

Sarcoma L-1 in mice as a model for the study of experimental angiogenesis

EWA SKOPIŃSKA-RÓŻEWSKA¹, HENRYK SKURZAK², ALEKSANDER WASIUTYŃSKI¹, LESZEK JUNG³, ANDRZEJ K. SIWICKI⁴, BARBARA J. BAŁAN¹, EWA SOMMER¹, MICHAŁ MAZURKIEWICZ¹, MARCIN SKORUPSKI³, ANTONI PRAŃNICKI¹, IRENA SOKOLNICKA⁵, PIOTR SKOPIŃSKI⁶

¹Department of Pathology, Center of Biostructure, Medical University of Warsaw, Poland; ²Department of Immunology, Cancer Center, Warsaw, Poland; ³Orthopedic Department, Institute of Rheumatology, Warsaw, Poland; ⁴Department of Microbiology and Clinical Immunology, Warmian and Mazurian University, Olsztyn, Poland; ⁵Department of Transfusion Immunology, The Children's Memorial Health Institute, Warsaw, Poland; ⁶Department of Histology and Embryology, Center of Biostructure, Medical University of Warsaw, Poland

Abstract

In this paper animal model for in vivo study of angiogenesis stimulators and inhibitors is described. The L-1 sarcoma, tumor which spontaneously arose in the lung of Balb/c mouse is maintained in vivo and was adapted to the growth in vitro. The L-1 cells from in vitro culture after injection under the Balb/c mice skin form tumors which then could be further transplanted, and used in the experiments in which we can observe vascularization, tumor growth and hemoglobin content in the tumors. The attitude of this model is that we used the same L-1 sarcoma cells in consecutive experiments from the stock L-1 cells obtained from in vitro culture, and saved in LN2. The paper describes some parameters of activity of L-1 sarcoma cells taken from LN2 collection, after various in vivo passages. Angiogenic activity of tumor cells was comparable in all studied passages except that it is a little higher during the third passage. The highest blood supply was present in the tumors during the second and fifth passages. The highest growth L-1 tumors during 14-days was observed in the third passage. For TIA tests cell suspensions from the passage 2nd to the passage 6th may be used interchangeably. However, use of the tumors of similar mass (about 300 mg) is recommended what should assure better standardization of the method.

L-1 sarcoma cells from 4th passage were used for estimation of the effect of two low-molecular weight heparins, enoxaparine and nadroparine, on tumor growth after subcutaneous grafting and on neovascular reaction after intradermal injection of these cells. Nadroparine significantly increased, and enoxaparine significantly decreased neovascular reaction and tumor growth.

Key words: L-1 sarcoma, mice, angiogenesis, tumor growth, low-molecular weight heparins.

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Introduction

Angiogenesis-dependence of solid tumor's growth is well documented. Without blood vessels, tumors can not grow beyond a critical size. The ability to regulate tumor angiogenesis presents an attractive new method for treatment of oncological patients in combination with conventional therapies. Various experimental models of tumor growth and various *in vivo* angiogenesis assays were established to test efficacy of anti-angiogenic agents [1].

L-1 sarcoma tumor employed in the present study arose spontaneously in the lung of Balb/c mouse and was described by Przemysław Janik from Warsaw Oncology Center [2]. This tumor has been maintained since then by subcutaneous serial passages in Balb/c mice and frozen and stored in Oncology Center Cells Collection. In the mean time isolated L-1 cells from tumors were adapted to the growth *in vitro*. It was found that L-1 sarcoma cells from culture, after grafting to animals, form tumors in *in vivo* conditions. Previously, we sometimes used L-1 sarcoma

cells for evaluation of pro- and anti-angiogenic activity of various substances of synthetic and natural origin [3-6]. However, it is not clear whether sarcoma cells recovered after various *in vivo* passages are similar in respect to their angiogenic potential and growth abilities, and may be used interchangeably for tumor-induced angiogenesis test (TIA). The purpose of the present study was to evaluate and compare growth of tumors (tumor mass after 14 days), blood supply (haemoglobin concentration in tumors), and proliferative and angiogenic activity of L-1 tumor cells, collected from *in vitro* culture saved in LN2, and after their defrosting and one or more passages *in vivo*. In our opinion, it is important for choosing the best time for collecting cells for angiogenesis tests.

