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Mitochondrial alterations induced by serum amine oxidase and spermine on human multidrug resistant tumor cells

G. Arancia[1], **A. Calcabrini**[1], **M. Marra**[1], **P. Crateri**[1], **M. Artico**[2], **A. Martone**[3], **F. Martelli**[3], and **E. Agostinelli**[3]

[1] Ultrastructures Laboratory, Istituto Superiore di Sanità, Rome, Italy
[2] Department of Pharmacology of Natural Substances and General Physiology and
[3] Department of Biochemical Sciences "A. Rossi Fanelli", University of Rome "La Sapienza" and CNR, Centre of Molecular Biology, Rome, Italy

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Summary. Multidrug resistance (MDR) has been studied extensively because it is one of major problems in cancer chemotherapy. The MDR phenotype is often due to overexpression of P-glycoprotein (P-gp), that acting as an energy-dependent drug efflux pump exports various anticancer drugs out of cells. The major goal of our investigation is to establish whether bovine serum amine oxidase (BSAO), which generates the products H_2O_2 and aldehyde(s), from the polyamine spermine, is able to overcome MDR of human cancer cells. The cytotoxicity of the products was evaluated in both drug-sensitive (LoVo WT) and drug-resistant (LoVo DX) colon adenocarcinoma cells. A clonogenic cell survival assay demonstrated that LoVo DX cells were more sensitive than LoVo WT cells. Exogenous catalase protected cells against cytotoxicity mainly due to the formation of H_2O_2. However, spermine-derived aldehyde(s) still induced some cytotoxicity. The cytotoxic effect was totally inhibited in the presence of both enzymes, catalase and NAD-dependent aldehyde dehydrogenase (ALDH). Transmission electron microscopy investigations showed that BSAO and spermine induced evident mitochondria alterations, more pronounced in MDR than in LoVo WT cells. The mitochondrial activity was checked by flow cytometry studies, labelling cells with the probe JC1, that displayed a basal hyperpolarized status of the mitochondria in multidrug-resistant cells. After treatment with amine oxidase in the presence of polyamine-spermine, the cells showed a marked increase in mitochondrial membrane depolarization higher in LoVo DX than in LoVo WT cells. Our findings suggest that toxic oxidation products formed from spermine and BSAO could be a powerful tool in the development of new anticancer treatments, mainly against MDR tumor cells.

Keywords: Polyamines – Amine oxidase – Multidrug resistance – Adenocarcinoma – Cytotoxicity – Mitochondria

Introduction

It is known that polyamines are polycations required for both eukaryotic and prokaryotic cell growth and differentiation (Pegg, 1986). The naturally occurring polyamines spermine and spermidine are the biosynthetic products of putrescine, which is derived from ornithine by the action of ornithine decarboxylase (ODC), the rate-limiting enzyme (William-Ashman and Canellakis, 1979; Tabor and Tabor, 1984). In *in vitro* studies, it was demonstrated that the addition of spermidine and spermine to culture medium, supplemented with calf serum, inhibits DNA and protein synthesis as well as cellular proliferation (Gaugas and Dewey, 1979; Bachrach et al., 1987; Averill-Bates et al., 1993). These effects are caused by hydrogen peroxide and aldehyde(s), formed during the enzymatic reaction of bovine amine oxidase, contained in the serum, with polyamines. Moreover, both the oxidation products of polyamines are implicated in programmed cell death (Parchment and Pierce, 1989) and can cause apoptosis in several cell types (Lindsay and Wallace, 1999).

The enzyme from bovine serum, a copper amine oxidase (BSAO, EC 1.4.3.6) with 6-hydroxydopa as organic cofactor, incorporated into the peptide chain within the active site, is a glycoprotein of 170 KDa MW that oxidatively deaminates the polyamines spermidine and spermine, to produce an aminoaldehyde or an aminodialdehyde, respectively, with H_2O_2 and ammonia. In the case of spermine, the monoaldehyde, the unstable dialdehyde or a further breakdown product, likely to be acrolein (CH_2=CHCHO), may be formed. Our previous studies demonstrated that both aldehyde(s) and hydrogen peroxide, formed by purified BSAO in the presence of exogenous spermine, are involved in cytotoxicity on several cultured cell lines (Averill-Bates et al.,

1993; Lord-Fontaine et al., 2001; Calcabrini et al., 2002). The activities of exogenous enzymes catalase and aldehyde dehydrogenase, simultaneously added to the incubation mixture, were high enough to transform the products of the amine oxidase catalyzed reaction at a rate that prevents their accumulation to cytotoxic levels (Averill-Bates et al., 1994; Calcabrini et al., 2002). In this context, it was demonstrated that the enzymatic oxidation products of exogenous spermine induced a cytotoxic effect in human colon adenocarcinoma cell lines, on both sensitive (LoVo WT) and its multidrug-resistant (MDR) variant (LoVo DX), selected by continuous exposure to doxorubicin (DOX) (Calcabrini et al., 2002).

The development of simultaneous resistance to multiple drugs appears to be a serious obstacle to the successful chemotherapy of human tumors. Various tissue culture systems have been established to study the physiologic and biochemical alterations induced during the development of multidrug resistance. Several MDR lines exhibit changes in the expression of high molecular weight glycoproteins on their cell surface.

