Macrophage Activation by Heparanase Is Mediated by TLR-2 and TLR-4 and Associates With Plaque Progression


Objective—Factors and mechanisms that activate macrophages in atherosclerotic plaques are incompletely understood. We examined the capacity of heparanase to activate macrophages.

Methods and Results—Highly purified heparanase was added to mouse peritoneal macrophages and macrophage-like J774 cells, and the levels of tumor necrosis factor-α, matrix metalloproteinase-9, interlukin-1, and monocyte chemotactic protein-1 were evaluated by ELISA. Gene expression was determined by RT-PCR. Cells collected from Toll-like receptor-2 and Toll-like receptor-4 knockout mice were evaluated similarly. Heparanase levels in the plasma of patients with acute myocardial infarction, stable angina, and healthy subjects were determined by ELISA. Immunohistochemistry was applied to detect the expression of heparanase in control specimens and specimens of patients with stable angina or acute myocardial infarction. Addition or overexpression of heparanase variants resulted in marked increase in tumor necrosis factor-α, matrix metalloproteinase-9, interlukin-1, and monocyte chemotactic protein-1 levels. Mouse peritoneal macrophages harvested from Toll-like receptor-2 or Toll-like receptor-4 knockout mice were not activated by heparanase. Plasma heparanase level was higher in patients with acute myocardial infarction, compared with patients with stable angina and healthy subjects. Pathologic coronary specimens obtained from vulnerable plaques showed increased heparanase staining compared with specimens of stable plaque and controls.


Key Words: heparanase ■ macrophages ■ TLR ■ TNFα ■ vulnerable plaque

Atherosclerosis represents a major cause of death and disability in adult population. Atherosclerotic lesions are asymmetric focal thickenings of the intima, consisting of inflammatory and immune cells, connective tissue elements, lipids, debris, and vascular endothelial and smooth muscle cells. Although the majority of these lesions remain stable, some undergo alterations that make them vulnerable to rupture. Inflammatory processes creates a thin cap of fibrous tissue over a lipid-rich and metabolically active core, which is the hallmark feature of vulnerable, high-risk plaques, associated with acute coronary syndrome and sudden cardiac death. The mechanism(s) underlying the progression from asymptomatic fibroatheromatous plaque to a lesion at high risk for rupture (vulnerable plaque [VP]) is largely unclear.

Proteoglycans are recognized to be associated with atherosclerotic lesions and lipid deposition in the vascular wall. Heparan sulfate proteoglycans (HSPG) derived from endothelial cells have been shown to be potent inhibitors of vascular smooth muscle cell proliferation and to inhibit the neointimal response to injury. Other reports suggest that heparan sulfate (HS) exerts proatherogenic effects. Although the mechanisms underlying the function of HS in the context of atherosclerosis are not entirely clear, they are likely to be regulated by HS-modifying enzymes.

Heparanase is an endo-β-D-glucuronidase capable of cleaving HS side chains at a limited number of sites. Heparanase activity correlates with the metastatic potential of tumor-derived cells, attributed to enhanced cell dissemination as a consequence of HS cleavage and remodeling of the extracellular matrix (ECM) barrier. Similarly, heparanase activity is implicated in neovascularization, inflammation, and autoimmunity, involving migration of vascular endothelial cells and activated cells of the immune system. We hypothesized that in addition to their mobilization, heparanase also activate macrophages. We provide evidence that transient transfection or exogenous addition of purified recombinant heparanase to primary macrophages resulted in a marked increase in the levels of monocyte chemotactic protein (MCP)-1, tumor necrosis factor (TNF)-α, interlukin (IL)-1, and matrix metalloproteinase (MMP)-9, mediators of plaque formation and rupture. Cytokine induction indistinguishable in magnitude was elicited by addition of mutated, enzymatically
inactive heparanase, pointing to a signaling feature that incorporates the phosphatidylinositol-3 kinase, mitogen-activated protein kinase, nuclear factor-xB (NFkB), and Toll-like receptor (TLR)-2 and -4 pathways. Notably, heparanase immunostaining was markedly increased in VP specimens compared with stable plaque or control artery, also reflected by immunostaining was markedly increased in VP specimens in the plasma of patients with acute myocardial infarction (MI) versus healthy subjects.

Materials and Methods

Heparanase Purification and Activity Assay

The 65-kDa latent heparanase protein was purified from medium conditioned by infected HEK-293 cells. Briefly, cells were grown in low serum (2.5%), until confluent. Cells were then grown under serum-free conditions for 24 hours; conditioned medium (1 L) was collected, filtered, and loaded (20 hours, 4°C) on a heparin column (Hi Tr Sep FF Heparin column, Pharmacia). Heparanase was eluted by a salt gradient (100 mM/L to 1.5 mol/L NaCl) in buffer containing 20 mM/L Heps pH 7.3 and 1 mM/L dithiothreitol. Heparanase is eluted from the column at 0.7 to 0.8 mol/L NaCl, and appears as a single, highly purified protein band by Coomassie blue and silver staining. Purified heparanase was assayed for the presence of bacterial endotoxin using the gel clot technique (Limulus amebocyte lysate, LAL test) and was found to contain <10 pg/mL endotoxin. Constitutively active heparanase (GS3) was purified from the condition medium of transiently transfected SP insect cells applying a similar purification procedure.