The experimental model of dermal angiogenesis described above, implemented for the first time by Sidky and Auerbach [7], was repeatedly used both in our research and studies conducted in others centers. The weakness of this model is a vast work consumption, nevertheless it has numerous advantages, generally it is much more humanitarian than other *in vivo* tests performed on mice, such as cornea test, tests on isolated cutaneous flap and tests with implantation of sponge or Matrigel. It allows using significantly smaller number of animals, as a test group numbers only 2-4 mice, each receiving 6 intracutaneous implants of neoplastic cells, and thus permitting to acquire sufficient number of results for statistical analysis. Transplanted cells secrete an array of pro-angiogenic factors, which leads to activation of endothelial cells of mouse blood vessels, and consequently their migration, proliferation and formation of new vessels. This is a suitable model for investigation of the effect of various substances on the earliest phase of vascularization of tumors and their metastases.

In this paper we present, as an example, the results of experiments with two low-molecular weight heparins, enoxaparine and nadroparine. Their effects on angiogenesis and L-1 sarcoma tumor growth were evaluated.

Materials and methods

The study was performed on 8-10 weeks old inbred male and female Balb/c mice, 20-25 g of body mass, delivered from own breeding colony, breeding material was obtained from Warsaw Cancer Center. L-1 sarcoma cells from *in vitro* culture stock were delivered from Warsaw's Oncology Center Collection. L-1 sarcoma cells were grafted subcutaneously (for evaluation of tumor growth, blood supply and cells proliferative activity) or intradermally (for evaluation of angiogenic activity).

Briefly, sarcoma cells were grafted (10⁶/0.1 ml) subcutaneously into subscapular region. After 14 days the tumours were excised, cut to smaller pieces, rubbed through sieve and suspended in 5 ml of PBS. The suspension was left for 10 min at room temperature. After sedimentation the supernatant was collected and centrifuged for 10 min at 1500 rpm. Obtained sarcoma cells were washed once with

PBS for 10 min, then centrifuged at 1500 rpm, and resuspended in Parker medium in concentration of 4x10⁶/ml or 10⁷/ml.

L-1 sarcoma cells proliferative activity was measured by use of 3H-thymidine incorporation test. Briefly, cells isolated from tumors were suspended in RPMI 1640 with addition of l-glutamine, 10% foetal calf serum, penicillin, and streptomycin, set in microplates and incubated in a humidified atmosphere at 7°C, with 5% CO₂ for 24 hours. Then, 10 µl of ³HTdR at dose of 0.2 µCi was added and cultures were incubated for 18 hours. After this time the cells radioactivity was measured in the scintillation counter (RackBeta, 1218, LKB Wallac). The results were shown in mean counts per min (cpm).

Angiogenesis induced in the skin of Balb/c mice after grafting of L-1 sarcoma cells. Cutaneous angiogenesis assay was performed according to Sidky&Auerbach [7] with own modifications [8]. Briefly, multiple 0.05 ml samples of 200 thousand of cells were injected intradermally into partly shaved, narcotised Balb/c mice (at least 2-4 mice per group). In order to facilitate the localisation of cell injection sites, the suspension was coloured with 0.1% of trypan blue. After 72 hours mice were sacrificed with lethal dose of Morbital. All newly formed blood vessels were identified and counted in dissection microscope, on the inner skin surface, at magnification of 6x, in 1/3 central area of microscopic field (figure 1). Identification was based on the fact that new blood vessels, directed to the point of cells injection are thin and (or) differ from the background vasculature in their tortuosity and divarications. All experiments were performed in anaesthesia (3.6% chloral hydrate, 0.1 ml per 10 g of body mass).

