

Superoxide Production by Phagocytes in Myeloid Graffi Tumor-Bearing Hamsters

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Superoxide Dismutase, Macrophages, Tumors

A progressive suppression of the phagocytic ability of peritoneal macrophages and polymorphonuclears (PMNs) in hamsters with transplanted myeloid tumors was previously established. The i.p. application of Cu/Zn SOD, isolated from the fungal strain *Humicola lutea* (HLSOD) (2 injections before and 5 injections after tumor transplantation) induced the mean survival time of the animals as well as a temporally stimulating action on the macrophage and PMNs phagocytosing indices. In the present work, the superoxide production of peritoneal macrophages and PMNs during 30 days of tumor progression was followed. Effects of the application of HLSOD in an optimal protective dose on the superoxide production in peritoneal macrophages and blood PMNs were examined. The spontaneous and phorbol-myristate acetate (PMA)-inducible O_2^- production in both types of phagocytes was 4–5-fold increased in tumor-bearing hamsters (TBH), as compared to the controls, at day 14 after tumor transplantation (the day of tumor appearance in transplanted animals). Furthermore, O_2^- production was also similar to the control values for the following days of observation. HLSOD treatment of TBH induced a normalization of superoxide production in macrophages and PMNs. Therefore, the established decrease of superoxide anions in phagocytosing cells of TBH indicates possible effects of HLSOD on the host antioxidant defense.

Introduction

Reactive oxygen species are formed constantly in the organisms, both, by deliberate synthesis (e.g. by activated phagocytes) or/and by chemical side reactions. They are removed by enzyme and non-enzyme antioxidant defense systems. Oxidative stress, occurring when antioxidant defenses are inadequate, can damage lipids, proteins, carbohydrates and DNA. Numerous clinical diseases are caused by oxidative stress, but more often the stress is a result from the diseases (Halliwell, 1991; Oberley and Oberley, 1997).

Free oxygen radicals may play an essential role in tumor initiation and tumor promotion as well. In high concentrations they have a toxic effect on the biological systems, including injure of DNA molecules and cell membranes, which is an important stage in the process of tumor genesis (Halliwell, 1991; Bittinger *et al.*, 1998).

Our previous investigation (Toshkova *et al.*, 2000) showed that *H. lutea* Cu/Zn SOD induced a protective effect on the survivability of myeloid tumor Graffi-bearing hamsters. This effect was partly explained by the established immunorestoring action of the preparations on the phagocytic activity of the peritoneal macrophages and polymorphonuclear leukocytes, as well as on the proliferating potential of spleen B-lymphocytes during the initial stage of tumor progression.

The aim of the present work was to examine the spontaneous and phorbol-myristate acetate (PMA)-induced superoxide production in macrophages and polymorphonuclears (PMNs) during tumor progression in hamsters with transplanted myeloid Graffi tumors as well as the possible influence of *H. lutea* Cu/Zn SOD (HLSOD) on the oxidant-antioxidant balance in these cells.

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Materials and Methods

Tumor

The myeloid tumor was induced by Graffi virus and was adapted to hamsters by subcutaneous inoculations of 1×10^5 viable tumor cells (Jakimov *et al.*, 1979). For the present experiments, 2×10^4 viable tumor cells were inoculated s.c. in the interscapular field of hamsters. According to our previous investigations, this quantity of cells induced 100% transplantability and 100% mortality of hamsters (Toshkova, 1995).

Experimental animals

“Golden Siberian” hamsters, two months of age, from both sexes and weighing 80–100 g, were used for the experiments. The animals were obtained from the animal house of the Institute of Microbiology, Bulgarian Academy of Sciences, Sofia. They were bred and grown under standard conditions, as accepted by the Bulgarian Veterinary Health Control Service. The animals were separated in 4 experimental groups (12 animals per group):

- *Group 1*: Hamsters with transplanted tumors, treated with *H. lutea* Cu/Zn SOD (SOD+T),
- *Group 2*: Hamsters with transplanted myeloid tumors (T),
- *Group 3*: Healthy hamsters, treated with HLSOD (SOD), and
- *Group 4*: Control group of healthy hamsters (Control).