Preparation of ECM-coated 35-mm dishes and determination of heparanase activity were performed, as described in detail elsewhere.14

Antibodies and Reagents

The following antibodies were purchased from Santa Cruz Biototechnology (Santa Cruz, CA): anti-inhibitor of kappa B, anti-Akt (sc-5298), anti-phospho-extracellular signal-regulated kinase (sc-7383), and anti-extracellular signal-regulated kinase-regulated kinase-2 (sc-154). Polyclonal antibodies to phospho-Akt (Ser473) and phospho-IkBα were purchased from Thermo Scientific (Fremont, CA); anti-inhibitor of kappa B, anti-EXV (sc-7383), and anti-extracellular signal-regulated kinase-2 (sc-154). Polyclonal antibodies to phospho-Akt (Ser473) and phospho-xKB were purchased from Cell Signaling (Beverly, MA). Anti-CD163 monoclonal antibody was purchased from Thermo Scientific (Fremont, CA); antiactin monoclonal antibody was purchased from Sigma (St. Louis, MO). Neutralizing antibodies to mouse TLR-2 (Invivogen, San Diego, CA) and TLR-4 (BioLegend, San Diego, CA) were also used. The selective phosphatidylinositol-3 kinase (LY 294002), mitogen-activated protein kinase (PD 98059), Src (PP2), NFkB (BAY 117082), and TLR inhibitors were purchased from Calbiochem or IMGENEX (San Diego, CA) and were dissolved in dimethyl sulfoxide as stock solutions. Dimethyl sulfoxide was added to the cell culture as control.

Cells and Cell Culture

The murine macrophage cell line J774 was kindly provided by Prof Michael Aviram (Rappaport Faculty of Medicine, Technion, Haifa, Israel). Mouse peritoneal macrophages (MPM) were harvested from thioglycolate-treated mice deficient for TLR-2, TLR-4, or both.15 The murine macrophage cell line J774 was kindly provided by Prof Michael Aviram (Rappaport Faculty of Medicine, Technion, Haifa, Israel). Mouse peritoneal macrophages (MPM) were harvested from thioglycolate-treated mice deficient for TLR-2, TLR-4, or both.15 The murine macrophage cell line J774 was kindly provided by Prof Michael Aviram (Rappaport Faculty of Medicine, Technion, Haifa, Israel). Mouse peritoneal macrophages (MPM) were harvested from thioglycolate-treated mice deficient for TLR-2, TLR-4, or both.15 The murine macrophage cell line J774 was kindly provided by Prof Michael Aviram (Rappaport Faculty of Medicine, Technion, Haifa, Israel). Mouse peritoneal macrophages (MPM) were harvested from thioglycolate-treated mice deficient for TLR-2, TLR-4, or both.15 The murine macrophage cell line J774 was kindly provided by Prof Michael Aviram (Rappaport Faculty of Medicine, Technion, Haifa, Israel). Mouse peritoneal macrophages (MPM) were harvested from thioglycolate-treated mice deficient for TLR-2, TLR-4, or both.15 The murine macrophage cell line J774 was kindly provided by Prof Michael Aviram (Rappaport Faculty of Medicine, Technion, Haifa, Israel). Mouse peritoneal macrophages (MPM) were harvested from thioglycolate-treated mice deficient for TLR-2, TLR-4, or both.15 The murine macrophage cell line J774 was kindly provided by Prof Michael Aviram (Rappaport Faculty of Medicine, Technion, Haifa, Israel). Mouse peritoneal macrophages (MPM) were harvested from thioglycolate-treated mice deficient for TLR-2, TLR-4, or both.15 The murine macrophage cell line J774 was kindly provided by Prof Michael Aviram (Rappaport Faculty of Medicine, Technion, Haifa, Israel). Mouse peritoneal macrophages (MPM) were harvested from thioglycolate-treated mice deficient for TLR-2, TLR-4, or both.15 The murine macrophage cell line J774 was kindly provided by Prof Michael Aviram (Rappaport Faculty of Medicine, Technion, Haifa, Israel). Mouse peritoneal macrophages (MPM) were harvested from thioglycolate-treated mice deficient for TLR-2, TLR-4, or both.15 The murine macrophage cell line J774 was kindly provided by Prof Michael Aviram (Rappaport Faculty of Medicine, Technion, Haifa, Israel). Mouse peritoneal macrophages (MPM) were harvested from thioglycolate-treated mice deficient for TLR-2, TLR-4, or both.15

Cells were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with glutaamine, pyruvate, antibiotics, and 10% fetal calf serum in a humidified atmosphere containing 5% CO2 at 37°C. J774 cells were transiently transfected with heparanase gene constructs by Amaxa nucleofection technology according to the manufacturer’s (Lonza, Walkersville, MD) instructions. Human monocytes were isolated from peripheral blood essentially as described.16 Briefly, peripheral blood mononuclear cells from normal individuals were collected from Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradients (<1.077 g/mL), resuspended in Roswell Park Memorial Institute 1640 medium containing 2% human serum (Hyclone Laboratories Logan, UT), and plated at a concentration of 4×106/mL into 24-well plates (1 mL/well). After incubation (37°C, 90 minutes), the cells were washed (x3) with PBS to remove nonadherent cells, yielding monolayers consisting of >95% monocytes, as determined by morphological analysis (Wright-Giemsa staining) and expression of CD14. The adherent cells (macrophages) were then incubated (37°C, 24 hours) in serum-free medium, in the absence or presence of heparanase (5 μg/mL). The medium was then collected, centrifuged, and aliquots of the supernatant taken for ELISA.