P-glycoprotein (P-gp), encoded by the *mdr-1* gene (Gottesman and Pastan, 1993), and multidrug resistance-associated protein (MRP) (Flens et al., 1996) are the best known of these membrane bound drug-transporter proteins. These proteins, acting as an energy dependent drug efflux pump, reduce the accumulation of drugs, including some anticancer agents, in MDR cells. These cells are also characterized by different intracellular drug distribution, compared with their parental sensitive counterparts (Gervasoni et al., 1991; Arancia et al., 1998).

Clinical resistance is generally acquired by the tumor cells following to the exposure to DOX or to other antineoplastic drugs, resulting in their loss of therapeutic efficacy. In order to reverse the drug resistance phenotype and to develop innovative chemotherapic strategies, effective against MDR tumor cells, various chemical compounds, such as verapamil, quinidine, cyclosporin A, etoposide and trifluoroperazine have been investigated, both *in vitro* and *in vivo*. Several clinical trials of cyclosporine, as a modulator of MDR, and etoposide have been described (Bertrand et al., 1992; Yahanda et al., 1992). Cyclosporine is a relatively potent competitive inhibitor of P-gp and high amounts of it may be achievable over a short term. Unfortunately, the high concentrations of these pharmacological agents, administered to the patients to reverse MDR, produce undesirable side effects that induce heart block and severe hypotension. While, etoposide is an MDR-related agent without major dose-limiting side effects other than myelosuppression (Yahanda et al., 1992).

It is therefore necessary, for cancer pharmacology, to clarify the cellular and molecular basis of drug resistance, and new therapeutic approaches that target P-gp should be useful to reverse the MDR phenotype. Recent promising investigations made it possible to admit genetic mutation of the cell to the area of application. The treatment of anti-sense oligonucleotide (Cucco and Calabretta, 1996; Motomura et al., 1998) against P-gp and anti P-gp ribozyme (Kobayashi et al., 1999) reversed MDR and increased the chemosensitivity of the treated tumor cells. It was also reported that single chain antibodies acted as highly specific tools for the intracellular modulation of target proteins, allowing several genetic therapies (Piche et al., 1998; Stackhouse et al., 1998).

Among the possibilities to suggest new anticancer strategies, this paper describes that the enzymatic oxidation products of spermine, hydrogen peroxide and aldehyde(s), can induce higher cytotoxicity in multidrug-resistant human colon adenocarcinoma LoVo DX cells than in sensitive LoVo WT ones. In particular, microscopic observations and cytofluorimetric studies showed that these toxic products cause mitochondrial damages more pronounced in MDR than in drug-sensitive cells.

Material and methods

BSAO purification

BSAO was purified from bovine blood as previously described (Turini et al., 1982). The purified enzyme moved as a single band on SDS/PAGE and all samples employed had a minimum specific benzylamine oxidase activity of 0.35 IU/mg, with IU defined as μmoles of substrate oxidized per min, assayed spectrophotometrically at 25°C by monitoring the formation of benzaldehyde at 250 nm absorbance ($\varepsilon = 12{,}500\,M^{-1} \cdot cm^{-1}$). The protein concentration was determined spectrophotometrically at 280 nm, assuming an absorption coefficient of $1.741\,g^{-1} \cdot cm^{-1}$.

Cell cultures

A human colon adenocarcinoma cell line (LoVo WT), isolated from a metastatic nodule, and its doxorubicin (DOX)-resistant variant (LoVo DX) were used in this study. Both cell lines were grown in monolayer in Ham's F12 medium (GIBCO BRL, Life Technologies, Paisley, UK) supplemented with 10% FBS (Hyclone, Cramlington NE23, UK), 1% L-glutamine (GIBCO BRL, Life Technologies), 1% penicillin (50 U/ml)-streptomycin (50 μg/ml) (GIBCO BRL, Life Technologies), 1% vitamins (GIBCO BRL, Life Technologies) in a humidified atmosphere of 5% CO_2 in a water-jacketed incubator at 37°C. The pleiotropic drug resistant cell line LoVo DX was obtained from its drug-sensitive parental LoVo cell line, by exposure to increasing concentrations of DOX (Adriblastina, Pharmacia and Upjohn, Milan, Italy) (Grandi et al., 1986).

Cell survival experiments

Cell survival experiments were carried out using confluent cells that had been incubated for 24 hr at 37°C with fresh culture medium. Cells were harvested with EDTA in phosphate buffer saline (PBS) and then by

addition of trypsin solution in PBS, washed by centrifugation and resuspended in PBS supplemented with 1% bovine serum albumine (BSA) (Sigma, St. Louis, MO). Freshly harvested LoVo WT and LoVo DX cells (10^5/ml) were incubated at 37°C for varying time intervals in the presence of the following reagents, used alone or in combination: BSAO (1.03×10^{-4} μmoles/ml corresponding to 6.98×10^{-3} U/ml), spermine (0–6 μM), catalase (240 U/ml) from bovine liver (Sigma), ALDH (EC 1.2.1.5) from yeast (0.4 U/ml) and NAD^+ (1.8 μg/ml; Boehringer-Mannheim, Mannheim, Germany). Spermine (Fluka, Buchs, Switzerland) was freshly prepared before each experiment and, if present, added last. Cells were then centrifuged, washed in PBS-BSA and finally resuspended in 1 ml PBS-BSA. The cells were then plated in tissue culture-coated Petri dishes (60×15 mm) and incubated at 37°C. Cytotoxicity was evaluated using a colony survival assay, thus determining the ability of cells to reproduce and form macroscopic colonies (>50 cells). After three weeks, colonies were fixed with 96% ethanol, stained with methylene blue and counted manually. Percentage cell survival was determined as the ratio between the mean number of colonies in treated and control samples.

