Are primed polymorphonuclear leukocytes contributors to the high heparanase levels in hemodialysis patients?

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1Bruce Rappaport School of Medicine, Technion, Haifa, Israel; 2Eliachar Research Laboratory, 3Pathologic Laboratory, and 5Nephrology Unit, Western Galilee Hospital, Nahariya, Israel; and 4Nephrology Research Laboratory, Division of Nephrology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

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Cohen-Mazor M, Sela S, Mazor R, Ilan N, Vlodavsky I, Rops AL, van der Vlag J, Cohen HI, Kristal B. Are primed polymorphonuclear leukocytes contributors to the high heparanase levels in hemodialysis patients? Am J Physiol Heart Circ Physiol 294: H651–H658, 2008. First published November 21, 2007; doi:10.1152/ajpheart.00952.2007.—Patients on chronic hemodialysis (HD) are at high risk for developing atherosclerosis and cardiovascular complications. Heparanase, an endoglycosidase that cleaves heparan sulfate (HS) side chains of proteoglycans, is involved in extracellular matrix degradation and, as such, may be involved in the atherosclerotic lesion progression. We hypothesize that heparanase is elevated in HD patients, partly due to its release from primed circulating polymorphonuclear leukocytes (PMNLs), undergoing degranulation. Priming of PMNLs was assessed by levels of CD11b and the rate of superoxide release. Heparanase mRNA expression in PMNLs was determined by RT-PCR. PMNL and plasma levels of heparanase were determined by immunoblotting, immunofluorescence, and flow cytometry analyses. The levels of soluble HS in plasma were measured by a competition ELISA. This study shows that PMNLs isolated from HD patients have higher mRNA and protein levels of heparanase compared with normal control (NC) subjects and that heparanase levels correlate positively with PMNL priming. Plasma levels of heparanase were higher in HD patients than in NC subjects and were further elevated after the dialysis session. In addition, heparanase expression inversely correlates with plasma HS levels. A pronounced expression of heparanase was found in human atherosclerotic lesions. The increased heparanase activity in the blood of HD patients results at least in part from the degranulation of primed PMNLs and may contribute to the acceleration of the atherosclerotic process. Our findings highlight primed PMNLs as a possible source for the increased heparanase in HD patients, posing heparanase as a new risk factor for cardiovascular complications and atherosclerosis.

heparan sulfate; atherosclerosis

The major cause of morbidity and mortality in chronic kidney disease (CKD) patients undergoing renal replacement therapy is atherosclerotic cardiovascular disease. The atherosclerotic lesion progresses due to a series of reactions that are induced by the repair of the injured intima and the degradation of extracellular matrix (ECM) (2). Matrix metalloproteinases (MMPs) were shown to be involved in the degradation of the ECM component (13, 22) and were also found to be significantly increased in CKD patients on chronic hemodialysis (HD) (5, 24). Heparanase is an endo-β-D-glucuronidase capable of cleaving heparan sulfate (HS) side chains of the ECM proteoglycans (PGs) at a limited number of sites, yielding HS fragments of −5–7 kDa (7, 23, 37, 41, 42). To the best of our knowledge, this enzyme has not been studied in clinical states associated with accelerated atherosclerosis, such as CKD treated with HD. Heparanase activity also results in the release of many HS-bound molecules including growth factors, ECM molecules, enzymes, enzyme inhibitors, chemokines, and cytokines, which are involved in processes such as extravasation, growth, and chemotaxis of cells (27). Cleavage of HS and the subsequent release of bound factors can have profound effects on a variety of normal and pathological processes, such as tissue repair, inflammation, tumor growth, and metastasis (2, 11, 16, 43). This inadvertent cleavage of HS, with its potential tissue damage, compels a tight regulation of heparanase expression, activation, and bioavailability (9). Under normal conditions, heparanase activity is restricted to the placenta, skin tissues, and blood-borne cells such as platelets, neutrophils, monocytes, mast cells, and T lymphocytes. In a number of immune cells, including neutrophils, macrophages, and lymphocytes (1, 23, 25, 44), heparanase is stored in specific granules; its release by degragation has been implicated in diapedesis and extravasation, strongly implying that heparanase is a proinflammatory mediator (41, 49). Heparanase is first synthesized as a latent, 65-kDa enzyme, containing a NH2-terminal signal peptide that directs the protein to the secretory pathway (10). This latent enzyme is subjected to rapid uptake, internalization, and subsequent proteolytic processing at the lysosomal compartment, removing a linker sequence of about 6 to 7 kDa, yielding 8- and 50-kDa protein subunits that heterodimerize to form an active enzyme (10, 42). In monocytes and polymorphonuclear leukocytes (PMNLs), heparanase is known to be stored in tertiary granules, readily secreted cellular compartments that contain, among others, a variety of matrix-degrading enzymes, enabling the efficient mobility of these cells to or in sites of inflammation (31).

