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

LESZEK JUNG [1], BARBARA J. BAŁAN [2], EWA SKOPIŃSKA-RÓŻEWSKA [2], JOANNA CHOROSTOWSKA-WYNIMKO [3], ANDRZEJ K. SIWICKI [4], EWA SOMMER [2], ADRIANA ROŻY [3], PIOTR SKOPIŃSKI [5]

[1]Orthopaedic Department, Institute of Rheumatology, Warsaw, Poland; [2]Pathology Department, Biostructure Center, Medical University, Warsaw, Poland; [3]Department of Molecular Diagnostics, National Institute of Tuberculosis and Lung Diseases, Warsaw, Poland; [4]Department of Microbiology and Clinical Immunology, Warmian and Mazurian Uniwersity, Olsztyn, Poland; [5]Department of Histology and Embryology, Biostructure Center, Medical University, Warsaw, Poland

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 in vivo angiogenic activity of serum, and its concentration significantly increased after 2-weeks enoxaparine treatment.*

***Key words:** VEGF, angiogenesis, orthopedic patients, enoxaparine.*

(Centr Eur J Immunol 2007; 32 (2): 61-65)

Introduction

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. However, a loss of the balance leads to generation or inhibition of neovascularisation, and this may be of utmost significance for the development and course of many diseases like psoriasis, age-related macular degeneration (AMD) and diabetic retinopathy, collagenoses, neoplasia or other illnesses connected with angiopathology. On the other hand lack of angiogenesis may be the reason of heart and other organs ischaemic diseases, or may result in disturbance of wound and bone healing [1].

VEGF, a potent and specific mitogen for endothelial cells in vessels, is one of the most important secreted

Correspondence: Ewa Skopińska-Różewska, Department of Pathology, Biostructure Center, Medical University of Warsaw, Chałubińskiego 5, 02-004 Warsaw, Poland. Phone/fax number: +48 22 629 98 92, Email: ewaskop@hotmail.com

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 (flk-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 sultate (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).

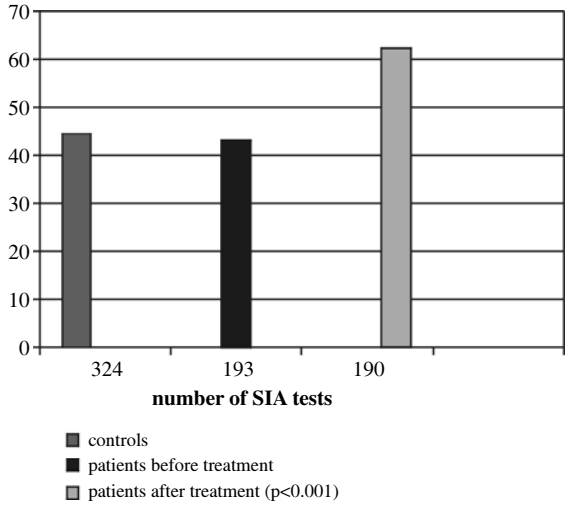

Fig. 1. Mean number of newly-formed blood vessels in *in vivo* SIA test

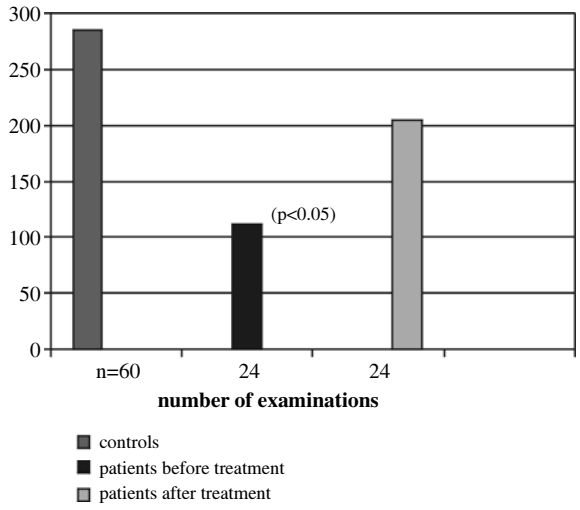

Fig. 2. Serum VEGF concentration (pg/ml). N – number of examinations

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).

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 (table 2).