Transmission electron microscopy

For transmission electron microscopy (TEM), cells were grown to near confluence and harvested as described above, then washed, centrifuged and resuspended in 2 ml of F12 medium-1% BSA, without serum. Cells were incubated for 60 min at 37°C in the presence of BSAO alone (6.98×10^{-3} U/ml), spermine alone (6 μM) or with both, BSAO and spermine. After incubation, cells were washed with F12 medium and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, added with 2% sucrose, at room temperature for 20 min. After postfixation with 1% OsO_4 in 0.2 M cacodylate buffer, pH 7.3, at room temperature for 30 min, cells were dehydrated with ascending concentrations of ethanol, and embedded in epoxy resin (TAAB Laboratories Equipment Limited, Aldermarton, UK). Ultrathin sections, obtained with an LKB Ultratome Nova ultramicrotome, were stained with uranyl acetate and lead citrate and then examined with a Philips 208 transmission electron microscope (FEI company, Eindhoven, The Netherland).

Flow cytometry

For determination of cell surface P-glycoprotein (P-gp), cells were harvested with 10 mM EDTA in PBS and then by addition of 0.25% trypsin solution in PBS, washed by centrifugation (3 min, 1,500 g) and resuspended in PBS-1% BSA. Cell suspensions were then incubated for 30 min at 4°C with monoclonal antibody MRK16 (Kamiya, Seattle, WA). After washing with PBS-1% BSA, cells were incubated for 30 min with a $F(ab')_2$ fragment of goat anti-mouse IgG fluorescein-conjugate, at 4°C. Finally, after washing with cold PBS, cells were immediately analysed by flow cytometer (Meschini et al., 2000).

The intracellular content of thiol groups was determined by using 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes Inc., Eugene, OR) (Täger et al., 2000). CM-H_2DCFDA stocks (10 mM) were prepared in ethanol. LoVo WT and LoVo DX cells were detached with EDTA and trypsin, resuspended in PBS containing 1% BSA (10^5 cells/ml) and treated with BSAO (6.98×10^{-3} U/ml) alone, spermine (6 μM) alone or with both for 10 and 60 min at 37°C. In the last 10 min of treatment, the dye was added at final concentration of 10 μM. Then, samples were washed with cold PBS and put on ice until analysis.

The mitochondrial membrane potential (MMP) was evaluated by using the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1, Molecular Probes) according to Cossarizza et al. (1993). LoVo cells were harvested with EDTA/trypsin, then washed, centrifuged and resuspended in PBS-1% BSA. Cells (10^5/ml) were treated for 10 and 60 min with BSAO (6.98×10^{-3} U/ml) alone, spermine (6 μM) alone or with both reagents. In the last 10 min of treatment, JC-1 was added at a final concentration of 5 ng/ml. After washing with cold PBS, the samples were analysed by the flow cytometer. As positive controls, LoVo WT and LoVo DX cells were treated with a 6 μM exogenous hydrogen peroxide solution (commercial H_2O_2, Baker Analysed Reagent, J.T. Baker, Deventer, The Netherlands) for 10 and 60 min and then labelled as described above.

The intracellular reactive oxygen species (ROS) content was determined by using the non fluorescent dye dihydrorhodamine 123 (DHR123, Molecular Probes). This compound represents a chemically reduced form of rhodamine 123 (R123) and freely diffuses into the cells. After oxidation by ROS, DHR123 is converted to green fluorescent R123 (Szûcs et al., 1998). Both LoVo cell lines were harvested with EDTA/trypsin, then washed, centrifuged and resuspended in complete F12 medium. Cells (5×10^5/ml) were labelled with 1 μM DHR123 for 20 min at 37°C. After washing with cold PBS, cells were analyzed by the flow cytometer.

Before adding the fluorescent dyes CM-H_2DCFDA, JC-1 and DHR123 both LoVo cell lines were treated for 10 min with 50 μM verapamil (VER, Sigma) in order to inhibit the efflux of the dyes from the MDR cells due to P-glycoprotein activity (Kühnel et al., 1997). Verapamil pre-treatment did not affect fluorescence in LoVo WT cells, while the emitted fluorescence was significantly increased in multidrug-resistant cells.

The samples were analyzed by a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with a 15 mW, 488 nm, air-cooled argon ion laser. By adding 2 μg/ml propidium iodide (PI, Sigma) to the cell suspensions, dead cells were excluded from the analysis.

The fluorescence emissions attributed to JC-1 monomers, DHR123 molecule was collected through a 530 nm band-pass filter, while the fluorescent signals ascribed to JC-1 aggregates and PI emission were collected through the 585 and 650 nm band-pass filters, respectively. At least 10 000 events per each sample were acquired in log mode. Results are presented as mean fluorescence channel (MFC) values calculated by using the Cell Quest software (Becton Dickinson).

Results

It is known that polyamines, spermine and spermidine, inhibit cell growth in the presence of fetal calf serum. This finding suggested that toxicity was mediated by H_2O_2 generated from amine oxidase activity present in the serum (Henle et al., 1986). Instead, in this study the inhibitory effect of polyamines was observed on sensitive and MDR human adenocarcinoma LoVo cells exposed to exogenous spermine in the presence of purified bovine serum amine oxidase. The cytotoxicity induced by the enzymatic oxidation products of polyamines was evaluated by plating efficiency assay and the mitochondrial alterations were investigated by transmission electron microscopy.