Cu/Zn superoxide dismutase application

Cu/Zn superoxide dismutase was applied i.p. during 7 days before and 14 days after tumor transplantation in a single 65U dose each time per animal (Fig. 1). This dose and scheme of application were determined to be optimal in our previous investigation (Toshkova *et al.*, 2000). Superoxide anion production by peritoneal macrophages and blood PMNs were examined at days 14, 26 and 30 after tumor transplantation.

Blood polymorphonuclears (PMNs)

Hamsters under ether narcosis were sacrificed by decapitation. Blood samples were collected in glass tubes and immediately diluted 1:3 with 2% sodium citrate. PMNs were separated from other leukocytes by gradient centrifugation on Histo-

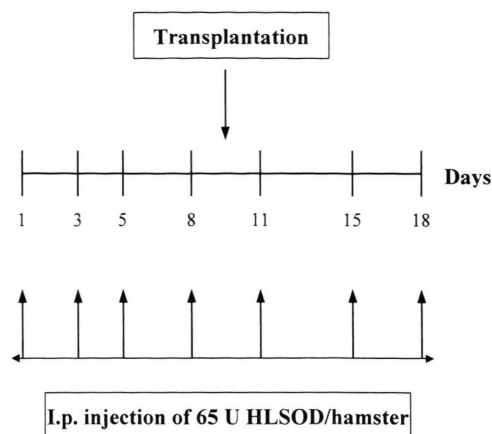


Fig. 1. Schedule of i.p. application of Cu/Zn SOD isolated from the fungal strain *Humicola lutea* 103.

paque (Sigma; Grünwald, Germany) according to a method described by Boyoum (1968). Briefly, citrate blood samples were subsequently carefully overlaid by two gradients (Histopaque 1077, overlaid with Histopaque 1119; the ratio of blood samples to gradients was 1:3) and centrifuged at 300xg for 30 min at room temperature. The granulocytes from the interphase between the two layers were collected and washed twice with cold phenol red-free Hanks balanced salt solution (HBSS) and the contaminating erythrocytes were destroyed by hypotonic lysis. The PMNs were then suspended in phenol red-free HBSS at a cell concentration of 3×10^6 cells/ml and prewarmed to 37 °C before assay. Cell viability was estimated to be 95–98% by the trypan blue excluding test, and the mononuclear cell contamination in Giemza-stained preparations was determined to be less than 1%.

Macrophages

Macrophages were collected by lavage of the peritoneal cavity of experimental animals with cold HBSS (pH 7.3). After centrifuging at 300xg for 10 min at 4 °C, erythrocyte contamination was eliminated with 0.84 M NH_4Cl . The cell pellets were washed with cold HBSS and resuspended to a final concentration of 5×10^6 cells/ml in RPMI 1640 medium (Sigma, Deisenhofen, Germany), supplemented with 5% fetal bovine serum (Sigma, Grünwald, Germany). Then, macrophages (60 μl /well) were distributed on a plastic plate (96 wells)

and allowed to adhere at 37 °C for 1 h in an atmosphere containing 5% CO₂. Before the O₂⁻ assay, adherent macrophages were washed twice with warmed HBSS.

O₂⁻ assay

Superoxide anion production by phagocytes was measured according to the superoxide dismutase-inhibiting cytochrome *c* method described by Leslie (1987). Briefly, cytochrome *c* (Cyt C; Fluka, Deisenhofen, Germany), 3.5×10^{-4} M, in a HBSS stock solution was prepared. Phorbol-myristate acetate (PMA) (Sigma, Deisenhofen, Germany), dissolved in DMSO at a concentration of 1 mg/ml stock solution, was stored at -70 °C. Prior to the assay, the cytochrome *c* (Cyt C) stock solution was diluted 1:3 with HBSS and thereof three solutions was prepared: (1) diluted Cyt C; (2) diluted Cyt C, containing PMA (200 ng/ml) and (3) diluted Cyt C, containing PMA (200 ng/ml) and SOD (800 U/ml). The enzyme used for solution (3) was Cu/Zn-SOD from bovine erythrocytes with an activity of 3000 U/mg dry weight (Fluka, Germany, Deisenhofen). One unit was defined as the amount of enzyme to cause a 50% inhibition in the rate of reduction of cytochrome *c* at 25 °C, pH 7.8.

