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Full Length Article

Heparanase procoagulant activity as a predictor of wound necrosis following diabetic foot amputation

Eli Peled [a], Eyal Melamed [a], Tali Banker Portal [a], Elena Axelman [b], Doron Norman [a], Benjamin Brenner [b], Yona Nadir [b,*]

[a] Division of Orthopedic, Rambam Health Care Campus, The Rappaport Faculty of Medicine, Technion, Haifa, Israel
[b] Thrombosis and Hemostasis Unit, Rambam Health Care Campus, The Rappaport Faculty of Medicine, Technion, Haifa, Israel

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ABSTRACT

Background: Trans-metatarsal operation to diabetic foot necrosis is a common procedure although only half of the patients do not need a second amputation due to surgery wound ischemia. No current tools are available for early prediction of surgery success and the clinical decision for a second operation may take weeks. Heparanase protein is involved in inflammation, angiogenesis and coagulation activation. The aim of the study was to evaluate heparanase level and procoagulant activity as an early predictor for success or failure of diabetic foot trans-metatarsal surgery.

Methods: The study group included 40 patients with diabetic foot necrosis requiring trans-metatarsal surgical intervention. Eighteen patients designated as necrotic group, developed post-surgery necrosis at the surgery wound within the first month, requiring a second more proximal amputation. Skin biopsies from the proximal surgery edge were stained for heparanase, tissue factor (TF), TF pathway inhibitor (TFPI) and by hematoxylin and eosin. Plasma samples were drawn pre-surgery and at 1 h, 1 week and 1 month post-surgery. Samples were tested for heparanase levels by ELISA and TF + heparanase activity, TF activity and heparanase procoagulant activity.

Results: Skin biopsy staining did not predict subsequent necrosis. In the non-necrotic group a significant rise in TF + heparanase activity, heparanase activity and heparanase levels were observed 1 h and 1 week post-surgery. The most significant increase was in heparanase procoagulant activity at the time point of 1 h post-surgery ($P < 0.0001$). Pre-surgery TF activity was significantly lower in the non-necrotic group compared to the necrotic group ($P < 0.05$).

Conclusions: Measuring heparanase procoagulant activity pre-surgery and 1 h post-surgery could potentially serve as an early tool to predict the procedure success. The present results broaden our understanding regarding early involvement of heparanase in the wound healing process.

1. Background

Diabetic foot necrosis is a severe and common complication in the western world. Diabetic foot ulcers can be divided to neuropathic and neuro-ischemic ulcers - feet with ischemia that are often associated with neuropathy. Both types can progress to ischemia and necrosis, requiring surgical orthopedic intervention [1]. Trans-metatarsal amputation is an operation designed to remove a limited area of irremediable tissue with ischemia and/or infection and preserve limb function. Patients are selected for trans-metatarsal operation based on degree of tissue loss/infection, adequacy of tissue perfusion at the trans-metatarsal level, current ambulatory status, and estimation of the likelihood of post-procedure ambulation. The validity of these selection criteria is very limited and nearly half of the patients require a second more proximal amputation due to necrosis at the wound edge [2]. Although the orthopedic selection criteria for the procedure are similar in all patients, currently the surgeons have no other tools to predict the operation outcome. The decision for a second amputation may take several weeks till the patient and the surgeon are convinced that it is inevitable. An early marker of the procedure success or failure would be helpful in shortening the decision-making time regarding a second amputation, hence reducing morbidity and costs. Heparanase, a β-D-endoglucuronidase is an enzyme that cleaves heparan sulfate side chains on the cell surface and in the extracellular matrix [3, 4]. It was later recognized as being a pro-inflammatory and pro-angiogenic protein that is abundant in platelets, granulocytes and monocytes [5, 6]. Our previous studies have demonstrated that heparanase may also affect the haemostatic system [7–9]. We showed that heparanase up-regulated the expression of the blood coagulation initiator- tissue factor (TF) [7] and interacted with the tissue factor pathway inhibitor (TFPI) on the cell surface membrane of

* Corresponding author at: Thrombosis and Hemostasis Unit, Rambam Health Care Campus, Haifa, Israel.
E-mail address: ynadir@netvision.net.il (Y. Nadir).

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endothelial and tumor cells, leading to dissociation of TFPI and resulting in increased cell surface coagulation activity [8]. Moreover, our findings demonstrated that heparanase directly enhanced TF activity which led to increased factor Xa production and subsequent activation of the coagulation system [9]. Elevated procoagulant activity of heparanase was demonstrated in five hypercoagulable clinical set-ups, using an assay developed at our laboratory [10–14]. Given the involvement of heparanase in angiogenesis and coagulation, the current study aimed to look for a possible pattern of heparanase expression in the plasma or surgery edge skin of diabetic patients hospitalized for trans-metatarsal amputation, that could serve as a predictor of the procedure outcome.

2. Materials and methods

2.1. Study group

The study was approved by the Institutional Review Board of the Rambam Health Care Campus. Between March 2012 and March 2014, 40 eligible patients were consecutively enrolled. Inclusion criteria were: age > 18, diabetic foot necrosis requiring trans-metatarsal surgical intervention. Eighteen patients developed post-surgery necrosis in the surgical wound area within the first month, requiring a second, more proximal trans-tarsal or below-knee amputation. In most patients with necrosis the second operation was performed at least a week after the first one. In the majority of these patients an infection process was also suspected and antibiotics were introduced. Twenty two patients were released with no signs of ischemia at the wound edge during the first month of follow-up at the orthopedic division and out-patients clinic.

