Enoxaparine treatment enhanced angiogenic activity of mouse and human serum

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Abstract

Regenerative processes play essential role in hard and soft tissues repair and are tightly connected with angiogenesis. During the wound healing angiogenic factors are released and found in the circulating blood. Enoxaparine (En) and nadroparine (Nad), low-molecular weight heparins (LMW) are routinely used as anti-coagulants for prophylaxis after bone surgery.

The aim of this study was to evaluate the in vivo angiogenic potency and VEGF and bFGF content of sera collected from seven patients after hip surgery, treated for 14 days with 40 mg of En daily. We also performed experiments with mice treated with En (80 μg) or Nad (8 IU) for 14 days. Treatment of the mice with En increased angiogenic activity and VEGF content of their sera, sera obtained from Nad mice did not differ from the controls.

Sera of patients treated with En presented higher angiogenic activity as well as higher bFGF and VEGF content after 14 injections than the values observed before the beginning of the treatment. Conclusion: beneficial effect of 14-days prophylaxis with enoxaparine on healing process might be expected in patients after hip surgery.

Key words: *enoxaparine, patients, hip surgery, angiogenesis, mice*

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Introduction

It was reported by Folkman more than 30 years ago, that the heparins may play important role in angiogenesis [1]. Since this time a lot of research concerning their angiomodulatory properties was done, but still this is a complex and controversial issue. In our previous study we investigated the effect of enoxaparine (clexane) and nadroparine (fraxiparine), administered to mice for 2 consecutive days, on the angiogenic activity of their sera. In these studies we have found significant stimulation of angiogenic ability of sera of mice treated with enoxaparine and significantly lower activity and VEGF content of the sera collected from fraxiparine-treated animals, in comparison to the controls [2]. The first aim of the present study was evaluation of the effect of 14 consecutive daily injections of these LMW heparins into the mice on the angiogenic activity of their sera.

The second aim was studying of angiogenic activity and angiogenic growth factors (vascular endothelial growth factor, VEGF and basic fibroblast growth factor, bFGF) content of the sera collected from patients with coxarthrosis, before and after hip surgery and prophylactic, lasting for fourteen days, enoxaparine treatment.

VEGF and bFGF are important cytokines implicated in the response to an injury. Healing of fractures is dependent on the vascularization of bone, promoted by VEGF [3]. The results of the study performed in rats, reported by Sojo et al. [4] confirm that angiogenesis is induced before osteogenesis. It was also documented on the experimental model (alloge-

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neic bone grafts in rabbits) that bFGF up-regulates the expression of VEGF during healing of grafted bone [5]. Other authors investigated the ability of recombinant human VEGF to stimulate the formation of bone in an experimental (rabbits) atrophic nonunion model. Standard nonunion operation was followed by interfragmentary deposition of 100 μg of VEGF, carrier alone or autograft. After seven weeks VEGF-treated osteotomies had united whereas the carrier-treated failed to unite [6].

Heparan sulfates and heparins modulate the binding of various growth factors to their receptors (among them bFGF and VEGF), changing endothelial cells response to these factors. It was reported by Norrby and Ostergaard [7] and others [8, 9] that LMWH suppress VEGF- and bFGF-induced angiogenesis, and inhibit chemokine CCL21-induced T cell adhesion and migration. Other authors reported in vitro inhibition of adhesion of granulocytes to endothelial cells by LMWH, what correlates with their activity on angiogenesis in mice [10].

All these findings prompted us to study the effect of two-weeks administration of LMWHs on overall angiogenic activity of mice and human sera, and their bFGF and VEGF content.

Material and methods

Patients

We collected blood samples from 1 male and 6 female patients, aged 64-71 years, with coxarthrosis (5 persons), gonarthrosis (1 person) and avascular necrosis of the femoral head (1 person). After clotting, the sera were separated and frozen at –70˚C until testing. Sera were collected for the first time immediately before beginning of enoxaparine (En) treatment, for the second time 12 hours after the first En injection, immediately before the surgery, and for the third time 14 days after the beginning of treatment (40 mg of En subcutaneous daily injections).