Subcutaneous tumour growth assay. Mice were injected subcutaneously in the dorsal scapular region with 1 million L-1 sarcoma cells. At the day 14-th mice were killed, tumours excised, and weighted. In experiments with low-molecular weight heparins, mice were treated with enoxaparine (clexane, Sanofi-Aventis) in daily dose of 80 µg, or with nadroparine (fraxiparine, Glaxo-Smith-Cline) in daily dose 8 IU, during 14 days after cells grafting. These doses corresponded to 40 mg (Clexane), and 4000 IU (Fraxiparine) given to 70 kg person (applying the counter 7 for differences between mouse and human in relation of the surface to body mass). Tumors volumes were measured since day eight with electronic calliper.

Estimation of Hb concentration in tumors was done according to the method described [9]. Briefly, tumors were homogenized in PBS using an ultrasonic sonicator (Virsonic, USA), then centrifuged for 20 min at 4000 x g. 20 µl of the supernatant was added to 5 ml of Drabkin reagent. The absorbance was read in a spectrophotometric reader Elx800 (Biotek Instruments, USA) at 570 nm. The reader for the Hb measurement was calibrated with haemoglobin standard solutions (Sigma). The results were shown as µg Hb in 1 mg of tumor mass.

Morphological examination was done on the cellular level using light – microscopic analysis. Immediately after

Table 1. Mass of tumors 14 days after grafting of 1×10^6 of L-1 sarcoma cells to syngeneic Balb/c mice

Passage after defrosting	Number of tumors	Mean tumor mass (mg) \pm SE	Significance of differences
2 nd	40	279 \pm 39	
3 rd	58	886 \pm 58	P<0.01 from all other groups
4 th	47	354 \pm 30	
5 th	31	285 \pm 44	
6 th	47	283 \pm 47	

Table 2. Hemoglobin content of tumors 14 days after grafting of 1×10^6 of L-1 sarcoma cells to syngeneic Balb/c mice

Passage after defrosting	Number of tumors	Mean Hb concentration (μ g/mg) \pm SE	Significance of differences
2 nd	40	37 \pm 2	P<0.01 from group 3, 4 and 6
3 rd	58	27 \pm 1	
4 th	47	27 \pm 2	
5 th	31	41 \pm 5	P<0.01 from group 3, 4 and 6
6 th	47	28 \pm 1	

Table 3. Angiogenic activity of tumor cells collected 14 days after grafting of 1×10^6 of L-1 sarcoma cells to syngeneic Balb/c mice

Passage after defrosting	Number of TIA tests	Mean number of newly-formed blood vessels \pm SE	Significance of differences
2 nd	94	21.9 \pm 0.57	
3 rd	31	25.7 \pm 0.52	P<0.05 from groups 2, 4, 5 and 6
4 th	19	22.8 \pm 0.62	
5 th	30	23.5 \pm 0.54	
6 th	54	21.8 \pm 0.85	

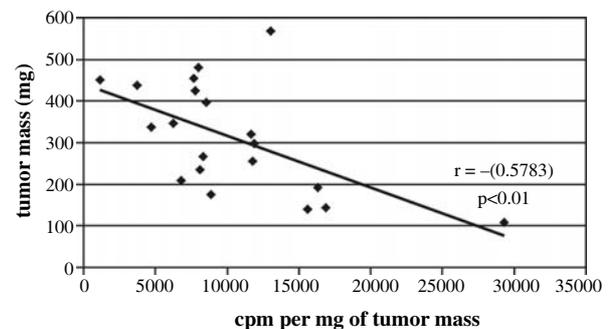
resection, tumor specimens were fixed in 10% formaldehyde solution. After fixation the specimens were dehydrated in increased concentrations of alcohol and embedded in paraffin. Paraffin tissue block was sectioned on 4 μ m thin sections. The specimens were contrasted by hematoxyline and eosine for first screening light microscopic examination.