In pathological disorders associated with atherosclerosis and cardiovascular diseases such as hypertension, diabetes, and end-stage renal disease treated with chronic HD, PMNLs are primed (18, 32, 33). In the primed state, PMNLs are more sensitive to local or systemic stimuli due to a previous exposure to a priming agent. Hence, upon encountering an additional stimulus, full cell activation occurs, resulting in a robust...
release of reactive oxygen species and tertiary granule content into the blood stream (6, 36).

Our hypothesis is that in CKD patients on HD, elevated circulating levels of heparanase will be present as a result of the activation and degranulation of primed PMNLs. Such levels may be hazardous, due to their capability in degrading ECM. This would add heparanase to the reported MMPs involved in the progression of the atherosclerotic process.

MATERIALS AND METHODS

Patients and Blood Samples

Blood was drawn from 20 patients (aged 52 ± 9 yr; 10 men and 10 women, 7 of which had diabetes) on chronic HD treatment (36 ± 9 mo) and 10 healthy normal control (NC) subjects (5 men and 5 women, aged 45 ± 7 yr). Blood for the determination of biochemical and hematological parameters and for the isolation of PMNLs was drawn from NC subjects after an overnight fast. For HD patients, blood was collected from the arterial line immediately before a dialysis session. Blood was also collected from 10 patients (5 men and 5 women, without diabetes) after the dialysis session. All patients underwent HD three times a week; each dialysis treatment lasted 4 h and was carried out with low-flux polysulfone membranes (F8; Fresenius Medical Care, Bad Homburg, Germany) and with heparin as the anticoagulant. The water for dialysis met the standards of the Association for the Advancement of Medical Instrumentation. Patients with evidence of acute or chronic infection or malignancy or who had received a blood transfusion within 3 mo before the blood sampling were excluded. All participants signed an informed consent for blood sampling, and the study was approved by the Institutional Committee in accordance with the Helsinki Declaration.

PMNL Isolation and Analysis

Blood was drawn into sodium citrate tubes, and PMNLs were isolated as described previously (33). Isolated PMNLs (>98% pure, ~10^7 cells/isolation) were resuspended in phosphate-buffered saline (PBS) containing 0.1% glucose, counted, and analyzed. PMNL priming was evaluated by the rate of superoxide release and the level of CD11b. The rate of superoxide release was determined after cell stimulation with 0.32 × 10^-7 M phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO). The assay is based on superoxide dismutase inhibitable reduction of 55.5 (PMA; Sigma, St. Louis, MO). The assay is based on superoxide dismutase and is mobilized within intracellular granules in resting granulocytes and is mobilized as described previously (18). CD11b/CD18 (Mac-1) integrin is stored in the ferrous form. The change in optical density was monitored at 549 nm, CD11b was expressed as mean fluorescence intensity (MFI) after incubation with anti-CD11b-fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (MAb) (IQ Products), and anti-CD16 conjugated goat anti-mouse IgG (1:200; Jackson Laboratories, Bar Harbor, ME) for 45 min in the dark. After washes, slides were mounted and visualized by confocal microscopy (Bio-Rad Radiance 2000 confocal).

Intracellular levels of heparanase in PMNLs measured in whole blood by flow cytometry. Cells were permeabilized with a cell permeabilization kit (Fix & Perm) according to the manufacturer’s (Caltag) instructions, and anti-CD16-PE was used for gating on PMNLs. Intracellular heparanase was detected in whole blood by the polyclonal antiheparanase antibody 1453 [affinity purified (AP); diluted 1:1,000] raised against the entire 65-kDa heparanase precursor isolated from the conditioned medium of heparanase-transfected 293 cells, as described (9), followed by the addition of a secondary specific primers. Heparanase-specific primers, sense (5'-CTGGCAAGAAGTCTGGT-3') and antisense (5'-AAACTATAGAAA-GCTGCG-3'), were used to amplify a fragment of 588 bp. A 200-bp fragment of actin (40) was amplified using the primers sense (5'-CCTCCCTGGGCAATGAGTCCTG-3') and antisense (5'-GGAG-CAATGATCTTGATCCTC-3'). Aliquots (15 μl) of the amplified cDNA were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining and compared with a housekeeping gene, β-actin. The density of the heparanase PCR product was calculated in each gel relative to the actin product.

