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Heparanase procoagulant activity is elevated and predicts survival in non-small cell lung cancer patients

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ABSTRACT

Background: Heparanase is implicated in angiogenesis and tumor progression. We had earlier demonstrated that heparanase may also affect the hemostatic system in a non-enzymatic manner. It forms a complex and enhances the activity of the blood coagulation initiator- tissue factor (TF). Although increased heparanase antigen level in the plasma and biopsies of cancer patients was previously demonstrated, in the present study we evaluated, for the first time, the heparanase procoagulant activity in the plasma of patients with lung cancer.
Materials and Methods: Sixty five patients with non-small cell lung cancer at presentation and twenty controls were recruited. Plasma was studied for TF / heparanase procoagulant activity, TF activity and heparanase procoagulant activity using chromogenic assay and heparanase antigen levels by ELISA.
Results: Heparanase antigen levels were higher in the study group compared to control ($P = 0.05$). TF / heparanase activity, and even more apparent, heparanase procoagulant activity were significantly higher in the study group compared to controls ($P = 0.008$, $P < 0.0001$, respectively). No significant difference was observed in the TF activity between the groups. Survival of patients with heparanase procoagulant activity higher than 31 ng/ml predicted a mean survival of 9 ± 1.3 months while heparanase procoagulant activity of 31 ng/ml or lower predicted a mean survival of 24 ± 4 months ($P = 0.001$). Heparanase procoagulant activity was higher than 31 ng/ml in the four cases of thrombosis detected during the follow-up period.
Conclusions: Elevated heparanase procoagulant activity in patients with lung cancer reveals a new mechanism of coagulation system activation in malignancy. Heparanase procoagulant activity can potentially be used as a predictor for survival.

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Introduction

Activation of the coagulation system can be demonstrated in the plasma of most cancer patients. Activated coagulation factors (*e.g.* thrombin and fibrin) serve as growth factors to the tumor and also induce clinical thrombotic events resulting in increased morbidity and mortality [1]. Thirty years ago, heparanase was discovered as an enzyme that cleaves heparan sulfate (HS) side chains on the cell surface and in the extracellular matrix (ECM) [2,3]. Over the years it was recognized that platelets and tumor cells have abundant amount of heparanase and that heparanase is a pro-angiogenic and a pro-metastatic protein [4,5]. Previously we have demonstrated that heparanase may also affect the hemostatic system in a non-enzymatic manner. Heparanase was shown to up-regulate the expression of the blood coagulation initiator - tissue factor (TF) [6] and interact with tissue factor pathway inhibitor (TFPI) on cell surface, leading to dissociation of TFPI from cell membrane of endothelial and tumor cells, resulting in increased cell surface coagulation activity [7]. Moreover, we have shown that heparanase directly enhances TF activity resulting in increased factor Xa production and activation of the coagulation system [8]. Additionally, a chromogenic assay to evaluate heparanase procoagulant activity, facilitation the research in the field, was developed by us and was tested in clinical setups of hypercoagulability [9–11]. The current study group of patients with lung cancer was previously assessed for soluble endothelial protein C receptor (sEPCR) and other coagulation parameters [12]. In the present study we evaluated heparanase level and heparanase procoagulant activity in this group of patients.

Materials and Methods

The study was approved by the Institutional Ethic Committee on human research at the Rambam Health Care Campus. One hundred and two consecutive patients with newly diagnosed non-small cell lung cancer (NSCLC) who were referred to the Rambam Oncology Institute were

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enrolled over 18 months period. As the samples were analyzed in our previous work [12], plasma was left from only 65 samples for the current analysis. Inclusion criteria for the patients group: histologically or cytologically confirmed diagnosis of NSCLC, presence of active malignancy, no prior malignancy apart from treated basal cell or squamous cell carcinoma of the skin, no prior chemotherapy or radiotherapy, no major surgery during the last month before investigation, no anticoagulant therapy during the last month before investigation, no evidence of active infectious disease, normal serum bilirubin, maximal serum transaminases of two folds of the upper limit of normal, maximal serum creatinine level of 1.5 mg/dL (maximal normal value 1.3 mg/dL). Control group included non-hospitalized volunteers. Inclusion criteria for the control group: as for the study group, except that no known active malignancy was present. After obtaining informed consent, a total of 6 ml of peripheral blood was collected, with 3.2% sodium citrate as an anticoagulant. Plasma was obtained by centrifugation (1500 g for 15 min at 4 °C) and all plasma samples were frozen and thawed once.

