Vascular endothelial growth factor (VEGF) in circulating blood of patients treated with enoxaparine after orthopaedic surgery

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Abstract

VEGF, a potent mitogen for endothelial cells, is one of the most important cytokines responsible for stimulating angiogenesis. Some data confirm an important role of VEGF in wound and bone healing. In the blood, VEGF is present in plasma and, as important intracellular pool, in neutrophils and platelets. Intracellular VEGF is released to the serum upon these cells activation during coagulation.

Low-molecular weight heparins (LMWHs) are routinely used as anti-coagulants for prophylaxis after bone surgery. In the previous study, performed in seven patients after orthopedic surgery, we have found that enoxaparine injections administered during post-operative two weeks, have increased angiogenic activity and VEGF level in their sera. To elucidate the origin of this cytokine, in the present study we compared VEGF level in matched plasma and serum samples collected from 12 persons before, and 14 days after surgery and daily enoxaparine (40 mg) injections. The results were also compared to the findings obtained for sera collected from 30 healthy blood donors.

Both serum and plasma samples collected after enoxaparine treatment presented higher in vivo angiogenic activity in mouse cutaneous test than before administration of this drug.

No differences in plasma VEGF were observed. However, VEGF concentration in matched sera samples was significantly higher after surgery and enoxaparine treatment than before the beginning of the study.

Conclusion: Intracellular VEGF, released during coagulation process plays an important role in vivo angiogenic activity of serum, and its concentration significantly increased after 2-weeks enoxaparine treatment.

Key words: VEGF, angiogenesis, orthopedic patients, enoxaparine.
glycoprotein, responsible for stimulating angiogenesis during development of embryo, as well as during tumor formation, growth and metastasis. VEGF serves as an endothelial cell survival factor, protecting endothelial cells against apoptosis, and it delays and may reverse endothelial cell senescence. There are data that confirm an important role of VEGF in wound and bone healing [2-10].

Members of VEGF family are the multifunctional cytokines that exert many effects on vascular endothelium and critically influence the angiogenic response. These include striking changes in cell morphology and cytoskeleton accompanied by stimulation of endothelial cell migration and proliferation. First and most distinctive biologic activity of VEGF is the increase of microvascular permeability. It results in leakage of plasma proteins, including fibrinogen and other clotting proteins. The clotting system is rapidly activated by means of the tissue factor pathways. VEGF is selective for vascular endothelium because its major tyrosine kinase receptors are mainly expressed on vascular endothelium. After binding to its receptors, VEGF initiates a cascade of signaling events that begins with autophosphorylation of receptor tyrosine kinases, followed by activation of numerous downstream proteins. There are 3 major transmembranous tyrosine kinase receptors for VEGF: VEGFR-1 (Flt-1), VEGFR-2 (flt-1/KDR) and VEGFR-3. Flt-1 and flk-1/KDR bind to all secreted VEGF-isoforms. This binding induces autophosphorylation and signal transduction. Endothelial cells express also neuropilin co-receptors, which bind selectively to the 165 aminoacid form of VEGF [11].

At a molecular level, VEGF reprograms endothelial cell gene expression, leading to increased expression of a number of different proteins, including the procoagulant tissue factor, proteins associated with the fibrinolytic pathway (urokinase, tissue-type plasminogen activator, type 1 plasminogen activator inhibitor, urokinase receptor), matrix metalloproteases, nitric oxide synthase, numerous mitogens, and a number of antiapoptotic factors. Degradation of heparan sulfate (HS) in the extracellular matrix (ECM) and on the cell surface by enzymes releases different polypeptides bound to HS and enables cellular invasion. Activation of mediators released from ECM and different cells like platelets, fibroblasts, macrophages, leukocytes, and others, promoting EC-receptors binding and intracellular signaling, leads to activation, proliferation and migration of endothelial cells (EC). Next step is tube and loop formation and ECM remodeling. Process is finalized by stabilization of newly-formed blood vessels [12, 13].

In our previous papers we have shown that enoxaparine (low-molecular weight heparin) enhanced in vivo angiogenic activity of mouse and human serum, evaluated by murine skin test, in comparison to the control. On the other hand, sera collected from nadroparine (fraxiparine) treated mice presented significantly lower neovascular reaction than the reaction observed in the skin of the recipients of control sera [14-16].

In the present study we compared angiogenic activity and VEGF content of plasma and sera of 12 patients undergoing orthopedic surgery, before and after 14 daily enoxaparine injections.

**Material and methods**

**Patients:** We collected blood samples (with or without EDTA) from 12 patients, aged 64-71 years, with coxarthrosis, gonarthrosis, avascular necrosis of the femoral head and similar others. Plasma and sera samples were separated and frozen at –70°C until testing. Blood samples were collected for the first time immediately before beginning of enoxaparine (En) treatment, and for the second time 14 days after the beginning of treatment (40 mg of En subcutaneous daily injections). We also collected, as a control, sera from 30 healthy people, aged 40-83 years.

**Angiogenic activity measurement of patients sera** was done by cutaneous test performed in mice (serum-induced angiogenesis, SIA test) according to Barcz et al. [17]. Briefly, multiple 0.05 ml of the sera and plasma samples were injected intradermally into partly shaved, narcotized, 8 weeks old Balb/c mice. At least 3 mice for one tested material were used. In order to facilitate the localisation of injection sites later on, each serum sample was colored 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 6 x, 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, are 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), and approved by Local Ethical Committee for Animal Experiments.

**Measurement of VEGF concentration:** Cytokine levels were determined in examined plasma and sera using sandwich ELISA kits (R&D Systems, USA) for human VEGF, 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 for human studies.