Localization of heparanase in PMNLs by confocal microscopy. Cytospin cells (5 × 10^6) were fixed and permeabilized with cold methanol (10 min), followed by incubation with 50 mM ammonium chloride at room temperature for 10 min to reduce autofluorescence. Five percent goat serum was then applied for blocking, followed by incubation with antiheparanase MAb 130 (10 μg/ml; 90 min), kindly provided by InSight Pharmaceuticals (Rehovot, Israel) (45). Slides were then washed three times with PBS supplemented with 0.05% Tween and incubated with Cy3-conjugated goat anti-mouse IgG (1:200; Jackson Laboratories, Bar Harbor, ME) for 45 min in the dark. After washes, slides were mounted and visualized by confocal microscopy (Bio-Rad Radiance 2000 confocal).

RNA isolation and RT-PCR. RNA was isolated using Tri-Reagent according to the manufacturer’s (Sigma) instructions. Complementary DNA (cDNA) was synthesized according to Krug and Berger (19) using an OmniGene thermal cycler (Hybaid, UK). The resulting cDNA was amplified by PCR using 2-μl aliquots of the RT reaction incubated with 0.2 mM dNTPs, 2 μl of Taq DNA polymerase buffer, 0.6 units of Taq DNA polymerase (BioLine), and 10 pmol of the specific primers. Heparanase-specific primers, sense (5'-CTGGCAAGAAGTCTGGT-3') and antisense (5'-AAACTATAGAAA-GCTGCG-3'), were used to amplify a fragment of 588 bp. A 200-bp fragment of actin (40) was amplified using the primers sense (5'-CCTCCCTGGGCAATGAGTCCTG-3') and antisense (5'-GGAGCAATGATCTTGATCCTC-3'). Aliquots (15 μl) of the amplified cDNA were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining and compared with a housekeeping gene, β-actin. The density of the heparanase PCR product was calculated in each gel relative to the actin product.

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**Fig. 1.** Heparanase mRNA expression in polymorphonuclear leukocytes (PMNLs). Total mRNA was extracted from PMNLs of control (NC) subjects and hemodialysis (HD) patients, and heparanase and actin transcripts levels were evaluated by RT-PCR analysis. Representative gel of PCR products of heparanase (588 bp) and actin (200 bp) are shown in A, and quantification of the relative mRNA heparanase expression is shown in B. *P < 0.05 HD vs. NC subjects; n = 10 subjects in each group.
**Intracellular levels of heparanase measured in separated PMNLs by protein extraction and immunoblotting.** Proteins from isolated human PMNLs (2 × 10⁶) were extracted using a lysis buffer containing 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, and 0.5% Triton X-100, supplemented with a cocktail of protease inhibitors (Sigma). Heparin-agarose beads (Sigma) were then added and the mixture was rotated for 2 h at 4°C. The beads were collected by centrifugation and washed three times with PBS. The loading buffer containing 3% β-mercaptoethanol was then added, and the samples were boiled at 100°C for 5 min. The supernatant was resolved by SDS-PAGE and transferred semidry (Bioimage) to nitrocellulose filters. The filters were blocked with low-fat milk for 1 h at room temperature and then incubated with anti-heparanase polyclonal antibody (1453-AP; diluted 1:1,000) for 2 h at room temperature, followed by incubation with goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (diluted 1:25,000) for 1 h at room temperature. The membranes were extensively washed after each step, and heparanase was visualized by enhanced chemiluminescence (EZ-ECL kit; Biological Industries, Beit-Haemek, Israel). The densities of the heparanase bands were determined with BioCapt and BioProfile (Bio-1D) software.

