Effect of Cu/Zn-Superoxide Dismutase from the Fungal Strain *Humicola lutea* 103 on Antioxidant Defense of Graffi Tumor-Bearing Hamsters

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A novel Cu/Zn-containing superoxide dismutase (SOD) was isolated from the fungal strain *Humicola lutea* 103. Previously, a protective effect of this enzyme (HLSOD) against tumor growth and also superoxide production in Graffi tumor-bearing hamsters (TBH) were established.

The aim of the present study was to investigate the effect of HLSOD on the activity of endogenous SOD and catalase in the cells from TBH during tumor progression. Our results point out that transplantation of Graffi tumor causes a significant decrease in SOD activity in the cells from liver of the hosts (from 35 to 59% compared to the control). In the tumor cells relatively low levels of SOD (about 7 U mg protein^{-1}) were found, and Cu/ZnSOD was the main isoenzyme in total SOD activity. Tumor growth resulted in a reduction of catalase activity, which correlated with the process of tumor progression. A single dose (65 U) treatment with HLSOD caused an increase in endogenous SOD and catalase activity in healthy animals and resulted in restoration of the antioxidant ability in liver cells of the hosts at the early stage of tumor progression. The results show the possible participation of HLSOD in the host oxidant-antioxidant balance, which is probably one of the factors of its immunoprotective action established earlier.

Introduction

There has been a considerable and continuing interest in the use of the antioxidant enzyme superoxide dismutase (SOD) in medicine over the last years, arising from its ability to reduce the deleterious effect of superoxide anion radicals ($^{\bullet}O_2^-$) in the cells. Reactive oxy-radicals (ROS), including $^{\bullet}O_2^-$, are continuously generated in aerobic organisms and can cause widespread damage to the cell (Fridovich, 1995). Their formation appears to be largely increased during stress conditions, so they are involved in creation of many diseases, including cancer.

The literature data about the levels of antioxidant enzymes during cancerogenesis concern different kinds of tumors in different stages of differentiation, as well different tissues from patients (Oberlay and Oberlay, 1997). Kahlos *et al.* (1999) established that malignant mesothelioma cells contained elevated levels of Mn SOD. They are more resistant to oxidants compared to non-malignant mesothelial cells. Janssen *et al.* (1999) established that colorectal adenomas expressed intermediate Mn-SOD levels, which increased significantly with the tumor diameter. Both, adenomas and carcinomas, and corresponding normal mucosa were found to have similar Cu/Zn-SOD, whereas liver metastases contained significantly more Cu/Zn-SOD as compared with normal tissue. Gallotti (2000) established that the Mn-SOD content was highly increased in cells which have undergone loss of tumor suppressor gene P_{53}, suggesting that antioxidants can under some circumstances favor tumor growth.

On the other hand Drane (2000) and Blasi *et al.* (1999) observed lower SOD activity in melanoma cells compared to the normal melanocytes. The

imbalance of the antioxidants in melanoma cells was related with the disease status. Nakada *et al.* (1988) found that the development of renal carcinoma in humans is not related to the abnormality of renal SOD. The enzymatic activities were similar in tumor and tumor-uninvolved renal tissue. Oxidative damage was found to be a factor in development of experimentally induced malignant changes in cell cultures (Ravid *et al.*, 1999; Emerit *et al.*, 1996).

The above-mentioned literature data suggest a possible new concept of cancer prevention and treatment by systematic modulation of the antioxidant defense system. Reduction of the radicals and oxidants with antioxidants, including SOD, was found to antagonize tumor promotion activity (Abu-Zeid *et al.*, 2000; Zhao *et al.*, 2000). Animal experiments have supported the concept in that SOD is protective during the promotional phase of cancer development. Recently, Li *et al.* (1998) demonstrated a protective effect of several antioxidants on tumor transformation of human keratinocytes. According to Zhu *et al.* (1994) SODs have an essential therapeutic effect during development of leukemia. Inoculation of Cu/Zn-SOD prolonged the survival of experimental animals with Ehrlich ascites and Sarcoma 180 tumors (Oberley and Buettner, 1979). Moreover, modern cancer therapy produces substantial acute and chronic toxicity that impairs quality of life and limits the effectiveness of treatment (Trotti, 1997).