The MDR colon adenocarcinoma cell line and its drug-sensitive parental LoVo WT were characterized for the expression of the transporter molecule P-gp. As shown in Fig. 1, LoVo WT cells were negative as concerns the surface P-gp expression determined by flow cytometry technique, while LoVo DX cells gave a highly positive answer for P-gp labelling. The intensity of their fluorescent signal increased about 2-log as compared to the LoVo WT cells.

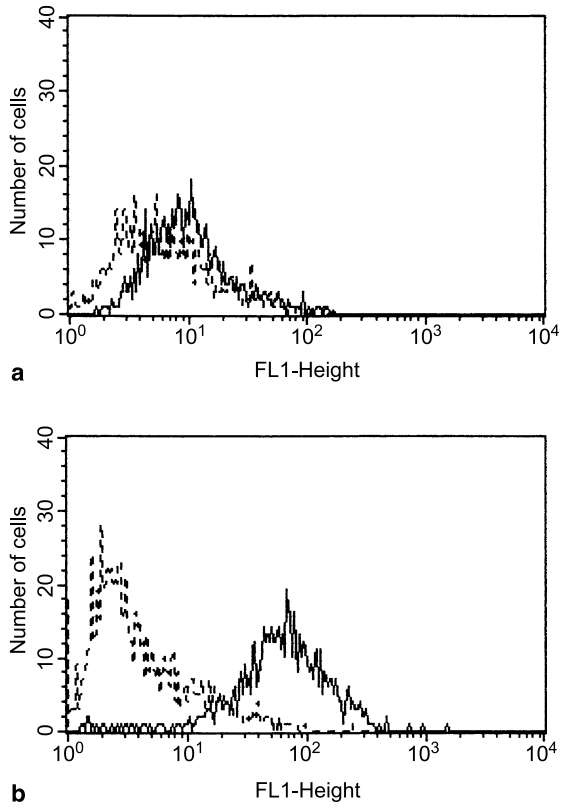

Fig. 1. Representative flow cytometric analysis of surface P-gp expression after incubation of (**a**) LoVo WT and (**b**) LoVo DX cells with MAb anti-P-gp (continuous line) or mouse isotypic globulins (dotted line)

Figure 2 shows the percentage cell survival versus the time of exposure to purified BSAO ($6{,}98 \times 10^{-3}$ U/ml) in the presence of exogenous spermine (6 μM), with and without the sole catalase or catalase and aldehyde dehydrogenase (ALDH), at 37°C. In the presence of BSAO and spermine alone a higher cytotoxicity was observed in LoVo DX than in LoVo WT cells. The percent cell survival decreased in both cell lines with increasing exposure time, resulting in about 18% in LoVo WT (curve a) and about 5% in LoVo DX cells (curve b), after 60 min of incubation. In order to evaluate the contribution of each enzymatic oxidation product in the inhibition of cell growth, the experiments were performed in the presence of exogenous catalase, an enzyme which decomposes H_2O_2, or catalase and aldehyde dehydrogenase (ALDH) added simultaneously to the incubation mixture. Catalase (240 U/ml) afforded a marked reduction of the cytotoxic effect, corresponding to about 80% cell survival, on LoVo WT and LoVo DX cells (curves c and d, respectively), probably due to the clearance of hydrogen peroxide, formed in the catalytic reaction by the enzyme. The result showed that H_2O_2 was not the sole toxic factor and that other products of the enzymatic oxidative deamination were involved, such as aldehyde(s), including acrolein spontaneously formed from the aminoaldehydes (Sharmin et al., 2001). The addition of exogenous NAD-dependent ALDH (0.4 U/ml) metabolized the aldehyde form to the corresponding carboxilic acid and prevented the toxic effects of acrolein. In fact, after addition of both exogenous enzymes, catalase and NAD-dependent ALDH, cytotoxicity was completely inhibited throughout the 60 min of incubation (curves e and f).

In order to reveal the intracellular targets involved in the mechanisms responsible for the higher cytotoxic effect induced on drug-resistant than on drug-sensitive cells, transmission electron microscopy observations were carried out. Both control untreated LoVo WT (Fig. 3a)

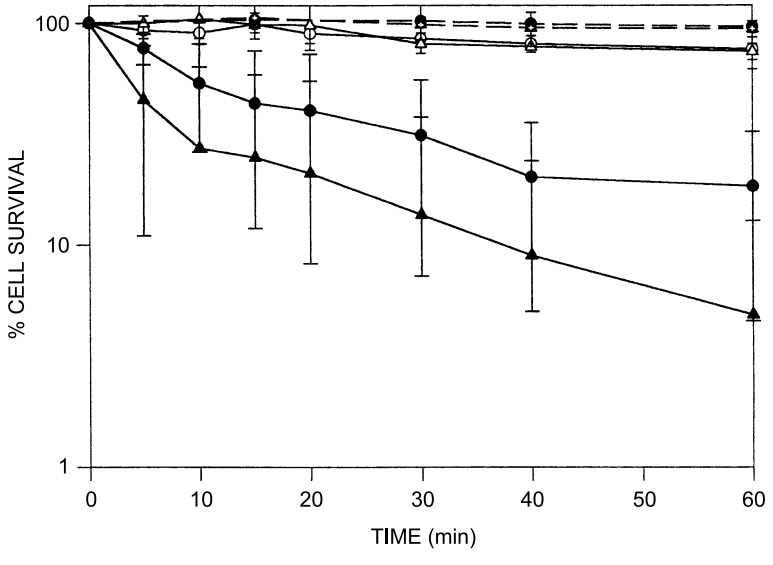

Fig. 2. Effect of catalase and ALDH on cytotoxicity induced by BSAO in the presence of spermine. LoVo WT (circles) and LoVo DX (triangles) cells were incubated at 37°C with purified BSAO ($6{,}5 \times 10^{-3}$ U/ml) and exogenous spermine (6 μM) (solid lines, ●; ▲), with catalase (240 U/ml) (○; △) and with catalase and ALDH (dashed lines, ●; ▲). Means and S.D. are shown for two to six estimations from four to six experiments. Where not shown, S.D. lie within the symbols