**Detection and quantification of heparanase in plasma.** Plasma diluted 1:10 in PBS was preabsorbed on heparin-agarose beads for 2 h. The beads were collected, and the samples were loaded onto SDS-PAGE, blotted onto a nitrocellulose membrane, and subjected to immunoblotting, as described in Intracellular levels of heparanase measured in separated PMNLs by protein extraction and immunoblotting. The results before dialysis are presented as measured. To correct for the hemoconcentration caused by water loss during HD, the values of heparanase measured after dialysis were corrected using the correction factor f: f = (1 - Hctₐ)/(1 - Hctᵦ) × (Hctᵦ/Hctₐ), where Hctₐ and Hctᵦ are the hematocrit values after and before dialysis, respectively (21).

**Quantification of Soluble HS in Plasma by a Competition ELISA**

Flat-bottom 96-well plates (Nunc A/S, Roskilde, Denmark) were coated with 5 μg/well HS from bovine kidney (Seikagaku, Tokyo, Japan) in PBS. The wells were blocked with 1% gelatin-PBS (BD Biosciences, Alphen a/d Rijn, The Netherlands). The plasma samples were incubated with the anti-HS antibody JM403 (38) for 1 h in a separate plate precoated with gelatin-PBS. In addition, different amounts of HS from bovine kidney were incubated with anti-HS antibody JM403 to prepare a standard curve. The HS-coated plate was washed with PBS-Tween, and the anti-HS antibody preincubated with the plasma samples or HS from bovine kidney was added and incubated for 1 h. Subsequently, the wells were washed with PBS-Tween. Anti-HS antibody binding was detected by incubating with the appropriate HRP-conjugated antibody for 1 h. Finally, the plates were washed with PBS-Tween and incubated with tetramethylbenzidine solution (SFRI, Berganton, France). After 15 min, the reaction was stopped with 2 M H₂SO₄, and absorption was measured at 450 nm. The amount of HS detected in plasma is expressed in arbitrary units, since HS from bovine kidney was coated and used to prepare the standard curve.

**Immunohistochemistry of Heparanase in Atherosclerotic Coronary Plaque**

Samples were taken from the arteries of three atherosclerotic patients and three patients who died from reasons other than cardiovascular disease. Formalin-fixed, paraffin-embedded autopsy specimens of coronary arteries were obtained from the Department of Pathology of Western Galilee Hospital-Nahariya. Four micrometer-thick serial sections from each representative paraffin block were prepared and then incubated with xylene, 100% ethanol, 2.5% H₂O₂ in methanol, 100% ethanol, 70% ethanol, and distilled water. The sections were boiled for 5 min in sodium citrate (pH = 6) by a pressure cooker, washed with PBS, and blocked in 10% goat serum. The sections were incubated overnight with a polyclonal rabbit anti-human heparanase antibody (α-733) (49) directed against the active enzyme, followed by incubation with HRP-conjugated goat-FITC-conjugated goat anti-rabbit IgG (Chemicon International). Irrelevant IgG-FITC antibody served as the control. The results are presented as MFI after the subtraction of the nonspecific background.

**Coronary Plaque Immunohistochemistry of Heparanase**

Fluorescence immunostaining with the antiheparanase monoclonal antibody (MAb 130) from Biosciences, Alphen a/d Rijn, The Netherlands, was used to analyze the subcellular localization of heparanase in human coronary plaques. The sections were incubated with the anti-HS antibody JM403 (38) for 1 h at room temperature and examined by confocal microscopy (×100) or subjected to fluorescence immunostaining with antiheparanase monoclonal antibody (MAb 130) and examined by confocal microscopy (×100).
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anti-rabbit IgG antibody (Dako). Color was developed using a Zymed aminoethyl carbozole substrate kit (Zymed, San Francisco, CA) for 10 min, followed by counterstain with Mayer’s hematoxylin. Foam cells were stained by monoclonal anti-human CD68 (1:20 dilution; Biogenics). A histofine simple stain kit (Nichirei) was used for immunohistochemical staining according to the manufacturer’s instructions.

Statistical Analysis

Data are expressed as means ± SE. In the bar and error bars presentations (Figs. 1, 3, and 6), the horizontal line in the middle shows the median (50th percentile), the top and bottom of the bar show the 75th and 25th percentiles, respectively, and the error bars show the maximum and the minimum values. The nonparametric Mann-Whitney test was used for comparing two independent groups. The two-paired Wilcoxon’s rank sum test was used for comparing two dependent groups. Statistical significance was considered at P < 0.05.

