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Minireview ■

Spermine Oxidation Products Induce Mitochondrial Alterations on Tumor Cells

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SUMMARY

Cytotoxic products of polyamines generated *in situ* by an enzyme-catalyzed reaction may be useful as a new avenue in combating cancer. This study demonstrates that multidrug resistant (MDR) cancer cells (colon adenocarcinoma and melanoma) are significantly more sensitive than the corresponding wild type (WT) ones to hydrogen peroxide and aldehydes, the products of bovine serum amine oxidase (BSAO)-catalyzed oxidation of spermine. Transmission electron microscopy (TEM) observations showed the major ultrastructural alterations of the mitochondria. These were more pronounced in MDR than in WT cells. After treatment with BSAO/spermine a higher mitochondrial membrane depolarization and an increased mitochondrial activity in drug-resistant cells were observed. The results suggest that enzymatically formed cytotoxic agents activate stress signal transduction pathways, leading to apoptotic cell death, mainly in multidrug resistant cell lines.

***Key words*: polyamines, multidrug resistance (MDR), bovine serum amine oxidase (BSAO)**

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INTRODUCTION

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, they can induce toxic effects. These molecules are substrates of a family of enzymes, the amine oxidases, that includes copper containing amine oxidases isolated from serum (1). These enzymes are important because they contribute to the regulation of the levels of mono- and polyamines. Amine oxidases catalyze the oxidative deamination of polyamines to generate the reaction products hydrogen peroxide and aldehyde(s) (1). Such toxic products are able to induce stress-activated signal transduction pathways, leading to cell death, necrosis or apoptosis in several tumor-cultured cell lines (2-4). The diversity between normal and tumor cells is related with polyamines content and metabolism. Polyamine concentrations are high in growing tissues such as tumors, for example, breast and colon cancer (5).

Cytotoxic products of spermine formed *in situ* by an enzyme-catalyzed reaction might be useful for the destruction of tumors. Therefore, this research explores the possibility of using purified BSAO in the presence of exogenous spermine or endogenous polyamines, after injection of the enzyme into the tumor, to induce cytotoxicity (6). BSAO (EC 1.4.3.6) is a copper-containing glycoprotein weighing 170 KDa, which oxidatively deaminates the primary amino groups of polyamines, such as spermine and spermidine. The reaction involves dioxygen and water as substrates (7). The products are H_2O_2, aldehydes and ammonia (8). In the case of spermine, the monoaldehyde, the unstable dialdehyde, and a further break-down product, likely to be acrolein, may be formed (9, 10) (Figure 1).

In this study the mechanism of cell death of drug sensitive and MDR human cancer cells, induced by the enzymatic toxic products, was investigated.

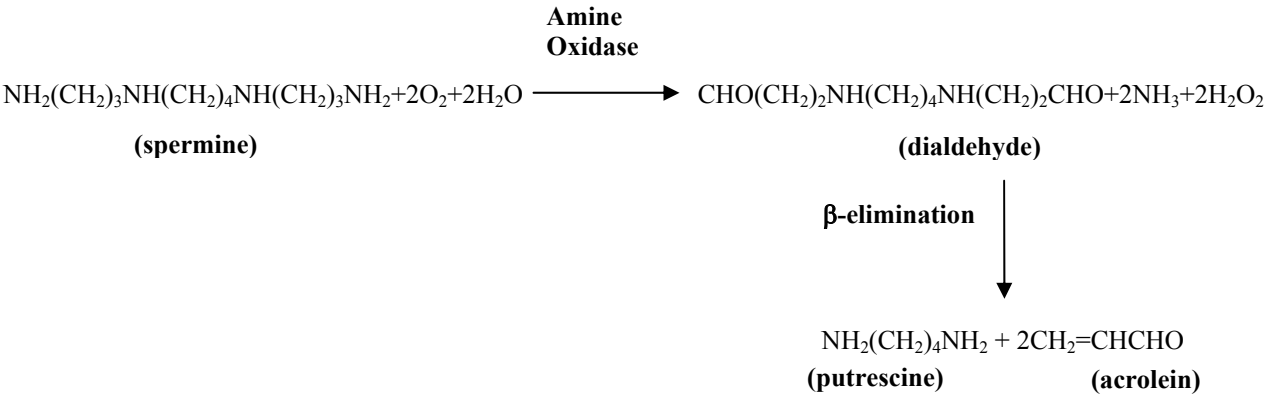

Figure 1. *Reaction scheme for spermine oxidation in the presence of BSAO*

Role of cytotoxic polyamine metabolites in inducing cell death

Our findings showed the possibility of using purified BSAO in the presence of exogenous spermine or endogenous polyamines, after injection of the enzyme into the tumor, to induce cytotoxicity (4, 6, 11). The mechanism of cell death induced by BSAO and spermine, in the extracellular environment, was examined on human melanoma and colon adenocarcinoma cell lines, either drug-sensitive or MDR (12, 13) .