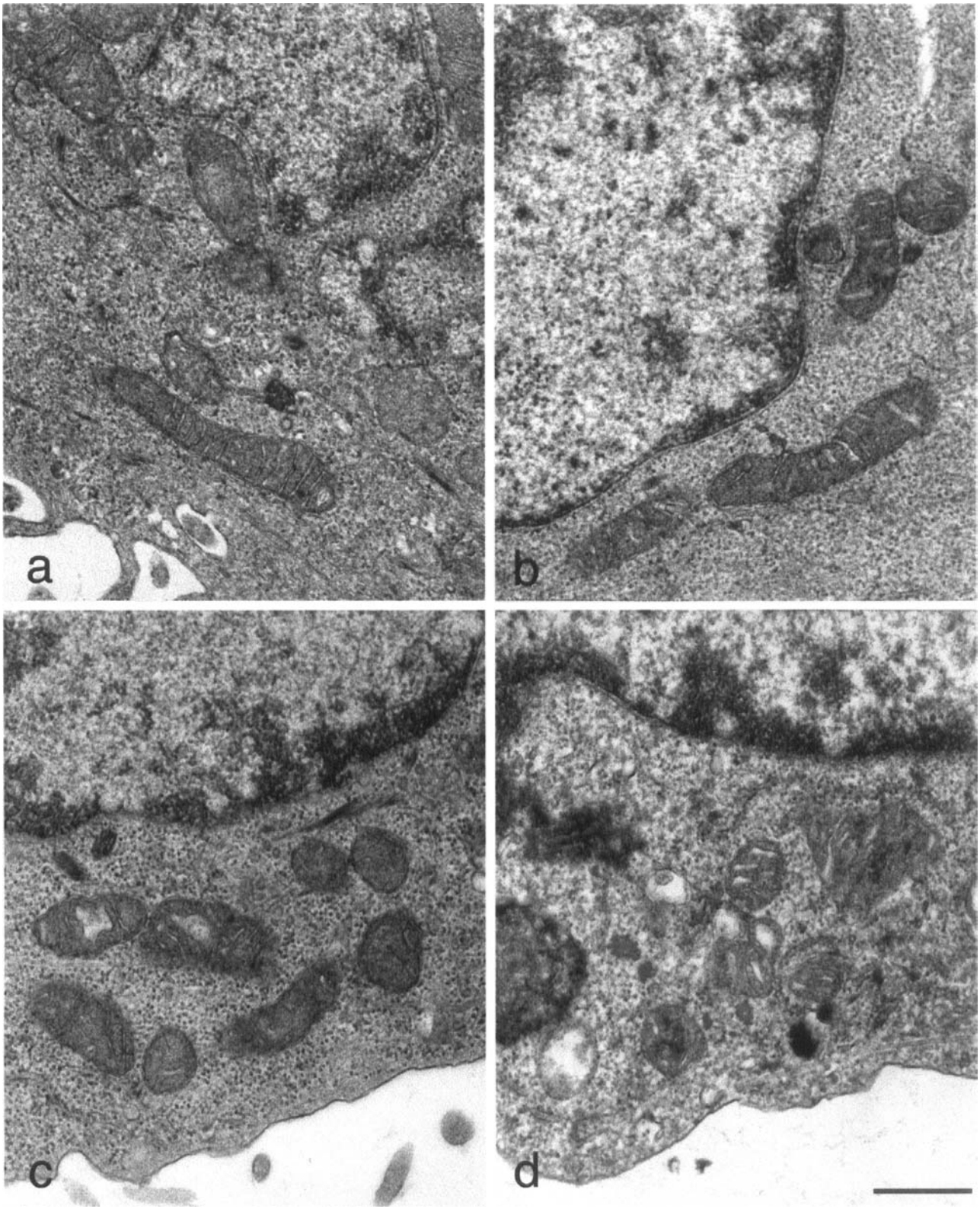

Fig. 3. Transmission electron microscopy observations. (**a**) LoVo WT and (**b**) LoVo DX control cells; (**c**) LoVo WT and (**d**) LoVo DX treated cells (BSAO and 6 μM spermine for 60 min). The alterations of the mitochondrial structure are more evident in treated LoVo DX cells. Scale bar = 0.5 μm

