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Anti-tumoral effect of native and immobilized bovine serum amine oxidase in a mouse melanoma model

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Abstract

Bovine serum amine oxidase (BSAO) oxidatively deaminates polyamines containing primary amine groups, spermidine and spermine, to form the cytotoxic products hydrogen peroxide and aldehyde(s). Polyamines are present at elevated levels in many tumor tissues. The aims of the study were to evaluate the anti-tumoral activities of native and immobilized BSAO in mouse melanoma and also to determine the mechanism of tumor cell death. C57BL mice received a subcutaneous injection of B16 melanoma cells to induce formation of tumors, prior to antitumor treatments with native and immobilized BSAO. The enzyme was immobilized in a poly(ethylene glycol) (PEG) biocompatible matrix. Antitumor treatments consisted of a single injection of enzyme into the tumor. When immobilized BSAO (2.5 mU) was injected into the tumor, there was a marked decrease of 70% of the tumor growth. This was compared with a decrease of only 32% of tumor size when the same amount of native BSAO was administered. The type of cell death was analysed in tumors that were treated with native or immobilized BSAO. When tumors were treated with immobilized BSAO, there was induction of a high level of apoptosis (around 70%), compared to less than 10% with the native enzyme. Apoptotic cell death was assessed by nuclear chromatin condensation using Hoechst staining and labelling of externalized phosphatidylserine using Annexin V. However, native BSAO, probably due to a burst of cytotoxic products, induced a high level of necrosis of about 40%, compared to less than 10% with immobilized BSAO. In conclusion, the advantage is that immobilized BSAO can act by allowing the slow release of cytotoxic products, which induces tumor cell death by apoptosis rather than necrosis.

Keywords: Amine oxidase; Apoptosis; Immobilized; Mouse melanoma; Necrosis; Polyamine

1. Introduction

The naturally occurring polyamines (spermine, spermidine and putrescine) play an essential role in cellular growth and differentiation [1]. Recently, they have also been implicated in apoptosis, which is a highly regulated process of cell death [2], and in cellular signal transduction [3]. The polyamine biosynthetic pathway is very active during the growth of various cancer cells. Polyamines are often present at increased concentrations in tumor cells and tissues, for example, breast and colon cancers [4]. Reasons for these increased levels include enhanced putrescine synthesis by ornithine decarboxylase and increased uptake of polyamines [5]. In addition, depletion of polyamines leads to inhibition of tumor growth [6].

Given the role of the natural polyamines in growth-related processes, enormous efforts have been made to synthesize inhibitors for the different enzymes involved in polyamine biosynthesis: spermidine and spermine synthase, ornithine decarboxylase and *S*-adenosyl-methionine decarboxylase [7]. In fact, new strategies for cancer treatment are currently under development [8] using (i) inhibitors of polyamine synthesis such as DFMO, a specific inhibitor of ornithine decarboxylase [9], and methylglyoxal-bis-guanidylhydrazone, an inhibitor of *S*-adenosyl-methionine decarboxylase,

Abbreviations: BSAO, bovine serum amine oxidase; DAH, 1,6-diaminohexane; DFMO, α-difluoromethylornithine; DMEM, Dulbecco's modified Eagle medium; GPx, glutathione peroxidase; GSH, reduced glutathione; GST, glutathione S-transferase; IU, international unit; PEG, polyethylene glycol; PBS, phosphate buffered saline; PI, propidium iodide; SD, standard deviation; S.E.M., standard error of the mean

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(ii) analogues of polyamines [10,11], which can deplete polyamine content and interfere with polyamine metabolism and/or function, (iii) polyamine transport inhibitors which can prevent uptake of exogenous polyamines by blocking membrane transporters [12] and (iv) the use of polyamine-degrading enzymes such as BSAO [13–18]. A recent development is that the polyamine oxidase inactivator, N^1,N^4-bis (2,3-butadienyl)-1,4-butanediamine (MDL 72527), can improve the anticancer effect of DFMO [7]. Currently, DMFO is undergoing clinical evaluation as a chemoprevention agent [19].

BSAO (EC 1.4.3.6) is a copper enzyme, Mw 170 kDa, which oxidatively deaminates polyamines containing primary amine groups (putrescine, spermidine and spermine) in the presence of oxygen and water [20]. The reaction products are hydrogen peroxide (H_2O_2), the corresponding aldehydes and ammonia [21]. Acrolein is formed by the spontaneous β-elimination of the dialdehyde, an unstable intermediate oxidation product of spermine [22,23]. Fig. 1 shows the reaction scheme for the enzymatic action of BSAO on spermine and the formation of cytotoxic products. Products of polyamine oxidation have been implicated in programmed cell death [24] and inhibition of DNA and protein synthesis [25].

We previously reported that spermine oxidation products could inhibit mammalian cell proliferation and that both H_2O_2 and aldehyde(s) were involved [18,26]. Furthermore, an important finding is that the BSAO/spermine enzymatic system was able to eliminate multidrug-resistant cells with overexpression of P-glycoprotein [17,27]. These findings suggest that BSAO could prove to be useful in cancer treatment. To take advantage of the higher levels of polyamines in tumor versus normal tissues [4], toxic

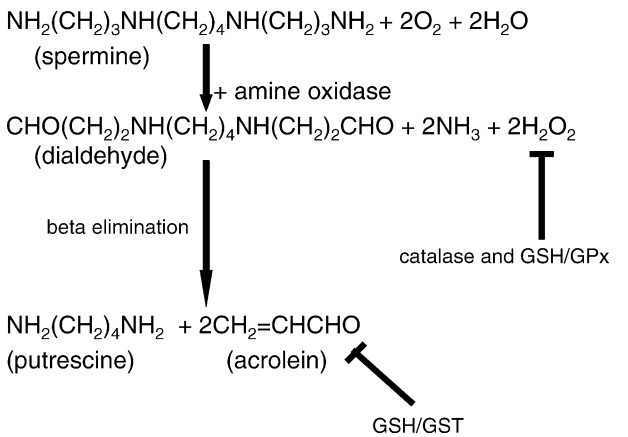

Fig. 1. Reaction scheme for the enzymatic oxidation of the polyamine spermine by BSAO. Spermine undergoes oxidative deamination in the presence of oxygen and water to form hydrogen peroxide (H_2O_2), ammonia and the unstable aminoaldehyde intermediate [*N*,*N*′-bis(3-propionaldehyde)-1,4-butanediamine]. The aminoaldehyde undergoes spontaneous β-elimination to form acrolein and putrescine. H_2O_2 is detoxified by two cellular defence systems: (i) by catalase and (ii) by reacting with reduced glutathione (GSH), catalyzed by glutathione peroxidase (GPx). Acrolein is detoxified by conjugation with GSH, catalyzed by glutathione *S*-transferase (GST).

products such as H_2O_2 and aldehyde(s) could be generated in situ by delivering amine oxidases into tumors to induce cytotoxicity [18,27]. Furthermore, BSAO could also act by depleting tissue levels of polyamines, necessary for tumor growth.

