

The effect of a single dose of enoxaparine on the angiogenic potential of human serum and plasma

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Abstract

Enoxaparine (En) – the low-molecular weight heparin (LMW) is routinely used as anti-coagulant in the thrombo-embolic prophylaxis prior to and following any surgical intervention. Our preliminary observations demonstrated that single enoxaparine dose administered prior to the hip surgery resulted in the increased angiogenic activity and basic fibroblast growth factor (bFGF) levels in patients' sera without any corresponding alterations in the vascular endothelial growth factor (VEGF) concentration.

The aim of the present study was to further analyze the mechanism of En proangiogenic effect. Plasma and serum samples have been collected from 12 patients prior to the hip surgery – before and 12 hours following single enoxaparine (40 mg) dose. In both materials serum and plasma, increased in vivo angiogenic activity has been demonstrated in the mouse cutaneous test following En administration. Also, plasminogen activator inhibitor-1 (PAI-1) levels increased while insulin-like growth factor-1 (IGF-1) concentrations decreased after single enoxaparine dose. No significant alterations in plasma VEGF, fibrinogen and antithrombin III levels were seen.

Key words: enoxaparine, angiogenesis, IGF-1, VEGF, PAI-1, fibrinogen, AT-III.

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Introduction

Angiogenesis is a complex process, affected by many diverse stimulatory or inhibitory factors. In response to a local pro-angiogenic stimuli activation of endothelial cells in the capillaries or postcapillary venules ensues, leading to dilatation and increased permeability of their walls. Afterwards, accumulation of extravasated fibrin in the extracellular matrix, proteolytic degradation of vessels' basement membrane and migration of endothelial cells towards angiogenic stimulus are following. Migrating cells are dividing, and this process supports elongation of a new vessel. Basement membrane and other elements are recreated in a later stage [1].

Angiogenesis is controlled by endogenous inhibitors, which are specific for individual stages of that process.

Considerable number of stimulators from the class of cytokines, chemokines and growth factors participate in this process as well. Also, the important role is played by proteolytic enzymes and various components of extracellular matrix interacting with activated endothelial cells through adhesion molecules, emerging on their cellular membranes. The central part in this process hold $\alpha_5\beta_3$ integrins, whose antagonists inhibit angiogenesis induced by bFGF (basic fibroblast growth factor), IGF-1 (insulin-like growth factor-1) and TNF-alpha (tumor necrosis factor) as well as $\alpha_5\beta_1$ integrins, whose antagonists inhibits angiogenesis induced by vascular endothelial growth factor VEGF [2, 3]. Recent data have also indicated that coagulation and fibrinolytic systems play important role in regulating the pro-/anti-angiogenic

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balance. Fibrin, cleaved from fibrinogen by thrombin, strongly stimulates capillary tube formation, while the cleaved form of antithrombin III (AT-III) has a potent anti-endothelial and anti-angiogenic activity [4]. Moreover, urinary – type plasminogen activator (urokinase, uPA) and its inhibitor plasminogen activator inhibitor-1 (PAI-1) interplay is critical for the endothelial cells proliferation, migration and sprouting, defining as a result the process of new vessels formation [5].

Folkman was the first to suggest in the 80', that heparins might play an important role in the angiogenesis [1]. However, despite considerable research their angiomodulatory activity remains a complex and controversial issue. Our previous studies demonstrated that low molecular weight (LMW) heparin enoxaparine (Clexane) administered for two consecutive days significantly increased the angiogenic activity of treated mice sera while nadroparine (Fraxiparine) had the opposite effect [2]. Similar results have been observed in mice treated with enoxaparine or nadroparine for 14 days. In addition, our preliminary observation in a group of seven patients proved that single dose of enoxaparine administered prior to the hip surgery resulted in the increased angiogenic activity and basic fibroblast growth factor (bFGF) level in patients' sera without any corresponding alterations in the vascular endothelial growth factor (VEGF) concentration. Consequently, the aim of present study was to confirm and further analyse the mechanism of En proangiogenic effect in humans. In particular, key growth factors (VEGF, IGF-1) as well as coagulation and fibrinolytic system markers (AT-III, fibrinogen, PAI-1) have been assessed. Additionally, in vivo angiogenic activity and IGF-1 content of matching sera and plasma patients' samples have been assessed.