2.2. Reagents and antibodies

Reagents for the heparanase enzyme-linked immunosorbent assay (ELISA): A single chain GS3 heparanase gene construct, comprising the 8 and 50 kDa heparanase subunits (8 + 50) was purified from the conditioned medium of baculovirus-infected cells. GS3 heparanase was assayed for the presence of bacterial endotoxin by Biological Industries (Beit Haemek, Israel), using the gel-clot technique (limulus amebocyte lysate – LAL test) and was found to contain <10 pg/ml endotoxin [7]. Polyclonal antibody 1453 was raised in rabbits against the entire 65 kDa heparanase precursor isolated from the conditioned medium of heparanase-transfected HEK-293 cells. The antibody was affinity-purified on immobilized bacterially expressed 50 kDa heparanase glutathione-S-transferase (GST) fusion protein [15]. Monoclonal anti-heparanase antibody 1E1 was generated by immunizing BALB/C mice with the entire 65 kDa heparanase protein.

Reagents for the heparanase procoagulant activity assay: Recombinant human factor VIIa and plasma-derived human factor X were purchased from American Diagnostic Inc. (Stamford, CT, USA). All coagulation factors were dissolved in double-distilled water. Chromogenic substrate to factor Xa (I.D. 222, solubility: Tris buffer, pH – 8.4) was purchased from American Diagnostica Inc. (Stamford, CT, USA). The calibration curve was performed with bovine factor Xa obtained from Sigma (St. Louis, MO, USA).

Antibodies for immunohistochemistry: Antibody 733 was raised in rabbits against a 15 amino acid peptide that maps at the N-terminus of the 50 kDa heparanase subunit. The 733 antibody preferentially recognizes the 8 + 50 kDa heterodimer vs. the 65 kDa latent proenzyme [16]. Polyclonal anti-human TF and polyclonal anti-human TFPI antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA).

2.3. Immunohistochemistry

After obtaining written informed consent a skin biopsy from the proximal edge of trans-metatarsal amputation was taken during the procedure. Staining of formalin-fixed, paraffin-embedded 5-μm sections was performed. Slides were deparaffinized with xylene, rehydrated and endogenous peroxidase activity was quenched for 30 min by 3% hydrogen peroxide in methanol. Slides were then subjected to antigen retrieval by boiling (20 min) in 10 mM citrate buffer, pH 6. Slides were incubated with 10% normal goat serum in phosphate buffered saline (PBS) for 60 min to block non-specific binding followed by incubation (20 h, 4 °C) with anti-heparanase, anti-TFPI or anti-TF antibodies, diluted 1:100 in blocking solution. Slides were then extensively washed with PBS containing 0.01% Triton X-100 and incubated with a secondary reagent (Envision kit; Dako, Glostrup, Denmark) according to the manufacturer's instructions. Following additional washes, color was developed with the AEC reagent (Sigma, St. Louis, MO, USA). Analyses of BM biopsy immunohistochemistry results were performed by two of the authors unaware of the slide allocation. Discrepancies in the analyses were reconciled following the assessment by a third reviewer. Five high power fields were evaluated in each stained slide. Staining intensity was scored as follows: 0, no staining; 1, weak intensity; 2, moderate intensity; and 3, marked intensity.

2.4. Blood sample collection

After obtaining written informed consent, a total of 6 ml of peripheral blood was collected, with 3.2% sodium citrate used as an anticoagulant. Blood was drawn immediately before operation, 1 h post-operation, 1 week post-operation and 1 month post-operation. Plasma was obtained by centrifugation (1500 *g* for 15 min at room temperature), and samples were frozen in aliquots at −70 ± 5 °C. Frozen plasma samples were thawed only once and each sample was analyzed in duplicates. As at the time of third sample withdrawal (one week post-surgery) most patients were receiving prophylactic dose of enoxaparin (40 mg) once daily, the samples were taken immediately prior to the next injection.

2.5. Heparanase procoagulant activity assay

Prior to testing, plasma aliquots were thawed in a water bath at 37 ± 0.5 °C for 15 min. As previously reported [10], a basic experiment of factor Xa generation was performed in the following manner. The concentrations mentioned are the final ones. Twenty-five μl of plasma, recombinant human factor VIIa (0.04 μM) and plasma-derived human factor X (1.4 μM) were incubated in a 50 μl assay buffer [0.06 M Tris, 0.04 M NaCl, 2 mM CaCl2, 0.04% w/v bovine serum albumin (BSA), pH 8.4] to a total volume of 125 μl in a 96-well sterile plate. After 15 min at 37 °C, chromogenic substrate to factor Xa was added (1 mM). Following 20 min, the reaction was stopped with 50 μl of glacial acetic acid and the level of Xa generation was determined using an ELISA plate reader (Power Wave XS, BIO-TEK, VT, USA). Heparins were shown to abrogate the TF/heparanase complex [9], therefore, in parallel, the same assay was performed except that fondaparinux (2.5 μg/ml) was added to the assay buffer. Bovine factor Xa diluted in the assay buffer was used to generate a standard curve. The heparanase procoagulant activity was calculated by subtraction of the first assay result from the second assay result. Thus, the assay gives three results: heparanase

Table 1
Demographic characteristics of study group.

	Non-necrotic n = 22	Necrotic n = 18	*P* value
Age (mean ± range)	73 (58–83)	72 (49–84)	NS
Male gender % (n)	86 (19)	89 (16)	NS
PVD[a] % (n)	27 (6)	33 (6)	NS
Hypertension % (n)	63 (14)	55 (10)	NS
Smokers % (n)	36 (8)	28 (5)	NS
Active cancer (n)	1	2	NA

[a] PVD – peripheral vascular disease.