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Effect of Glutathione Depletion on Antitumor Drug Toxicity (Apoptosis and Necrosis) in U-937 Human Promonocytic Cells

THE ROLE OF INTRACELLULAR OXIDATION*

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Alfonso Troyano‡§, Carlos Fernández‡, Patricia Sancho‡¶‖, Elena de Blas‡, and Patricio Aller‡**

From the ‡Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, and ¶Departamento de Biología Celular y Genética, Universidad de Alcalá, Madrid 28006, Spain

Treatment with the DNA topoisomerase inhibitors etoposide, doxorubicin, and camptothecin, and with the alkylating agents cisplatin and melphalan, caused peroxide accumulation and apoptosis in U-937 human promonocytic cells. Preincubation with the reduced glutathione (GSH) synthesis inhibitor L-buthionine-(*S*,*R*)-sulfoximine (BSO) always potentiated peroxide accumulation. However, although GSH depletion potentiated the toxicity of cisplatin and melphalan, occasionally switching the mode of death from apoptosis to necrosis, it did not affect the toxicity of the other antitumor drugs. Hypoxia or preincubation with antioxidant agents attenuated death induction, apoptotic and necrotic, by alkylating drugs. The generation of necrosis by cisplatin could not be mimicked by addition of exogenous H_2O_2 instead of BSO and was not adequately explained by caspase inactivation nor by a selective fall in ATP content. Treatment with cisplatin and melphalan caused a late decrease in mitochondrial transmembrane potential ($\Delta\Psi m$), which was much greater during necrosis than during apoptosis. The administration of the antioxidant agents *N*-acetyl-L-cysteine and butylated hydroxyanisole after pulse treatment with cisplatin or melphalan did not affect apoptosis but attenuated necrosis. Under these conditions, both antioxidants attenuated the necrosis-associated $\Delta\Psi m$ decrease. These results indicate that oxidation-mediated alterations in mitochondrial function regulate the selection between apoptosis and necrosis in alkylating drug-treated human promonocytic cells.

Apoptosis and necrosis are two different forms of cell death with well defined morphological characteristics (1–3). Among other aspects, during apoptosis the cells undergo nuclear and cytoplasmic shrinkage, the chromatin is condensed and partitioned into multiple fragments, and the cells are finally broken into multiple membrane-surrounded bodies (apoptotic bodies). However, the plasma membrane retains the integrity during the process. By contrast, necrosis is characterized by cell swelling, lysis of intracellular organella, and rapid disintegration of the plasma membrane. Apoptosis seems to be clearly advantageous for the organism, because the elimination of the apoptotic cells or the resulting apoptotic bodies by phagocytosis prevents the release of intracellular content and the consequent damage of the surrounding tissue, as it occurs during necrosis. Hence, it seems very important to elucidate the mechanisms that regulate apoptosis and necrosis and the factors that may decide the selection between one or the other mode of death.

One of the most complex aspects in the regulation of cell death is the role of intracellular oxidation. It was initially proposed that oxidation could be a general mediator of apoptosis (4). In fact, (i) exposure to reactive oxygen species (ROS),[1] such as hydrogen peroxide (H_2O_2) or nitric oxide (NO), induces apoptosis in different cell types (5, 6); (ii) many apoptotic inducers, which are not ROS themselves, cause intracellular oxidation, *e.g.* growth factor deprivation, glucocorticoids, UV irradiation, and some cytotoxic drugs (7–11); and (iii) overexpression of Bcl-2 reduces both ROS generation and apoptosis induction by different stimuli (8, 12). However, the relationship between oxidation and apoptosis is far from being clear. In fact, (i) some forms of apoptosis may take place under very low oxygen tensions, in which ROS generation is expected to be absent or greatly reduced (13, 14), or in the presence of antioxidants (13); (ii) pre-exposure to hyperoxia inhibited H_2O_2-provoked apoptosis in lung adenocarcinoma cells (15); and (iii) low ROS concentrations may promote proliferation and prevent apoptosis in some cell models (16, and references therein). An additional factor of interest is given by the fact that the intensity of oxidation may be determinant for the mode of death. For instance, treatment with H_2O_2 provoked apoptosis or necrosis, depending on the concentration used (5), and the administration of low concentrations of H_2O_2 sufficed to inhibit apoptosis and cause necrotic-like death in antitumor drug-treated Burkitt's lymphoma cells (17).

It is known that the toxicity of antitumor drugs may largely depend on the intracellular level of reduced glutathione (GSH).

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‖ Recipient of a pre-doctoral fellowship from the Universidad de Alcalá, Spain.

** To whom correspondence should be addressed: Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Velázquez 144, 28006 Madrid, Spain. Tel.: 34-9156-44562 (Ext. 4247); Fax: 34-9156-27518; E-mail: aller@cib. csic.es.

[1] The abbreviations used are: ROS, reactive oxygen species; BHA, butylated hydroxyanisole; BSO, DL-buthionine-(*S*,*R*)-sulfoximine; cisplatin, *cis*-platinum(II)-diammine dichloride; DAPI, 4,6-diamidino-2-phenylindole; DHE, dihydroethidium; GSH, reduced glutathione; H_2DCFDA, dichlorodihydrofluorescein diacetate; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; NAC, *N*-acetyl-L-cysteine; PBS, phosphate-buffered saline; PI, propidium iodide; R123, rhodamine 123; FCS, fetal calf serum; Z-VAD-Fmk, *Z*-Val-Ala-Asp-CH_2F; PKC, protein kinase C.

Thus, depletion of GSH by prolonged incubation with L-buthionine-(*S*,*R*)-sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase, increased the lethality of the DNA topoisomerase I inhibitor CPT-11 in V79 hamster lung fibroblasts (18), of the DNA topoisomerase II inhibitor etoposide in K562 human erythroleukemia cells (19), and of the anthracycline doxorubicin in different cell types (20–23). The influence of GSH was particularly evident in the case of alkylating agents, where BSO was occasionally able to change the mode of death from apoptosis to necrosis (24, 25). Because GSH is the main antioxidant system in the cell, a possible explanation is that GSH depletion facilitates ROS accumulation in cells treated with antitumor drugs (26), which in turn increases their lethality.

To test the validity of this hypothesis, in the present work we comparatively examined the capacity of BSO to modulate ROS production and cell death in U-937 human promonocytic cells treated with different antitumor drugs. The effects of exogenous H_2O_2 and antioxidant agents, and the possible role of oxidation-related events, such as caspase inactivation, ATP depletion, and mitochondrial dysfunction, were also considered.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—U-937 promonocytic leukemia cells (27) were grown in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS, Life Technologies, Inc.) and 0.2% sodium bicarbonate and antibiotics in a humidified 5% CO_2 atmosphere at 37 °C. Monochlorobimane, dichlorodihydrofluorescein diacetate (H_2DCFDA), rhodamine 123 (R123), and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), were obtained from Molecular Probes (Eugene, OR); benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-Fmk) from Enzyme Systems Products (Dublin, CA); and 4,6-diamidino-2-phenylindole (DAPI) from Serva (Heidelberg, Germany). All other reagents were obtained from Sigma Chemical Co. (Madrid, Spain). Stock solutions of etoposide (20 mM), camptothecin (10 mM), monochlorobimane (200 mM), *N*-acetyl-L-cysteine (NAC, 3 M), dihydroethidium (DHE, 10 mM), Z-VAD-Fmk (20 mM), JC-1 (0.3 mM), carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (10 mM), and oligomycin (30 mM) were prepared in dimethyl sulfoxide; butylated hydroxyanisole (BHA, 0.5 M) and H_2DCFDA (5 mM) in ethanol; melphalan (164 mM) in a mixture of ethanol/HCl (40/1, v/v); and *cis*-platinum(II)-diammine dichloride (cisplatin, 3.3 mM) and doxorubicin (20 mM) in distilled water. All these solutions were stored at −20 °C. In some experiments we used a commercial preparation of cisplatin (PLACIS, Chiesi Wasserman, Barcelona, Spain), with similar results. Stock solutions of DAPI (10 μg/ml), propidium iodide (PI, 1 mg/ml), and R123 (1 mg/ml) were prepared in phosphate-buffered saline (PBS) and stored at 4 °C. BSO (50 mM) was freshly prepared in distilled water, just before use. For GSH depletion, the cells were incubated for 24 h with 1 mM BSO, as earlier described by Ghibelli *et al.* (28). Under these conditions BSO did not affect cell proliferation nor viability, at least during 30 h of incubation. For ATP depletion, the cells were incubated in the presence of 10 μM oligomycin in glucose-free RPMI medium (Life Technologies, Inc.) supplemented with 1 mM sodium pyruvate and 10% dialyzed FCS. Oligomycin inhibits mitochondrial F_0- or F_1-ATPases and depletion of glucose blocks glycolysis, suppressing all sources of ATP (29). Hypoxia was induced with the use of a cell culture incubator perfused with 1% O_2/5% CO_2/94% N_2.