The cytotoxicity induced by BSAO in the presence of exogenous spermine was evaluated in both colon adenocarcinoma LoVo WT and LoVo DX cell lines as a function of spermine concentration as well as of exposure time, at 37 °C. The plating efficiency assay, a method which determines the ability of the cells to reproduce and form macroscopic colonies in culture, was applied to determine the cytotoxic effect. Figure 2 shows the percentage of cell survival as a function of exogenous spermine concentration up to 15 μM in the presence of BSAO, after 60 min of incubation. Multidrug-resistant cells are more affected, by the treatment in the presence of different spermine concentrations than their sensitive counterparts. For instance, at 6 μM spermine concentration, the survival of LoVo WT cells was approx. 45%, while only a very lower percentage approx. 7.5% in LoVo DX cells maintained their viability. To evaluate the contribution of H_2O_2 to cytotoxicity with respect to other enzymatic oxidation products, experiments were carried out in the presence of catalase. Catalase is a hydrogen peroxide-scavenging enzyme which converts H_2O_2 into water and oxygen. Therefore, a drastic reduction of the cytotoxic effect approx. 80% occurred in both cell lines, apparently due to the clearance of H_2O_2 by catalase. However, this result demonstrated that H_2O_2 is not

the exclusive toxic agent and that other species are involved, such as aldehyde(s) including acrolein. To determine the aldehyde's contribution in inducing the cytotoxicity by BSAO/spermine, both enzymes, catalase and NAD-dependent ALDH were added to the incubation mixture. In these experimental conditions, cytotoxicity was completely inhibited throughout the 60 min of incubation (Figure 3). Also the MDR human melanoma cells were more sensitive to the treatment at all concentrations of spermine than the corresponding wild type cells. At the 6 μM spermine concentration, the survival of M14 WT cells was approx. 37.1%, while only 18.8% of the M14 ADR cells remained viable (Figure 2). BSAO alone or spermine alone up to 15 μM were not toxic to either cell line.

Electron microscopy was used to reveal eventual cellular targets of the cytotoxic polyamine metabolites. To gain insight into the mechanisms responsible for the higher cytotoxic effect in MDR cells than the sensitive ones, the morphological and ultrastructural changes induced by the treatment with BSAO/spermine were investigated by scanning (SEM) and transmission electron microscopy (TEM). Control M14 WT and M14 ADR cells grown at 37°C, observed by SEM, show elongated or polygonal shape and their surface is covered by randomly disseminated microvilli. After treatment with BSAO/spermine (6 μM) at 37°C, the cells of both lines appeared less elongated than the untreated controls; some of them tended to become rounded with numerous blebs on their surface. These cells had a tendency to detach from the substrate.

Both M14 WT and M14 ADR control cells grown at 37°C showed a well-preserved ultrastructure when observed by TEM. The cytoplasm was characterized by the presence of numerous mitochondria with parallel cristae in a dense and uniform matrix. After exposure to BSAO/spermine (6μM) at 37°C, M14 WT cells did not show any consistent aberration but some mitochondria display dilated cristae. The alterations of mitochondria structure were much more evident in MDR cells; in particular, they showed a highly condensed matrix and vacuolised cristae (14).

Similar morphological modifications and ultrastructural alterations were also observed in both LoVo colon adenocarcinoma cell lines, where MDR cells showed all the mitochondria visibly damaged.

Since mitochondria appear to play a pivotal role in determining the differential response between sensitive and drug-resistant cells, a flow cytometric study was carried out on LoVo cells in order to get information on the mitochondrial activity. The results showed a basal hyperpolarized status of the mitochondria in control MDR LoVo cells. After the treatment with BSAO/spermine, the higher sensitivity to cytotoxic spermine derivatives observed in adenocarcinoma LoVo DX cells, compared to their sensitive counterparts, has been therefore attributed to an earlier and higher mitochondrial membrane depolarisation. Moreover, a higher basal production of ROS in MDR cells than in the drug-sensitive cells was detected, suggesting an increased METC activity in MDR cells (3, 15).