Material and Methods

Patients

Twelve patients (aged 64-71 years, 9 women and 3 men) with diagnosed coxarthrosis, gonarthrosis and avascular necrosis of the femoral head, hospitalised due to the planned surgery, were included into the study group. Plasma (EDTA) and serum samples were collected, isolated by centrifugation, aliquoted and frozen at -70°C for further experiments. Blood samples were collected twice – immediately before enoxaparine (Clexane, Sanofi-Aventis, 40 mg) subcutaneous injection, and subsequently – 12 hours later.

All experiments were approved by the Local Ethical Committee.

Methods

Serum-induced angiogenesis test (SIA)

SIA tests were performed on 8-weeks old inbred Balb/c mice, females, about 20 g of body mass, delivered from Polish Academy of Sciences breeding colony.

Angiogenic activity of patients plasma and sera samples was evaluated in the mice cutaneous assay according to

Skopiński et al. [6] and Barcz et al. [7], as described before. Briefly, multiple 0.05 ml samples of sera or plasma were injected intradermally into partly shaved, narcotised Balb/c mice. At least 3 mice for one plasma or serum sample were used. In order to facilitate the further localisation of injection sites, each sample was dyed with 0.1% of trypan blue. After 72 hours mice were sacrificed with lethal dose of Morbital (Biowet, Poland). All newly formed blood vessels were identified and counted in dissection microscope, on the inner skin surface, at a magnification of 6 \times , 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, were thin and 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). 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.

Growth factors and PAI-1 measurement

VEGF, IGF-1 and PAI levels were determined in examined materials by sandwich ELISA method using commercially available kits for human VEGF, IGF-1 (R&D Systems, USA) and PAI-1 (American Diagnostica, USA). Measurement was performed according to the manufacturer's instructions. Optical density was measured at 450 nm in spectrophotometric reader Elx800 (Biotek Instruments, Inc., USA). Cytokines concentration was expressed as pg/ml.

AT-III measurement

AT-III levels in plasma were assessed by the kinetic method using commercially available reagents (Dade Behring Marburg, Germany) according to manufacturer's instructions. Results were expressed as % of reference normal value.

Fibrinogen measurement

Fibrinogen concentration in plasma was assessed by the modification of the Claus method using commercially available reagents (Dade Behring Marburg, Germany) according to manufacturer's instructions. Results were expressed as g/l.

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

Results

Significant stimulation of the angiogenic activity after single subcutaneous dose (40 mg) of enoxaparine has been observed, both in plasma and serum evaluated in the SIA test. Moreover, the up-regulation of new blood vessels formation induced by serum was significantly stronger in comparison to plasma (Table 1).

The plasma concentration of VEGF, AT-III and fibrinogen were not affected by the single dose of En, while PAI-1

Table 1. The effect of a single enoxaparine (En) dose (40 mg s.c.) on the angiogenic activity of serum and plasma in the group of 12 patients prior to the orthopaedic surgery

	Number of SIA tests	Mean number of blood vessels $\bar{x} \pm SE$		Statistical significance of differences
		before treatment	after treatment	
Serum	381	43.3 \pm 0.61	57.6 \pm 0.87	p<0.001
Plasma	70	14.8 \pm 0.3	19.2 \pm 0.8	p<0.01
Statistical significance of differences		P<0.001	P<0.001	

Table 2. The effect of a single enoxaparine (En) dose (40 mg s.c.) on the pro- and anti-angiogenic factors concentration in serum and plasma of 12 patients prior to the orthopaedic surgery

	Before treatment $\bar{x} \pm SE$ (N)	After treatment $\bar{x} \pm SE$ (N)	Statistical significance of differences
IGF-1 (serum, pg/ml)	76.6 \pm 8.4 (24)	57.3 \pm 6.5 (24)	P<0.05 ↓
VEGF (plasma, pg/ml)	10.1 \pm 0.4 (24)	11.4 \pm 0.8 (24)	n.s.
Fibrinogen (plasma, g/l)	402.4 \pm 18 (64)	403.4 \pm 21 (64)	n.s.
PAI-1 (plasma, pg/ml)	38.6 \pm 5.1 (24)	52.2 \pm 3 (24)	P<0.05 ↑
AT-III (plasma, %)	98.7 \pm 2.5 (12)	93.4 \pm 2.7 (12)	n.s.