The aim of this study is to evaluate in vivo, using a mouse melanoma model, whether BSAO, when injected directly into the tumor, is able to induce tumoricidal activity by converting polyamines to toxic products in situ. It has been well established that immobilization of enzymes such as asparaginases into polymeric matrices such as PEG increases their structural stability and functional activities in vitro and in vivo [28]. Immobilization of BSAO into a biocompatible matrix made of bovine serum albumin and PEG was reported recently [29] and the enzyme showed a high operational stability relative to its native form. Therefore, both native and immobilized BSAO will be compared in vivo in terms of their antitumor efficacy against mice melanomas.

For cancer therapies, it is also important to establish the mechanism(s) by which cytotoxic agents cause tumor cell death. Apoptosis is a highly regulated form of cell death involving many different genes and proteins [30]. During apoptosis, caspase enzymes are activated, chromatin condensation and internucleosomal degradation of DNA occur in the nucleus and blebs appear on the surface of the cell membrane. An early morphological event in apoptosis is the loss of plasma membrane asymmetry, resulting in exposure of phosphatidylserine at the outer membrane leaflet. Cells are subsequently dismantled in an orderly manner into apoptotic bodies, which are removed by phagocytosis. This process avoids liberation of cellular contents into surrounding tissue and induction of inflammation, as occurs when cells lose membrane integrity and die by necrosis. Therefore, native and immobilized BSAO will be compared in vivo in terms of their respective abilities to induce tumor cell death by either apoptosis or necrosis.

2. Materials and methods

2.1. Materials

Benzylamine, Hoecsht (# 33258), Annexin V-FITC, propidium iodide, bovine serum albumin, epidermal growth factor, hydrocortisone, insulin, polyethylene glycol of 3.5 kDa and transferrin were purchased from Sigma Chemical Co. Triethylamine, perchloric acid (70%) and DAH were purchased from J.T. Baker. DMEM, foetal bovine serum, penicillin, streptomycin and trypan blue were from Gibco Canada. Mouse melanoma cells B16-F0 (CRL-6322) were purchased from the American type culture collection. Female C57BL mice, aged 6–8 weeks and weighing 16–20 g, were purchased from Charles River Inc.

2.2. Methods

2.2.1. Tissue culture

Melanoma cells (B16-F0) were grown in monolayer in DMEM supplemented with 10% foetal bovine serum, 4 mM L-glutamine, 1% penicillin (50 units/ml)-streptomycin (50 μg/ml), 10 μg/ml insulin, 10 μg/ml transferrin, 10 ng/ml epidermal growth factor and 1.4 μM hydrocortisone, in an atmosphere of 5% CO_2 in a water-jacketed incubator at 37 °C. Confluent cells were harvested with 0.25% (w/v) trypsin-0.02% (w/v) EDTA solution and centrifuged at 1000 × *g* for 3 min. The cell pellet was resuspended in sterile PBS. Cell viability was estimated by the trypan blue exclusion test [27].

2.2.2. Purification of BSAO

Bovine blood was withdrawn at a slaughterhouse and mixed with 3.8% sodium citrate solution (an anticoagulant) and then treated according to Turini et al. [31] to purify the enzyme amine oxidase. Some modifications were added to the method:

(a) CM-Cellulose column, equilibrated with phosphate buffer (0.01 M) at pH 5.8, to remove haemoglobin, followed by (b) an AE-Agarose column, in phosphate buffer (0.01 M) at pH 7.2 to eliminate ceruloplasmin and then, as the last steps were added two ionic exchange chromatographies performed using a Q-Sepharose column, in phosphate buffer (0.025 M) at pH 6.8 and a Q-Sepharose column, in phosphate buffer (0.02 M) at pH 8.0, according to Janes et al. [32]. The enzyme was eluted highly purified with a NaCl gradient. All purification steps were carried out in a cold room, at 4 °C.

2.2.3. Measurement of soluble and immobilized BSAO activities

The benzylamine oxidase activity of native BSAO, diluted at 4 μg of protein/ml, was measured at 25 °C in the presence of 5 mM benzylamine in 100 mM phosphate buffer, pH 7.2 [33]. After 5 min of incubation at room temperature, the quantities of benzaldehyde formed were monitored at 250 nm ($\varepsilon = 12\,500\ M^{-1}\ cm^{-1}$). The native enzyme had a specific activity of 0.24 IU/mg, where 1 IU was the amount of enzyme required to catalyze the oxidation of 1 μmol of substrate per min.

Immobilization of BSAO was performed into a film of hydrogel made from rat serum albumin crosslinked by di-4-nitrophenylcarbonate PEG [29]. Microbeads were obtained by crunching the film through a series of mesh filter sheets to obtain a microbead size which is injectable into the tumor [34]. The hydrogel slab was first crunched through a 100 μm polyethylene Spectra/Mesh filter sheet (Spectrum Laboratories) and then after the particles were crunched again through a 10 μm filter sheet. The microbeads looked like elongated spheres and their final size was greater than the sieve used, due to swelling during the washing procedure. The exact size of the microbeads was not evaluated, but their size would be between 10 and 15 μm. The size of the microbeads obtained was small enough to pass through a 21 g needle mounted on a syringe, for tumor injection. The activity of immobilized BSAO was evaluated by incubating 100 mg of wet microbeads for 5 min at room temperature in the presence of 5 mM benzylamine in 100 mM phosphate buffer, pH 7.2, as previously described [29]. After centrifugation (16,000 × *g*, 2 min) (Biofuge Pico, Heraeus), the absorbance of the supernatant was measured at 250 nm following the formation of benzaldehyde during 5 min.

2.2.4. Analysis of plasma polyamines

The analysis of polyamines in blood samples was carried out according to De Vera and co-workers [35]. Briefly, blood was collected in heparinized microvettes CB 300 (Sarstedt) from the saphen vein of the mice. Polyamines were extracted and derivatized with dansyl chloride (5 mg/ml) solution. The dansyl-polyamines (putrescine, cadaverine, spermidine and spermine) were analyzed by HPLC equipped with a Phenomenex Synergi Hydro-RP reverse-phase column (150 × 4.60 mm, particle size 4 μm). 1.0 mM DAH was added as internal standard. The column eluate was monitored using a Shimadzu RF-551 fluorescence detector at λ excitation (λ_{ex}) = 350 nm and λ emission (λ_{em}) = 520 nm. A gradient of solution A (75:25, methanol: 1.2 mM Na_2HPO_4/12 mM NaCl buffer solution, pH 7.0) and solution B (methanol) was used. At initial time, the composition of the eluate was 100:0, A:B and at $t = 4.5$ min the eluate composition was 0:100, A:B. The retention times of the polyamines, putrescine, cadaverine, DAH, spermidine and spermine, were 3.85 ± 0.4, 4.38 ± 0.7, 4.77 ± 0.4, 5.97 ± 0.4 and 6.67 ± 0.4 min, respectively. Amounts of polyamines were determined using the internal standard method.

