Comparison of the effects of enoxaparin and nadroparin on tumor angiogenesis in mice

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Abstract

Angiogenesis-dependence of tumor's growth is known for more than 30 years. Studies on the influence of heparin and its derivatives (as low molecular weight heparins /LMWH/) on tumor angiogenesis were done and given different results.

As LMWH are widely used in deep venous thrombosis /DVT/ prophylaxis in patients with neoplastic disease, we tried to check influence of enoxaparin and nadroparin on tumor angiogenesis and tumor growth in mice.

Experiments were done in inbred Balb/c mice whom L-1 sarcoma cells were grafted subcutaneously or intradermally. Nadroparin highly significantly increased, and enoxaparin significantly decreased neovascular reaction induced in syngeneic mice skin on the 3-rd day after cells grafting. Cytokines concentration (VEGF and bFGF) in 5-th days lesions behaved similarly as neovascular reaction (enoxaparin decreased VEGF and bFGF content in comparison to the control, nadroparin increased bFGF concentration). Fourteen days after sarcoma cells grafting VEGF concentration was similar in all groups, and the only one difference from the control it was higher bFGF content of tumors collected from nadroparin – treated mice. Histological examination of the lesions didn't revealed serious changes between control and experimental groups of animals.

Key words: *low-molecular-weight heparins, angiogenesis, mice, L-1 sarcoma, cytokines*

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Introduction

Folkman et al revealed, more than 30 years ago, that tumor growth is angiogenesis-dependent, and that heparin released by mast cells accumulating at a tumor site, increased the migration of the capillary endothelial cells in vitro and enhanced tumor angiogenesis in vivo [1]. Later studies presented evidence of opposite, inhibitory effect of low-molecular weight fragments of heparin (LMWH) on angiogenesis [2].

Low-molecular weight heparins (LMWH) are widely used as anti-coagulants for prophylaxis and therapy of thrombo-embolic complications in many clinical situations, also in patients with neoplastic disease. Some experimental and clinical studies have shown their anti-cancer effect. LMWH may influence various parameters of tumor cells and immune system cells activity, and they may interfere with various steps of tumor angiogenesis. LMWH can reduce binding of bFGF (fragments of less than 10 saccharides) and VEGF (fragments of less than 18 saccharides) to their receptors, and inhibit bFGF- and VEGF-mediated angiogenesis in vivo [3-6].

Enoxaparin and nadroparin are commonly used in Poland since 15 years. They often are used interchangeably because of their similar molecular weight and anti-coagulant

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properties. In our previous study we presented evidence of opposite effects of these two LMWH on the angiogenic activity of serum in mice [7]. The aim of the present study was to compare the effect of these two LMWH on tumor angiogenesis and tumor growth in mice.

Materials and methods

The study was performed on 8-10 weeks old inbred Balb/c and Balb/c x DBA2 F1 mice, weighing about 20 g, of both sexes, delivered from Polish Academy of Sciences, Mazurian University and own breeding colony. Enoxaparin (Clexane, Sanofi-Aventis) and nadroparin (Fraxiparine, Glaxo-Smith-Kline) were administered to mice in daily doses 80 μg and 8 IU, respectively. 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). Mice received drugs subcutaneously in 0.1 ml of saline, for 3 days (when grafted with tumour cells intradermally), or for 14 days (after subcutaneous tumour cells grafting). Sarcoma cells were delivered from Warsaw's Oncology Center Bank and then passaged through several generations of Balb/c mice. Briefly, sarcoma cells were grafted (10^6 /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 1400 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 $4x10^6$/ml or 10^7/ml. Some samples of cell suspensions were incubated with LMWH before grafting to recipient mice. Briefly, cells were mixed with heparins in final concentration of 10^7 cells with 10 μg of Clexane, or 1 IU of Fraxiparine per ml of Parker medium, and left for 60 min at 37°C, 5%CO_2 and humidified atmosphere.

Angiogenesis induced in the skin of Balb/c or Balb/c x DBA2 F1 mice after grafting of L-1 sarcoma cells

Cutaneous angiogenesis assay was performed according to Sidky&Auerbach [8] with own modifications [9]. Briefly, multiple 0.05 ml samples of 200 thousand of cells were injected intradermally into partly shaved, narcotised Balb/c mice (at least 3 mice per group). 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 injected with LMWH (if tumour cells were not preincubated with LMWH). 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. Identification was based on the fact that new blood vessels, directed to the point of cells injection, 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).