ELISA

PM and J774 cells (1×106) were plated into 24-well plate and grown to 70% to 80% confluence. After 24-hour incubation in serum-free medium, cells were incubated with latent 65-kDa, inactive (mutated at Glu203 and Glu182; DM), constitutively active (GS3),13 or the C-terminus domain (8C) heparanase proteins (0–10 μg/mL; 37°C). Medium was collected after 24 hours and examined for the level of TNFα, MMP-9, IL-1, and MCP-1 using ELISA plates, according to the manufacturer’s (R&D Systems, Minneapolis, MN) instructions. In inhibition studies, selective inhibitors were added to cell cultures 30 minutes before the addition of heparanase.

RT-PCR Analysis

Total RNA was extracted with TRizol (Sigma) and RNA (1 μg) was amplified using 1-step PCR amplification kit, according to the manufacturer’s (ABGene, Epsom, UK) instructions. The PCR primer sets were as follows:

- Heparanase F-5'-AGGTCCTGACATAGGACGGC-3'
- Heparanase R-5'-TGAACCTTCTCTGGCCGAGAG-3'
- GAPDH F-5'-CCAGCGCGACACATCGGTC-3'
- GAPDH R-5'-ATGGACCGGAGCCGTCTCCAT-3'
- TNFa F-5'-GATCTCAAAGACAAACACTCA-3'
- TNFa R-5'-CTCCAGCTGGAAGACCTCCAG-3'
- MMP-9 F-5'-TAGTGAGAGACTCTCACACAG-3'
- MMP-9 R-5'-CCACCTTCTTGTACGTGTCGA-3'

Cell Fractionation and Immunoblotting

Cell fractionation was carried out using NE-PER nuclear and cytoplasmic extraction reagents, according to the manufacturer’s (Pierce, Rockford, IL) instructions. Cell extracts were prepared using a lysis buffer containing 50 mM/L Tris-HCl, pH 7.4, 150 mM/L NaCl, 0.5% Triton X-100, and a cocktail of protease inhibitors (Roche, Mannheim, Germany). Protein concentration was determined (Bradford reagent, BioRad), and 30 μg protein were resolved by SDS-PAGE under reducing conditions. After electrophoresis, proteins were transferred to polyvinylidene fluoride membrane (BioRad) and probed with the appropriate antibody followed by horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) and an enhanced chemiluminescent substrate (Pierce), as described.18,19

Immunostaining

Staining of formalin-fixed, paraffin-embedded 5-μm sections was performed essentially as described.18,20 Briefly, slides were deparaffinized, rehydrated, and endogenous peroxidase activity was quenched (30 minutes) by 3% hydrogen peroxide in methanol. Slides were then subjected to antigen retrieval by boiling (20 minutes) in 10 mM/L citrate buffer, pH 6.0. After washes with PBS, slides were incubated with 10% normal goat serum in PBS for 60 minutes to block non-specific binding, and incubated (20 hours, 4°C) with antiheparanase polyclonal antibody (#733),21 diluted 1:100 in blocking solution. Antibody 733 was raised in rabbits against a 15 amino acid peptide (KFKFNSTYSKSSVDC) that maps at the N terminus of the 50-kDa human heparanase subunit and preferentially recognizes the 50-kDa heparanase subunit versus the 65-kDa latent proenzyme.21 Slides were extensively washed with PBS containing 0.01% Triton X-100 and
incubated with a secondary reagent (Envision kit), according to the manufacturer’s (Dako) instructions. After additional washes, color was developed with the AEC reagent (Dako), sections were counterstained with hematoxylin and mounted, as described.19,20

**Patients**

In the study, 106 patients were included. Inclusion criteria were patients with MI according to clinical grounds and troponin I elevation, with or without electrocardiogram changes 2 to 10 hours from symptoms onset (n=50); patients with stable angina (SA) and angiographic chronic lesion admitted for coronary angiography (n=38); and healthy subjects (n=18). Informed consent was obtained from all individuals, with the approval of the Rambam Ethical Committee. Patients that have been treated with anticoagulation or thrombolytic therapy before the enrollment, patients with any sign of interfering noncardiac disease, such as inflammation, malignancy, infection, and surgery or trauma, in the past 4 weeks were excluded. Coronary plaques were identified by angiography, according to previously used criteria: thrombus, plaque ulceration, irregularity, and impaired flow.23 Eccentric irregular lesion with a thrombus was concluded in 64% of the patients. In addition, 46% of the patients had thrombolytic in myocardial infarction flow 0 on admission, thus indicating the existence of unstable plaque that became vulnerable.

