechanisms within the mitochondrial matrix space, as has been reported previously (13, 14, 30).

At all time points examined, there was a strong correlation between the loss of MMP and phosphatidylserine exposure on the cell surface. Phosphatidylserine is an aminophospholipid that is normally confined to the inner leaflet of the cell membrane. Translocation to the outer leaflet is an active process that allows phagocytes to recognize apoptotic cells in vivo (18—20). Costedo et al. found that phosphatidylserine exposure occurred approximately 1 h after the mitochondrial permeability transition in dexamethasone-treated thymocytes (10). However, in our experiments, almost all propidium iodide-excluding cells were either annexin V positive/low MMP or annexin V negative/high MMP. Furthermore, as seen in Figs. 3 and 5, the proportion of cells that were in the transition between these two states was very small. This indicates that the loss of MMP and the exposure of phosphatidylserine on the outer membrane take place rapidly and occur within a few minutes of each other.

High levels of ROI generation were seen in some cells that had lost MMP, as has been reported by others (16, 17). ROI s such as superoxide anion are normally generated as byproducts of mitochondrial respiration (31). Because respiration is feedback-inhibited by cellular ATP levels, it is likely that the high ROI generation seen after loss of MMP was due to the uncoupling of oxidative phosphorylation, with increased respiratory chain activity occurring as ATP levels decrease (16, 17).

The final event observed before the loss of surface membrane integrity was a large increase in intracellular ionized calcium. Low calcium values were found in approximately half of the cells that had lost MMP, indicating that a significant time interval occurs between the permeability transition and the loss of calcium ion regulation. The most likely explanations for the increase in [Ca^{2+}] are the failure of ATP-dependent calcium pumps or lipid peroxidation damage to membrane sites of calcium regulation (32). Internucleosomal fragmentation of DNA by the activation of calcium-dependent endonucleases is
considered to be a major late event during apoptosis (32), although its relationship with more recently described death effectors, such as the interleukin 1β converting enzyme-like proteases, remains to be established.

A large number of genes and their products are implicated in the regulation of apoptosis, but there are apparent contradictions in the published literature, and it is difficult to fit all of the potential effector mechanisms into a unifying scheme (33). In this paper, we have taken the alternative approach of describing ara-C-induced apoptosis in terms of the relationships between the important physiological events that occur during apoptosis, including changes in cellular redox state, calcium ion regulation, and the mitochondrial permeability transition. This physiological approach provides a framework in which specific genetically controlled events such as Bcl-2 alterations could be fitted. In addition, it may give insights into potential new types of cancer treatment that are designed to overcome drug resistance caused by the failure to undergo apoptosis. For example, in the present study, we have shown that an early event after ara-C treatment is an increase in GSH content, which may inhibit the mitochondrial permeability transition. Clinical trials of the GSH-depleting agent buthionine sulfoximine have shown that this can produce up to 90% loss of peripheral blood leukocyte GSH with minimal toxicity (34, 35). Although buthionine sulfoximine is currently under consideration...
as a modulator of drug resistance to alkylating agent, our results suggest that it may also have an adjunctive role in ara-C-containing regimens.

REFERENCES


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