Immediately, 100 µl/well of each solution were distributed to the 96 wells plate, containing adherent macrophages (60 µl; 3×10^5), a PMN suspension (60 µl; 1.6×10^5 cells) or HBSS (60 µl; for cell-free control). The plate was then incubated at 37 °C in an atmosphere of 5% CO₂ for 30 min and finally cooled on ice to stop the reaction. Cytochrome C reduction was measured at 550 nm with an ELISA reader (Packard, England) after 15 and 30 min of incubation.

Data analysis

Superoxide production was determined from the difference in the amount of cytochrome *c* reduction by PMA-stimulated cells in the absence or presence of SOD according to the formula, proposed by Leslie (1987):

$$\Delta E_{550} \text{ (PMA-inducible)} = E_{550} \text{ (Cyt C+ PMA)} - E_{550} \text{ (Cyt C+ PMA+SOD)};$$

$$\Delta E_{550} \text{ (spontaneous)} = E_{550} \text{ (Cyt C)} - E_{550} \text{ (Cyt C+ PMA+ SOD)}$$

The extent of Cyt C reduction was converted to nmol of Cyt C reduced per 10⁶ cells, using the formula:

$$\text{nmol O}_2^- = \Delta E_{550} / (8.61 \times 10^3 \text{ M}^{-1})$$

Cu/Zn superoxide dismutase

The fungal strain, *Humicola lutea* 103, from the Mycological Collection of the Institute of Microbiology, Sofia, was used for the production of Cu/Zn-SOD. The fermentation conditions were the same as described earlier (Pashova *et al.*, 1999). Cultivation was performed in a 3-l bioreactor ABR-09, equipped with a pH-monitoring system, an automatic DO-monitoring and controlling system. The purified HLSOD was prepared according to a method described by Dolashka-Angelova *et al.* (1999). It is a water-soluble homodimeric glycoprotein with a molecular mass of approximately 31 700 Da (unpublished data). The SOD activity was measured by the nitroblue tetrazolium (NBT) reduction method of Beauchamp and Fridovich (1971). One unit of SOD activity was defined as the amount of SOD required for a 50% inhibition of the reduction of NBT and is expressed as units per mg protein (U/mg protein). Sodium cyanide (2 mM) was used to distinguish between the cyanide-sensitive isoenzyme Cu/ZnSOD and the cyanide-resistant MnSOD. The Cu/ZnSOD activity was obtained as total activity minus the activity in the presence of 2 mM sodium cyanide. The protein content was estimated according to Lowry *et al.* (1951), using crystalline bovine serum albumin as standard.

Statistical methods

The results of experimental groups were analyzed by the Student's T test. Data are presented as mean arithmetical values ± SD, and P<0.05 was accepted to be significant.

Results

The O₂⁻ production in both types of cells, macrophages and PMNs, does not strongly depend on the time of incubation, as no considerable differences between the extinction coefficients after 15 and 30 minutes are observed. Therefore, in the present study, results obtained after 30 minutes of

incubation of the macrophages and PMNs with the three Cyt C-containing solution are given.