In some experiments mice were killed 5 days after tumour cells grafting, lesions were harvested and fixed in 10% formaline for histopathology, or frozen at –78°C (suspended in PBS in proportion 100 mg per 1 ml) for later angiogenic cytokines measuring.

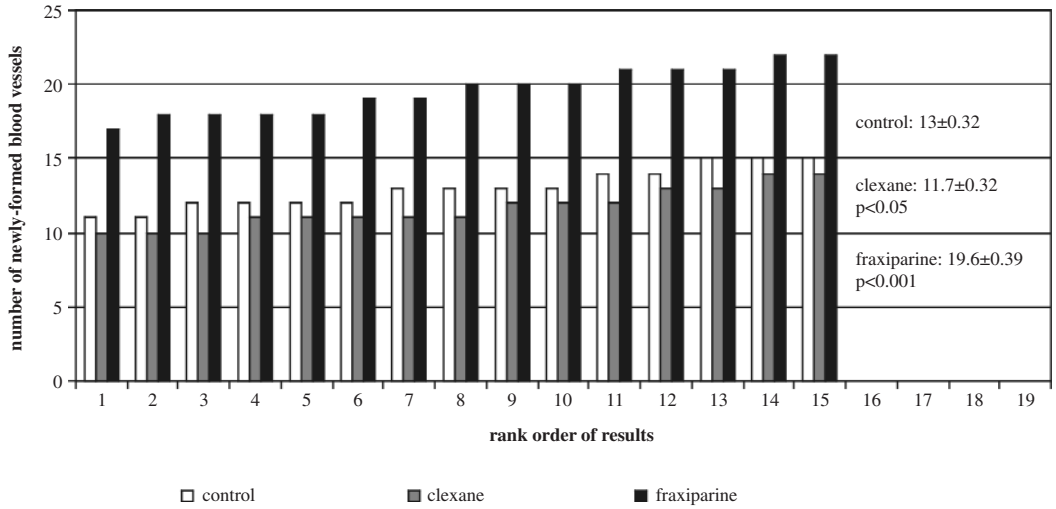

Fig. 1. Various effects of Clexane and Fraxiparine administered for 3 days after L-1 Sarcoma cells intradermal grafting on neovascular reaction induced by these cells in syngeneic mice

Measurement of cytokines concentration

The tumour samples collected on the day five were pooled within groups, homogenized with an ultrasonic disrupter VirSonic (Virtis) for 2 minutes, at frequency 22.5 KHz. Cytokine levels were determined using standard ELISA R&D kits for mouse VEGF and human bFGF (high sensitivity), according to producer instructions. Optical density was measured at 450 nm using spectrophotometric reader Elx800 (Biotek Instruments, Inc., USA). Cytokines concentration was expressed as pg/ml. Inhibition/stimulation indices were calculated dividing results of tests performed with tumours collected from heparins-treated mice by mean concentration of cytokines in control tumours.

Table 1. Angiogenic cytokines in tumors 5 days after intradermal grafting of L-1 sarcoma cells to syngeneic mice (inhibition/stimulation indices +/- SE)

Group of mice	number of tumors	number of tests	VEGF	bFGF
Enoxaparine treated	36	8	0.62±0.07**	0.79±0.08*
Nadroparine treated	32	8	1.01±0.1	1.27±0.08**
Controls	36	8	1±0.14	1±0.04

** 0.05<p<0.1 ** p<0.05*

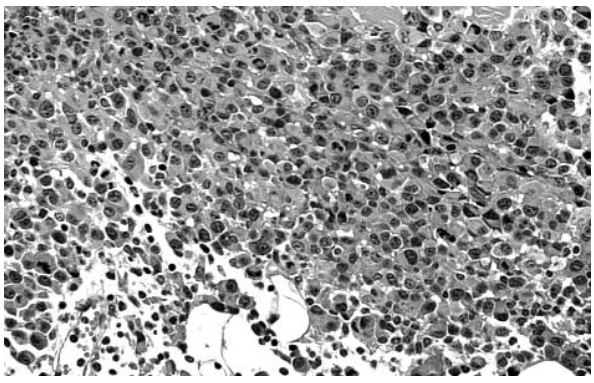

Fig. 2. Microscopic picture of L-1 Sarcoma 5 days after intracutaneous transplantation (magnification 500 x). HE stain. Recipient mice were treated with enoxaparin after tumor cells grafting