The concentrations of the different polyamines were evaluated in the sera of the various experimental groups of mice to ensure that the enzyme substrates, spermine and spermidine, were present in sufficient quantities. The quantification of polyamines showed a mean plasma concentration of 250 pmol/ml for spermine, 1700 pmol/ml for spermidine, 388 pmol/ml for putrescine and 163 pmol/ml for cadaverine. A chromatogram representing the polyamines in mice plasma is shown in Fig. 2. The quantities of polyamines in the mouse chow ($n = 3$) were 6.7 ± 0.5 μmol/g of dry food for spermine, 29.9 ± 2.1 μmol/g for spermidine, 55.2 ± 3.3 μmol/g for putrescine and 10.7 ± 1.8 μmol/g for cadaverine.

2.2.5. Treatment of mice tumors

Animal experiments were carried out according to the guidelines of the Canadian Council for Animal Care and with approval by the institutional animal care committee. All mice, with the exception of the negative control groups, were injected subcutaneously in the dorsal position with 1×10^6 B16-F0 cells suspended in 120 μl of sterile PBS.

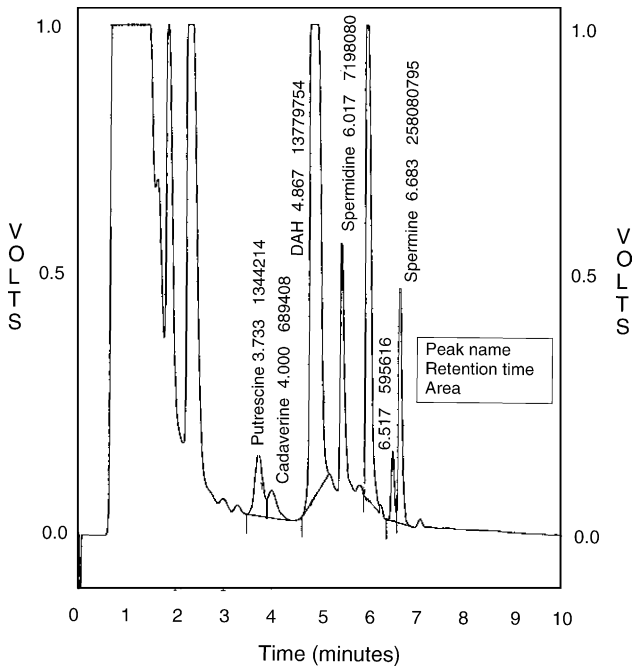

Fig. 2. Analysis of polyamine content in plasma of mice by reversed-phase HPLC. The separation of different polyamines was carried out by reversed phase HPLC using dansyl precolumn derivatives as described in Section 2. Putrescine, cadaverine, DAH (internal standard), spermidine and spermine had retention times of 3.7, 4.0, 4.87, 6.0 and 6.5 min, respectively. A representative chromatogram is shown from at least four experiments.

Negative control groups were injected with 120 μl of PBS alone.

A series of three experiments (4–5 mice per treatment) was carried out to determine the optimal parameters such as (1) enzyme dosage and (2) tumor size before starting the treatment, as well as (3) the effect of adding exogenous spermine to the drinking water. Doses of enzyme tested were 2.5, 5 and 15 mU. Treatment of mice with enzyme was started at tumor sizes of 0.02 g, 0.05 g or 0.08 g. Spermine was added in the drinking water at a concentration of 100 mg/l, once BSAO was injected into the tumor, and remained in the water throughout the duration of the treatment. Once the optimal parameters were determined, four series of experiments were carried out using the optimal dose (2.5 mU) for native or immobilized BSAO and a tumor size of 0.02 g (e.g. 26.0 mm^3), before beginning the treatment. This size was reached approximately 6–8 days after inoculation with the tumor cells. For these subsequent four experiments, spermine was not added to the drinking water.

Tumor size was determined four times per week, until mice were sacrificed. Euthanasia of mice was performed using CO_2 inhalation when the size of the tumor exceeded 3.5 g. The length (l) and the width (w) were measured with a digimatic calliper (Mitutoyo). From these dimensions, the tumor volume (v) (mm^3) was calculated using the formula $(w/2)^2 l\pi$ [36].

To assess the effect of BSAO on tumor growth rate, four series of experiments were carried out in this study and 26 mice were used in each series. For each series, there were three control groups. For the negative control group, mice received an injection of only 120 μl of PBS alone, but were not inoculated with tumor cells. The mice in the positive control group were inoculated with tumor cells and received an injection of PBS as treatment. The third control group of mice was inoculated with tumor cells and received a naked hydrogel as treatment. The experimental groups were composed of two groups of mice carrying the tumor. The first group was treated with an intratumoral injection of native BSAO (2.5 mU), and the second group was treated with an intratumoral injection of BSAO immobilized in a hydrogel (2.5 mU). All groups were composed of 5–6 mice. The development of cutaneous wounds at the site of tumor injection was also evaluated under these conditions in 3–7 independent experiments with 5–6 mice per group.

2.2.6. Viability test and death mechanism of tumoral cells

In this study, four series of experiments were carried out. In each series, 18 mice were used and were subdivided into subgroups of 5–6 mice to determine the time course for kinetics of cell death. The tumors were excised from euthanized mice at specific times after the beginning of the treatment with either PBS alone, native BSAO or immobilized BSAO. The tumor mass was chopped finely into small pieces and incubated with 200 units of collagenase IV for 60 min under gentle agitation. After the incubation, cells were detached by gentle passage through a pipette. The cells obtained from the tumors were suspended in DMEM medium and then centrifuged at 400 × g for 30 s. The cell pellet was then washed twice with PBS. Then, 15 μl of Hoechst solution (1 mg/ml) was added to the cells, along with 500 μl of PBS. The cells were incubated under weak agitation at 37 °C for 15 min and then centrifuged at 120 × g for 30 s. The resulting cell pellet was then washed in PBS and centrifuged at 120 × g for 30 s. Then, 500 μL of DMEM medium with PI (50 μg/ml) were added to the tumor cells before observation under a fluorescent microscope (Carl Zeiss Canada Ltd.). Pictures were taken by digital camera (camera 3CCD, Sony DXC-950P, Empix Imaging Inc.). Images were analysed by Northern Eclipse software. Cells were classified using the following criteria: (a) live cells (normal nuclei, pale blue chromatin with organized structure); (b) membrane-intact apoptotic cells (bright blue condensed or fragmented chromatin); (c) necrotic cells (red, enlarged nuclei with smooth normal structure) [37]. The fractions of apoptotic and necrotic cells were determined relative to total cells (obtained using bright field illumination). At least 400 cells were counted per tumor.

