Alpha-tocopheryl succinate (α-TOS) influences cell vitality and enzyme activity in Ehrlich ascites carcinoma cells

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SUMMARY

Background: *One of the most important strategies in research and development of new anticancer agents is the tumor-specific induction of apoptosis. The effects of semisynthetic derivative of vitamin E, (α-TOS, D-α-tocopheryl succinate), appear to be largely restricted to malignant cells.*

Methods: *We investigated the in vivo effects of intraperitoneally administered α-TOS on vitality of Ehrlich ascites carcinoma cells (EAC) in mice, as well as the influence of α-TOS on specific activity of enzymes involved in antioxidative mechanisms in EAC cells.*

Results: *According to our results, the intraperitoneal application of α-TOS induces the decrease of the EAC vitality, and the statistically significant alteration of the glutathione-dependent enzyme activity in EAC cells.*

Conclusion: *We may conclude that α-TOS is an important micronutrient, with significant impact on vitality and metabolism of malignant cells.*

Key words: *Carcinoma, Ehrlich Tumor; Vitamin E; Oxidative Stress; Enzymes; Mice*

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INTRODUCTION

The most important feature of an anti-cancer agent, preventing progression of the malignant disease, is to selectively control and prevent tumor growth, exerting low toxicity towards normal cells. Recent studies have shown that analogs of vitamin E (VE) are effective as anti-proliferative and pro-apoptotic agents, being tested on multiple cancer cell lines (1-3).

Vitamin E succinate (α-TOS, D-α-tocopheryl succinate, VES) is a micronutrient with important anti-tumor potential, preventing the progression of experimental colon (4), breast cancer (5), melanoma (6), mesothelioma (7), gastric cancer (8) and neuroblastoma (9) in pre-clinical settings. The vitamin E analogs inhibit proliferation of cancer cells by several mechanisms, including inhibition of DNA synthesis, induction of apoptosis and cellular differentiation, and by affecting the protein kinase C and the MAP kinase pathways (10, 11). More importantly, α-TOS exerts anti-proliferative/pro-apoptotic effects in malignant cell lines, but is largely nontoxic toward normal cells and tissues (11).

The pro-apoptogenic activity of α–TOS is shown in some 50–60 different malignant cell lines tested to date (12-14). In case of α-TOS, the presence of succinyl moiety is essential for the apoptosis-inducing activity of the agent, as suggested by the experiments showing that α-TOS acts as a stressor to which the cell responds by generation of ROS. In Jurkat and MCF7 cells, α-TOS acts as a typical inducer of apoptosis through the mithochondrial pathway (15).

Alpha-TOS has demonstrated an excellent safety and tolerability profile in clinical trials utilizing high doses of VES in children with vitamin E deficiency (1, 16). Recent study of Weber et al (15), have shown that the high sensitivity of malignant cells to α-TOS effects is based on its ability to efficiently induce the mitochondrial signal transmission mechanisms (including ROS generation), which triggers apoptosis. These authors believe that the major mode of action of α-TOS is mitochondrial destabilization, involving the formation of ROS, followed by the release of cytochrome c and activation of multiple caspases, without deregulation of tumor suppressor activity (15).

The effect of α-TOS appears to be largely restricted to malignant cells (17), a feature that may be associated with their high proliferation rates (18). The fast transition through the cell cycle appears to be positively correlated with cell susceptibility to apoptosis (4). It is also shown, in a pre-clinical model that the intraperitoneal (IP) injection of VE analogue into mice results in rapid accumulation at sites distal to tumors (4).

The results of Neuzil et al. (12) show that ROS are important mediators of apoptosis induced by VE analogues, with high selectivity towards malignant cells. These authors report that differentiated cells are well protected against α-TOS toxicity after the treatment with VE analogue with at least two different mechanisms of resistance, including higher expression of the anti-apoptotic Bcl-2 family proteins and decreased accumulation of ROS (12). Therefore, our principal hypothesis was that intraperitoneal application of α-TOS may influence the activity of antioxidative enzymes in EAC cells and their vitality accordingly. We studied the *in vivo* effects of α-TOS on Ehrlich ascites carcinoma (EAC) cells vitality and activity of enzymes involved in ROS attenuation.

MATERIAL AND METHODS

Ehrlich ascites carcinoma cells (EAC) were propagated in the abdominal cavity of Swiss mice. Two days after transplantation (cells in exponential growth phase) we commenced the intraperitoneal application of alpha-tocopheryl succinate (α-TOS). The total number of 24 animals was divided in three groups. Eight mice were left untreated (control group) while 16 animals (test group) were injected intraperitoneally, each third day, four doses in total. The first test subgroup of 8 mice received 0.05 ml of α-TOS dissolved in DMSO (0.2 mol/l), and the second test subgroup of 8 mice received 0.1 ml of α-TOS dissolved in DMSO (0.2 mol/l). Control mice received a corresponding dose of DMSO only. The dosing regimen was selected according to the pilot experiments, showing no decrease in survival of treated animals. Mice were observed for survival. All experiments were approved by and carried out in accordance with the ethics regulations of Medical faculty of Novi Sad.