It was found that the PMA-inducible O_2^- production by peritoneal macrophages in tumor-bearing hamsters (TBH) was significantly (5-fold) higher compared to that of the control group at the day of tumor appearance in most animals with transplanted tumors (day 14 after transplantation; 49.04 nmol/ 10^6 cells (T); 8.77 nmol/ 10^6 cells (control)). Macrophages from HLSOD-treated healthy and tumor-bearing hamsters showed indispensable enhanced PMA-inducible superoxide anion production (11.36 nmol/ 10^6 cells (SOD); and 11.87 nmol/ 10^6 cells (SOD+T), respectively). An unchanged O_2^- production of macrophages was obtained after PMA stimulation for the following days in HLSOD-treated healthy animals (5.05 nmol/ 10^6 cells (SOD)). Lacking of HLSOD treatment of tumor-bearing animals showed an extreme decrease in O_2^- secretion (3.2 nmol/ 10^6 cells and 0 nmol/ 10^6 cells (T)) at days 26 and 30, respectively) (Fig. 2).

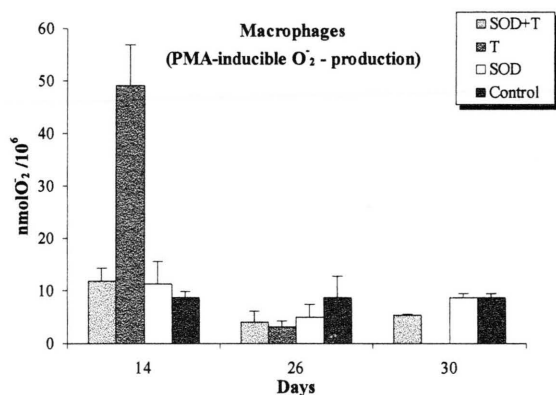


Fig. 2. Phorbol-myristate acetate (PMA)-inducible superoxide anion production (nmol O_2^- / 10^6 cells) by peritoneal macrophages from hamsters with progressing myeloid Graffi tumors (T). *Experimental groups*: (SOD+T): tumor-bearing animals, treated by Cu/Zn-SOD isolated from *Humicola lutea* 103 (HLSOD); (T): tumor-bearing hamsters without treatment; (SOD): healthy animals, treated by HLSOD; (control): healthy animals.

In the absence of PMA lower values of superoxide anion production was observed and the effectiveness of macrophages in antitumor immunity was examined. At the 14th day of observation, the level of O_2^- in TBH was strongly enhanced (3.7 \times) compared to the healthy control macrophages

(26.33 nmol/ 10^6 cells (T); 7.0 nmol/ 10^6 cells (control)). Spontaneous superoxide production by macrophages from TBH was significantly depressed or absent (4.03 nmol/ 10^6 cells and 0 nmol/ 10^6 cells) following the next days of observation (26th and 30th respectively). Not any significant differences in spontaneous superoxide production by macrophages from HLSOD-treated TBH (SOD+T), healthy animals (SOD) and the control group (control; Fig. 3) were observed.

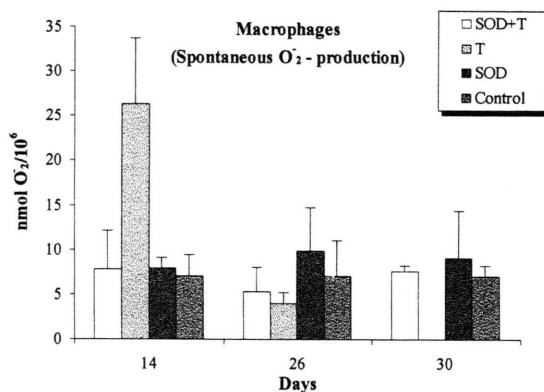


Fig. 3. Spontaneous superoxide anion production (nmol O_2^- / 10^6 cells) by peritoneal macrophages from hamsters with progressing myeloid Graffi tumors. *Experimental groups*: see Fig. 2.

Increased (2.0 \times) superoxide anion production in PMA-stimulated blood PMNs from TBH at day 14 (15.87 nmol/ 10^6 cells (T) and 7.84 nmol/ 10^6 cells (control)) was followed by a twofold decrease or even absence of O_2^- secretion during the tumor progression (Fig. 4). The levels of the inducible superoxide production by PMNs from healthy and tumor-bearing animals, treated with SOD, were lower than that of the controls for the whole time of observation (Fig. 4).