2.2.7. Determination of cell death by Annexin V-FITC staining

Externalized phosphatidylserine on the outer surface of the cytoplasmic membrane becomes labelled by fluores-

cein-labelled Annexin V, which has a high affinity for phosphatidylserine-containing phospholipid bilayers [38]. To analyze apoptotic cell death by flow cytometry, B16-F0 cells (1×10^6/ml) were incubated for 4 h with empty PEG hydrogels, or with either immobilized or native BSAO (6.0 mU/ml) and spermine (50 μM). Cells were then washed twice with PBS and resuspended in 1 ml of binding buffer (10 mM Hepes/NaOH, pH 7.5, 140 mM NaCl, and 2.5 mM $CaCl_2$). Five hundred microliters of cell suspension were then incubated with 5 μl of Annexin V-FITC and 10 μl of PI for 10 min at room temperature in the dark. The populations of Annexin V-positive/PI-negative cells (early apoptosis) and Annexin V-positive/PI-positive cells (late apoptosis) were evaluated by flow cytometry [38]. Data were collected using a FACS scan equipped with an argon laser emitting at 488 nm and analyzed using Lysis II software (Becton Dickinson).

2.3. Statistical analysis

Data represent the mean value and S.E.M. The effects of the different treatments on the rate of tumor growth were compared to the growth of the tumor from the positive control group by the extra sum of squares (log% of tumor growth versus day post treatment). The percentages of apoptosis at various times of tumor excision for the following groups: untreated, treated with native or immobilized BSAO, were compared using the Bartlett's test for homogeneity of variance, followed by a Tukey multiple comparison test. For significant differences, $P < 0.05$.

3. Results

3.1. Effect of dietary supplementation of spermine on tumor growth rate in mice

It was first necessary to establish whether sufficient levels of polyamines were present in mice to allow the enzymatic reaction to occur, or whether dietary supplementation would be necessary. Following inoculation of mice with tumor cells, the effect of dietary addition of spermine on the rate of tumor growth was determined. One would expect that addition of spermine would allow an increased BSAO kinetic reaction rate, which should be reflected by a lower growth rate of the tumor, due to increased generation of toxic products. In fact, this was not the case. Instead, dietary addition of spermine caused marked acceleration of the rate of tumor growth (Fig. 3). Furthermore, in the presence of dietary spermine and immobilized BSAO (5 mU), a higher percentage of wound tissue was observed at the tumor site (group 7), when compared to mice bearing tumors but without addition of spermine in their water (group 1) (Fig. 4). Effectively, up to 80% of the mice having tumors and receiving dietary spermine and immobilized BSAO treatments developed

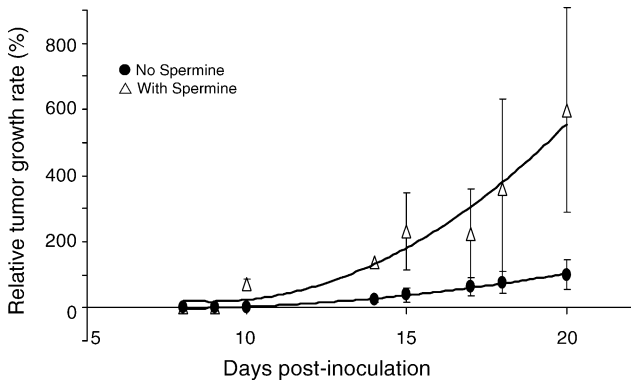

Fig. 3. Dietary supplementation with spermine accelerates rate of tumor growth. All of the animals were injected subcutaneously in the dorsal position with melanoma B16-F0 cells (1×10^6 cells) to induce formation of tumors. Mice received water either with ((△), open triangle) or without ((●), solid circle) addition of spermine (100 mg/l). Data represent means and S.D. from three to five mice and the experiment was repeated three times.

severe skin wounds at the site of tumor implantation (Fig. 4). The severity of the tissue wound was in many cases high enough to require euthanasia of mice before completing the experiment. This high percentage has to be compared with a level of 20–40% for all other groups of mice bearing tumors and drinking regular water (Fig. 4). These findings indicate that increasing polyamine substrate availability for the BSAO kinetic reaction by dietary addition of spermine would not provide an advantage

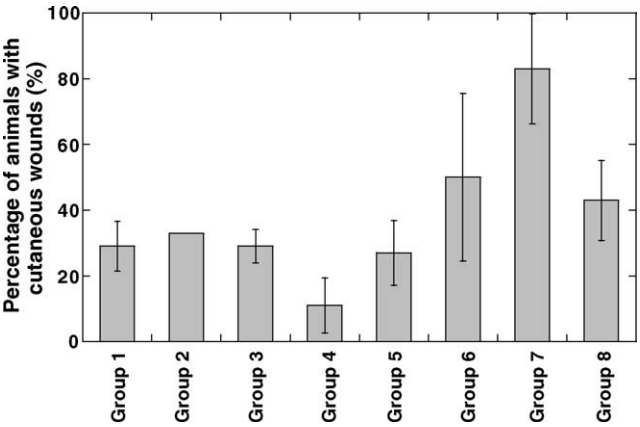

Fig. 4. Percentage of cutaneous wounds for the various groups of mice following different treatments. All of the animals were injected subcutaneously in the dorsal position with melanoma B16-F0 cells (1×10^6 cells) to induce formation of tumors. The various treatments began when tumors had grown to a weight of 0.02 g. The control groups 1 and 2 were treated with PBS, however, group 2 had spermine (100 mg/l) in its drinking water. Group 3 control received a naked hydrogel as treatment. Groups 4 and 5 received native BSAO at concentrations of 5 and 2.5 mU, respectively. Groups 6 and 7 received immobilized BSAO at a concentration of 5 mU, but group 7 had spermine (100 mg/l) in its drinking water. Group 8 received immobilized BSAO at a concentration of 2.5 mU. The data represent means and S.E.M. from at least 3 (groups 1, 3, 4, 6) to 7 (groups 2, 5, 8) independent experiments, each with 5–6 mice per group. For group 7, data are the mean and standard deviation of only one experiment (five mice).

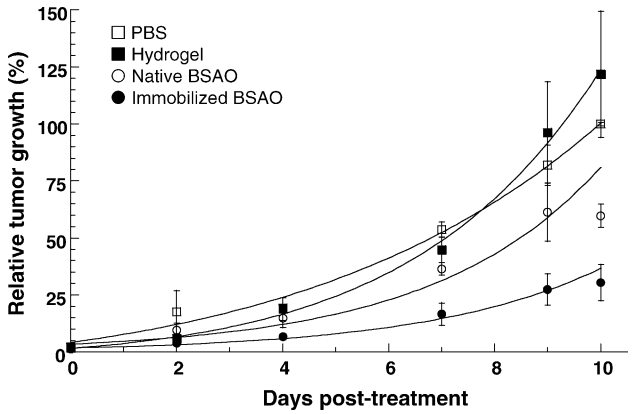

Fig. 5. Effect of different treatments with native or immobilized BSAO on tumor growth rate. All of the animals were injected with melanoma B16-F0 cells (1×10^6 cells) and groups received different treatments when their tumor reached 0.02 g. All measurements of tumor size were made from day 0 post-treatment. Animals were sacrificed at day 10 after the beginning of the treatment. The data represent means and S.E.M. from four independent experiments, each with 4–6 mice per group. The positive control groups were injected with PBS ((□), open square) or with a naked hydrogel ((■), solid square). The two other groups were treated with a single injection of 2.5 mU of native BSAO ((○), open circle) or with immobilized BSAO ((●), closed circle), respectively.

for the anticancer strategy since it was, in fact, deleterious to health of the animals. Furthermore, spermine was previously reported to cause renal toxicity [39]. Therefore, further experiments were carried out without dietary addition of spermine.