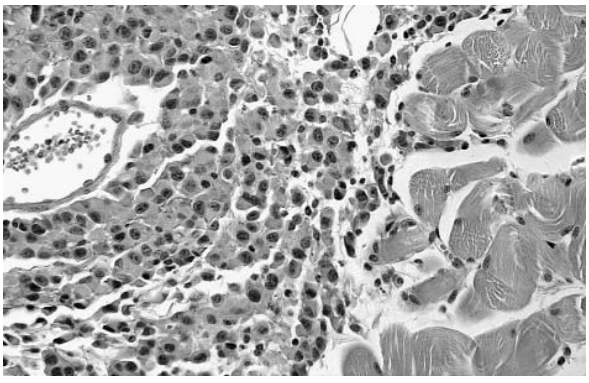

Fig. 3. Microscopic picture of L-1 Sarcoma 5 days after intracutaneous transplantation (magnification 500 x). HE stain. Recipient mice were treated with nadroparin after tumor cells grafting

Subcutaneous tumour growth assay

Mice were injected subcutaneously in the dorsal scapular region with 1 million L-1 sarcoma cells (10 animals per group). At the day 14-th mice were killed, tumours excised, weighted and homogenized in 12 ml of PBS for 30 sec at frequency 22.5 KHz. After double washing with PBS sediment was resuspended in 2 ml of PBS, divided to 0.5 ml samples and frozen at –78°C. VEGF and bFGF levels in the tumours homogenates were measured by ELISA. Cytokines concentration were expressed as pg/mg of tumour tissue. Inhibition/stimulation indices were calculated as above.

Morphological examination was done on the cellular level using light – microscopic analysis. Immediately after 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 μm 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* and Mann-Whitney tests.

Results

The results of LMWHs administration to mice after transplantation of L-1 sarcoma cells are presented on Fig. 1. Nadroparin highly significantly ($p<0.001$) increased, and enoxaparin significantly ($p<0.05$) decreased neovascular reaction induced in syngeneic mice skin 3 days after cells grafting. Cytokine examinations of lesions collected on the day 5-th after grafting revealed lower content of VEGF and bFGF in tumours of enoxaparin-treated mice, and higher content of bFGF in tumors collected from nadroparin-treated animals (Table 1). Histological examination revealed no major differences between tumors collected from both experimental groups of mice. The dominant picture of tumors morphology was mass of poorly differentiated oval or fusiform atypic cells with features of sarcoma (Fig. 2 and Fig. 3). Tumors collected

from control mice were composed of similar cells, however, they also contained bizarre and giant cells, some of them multinuclear (Fig. 4).

The results of experiments performed with L-1 sarcoma cells preincubated with LMWHs are presented on Fig. 5. In this experimental model, both nadroparin and enoxaparin diminished angiogenic activity of tumor cells (neovascular reaction induced by intradermal grafting of cells preincubated with heparins was significantly lower than that induced by control cells).

Examination of cytokines 14 days after subcutaneous sarcoma cells grafting revealed that VEGF concentration was similar in all groups, and the only one difference from the control it was higher bFGF content of tumors collected from nadroparin – treated mice (Table 2). There were no differences between tumor mass of mice treated for 14 days with nadroparin, enoxaparin or PBS (Table 3).

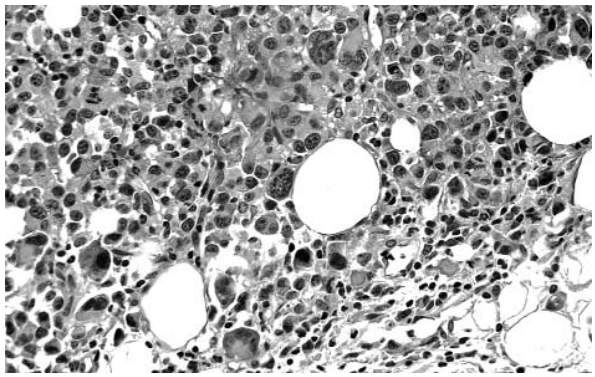

Fig. 4. Control (untreated) mice. Microscopic picture of L-1 Sarcoma 5 days after intracutaneous transplantation (magnification 500 x). HE stain. Variety of tumor cells of different size and nuclei, some of them are multinuclear