For all experiments animals were handled according to the Polish law on the protection of animals and NIH standards. All experiments were accepted by the local Ethical Committee.

Statistical evaluation of results was performed by Student's *t*, Mann-Whitney's and Pearson's tests.

Results

After defrosting and short-term *in vitro* culture of L-1 sarcoma cells, the highest *in vivo* growth during 14-days was observed in the third passage (table 1). The highest blood supply was present in the tumors during the second and fifth passages (table 2). Angiogenic activity of tumor cells was comparable in all studied passages except that it is a little higher during the third passage (table 3).

**Fig 1.** Typical picture of neovascular reaction 3 days after intradermal injection of 0.2×10^6 L-1 sarcoma cells to Balb/c mice**Fig. 2.** Strong negative correlation between tumor mass and proliferative activity of cells (passage 4th)

Statistical analysis revealed negative correlation between tumor mass and amount of 3 H-thymidine incorporation by cells present in 1 mg of tumor (figure 2), and also negative correlation between tumor mass and hemoglobin concentration, but not in all tumor passages (figures 3-5).

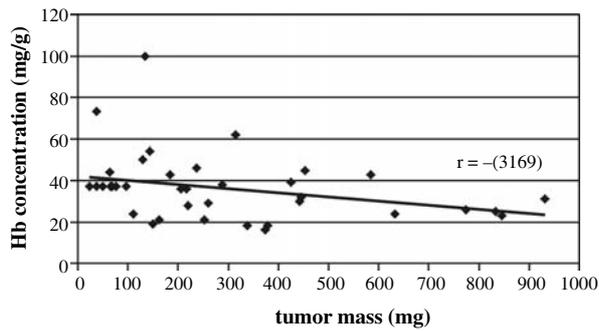


Fig. 3. Weak negative correlation between tumor mass and hemoglobin concentration (passage 2nd)

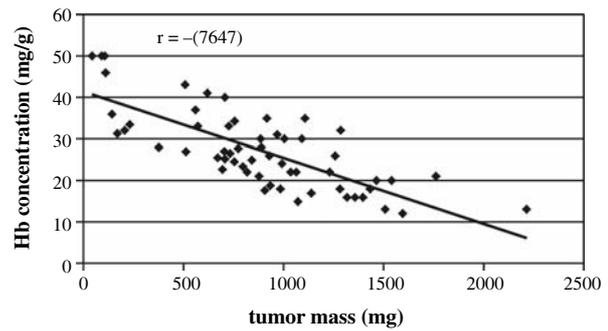


Fig. 4. Strong negative correlation between tumor mass and hemoglobin concentration (passage 3rd)

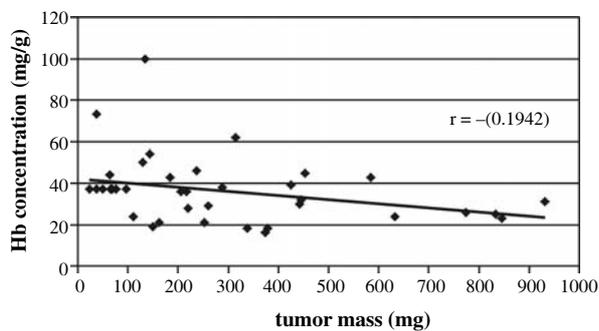


Fig. 5. Lack of correlation between mass of tumors and their hemoglobin concentration (passage 4th)