**Results**

Initial angiogenic activity of sera from the patients was similar to the angiogenic activity of sera collected from control people. Significant increase of neovascular response was observed when sera from enoxaparine-treated patients are injected into mice skin (figure 1).
Both serum and plasma samples collected after enoxaparine treatment presented higher in vivo angiogenic activity in mouse cutaneous test than before administration of this drug. However, the reaction induced by sera was significantly higher than that induced by plasma samples (table 1). Initial VEGF concentration in patients sera was significantly lower than that of the control group and have increased after enoxaparine treatment (figure 2).

Table 1. The effect of fourteen daily enoxaparine (En) injections on the angiogenic activity of serum and plasma collected from 12 patients

<table>
<thead>
<tr>
<th>Number of SIA tests</th>
<th>Mean number of blood vessels ±SE</th>
<th>Statistical significance of differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>before En</td>
<td>after En</td>
<td></td>
</tr>
<tr>
<td>serum 383</td>
<td>43.3±0.61</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>plasma 72</td>
<td>14.8±0.36</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>statistical significance of differences</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

Table 2. The effect of fourteen daily enoxaparine (En) injections on VEGF levels in serum and plasma collected from 12 patients. Number of examinations in parentheses

<table>
<thead>
<tr>
<th>VEGF (serum, pg/ml)</th>
<th>Before En</th>
<th>After En</th>
<th>Statistical significance of differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>112.2±16 (24)</td>
<td>205.3±38 (24)</td>
<td>P&lt;0.05</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>VEGF (plasma, pg/ml)</th>
<th>Before En</th>
<th>After En</th>
<th>Statistical significance of differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.1±0.4 (24)</td>
<td>10.6±0.6 (24)</td>
<td>n.s.</td>
<td></td>
</tr>
</tbody>
</table>

was significantly higher after surgery and enoxaparine treatment than before the beginning of the study (table 2).

Discussion

Our present study revealed that VEGF in sera of our patients was significantly lower than in the controls. However, angiogenic activity of the sera was similar to that observed in the control group. This suggests, that in vivo angiogenic activity of human serum in mouse cutaneous test (SIA) is dependent on some other factors also. In fact, we have shown important role of bFGF in this test [18]. Sera of our patients contained amounts of bFGF comparable to the concentrations present in the sera of age-matched control group [16, 19], and significant increase of bFGF amount was observed after one and fourteen enoxaparine 40 mg daily doses. Paralelly, we have shown that enoxaparine enhanced angiogenic activity of mouse and human serum [14-16]. However, enoxaparine may also behave as inhibitor of angiogenesis, what we have shown after preincubation of murine L-1 sarcoma cells with enoxaparine, or during administration of drug to L-1 sarcoma cells recipients [20, 21]. These opposite action of
heparins, used in different experimental models of angiogenesis may perhaps be explained by the fact that heparin and LMWHs may exert polypharmacological actions on each level of this cascade process, and they represent complex natural mucopolysaccharide drugs that have undergone different chemical and enzymatic modifications [22-24].

We have also compared the effect of different LMWHs – enoxaparin (Clexane) and nadroparin (Fraxiparine) which were administrated for 2 or 14 days to mice, on angiogenic activity of their sera. We have found that enoxaparin stimulates angiogenic activity of sera and nadroparin inhibits such activity [14-16].

One may suppose, that enoxaparine treatment would positively influence bone healing after surgery, what was experimentally confirmed in rats [25]. However, some experimental and clinical studies revealed that longer treatment with heparin or nadroparin of rats (4 weeks) caused disorder of bone formation and intensification of bone resorption [26], and long-term exposure (1 year) to enoxaparin caused the decrease in bone mineral density in humans [27].

In the present paper we have observed higher in vivo angiogenic activity and higher VEGF levels in serum samples than in matched plasma samples. Dependence of VEGF concentration on blood collection procedures was described. Blood coagulation initiates events leading to the activation of platelets and granulocytes and to the release intracellular pool of VEGF [28-32]. Isolated neutrophils contain considerable amounts of VEGF. It was also reported that the number of platelets is significantly correlated to VEGF concentration in serum, but not to the VEGF concentration in plasma. Some authors demonstrated that higher VEGF concentration in serum samples than in matched plasma samples was due to the presence of VEGF within platelets and its release upon their activation during coagulation [33-36].

The results of our present study suggest that enoxaparin treatment increased intracellular pool of VEGF in blood leukocytes and (or) platelets. We feel, that this increase in VEGF content and angiogenic activity of serum is mainly connected with enoxaparin treatment, and only partly dependent on the effect of surgery itself, because we have obtained similar findings in mice treated with 14 doses of enoxaparin and not subjected to surgical procedures [16].

Perhaps, the influence of heparins on the process of angiogenesis may be connected with their affinity to VEGF. The several VEGF isoforms encoded by alternative splicing have distinct physical properties. VEGF 121 is freely soluble and does not bind heparin. By contrast, isoforms 165 and 189 have increasing basic charge and bind heparin with increasing affinity; in fact, VEGF 165 was originally purified on the basis of its affinity for heparin. Heparin, heparan sulfate, and angiogenesis may be connected with their affinity to VEGF. Therefore, in this and likely other instances, proteases are required to push the “angiogenic switch”.

**Conclusion:** Intracellular VEGF, released during coagulation process plays an important role in in vivo angiogenic activity of serum, and its concentration significantly increased after 2-weeks enoxaparin administration. Short-term enoxaparine treatment after surgery could improve the healing process.

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**References**


