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Inhibitory effect of shark liver oil on cutaneous angiogenesis induced in Balb/c mice by syngeneic sarcoma L-1, human urinary bladder and human kidney tumour cells

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Abstract. The effect of shark liver oil on cutaneous angiogenesis induced in mice by intradermal grafting of tumour cells was evaluated. It was shown that this substance (Ecomer) suppressed neovascular response in mice grafted with sarcoma L-1 syngeneic cells, human kidney cancer and human urinary bladder cancer cells. In addition, strong stimulatory effect of this drug on mice blood granulocyte number and their metabolic activity was observed.

Introduction

Angiogenesis, the formation of new blood vessels, is an important step in tumour growth, invasion and metastasis (1,2). Anti-angiogenic therapy, in addition to the traditional treatment, is currently one of the most promising strategies for restricting tumour growth. Many potent angiogenesis inhibitors, more or less toxic, have recently been discovered, some of them are tested in clinical trials. It could be of great importance to find new, non-toxic anti-angiogenic agents among substances plant or animal origin, widely used in traditional medicine.

Ether lipids (also referred to as glyceryl ethers, alkylglycerols or alkoxyglycerols) and their derivatives represent a new class of compounds for treatment of experimental tumours (3). They occur, mostly as di-esters of fatty acids, in the lipids of various animal organs. The most important source is the liver oil of certain elasmobranch fish, i.e. sharks and rays (4). The ether lipid analogues are known to be incorporated into cell membranes. They block intracellular signaling at several sites, including the inhibition of inositol phosphate (IP3) formation, the inhibition of the release of intracellular Ca^{2+} by IP3, as well as inhibition of protein kinase C (5).

They are known to increase the fluidity of cell membranes and they increase cell susceptibility to lipid peroxidation (6). Inhibition of phosphatidylinositol-3-kinase by antitumour ether lipid analogues contribute to their antiproliferative activity (7). Recently, it was shown that the antineoplastic ether lipid, s-phosphonate, selectively induces apoptosis in human leukemic cells and exhibits anti-angiogenic and apoptotic activity on the chorioallantoic membrane of the chick embryo (8).

In this study, we demonstrate that shark liver oil, rich in alkylglycerols exhibited anti-angiogenic activity in mice, which have been grafted intradermally with sarcoma L-1 tumour cells, human kidney cancer cells or human bladder cancer cells. Simultaneously, we observed strong stimulatory effect of this drug on mice blood granulocyte number and their metabolic activity.

Materials and methods

Studies on the effect of Ecomer (Exposan AB) were performed in 2-month old, male inbred Balb/c mice. Mice were of local laboratory breed, weighing 20 g each. Each mouse received per os Ecomer in dose of 12.5 mg/mouse/24 h, or water-during 7 days. On day 8th mice were subjected to narcosis by means of chloral hydrate, bled from the retro-orbital plexus and sacrificed.

The following tests were performed in the study of heparinized blood specimens. i) Counting of granulocytes; ii) Measurements of their metabolic activity using chemiluminescence stimulated by Zymosan, according to the method described by Easmon *et al* (9). Chemiluminescent activity was measured in scintillation counter (RackBeta 1218, LKB Wallac, Sweden) and expressed as cpm per 1000 granulocytes.

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Table I. Blood granulocyte number and activity in mice fed Ecomer for 7 days.

	No. of mice	No. of granulocytes in mm^3 ± SE	p-value	Chemiluminescence activity cpm ± SE	p-value
Control group (water)	5	552±40		8030±401	
Experimental group (Ecomer)	6	873±26	p<0.001	12881±1751	p<0.05

Angiogenesis induced by cells isolated from mice tumour-Balb/c sarcoma L-1. Sarcoma cells were delivered from Warsaw's Oncology Center Bank and then passaged through several generations of locally bred Balb/c mice. Briefly, sarcoma cells were grafted ($5x10^5$/0.1 ml) subcutaneously into mice. After 14 days tumours were removed, weighed and measured.