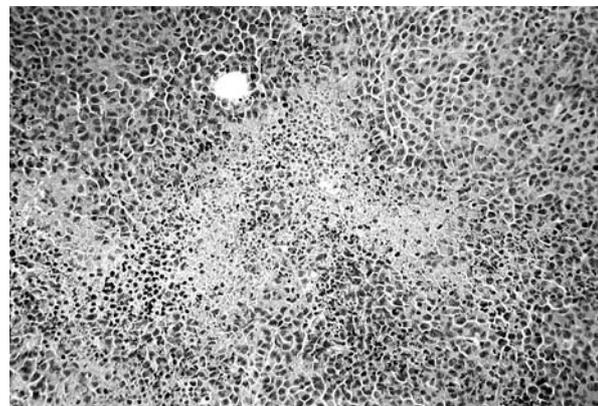


Fig. 6. Area of necrosis in the tumor (photo taken under 10x lens)

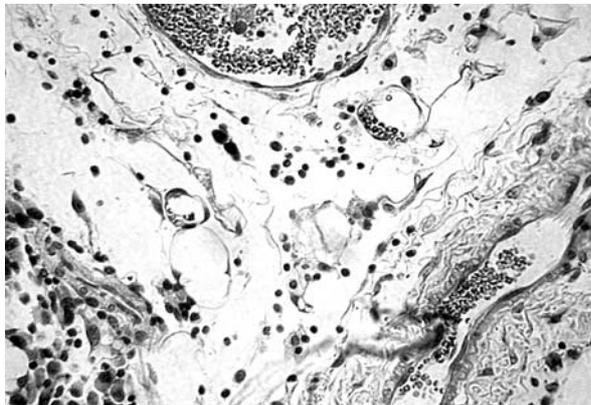


Fig. 7. Very sparse inflammatory infiltration at the tumor margin and around the vessels (photo taken under 20x lens)

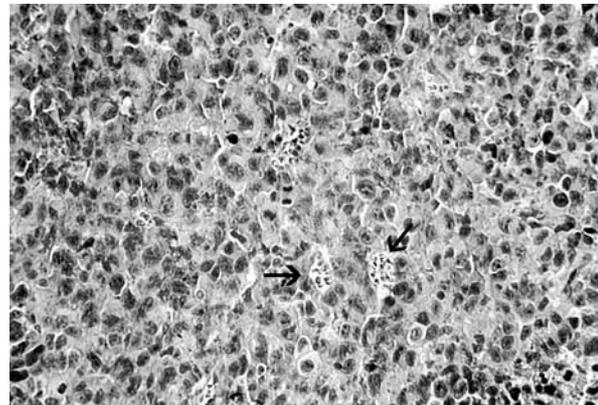


Fig. 8. Newly-formed vessels within the tumor (arrows) (photo taken under 20x lens)

Histological studies revealed no major differences between tumors deriving from different passages. No differences were also observed between tumors from control and low-molecular-weight heparin treated mice. The dominant picture of tumors morphology was mass of poorly differentiated oval or fusiform atypic cells with features of

sarcoma, some of them multinuclear. Either necrosis and very sparse inflammatory infiltrations at the tumor margins were seen in all groups. There was also numerous small vessels seen in the tumor (figures 6-8). Nadroparine, administered for 3 days after intradermal L-1 sarcoma cells injection, significantly increased, and enoxaparine significantly