**Venous Blood Samples and Heparanase Determination**

Blood was collected in EDTA-containing tubes on admission from patients with SA and acute MI. A second blood sample was taken 3 to 5 days after admission from patients with acute MI. Blood samples were taken from a cuffed antecubital vein. Blood samples were placed immediately on ice; plasma was separated by centrifugation (5 minutes, 1200g, 4°C), and samples were kept at −70°C, until analyzed. Heparanase levels in the plasma of patients with MI, SA, and healthy control individuals were determined, according to a previously described ELISA method.24,25

**Pathological Evaluation**

Coronary specimens were obtained from postmortem analyses of patients with clinical and pathologic manifestation of acute MI, patients with clinical and pathologic diagnosis of SA, and relatively normal coronary specimens. The lesions were classified into categories of plaque progression as proposed by Virmani et al2: VP with thin fibrous cap infiltrated by macrophages and lymphocytes with rare smooth muscle cells (SMC) and an underlying necrotic core with or without thrombus and intraplaque hemorrhage, and stable plaque with collagen-rich plaque, areas of calcification, few inflammatory cells, or SMC in a proteoglycan-rich matrix with areas of extracellular lipid accumulation, with or without necrosis.

**Statistics**

Results are shown as means±SD or ±SE. The significance of paired data was determined by t test; χ² or Fisher exact test for comparing categorical variables. The Mann–Whitney and Kruskal–Wallis tests were used to test for statistical differences in continuous nonparametric parameters. Correlation between the number of macrophages and percentage of heparanase staining was determined by Spearman coefficient of correlation. A value of P<0.05 was considered statistically significant.

**Results**

**Heparanase Elicits Cytokines Expression in J774 Cell Line and Mouse Peritoneal Macrophages**

We have purified the latent 65-kDa heparanase protein to homogeneity by heparin chromatography (Figure I in the online-only Data Supplement). Addition of this heparanase preparation (5 μg/mL) to the culture medium of J774 mouse macrophage-like cells resulted in marked increase in MCP-1 levels (Figure 1B; J774; P=0.05), in agreement with increased MCP-1 expression in injured neointima of transgenic mice overexpressing heparanase.26 Heparanase addition elicited even higher increase in TNFα (Figure 1A; J774; P=0.0001) and IL-1 (Figure 1D; J774; P=0.004) levels in a dose-dependent manner (Figure II A and II B in the online-only Data Supplement), whereas MMP-9 was elevated to a lesser degree (Figure 1C; P=0.0001). To further substantiate these results, heparanase was similarly added to primary macrophages isolated from the peritoneum of thioglycolate-treated mice, resulting in a comparable increase in the levels of TNFα, MCP-1, MMP-9, and IL-1 (Figure 1A–AD; MPMs). Marked increase of TNFα (Figure IIC in the online-only Data Supplement) and IL-1 (data not shown) was similarly obtained after addition of heparanase to monocytes isolated from human peripheral blood. Indistinguishable increase was observed after the addition of mutated (glutamic acid residues 225 and 343),27 enzymatically inactive heparanase to J774 cells (Figure 2A; DM), thus strongly implying that HS-degrading activity is not required for cytokines elevation by heparanase. Moreover, inclusion of heparin, which inhibits the catalytic activity of heparanase and its binding to HS,28 together with heparanase did not interfere with cytokine induction (Figure IIIA and IIIB in the online-only Data Supplement), suggesting that neither enzymatic activity nor association with HS are required for this function of heparanase. Notably, induction of TNFα (Figure 2A; lower panel), MCP-1, and IL-1 (not shown) was abolished after preincubation of heparanase with pronase, conferring the protein nature of the inducing component. Pronase (Figure 2A; lower panel) or BSA (data not shown) by themselves had no effect on cytokine expression. RT-PCR analyses revealed that the observed increase in cytokine levels in the cell-conditioned medium (Figures 1 and 2A) is because of enhanced gene transcription of MMP-9 (Figure 2B) and TNFα (Figure 2C). TNFα transcription was similarly induced by the heparanase C terminus protein domain (8C; Figure 2C) shown previously to mediate signaling properties of heparanase,21,29 further supporting this mode of action. To ascertain that the induction of TNFα and MMP-9 is not a result of contamination in the heparanase preparations, J774 were transiently transfected with heparanase gene constructs, total RNA was extracted after 24 hours and subjected to PCR analysis. Overexpression of wild-type (Hepa), enzymatically inactive (DM), and heparanase-signaling domain (8C) markedly stimulated the expression of TNFα (Figure 2D; third panel) and MMP-9 (Figure 2D; fourth panel) compared with control cells (Vo). These results clearly imply that overexpression or exogenous addition of heparanase activates macrophages and stimulates the transcription of selected genes.