RESULTS

PMNL Priming in HD Patients Versus NC Subjects

The rate of superoxide release and surface levels of CD11b served as PMNL priming indices (32, 48). The rate of superoxide release following PMA stimulation was higher in PMNLs isolated from HD patients compared with NC subjects (29.73 ± 1.91 vs. 18.43 ± 1.8 mmol·10⁶ cells⁻¹·10 min⁻¹, respectively; P < 0.05). CD11b expression was higher in PMNLs from HD patients compared with NC subjects (3.6 ± 0.4 vs. 2 ± 0.3 MFI, respectively; P < 0.05). Both indices indicate a higher priming state of PMNLs from HD patients.

PMNL Heparanase in HD Patients Versus NC Subjects

Transcriptional levels of heparanase in PMNLs. Representative PCR products of heparanase (588 bp) and actin (200 bp) from PMNLs are shown in Fig. 1A. Heparanase mRNA expression was significantly higher in PMNLs isolated from HD patients than from NC subjects (Fig. 1A). Densitometry analysis revealed a 2.4-fold increase of heparanase mRNA expression in PMNLs from HD patients compared with PMNLs from NC subjects (Fig. 1B).

Localization of heparanase in PMNLs. Separated PMNLs visualized by an inverted microscope showed an equal dispersal of cells in NC and HD slides (Fig. 2, A and B). Confocal microscopy revealed that although only a few PMNLs from NC subjects were stained positively for heparanase (Fig. 2C), heparanase was abundantly expressed at membranal and cytoplasmic localizations in PMNLs from HD patients (Fig. 2D).

Intracellular levels of heparanase in PMNLs. Quantification of heparanase by flow cytometry in nonseparated PMNLs (whole blood) revealed elevated levels of heparanase in PMNLs of HD patients compared with NC subjects (0.85 ± 0.12 vs. 0.53 ± 0.05 MFI, respectively; Fig. 3A). The heparanase protein level in PMNLs correlated with CD11b levels (Fig. 3B; r = 0.66; P < 0.05), showing a positive linear correlation with the priming state of PMNLs; the increased priming of PMNLs is associated with higher intracellular heparanase levels.

Expression of heparanase in cell extracts (Fig. 4) revealed that although the levels of the 65-kDa latent heparanase were not significantly different in PMNLs from NC versus HD patients (Fig. 4B), the active 50-kDa heparanase was elevated twofold in PMNLs from HD patients, as measured by densitometry analysis (Fig. 4B).

Plasma Heparanase and Soluble HS Levels

A representative gel shows plasma samples from NC and HD patients that were preabsorbed on heparin-agarose beads to reduce the nonspecific reactivity with the antiheparanase antibodies during immunoblotting (Fig. 4A; plasma). Plasma collected from HD patients contained significantly higher levels of both latent (65 kDa) and active (50 kDa) forms of heparanase compared with control plasma (0.95 ± 0.1 vs. 0.59 ± 0.07, respectively; Fig. 4, A and C). Furthermore, the amount of active plasma heparanase correlated with active heparanase levels in PMNLs (r = 0.685; P < 0.05; Fig. 4D), suggesting that in HD patients plasma-active heparanase originates, at least in part, from PMNLs. Interestingly, we found that in most patients, the levels of plasma heparanase were significantly elevated immediately following the dialysis sessions (P < 0.05; Fig. 5).

Finally, we determined the amount of HS in the plasma of NC and HD patients, utilizing a competition ELISA. HS levels found in the plasma of HD patients were significantly lower than those of NC subjects (Fig. 6A) and inversely correlated with heparanase levels expressed by PMNLs.
It appears that the increased heparanase intracellular levels in primed PMNLs from HD patients results in the elevation of active heparanase in the plasma and the concomitant degradation of HS in the plasma.

Immunohistochemistry of Heparanase in Normal and Atherosclerotic Coronary Arteries

All autopsy specimens of the nonatherosclerotic coronary artery (control; Fig. 7A) showed a well-preserved, relatively thick-wall vascular artery with signs of moderate intimal fibrosis and no evidence of atherosclerosis. All cross sections of an atherosclerotic coronary artery (Fig. 7B) showed atherosclerotic plaque with fibrous cap, clefts of lipid crystals associated with local calcifications, and multiple foamy cell macrophages (Fig. 7C).