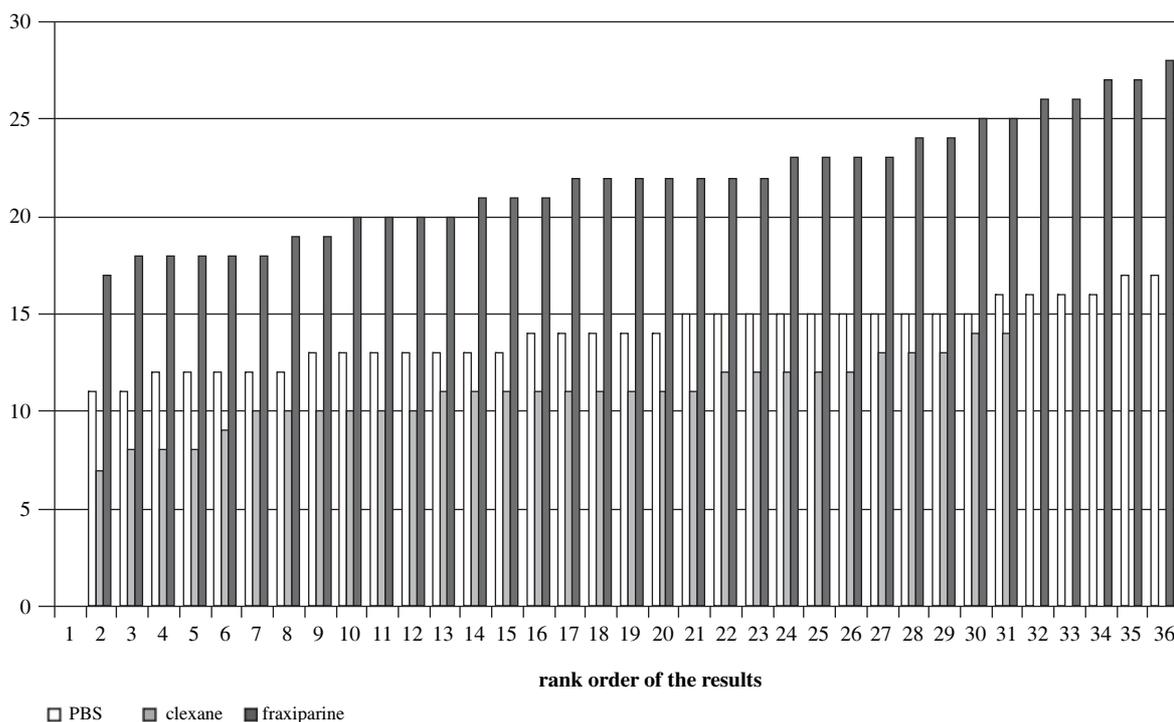


Fig. 9. Number of newly-formed blood vessels 3 days after intradermal injection of L-1 sarcoma cells to Balb/c recipients. Mice were treated for 3 days with Clexane, or Fraxiparine, or PBS (controls)

decreased neovascular reaction induced in recipients Balb/c mice skin on the third day after cells grafting (figure 9).

The results of volume measurements are presented in figure 10.

Tumor volume on the 8th day was the lowest in the control group, significantly higher in enoxaparine group ($p < 0.05$) and even higher in nadroparine group ($p < 0.01$). On the following days tumors in mice receiving nadroparine were significantly larger than in two remaining groups up to the 13th experimental day ($p < 0.05$).

Between day 13 and 14 there was a rapid tumor growth in control and enoxaparine group, and much less extensive in nadroparine group. The results of volume measurements performed on the 14th day did not reveal differences among groups. In qualitative observations we did not see any differences of morphology and number of blood vessels between examined groups.

Discussion

On the basis of the results obtained in this study, we consider, that for use of L-1 sarcoma cells in TIA test, tumor cells from the majority of tested passages could be used interchangeably. However, having in mind some differences in relation of tumor mass to hemoglobin concentration observed in tumors belonging to various passages, we feel

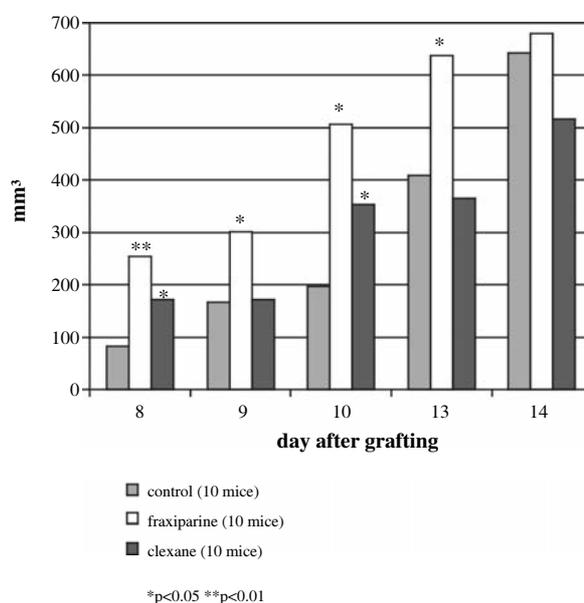


Fig. 10. The effect of Clexane or Fraxiparine treatment on tumor volume in various days after L-1 sarcoma cells grafting

that use of the tumors of similar mass (about 300 mg) is recommended, and use of the cells from the third passage should be avoided, what would assure better standardization of the method.

