

PAI-1, fibrinogen, AT-III and angiogenic activity of plasma in patients before and 14 days after orthopaedic surgery and 14 daily enoxaparine injections

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Abstract

We previously reported that single enoxaparine dose administered prior to the hip surgery resulted in an increase of angiogenic activity and basic fibroblast growth factor (bFGF) level in patients' sera without any corresponding alterations in the vascular endothelial growth factor (VEGF) concentration. Increased VEGF serum level, however, was observed in sera after 14 enoxaparine injections. Plasminogen activator inhibitor-1 (PAI-1) levels increased after single enoxaparine dose, and no significant alterations in plasma VEGF, fibrinogen and antithrombin III (AT-III) levels were seen. In the present paper we have study the same parameters in plasma of patients treated with enoxaparine for 2 weeks after surgery.

Significant increase of plasma PAI-1 and fibrinogen concentration, as well as angiogenic plasma activity *in vivo* in PIA mice cutaneous test, were observed. No alterations in plasma VEGF and AT-III were seen.

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

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Introduction

Angiogenesis is the formation of new blood vessels from the existing vasculature. It is a multistage process in which activated endothelial cells (EC) degrade basement membrane and the fibrin or interstitial matrix, migrate, proliferate, and form new capillary tubes as well as a new basement membrane [1]. The process of angiogenesis relies on a coordinated expression of pro- and anti-angiogenic factors, released by various cells and tissues, which in normal physiological conditions are maintained in balance. A loss of equilibrium leads to generation or inhibition of neovascularisation that might be of utmost significance for the development and course of many diseases [2].

The key molecules in the *in vivo* control of angiogenesis, vasodilation and increased microvascular permeability, are growth factors vascular endothelial (VEGFs) [3]. However, the coagulation and fibrinolytic systems also play important role in regulating the pro-/anti-angiogenic balance. Fibrin, effectively cleaved from fibrinogen by thrombin, stimulates capillary tube formation, while the active, cleaved form of antithrombin III (AT-III) – holds distinct anti-endothelial and anti-angiogenic activity [4]. Also, the urokinase-type plasminogen activator (uPA) and its inhibitor, the plasminogen activator inhibitor-1 (PAI-1)

are involved in the regulation of angiogenesis, primarily endothelial cells proliferation, migration and new vessels sprouting [5].

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Folkman was the first to suggest, that heparins might play an important role in the angiogenesis processes [6]. Our previous studies demonstrated that administration of low-molecular weight heparins (LMWHs) enoxaparine and nadroparine (fraxiparine) exerted opposite effects on the angiogenic activity of mice sera [7]. We also observed increase of human serum angiogenic activity in mouse cutaneous test (serum induced angiogenesis, SIA) and serum VEGF concentration after 14 enoxaparine doses administered after orthopaedic surgery. Therefore, the aim of present study was to further analyse the mechanism of enoxaparine proangiogenic effect in humans focusing mostly on the plasma elements of the coagulation and fibrinolytic systems AT-III, fibrinogen and PAI-1 as well as key growth factor – VEGF.

Material and Methods

Patients

12 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) samples were collected, isolated by centrifugation, aliquoted and frozen at -70°C for further experiments. Blood samples were collected twice – immediately before enoxaparine (En) (Clexane, Sanofi-Aventis, 40 mg) subcutaneous injection, and subsequently – at the 14th day after surgery and daily treatment with En (40 mg in subcutaneous daily injections).

All experiments were approved by the Local Ethical Committee.

Plasma-induced angiogenesis test (PIA)

PIA test was performed in mice according to Skopiński et al. [8] and Barcz et al. [9], as described before for serum-induced angiogenesis (SIA) test. 8-weeks old inbred Balb/c mice, females, about 20 g of body mass were delivered from Polish Academy of Sciences breeding colony.

Briefly, multiple 0.05 ml samples of the plasma were injected intradermally into partly shaved, narcotized mice. At least 3 mice for one plasma sample were used. In order to facilitate the localization of injection sites later on, each plasma 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 the 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.

Measurement of VEGF and PAI-1 concentrations

VEGF and PAI levels were determined in examined materials by sandwich ELISA method using commercially available kits for human VEGF (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 Infinite M200 (Tecan, Austria). Cytokines concentration was assessed in pg/ml.

AT-III and fibrinogen 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 concentration in plasma was assessed in g/l by the modification of the Claus method using commercially available reagents (Dade Behring Marburg, Germany) according to manufacturer's instructions.

For some statistical comparisons the results were also expressed as the stimulation index calculated as a ratio of the value obtained after 14 days to the value obtained before treatment.

Data analysis

Statistical analysis was performed using Student-*t* test and ANOVA (software system data analysis Statistica PL version 5, Statsoft Inc. 1997).

Results

Significant increase in proangiogenic plasma activity following surgery and En treatment was observed in the PIA test (Fig. 1 and Table 1). The plasma concentration of PAI-1 and fibrinogen also significantly increased, while AT-III and VEGF levels were not significantly changed in examined subjects after 14 days standard therapy with En (Fig. 2 and Table 1).