Previously we have selected the fungal strain *Humicola lutea* 103 as a producer of a Cu/Zn-containing SOD mainly. The novel enzyme was purified to electrophoretic homogeneity and its high degree of structural homology with Cu/Zn-SODs from other pro- and eukaryotic sources was proven (Angelova *et al.*, 2001). Moreover, *H. lutea* SOD is a naturally glycosylated enzyme (Angelova *et al.*, 2001) found so far in very few cases only. The major advantage of such modified SODs is their longer half-lives in plasma and blood (Maksimenko *et al.*, 1993).

H. lutea SOD (HLSOD) was used in an *in vivo* model for the demonstration of its protective effect against myeloid Graffi tumor. Our studies on hamsters with transplanted Graffi tumors revealed that treatment with HLSOD produced: (i) elongation of the latent time for tumor appearance; (ii) inhibition of tumor growth in the early stage of tumor progression; (iii) increase in the mean survival time of Graffi tumor-bearing hamsters (Toshkova *et al.*, 2000). This protective effect was partly explained by the stimulation by fungal SOD of the phagocytic abilities of peritoneal macrophages and blood polymorphonuclear leukocytes, as well as on the *in vitro* proliferative ability of spleen B lymphocytes (Toshkova *et al.*, 2000).

Hypothesizing that the protective effect of the HLSOD on Graffi-tumor progression in hamsters is due to the restoration of the host oxidant-antioxidant balance, we have investigated in the present work the changes in endogenous antioxidant enzyme activities (SOD and catalase) in tumor-bearing hamsters (TBH), treated or not treated with HLSOD.

Materials and Methods

Experimental animals

"Golden Siberian" hamsters (60 animals), 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 from the Bulgarian Veterinary Health Control Service. The animals were separated in 4 experimental groups: 1st – hamsters with transplanted myeloid Graffi tumors, treated by Cu/Zn SOD, isolated from the fungal strain *Humicola lutea* (HLSOD); 2nd – hamsters with transplanted myeloid tumors without treatment; 3rd – healthy hamsters, treated by HLSOD; 4th – healthy hamsters for control.

Tumor

The myeloid tumor, preliminary induced by Graffi virus, was maintained *in vivo* in hamsters by subcutaneous inoculation of 5.10^4 viable trypan blue excluded tumor cells in the interscapular field (Jakimov *et al.*, 1979). A 100% transplantability till day 15 and 100% lethality within 45 days after transplantation of the tumor were previously established (Toshkova, 1995). For the present experiments, 10^4 tumor cells were inoculated subcutaneously as just described.

Doses and application of HLSOD

The animals from groups 1 and 3 were injected intraperitoneally (i.p.) with a 65 U single dose of HLSOD, applied two times a week, during 3 weeks, starting 1 week before the day of transplantation. The dose and scheme of application were estimated as optimal in our previous investigation (Toshkova, 1995).

Tissue homogenates

For SOD and catalase assays, tissues and cell homogenates were prepared by a modified method of Nandi and Chatterjee (1988). Briefly:

a) Liver tissue and tumor tissue homogenates

Tissue pieces of about 1 g were desintegrated in ice-cold phenol red-free Hanks balanced salt solution (HBSS) at pH 7.2 (1:9, w/v) by mechanical way and subsequently by an ultrasound desintegrator (MSE, England) for 3 min (interrupting sonifications every 15 sec). The homogenates were then centrifuged at 4000 rpm for 20 min at 0 °C and the supernatants examined for antioxidant enzyme activities.

b) Peritoneal macrophages

Peritoneal macrophages were collected from the peritoneal cavities by washing with ice-cold HBSS (pH 7.2). The cells were desintegrated by an ultrasound desintegrator (MSE) under conditions as described above and examined for their activities of antioxidant enzymes.

c) Blood polymorphonuclear cells (PMNs)

PMNs were separated according to the method of Yamamoto *et al*. (1993). Briefly, blood from animals was immediately mixed with a sodium citrate (2%) solution (1:10 v/v). Immediately before separation of the PMN cells, the citrate blood was diluted (1:1) with PBS (phosphate-buffered saline), layered on Ficol-paque (Pharmacia, Sweden) (2:1, v/v) and centrifuged at 1750 rpm for 35 min at room temperature. The PMNs, located as a visible ring in the Ficol supernatant, were than carefully collected. The cell suspension was washed twice with HBSS, resuspended in HBBS and the PMN cells were desintegrated the same way as described before.