3.2. Antitumor effect of native and immobilized BSAO in mice

The various treatments of the tumors consisted of a single injection (PBS, empty hydrogel, native BSAO or immobilized BSAO) in the center of the tumor. The day at which the treatment begins corresponds to day 0 post-treatment. When native BSAO (2.5 mU) was used for the treatment, the size of the tumor was 40–50% smaller than the size of tumor in the positive control group at day 10 (Fig. 5). A more striking reduction of tumor size was observed in the mice treated with immobilized BSAO (2.5 mU). Effectively, the tumor was 70% smaller than the tumor in the positive control group at day 10. The tumor growth curves for the groups that received native or immobilized BSAO as treatments were statistically compared to the tumor growth curve of the positive control group. A significant difference was observed in terms of growth rate between mice receiving native BSAO and the positive control group ($P = 0.0409$). However, a highly significant difference was obtained for a similar comparison with the group treated with immobilized BSAO and the positive control group ($P = 0.0001$). When a higher dose (15 mU) of immobilized or native BSAO was used, there was no variation in the rate of the tumor growth, relative to control groups (Table 1).

Table 1
Lack of effect on tumor growth by a higher concentration of immobilized BSAO

Days post-treatment	Relative tumor growth (%)	
	Control	Immobilized BSAO (15 mU)
0	0	0
1	0	8.30
3	4.53	5.43 ± 3.50
4	4.38 ± 2.85	11.70 ± 6.03
5	9.96 ± 3.55	19.25 ± 7.43
6	17.58 ± 5.71	20.75 ± 5.87
8	36.83 ± 6.46	28.15 ± 15.03
9	40.75 ± 10.50	48.83 ± 21.07
10	44.38 ± 9.70	46.64 ± 12.34
11	54.71 ± 10.09	49.74 ± 5.00
12	70.19 ± 14.06	67.17 ± 0.75
13	69.81 ± 9.48	79.47 ± 10.45
15	100.00 ± 12.17	78.49

Tumor growth was determined in melanoma-bearing mice at different days post-treatment with PBS (controls) or immobilized BSAO, which were both administered by subcutaneous injection. Data represent mean and S.D. from two experiments each with three mice per group.

3.3. Mechanism of tumor cell death following treatment with native and immobilized BSAO

Subsequently, the mechanism by which the tumor cells died was determined. The tumor mass was excised at different times following the treatment with both forms of BSAO from the various groups of mice. The cells obtained from the untreated tumors excised from the positive control group showed a constant viability of around 80% and few apoptotic (5%) or necrotic (15%) cells were observed at any time after the saline injection (Fig. 6A and D).

In contrast, cells isolated from tumors treated with 2.5 mU of native BSAO showed a gradual decrease in cell viability from around 80% to 55%, 48–72 h after the injection (Fig. 6B). At 96 h post-injection, the tumor cell viability increased gradually and returned to initial levels (approx. 80%) at 120 h after the administration of native BSAO. There was a corresponding gradual increase in necrotic cells (Fig. 6B and E) during the first 72 h after the treatment, to reach a maximum close to 40%. The percentage of cell death by apoptosis remained constant at low levels (Fig. 6B), which were similar to those observed in the control group (Fig. 6A).

Interestingly, Fig. 6C and F highlight a different mechanism of cell death in the group of mice treated with immobilized BSAO. Effectively, after the injection of immobilized BSAO, tumor cells underwent cell death by apoptosis. Levels of apoptotic cells increased to 35% at 4 h and reached a maximal value of about 70% at 120 h after the treatment (Fig. 6C). Subsequently, there was a gradual decrease to 30% apoptotic cells at 216 h. Relative to native BSAO, there was a more pronounced decrease in cell viability following treatment of mice with immobilized BSAO. A minimal viability of less than 20% was observed