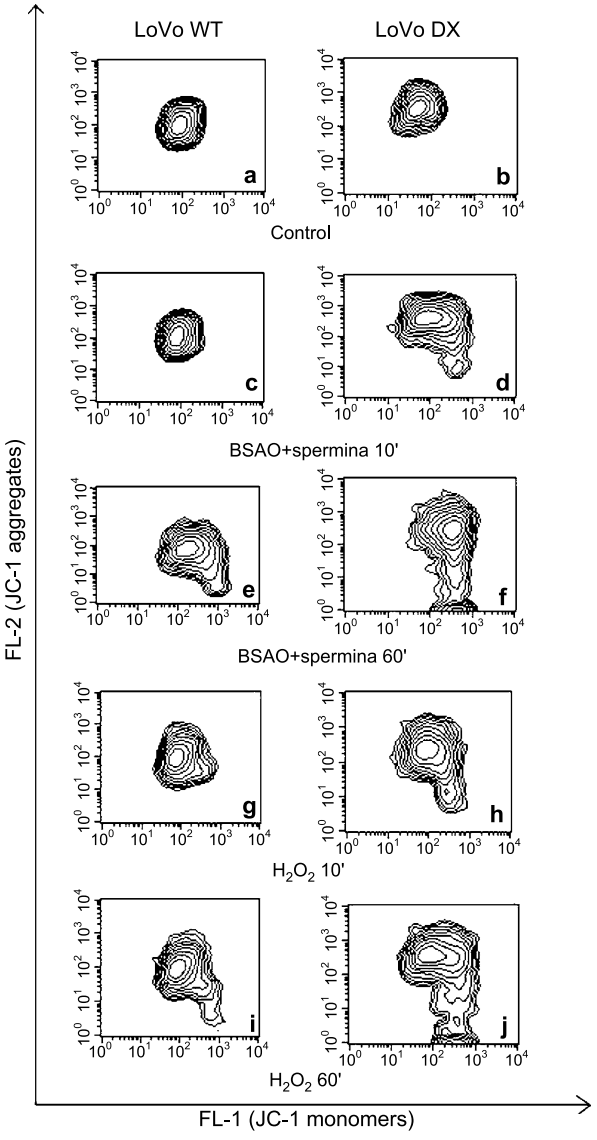

Fig. 4. Mitochondrial membrane potential assessed by flow cytometry after JC-1 staining. Results are represented as contour plots. (**a**, **c**, **e**, **g**, **i**) LoVo WT and (**b**, **d**, **f**, **h**, **j**) LoVo DX cells. A representative experiment of five is reported

and LoVo DX cells (Fig. 3b) showed a well preserved ultrastructure. In particular, numerous mitochondria with elongated shape and parallel cristae were present in the cytoplasm. After treatment with BSAO and spermine, most LoVo WT cells still exhibited a quite good general morphology but some mitochondria showed condensed matrix and altered cristae with a beginning of intracristal swelling (Fig. 3c). Such changes appeared to be much more pronounced in MDR cells (Fig. 3d): all mitochondria showed marked modifications consisting in altered shape, highly condensed matrix and dilated cristae.

A flow cytometric study was carried out in order to get information on the mitochondrial activity, adding a fluorescent dye, the probe JC-1, to the control and treated cells (Fig. 4). The probe JC1 is a monomeric molecule with green fluorescence (FL1, 530 nm emission after excitation at 488 nm) which is able to selectively enter the mitochondria. When the mitochondrial membrane becomes hyperpolarized, the dye forms aggregates that emit red fluorescence (FL2, 590 nm emission). Control MDR cells (Fig. 4b) showed a higher FL2 emission than control LoVo WT cells (Fig. 4a). This result might be attributed to a basal hyperpolarized status of the mitochondria in multidrug-resistant cells. While the monomeric JC-1 form gave a very similar fluorescent signal in both control LoVo WT and LoVo DX cells (Fig. 4a and 4b). The cell samples treated with amine oxidase or spermine alone did not show any variation of the fluorescent signals (data not shown). Instead, the cells treated with BSAO in the presence of spermine showed an evident mitochondrial membrane depolarization. LoVo DX cells (Fig. 4d and 4f) showed a marked increase in mitochondrial membrane depolarization respect to LoVo WT ones (Fig. 4c and 4e), as represented by the alterations of the contour plot, after 10 min and 60 min of incubation. The cells with depolarized mitochondria are those from the middle of the quadrant to the lower right corner, as they lose greenish orange fluorescence (in FL2). After 10 min of incubation LoVo DX cells already showed a detectable mitochondrial membrane depolarization in the presence of both BSAO and spermine (Fig. 4d) that became much more evident after 60 min (Fig. 4f). Similar variations were also

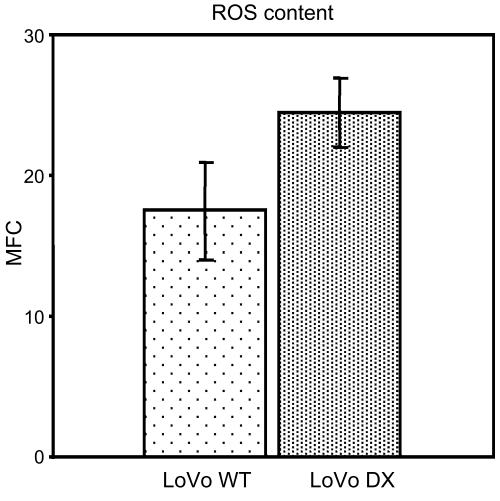

Fig. 5. ROS content evaluated by flow cytometry after DHR123 staining. Results are represented as mean fluorescence channel (MFC) values; the mean ± SD of five independent experiments is reported

observed when the cells were incubated with exogenous H_2O_2 (Fig. 4g–4j).

Intracellular ROS content in LoVo WT and LoVo DX cells, determined using DHR123, is reported in Fig. 5. A higher fluorescent signal was obtained from the drug-resistant cells than from the sensitive ones ($p < 0.05$), suggesting an increased basal production of ROS in multidrug-resistant cells.