The spontaneous superoxide anion production by blood PMNs was increased twofold in TBH at day 14 (18.58 nmol/ 10^6 cells (T), 8.13 nmol/ 10^6 cells (control)) and then superoxide production strongly decreased up to 0 nmol/ 10^6 cells at the 30th day of observation (Fig. 5). In the groups of healthy and tumor-bearing animals, treated with HLSOD, the values of spontaneous production of O_2^- anions by blood PMNs were similar to that of the healthy untreated hamsters (control, Fig. 5) at the final day of observation.

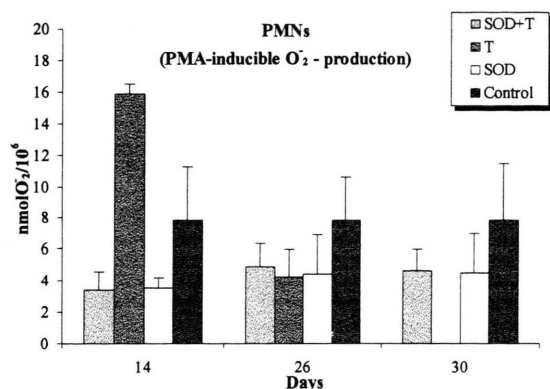


Fig. 4. Phorbol-myristate acetate (PMA)-inducible superoxide anion production (nmol O_2^- per 10^6 cells) by polymorphonuclears (PMNs) from hamsters with progressing myeloid Graffi tumors. *Experimental groups:* see Fig. 2.

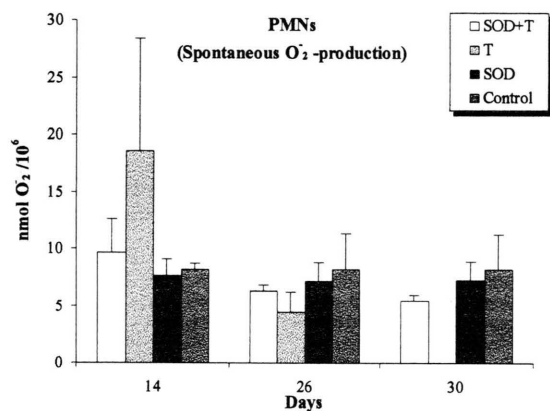


Fig. 5. Spontaneous superoxide anion production (nmol O_2^- per 10^6 cells) by polymorphonuclears (PMNs) from hamsters with progressing myeloid Graffi tumors. *Experimental groups:* see Fig. 2.

Discussion

Our experiments show that production of superoxide radicals by phagocytes (spontaneous and PMA-inducible) is extremely increased (3–4 fold) in TBH at the initial stage of tumor progression (day 14, the day of tumor appearance) (Figs 2–4). This enhanced anion secretion might cause the initial elimination of small quantities of malignant cells at the site of tumor transplantation. Furthermore, the imbalance, induced by the increase of reactive oxygen radicals without the possibility for adequate antioxidant dismutation is probably one

of the reasons for the subsequent rapid growth of the implanted Graffi tumor cells. In the group of HLSOD-treated tumor-bearing animals, the superoxide production by phagocytes was similar to that of the healthy animals (control, Figs. 2–4). The low values or absence of superoxide radicals in phagocytes of TBH at the final stages of observation (day 26 and 30) correlate with the decreased phagocytic indices of PMNs and macrophages, established earlier (Toshkova *et al.*, 2000). This suppression could be explained by circulation of immunosuppressive factors, probably produced by tumor tissue in the tumor-bearing hamsters. Our previous investigations showed a progressive suppression of the migration, cytotoxic and phagocytic ability of peritoneal macrophages as well as of the phagocytic and adherence ability of blood PMNs in hamsters with transplanted myeloid Graffi tumors (Toshkova, 1995).