The studies we previously conducted on this experimental model [3] demonstrated the effect of administration of enoxaparine and nadroparine to mice (L-1 sarcoma cells recipients) on neovascular reaction observed on the third day following implantation of cells. In the current study we repeated the same experimental schema with the equal results: reaction in mice receiving nadroparine was significantly larger than in control group, while in mice receiving enoxaparine significantly lesser. In the earlier research [3] we have not observed the effect of these two heparins applied to mice – tumor cells recipients – on tumor mass on the 14th day of experiment, although we noticed a certain difference in concentration of VEGF and bFGF cytokines (decreased concentration of VEGF and bFGF in enoxaparine group and increased concentration of bFGF in nadroparine group on the 5th day after L-1 sarcoma cells transplantation, and increased content of bFGF in the tumors from nadroparine group on the 14th experimental day). In the present study we observed larger volumes of tumors belonging to nadroparine group in comparison to the tumors growing in mice treated with enoxaparine or untreated.

Folkman research on the role of heparin in angiogenesis, and later Norrby research on function of mast cells and heparin and its low molecular weight fractions in this process have proved an important role, that the mentioned agents may play in tumor angiogenesis, and therefore in the development of neoplasm [10-14]. Enoxaparine and nadroparine, the two low molecular weight heparins (LMWH) are available on the polish market for over 15 years and used in many conditions in order to avoid thromboembolic complications. 'Non-fractionated' heparins are produced from mucous membrane of porcine bowels. The molecular weight of non-fractionated heparin is around 15 000 Da. Polysaccharide chains with molecular weight about 5 000 Da are obtained by enzymatic or chemical depolymerization method. Depolymerization changes biologic properties of heparin and nascent low molecular weight heparins vary depending on the method applied in their production.

They are currently widely used, e.g. for prevention of thrombotic complications in neoplastic patients. They are better tolerated and give less complication than non-fractionated heparin. Recent publications indicate anti-neoplastic properties of heparin oligosaccharides in experimental models. They act via inhibition of activity of two important angiogenic growth factors, VEGF and bFGF [15-17].

Neoplastic tumors secrete many pro-angiogenic factors, which stimulate angiogenesis through their affinity to receptors on endothelial vascular cells. Low molecular weight heparins are more effective in inhibition of these factors than non-fractionated heparin. It is postulated that they decrease

incidence of metastases by diminishing invasiveness of tumor cells, which in turn is the result of inhibition of heparinase, secreted by neoplastic cells. Heparinase activity is correlated with metastases occurrence [14].

Noteworthy, the authors of majority of the reports seem to treat the problem of influence of low-molecular weight heparins on neoplasms as homogenous and pertinent to a group of compounds with similar molecular weight, conversely they do not devote enough attention to chemical and structural differences, probably resulting from manufacturing methods of this biological agents.

In our previous studies on influence of two low molecular weight heparins – enoxaparin and nadroparin – on angiogenic activity and concentration of VEGF in mouse plasma, we demonstrated opposing effects of afore mentioned heparins. Enoxaparine (Clexane) stimulated, whereas nadroparine (Fraxiparine) inhibited angiogenic activity of plasma, additionally decreased concentration of VEGF in mouse plasma samples [15].

We feel, that more studies are necessary to resolve the problem of different action of enoxaparine and nadroparine on tumor angiogenesis. However, we are sure that these two low-molecular weight heparins cannot be used interchangeably.

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