Flow Cytometry—The analysis of samples was carried out using an EPICS XL flow cytometer (Coulter, Hialeah, FL) equipped with an air-cooled argon laser tuned to 488 nm. The specific fluorescence signals corresponding to H_2DCFDA and R123 were collected with a 525-nm band pass filter, the signal corresponding to JC-1 with a 575-nm band pass filter, and the signals corresponding to PI and DHE with a 620-nm band pass filter.

Determination of Apoptosis—To analyze changes in nuclear morphology, cells were collected by centrifugation, washed with PBS, resuspended in PBS, and mounted on glass slides. After fixation in 70% (v/v) ethanol, the cells were stained for 20 min at room temperature in PBS containing 1 μg/ml DAPI and examined by fluorescence microscopy. Apoptosis was characterized by chromatin condensation followed by partition into multiple bodies. Within the experimental time periods used in this work, non-apoptotic, primary necrotic cells still exhibited diffuse and uniform chromatin staining, as untreated cells.

To measure loss of DNA, cells were collected by centrifugation and incubated for 30 min in PBS containing 0.5 mg/ml RNase A. After the addition of PI (final concentration of 50 μg/ml) and permeabilization with Nonidet P-40 (0.1%, w/v), the cells were analyzed by flow cytometry. Late apoptotic cells exhibited sub-G_1 PI incorporation (hypo-diploid cells). Within the experimental time periods used, non-apoptotic, primary necrotic cells did not exhibit significant loss of DNA nor significant alterations in the cell cycle distribution in relation to untreated cells.

Determination of Necrosis—The criterion currently used to examine necrosis was the loss of membrane integrity, as measured by massive influx of either trypan blue or PI in non-permeabilized cells. In the first case, cells were incubated for 5 min with 0.2% (w/v) trypan blue and examined by microscopy using a Neubauer hemacytometer. Under these conditions, only necrotic cells were clearly stained. In the second case, non-permeabilized cells were suspended in PBS containing 50 μg/ml PI, and the fluorescence was analyzed by flow cytometry. Under these conditions only necrotic cells exhibited great fluorescence, whereas the fluorescence was null or very low in apoptotic cells (30).

Measurement of Reactive Oxygen Species—The intracellular accumulation of ROS was determined using the fluorescent probes DHE and H_2DCFDA. DHE preferentially measures $O_2^{\cdot-}$ (31). H_2DCFDA was commonly used to measure H_2O_2 (32), but it is now accepted that this probe is also sensitive to other peroxides (33). With this aim, 1 h prior to treatment with the cytotoxic agents the cells were collected by centrifugation, resuspended in RPMI medium without red phenol, and loaded with either 5 μM H_2DCFDA or 2 μM DHE. The fluorescence was measured at the desired time intervals by flow cytometry. Control cells were subjected to the same manipulation, except for treatment with the cytotoxic agents.

Measurement of Mitochondrial Transmembrane Potential (ΔΨm)—Cells were washed with PBS and then incubated for 20 min at 37 °C with PBS containing either 50 nM JC-1 or 1 μg/ml R123. After washing twice with FCS (in the case of JC-1) or once with PBS (in the case of R123), the cells were resuspended in PBS and the fluorescence was measured by flow cytometry. Under these conditions, incubation with the depolarizing agent carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (100 μM) greatly decreased ΔΨm.

Measurement of GSH Levels—To determine the total intracellular GSH content, samples of 5×10^6 cells were washed and resuspended in 400 μl of PBS containing 2 mM monochlorobimane (34). Upon incubation for 30 min at 37 °C in the dark, the cells were centrifuged and resuspended in 400 μl of PBS. Aliquots of 100 μl were taken to estimate the fluorescence, using a POLARstar Galaxy fluorometer (BMG Labtechnologies, Offenburg, Germany) at excitation wavelength of 390 nm and emission wavelength of 520 nm.

To determine the mitochondrial GSH, samples of 1.5×10^8 cells were washed with PBS and resuspended in 8 ml of ice-cold buffer A (20 mM Hepes-KOH, 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM KH_2PO_4, 1 mM EGTA, 250 mM sucrose, 10 mM Tris-HCl, pH 7.6), and homogenized by 10 strokes in an ice-cold Dounce homogenizer. Non-lysed cells and nuclei were pelleted by centrifugation at $750 \times g$ for 10 min at 4 °C, the supernatant centrifuged again at $10{,}000 \times g$ for 15 min at 4 °C, and the resulting mitochondrial pellet was resuspended in 500 μl of ice-cold buffer A. Aliquots of 100 μl were taken to estimate the mitochondrial GSH content, as indicated above.

Measurement of ATP Levels—To estimate the intracellular ATP content, aliquots of 2×10^6 cells were collected in a pre-heated (70 °C) buffer consisting of 100 mM Tris and 4 mM EDTA, pH 8, and heated for 2 min at 100 °C. After cooling on ice and centrifugation at $1500 \times g$ for 1 min 4 °C, the ATP content in the supernatants was determined using an ATP Bioluminescence Assay kit CLSII (Roche Diagnostics, Barcelona, Spain), following the procedure indicated by the manufacturer, and a TD-20/29 luminometer (Turner Designs, Sunnyvale, CA). ATP standard curves (linear in the range of 5–500 nM) were carried out in all experiments. Extracts from cells depleted of ATP by incubation with oligomycin-containing glucose-free medium were used as control of the technique.

Immunoblot Assays—The whole procedure was as previously described (35). The antibody used was rabbit anti-human PKCδ polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

RESULTS

ROS Production—First, we investigated the capacity of antitumor drugs to cause intracellular oxidation, either under