Production of the HLSOD

The fungal strain *Humicola lutea* 103 from the Mycological Collection of the Institute of Microbiology, Sofia, was used for the production of Cu/ZnSOD. The fermentation conditions and preparation of the purified enzyme as a water-soluble homodimeric glycoprotein with a molecular mass of approximately 31700 Da were the same as described earlier (Angelova *et al.*, 2001).

Analytical assays

SOD and catalase were examined at days 14, 26, and 30 after tumor transplantation. 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. Catalase was assayed by the method of Beers and Sizer (1952). 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.

Results

We compared the activities of the antioxidant enzymes SOD and catalase in the liver cells removed from hamsters of all experimental groups (Figures 1, 2, and Table I). Our results demonstrate that antioxidant defense in liver tissue from hamsters with transplanted Graffi tumor (2nd group) changes significantly in the process of tumor progression. As shown in Fig. 1., the total liver SOD activity in TBH was lower (35–59%) compared to that of the control animals. Moreover, the enzyme activity in TBH decreased con-

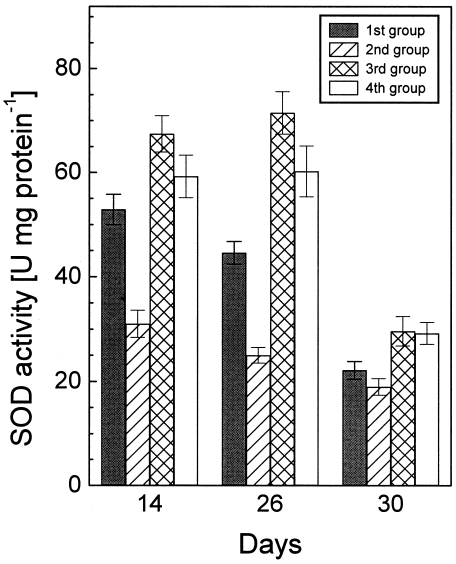

Fig. 1. Effects of treatment of healthy and tumor-bearing hamsters with HLSOD on total SOD activity in liver tissue during Grafii tumor progression.
Experimental groups: 1 – Graffi tumor-bearing hamsters, treated with HLSOD; 2 – Graffi tumor-bearing hamsters; 3 – healthy hamsters, treated with HLSOD; 4 – healthy hamsters as a control.

tinuously during the experimental period (31.0±2.6, 25.0±0.8, 19.0±1.6 U mg protein^{-1} at day 14, 26 and 30 respectively). Treatment of TBH with HLSOD (1st group) resulted in an increase (70–78%) in total endogenous SOD activity in the earlier stage of tumor progression (52.9±1.9 and 44.6±2.2 U mg protein^{-1} at day 14 and 26, respectively) compared to the TBH (2nd group). Later (on day 30) an about 16% increase was determined (22.2±1.4 vs. 19.0±1.6 U mg protein^{-1}). The healthy animals injected with HLSOD (3rd group) also showed an enhanced level of the endogenous SOD activity in liver tissues for a long period of time (67.5±3.5 and 71.5±4.1 at days 14 and 26 respectively) compared to that of the control group (4th group). The enzyme activity at day 30 was almost equal in the hamsters from both, groups 3 and 4.

As next we were interested in changes of the isoenzyme profile of SOD in the liver tissue during the tumor progression. Figure 2 shows the presence of both, Cu/Zn- and Mn-containing isoenzymes in the liver tissue of all experimental groups. The mainly cytosolic Cu/ZnSOD accounted for the $^{\bullet}O_2^-$ scavenging activity of the liver tissue from healthy as well as tumor-bearing hamsters was found. Moreover, the enhanced level of SOD in treated healthy hamsters was due to this isoenzyme also.