Cells of EAC were washed in Krebs-Ringer phosphate solution of the following composition: 145 mM NaCl, 5 mM KH_2PO_4, 1.5 mM $MgSO_4$ and 6 mM KCl. The final pH was 7.4 and the temperature was 0°C.

Viability of the cells was tested by measuring leakage of LDH into the incubation medium. The average viability of the cells was 95-96% of total cell number in thick suspension. Cell weight was measured in 1.5 ml Eppendorf 3200 centrifuge tubes after centrifugation for 2.5 minutes and removal of supernatant. It was assumed that cell pellet contains 25% of incubation medium. All measurements are expressed per mg wet wt. of cells and protein content. The measurements of all parameters were done in triplicate, and means of three determinations were recorded. Preliminary experiments were carried out in order to find proper experimental conditions. Results are expressed as mean ± standard error of the mean (SEM). Differences among means were tested for statistical significance by student's two-tailed t-test. Statistical significance was set at $p<0.05$.

In cell homogenate obtained by described method, we determined the protein content using the Biuret method. The activities of enzymes glutathione reductase (GR), glutathione peroxidase (GPX) and γ – glutamyl transferase (γ – GT) were determined by kinetic method (19-21).

RESULTS

Our main goal was to investigate the effects of the intraperitoneal application of α-TOS on Ehrlich ascites carcinoma cells (EAC) vitality, as well as on the activity of enzymes involved in antioxidative protection of EAC.

Our results of protein concentration testing in EAC, both in control group of animals (only DMSO was injected), and in experimental group (intraperitoneal application of 50 μl, and 100 μl of 0.2 M α-TOS, respectively), are shown in Figure 1. These results are showing statistically significant decrease in protein concentration in both experimental groups (receiving α-TOS) in comparison with the control group, which may indicate the process of protein synthesis inhibition in EAC cells, induced by α-TOS.

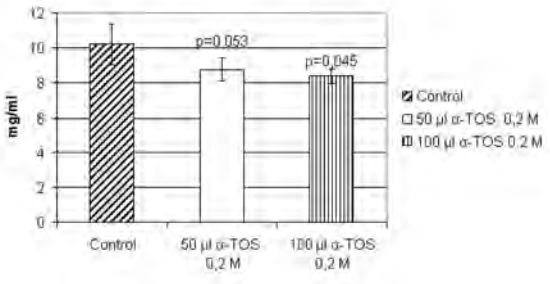

Figure 1. Protein concentration in Ehrlich ascites carcinoma cells

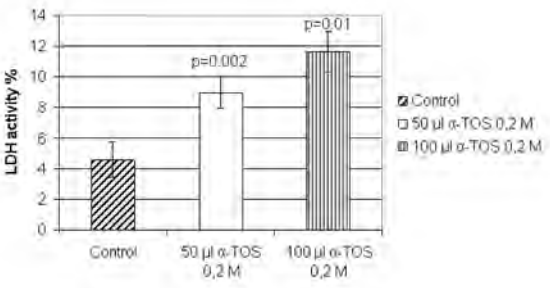

Figure 2. The percentage of LDH enzyme activity as an indicator of Ehrlich ascites carcinoma cells vitality

Testing of the vitality in Ehrlich ascites carcinoma cells in control and in both experimental groups is showing the statistically significant differences between these groups. The leakage of the enzyme lactate dehydrogenase (LDH) into the extracellular compartment is a consequence of the cell membrane damage. In control group, the percentage of damaged EAC cells was only 4.56%, whereas in experimental groups, this percentage was 8.98% and 11.63%, respectively (Figure 2).

The Figure 3 shows the results of the specific activity measurement of following enzymes: glutathione reductase (GR), glutathione peroxidase (GPX) and γ – glutamyl transferase (γ – GT). The statistically significant differences in enzyme activity between control and experimental groups are detected only in GPX activity, where we found the statistically significant decrease in GPX activity in EAC cells in experimental groups of animals, after the intraperitoneal application of 50 μl, and 100 μl of 0.2 M α-TOS. These results are in accordance with the data in Figure 1, showing the statistically significant decrease in total protein concentration in EAC cells in experimental groups of animals after the IP application of α-TOS. The differences in GR and γ – GT specific activity, observed between control and experimental groups, are not statistically significant.