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

	Number of SIA tests	Mean number of blood vessels ±SE		Statistical significance of differences
		before En	after En	
serum	383	43.3±0.61	62.3±0.92	P<0.001
plasma	72	14.8±0.36	17.2±0.78	P<0.01
statistical significance of differences		P<0.001	P<0.001	

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

	Before En	After En	Statistical significance of differences
VEGF (serum, pg/ml)	112.2±16 (24)	205.3±38 (24)	P<0.05
VEGF (plasma, pg/ml)	10.1±0.4 (24)	10.6±0.6 (24)	n.s.
statistical significance of differences	P<0.001	P<0.001	

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 – enoxaparine (Clexane) and nadroparine (Fraxiparine) which were administrated for 2 or 14 days to mice, on angiogenic activity of their sera. We have found that enoxaparine stimulates angiogenic activity of sera and nadroparine 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 nadroparine of rats (4 weeks) caused disorder of bone formation and intensification of bone resorption [26], and long-term exposure (1 year) to enoxaparine 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 enoxaparine 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 heparinase all displace the larger VEGF isoforms from proteoglycan-binding sites; plasmin cleavage also activates these isoforms by freeing them from cells or matrix. Despite these physical differences, the several VEGF isoforms apparently have identical biologic activities when free in solution. Angiogenesis is initiated only at a stage when cells express metalloproteases that liberate VEGF from matrix. 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 enoxaparine administration. Short-term enoxaparine treatment after surgery could improve the healing process.

Acknowledgment

This work was partly supported by Polish Scientific Committee, grant 3PO5B17223.

References

1. Skopińska-Różewska E, Skopiński P (2001): Modulation of angiogenesis-new field of clinical immunology. Terapia 9, 3 (2): 3-6.
2. Colwell AS, Beanes SR, Soo C, et al. (2005): Increased angiogenesis and expression of vascular endothelial growth factor during scarless repair. Plast Reconstr Surg 115: 204-212.
3. Eckardt H, Ding M, Lind M, et al. (2005): Recombinant human vascular endothelial growth factor enhances bone healing in an experimental nonunion model. J Bone Joint Surg Br 87: 1434-1438.
4. Geiger F, Bertram H, Berger I, et al. (2005): Vascular endothelial growth factor gene-activated matrix (VEGF165-GAM) enhances osteogenesis and angiogenesis in large segmental bone defects. J Bone Miner Res 20: 2028-2035.
5. Karayiannakis AJ, Zbar A, Polychronidis A, Simopoulos C (2003): Serum and drainage fluid vascular endothelial growth factor levels in early surgical wounds. Eur Surg Res 35: 492-496.
6. Hu J, Zou S, Li J, et al. (2003): Temporospatial expression of vascular endothelial growth factor and basic fibroblast growth factor during mandibular distraction osteogenesis. J Craniomaxillofac Surg 31: 238-243.
7. Knabe C, Nicklin S, Yu Y, et al. (2005): Growth factor expression following clinical mandibular distraction osteogenesis in humans and its comparison with existing animal studies. J Craniomaxillofac Surg 33: 361-369.
8. Eppley BL, Woodell JE, Higgins J (2004): Platelet quantification and growth factor analysis from platelet-rich plasma: implications for wound healing. Plast Reconstr Surg 114: 1502-1508.
9. Anitua E, Andia I, Ardanza B, et al. (2004): Autologous platelets as a source of proteins for healing and tissue regeneration. Thromb Haemost 91: 4-15.
10. Appleton I (2003): Wound healing: future directions. IDrugs 6: 1067-1072.
11. eufeld G, Cohen T, Gengrinovitch S, Poltorak Z (1999): Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 13: 9-22.
12. Quinn TP, Peters KG, De Vries C, et al. (1993): Fetal liver kinase 1 is a receptor for VEGF and is selectively expressed in vascular endothelium. Proc Natl Acad Sci USA 90: 7533-7537.
13. Arisato T, Hashiguchi T, Sarker KP, et al. (2003): Highly accumulated platelet VEGF in coagulant thrombotic region. J Thromb Haemost 12: 2589-2593.