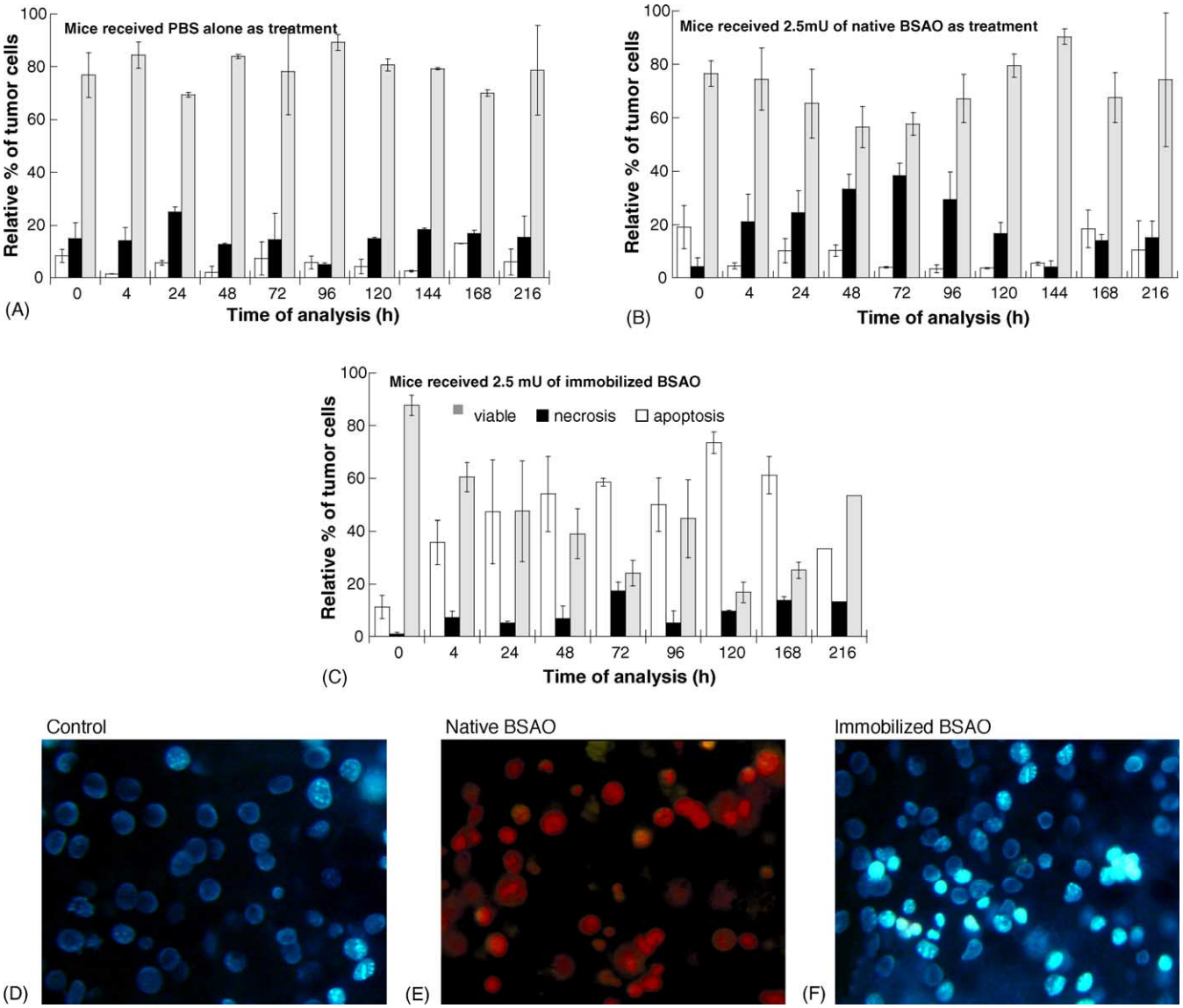

Fig. 6. Determination of cellular death pathway in tumors excised following a treatment with native or immobilized BSAO. Mice had received, by subcutaneous injection in the dorsal position, melanoma B16-F0 cells (1×10^6 cells) and tumors were allowed to grow to 0.02 g before beginning the various treatments. Tumor cells were obtained by excision of the tumor at various times following one of the treatments, with (A) PBS alone, (B) native BSAO or (C) immobilized BSAO. Apoptotic ((□), open square), necrotic ((■), solid square) and viable cells ((▭), grey) were determined by fluorescence microscopy and the total number of cells under bright field illumination. The fractions of apoptotic and necrotic cells are given relative to total cells. A minimum of 400 cells was counted per tumor. Representative images (320×) are shown of cells from tumors of mice treated with (D) PBS alone, or with either (E) native or (F) immobilized BSAO, after 72 h. The data represent the mean and S.E.M. from four independent experiments, each with 4–6 animals per group. The percentage of apoptosis showed significant differences ($P < 0.05$) between groups of mice treated with immobilized BSAO and groups treated with native BSAO or untreated controls at 4, 24, 48, 72, 96, 120 and 168 h after the beginning of the various treatments.

in tumor cells in this group of treated mice at 120 h (Fig. 6C). The percentage of necrotic cells remained low and was similar to that in the positive control group of mice. Statistical analysis of the percentage of apoptosis showed significant differences ($P < 0.05$) between groups of mice treated with immobilized BSAO (Fig. 6C) relative to groups treated with native BSAO (Fig. 6B) or untreated controls (Fig. 6A) at 4, 24, 48, 72, 96, 120 and 168 h after the beginning of the various treatments. The percentage of apoptotic cells in the group treated with native BSAO (Fig. 6B) was significantly different ($P < 0.05$) from that observed in the untreated group (Fig. 6A) at the experimental time of 24 h only. The level of apoptotic cells was similar for the three groups at the beginning ($T = 0$) and at the end of the experiment ($T = 216$ h) (Fig. 6A, B and C).

To further confirm that the BSAO/spermine enzymatic system can in fact cause cell death by apoptosis, Annexin V-FITC/PI fluorescence was analyzed by flow cytometry (Fig. 7). B16-F0 melanoma cells were exposed to either immobilized BSAO (Fig. 7C) or native (Fig. 7D) BSAO and spermine for 4 h, followed by Annexin V/PI staining. When cells were treated with immobilized or native BSAO,