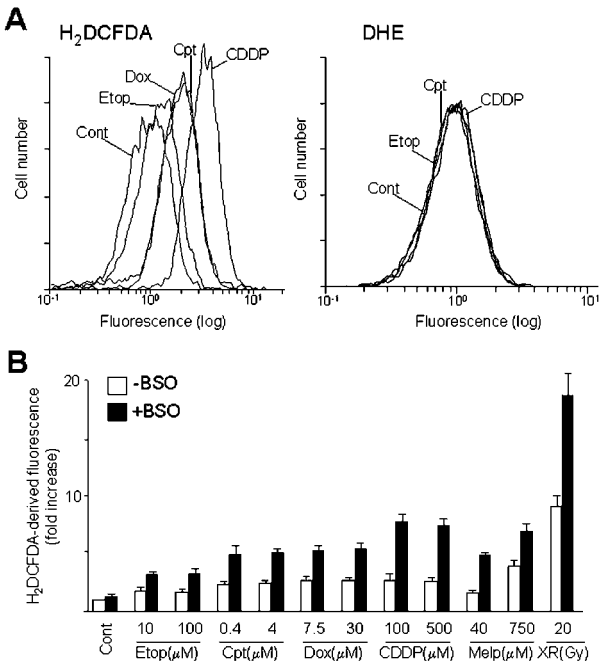

FIG. 1. **ROS generation by antitumor drugs and x-rays.** To determine the intracellular content of peroxides and anion superoxide, U-937 cells were loaded with H_2DCFDA and DHE, respectively, and the fluorescence was measured by flow cytometry. *A*, example of cell distribution according to their H_2DCFDA- and DHE-derived fluorescence in untreated cultures (*Cont*) and in cultures treated for 1 h with 10 μM etoposide (*Etop*), 0.4 μM camptothecin (*Cpt*), 7.5 μM doxorubicin (*Dox*), and 100 μM cisplatin (*CDDP*). *B*, increase in H_2DCFDA-derived fluorescence at 30 min of treatment with the indicated concentrations of etoposide, camptothecin, doxorubicin, cisplatin, and melphalan (*Melp*) or at 30 min of recovery after x-ray pulse treatment (*XR*), with and without preincubation with BSO. The values (mean ± S.D. of at least three determinations) are expressed in relation to untreated cells, which received the arbitrary value of one. BSO (1 mM) was applied 24 h before treatment with antitumor drugs and x-rays and maintained during the treatment and recovery periods.

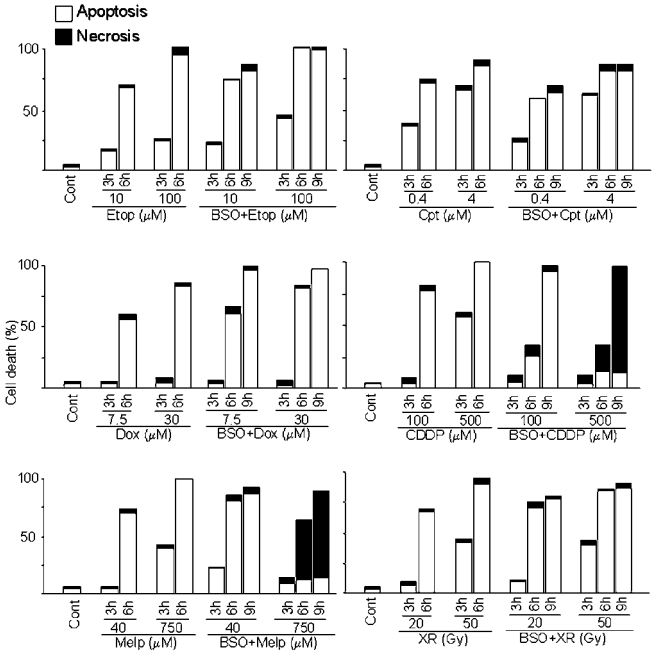

FIG. 2. **Cell death induction by antitumor drugs and x-rays.** The *histograms* represent the frequency of apoptotic and necrotic cells, as determined by chromatin fragmentation and trypan blue permeability, respectively, in cells treated for the indicated time periods with the antitumor drugs, or allowed to recover for the indicated time periods after x-rays pulse-treatment, with and without preincubation with BSO. The values are representative of one of at least three determinations with similar results. All other conditions were as in Fig. 1.

standard culture conditions or following GSH depletion. The antitumor drugs used were as follows: the DNA topoisomerase II inhibitors etoposide and doxorubicin, the DNA topoisomerase I inhibitor camptothecin, and the alkylating agents cisplatin and melphalan. As a positive control, cells were treated with x-rays (20 grays), a well-known oxidation-inducing agent (36). For GSH depletion, the cells were preincubated for 24 h with 1 mM BSO, which was maintained during treatment with the antitumor drugs and during recovery after x-rays treatment. Measurements using the GSH-sensitive probe monochlorobimane revealed that BSO caused an ~70% decrease in both the total intracellular and mitochondrial GSH pools (from 8.9 and 0.06 nmol/10^6 cells for total and mitochondrial GSH, respectively, in untreated cells; to 2.6 and 0.019 nmol/10^6 cells for total and mitochondrial GSH, respectively, in BSO-treated cells). Oxidation was determined by measuring the H_2DCFDA- and DHE-derived fluorescence, as an indication of peroxides and anion superoxide accumulation, respectively. Fig. 1 shows the results obtained with 10 and 100 μM etoposide, with 7.5 and 30 μM doxorubicin, with 0.4 and 4 μM camptothecin, with 100 and 500 μM cisplatin, and with 40 and 750 μM melphalan. Treatment with all antitumor drugs increased the H_2DCFDA-derived fluorescence, which was roughly independent of drug concentration (with the only exception of melphalan), and was always potentiated by preincubation with BSO. The increase in fluorescence was always inhibited in a 50–70% by the H_2O_2-specific scavenger catalase (results not shown; see also Fig. 4), indicating that it represented at least in part H_2O_2 accumulation. By contrast, we were unable to detect any increase in DHE-derived fluorescence in cells treated for 30–120 min with the antitumor drugs, either with or without preincubation with BSO (Fig. 1*A*, and results not shown). In this later assay doxorubicin was omitted, due to its strong autofluorescence at 620 nm.

Cell Death—Then, we analyzed the capacity of the antitumor drugs and x-rays, with and without preincubation with BSO, to cause cell death. The results, indicated in Fig. 2, were as follows: (i) Treatment with all agents in the absence of BSO caused death by apoptosis, as revealed by the presence of cells with fragmented chromatin. Increasing the drug concentration, or the x-ray dosage, increased or accelerated apoptosis but did not significantly cause necrosis, as revealed by the low frequency of trypan blue-stained cells. (ii) Preincubation with BSO did not affect the frequency nor the mode of death (apoptosis) caused by etoposide, camptothecin, doxorubicin, and x-rays. (iii) By contrast, BSO inhibited apoptosis and occasionally caused necrosis in cells treated with cisplatin. Thus, when used with 100 μM cisplatin BSO delayed cell death, but the mode of death was mostly apoptotic; when used with 500 μM cisplatin, BSO almost totally switched the mode of death from apoptosis to necrosis; and when used with 250 μM cisplatin, BSO caused a mixed situation, *i.e.* approximately equal amounts of apoptosis and necrosis (results not shown; see also Fig. 6*A*). The generation of necrosis by cisplatin plus BSO was slightly delayed (~2–3 h) in relation to the generation of apoptosis by cisplatin alone. Thus, although 500 μM cisplatin alone caused ~95% apoptotic cells at 6 h of treatment, a similar amount of necrotic cells was obtained at 8–9 h of treatment with BSO plus 500 μM cisplatin. (iv) As in the case of cisplatin, BSO almost totally switched the mode of death from apoptosis to necrosis when used with 750 μM melphalan, suggesting that this phenomenon is a common characteristic of alkylating agents. However, BSO slightly accelerated or potentiated the