N – number of measurements, n.s. – not significant.

significantly increased in examined subjects. Simultaneously, serum levels of IGF-1 considerably decreased after the single dose of the En (Table 2).

Also, while prior to the En injection the serum angiogenic activity weakly correlated to the fibrinogen level ($r=0.4675$, $P<0.1$ on the border of statistical significance) and PAI-1 levels negatively correlated to the AT-III in plasma ($r=-0.49$, $P<0.1$ on the border of statistical significance) these relationships has not been further observed after a single enoxaparine dose (40 mg).

Discussion

Present study confirms the considerable effect exerted by the low-molecular-weight heparin (LMWH), enoxaparine, on the formation of new blood vessels (angiogenesis). We have clearly demonstrated that single dose of En resulted in the direct up-regulation of serum/plasma angiogenic activity. These data correspond very well with the considerable proangiogenic effect of En observed by Norman et al. simultaneously with amplification of other bone reparative processes [8]. Moreover, in patients with ischemic heart disease one dose of heparin resulted in the augmented serum bFGF concentration [9].

However, apart from the above data on the En pro-angiogenic activity, there is number of publications proving its opposite effect [10-12]. Several *in vitro* studies showed significant anti-angiogenic effects of En and other LMW heparins. Also, recent meta-analyses of clinical trials

demonstrated an improved survival of cancer patients receiving LMWHs and implied their significant influence on tumor angiogenesis and metastasis formation [13]. It has been suggested that potential anticancer activities of En and LMW heparins might be mediated, at least in part, by their anti-coagulant effect (for example: by release of Tissue Factor Pathway Inhibitor (TFPI) and alterations in the fibrin structure), but also by the non-anticoagulant effects, including interaction with growth factors/growth factors receptors as well as endothelial cells and extracellular matrix [14].

Therefore, in order to analyse the mechanism of observed En proangiogenic activity, key growth factors (VEGF, IGF-1) as well as coagulation and fibrinolytic system markers (AT-III, fibrinogen, PAI-1) have been assessed in present study. Preliminary experiments had demonstrated considerable increase in the serum bFGF level and no alteration in the VEGF concentration following the single dose of En. Consequently, evaluation of IGF-1 – proangiogenic growth factor acting similarly to bFGF via the $\alpha_5\beta_1$ integrins has been performed and eventually significant decrease in its serum concentration shown. The biological basis of this phenomenon is not clear and no correlation was observed between serum angiogenic potential and IGF-1 levels. However, it should be pointed out that heparin as well as LMW heparins inflict considerable effect on IGF-1 functional activity changing its affinity to the IGF-binding proteins (BP) and dissociating IGF-1-BP complexes [15]. Importance of this phenomenon has been elegantly described by Mnich

et al. who demonstrated that in healthy rats heparin caused release of IGF-1 from the serum complexes with high molecular weight BP, making it therefore available for LMW BP known as the inhibitor of IGF-1 dependent functions. Parallely, the same experimental set-up in diabetic rats resulted in opposite – extensive down-regulation of the IGF-1 affinity to the – LMW BP with subsequent up-regulation of its biological activity. For certain, IGF-1 interaction with heparins is extremely complex and to considerable extent unclear. But to make reported results even more difficult to explain, it should be mentioned that IGF-1-BPs have been reported to bind to and inactivate PAI-1 [16]. Therefore, it might be speculated that while in general increased PAI-1 concentrations would inflict potent anti-angiogenic effect, in this particular setting with elevated number of BPs (from heparin disrupted IGF-1-BP complexes), PAI-1 might become inactivated and therefore achieve pro-angiogenic potential. That is in accordance with strong data proving the opposite effects of active and non-active PAI-1 molecules on the new blood vessels formation, respectively anti- and pro angiogenic [5].

Conclusion

Reported pro-angiogenic effect of a single En dose is in need of further analysis to explain its exact mechanism. However, it should be also extensively examined for its clinical significance, as the postoperative wound healing depends greatly on the angiogenesis.

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