Since SOD levels often affect other antioxidant enzymes, we determined also the level of catalase in the liver tissue. The enzyme activity in the control animals (4th group) did not change significantly during the investigation (117.4±4.1,

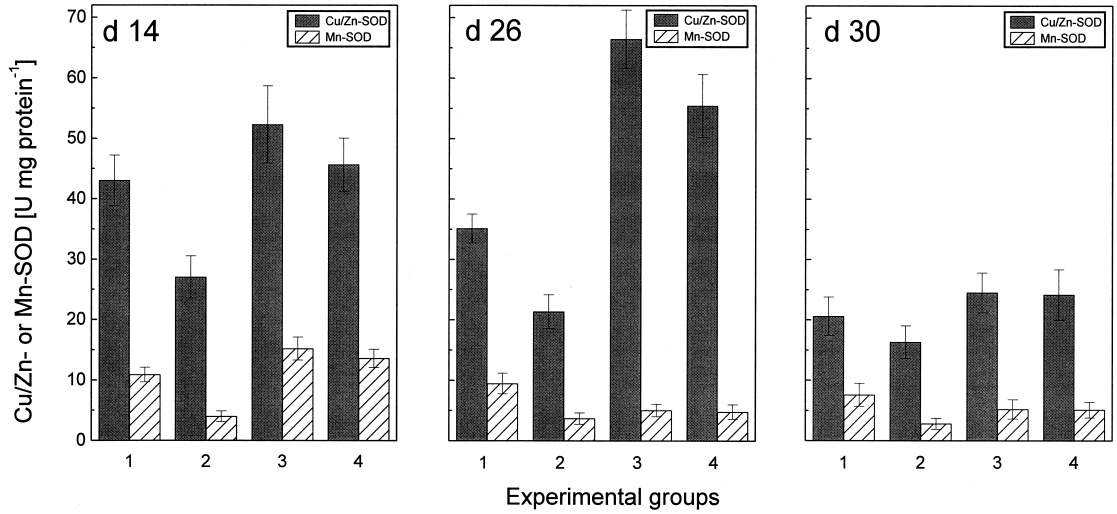

Fig. 2. Isoenzyme profile of endogenous SOD (Cu/Zn- and Mn-SOD) in liver tissue of hamters at different stages of tumor development. Experimental groups: as in Figure 1.

93.7±6.4 and 104.3±5.1 U mg protein-1 at day 14, 26 and 30, respectively). Tumor transplantation (2nd group) caused about 16 to 67% decrease in catalase activity compared to healthy hamsters (4th group) and these changes showed a correlation with the process of tumor progression. When TBH were injected with HLSOD (1st group), catalase activity in cells, removed from liver, increased about 1.4 to 2.5-fold compared to TBH without treatment (2nd group) during the entire experimental period. Moreover, in the earlier stages (days 14 and 26) the addition of HLSOD (1st group) increased the catalase activity over or back to levels of untreated controls (4st group). The same trends in the changes of catalase activity in the liver from healthy hamsters treated with HLSOD (3rd group) were observed (Table I).

It was found, that the antioxidant enzyme defense (SOD and catalase) in macrophages and PMNs from all experimental groups is much lower than in liver cells. Evaluation of both assessed phagocytic cells removed from healthy hamsters (4th group) indicated that SOD level was about 2 U mg protein^{-1} (data not shown). Tumor transplantation (2nd group) caused a complete reduction of the enzyme activity, whereas application of exogenous HLSOD to TBH (1st group) resulted in a minor enhanced level of SOD in the earlier stage of tumor progression. Similarly was the effect of application of HLSOD on the endogenous SOD levels in macrophages and PMNs in healthy hamsters (3rd group) (data not shown).

As shown in Table I, the tumor process and treatment with HLSOD significantly influences catalase activity in macrophages. Inhibition of catalase synthesis was observed in TBH (2nd group) as compared to the control group (5.1±1.2 vs. 7.8±1.9, 4.4±2.1 vs. 5.9±1.4 and 1.2±0.6 vs. 4.6±1.5 at days 14, 26 and 30, respectively). Macrophages from TBH under HLSOD treatment (1st group) showed a strong increase in catalase levels throughout the whole experimental period (from 2.2 to 4-fold) compared to the levels in untreated TBH (2nd group). It is interesting to mention that at day 14 and 26 the catalase activity was much higher compared to that in control group of healthy animals. A similar trend was found for the 3rd group (healthy hamsters treated with HLSOD), in comparison to the control group.

Catalase activity in PMNs from healthy hamsters showed a tendency to decrease with time during the experiments. At the same time, a significant drop of the enzyme level was observed in PMNs from TBH compared to those from healthy hamsters (Table 1). This reduction correlated with tumor progression and was very pronounced at day 30 (0.65±0.4 U mg protein^{-1}). At each of the time points indicated, the catalase activity was significantly higher in TBH treated with HLSOD (1st group) vs. untreated TBH. Catalase levels in PMNs from healthy hamsters treated with HLSOD (3rd group) showed always higher levels compared to those in control animals (Table I).