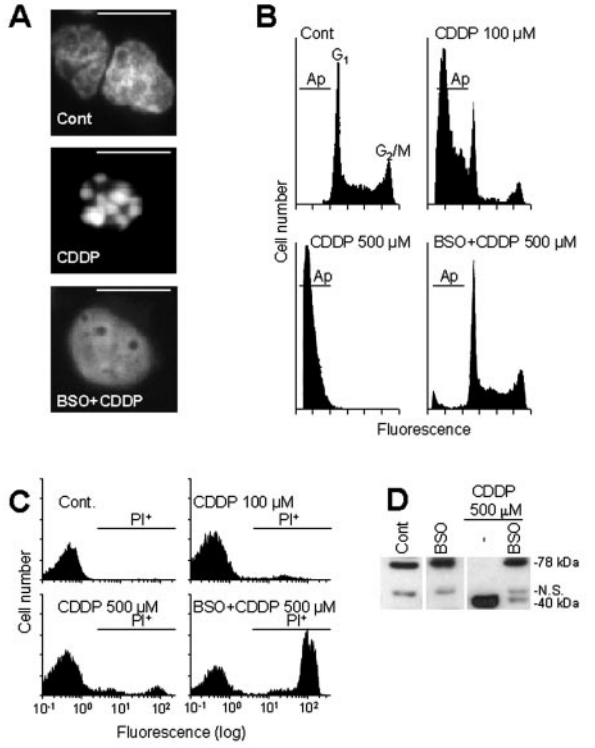

FIG. 3. **Expression of apoptotic and necrotic markers in cells treated with cisplatin, with and without preincubation with BSO.** *A*, examples of chromatin structure in nuclei from untreated cells (*Cont*), from cells treated with cisplatin alone (*CDDP*), and from cells preincubated with BSO and treated with 500 μM cisplatin (*BSO+CDDP*). *Bars*, 10 μm. *B*, cell distribution according to their DNA content, as measured by flow cytometry after cell permeabilization and PI staining. The fraction of cells with sub-G_1 DNA content (apoptotic cells) is indicated in each profile (*Ap*). *C*, free PI uptake, as determined by flow cytometry after addition of PI to non-permeabilized cells. The *bar* represents the region corresponding to PI-stained cells (*above the background* given by control cells). *D*, PKCδ cleavage, as determined by immunoblotting. The positions of the whole protein (~78 kDa) and the caspase-3-mediated cleavage fragment (~40 kDa) are indicated. *N.S.*, nonspecific band. All determinations were carried out at 6 h of treatment. The experiments in *B* and *C* were repeated three times, and those in *D* twice, with similar results. All other conditions were as in Fig. 1.

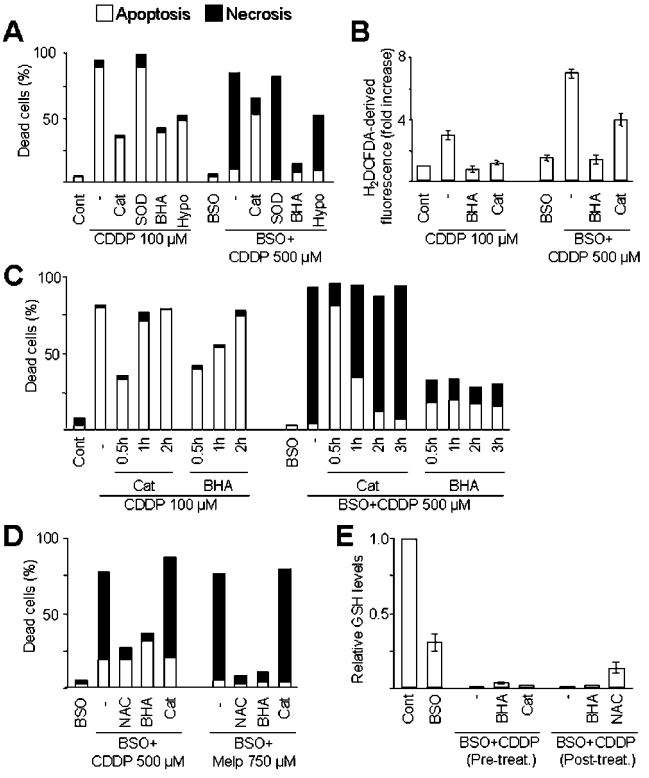

FIG. 4. **Effects of hypoxia and antioxidant agents.** For hypoxia (*hypo*), the cells were incubated at 1% O_2 atmosphere. The antioxidant agents were: catalase (*Cat*, 500 units/ml), SOD (400 units/ml), BHA (200 μM), and NAC (15 mM). *A*, frequency of apoptotic and necrotic cells in untreated cultures (*Cont*); in cultures incubated for 24 h with BSO (*BSO*); and in cultures treated for 8 h with cisplatin, with or without preincubation with BSO, and in the absence (−) or the presence of antioxidants or under hypoxic conditions. The antioxidants were applied 2 h before cisplatin and maintained during the treatment period. *B*, modulation of the H_2DCFDA-derived fluorescence at 30 min of treatment with cisplatin, following the same experimental conditions as in *A*. The values (mean ± S.D. of three determinations) are expressed in relation to untreated cells (*Cont*), which received the arbitrary value of one. *C*, changes in the frequency of apoptosis and necrosis by addition of antioxidant agents at 0.5, 1, 2, and 3 h of treatment with cisplatin. *D*, frequency of apoptotic and necrotic cells in BSO-preincubated cultures pulse-treated for 3 h with cisplatin or melphalan, then washed, and finally allowed to recover for 5 h in the absence or presence of antioxidant agents. BSO was always present during the recovery period. *E*, relative intracellular GSH content in BSO-preincubated cells treated for 6 h with cisplatin, with and without pre-treatment with antioxidant agents (*Pre-treat*); and in BSO-preincubated cells pulse-treated for 3 h with cisplatin and allowed to recover for 3 h with and without antioxidant agents (*Post-treat*). The values (mean ± S.D. of three determinations) are expressed in relation to untreated cells, which received the arbitrary value of one. All other conditions were as in Fig. 1. Approximate GSH content in untreated cultures: 9 nmol/10^6 cells.

generation of apoptosis without causing necrosis when used with a low concentration of melphalan (40 μM) and caused a mixed situation (approximately equal amounts of apoptosis and necrosis) when used with 400 μM melphalan (result not shown).

The necrotic cells obtained by treatment with BSO plus cisplatin or BSO plus melphalan exhibited cytoplasmic swelling and diffuse non-fragmented chromatin (Fig. 3*A*, and results not shown), suggesting that the observed necrosis is primary necrosis instead of apoptosis-derived secondary necrosis. To corroborate this fact, we measured other apoptotic and necrotic markers. It was observed that treatment with 500 μM cisplatin alone caused accumulation of cells with sub-G_1 DNA content, which is a typical marker of apoptosis, but this characteristic was absent in BSO plus 500 μM cisplatin-treated cells (Fig. 3*B*). By contrast, although free PI uptake was very low in cells treated with cisplatin alone, it was greatly increased in cells treated with BSO plus 500 μM cisplatin, indicating loss of plasma membrane integrity (Fig. 3*C*). Finally, although cisplatin alone elicited the typical caspase-3-mediated PKCδ cleavage to give a fragment of ~40 kDa, characteristic of apoptosis (37), BSO plus 500 μM cisplatin did not (Fig. 3*D*). Taken together, these results indicate that the observed necrosis is a *bona fide* apoptosis-independent, primary necrosis.

Effect of Antioxidants—To investigate the possible relevance of oxidation for apoptosis and necrosis induction, cells were treated with cisplatin either under hypoxic conditions or in the presence of antioxidant agents. The antioxidants used were catalase (500 units/ml, specific for H_2O_2), SOD (400 units/ml, specific for $O_2^{\cdot-}$), and the nonspecific ROS scavenger BHA (200 μM). Under these conditions, both hypoxia and the antioxidant agents were innocuous, at least during 9 h of incubation. Some of the obtained results are represented in Fig. 4*A*. It was observed that hypoxia and preincubation with catalase and BHA attenuated the generation apoptosis by cisplatin, as well as the generation of necrosis by BSO plus cisplatin. However, although hypoxia and BHA reduced necrosis without concomitant increase in apoptosis, catalase caused a partial reversion from necrosis to apoptosis. Under these conditions both BHA

and catalase inhibited, albeit to different extent, the increase in H_2DCFDA-derived fluorescence caused by both cisplatin alone and BSO plus cisplatin (Fig. 4*B*). Preincubation with SOD did not prevent apoptosis nor necrosis, which is consistent with the apparent failure of the antitumor drugs to cause $O_2^{\cdot-}$ accumulation (Fig. 1). The antioxidant agents caused the same effects on both cell death and ROS generation when cisplatin was substituted by melphalan (results not shown). Catalase also decreased the generation of apoptosis and the increase in H_2DCFDA-derived fluorescence in cells treated with etoposide and camptothecin, whereas SOD was ineffective (results not shown).