Suppressing factors in tumor immunology have been intensively studied. Interleukin 10 was identified in patients with renal cell carcinoma by Wittke *et al.* (1999). Dai *et al.* (1998) found that a variant of Freund leukemia virus FIS-2 is responsible for a rapid immunosuppressive action. Leigh *et al.* (1995) and Brown *et al.* (1994) explained the immunosuppression in skin cancer patients by the presence of human papilloma virus. Scheeren *et al.* (1992) found that retroviral p15E-related proteins are responsible for the immunosuppression in patients with neck and head carcinomas. Probably the Graffi virus could be an important factor responsible for some immunosuppression in the case of the myeloid tumor in our experimental model in hamsters.

During the last decade, a large number of investigations on the effect of antioxidants and free radicals in anticancer therapy were performed. The results are contradictory because they concern different tumors, human cancer diseases, tumor cell cultures, different stages of tumor progression, etc.

A large number of experiments showed a cancerogenic role of high levels of free oxygen radicals. Yamanaka and Deamer (1974) and Lu *et al.* (1998) proved that antioxidants prevent tumor transformation, both, *in vivo* and *in vitro*. It was shown that the inoculation of Cu/ZnSOD prolonged the survivability of experimental animals with Ehrlich ascites and Sarcoma 180 tumors (Oberley and Buettner, 1979).

During the last years, clinical investigations have indicated changes in the oxidant-antioxidant balance during the oncogenic process. Zhong *et al.* (1997) established a suppressed malignant phenotype of human glioma cells, connected with overexpression of MnSOD. Experimental data of Janssen *et al.* (1999) showed that MnSOD levels in colorectum carcinoma cells and liver metastases are 2–4 times higher than in normal mucosa. They suppose that MnSOD has an important role in human colorectal carcinogenesis. Ambrosone *et al.* (1999) demonstrated that antioxidants (dietary and MnSOD) could ameliorate the genetically based risk for human breast cancer. Bittinger *et al.* (1998) found large O_2^- production without stimulants in melanoma cells at an increasing rate in relation to time and formation of metastases which can be inhibited by SOD. They supposed that O_2^- is implicated in the mechanism of formation of metastases. Similarly, Grammatico *et al.* (1998) found an increased sensitivity to oxidizing agents, correlated with the imbalance of antioxidants in normal melanocytes from melanoma patients. Liaw *et al.* (1997) found significantly lower Cu/ZnSOD activity in human hepatocellular carcinoma cells, compared to the surrounding “normal” cirrhotic tissue. Oberley and Oberley (1997) have demonstrated that antioxidant levels in most human and animal cancers are low. However, a few cancer types have been found to have elevated levels of antioxidant enzymes, particularly MnSOD. The granular cell variant of human renal adenocarcinoma was shown to express low catalase and glutathione peroxidase levels, suggesting

that most cancer types cannot detoxify hydrogen peroxide. Jung *et al.* (1997) established that in 3 permanent malignant prostatic epithelial cell cultures the antioxidant enzyme activities, SOD, catalase or glutathione-reductase were higher and glutathione S-transferase, glutathione peroxidase were lower than in primary cell cultures. On the other hand, Kahlos *et al.* (1999) established lower superoxide levels in mesothelioma cells, due to the higher MnSOD activity of mitochondria. As a result, mesothelioma cells are highly resistant to oxidant components compared to the non-malignant cells. Investigations of Roller and Weller (1998) showed that antioxidants inhibited cisplatin cytotoxicity in human malignant glioma cells.

Conclusions

- The macrophages and PMNs in tumor Graffi-bearing hamsters significantly decrease the superoxide production during tumor progression.
- At the day of tumor appearance, the superoxide production is extremely enhanced, both, in macrophages and PMNs.
- HLSOD, applied i.p., before and after tumor transplantation in optimal doses, participates in the oxidant-antioxidant balance at the beginning of tumor progression.

Acknowledgments

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