Then, each tumour was cut to smaller pieces, rubbed through sieve and suspended in 5 ml of phosphate buffered saline (PBS, Polfa, Kutno). The suspension was left for 15 min in room temperature. After sedimentation the supernatant was collected and centrifuged for 10 min. at 1400 rpm. Obtained cancer cells were washed once in PBS for 10 min. then centrifuged at 1500 rpm. Cells were suspended in Parker medium in concentration of $4x10^6$/ml.

Multiple samples of ~200,000 sarcoma cells were implanted (while suspended in 0.05 ml of Parker medium) into partly shaved, narcotised Balb/c mice. In order to facilitate the localisation of cell injection sites, the suspension was coloured with 0.1% of trypan blue. On the day of cells grafting and on the following two days mice were fed Ecomer 12.5 mg per mouse, per day, or water, and angiogenesis was estimated quantitatively after 3 days of implantation, on the inner skin surface, as described previously (10).

Cutaneous angiogenesis induced in mice by cells isolated from human kidney and bladder tumours. Material was obtained surgically from 5 patients with kidney tumours and 5 patients with bladder tumours. About 5 g of tumour tissue was dispersed mechanically and subjected to enzymatic digestion by use of collagenase 0.1 mg/ml (Sigma) and DNAse 0.001 mg/ml (Serva) dissolved in PBS for 45 min on magnetic shaker in room temperature. Then obtained suspension was filtered through a sieve, washed twice in PBS and suspended in Parker medium in concentration of $10x10^6$/ml. Viability of tumour cells was assessed by 0.5% trypan blue exclusion test.

Cutaneous angiogenesis assay was performed according to the method of Sidky and Auerbach with some modifications (11,12). Briefly, 6 to 8-week-old inbred female Balb/c mice were anesthetized with chloral hydrate. Cancer cells were grafted intradermally into shaved mice flanks ($5x10^5$ cells/0.05 ml). In order to facilitate the localisation of cell injection sites later on the suspension was coloured with 0.1% of trypan blue. Then, mice were fed Ecomer for 3 days at 12.5 mg/mouse/day or Ecomer at the same dose was applied topically in the site of cell grafting. Control mice obtained water. After 72 h mice were sacrificed with lethal dose of Morbital. All newly formed blood vessels were identified and counted in dissection microscope using criteria suggested (11).

Table II. The influence of Ecomer on cutaneous angiogenesis reaction induced in Balb/c mice with syngeneic sarcoma L-1 cells.

	No. of tests	Mean no. of newly formed blood vessels ± SE	p-value
Control group	39	19.8±0.68	
Ecomer applied locally (12.5 mg daily for 3 days)	21	14.7±0.61	p<0.001
Ecomer applied orally (12.5 mg daily for 3 days)	34	13.9±0.59	p<0.001

Results

Results of the studies on mice blood are presented in Table I. Both number of circulating granulocytes (p<0.001) and their chemiluminescence activity, when measured in a scintillation counter increased significantly (p<0.05) after Ecomer treatment.

In the study of angiogenesis, it was shown, that administration of Ecomer following implantation of sarcoma L-1 cells, significantly reduced sarcoma-induced angiogenesis (Table II). As well, Ecomer administered to mice per os or topically, significantly (p<0.001) inhibited mice cutaneous angiogenesis induced by human kidney and bladder tumour cells (Tables III and IV).

Discussion

The results of the performed studies indicate that Ecomer treatment induces an increase in cell-mediated non-specific

Table III. The effect of Ecomer (12.5 mg/mouse/day for 3 days) on cutaneous angiogenesis reaction induced in Balb/c mice by cancer cells isolated from human kidney tumours.