In nonatherosclerotic coronary arteries, scattered fibroblasts in the deep intima showed very weak positive immunostaining for heparanase. This was totally absent in luminal endothelial cells. With local positive immunostaining of endothelial lining, heparanase was strongly and consistently positive in all sections of atherosclerotic arteries, and especially in foamy cell macrophages in the tunica media.

DISCUSSION

The present study shows that PMNLs of CKD patients treated with HD contain a two- to threefold higher expression of intracellular heparanase mRNA and protein compared with healthy subjects. The intracellular heparanase levels correlate directly with the priming state of PMNLs. In addition, the blood levels of the circulating active form of heparanase correlate with its intracellular levels in PMNLs, suggesting that PMNLs are a new source for the active form of heparanase in
HD patients. The higher levels of heparanase in HD blood are further elevated by the dialysis session, suggesting that the HD process, per se, causes the activation and degranulation of primed PMNLs, resulting in an increased release of this intracellular endo-β-1,3-glucuronidase from PMNLs.

The increased priming of PMNLs isolated from HD patients was shown by the elevated levels of CD11b and the enhanced superoxide release upon PMA stimulation. The higher priming of PMNLs from patients on HD is in agreement with previous findings (32, 48). The elevated intracellular heparanase levels in primed PMNLs from HD patients can be explained by the elevated mRNA levels found in these cells, followed by protein synthesis, a fact that is supported by a study that demonstrated the capability of de novo protein synthesis by mature PMNLs (8). We suggest that the activation of the heparanase transcriptional cascade by various stimulation agents in HD results in protein synthesis and the release of heparanase into the circulation.

The association between PMNL priming, de novo protein synthesis, and degranulation is supported by studies showing that another PMNL constituent, the enzyme myeloperoxidase, is significantly elevated in plasma from HD patients compared with NC subjects (4).

Interestingly, tumor necrosis factor (TNF-α) was found to induce PMNL activation (36). Since the TNF-α level is increased in the blood of HD patients (29), we suggest that the elevated heparanase plasma levels in these patients may be derived from increased secretion of heparanase from activated PMNLs. Nevertheless, other mechanisms, such as reduced clearance of the released enzyme, should also be considered. In this context, hepatic expression of LDL receptor-related protein, which is a multifunctional receptor for removal of numerous molecules including circulating enzymes such as heparanase, is markedly downregulated in chronic kidney failure and can contribute to the elevation of its plasma concentration (15, 46).

Although PMNLs from HD patients overexpress the active form of heparanase, plasma of HD patients contains higher levels of both active and nonactive heparanase compared with the plasma of NC subjects. The high levels of nonactive heparanase in plasma of HD patients can be explained by a significant increase in PMNL counts and degranulation detected in HD patients (32, 48). The increased number of PMNLs undergoing degranulation results in higher levels of both forms of heparanase in the circulation. The further increase in plasma levels of heparanase during dialysis shown herein is reminiscent of the reported release of granule enzymes from PMNLs, such as elastase and cathepsin G, to the blood stream by the dialysis (30). The activation of neutrophils during low-flux dialysis (47) probably causes the release of heparanase from these activated cells. This is in agreement with other recent findings describing enhanced superoxide and hydrogen peroxide production by granulocytes and monocytes.

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Fig. 6. Plasma levels of heparan sulfate (HS) in NC subjects and HD patients. A: levels of soluble HS in plasma of NC and HD patients measured by an inhibition ELISA (*P < 0.05 HD vs. NC subjects; n = 10 subjects in each group). B: correlations between the soluble HS levels in plasma of NC and HD patients and 1) 50-kDa plasma active heparanase (r = -0.66; P < 0.05; dashed line) and 2) PMNL intracellular active heparanase (r = -0.53; P < 0.05; continuous line).

Fig. 7. Immunostaining of heparanase in normal and atherosclerotic coronary artery tissue sections. A: light microscopy picture of coronary artery control (×100) stained for heparanase. B: light microscopy picture of coronary artery with atherosclerotic plaque stained for heparanase (×100). C: light microscopy picture of coronary artery with atherosclerotic plaque stained for foam cells (CD68).
following dialysis and a concomitant decrease of cell granularity, regarded as spontaneous activation (48).