To evaluate the effect of exogenous HLSOD on the oxidant-antioxidant balance in tumor cells we investigated the levels of SOD and catalase in these cells removed from TBH treated and untreated with the preparation. It was found that the activity of the endogenous SOD enzyme in tumor cells from untreated hamsters (2nd group) was low and decreased continuously from 6.9±2.5 and 5.7±2.2 U mg protein^{-1} at day 14 and 26, respec-

Table I. Catalase activity in liver cells, peritoneal macrophages and PMNs from healthy and tumor bearing hamsters treated or not treated with HLSOD.

Gr.	Catalase activity [U mg protein^{-1}]								
	Liver			Peritoneal macrophages			PMNs		
	14 d	26 d	30 d	14 d	26 d	30 d	14 d	26 d	30 d
1st	161.0±8.2	96.4±4.1	49.3±3.9	12.4±1.8	9.9±1.5	4.8±1.1	14.5±1.8	8.3±0.8	1.2±0.4
2nd	98.7±5.3	39.0±2.1	35.0±3.8	5.1±1.2	4.4±2.1	1.3±0.6	3.1±0.7	1.6±0.5	0.7±0.4
3rd	239.7±7.9	157.8±6.2	107.2±6.5	14.6±2.8	11.8±2.4	7.1±2.3	17.8±2.1	11.0±1.8	7.3±1.4
4th	117.4±4.1	93.7±6.4	104.3±5.1	7.8±1.9	5.9±1.4	4.6±1.5	10.5±2.3	8.4±1.9	5.6±1.3

Experimental groups: 1st – Graffi-tumor bearing hamsters, treated with HLSOD; 2nd – Graffi-tumor bearing hamsters without treatment; 3rd – healthy hamsters, treated with HLSOD; 4th – healthy hamsters as a control.

tively, to 1.5±1.0 U mg protein^{-1} at day 30. Treatment of TBH with HLSOD (1st group) resulted in a marked enhancement of the SOD activity reaching 1.9-, 1.8- and 1.4-fold levels compared to the untreated animals (2nd group) at days 14, 26 and 30, respectively (Table II). The izoenzyme profile showed that the total SOD activity is due to the presence of Cu/ZnSOD. MnSOD was not found in tumor cells, neither in those from TBH nor in those from TBH treated with HLSOD (data not shown).

The production of catalase in tumor cells, removed from TBH (2nd group), showed a marked reduction that correlated with the process of tumor progression (Table II). Whereas the catalase activity on day 14 was approx. 45.8±3.9 U mg protein^{-1}, it decreased 3.8- and 9.8-fold on days 26 (12.1±2.3 U mg protein^{-1}) and 30 (4.7±0.9 U mg protein^{-1}), respectively. Tumor cells from TBH treated with HLSOD, showed a higher catalase profile compared to experimental group 2 (Table II). The highest enzyme activity was found at day 14 (76.1±4.2) and decreased on days 26 and 30 to levels of 27.1±2.6 and 8.7±1.1 U mg protein^{-1}, respectively (Table II).

Discussion

In this study we attempted to enhance the antioxidant defense system of Graffi tumor-bearing hamsters by treatment with HLSOD. Transplantation of tumors results in a series of events that led to deleterious changes in the activities of antioxidant enzymes in different tissues. Moreover, the levels of these activities were cell-type specific. Whereas the total SOD activity in macrophages and PMNs was found in minor amounts in all experimental groups, the liver cells produced this enzyme at comparable high levels, and Graffi tumor progression caused a significant decrease in this activity. In tumor cells, endogenous SOD production was found to be low and depressed supplementary during tumor development both in treated and in untreated TBH.

Other studies have also indicated that tumor growth can cause antioxidant disturbances, related to the total SOD level, in different of tissues including the liver tissue (Abu-Zeid *et al.*, 2000), transformed mouse liver cells (Sun *et al.,* 1989), cancerous thyroid and kidney tissues (Durak *et al.,* 1996, 1997). Spier and Newburger (1986) showed progressively declined total SOD activity in leukemia cell line during *in vitro* cultivation. It was reported that different isoforms of the enzyme SOD were involved in the progression of human renal cell carcinoma (Sarto *et al.*, 1999), and in human breast cancer (Portakal *et al.*, 2000). In most cases, cancer development is associated with the expression of MnSOD mainly (Janssen *et al.*, 1999; Portakal *et al.*, 2000), which don't coincide with our results (Fig. 2.). However, there are also data pointing out that Cu/ZnSOD as the isoenzyme, involved in the noneffective lipid peroxidation in different types of leukemia (Devi *et al.*, 2000).