**Signaling Pathways Underlying Cytokine Induction by Heparanase**

To appreciate signaling pathways that may be involved in cytokine gene regulation, heparanase was added to J774 cells and lysate samples were subjected to immunoblotting applying phosphospecific antibodies. Heparanase stimulated the phosphorylation of Akt (Figure 3A; left panel) and extracellular
mitogen-activated protein kinase signaling pathways. Addition of the 8C heparanase protein yielded similar results (not shown). Although J774 cells markedly respond to heparanase addition (Figure 3B; Hepa), elevation of TNFα, MCP-1, and IL-1β was practically diminished in cells that were pretreated with selective mitogen-activated protein kinase (PD; Figure 3B) or phosphatidylinositol-3 kinase (LY; Figure 3B) inhibitors, before the addition of heparanase (Figure 3B) or 8C (Figure III C and IIID in the online-only Data Supplement) proteins, with MMP-9 being somewhat less susceptible to PD and LY treatment (Figure 3B, lower right panel; Table I in the online-only Data Supplement). RT-PCR analysis further showed that TNFα induction is attenuated in cells that were pretreated with PD and LY, before the addition of heparanase (Figure 3C).

We have next examined the possible involvement of NFκB in the induction of cytokines by heparanase, as this pathway is highly implicated in cytokine regulation associated with atherosclerosis and VP.30,31 We found that cytokine elevation was markedly reduced in cells that have been preincubated with BAY117082, a selective inhibitor of NFκB, before addition of heparanase (Figure 4A; Hepa+Bay). Similarly, IL-1β induction by heparanase was abolished in cells that were treated with BAY117082 (Figure 4A, lower panel; Hepa+Bay; Table II in the online-only Data Supplement). In line with these results, we found that heparanase enhances the phosphorylation of IκB signal-regulated kinase (Figure 3A; right panel) in a time-(Figure 3A) and dose (not shown)-dependent manner, signifying activation of the phosphatidylinositol-3 kinase and

**Figure 1.** Increased cytokine levels after heparanase addition. Primary mouse macrophages (MPMs) and J774, a mouse macrophage cell line, were incubated without (white bars) or with heparanase (5 μg/mL; black bars). Medium was collected after 24 hours and the levels of tumor necrosis factor (TNF)-α (A), MCP-1 (B), matrix metalloproteinase (MMP)-9 (C), and interleukin (IL)-1 (D) were quantified by ELISA.

**Figure 2.** Elevation of cytokine levels by heparanase does not require heparan sulfate (HS)-degrading activity and involves increased gene transcription. A, J774 macrophages were left untreated as control (Con) or were incubated with heparanase (Hepa; 5 μg/mL) or mutated, enzymatically inactive heparanase (mutated at glutamic acids residues 225 and 343; DM, 5 μg/mL). Tumor necrosis factor (TNF)-α (upper panel), MCP-1 (second panel), and interleukin (IL)-1 (third panel) levels were determined as above. TNFα levels were similarly quantified in control untreated cells, cells that were incubated with pronase, and cells incubated with heparanase, before (Hepa) and after its preincubation with pronase (Hepa+pronase; lower panel). Note lack of TNFα elevation after treatment with pronase. B and C, PCR analysis. J774 cells were left untreated (C) or were incubated with heparanase (Hepa, left panel) or 8C domain (8C, right panel); total RNA was extracted after 6 hours and subjected to RT-PCR analyses, applying primers specific for TNFα (third panel), MMP-9 (fourth panel), and GAPDH (lower panel). D, Transfection. J774 cells were transiently transfected with an empty vector (Vo), wild-type heparanase (Hepa), heparanase mutated at glutamic acid residues 225 and 343 critical for catalysis (DM), or the heparanase C terminus domain (8C). Total RNA was extracted after 24 hours and subjected to RT-PCR analysis, applying primers specific for heparanase (amplifying wild-type and DM transcripts, upper panel), 8C (second panel), TNFα (third panel), MMP-9 (fourth panel), and GAPDH (lower panel).
Of note, lipopolysaccharide stimulated marked induction of cytokines in untreated control cells (Con.; Figure 4D; middle panel). Induction of MMP-9 (Figure 4D; middle panel) and cytokines in untreated control cells (Con.; Figure 4D; middle panel) were similarly abolished in TLR-4–deficient cells, possibly because of high levels of these cytokines in untreated control cells (Con.; Figure 4D; middle and lower panels; Table III in the online-only Data Supplement). Notably, heparanase failed to stimulate TNFα elevation in Ly- and cytokine levels were quantified as pg/mL (0) or were incubated with heparanase addition. Vehicle was added as control (dimethyl sulfoxide). Culture medium was collected after 6 hours and subjected to RT-PCR, applying primers for TNFα (upper panel) or GAPDH (lower panel). Table I in the online-only Data Supplement summarizes the statistical analyses.