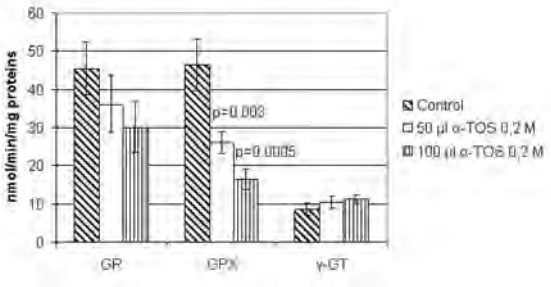

Figure 3. Specific activity of the enzymes glutathione-reductase, glutathione-peroxidase and gamma-glutamyl transferase in Ehrlich ascites carcinoma cells

DISCUSSION

The recent *in vivo* studies have shown that alpha-tocopheryl succinate (α-TOS) induces apoptosis and inhibits proliferation of malignant cells. The delivery of the intact molecule of α-TOS is necessary to promote apoptosis and inhibit cellular proliferation. These authors showed that delivery of the intact molecule of α-TOS to the tumor was best accomplished via intraperitoneal injections (5). While oral dosing would be ideal, the intact VES molecule does not reach the tumor because the succinic side branch is cleaved by esterases in gastrointestinal tract (22).

The pro-apoptotic activity of α-TOS requires the compound to be intact, and malignant cells, indeed, appear to be unable to significantly hydrolyze the ester. In contrast, relevant esterase activity is present in certain normal cells, including hepatocytes, fibroblasts, cardiomyocytes and the intestinal epithelial cells, which cleaves α-TOS to produce vitamin E (18,23).

The second reason for the cancer cell-specific toxicity of a-TOS may be related to the inherent property of many inducers of apoptosis to trigger programmed cell death by initially inducing cells to accumulate reactive oxygen species (ROS), which in turn cause a cascade of subsequent reactions leading to transition of the cell into the commitment phase (24). α-TOS has been reported to induce ROS accumulation in many different cancer cell lines,

most probably resulting in the generation of superoxide anion radicals (25). In addition, it has been reported that cancer cells have lower antioxidative capacity than normal cells: for example, malignant cells have lower levels of expression of manganese superoxide dismutase (MnSOD) compared to their non-malignant equivalents (25).

In last few years, numerous articles have shown that the intraperitoneal application of α-TOS, which we have applied in our study, induces tumor growth inhibition (5, 7, 26). Neuzil et al. (16) showed in 2001, that *in vitro* treatment of several normal human cell lines with α-TOS did not influence their proliferation, whereas it was able to induce the growth inhibition of several malignant human cell lines. These results are in agreement with our data, showing the reduced vitality of Ehrlich ascites carcinoma (EAC) cells after IP application of α-TOS in tumor-bearing mice.

Recent studies show that α-TOS induces tumor-specific alterations in expression of growth factors, prostaglandins, enzymes, apoptosis mediators and executioners, and increased production of reactive oxygen species (ROS) (27). In light of these results, we might interpret our data, showing alterations in specific activity of glutathione reductase, glutathione peroxidase and gamma-glutamyl transferase, induced by several possible mechanisms, which we will further investigate. These changes in enzyme activity might be due to the alterations in gene expression, possibly induced by the α-TOS influence on ROS production or other signal transduction mechanisms. Several reports suggest that doxorubicin induces ROS generation in cells, (28) in a p-53 dependent manner, similarly to α-TOS. Kwiecien et al. (29) could not detect changes in γ – glutamyl transferase (γ – GT) activity in cells after doxorubicin treatment, which is in accordance with our results.

It has been demonstrated that the generation of ROS is related to α-TOS induced malignant cells cytotoxicity, followed by the diminished antioxidative protection capacity in malignant cells, in comparison with normal cells (30). The results of Fukuzawa et al. (30) are in concordance with our data of decreased activity of glutathione reductase and glutathione peroxidase in EAC cells after IP application of α-TOS in tumor-bearing mice.

Our results of decreased specific activity of enzymes involved in glutathione (GSH) metabolism and decreased protein synthesis in EAC cells are in accordance with the results of the authors who studied the GSH metabolism regulation in EAC cells in mice (31). The observed decrease in antioxidative potential (decrease in glutathione-dependent enzyme activity), may have the important influence on EAC cells increased susceptibility towards apoptosis, which has been shown by their decreased vitality after IP application of α-TOS.

Numerous *in vivo* and *in vitro* studies have shown that the increased ROS generation may induce the increased mRNA levels of antioxidative enzymes (32). However, this increase in expression is not followed by the increase in enzyme activity, showing the important role of post-transcriptional and post-translational regulation in protein synthesis upon stress signaling. It has been also shown that the increased levels of ROS may induce the protein inactivation and consecutive decrease in activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), which is in accordance with our results of decreased GPX activity (32). Recent reports that α-TOS can lower the intracellular GSH concentration in Jurkat cells (15, 33), may also provide another possible explanation for decrease of GPX activity (34).

According to our results, we may conclude that intraperitoneal application of α-TOS induces the decrease of the EAC vitality and statistically significant alteration of the glutathione-dependent enzymes activity in EAC cells. Our results are in accordance with the results of other authors, which show that α-TOS is important micronutrient with significant pro-apoptotic and anti-proliferative effects on malignant cells.

Conflict of interest

We declare no conflict of interest.

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