The established by our study gradually decrease of the endogenous Cu/Zn SOD during tumor progression in hamsters may be due to enzyme active site inactivation by H_2O_2- a mechanism suggested by Diplock *et al.* (1994). It is known, that under normal cell conditions the majority of H_2O_2, generated by the dismutation reaction of superoxide, is removed by a series of antioxidant enzymes and compounds preventing their attack on the active site of Cu/ZnSOD (Yim *et al.*, 1993).Our results point, that Grafii tumor growth was accompanied with a reduction of catalase activity and this reduction correlated with the process of tumor progression. Thus, a diminished removal of H_2O_2, generated in the cells of TBH, could cause above described inhibition of Cu/ZnSOD (Diplock *et al.*, 1994).

Table II. SOD and catalase activity in tumor tissue from hamsters with implanted myeloid Graffi tumors.

Group	SOD activity [U mg protein^{-1}]			Catalase activity [U mg protein^{-1}]		
	14 d	26 d	30 d	14 d	26 d	30 d
1st	13.1±2.0	10.1±3.6	2.0±0.6	76.1±4.2	27.1±2.6	8.7±1.1
2nd	6.9±2.5	5.7±2.2	1.4±1.0	45.8±3.9	12.1±2.3	4.7±0.9

Experimental groups: 1st – – Graffi-tumor bearing hamsters, treated with HLSOD;
2nd – Graffi-tumor bearing hamsters without treatment.

On the other hand, it is known that $^{\bullet}O_2^-$ can inactivate catalase (Folz *et al.*, 1999). So, we can suppose that the previously established enhanced accumulation of $^{\bullet}O_2^-$ in tissues of Grafii tumor-bearing hosts (Dimitrova *et al.,* 2000) probably contribute to the decrease in antioxidant defense, inactivating catalase.

As suggested, an unbalanced oxidant-antioxidant status of host cells is the key to the development and progression of cancer (Mathes *et al.*, 1999). Also, our present and previous findings show a correlation between decreased activity of antioxidant enzymes, enhanced levels of $^{\bullet}O_2^-$ and the process of tumor progression (Toshkova *et al.*, 2000; Dimitrova *et al.*, 2000). These results are consistent with the general tendency in the scientific literature (Spier *et al.*, 1986; Durak *et al.*, 1997; Oberley and Oberley, 1988).

The next main finding of this study is that treatment with 65 U mg protein^{-1} HLSOD plays a beneficial role in enhancing the antioxidative ability of the cells in TBH. We have demonstrated that SOD and catalase activity significantly raised. The presence of exogenous SOD contributes to the antioxidant defense against the oxidative damage mediated by superoxide radicals. If removes superoxides by catalyzing the dismutation of two $^{\bullet}O_2^-$ radicals to yield H_2O_2 and oxygen resulting in a decrease of the concentration of $^{\bullet}O_2^-$ (Fridovich, 1995). These suggestions are in good agreement with our previous conclusion that HLSOD treatment of TBH induces a normalization of superoxide production (Dimitrova *et al.*, 2000) and thereby reducing inactivation of catalase (Kono and Fridovich, 1982). Under conditions of the regulation by the above mentioned processes, excess H_2O_2 will be destroyed and protection given (Tiedge *et al.*, 1998). The presented results could explain our previous data about the protective effect of treatment of TBH with HLSOD on the development of Graffi tumor (Toshkova *et al.*, 2000).

Our results about the protective effect of HLSOD during Graffi tumor development confirm the advantages of antioxidant therapy at early stage of cancer progression (Portakal *et al.*, 2000). Exogenously added antioxidants and antioxidant enzymes boost the cellular antioxidant system and thereby offer protection of the cells (Zhao *et al.*, 2000; Ravid *et al.*, 1999).

The presented findings may provide an additional and supporting alternative to cancer therapy with conventional anti-tumor drugs.

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