Clinical Relevance
To reveal the clinical relevance of these results, we examined by immunostaining heparanase expression in specimens of stable plaque and VP compared with control arteries. Histological features typical of VP are presented in the online-only Data Supplement Figure V. Weak staining of heparanase was observed in the media of control (Figure 5E–5H). Morphometric analysis revealed a significant increase in the staining percentage for heparanase in specimens of VP, as compared with specimens of stable plaque. Mean optical density (255-pixel gray level) was significantly higher in VP compared with stable plaque (Table IV in the online-only Data Supplement). To identify heparanase-positive cells in the intima of VP, specimens were double stained for CD163, a macrophage cell surface marker (Figure 5I, red), and heparanase (Figure 5J, green). Merge image (Figure 5K) suggests that most of the plaque-residing cells stained positively for heparanase are macrophages. Notably, heparanase staining was significantly associated with macrophage content (Spearman coefficient of correlation; R=0.72, P=0.01). We next evaluated the levels of heparanase in the plasma of patients with acute MI, SA, and healthy subjects by ELISA. Demographic and clinical description of patients enrolled is summarized in Table I. Heparanase levels were increased nearly 9-fold in patients with acute MI, and 3-fold in patients with SA as compared with healthy subjects (71, 237, and 620 pg/mL for control, SA, and acute MI, respectively; Figure 5L), increase that is...
statistically highly significant ($P=0.0006$). Subsequently, on day 3 to 5, after the admission with acute MI, mean heparanase levels were reduced significantly (569 pg/mL versus 233 pg/mL during admission and postadmission, respectively).

We next compared the clinical manifestation of patients exhibiting low (<320 pg/mL) versus high (>320 pg/mL; 2 standard deviation above the level of heparanase in healthy subjects) levels of plasma heparanase. High levels of heparanase were associated with acute MI and elevated white blood cell count (Table 2). Heparanase may thus be considered diagnostic marker and potentially therapeutic target in acute heart diseases.

**Discussion**

Coronary artery disease continues to be a major cause of morbidity and mortality throughout the world. It is now evident that activation of the atherosclerotic plaque rather than stenosis causes ischemia and infraction. Major advance in prevention of the disease will thus require early detection of rupture-prone, VP. The results of this study imply that heparanase levels are associated with plaque vulnerability and progression.

Several lines of evidence tie heparanase with atherosclerosis. Heparanase expression was increased after balloon or stent injury in rabbit and rat models, correlating with increased neointimal thickness. Likewise, increased fatty streaks deposition and arterial thickness was observed in transgenic mice overexpressing heparanase. Furthermore, increased heparanase expression and activity was noted in lesions with thin cap fibroatheroma and in macrophages treated with oxidized low-density lipoprotein or angiotensin. Noteworthy, macrophages appeared more abundant in the neointimal lesion of...
heparanase transgenic mice and the arterial lysate contained higher levels of MCP-1.\textsuperscript{26} Here, we provide evidence for the clinical significance of heparanase in atherosclerosis. We found an abundant immunostaining of heparanase in the intima of VP compared with stable plaque (Figure 5), associated with a marked increase of heparanase levels in the plasma of patients with acute MI (Figure 5L). Moreover, most patients (74%) harboring high levels of plasma heparanase (ie, >320 pg/mL) experienced acute MI (Table 2), further delineating a casual association between heparanase and infraction. Noteworthy, presence of high levels of heparanase systemically may further damage the vasculature. For example, accumulating evidence suggest that heparanase functions also as a procoagulation mediator, enhancing expression of tissue factor and generation of factor Xa, 2 critical components in blood coagulation,\textsuperscript{38–40} thus providing another mode by which heparanase affects the vasculature, in general, and plaque development and progression, in particular.

Extensive research in the last decade has shown that inflammation plays a key role in coronary artery disease. Activated immune cells are abundant at sites of rupture and produce numerous inflammatory molecules and proteolytic enzymes that transform the stable plaque into vulnerable, unstable structure.\textsuperscript{3,34,41,42} Notably, heparanase staining colocalized in part, to CD163-positive cells (appears yellow–orange in K). Original magnification, ×63. L, Elevation of heparanase levels in the plasma of atherosclerotic patients. Plasma samples of control healthy donors (Con) and patients exhibiting stable angina (SA) or acute myocardial infarction (AMI) were collected on admission, as described in Materials and Methods, and heparanase levels were quantified by ELISA. Of note, marked increase of heparanase levels in patients exhibiting SA, and even more so in patients exhibiting acute MI ($P$=0.0006).
not only functions to recruit, but also activates macrophages. Clearly, the expression of TNFα, MCP-1, and IL-1, hallmark of cytokine associated with macrophage activation and plaque progression,3,34,41,42 was markedly increased following exogenous addition (Figures 1 and 2) or overexpression (Figure 2D) of heparanase variants. We have also found that macrophages, and especially activated macrophages (ie, phorbol 12-myristate 13-acetate-treated), exhibit high-heparanase activity (Figure 2B; Figure IIIC) that can also get secreted (Figure VIB in the online-only Data Supplement). Altogether, these results imply that macrophages exhibit heparanase activity that can be detected inside and outside the cells, resulting in macrophage activation in an autocrine manner. Macrophages can also get activated by heparanase originating from other cell types residing in the atherosclerotic plaque. For example, treatment of human microvascular endothelial cells with the proinflammatory cytokines, TNFα and IL-1β, resulted in a marked increase of heparanase secretion.44 This suggests cooperation between cellular compartments of the atherosclerotic plaque, in which cytokines secreted by activated macrophages (ie, TNFα) stimulate the secretion of heparanase from endothelial cells, leading to further augmentation of macrophage activation. Subsequently, latent and active heparanase secreted by macrophages, together with enzymatic activity responsible for proteolytic cleavage of protein constituents of the ECM (ie, MMPs), likely cooperate in remodeling the ECM, leading to plaque rupture. Induction of MMP-9 expression by macrophages after heparanase overexpression or exogenous addition (Figure 2B and 2D) is in agreement with MMP-9 induction in myeloma cells overexpressing heparanase49 and suggests an intimate cooperation between the 2 distinct enzymatic activities in cancer, inflammation, and atherosclerosis. Endothelial and vascular SMC are 2 other cellular constituents of the atherosclerotic plaque. Heparanase exerts strong angiogenic response, and elevation of microvessel density correlated with heparanase induction in solid11 and hematological malignancies, in tumor xenografts produced by cells overexpressing heparanase47-50 and in heparanase-treated wounds.51,52 Heparanase was also noted to accelerate the proliferation of SMC53 implying that this enzyme affects all major cellular components (ie, endothelial cells, vascular smooth muscle cells, macrophages) of the atherosclerotic lesion.