In the experiments performed above, the antioxidant agents were applied prior to treatment with antitumor drugs, to inhibit the trigger of oxidation. Hence, new experiments were performed in which the antioxidants were applied at different times of treatment with cisplatin, to allow ROS to accumulate for limited time periods. The results obtained are represented in Fig. 4*C*. Catalase was still able to attenuate apoptosis and to switch back necrosis to apoptosis when added at 0.5 and 1 h of treatment with cisplatin, respectively, but was ineffective when applied at later times. BHA also exhibited a time limitation (0.5–1 h) to attenuate apoptosis but was still able to inhibit necrosis when applied at later times (at least until 3 h, the maximum examined time period).

Because cisplatin is a very reactive molecule, we may not exclude direct cisplatin-BHA interactions, which could explain the inhibition of necrosis by BHA. For this reason new experiments were carried out in which BSO-preincubated cells were pulse-treated for 3 h with 500 μM cisplatin, then washed and allowed to recover in the absence or presence of catalase, BHA, or the nonspecific ROS scavenger NAC; or pulse treated for 1 h with cisplatin, then maintained for 2 h in cisplatin-free medium (with occasional washing to facilitate drug extrusion), and finally treated with the antioxidant agents. It was observed in all cases that BHA and NAC were still able to attenuate necrosis induction, whereas catalase was ineffective (Fig. 4*D* and results not shown). Moreover, allowing for minor quantitative differences, similar results were obtained using 750 μM melphalan, a drug with a different structure than cisplatin (Fig. 4*D*).

Finally, Fig. 4*E* shows the modulation of the total intracellular GSH content caused by BSO plus cisplatin, with and without pre-treatment or post-treatment with antioxidant agents. Although BSO alone caused a partial GSH decrease, BSO plus cisplatin caused an almost total depletion. The GSH depletion was not significantly affected by pre-treatment with BHA and catalase nor by post-treatment with BHA but was slightly reverted by post-treatment with NAC. This later effect seems to be consistent with the well-known role of NAC as a precursor of GSH synthesis (38).

Effects of Exogenous H_2O_2—The results in Fig. 4*A* indicate that oxidation mediates both apoptosis and necrosis induction by alkylating drugs, but they do not indicate the relevance of oxidation in determining the mode of death. Recent observations demonstrated that the administration of low concentrations of H_2O_2 inhibited apoptosis and caused death with features of necrosis in Burkitt's lymphoma cells treated with antitumor drugs (17). Hence, we wanted to know whether H_2O_2 could mimic the action of BSO in U-937 cells. The results are indicated in Fig. 5. (i) H_2O_2 caused apoptosis when used in the range of 100–500 μM, whereas higher concentrations caused necrosis (Fig. 5*A*). (ii) Although the combination of H_2O_2 plus cisplatin was more toxic than cisplatin alone, measured by apoptosis induction (Fig. 5*B*), H_2O_2 plus 500 μM cisplatin was unable to cause necrosis (Fig. 5*C*). (iii) The combination H_2O_2

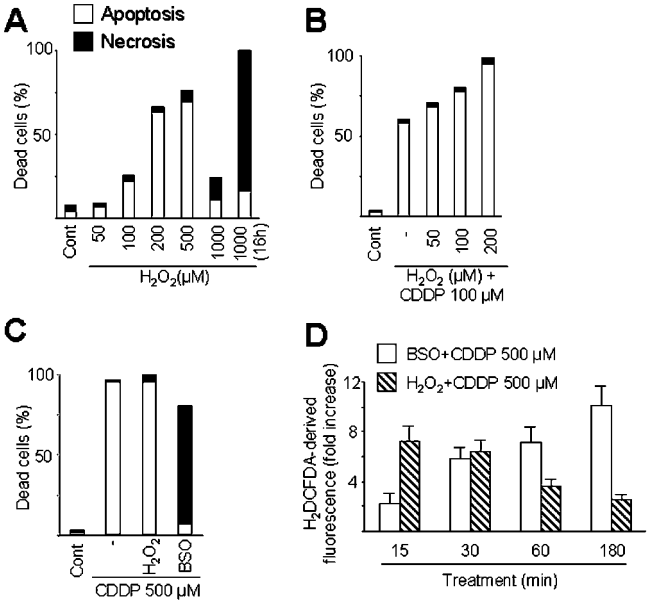

FIG. 5. **Effects of hydrogen peroxide.** *A*, frequency of apoptotic and necrotic cells in untreated cultures (*Cont*) and in cultures treated with the indicated concentrations of H_2O_2. Except when otherwise is indicated, the determinations were carried out at 6 h of treatment. *B*, frequency of apoptotic and necrotic cells in untreated cultures (*Cont*) and in cultures treated for 6 h with 100 μM cisplatin, either alone (−) or with the indicated concentrations of H_2O_2. *C*, frequency of apoptotic and necrotic cells in untreated cultures (*Cont*), in cultures treated for 8 h with 500 μM cisplatin alone (−), with 500 μM cisplatin plus 500 μM H_2O_2, and with 500 μM cisplatin following preincubation with BSO. All determinations were repeated at least three times, with similar results. *D*, H_2DCFDA-derived fluorescence at the indicated times of treatment with H_2O_2+CDDP and with BSO+CDDP, following the same conditions as in *C*. The values (mean ± S.D. of four determinations) are expressed in relation to untreated cells, which received the arbitrary value of one. All other conditions were as in Fig. 1.

plus 500 μM cisplatin caused a rapid increase in H_2DCFDA-derived fluorescence, which was initially (15 min) higher than that caused by BSO plus 500 μM cisplatin. However, although the fluorescence declined later in H_2O_2 plus cisplatin-treated cells, it was still augmented in BSO plus cisplatin-treated cells, at least until 3 h of treatment (Fig. 5*D*). Hence, the induction of necrosis by BSO plus cisplatin seems to be associated to the permanence of the oxidant state.

Caspase Inhibition—Caspases are oxidation-sensitive proteases required for the execution of apoptosis, in such a manner that caspase inactivation may suppress apoptosis and lead the cells into necrosis (39, 40). Because BSO plus 500 μM cisplatin failed to elicit the typical caspase-3-mediated PKCδ cleavage (Fig. 3*D*), we asked whether the induction of necrosis could be a mere consequence of caspase inactivation. To investigate this possibility, we analyzed the effect of the nonspecific caspase inhibitor Z-VAD-Fmk (50 μM) in cells treated with cisplatin, either with or without BSO. The results were as follows: (i) Z-VAD-Fmk did not inhibit but potentiated the induction of necrosis by BSO plus cisplatin. This later effect was observed using BSO plus an intermediate concentration of cisplatin (250 μM), which caused both apoptosis and necrosis (Fig. 6*A*). (ii) Z-VAD-Fmk suppressed the generation of apoptosis by 500 μM cisplatin alone, leading to necrosis (Fig. 6*B*). However, under these conditions necrosis was slightly manifested at 24 h, *i.e.* at a much later time than in the case of BSO plus cisplatin (Fig. 2*B*). Hence, although it is clear that caspase inhibition influences the mode of death, the switch from apoptosis to necrosis in BSO plus cisplatin-treated U-937 cells may not be merely explained as a consequence of caspase inactivation.