Figure 6 shows the results of the cytofluorimetric assays of reduced glutathione (GSH) content at the single cell level by using the GSH-sensitive fluorescent dye CM-H_2DCFDA. Control LoVo WT (Fig. 6a) and LoVo DX cells (Fig. 6b) showed very similar basal GSH content, as demonstrated by the corresponding MFC values (27.1 and 26.0, respectively). In addition, both cell lines did not show any statistically significant variation of the fluorescent signal after treatment for 60 min. with BSAO and spermine or with exogenous H_2O_2.

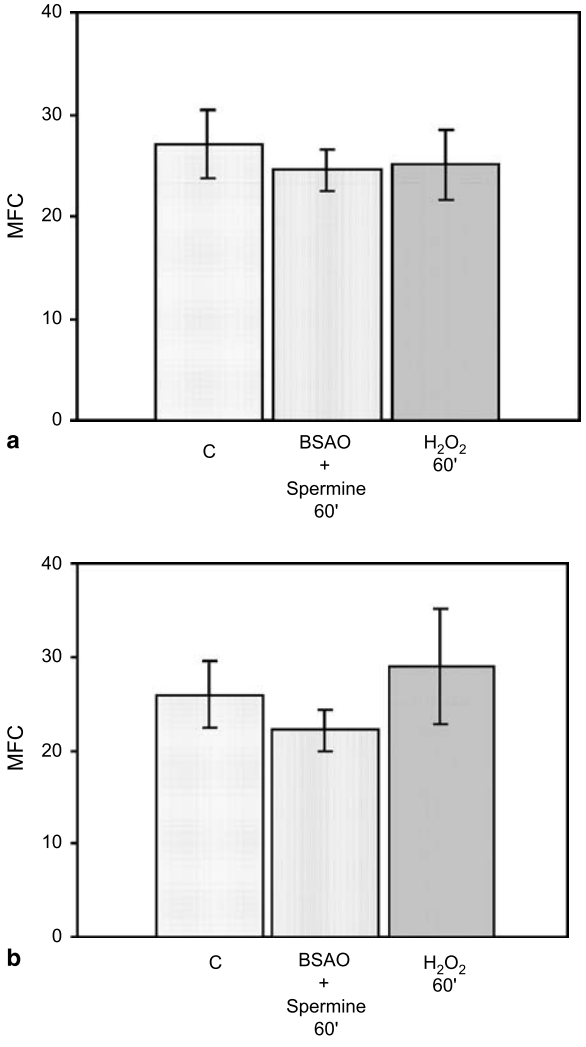

Fig. 6. GSH content evaluated by flow cytometry after CM-H_2DCFDA staining in (**a**) LoVo WT and (**b**) LoVo DX cells. Results are represented as mean fluorescence channel (MFC) values; the mean ± SD of five independent experiments is reported

Discussion

The polyamines spermine, spermidine and putrescine are ubiquitous cell components. If they accumulate excessively within the cells, either due to very high extracellular amount or to deregulation of the systems which control polyamine homeostasis, can induce toxic effects. These molecules are substrates of a family of enzymes that includes monoamine oxidases, diamine oxidases, polyamine oxidases and copper containing amine oxidases isolated from serum. Amine oxidases are important because they contribute to regulate levels of mono- and polyamines. These enzymes catalyze the oxidative deamination of polyamines to generate the reaction products hydrogen peroxide and aldehyde(s) that are able to induce cell death in several tumor cultured cell lines. The diversity between normal and tumor cells is related with polyamines content and metabolism. Polyamine concentrations are high in growing tissues such as tumors. Therefore, this research explores the possibility of using purified bovine serum amine oxidases to produce toxic products, able to induce a cytotoxic effect on human tumor cells and to compare such an effect between drug sensitive and drug resistant cells. The study was therefore undertaken to test the hypothesis that the cytotoxic effect of spermine is dependent upon the presence of amine oxidase, which is known to be present in fetal calf serum (FCS). The findings provide direct evidence for the role of amine oxidase in converting spermine into a toxic agent. The experiments, in this research, were performed in the medium PBS-BSA which did not have any contaminating amine oxidase activity, but 6.98×10^{-3} U/ml of BSAO were added to the incubation mixture as purified enzyme. The cytotoxic effect induced by spermine and BSAO could be mediated by the oxidation products, H_2O_2 and aminodialdehyde or acrolein. In our experimental conditions, the main factor to cause cytotoxicity resulted to be H_2O_2, since the addition of catalase protected both cell lines almost completely (80%). The residual cytotoxicity was attributed to the other reaction product, aminodialdehyde or acrolein. The simultaneous presence in the incubation mixture of both exogenous enzymes, catalase and NAD-dependent aldehyde dehydrogenase (ALDH), completely inhibited cytotoxicity, as already demonstrated in our previous papers (Averill-Bates et al., 1994; Calcabrini et al., 2002). It has been

recently reported that acrolein is more strongly involved than hydrogen peroxide in the inhibition of cell growth by spermine (Sharmin et al., 2001). The concentration of spermine (15 to 30 μM) that induced cytotoxicity was slightly higher than that of acrolein (7.5 to 15 μM), but was much lower than that of H_2O_2 (0.2 to 0.4 mM). Moreover, the cytotoxic effects of spermine on cell growth could be prevented by the addition of ALDH, but not catalase. However, it is important to consider that in these and other studies the polyamine oxidation products were formed in the medium with FCS, that contains the crude serum, rather than in the presence of highly purified BSAO. Such a condition does not exclude the occurrence of secondary enzymatic reactions.