Patients initials/ Diagnosis	Mean number of newly formed blood vessels ± SE		p-value
	Control mice (n)	Ecomer treated mice (n)	
S.B. Ca. clarocellulare	14.00±0.76 (12)	10.83±0.48 (12)[a]	p<0.001
W.D. Ca. clarocellulare	14.58±0.48 (12)	10.44±0.45 (18)[b]	
	11.93±0.32 (14)[a]		p<0.001
B.J. Ca. clarocellulare	15.58±0.48 (7)	11.20±0.38 (10)[b]	p<0.001
R.S. Ca. clarocellulare	17.80±0.46 (15)	13.66±0.50 (6)[b]	p<0.001
S.W. Ca. clarocellulare	15.43±0.43 (7)	12.17±0.74 (6)[b]	p<0.001

[a]Topical application; [b]feeding; n, number of tests.

Table IV. The effect of Ecomer (12.5 mg/mouse/day for 3 days) on cutaneous angiogenesis reaction induced in Balb/c mice by cancer cells isolated from human bladder tumours.

Patients initials/ Diagnosis	Mean number of newly formed blood vessels ± SE		p-value
	Control mice (n)	Ecomer treated mice (n)	
F.S. Ca. transitionale	9.92±0.53 (12)	6.50±0.65 (10)[a]	p<0.001
W.L. Ca. transitionale	13.53±0.38 (79)	8.50±0.86 (4)[a]	
		11.93±0.32 (14)[b]	p<0.001
Z.P. Ca. transitionale	32.36±1.21 (11)	21.27±0.97 (13)[b]	p<0.001
R.R. Ca. transitionale	17.25±0.53 (8)	13.30±0.47 (10)[b]	p<0.001
D.S. Ca. transitionale	12.50±0.62 (8)	9.40±0.60 (5)[b]	p<0.001

[a]Topical application; [b]feeding; n, number of tests.

immunological defence mediated by granulocytes (the number and activity of granulocytes which constitute the first line of immunological defence). Ecomer seems therefore to be a suitable preparation for patients with decreased cell-dependent immunological defence in the course of different diseases, in particular neoplasmatic diseases.

The application of Ecomer in cancer disease and its beneficial effect is indicated by its inhibitory effect on tumour angiogenesis. In our present study, mean number of newly formed blood vessels in mice treated with Ecomer was found much lower, than in controls, for various forms of cancer (murine L-1 sarcoma, human renal and urinary bladder carcinoma).

Anti-angiogenic effects of substances isolated from shark has been previously described by Sills *et al* (13). Some ether lipids have been shown to exhibit anti-angiogenic activity. An immortalised human endothelial cell line that can be stimulated to form tubules *in vitro* has been used to show that this activity is inhibited by sublethal concentration of ET-18-OCH_3 (edelfosine) (14).

In addition, the drug causes downregulation of four endothelial cell adhesion molecules (15) and weakness in the integrity of endothelial cell junctions. Recently, the same group reported that edelfosin acted directly on capillary endothelial cell, inhibiting their migration toward the angiogenic factor (basic fibroblastic growth factor-bFGF).

When given systematically to rats (20 mg/kg intraperitoneally twice daily), edelfosine was found clearly antiangiogenic. The majority of treated animals were not able to mount a corneal neovascular response to a pellet releasing

bFGF, whereas vigorous vessel growth was seen in untreated controls (16).

Another antineoplastic ether lipid, s-phosphonate was shown to induce apoptosis in human leukemic cells and to exhibit anti-angiogenic activity on chorioallantoic membrane of the chick embryo (17).

The anti-angiogenic effect of Ecomer may be connected with its ability to induce free oxygen radicals and, subsequently, apoptosis of endothelial cells. Hovewer, substances known as free radical scavengers, for example polyphenolics, induce apoptosis and suppress tumour angiogenesis. Another possibility is, that Ecomer influences activity of some enzymes involved in cellular activation and proliferation processes, and/or that it inhibits binding of angiogenic growth factors to their membrane receptors. Tyrosine kinase receptors, also known as growth factor receptors, being good candidates. They exist as monomers in the cell membrane and when activated by ligand binding, they form dimers, causing activation of thyrosine kinase and autophosphorylation. Moreover, the second messengers, i.e. diacylglycerol and inositol triphosphate (IP3), have been shown to be required for activation of protein kinase C and for release of calcium from intracellular stores.

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