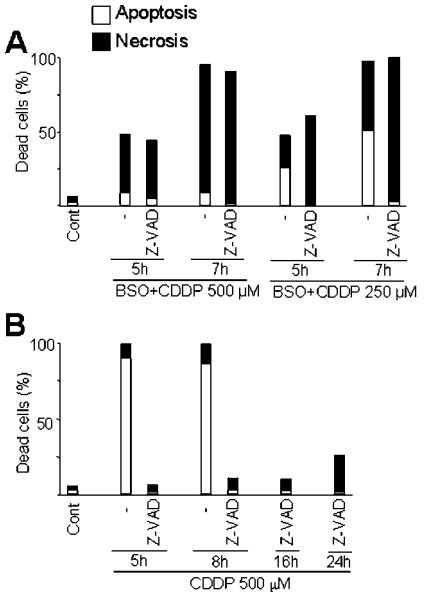

FIG. 6. **Effect of the caspase inhibitor Z-VAD-Fmk on cell death.** *A*, frequency of apoptotic and necrotic cells in untreated cultures (*Cont*) and in cultures treated for the indicated time periods with BSO plus the indicated concentrations of cisplatin, either in the absence (−) or presence of 50 μM Z-VAD-Fmk. *B*, frequency of apoptotic and necrotic cells in untreated cultures (*Cont*) and in cultures treated for the indicated time periods with 500 μM cisplatin alone, either in the absence (−) or presence of 50 μM Z-VAD-Fmk. All determinations were repeated three times with similar results. All other conditions were as in Fig. 1.

ATP Levels—It has been reported that the adoption of the apoptotic or the necrotic pathway may be determined by the availability of intracellular ATP (29, 41). Hence, we wanted to measure the changes in ATP levels after treatment with antitumor drugs, with and without preincubation with BSO. The treatments were carried out for a maximum of 3 h, to prevent possible ATP leakage through damaged plasma membrane in cells undergoing necrosis. Fig. 7 shows the obtained results, using a luciferin/luciferase-based detection procedure. (i) As expected, incubation with oligomycin in glucose-free medium caused an almost complete depletion of ATP. However, incubation with oligomycin in glucose-containing medium (either standard RPMI medium, or commercial glucose-free RPMI medium supplemented with glucose) only slightly decreased the ATP level (Fig. 7*A*). This later result agrees with earlier observations in U-937 cells, indicating that glycolysis is the main source of ATP in this cell type (42). (ii) Allowing for quantitative differences, treatment with etoposide, melphalan, and cisplatin alone caused a decrease in ATP content (Fig. 7*B*). (iii) By contrast, the results were markedly different when the cells were preincubated with BSO. In fact, BSO did not significantly modify the ATP decrease caused by etoposide; it apparently accelerated the decrease caused by melphalan, as observed at 2 h of treatment; and, surprisingly, it prevented the decrease caused by cisplatin, in such a manner that at 2 and 3 h the ATP level was even higher in BSO plus cisplatin-treated cells than in control cells (Fig. 7*B*). Of note, this later result (which was repeated in eight independent experiments) represented a *bona fide* measurement of ATP instead of a possible artifact of the used technique, because the luminescence was almost null when the treatments with cisplatin alone and with BSO plus cisplatin were carried out under ATP-depleting (oligomycin-containing, glucose-free) culture conditions (Fig. 7*B*). (iv) For comparative purposes, we also measured the effect of H_2O_2, alone and in combination with cisplatin. Treatment with 500 μM H_2O_2 alone, which caused apoptosis, only slightly reduced the ATP content, whereas treatment with 1 mM, which provoked necrosis, caused a great decrease. Treatment with H_2O_2 plus cisplatin accelerated the decrease in ATP content, when compared with the action of either cisplatin or H_2O_2 alone (Fig. 7*B*).

To determine whether the accelerated ATP depletion caused by BSO plus melphalan could be determinant for necrosis induction, we analyzed the effect of catalase and BHA on the ATP content. The results in Fig. 7*C* show that preincubation with the antioxidants did not reduce the drop in ATP, which contrasts with their capacity to attenuate the generation of necrosis as indicated above.

Finally, we wanted to know whether the action of BSO plus alkylating agents on cell death could be mimicked by treatment with the alkylating drugs alone under ATP-depleting culture conditions. The results are indicated in Fig. 7*D*. Cell culture in deficient (oligomycin-containing, glucose-free) medium was *per se* toxic, as evidenced by the presence of both apoptosis and necrosis at 6 h. The generation of apoptosis by 500 μM cisplatin and 750 μM melphalan was lower in this deficient medium than in standard culture conditions. However, the decrease in apoptosis was not compensated by an increase in necrosis, which contrasts with the results obtained with BSO plus cisplatin or BSO plus melphalan (Fig. 2*B*). Moreover, treatment with etoposide (100 μM) in deficient medium caused the same effect as cisplatin and melphalan. Longer treatments in deficient medium had to be avoided, because the basal toxicity was very high. Taken together, these results demonstrate that the suppression of apoptosis and the generation of necrosis by BSO plus alkylating agents may not be explained by a selective suppression of ATP.

Mitochondrial Transmembrane Potential—The induction of cell death (apoptotic and necrotic) is generally associated with, and probably mediated by perturbations in the mitochondrial function, a manifestation of which is the dissipation of the transmembrane potential (ΔΨm). In some models, the ΔΨm decay was more rapid and prominent during necrosis than during apoptosis (43, 44). Hence, we wanted to analyze ΔΨm during apoptosis and necrosis induction in antitumor drug-treated U-937 cells. Fig. 8*A* shows the results obtained with cisplatin using two different fluorescent probes, namely JC-1 and R123 (45). Treatment with 500 μM cisplatin alone caused an early increase in JC-derived fluorescence (1 h), which was followed by a slight decrease at 6 h. BSO plus 500 μM cisplatin also caused an early increase in JC-1-derived fluorescence (1 h), which was rapidly followed by a great decrease. In this case, the reduction of ΔΨm was clearly observed at 3 h, a time at which cell membrane damage (manifested by trypan blue uptake) was still negligible (Fig. 2*B*). The use of R123 produced some differences in relation to JC-1, namely the lack of the early increase in fluorescence, and a more rapid and prominent decrease during apoptosis (detected at 3 and 6 h of treatment with cisplatin alone). Nonetheless, R123 also revealed a deeper decrease in fluorescence under necrosis-inducing (BSO plus cisplatin) than under apoptosis-inducing conditions. The results obtained with BSO plus melphalan were the same as with BSO plus cisplatin (results not shown). Treatments with etoposide and camptothecin also caused a late decrease in ΔΨm, but the decrease was not influenced by preincubation with BSO (results not shown).

To analyze whether ΔΨm dissipation and necrosis induction are correlated, we examined the capacity of antioxidant agents to modulate ΔΨm. The results, using the JC-1 probe, are indicated in Fig. 8 (*B* and *C*). The administration of catalase and BHA prior to treatment with BSO plus cisplatin attenuated the ΔΨm decrease (Fig. 8*B*). This result correlates with the capac-