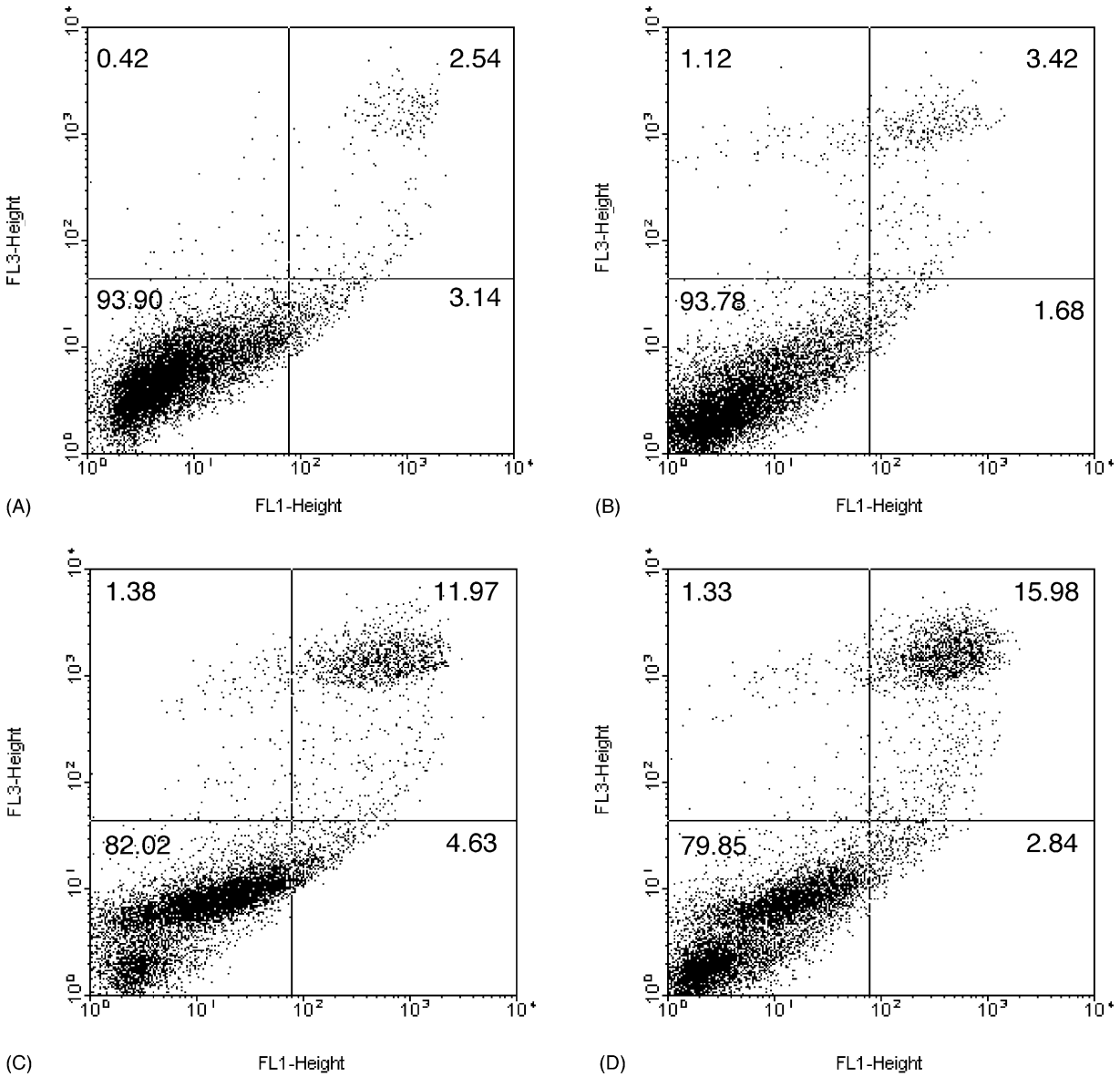

Fig. 7. Flow cytometry analysis of apoptosis using Annexin V in melanoma cells following treatment with immobilized and native BSAO. Cells (1×10^6/ml) were either (A) untreated controls, or they were treated with (B) empty PEG hydrogels, or spermine (50 μM) and either (C) immobilized BSAO (6.0 mU/ml) or (D) native BSAO (6.0 mU/ml), for 4 h. Cells were subsequently stained with Annexin V-FITC (*x*-axis) and PI (*y*-axis). Twenty thousand cells were then analyzed using a FACS scan to determine the percentage of Annexin V/PI negative (early apoptosis) and Annexin V/PI positive cells (late apoptosis). BSAO alone and spermine alone were similar to controls (data not shown). One representative experiment is shown from three experiments.

there was an increase in Annexin V positive cells, compared to untreated controls (Fig. 7, Table 2). The fraction of Annexin V positive/PI negative cells in early apoptosis was higher in cells exposed to immobilized BSAO, compared to native BSAO. However, there were more Annexin V positive/PI positive cells in late apoptosis when cells were

Table 2
Fraction of melanoma cells in early and late stages of apoptosis following treatment with native or immobilized BSAO

Treatment	Type of cell death (% of total cells)	
	Annexin V, PI −ve (early apoptosis)	Annexin V, PI +ve (late apoptosis)
Control	2.31 ± 0.84	3.01 ± 0.88
Naked hydrogel	1.97 ± 0.29	4.52 ± 1.10
Immobilized BSAO	4.27 ± 0.37	10.02 ± 1.96
Native BSAO	2.69 ± 0.16	13.70 ± 2.29

B16 melanoma cells (1×10^6/ml) were incubated for 4 h with empty PEG hydrogels, or with 6 mU/ml of native BSAO or immobilized BSAO in the presence of 50 μM spermine, relative to untreated controls. Cells were labeled with Annexin V and propidium iodide (PI) and then analyzed by FACscan. Data represent means and S.E.M. from three separate experiments.

exposed native BSAO, relative to immobilized BSAO. The naked PEG hydrogels (Fig. 7B) showed similar effects to untreated controls (Fig. 7A, Table 2).

4. Discussion

In this study, native and immobilized forms of the BSAO enzyme were used as anti-tumoral drugs on mice bearing melanomas and their efficiencies were evaluated in terms of inhibition of tumor growth. Also, the type of death pathway of tumor cells exposed in vivo to the enzyme was evaluated with the aim of determining whether cell death was related to an apoptotic or necrotic mechanism. BSAO could be useful as an antitumor agent, by causing inhibition of proliferation by exerting two separate mechanisms. Firstly, it is well known that BSAO is able to generate highly cytotoxic products, including hydrogen peroxide and aldehydes such as acrolein and aminoaldehydes, by deaminating polyamine substrates such as spermine and spermidine [6,22,26]. When BSAO is directly injected into the tumor, it is likely that polyamines may be secreted by membrane-leaky necrotic cells in the tumor core, thus rendering them available for the enzymatic reaction. Secondly, when injected directly into a solid tumor, the BSAO enzyme could modify polyamine concentrations in the tumor, leading to polyamine deprivation to a sufficient level that the proliferation of the tumor cells could be affected. In this case, a decrease in polyamine levels could lead to decreased tumor growth.

The polyamine biosynthetic pathway is very active during the growth of various cancer cell lines in vitro [4] and also in human cancers in vivo [40–42]. The addition of spermine in the drinking water of C57BL mice caused a marked increase in the size of untreated melanoma tumors, when compared to tumors of mice drinking regular water. This finding is consistent with the necessity of polyamines for tumor growth. Dietary supplementation of spermine (group 2) did not increase the frequency (35%) of skin wounds when compared to controls (group 1) (Fig. 4). However, marked increases in skin wound frequencies, up to 50% and 80%, occurred in mice treated with 5 mU of immobilized BSAO and either regular (group 6) or spermine-enriched water (group 7), respectively (Fig. 4). At lower doses of BSAO, 2.5 mU, there were no major differences in skin wound frequencies compared to controls. It appears that the higher concentration of immobilized BSAO (5 mU) produced cytotoxic products in concentrations high enough to diffuse to the normal skin tissue surrounding the melanoma and to induce severe wounds. This effect was more pronounced when spermine was added to the drinking water, in combination with the high dose of BSAO. Under these conditions, the velocity of the kinetic reaction would be more rapid, generating higher concentrations of cytotoxic products. Hydrogen peroxide is detoxified by two cellular defence systems: by the enzyme catalase and by reacting with reduced glutathione (GSH), catalyzed by glutathione peroxidase (Fig. 1). Acrolein is detoxified by a conjugation reaction with GSH, catalyzed by glutathione *S*-transferase (Fig. 1). These higher levels of cytotoxic products would overwhelm the detoxification ability of the cellular defences, therefore contributing to normal tissue toxicity. For these reasons, addition of exogenous spermine and high doses of BSAO were not studied in the subsequent series of experiments.

Treatment of mice bearing subcutaneous melanoma tumors with a low dose (2.5 mU) of native (group 5) or immobilized (group 8) BSAO, induced inhibition of tumor growth by 40% and 70%, respectively, during a period of 10 days after a single injection of the enzyme into the center of the tumor, compared to the positive control group (group 1) (Fig. 5). The PEG-albumin matrix used for enzyme immobilization had no significant effect on tumor growth. The difference in the decrease of tumor growth between the two forms of BSAO could be explained by several reasons. Firstly, the native enzyme is more susceptible to deactivation and degradation in the physiological environment than the immobilized form of the enzyme [43]. Secondly, it was previously reported that the immobilization of BSAO into a hydrogel matrix of PEG-albumin increased its operational stability in vitro from 1.5 h to 70 h [29]. Operational stability was defined as the half-life of the enzyme continuously exposed to a constant substrate concentration at 25 °C. Also, the apparent K_m (binding affinity between enzyme and substrate) of the immobilized BSAO was very similar to the K_m of the native BSAO, when benzylamine was the substrate. However, the V_{max} (maximum velocity of enzymatic reaction) of the immobilized BSAO was two times lower than the V_{max} of the native soluble enzyme. This was explained by the fact that the rate of catalysis was diffusion limited by the matrix, i.e. the polyamine substrates need to diffuse inside the PEG matrix in order to have access to the enzyme. Similar results of catalytic stability in vivo were obtained when immobilized asparaginase was injected into the rat peritoneal cavity to eliminate blood levels of asparagine [28]. The lower V_{max} of the immobilized form of BSAO would allow a prolonged, slow release of cytotoxic products compared to more rapid generation of higher levels of cytotoxic products with the native enzyme.