Animals

Sera were collected from 21 mice Balb/cxDBA2F1, 6-8 weeks old, about 20 g of body mass, females. Seven of them were treated subcutaneously with 80 μg of En (Clexane, Sanofi-Aventis), seven obtained 8 IU of Nad (Fraxiparine, GlaxoSmithCline), and seven were treated with subcutaneous injections of 0.1 ml of PBS (control group). Mice were treated for 14 days. After blood clotting, the sera were separated, collected within groups, and frozen until used.

Angiogenic activity measurement of patients and mice sera was done by cutaneous test performed in mice (serum-induced angiogenesis, SIA test) according to Skopiński et al [11] and Barcz et al [12]. Briefly, multiple 0,05 ml samples of the sera were injected intradermally into partly shaved, narcotised Balb/c (in the case of patients sera) or Balb/cx DBA2F1 (in the case of animals sera) mice. At least 3 mice for one human serum and 3-4 mice for each murine serum pool were used. In order to facilitate the localisation of injection sites later on, each serum sample 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 a magnification of 6x, in 1/3 central area of the 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).

Measurement of VEGF and bFGF concentration

Cytokine levels were determined in examined sera using sandwich ELISA kits (R&D Systems, USA) for human VEGF, mouse VEGF and human bFGF (high sensitivity), according to the producer instructions. Optical density was measured at 450 nm using spectrophotometric reader Elx800 (Biotek Instruments, Inc., USA). Cytokines concentration was expressed as pg/ml.

Statistical analysis was performed using Student-*t* test.

All experiments were approved by Local Ethical Committee.

Results

The results of the experiments performed with mice sera are presented on the Table 1 and on the Fig. 1. Sera from

Table 1. Neovascular response induced in Balb/cxDBA2 F1 mice skin by sera collected from syngeneic mice injected previously with enoxaparine or fraxiparine for 14 days

Sera donors	number of SIA tests	Mean number of newly-formed blood vessels (±SE)	Statistical significance of difference from the control
7 control mice	17	14.8±0.41	–
7 Enoxaparine – treated mice (80 μg daily)	15	17.8±0.42	p<0.001
7 Fraxiparine – treated mice (8 IU daily)	16	13.8±0.37	non significant

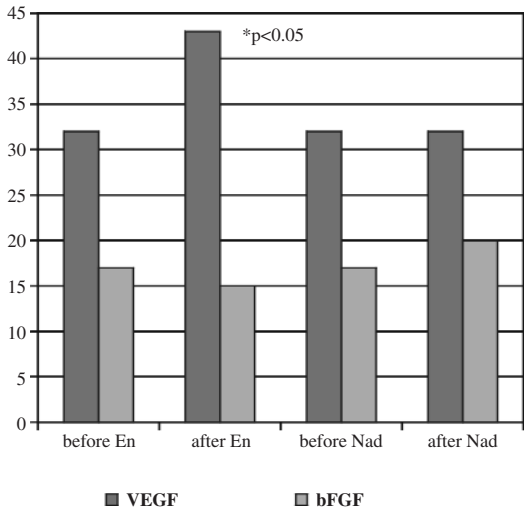

Fig. 1. Concentration of VEGF and bFGF in sera collected from mice injected for 14 days with En or Nad

the mice which obtained fourteen En injections induced highly significantly more blood vessels than the sera from the Nad – treated or control mice.

Sera collected from the En group contained higher VEGF concentration than the sera obtained from the animals belonging to the Nad and control groups. Concentration of bFGF in both experimental groups did not differ from the control.

In the patients sera we observed significant stimulation of the angiogenic response after one En dose, and highly significant stimulation after fourteen En injections (Table 2). The VEGF level measured after the first injection of En did not differ from the initial value. However, the sera collected after fourteen En doses contained significantly more VEGF, than that was observed before and after the first dose of this drug (Table 3).

The second evaluated cytokine, bFGF, presented different time-course. Concentration of this growth factor highly significantly increased after the first dose of the En, and have remained elevated up to the 14-th day after the beginning of treatment (Table 4).