Discussion

Angiogenic processes are essential in hard and soft tissues repair. During the wound healing proangiogenic factors are released and found in circulating blood. Meanwhile, low-molecular weight heparins (LMWH) are routinely used as anti-coagulants for prophylaxis after surgery. Their modulatory effect on the regenerative processes as well as angiogenesis is not well known or understood.

Present study reconfirmed the regulatory effect exerted by the LMWH enoxaparine (En) on the new blood vessels

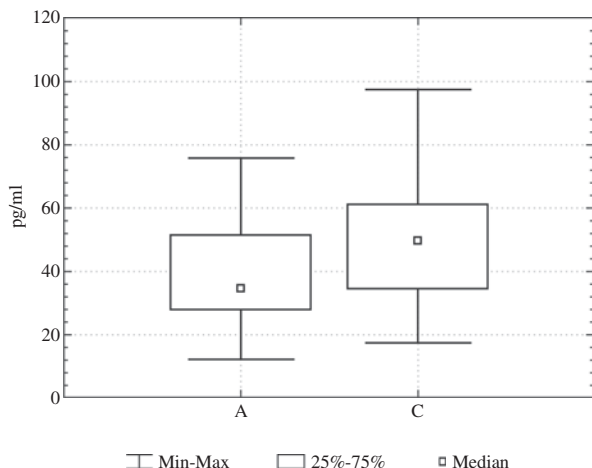


Fig. 1. PAI-1 concentration in plasma of patients before (A) and 14 days after (C) the surgery and daily enoxaparine treatment

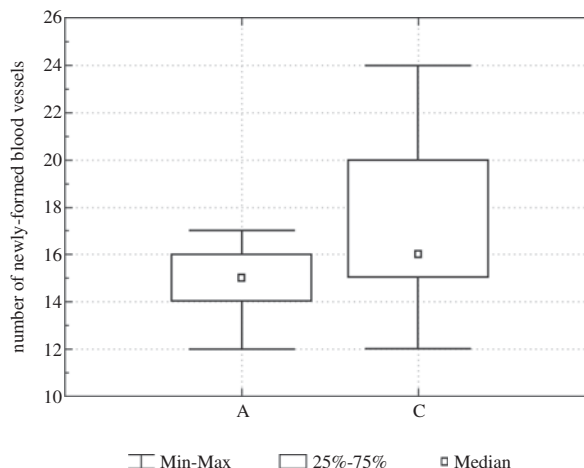


Fig. 2. Angiogenic activity of plasma collected from patients before (A) and 14 days after (C) the surgery and daily enoxaparine treatment

Table 1. The effect of enoxaparine administered to 12 patients for 14 days on the angiogenic activity of their plasma

	Before treatment (mean I.S. ± SE)	After treatment (mean I.S. ± SE)	Statistical significance of differences
PAI-1	1±0.12	1.40±0.13	p<0.05
Fibrinogen	1±0.04	1.29±0.05	p<0.001
AT-III	1±0.02	0.98±0.03	n.s.
VEGF	1±0.04	1.05±0.06	n.s.
In vivo PIA test (n=72)	1±0.02	1.23±0.05	p<0.001

I.S. – index of stimulation; n.s. – not significant

formation in the in vivo PIA assay. We have clearly demonstrated that 14 days treatment with enoxaparine (En) resulted in the direct up-regulation of serum proangiogenic activity. These data correspond with the considerable proangiogenic effect of En observed by Norman et al. that was simultaneous with amplification of other bone reparative processes [10]. It should be however mentioned that several authors observed contradictory results, demonstrating antiangiogenic effects of En in endothelial tube formation assay as well as FGF-2 and VEGF¹⁶⁵ inhibition [11-13].

In the present study, key growth factor (VEGF) as well as coagulation and fibrinolytic system markers (AT-III, fibrinogen, PAI-1) have been also analyzed. Plasma concentration of VEGF and AT-III were not affected by 14 days treatment with En, while PAI-1 and fibrinogen significantly increased in examined subjects.

Quite divergent outcomes of En treatment on VEGF serum levels have been observed, both up-regulation of its serum levels after 14 enoxaparine doses as well as no effect

following the single En dose. This suggests that enoxaparine have up-regulated intracellular pool of VEGF, released from activated granulocytes and platelets during separation of sera by coagulation, and not released from the blood cells in EDTA samples.

Similarly to prior results of one enoxaparine dose, AT III serum concentration has not been affected [14, 15].

On the other hand, significant increase of fibrinogen – the key element of the coagulation system and angiogenesis mechanism has been observed in our study. That goes along with phenomenon demonstrated by Salih et al. who has shown the significant strong correlation between fibrinogen and angiogenic activity [16].

Parallely to fibrinogen, PAI-1 plasma concentration significantly increased after En 14-day treatment. Again, data concerning En-PAI-1 interaction are quite divergent. Gori et al. observed no effect of single-dose En treatment, while Shafiq et al significant suppression of PAI-1 synthesis following 30 days of standard En treatment [17, 18].

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