Several in vitro studies have shown that the cytotoxic products of BSAO and spermine, H_2O_2 and acrolein, can induce either apoptosis or necrosis, depending on their concentrations and the cell type [44–46]. However, there are few, if any, in vivo studies highlighting the mechanism of cell death caused by polyamine-mediated generation of these cytotoxic products. To determine the type of cell death induced by BSAO, tumors were excised at different times from the various groups of mice after the single injection of saline, 2.5 mU of native BSAO or 2.5 mU of immobilized BSAO. Tumors from the untreated mice showed a high level of cell viability and the few remaining

cells were necrotic or apoptotic. On the other hand, when mice were treated with BSAO, the viability of tumor cells decreased, but the response was more rapid and for a longer time (5 days) with immobilized rather than native BSAO (2–3 days). However, an important finding is that immobilized BSAO caused cell death mainly by apoptosis. These findings with immobilized BSAO could be beneficial since the ultimate aim of anticancer treatments is to induce tumor cell death by apoptosis, a process which avoids inflammatory damage to surrounding tissue.

The difference in the mechanisms of cell death observed when native or immobilized BSAO were used to treat the mice can be explained in terms of the kinetics of generation of cytotoxic products. As discussed previously, native BSAO has a higher V_{max} than immobilized BSAO. In addition, when using native BSAO, the reaction products do not have to cross a matrix to reach the tumor tissues, nor does the substrate have to enter the matrix to have access to the enzyme. It is also possible that the area of enzyme distribution covers a larger volume of the tumor than that for the immobilized one. Under these conditions, the native BSAO generates a burst of hydrogen peroxide and aldehyde(s) at such concentrations that necrosis is favoured. On the other hand, a lower V_{max} and diffusional constraints due to the matrix should allow a more gradual release of the cytotoxic products generated by the immobilized enzyme in the tumor. Effectively, it is well known that during the translocation of the substrate to the center of the bioreactor to reach the deeply embedded enzyme, the substrate is transformed into the product(s). So, under these conditions only a part of the enzyme is operational. These results are consistent with findings generally observed in vitro, where toxic compounds usually cause necrosis at higher doses and apoptosis at lower doses. Effectively, it was demonstrated that higher concentrations of acrolein favoured necrosis by inhibiting caspases in lymphocytes [46]. Also, high concentrations of H_2O_2 (1–10 mM) induced necrosis in lung fibroblasts, whereas lower amounts (10–100 μM) caused cell death by apoptosis [45].

Native BSAO caused tumor cell toxicity during a shorter period of time, compared to immobilized BSAO (Fig. 6B and C). Native BSAO caused toxicity by necrosis during 5 days, with a maximum level of toxicity after 2–3 days (Fig. 2B). There was a gradual recovery of viable cells starting from day 4, indicating that the native enzyme was no longer functionally active. Immobilized BSAO caused toxicity by apoptosis throughout the 9 days, with a maximum level of toxicity after 5 days (Fig. 6C). It was demonstrated that native BSAO can be inactivated by H_2O_2 formed during the enzymatic reaction [47]. Furthermore, aldehydes such as acrolein can react with proteins and generate a protein-linked carbonyl derivative [48], which could alter protein function. Thus, the initial burst release of the cytotoxic products could inactivate native BSAO, which could partly explain the shorter duration of tumor cell toxicity, compared to the immobilized form of BSAO. Furthermore, the tumor environment can contain high levels of proteases, which would be detrimental to the structural integrity of the enzyme. Immobilization into a matrix protects BSAO against such detrimental factors and this could result in the more sustained cytotoxic action of the immobilized enzyme, which was at least two times longer (minimum viability observed at day 5 compared to days 2–3 for native BSAO).

PEG is a non-toxic, non-immunogenic biocompatible polymer which has been approved for clinical use, conjugated to certain enzymes and drugs, by the Food and Drug Administration. PEG conjugation increases the circulating half-life of proteins and drugs and reduces their renal clearance, while maintaining their biological activity, compared to the parent compound. The immunogenicity of certain proteins can be reduced, by masking their antigenic sites by the polymeric chains of PEG [43]. In cancer treatment, PEG-asparaginase is useful in acute lymphocytic leukemia [49], PEG is used to reduce the immunogenicity of liposome-encapsulated doxorubicin and pegfilgrastim is a pegylated form of granulocyte colony-stimulating factor filgrastim, used in the management of chemotherapy-induced neutropenia [50].

The ultimate aim of the current research is to develop a new type of cancer treatment for solid tumors, which takes advantage of the fact that levels of polyamines are higher in many solid tumor tissues, such as breast, colon, melanoma, brain and kidney [4]. The major objective of this paper is to demonstrate anti-tumor activity in vivo with the spermine/amine oxidase enzymatic system, following the promising results that were previously obtained in vitro in several tumor cell lines [15–18,26]. The microbeads used in this study were prepared with the objective of direct injection into solid tumors. This allows targeting of the toxic treatment to the solid tumor, which is not yet attainable by intravenous injection. Direct injection of a protein/enzyme into solid tumors is applicable to melanomas as well as to other inoperable solid tumors (e.g. brain), which are accessible by needle injection in some cases. This approach is also applicable to solid tumors which develop resistance following treatment with chemotherapy or radiotherapy. We also reported that multidrug resistant cells are sensitive to the toxic effects of the BSAO/spermine enzymatic system [17,27]. Multidrug resistance is a major reason for the failure of chemotherapy in tumors which are often initially responsive to treatment. If drug resistance could be overcome, the impact on cancer patient survival would be highly significant [51]. Targeting of solid tumors is an important objective for improved treatment as well as for decreasing undesirable side effects in the cancer clinic.

In conclusion, the treatment of melanomas in mice with BSAO immobilized in a matrix of PEG hydrogel represents an effective strategy to eliminate this type of cancer. The immobilization allows an improved performance of the enzyme by maintaining its activity for 9

days and represents a 'cleaner' treatment since the cells die by apoptosis and not by necrosis. Compared to the native form of the enzyme, the advantage with respect to anticancer treatment is that immobilized BSAO can act by allowing the slow release of cytotoxic products within the tumor.

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