An interesting result is the higher cytotoxicity observed in LoVo DX cells than in their sensitive counterparts. The authors already observed this finding on chinese hamster ovary (CHRC5) cells resistant to colchicine (Lord-Fontaine et al., 2001). They attributed this phenomenon to a different content of GSH in multidrug-resistant cells when compared to the parental sensitive ones. Instead, in human colon adenocarcinoma cells, flow-cytometric studies revealed a similar fluorescent signal in control LoVo WT and LoVo DX cells, and in the samples treated with BSAO/spermine enzymatic system or exogenous H_2O_2. The fluorescent signal was due to the presence of the intracellular thiol groups. The cell survival data, obtained by a clonogenic assay, were supported by transmission electron microscopy and fluorescence studies where MDR cells showed more pronounced ultrastructural modifications than LoVo WT cells. The structural changes mainly observed in the mitochondrial organules might cause functional alterations. Such an investigation demonstrated that the effects of the enzymatic oxidation products of spermine (6 μM), formed in our experimental conditions, were not reflected in damage of DNA, i.e. condensation of chromatin and/or fragmented nuclei, typical signals of apoptotic death.

Infact, Henle et al. (1986) showed that spermidine added to the culture medium containing fetal bovine serum resulted in extensive DNA damage and that the amount of damage increased rapidly as a function of the polyamine concentration (from 30 to 100 μM). Damage from spermidine was markately diminuished after addition of exogenous catalase to the culture medium. The data supported that the enzymatic oxidation of spermidine resulted in the formation of both H_2O_2 and aldehyde(s), or acrolein. The aldehyde(s) toxicity may not be involved in DNA damage but only to contribute to the decreasing of cell survival. Moreover, Gardner et al. (1997) observed that in human CAL-27 tumor cells DNA integrity was not affected after 24 or 48 h of incubation in 0.1 or 0.5 mM H_2O_2; concentrations between 1–10 mM induced ladder-like DNA cleavage followed by a cytotoxic effect associated with characteristic morphologic evidence of apoptosis, while the highest concentration of H_2O_2 (>10 mM) rapidly killed the cells producing evident smeared DNA profiles indicative of necrotic death, without signals of apoptosis.

The ultrastructural alterations might be associated with a depolarization of the mitochondrial membrane, that was immediately observed when the cells were treated with BSAO in the presence of spermine or with exogenous H_2O_2. Living cells previously labelled with a specific probe, JC-1 dye (Cossarizza et al., 1993), allowed us to reveal alterations of mitochondrial membrane potential by flow cytometry technique. The mitochondrial membrane depolarization was earlier (after 10 min) and more evident in MDR colon adenocarcinoma cells than in LoVo WT ones, in agreement with both cell survival and electron microscopy results. It was supposed that the number of mitochondria might affect the cell response to the treatments. The analysis of the mitochondrial mass performed on the cells by NAO labelling assay, demonstrated no significant difference between the two cell lines (data not shown). Therefore, the greater cytotoxic effect caused in LoVo DX cells was not attributed to a lower number of mitochondria. This finding suggested different structural and/or functional properties of the mitochondria that were present in sensitive and MDR cell lines. The study of the mitochondrial functionality was performed by flow cytometry. The cells previously labelled with the probe JC-1, exhibited an hyperpolarization status of the mitochondria of multidrug resistant cells. Since the mitochondrial membrane potential is related to the activity of the mitochondrial electron transport chain (METC), it is supposed that LoVo DX cells can show a higher METC activity than LoVo WT ones. It was previously observed (Jia et al., 1996) an increased mitochondrial electron transport chain activity in several multidrug resistant cell lines when compared to their sensitive counterparts. Thus, it was hypothesized that multidrug-resistant cells present an increased METC activity because they highly express ATP-dependent P-gp (Jia et al., 1997). The METC activity led to the formation of reactive oxygen species (ROS) such as superoxide radical, H_2O_2 and hydroxyl radical (Sohal, 1997) which are usually removed by cells (Kowaltowsky and Vercesi, 1999). It was observed an increased basal ROS production in LoVo DX cells and this could be considered the direct consequence of the

higher METC activity in resistant cells when compared to that revealed in LoVo WT cells. The treatments with BSAO/spermine enzymatic system or exogenous H_2O_2 increased the amount of ROS inside the cells. Thus, it could be postulated that multidrug-resistant cells resulted more sensitive than LoVo WT cells as they contained a high concentration of ROS which could not be removed by cellular defences. The accumulation of these molecules induced a higher impairment of the mitochondrial structure and function in LoVo DX than LoVo WT cells. In this experimental model, morphological alterations and the depolarization effect observed in the mitochondria did not represent the typical features of apoptosis.

The enhancement of amine oxidase activity in tissues undergoing pathological proliferative phenomena may reasonably be used in the treatment of neoplastic diseases. It is well known that reduced polyamine and increased H_2O_2 and aldehyde(s) levels exert an inhibitory effect on the cellular growth and division (Bachrach, 1970). The enhanced formation of aldehyde(s) or acrolein derived from endogenous polyamines may increase the effect of currently used antineoplastic therapies such as hyperthermic treatment. The results reported using an extracellular application of BSAO appear promising and it is expected that the same enzyme delivered inside tumor cells might be even more effective. If the results of further investigations will be up to expectations, we think that the handling of amine oxidase activity, in the presence of polyamines, will undoubtedly turn out to be a powerful tool in the development of new anticancer treatments.

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Authors' address: Prof. Enzo Agostinelli, Department of Biochemical Sciences "A. Rossi Fanelli", University of Rome "La Sapienza", Piazzale A. Moro 5, 00185 Rome, Italy,
Fax: +39064440062, E-mail: enzo.agostinelli@uniroma1.it