Discussion

The results presented in this study confirm the important role of one of the low-molecular-heparins, enoxaparine, in modulating the angiogenesis processes. It is noteworthy, that in our experimental model we observed stimulation of angiogenic potential of the sera. One may suppose, that the stimulatory effect of enoxaparine treatment on overall angiogenic potential of sera visualised by the SIA test, might be also partly connected with performed surgery, as it is known that increasing levels of VEGF may be detected in the serum during a wound healing and bone repair [5, 13-15]. However, we observed similar rise of angiogenic activity and VEGF level in mice sera, collected after fourteen enaxaparine doses. In our patients, bFGF level rose before surgery, after one injection of enoxaparine. It is in agreement with the results of East et al, obtained for unfractionated heparin. These authors demonstrated, that one shot of heparin given to the patient with ischaemic heart disease, has risen the concentration of bFGF in serum [16], what was probably connected with release of bFGF from the extracellular matrix.

In the other paper [17] we present an evidence, that enoxaparine may also behave as angiogenesis inhibitor. Preincubation of murine L-1 sarcoma cells with the En, or administration of En to L-1 sarcoma cells recipients, significantly diminished a neovascular response in a tumor-induced cutaneous angiogenesis test in mice. These

Table 2. The effect of enoxaparine (En) injections for 1 or 14 days (40 mg daily) on the angiogenic activity of human serum (measured as a number of newly-formed blood vessels in SIA test)

	Before first dose of En		After first dose of En (before surgery)		After 14 injections	
Patient initials	**Number of SIA tests**	**Mean ± SE**	**Number of SIA tests**	**Mean ± SE**	**Number of SIA tests**	**Mean +/- SE**
MZ	17	44.6±2.4	17	49.4±3.3	17	59.1±2.7
SA	17	39.1±2.4	20	53.8±2.9	16	52.4±2.2
KA	18	58.9±3.6	15	62.3±6.1	15	74.5±3.9
JA	15	43.5±3.4	16	62.4±3.6	16	94.2±5.2
CM	14	39.7±2.1	15	57.3±4.2	18	77.6±4.9
SC	16	51±1.6	14	68.5±3.7	14	79.4±1.9
KB	16	37.8±2.2	15	49±2.3	16	67.6±2.7
Total	**113**	**44.6±2.5**	**112**	**57.3±2.4 p<0.001**	**112**	**72±4.9 p<0.001**

Table 3. The effect of enoxaparine (En) injections for 1 or 14 days (40 mg daily) on the VEGF content of human serum (pg/ml)

	Before first dose of En		After first dose of En (before surgery)		After 14 injections	
Patient initials	**Number of tests**	**Mean**	**Number of tests**	**Mean**	**Number of tests**	**Mean**
MZ	3	203	3	162	3	364
SA	3	50	3	52	3	71
KA	3	146	3	119	3	251
JA	3	279	3	312	3	671
CM	3	230	3	264	3	494
SC	3	26	3	20	3	40
KB	3	66	3	70	3	141
Total	**21**	**142.8±30**	**21**	**142.7±35**	**21**	**290.3±60 p<0.05**

Table 4. The effect of enoxaparine (En) injections for 1 or 14 days (40 mg daily) on the bFGF content of human serum (pg/ml)

	Before first dose of En		After first dose of En (before surgery)		After 14 injections	
Patient initials	**Number of tests**	**Mean**	**Number of tests**	**Mean**	**Number of tests**	**Mean**
MZ	3	13.6	3	16	3	37
SA	3	1.6	3	5.5	3	6
KA	3	1.4	3	15.7	3	4.1
JA	3	4.7	3	39	3	2
CM	3	3.3	3	5.9	3	8
SC	3	1.4	3	10.5	3	8.8
KB	3	1.6	3	8.6	3	6.7
Total	**21**	**3.9±1.3**	**21**	**14.5±3.2 p<0.01**	**21**	**10.4±3.1 p<0.05**

various effects of the enoxaparine may be explained by the fact, that the heparin and low-molecular-weight heparins have polypharmacological actions at various levels. Besides their effects on the activity of angiogenic growth factors VEGF and bFGF, they modulate a tissue factor/VIIa noncoagulant activities, and inhibit the matrix-degrading enzymes [18, 19].

Conclusion

In the light of the results obtained, one may expect a beneficial effect of fourteen-days prophylaxis with enoxaparine on the healing process in patients undergoing orthopaedic surgery.

Experiment performed in the mice confirmed our previous report on different action of enoxaparine and nadroparine on angiogenesis.

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