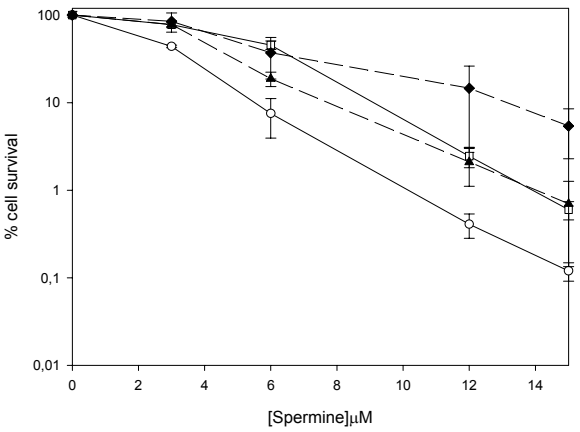

Figure 2. *Effect of exogenous spermine concentration (0-15 μM) on percentage cell survival in the presence of purified BSAO (6,54 x 10^{-3} U/ml) in LoVo WT (open circles, ○), LoVo DX (open squares, □), M14 WT (solid rhombics, ♦) and M14 ADR (solid triangles, ▲) cells during 60 min at 37 °C. Means and SD are shown for two to six estimations from four to six experiments. Where not shown, SD lies within the symbols.*

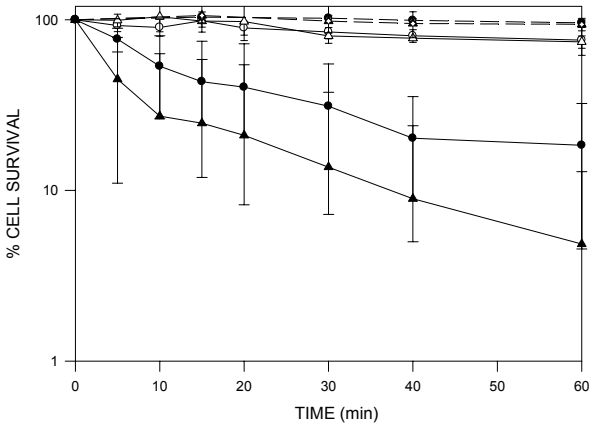

Figure 3. *Effect of catalase and ALDH on cytotoxicity induced by BSAO in the presence of spermine. LoVo WT (circles) and LoVo DX (triangles) cells were incubated at 37°C with purified BSAO (6,5 x 10^{-3} U/ml) and exogenous spermine (6 μM) (solid lines, ●;▲), with catalase (240 U/ml) (○;Δ) and with catalase and ALDH (0.4 U/ml) (dashed lines, ●;▲). Means and SD are shown for two to six estimations from four to six experiments. Where not shown, SD lies within the symbols.*

Bovine serum amine oxidase in cancer therapy: Perspectives

On the basis of the above described findings, the use of amine oxidase in cancer therapy deserves to be

considered (4). In the previous studies, H_2O_2 and aldehydes were produced outside the cells and subsequently they entered the cells, inducing cytotoxic effects. Catalytically liberated cytotoxic agents have the advantage to require only a few enzymatic units of the protein for toxin formation, and that the cytotoxic reaction products are continuously formed over an extended period of time (12).

Since endogenous polyamines are present at high concentrations in tumour cells and growing tissues, it is expected that by delivering BSAO into cancer cells, toxic enzymatic oxidation products could be produced intracellularly for selective *in situ* killing of the same cells. Therefore, strategies could be developed to find out how the enzyme could be delivered *in vivo*, for possible clinical application. In fact, in cultured normal chick fibroblasts or in fibroblasts transformed by Rous sarcoma viruses, Bachrach et al. (16) observed an inhibition of the synthesis of proteins and nucleic acids when the cells were enriched with amine oxidase by microinjection. Transformed cells were more sensitive than normal controls, presumably due to higher polyamine content. Moreover, attempts were made to incorporate the enzyme into liposomal vesicles (17) and to prepare amine oxidase-gold complexes that are bound and incorporated by hepatocytes (18). Thus, endogenous polyamines could be targeted and oxidized by the enzyme.