14. Jung L, Siwicki AK, Skopińska-Różewska E, et al. (2005): Enoxaparine increases and fraxiparine decreases angiogenic activity of murine serum independently of the strain of mice. Pol J Environm Studies 14 (Suppl 2): 564-568.
15. Skopińska-Różewska E, Jung L, Skopiński P, et al. (2005): Various effects of low-molecular-weight heparins on serum angiogenic activity and tumor-induced angiogenesis in mice. Int J Molec Med 16 (Suppl 1): S27.
16. Jung L, Skopińska-Różewska E, Małdyk P, et al. (2006): Enoxaparine treatment enhanced angiogenic activity of mouse and human serum. Centr Eur J Immunol 31: 6-10.
17. Barcz E, Skopińska-Różewska E, Kamiński P, et al. (2002): Angiogenic activity and IL-18 concentration in peritoneal fluid and sera in endometriosis. Int J Gynaecol Obstet 79: 229-235.
18. Skopińska-Różewska E, Chorostowska-Wynimko J, Skopiński P, et al. (2007): Important role of bFGF in the angiogenic activity of human serum evaluated by the mouse cutaneous test. Centr Eur J Immunol 32: 45-47.
19. Skopiński P: Poszukiwanie źródeł aktywności pro- i anty-angiogennej w surowicach osób chorych na cukrzycę powikłaną retinopatia prostą. In: Naturalne i syntetyczne modulatory odpowiedzi immunologicznej i angiogenezy. Ed. AK Siwicki, E Skopińska-Różewska. SPW EDYCJA, Olsztyn 2007, 261-279.
20. Wasiutyński A, Skopińska-Różewska E, Jung L, et al. (2006): Comparison of the effects of enoxaparin and nadroparin on tumor angiogenesis in mice. Centr Eur J Immunol 31: 70-74.
21. Skopińska-Różewska E, Sommer E, Bany J (2006): In vivo i in vitro wpływ enoksaparyny i nadroparyny na wybrane parametry aktywności komórek izolowanych z mysiego mięsaka L-1. In: Naturalne i syntetyczne modulatory odpowiedzi immunologicznej i angiogenezy. Ed. AK Siwicki, E Skopińska-Różewska. SPW EDYCJA. Olsztyn. 2007, 281-291.
22. Fareed J, Leong WL, Hoppensteadt DA, et al. (2004): Generic low-molecular weight heparins – some practical considerations. Semin Thromb Hemost 30: 703-713.
23. Folkman J, Shing Y (1992): Control of angiogenesis by heparin and other sulfated polysaccharides. Adv Exp Med Biol 313: 355-364.
24. Iozzo RV, San Antonio JD (2001): Heparan sulfate proteoglycans: heavy hitters in the angiogenesis arena. J Clin Invest 108: 349-355.
25. Norman D, Miller Y, Sabo E, et al. (2002): The effects of enoxaparin on the reparative processes in experimental osteonecrosis of femoral head of the rat. APMIS 110: 221-228.
26. Folwarczna J, Janiec W, Barej M, et al. (2004): Effects of nadroparin on bone histomorphometric parameters in rats. Pol J Pharmacol 56: 337-343.
27. Wawrzyńska L, Tomkowski WZ, Przedlacki J, et al. (2003): Changes in bone density during long-term administration of low--molecular-weight heparins or acenocoumarol for secondary prophylaxis of venous thromboembolism. Pathophysiol Haemost Thromb 33: 64-67.
28. Larsson A, Skoldenberg E, Ericson H (2002): Serum and plasma levels of FGF-2 and VEGF in healthy blood donors. Angiogenesis 5: 107-110.
29. Dittadi R, Meo S, Fabris F, et al. (2001): Validation of blood collection procedures for the determination of circulating vascular endothelial growth factor (VEGF) in different blood compartments. Int J Biol Markers 16: 87-96.
30. Fischer JE, Janousek M, Fischer M, et al. (1998): Effect of collection and preprocessing methods of neutrophil elastase plasma concentrations. Clin Biochem 31: 131-136.
31. Gaudry M, Bregerie O, Audrien V, et al. (1997): Intracellular pool of vascular endothelial growth factor in human neutrophils. Blood 90: 4153-4161.
32. Gerasimov IG, Ignatov DY (2005): Blood coagulation initiates respiratory burst in neutrophils. Bull Exp Biol Med 140: 77-79.
33. Kusumanto YH, Dam WA, Hospers GA, et al. (2003): Platelets and granulocytes, in particular the neutrophils, form important compartments for circulating vascular endothelial growth factor. Angiogenesis 6: 283-287.
34. Scapini P, Morini M, Tecchio C, et al. (2004): CXCL1/macrophage inflammatory protein-2-induced angiogenesis in vivo is mediated by neutrophil-derived vascular endothelial growth factor-A. J Immunol 172: 5034-5040.
35. Banks RE, Forbes MA, Kinsey SE, et al. (1998): Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: significance for VEGF measurements and cancer biology. Br J Cancer 77: 956-964.
36. Werther K, Christensen IJ, Nielsen HJ (2002): Determination of vascular endothelial growth factor (VEGF) in circulating blood: significance of VEGF in various leukocytes and platelets. Scand J Clin Lab Invest 62: 343-350.