The molecular mechanism underlying cytokine induction by heparanase is not entirely clear but emerges to involve TLRs. TLRs are family of type I transmembrane proteins that bind and are activated by a range of bacterial products, leading to downstream signaling via NFκB that activates the transcription of proinflammatory mediators, such as TNFα, interferon, MCP-1, and IL-1β. TLR-2 and TLR-4 are expressed by macrophages and are the best characterized in terms of their contribution to macrophages and are the best characterized in terms of their contribution to atherosclerotic lesion development.55 Cytokine induction by heparanase appears to involve TLR-2, TLR-4, and NFκB. Activation of NFκB by heparanase is revealed by decreased cytokine induction in cells treated with NFκB inhibitor (Bay; Figure 4A); increased phosphorylation of IκB and its subsequent degradation (Figure 4B; upper panels); and higher amounts of p65 in the nuclear fraction after addition of heparanase (Figure 4B; lower panels). The involvement of TLRs is concluded, as TNFα elevation was markedly attenuated in cells treated with MyD88 inhibitor (Figure 4C; right), neutralizing anti–TLR-2 antibody, or anti–TLR-2 and anti–TLR-4 neutralizing antibodies (Figure 4C; left) before heparanase addition. Even more striking were the results using MPM derived from TLR-knockout mice. Clearly, induction of TNFα, MMP-9, and MCP-1 by heparanase was not seen in MPM deficient for TLR-2 or TLR-2 and TLR-4 (Figure 4D). Interestingly, whereas TNFα was not induced by heparanase in TLR-4–deficient cells, MMP-9 and MCP-1 induction in these cells was indistinguishable from control cells (Figure 4D). This is, apparently, because of a marked increase of MMP-9 and MCP-1 levels in control, untreated TLR-4–deficient cells (Figure 4D; middle and lower panels; Con). The reason for increased cytokine levels in TLR-4–deficient macrophages is unclear but may result from as yet uncharacterized compensatory effect. The molecular mechanism leading to TLR activation by heparanase is largely obscure but appears not to involve binding or degradation of HS. This is concluded because induction of cytokines indistinguishable in magnitude was observed after the addition or overexpression of mutated enzymatically inactive heparanase (DM; Figure 2A and 2D), or its C terminus domain thought to be held responsible for signaling properties of heparanase (8C; Figure 2C and 2D; Figure IIIC

### Table 1. Demographic and Clinical Description of Patients Enrolled

<table>
<thead>
<tr>
<th>Value</th>
<th>MI (n=50)</th>
<th>SA (n=38)</th>
<th>P Value (MI vs. SA)</th>
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<tr>
<td>Age, mean±SD</td>
<td>58±14.7</td>
<td>64.6±7.7</td>
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<td>Men, %</td>
<td>86%</td>
<td>89%</td>
<td>N/S</td>
</tr>
<tr>
<td>HTN, %</td>
<td>50%</td>
<td>81.5%</td>
<td>0.002</td>
</tr>
<tr>
<td>DM, %</td>
<td>16%</td>
<td>42%</td>
<td>0.006</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>58%</td>
<td>31.6%</td>
<td>0.01</td>
</tr>
<tr>
<td>IHD, %</td>
<td>28%</td>
<td>58%</td>
<td>0.04</td>
</tr>
<tr>
<td>LDL, mean±SD</td>
<td>121±42.4</td>
<td>102±44.2</td>
<td>0.09</td>
</tr>
<tr>
<td>FH, %</td>
<td>24%</td>
<td>18.4%</td>
<td>N/S</td>
</tr>
<tr>
<td>HDL, %</td>
<td>27%</td>
<td>58%</td>
<td>0.04</td>
</tr>
<tr>
<td>MI, %</td>
<td>18%</td>
<td>42%</td>
<td>0.01</td>
</tr>
<tr>
<td>Cr, mean±SD</td>
<td>1.3±0.8</td>
<td>6.9±3.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>WBC, mean±SD</td>
<td>12.7±2.7</td>
<td>5±1.4</td>
<td>N/S</td>
</tr>
</tbody>
</table>

MI indicates myocardial infarction; SA, stable angina; HTN, hypertension; DM, diabetes mellitus; LDL, low-density lipoprotein; FH, family history of ischemic heart disease; IHD, ischemic heart disease; Cr, serum creatinine level; and WBC, white blood cell count.