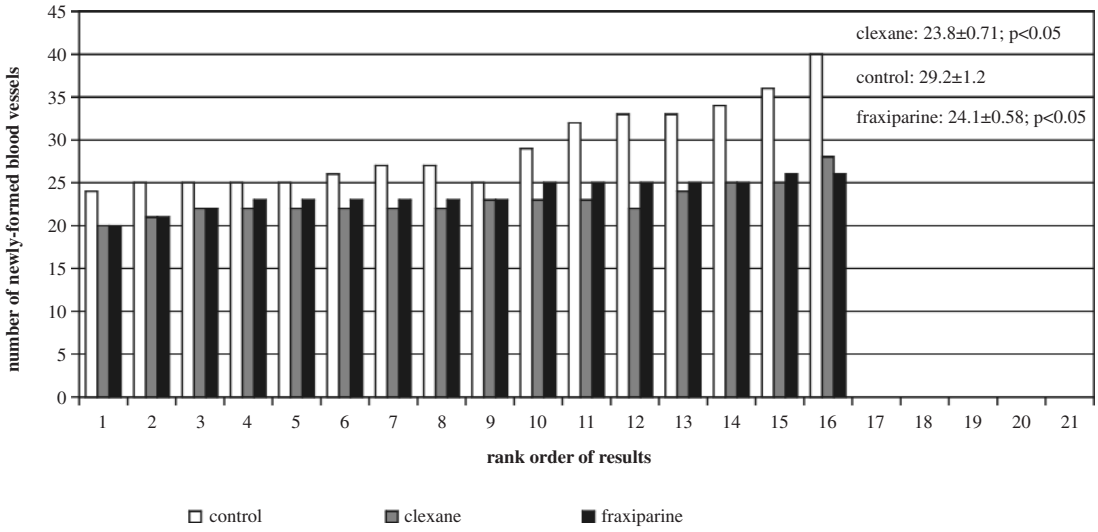

Fig. 5. Inhibitory effect of preincubation of L-1 sarcoma cells with low-molecular weight heparins on neovascular response induced in syngeneic mice skin

Table 2. Angiogenic cytokines in tumors 14 days after subcutaneous grafting of L-1 sarcoma cells to syngeneic mice (inhibition/stimulation indices ±SE)

Group of mice	number of tumors	number of tests	VEGF	bFGF
Enoxaparine treated	10	10	1.11±0.20	1.08±0.15
Nadroparine treated	10	10	1.02±0.06	1.28±0.07**
Controls	10	10	1±0.13	1± 0.11

*** p<0.05*

Table 3. Mass of tumors 14 days after subcutaneous grafting of L-1 sarcoma cells to syngeneic mice

Group of mice	number of tumors	mean (mg)±SE
Enoxaparine treated	10	527±75
Nadroparine treated	10	538±60
Controls	10	514±82

Discussion

Low-molecular weight heparins are now commonly used for the prevention and treatment of thromboembolic events that often accompany malignancies. It was reported recently, that heparin and LMWHs may have anti-metastatic activity [5, 10], and it was reported that nadroparin and enoxaparin may favourably influence the survival in patients with advanced malignancy [5, 11]. Also animal studies suggested, that heparins may play a role in cancer growth and dissemination [12]. Heparins can interfere with immunity system, cancer cell adhesion and motility, and tumour angiogenesis [6, 13, 14]. Various LMWHs exhibit specific structural characteristics that may contribute to their various unique biological properties, not connected with their anticoagulant activity, which may be similar. In this paper, as well as in some others, we present evidence of fundamental differences between influence of enoxaparin and nadroparin on some aspects of experimental angiogenesis [7, 15]. In the present study we observed stimulation of new blood vessel formation and increase in bFGF content in the sites of L-1 sarcoma cells grafting into mice skin, when mice obtained 3 subcutaneous injections of nadroparin after cells grafting. Contrary to the effect of nadroparin, enoxaparin treatment resulted in diminished neovascular response and lower bFGF, as well as VEGF, content of 5-th day old tumours. This discrepancy evidently depended on the opposite action of these two LMWHs on host microenvironment of tumour cells, as cells preincubated with nadroparin and cells preincubated with enoxaparin behaved similarly, expressing lower angiogenic activity than cells preincubated in medium alone. Moreover, L-1 sarcoma cells cultured in vitro in the presence of enoxaparin or nadroparin for 24 hours, produced the same amounts of bFGF and VEGF as control cells [in preparation]. We also observed no difference in histological picture and tumour mass after 14 daily injections of enoxaparin and nadroparin, in comparison to the controls, what may suggest, that presently observed differences were connected with the influence of these LMWHs on early steps of angiogenesis and tumour development only, and have no impact on later L-1 sarcoma tumour growth.

Recently, it has become clear, that microenvironment plays important role in tumour angiogenesis [16]. Endothelial cells interact not only with tumor cells, but also with pericytes, leukocytes, fibroblasts and factors released from extracellular matrix (ECM). We may only hypothesize, that nadroparin and enoxaparin may react with different elements of this multidirectional inter-cellular cross-talk

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