Reagents and Antibodies

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 [6]. 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 [13]. Monoclonal anti-heparanase antibody 1E1 was generated by immunizing BALB/C mice with the entire 65 kDa heparanase protein. Recombinant human factor VIIa and plasma-derived human factor X were purchased from American Diagnostica (Stanford, CT). 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 (Stanford, CT). Bovine factor Xa was obtained from Sigma (St. Louis, MO).

Heparanase Procoagulant Activity Assay

As previously reported [9], we performed a basic experiment of factor Xa generation in the following manner: the concentrations mentioned are the final concentrations. Twenty-five micro-liters 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 [8], so 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 assay buffer was used to generate a standard curve. The subtraction of the first assay result from the second assay result determined heparanase procoagulant activity. Thus, the assay gives three results: heparanase procoagulant activity, TF activity and TF / heparanase procoagulant activity.

Heparanase Enzyme-linked Immunosorbent Assay (ELISA)

Wells of microtiter plates were coated (18 h, 48C) with 2 μg/ml of anti-heparanase monoclonal antibody in 50 ml coating buffer (0.05 M Na2CO3, 0.05 MNaHCO3, pH9.6) and were then blocked with 2% BSA in phosphate-buffered saline (PBS) for 1 h at 37 °C. Samples (200 μl) were loaded in duplicates and incubated for 2 hours at room temperature, followed by the addition of 100 μl antibody 1453 (1 μl/ml) for an additional period of 2 hours at room temperature. HRP-conjugated goat anti-rabbit IgG (1:20,000) in blocking buffer was added (1 hour, room temperature) and the reaction was visualized by the addition of 50 μl chromogenic substrate (TMB; MP Biomedicals, Germany) for 30 minutes. The reaction was stopped with 100 μl H2SO4 and absorbance at 450 nm was measured using an ELISA plate reader (PowerWave XS, BIO-TEK). Plates were washed five times with a washing buffer [PBS, pH 7.4 containing 0.1% (w/v) Tween 20] after each step. As a reference for quantification, a standard curve was established by serial dilutions of recombinant 8 + 50 GS3 heparanase (390 pg/ml-25 ng/ml)[14].

Statistics

Data were evaluated by SPSS software for Windows version 13.0 (SPSS Inc., Chicago, IL). Statistics was calculated by T-TEST for independent variables. Values were reported as mean ± standard deviation (SD). Pearson correlation analysis was evaluated between stage of disease and heparanase levels or heparanase procoagulant activity and between heparanase procoagulant activity and platelets number at presentation. Kaplan–Meier survival analysis (log-rank test) was used to identify the covariates that were significantly associated with survival. All parameters were analyzed with the median, high or low cut-off level of the normal range for each parameter. Survival duration was determined as the time from entering the study to the death of the patient. Significance level was set at $P < 0.05$.

Results

Demographic characteristics of the study group and controls are presented in Table 1. The patients' mean age was 65 ± 10 years and mean controls age was 63 ± 12 years ($P = 0.9$). 77% of patients were males and 75% of controls were males ($P = 0.6$). There was no difference in rate of anti-platelets therapy (23% *vs.* 20%, $P = 0.7$) or in rate of previous arterial or venous thrombotic events (1/20 *vs.* 3/65, $P = 0.9$). 85% of the study group smoked cigarettes regularly (more than 5 cigarettes a day) and only 65% smoked cigarettes regularly in the controls ($P = 0.05$). Characteristics of the study group are detailed in Table 2. In the study group 32 patients had NSCLC of adeno-carcinoma type, 25 patients had NSCLC of squamous cell type and 8 patients had other types of NSCLC. 82% of patients were in advanced stage at presentation (stage 3B and stage 4). Overall mean survival of the study group was 15.5 ± 13.2 months. Follow up was *via* visits in the Oncology outpatient's clinic or *via* telephone calls. Except 2 patients who survived beyond 48 months, all other patients died during the follow up period. Five symptomatic thrombotic events were documented, all occurred in the first 6 months since presentation.

Heparanase antigen levels were higher in the study group compared to control (mean 465 pg/ml ± 74 vs. 514 pg/ml ± 186, $P = 0.05$) (Fig. 1A). TF / heparanase activity, and even more apparent, heparanase procoagulant activity were significantly higher in the study group compared to controls (mean 62 ng/ml ± 20 vs. 87 ng/ml ± 38, $P = 0.008$, mean 14 ng/ml ± 10 vs. 48 ng/ml ± 32, $P < 0.0001$, respectively)

Table 1
Demographic characteristics of the study group and control group.

	Patients (n = 65)	Controls (n = 20)	*P*
Age (years)	65 ± 10	63 ± 12	NS
Males	77%	75%	NS
Anti-platelet therapy use	23%	20%	NS
Current smoking	82%	65%	0.05
Thrombotic event in the past	3/65	1/20	NS

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