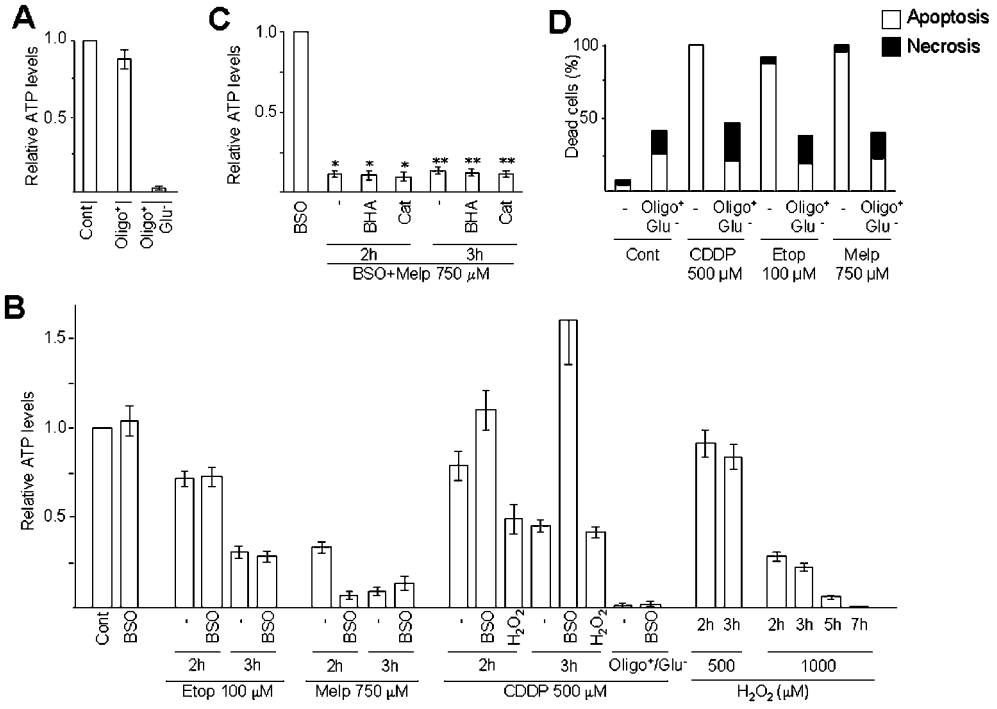

FIG. 7. **Modulation of ATP levels and effects of ATP depletion.** The histograms in *A–C* show the relative ATP content in cells extracts, using a luciferin/luciferase-based procedure. The results (mean ± S.D. of at least three determinations) are represented in relation to the corresponding control (untreated cells (*A* and *B*) or cells incubated with BSO (*C*)), which were given the arbitrary value of one. The ATP content in untreated cells was 24.2 ± 3.1 nmol/10^6 cells. *A*, ATP levels in untreated cells (*Cont*), in cells treated for 3 h with 10 μM oligomycin (*Oligo*$^+$), and in cells treated with oligomycin in a glucose-free medium (*Oligo*$^+$/*Glu*$^-$). *B*, ATP levels in untreated cells (*Cont*); in cells incubated for 24 h with BSO (*BSO*); in cells treated for the indicated time periods with the indicated concentrations of H_2O_2; in cells treated for the indicated time periods with the indicated concentrations of etoposide, melphalan, and cisplatin, either with (BSO) or without (−) preincubation with BSO; and in cells treated with cisplatin plus 500 μM H_2O_2. As an internal control, cells (with or without preincubation with BSO) were treated for 3 h with 500 μM cisplatin and 10 μM oligomycin in glucose-free medium. *C*, ATP levels in cells incubated with BSO (*BSO*), and in cells preincubated with BSO and treated for the indicated time periods with 750 μM melphalan, either alone (−) or with BHA and catalase (*BHA*, *Cat*). The antioxidants were applied 2 h before melphalan and maintained during the treatment period. *Asterisks*, no significant differences ($p > 0.1$, Student's t test). *D*, frequency of apoptotic and necrotic cells at 6 h of incubation under standard culture conditions (−) or in oligomycin-containing glucose-free medium (*Oligo*$^+$/*Glu*$^-$), either in the absence (*Cont*) or the presence of the indicated concentrations of etoposide, cisplatin, and melphalan. The determinations were repeated four times with similar results. All other conditions were as in Figs. 1 and 4.

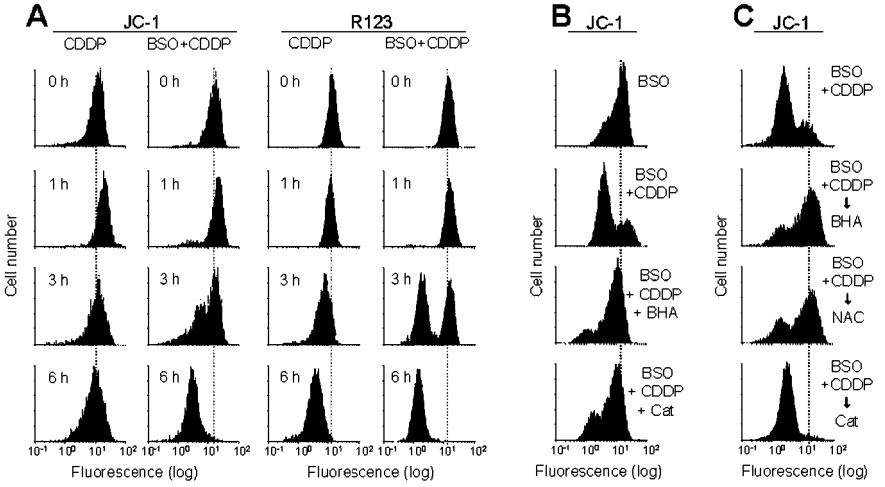

FIG. 8. **Modulation of mitochondrial transmembrane potential (ΔΨm).** Modulation of ΔΨm, as determined by changes in fluorescence upon JC-1 or R123 loading. *A*, cells were treated for the indicated time periods with 500 μM cisplatin, with or without preincubation with BSO. *B*, cells were incubated for 24 h with BSO (*BSO*) or preincubated with BSO and treated with 500 μM cisplatin, alone and in the presence of BHA or catalase. The antioxidants were applied 2 h before cisplatin and maintained during the treatment period. The determinations were carried out at 6 h. *C*, cells preincubated with BSO were firstly pulse-treated for 3 h with 500 μM cisplatin, then washed and allowed to recover for 5 h in the absence or the presence of BHA, NAC, and catalase. BSO was present during the recovery period. The *vertical*, *dotted lines* represent the mean fluorescence value in the corresponding control (untreated cells, or cells incubated with BSO alone), to better discern the displacement caused by the treatments. All determinations were repeated at least twice with the same results. All other conditions were as in Figs. 1 and 4.

ity of both antioxidants to reduce necrosis, when used under similar conditions (Fig. 4*A*). By contrast, when the antioxidants were applied after a 3-h pulse treatment with BSO plus cisplatin, only BHA and NAC were able to attenuate the ΔΨm decrease, whereas catalase was ineffective (Fig. 8*C*). Again, this result fully correlates with the capacity of BHA and NAC, and

with the inability of catalase, to reduce necrosis, when used under similar conditions (Fig. 4*D*). The administration of catalase, BHA, and NAC with BSO plus 750 μM melphalan produced exactly the same effects as that found with BSO plus cisplatin (results not shown).

Looking for possible targets for alkylating drugs in the mitochondria, we examined the expression of Bax, Bcl-2, and Bcl-X_L, because the relative levels of these proteins might influence the mode of death (46). It was found that the expression of these proteins remained unaltered upon treatment with cisplatin, with and without preincubation with BSO, and with and without post-treatment with BHA and NAC (results not shown). The possibility that necrosis could be due a specific disruption of the respiratory chain may also be discarded, because treatment with oligomycin (which inhibits the mitochondrial F_0/F_1-ATPase) in glucose-containing medium only had minor effects on the ATP level (Fig. 7*A*) and did not cause cell death (result not shown).

DISCUSSION

The present results indicate that antitumor drugs (topoisomerase inhibitors and alkylating agents) rapidly induce intracellular oxidation in U-937 human promonocytic cells, as measured by peroxide accumulation. Oxidation effectively mediates apoptosis and necrosis induction, as revealed by the capacity of the H_2O_2-specific antioxidant catalase and the nonspecific antioxidant BHA, and by incubation under hypoxic conditions, to attenuate both forms of death. However, we did not detect significant accumulation of O_2^-, and the antioxidant SOD (specific for O_2^-) failed to protect the cells. Although we may not totally exclude that the inability to detect O_2^- could be due to technical reasons, it must be noted that similar results (concerning both the lack of O_2^- detection and the ineffectiveness of SOD) were obtained by other authors using HL-60 human promyelocytic cells treated with antitumor drugs (11). These results seem to emphasize the pivotal importance of H_2O_2 for death regulation in myeloid cells, as reported earlier (8, 11, 47).