In this context, our attention was particularly focused on another strategy, currently under further investigation, to produce an immobilized BSAO with the aim to increase its plasmatic half-life and therapeutic efficacy and to decrease drug toxicity. The enzyme was conjugated to a bio-compatible non-immunogenic polymer, polyethylene glycol (PEG), and then immobilized into a hydrogel-type matrix (19). The immobilized BSAO exhibited considerable advantages over the free enzyme. Both native and immobilized BSAO were then compared *in vivo,* in terms of their respective abilities to induce melanoma regression in mice by either apoptosis or necrosis. In fact, the growth of a mouse melanoma (B16-F0) was reduced by 70% after a single injection of the immobilized enzyme, in comparison with 32% inhibition after injection of the same amount of native BSAO. While the immobilized enzyme induced a high level (70%) of apoptosis, non-apoptotic cell death prevailed in the case of the native enzyme (6). The difference of cell death ratio was attributed to the slow, gradual release of spermine enzymatic oxidation products from the hydrogel, i. e. the long-term exposure of the tumour to ROS and aldehydes, as compared with the shorter, though more rapid release of toxic metabolites by the native enzyme.

CONCLUSIONS

Numerous studies have demonstrated that H_2O_2, as other ROS, are able to affect cell cycle progression, inducing inhibition of cell proliferation and a block in G_1, S or G_2 phases of the cell cycle (20). The growth arrest can be transient or permanent and, in the latter case, the process may end in cell death by apoptosis or necrosis, depending on the entity of the oxidative stress, time of treatment and cell type.

Biogenic amines in cell redox balance may behave directly as scavengers against specific types of ROS, or may indirectly cause an increase in ROS production, via H_2O_2 generation, mediated by their oxidative deamination by amine oxidases. These enzymes are important because they contribute to regulate the levels of polyamines.

However, therapeutic applications of radical generating systems are still at the beginning. It is our hope that, if the results of further studies of these approaches will be up to expectations, the handling of amine oxidase activity, in the presence of biogenic amines, will undoubtedly turn out to be a powerful tool in the development of new anticancer treatments (6).

The new approach shows a higher sensitivity to the cytotoxic spermine metabolites H_2O_2 and aldehydes, of MDR human adenocarcinoma and melanoma cells, as compared with their wild type counterparts. This finding has been previously attributed to an earlier and higher mitochondrial membrane depolarization, and a higher basal production of ROS (15). In fact, H_2O_2 could directly interact with some iron of Fe/S centres located in the respiratory chain, raising the highly reactive hydroxyl radical ($HO^{\cdot}$) by means of Fenton reaction, which induces some thiol (SH) groups, proteins and lipids oxidation (14).

In conclusion, the use of BSAO/polyamine metabolites in therapeutic applications is of great interest since it might represent a promising strategy to overcome MDR of cancer cells.

Abbreviations: MDR, multidrug resistance; WT, wild type; BSAO, bovine serum amine oxidase; SEM scanning electron microscopy; TEM, transmission electron microscopy; P-gp, P-glycoprotein; ALDH, aldehyde dehydrogenase; ROS, reactive oxygen species; PEG, poly (ethylene glycol).

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PRODUKTI OKSIDACIJE SPERMINA IZAZIVAJU PROMENE NA MITOHONDRIJAMA KOD TUMORSKIH ĆELIJA

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Sažetak

Citotoksični produkti poliamina stvorenih *in situ* u toku reakcija katalisanih enzimima mogu biti od koristi u pronalaženju novih načina borbe protiv kancera. Ova studija pokazuje da su ćelije kancera sa multiplom rezistencijom na lekove (MDR) (adenokarcinom kolona i melanom), u poređenju sa divljim tipom (WT) ćelija, značajno osetljivije na hidrogen peroksid i aldehide, i na produkte oksidacije spermina katalisane amino oksidazom goveđeg seruma (BSAO). Transmisiona elektronska mikroskopija (TEM) je ukazala na velike ultrastrukturalne promene na mitohondrijama. One su bile izraženije kod MDR nego kod WT ćelija. Nakon tretmana BSAO/sperminom, kod ćelija rezistentnih na lekove primećena je veća depolarizacija membrane mitohondrija kao i pojačana aktivnost mitohondrija. Rezultati ukazuju da citotoksični agensi, nastali pomoću enzima, aktiviraju puteve signalne transdukcije stresa, što dovodi do apoptoze ćelija, prvenstveno kod ćelijskih linija sa multiplom rezistencijom na lekove.

Ključne reči: **poliamini, multipla rezistencija na lekove (MDR), amino oksidaza goveđeg seruma**