We have previously shown that the addition of heparin results in the accumulation of heparanase in the culture medium of heparanase-transfected cells (9). Therefore, heparin, which was used as an anticoagulant during the dialysis sessions, may also have an effect on heparanase release from heparanase-containing cells. In addition, although we have adjusted our results to hematocrit, the possibility that fluid removal by ultrafiltration during dialysis can alter the concentration of macromolecules in the plasma should be considered. The decrease in plasma heparanase levels before dialysis (48–72 h after the previous dialysis session) could be related to its uptake by HS-containing cells (9) or by degradation, since the $t_{1/2}$ of the active enzyme is $\sim 30$ h (9). Nonetheless, the levels of heparanase before dialysis were still higher compared with NC subjects, implying that the PMNLs are continuously primed in HD patients and undergo spontaneous degranulation.

The levels of plasma-soluble HS are significantly lower in HD patients, probably due to its degradation by the high levels of the soluble-active heparanase found in the plasma of these patients. The specific anti-HS antibody used in this assay recognizes a specific domain within HS, which is lost after cleavage by heparanase (17, 39). Hence, the disappearance of this specific HS domain is an additional indirect measure for heparanase activity. Furthermore, this is supported by the significant negative correlation between soluble HS in plasma and plasma-active heparanase.

We previously reported that primed PMNLs from HD patients initiate endothelial dysfunction, leading to a disruption of the endothelial cell monolayer, and increased P-selectin, tissue factor, and endothelial nitric oxide synthase levels (12). In addition, cocultivation of PMNLs from HD patients with human umbilical vein endothelial cells caused a decrease of HS levels on endothelial cells (unpublished data). The continuous circulation of active heparanase near the vascular endothelium may lead to proteoglycan degradation, accompanied by increased vascular permeability, most likely through the disruption of the vascular endothelium and subendothelial basement-membrane integrity (3). In addition, Pillarisseti et al. (26) have shown that exposure of cultured endothelial cells to oxidized LDL (OxLDL) leads to a reduction in matrix HSPG and the production of an endothelial heparanase (26). Since HD patients are known to have significantly higher concentrations of OxLDL in blood (20), it also can affect heparanase levels within the vessel endothelium and in plasma.

Thus heparanase, originated either from endothelial cells or activated PMNLs or heparanase available systemically and subjected to cellular uptake and activation (9, 42, 46), may contribute to chronic injury conditions of the endothelium, that may lead to atherosclerosis, a major complication prevalent in HD patients (14, 35, 50). Indeed, heparanase expression was found in coronary artery atherosclerotic plaques, similar to the staining pattern that was observed in aortic tissue sections of apolipoprotein E-null mice (3). Possible mechanisms for increased heparanase expression in atherosclerotic lesions are local production by vascular cells (26) and endocytosis of plasma heparanase by the HS-containing cells in the plaque (9).

The profound expression of heparanase in the atherosclerotic vessel wall and plaque, as shown here, suggests that this enzyme is also involved in the late stages of the atherosclerotic process. The composition and content of the vessel wall proteoglycans change during the development of the atherosclerotic lesion, with lower levels of HSPG found in atherosclerotic vessels (34). In normal intima, it was shown that basement membrane proteins, such as fibronectin, laminin, and collagen, are masked by HSPG present in ECM, preventing the binding and retention of monocytes within the intima, thereby protecting against the initiation of the atherosclerotic process.

Therefore, an additional effect of heparanase activity is the decrease in vessel wall HSPG, enabling a retention of monocytes within the vessel wall and allowing them to convert into macrophage-rich foam cells, as has been shown in the early stage of the atherosclerosis process (34). The continuous interaction of primed PMNLs with the blood vessel endothelial monolayer implies that the vascular wall may be chronically exposed to the uncontrolled release of the granular heparanase, which abnormally degrades HS from the endothelial surface and the ECM, causing endothelial injury/activation and ECM loosening. These mechanisms are involved in the early stages of the atherosclerotic process.

The increased heparanase expression in both PMNLs and plasma of HD patients, together with its presence in the atherosclerotic blood vessels, suggests a new direction for understanding accelerated atherosclerosis in HD patients and needs to be further studied in all clinical states associated with primed PMNLs and accelerated atherosclerosis, namely hypertension and diabetes.

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