In addition, our results indicate that preincubation with BSO potentiates the trigger of oxidation by all antitumor drugs, as revealed by the increase in H_2DCFDA-derived fluorescence. Nevertheless, this was not necessarily followed by an increase in toxicity. Thus, although BSO potentiated the toxicity of cisplatin and melphalan, manifested by the suppression of apoptosis and the induction of necrosis, it did not affect the mode (apoptosis) nor the extent of death caused by camptothecin, etoposide, and doxorubicin. The uncoupling between intensity of oxidation and toxicity was especially evident in the control experiments with x-rays and H_2O_2. In fact, the trigger of oxidation by BSO plus x-rays was higher than that caused by BSO plus cisplatin; nevertheless BSO did not affect the lethality of x-rays. In a similar manner, the trigger of oxidation, measured by the initial increase in H_2DCFDA-derived fluorescence, was higher in H_2O_2 plus cisplatin-treated cells than in BSO plus cisplatin-treated cells; nevertheless H_2O_2 plus cisplatin did not cause necrosis. Nevertheless, this later experiment revealed an additional fact, namely that the hyperoxidation was much more stable in BSO plus cisplatin-treated cells than in H_2O_2 plus cisplatin-treated cells. This observation, and the fact that the antioxidants BHA and NAC were able to attenuate necrosis even when added 3 h after treatment with BSO plus cisplatin (or with BSO plus melphalan), might indicate that necrosis is mainly determined by the permanence of the oxidant state rather than by its initial intensity. By contrast, a transient, initial increase in oxidation may suffice to trigger apoptosis, because antioxidants only prevented apoptosis when applied prior shortly (0.5–1 h) after treatment with cisplatin. Of note, we recently obtained similar conclusions using the heavy metal cadmium in appropriate combination with BSO and antioxidant agents (48). Another report indicated that BHA was able to prevent cell death when applied as late as 12 h after treatment with cisplatin or tumor necrosis factorα in human ovarian carcinoma cells, although in this case the mode of cell death was not indicated (49). Nevertheless, it must be noted that our results are not coincident with those obtained using Burkitt's lymphoma cells, where H_2O_2 suppressed the generation of apoptosis by cisplatin and other antitumor drugs, causing necrotic-like death (17). This may probably be explained by the unequal sensitivity of the different cell types to H_2O_2, because concentrations below 100 μM, which were clearly toxic for lymphoid cells (17), are innocuous for U-937 cells (our present results).

Looking for oxidation-sensitive factors that could regulate the selection between apoptosis and necrosis, we examined caspase inhibition, ATP levels, and the mitochondrial function. According to some models, necrosis is a defective mode of death that the cells are forced to adopt when the execution of apoptosis is hindered by caspase inactivation or by the lack of energy. Excessive ROS production may affect caspases, either by directly inhibiting their activity (39, 40) or preventing their activation (50). Earlier observations indicated that the caspase inhibitor Z-VAD-Fmk potentiated the induction of necrosis by tumor necrosis factor in L929 cells (51). In a similar manner, in our experiments Z-VAD-Fmk potentiated the induction of necrosis by BSO plus 250 μM cisplatin in U-937 cells, indicating that caspases are involved in the regulation of the mode of death in this model. However, caspase inactivation may not explain the switch from apoptosis to necrosis by BSO plus cisplatin, because this treatment caused necrosis much more rapidly (6 h) than Z-VAD-Fmk plus cisplatin (initiated at 24 h). Concerning the supply of energy, excessive ROS accumulation might affect ATP synthesis in different manners, *e.g.* by inhibiting mitochondrial ATP synthase activity (52) and by altering and inactivating glycolytic enzymes (53). Actually, oxidation-mediated ATP depletion was presented as the factor ultimately responsible for apoptosis inhibition and necrosis induction in H_2O_2 plus antitumor drug-treated Burkitt's lymphoma cells (54). In our experiments, H_2O_2 also caused a greater decrease in ATP content at the necrosis-inducing concentration (1 mM) than at the apoptosis-inducing concentration (500 μM). Moreover, we observed that BSO potentiated ATP depletion by melphalan. However, it seems clear that ATP depletion was not determinant for the switch from apoptosis to necrosis in BSO plus melphalan-treated cells, because (i) antioxidants attenuated necrosis induction without attenuating ATP depletion and (ii) treatment with melphalan under ATP-depleting culture conditions (oligomycin-containing, glucose-free medium) suppressed apoptosis but did not immediately cause necrosis, as was done by BSO plus melphalan. Moreover, although BSO plus cisplatin caused necrosis, this treatment did not decrease, and even augmented the ATP levels. However, this surprising result requires further investigation and, hence, must be considered with caution.

By contrast, our results show a close correlation between necrosis induction and alteration in the mitochondrial function, as revealed by $\Delta\Psi$m dissipation. After an occasional initial increase in fluorescence (which in our experiments was only observed using JC-1), interpreted by some authors as a transient mitochondrial hyperpolarization (55, 56), all treatments, apoptotic and necrotic, caused a late $\Delta\Psi$m depolarization. The late $\Delta\Psi$m decrease was more rapid and pronounced under necrosis-inducing than apoptosis-inducing conditions. Moreover, this late decrease was prevented by antioxidants when (and only when) the

antioxidants attenuated necrosis. Because the $\Delta\Psi m$ decrease apparently preceded other manifestations of necrosis, *e.g.* plasma membrane damage, we may speculate that mitochondrial dysfunction regulates necrosis induction. Actually, the extent of mitochondrial dysfunction, measured by $\Delta\Psi m$ decay, was also proposed to be the determinant for the selection between apoptosis and necrosis in other cell models (57).

What could be the relationship between GSH depletion, mitochondrial dysfunction, and alkylating drug toxicity? Although it is generally assumed that the toxicity of antitumor drugs is the consequence of their capacity to cause genomic DNA damage (DNA adducts in the case of alkylating drugs, and topoisomerase-mediated DNA strand breaks in the case of topoisomerase inhibitors), alkylating agents also exert important effects in other cellular compartments (58). In particular, cisplatin accumulates in mitochondria, altering the mitochondrial structure and function (59, and references therein). Because alkylating drugs are detoxified by GSH conjugation (60), it may be expected that the intracellular (and mitochondrial) free, acting drug concentration is increased following GSH depletion. In addition GSH is transported to the mitochondria, where it plays a protective role as an ROS scavenger (61). Both factors, increased local alkylating drug concentration and decreased local antioxidant activity, could result (among other effects) in a drastic and prolonged overaccumulation of ROS in the mitochondria of BSO-treated cells, causing irreversible mitochondrial damage and leading to necrosis. Actually, increased ROS accumulation in mitochondria has been proposed as the mechanism responsible for necrosis induction in some cell systems, *e.g.* in tumor necrosis factor-treated L929 cells (62). Under these conditions, BHA and NAC might enter into the mitochondria and operate as local ROS scavengers, attenuating mitochondrial damage (as revealed by the reduction of $\Delta\Psi m$ dissipation) and, hence, necrosis. In fact, there are multiple reports indicating that BHA and NAC prevent cell death by stabilizing the mitochondrial structure and function in different cell systems (62–64). This may also explain the inability of catalase to prevent $\Delta\Psi m$ decrease and necrosis induction, at least when applied as a post-treatment. Because exogenous catalase does not penetrate the plasma membrane, it may not directly protect the mitochondria, only acting as a scavenger of the H_2O_2 which diffuses out of the cell.

In summary, the present results indicate that GSH depletion potentiates to some extent oxidation induction by different antitumor drugs in human promonocytic cells, but only affects the toxicity of alkylating agents, occasionally switching the mode of death from apoptosis to necrosis. The induction of necrosis seems to be the consequence of severe mitochondrial damage, derived from the particular action mechanism of the alkylating drugs.

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Alfonso Troyano, Carlos Fernández, Patricia Sancho, Elena de Blas and Patricio Aller

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