### Table 2. Characteristics of Atherosclerotic Patients With Low Versus High Levels of Heparanase

<table>
<thead>
<tr>
<th>Heparanase &gt;320 pg/mL (n=23)</th>
<th>Heparanase &lt;320 pg/mL (n=65)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean±SD</td>
<td>56±12.4</td>
<td>61±11.3</td>
</tr>
<tr>
<td>LDL, mean±SD</td>
<td>130±37.8</td>
<td>96±24.5</td>
</tr>
<tr>
<td>WBC, mean±SD</td>
<td>11.7±3.4</td>
<td>9.8±3.7</td>
</tr>
<tr>
<td>Trl, mean±SD</td>
<td>15.4±17.7</td>
<td>14.4±10.7</td>
</tr>
<tr>
<td>MI, %</td>
<td>74%</td>
<td>26%</td>
</tr>
</tbody>
</table>

LDL indicates low-density lipoprotein; WBC, white blood cell count; Trl, troponin I; and MI, myocardial infarction.
and IIID in the online-only Data Supplement). Moreover, increased TNFα and IL-1 levels were noted when heparanase was added together with heparin (Figure IIIA and IIIB in the online-only Data Supplement). Heparin is not only a strong heparanase inhibitor, but also prevents its association with cell membrane HSPG (ie, syndecans), thus implying that cytokine induction does not involve clustering and activation of cell membrane HSPG. A growing body of evidence now suggests that TLR signaling is elicited in the absence of infection through endogenous ligands generated at sites of tissue remodeling and inflammation. Of note, ECM components and their degradation products generated during tissue injury were added together with heparin (Figure IIIA and IIIB in the online-only Data Supplement). Examples are hyaluronic acid, decorin, and soluble biglycan recognized as ligands for TLR-2 and TLR-4, and veriscan that activates tumor-infiltrating myeloid cells through TLR-2 and its coreceptors, TLR-6 and CD14. Thus, although activation of TLRs does not require binding or cleavage of HSPG, heparanase may activate TLRs by introducing conformational changes in cell membrane HSPG (ie, syndecans or glypicans) after their clustering and activation or by HS-cleavage products, in addition to its HS-independent function.

Taken together, the results provide indication for the clinical relevance of heparanase in plaque progression and rupture, and identify TLR family members as mediators of this function. The signal transduction initiated by heparanase is transmitted to the cell nucleus, actively inducing the transcription of proinflammatory genes that further fuels the inflammatory reaction. Heparanase inhibitors (ie, nonanticoagulant, glycol-split heparin) are thus expected to attenuate plaque progression. A proof-of-principle of this notion emerges by the ability of antiheparanase antibody delivered locally to inhibit neointima formation.

Acknowledgments

We thank Prof M. Aviram and Dr B. Fuhrman (Rappaport Faculty of Medicine, Technion) for their continuous support, advice and active collaboration, and Prof Vivian Barak (Hadassah-Hebrew University Medical Center) for the help in performing the experiment with human peripheral blood monocytes. A manuscript (Osterholm et al., 2013) supporting the involvement of heparanase in atherosclerosis and plaque instability, appeared just after the final acceptance of the current manuscript.

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Disclosures

None.

References


Macrophage Activation by Heparanase Is Mediated by TLR-2 and TLR-4 and Associates With Plaque Progression


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A

B

C

Blich et al
Suppl. Fig. II
A. 

![Graph showing TNFα levels](image)

- Control (Con)
- Hepa
- Hepa + heparin

P = 0.0001 (n = 14) 
ns (n = 3)

B. 

![Graph showing IL-1 levels](image)

- Control (Con)
- Hepa
- Hepa + heparin

p = 0.004 (n = 14) 
ns (n = 3)

C. 

![Graph showing MCP-1 levels](image)

- 8C + Ly
- 8C + PD
- 8C
- DMSO
- Control (Con)

D. 

![Graph showing TNFα levels](image)

- 8C + Ly
- 8C + PD
- 8C
- DMSO
- Control (Con)

Blich et al
Suppl. Fig. III
A

![Graph A: TNFα (pg/ml) vs LPS (ng/ml)]

- WT
- TLR2 KO
- TLR4 KO

B

![Graph B: TNFα (pg/ml)]

- WT
- TLR2 KO
- TLR4 KO

Blich et al,
Suppl. Fig. IV
Massive inflammatory infiltrates

Wall necrosis

Lumen

Thin fibrous cap

Underlying necrotic area with macrophages

Deeper zone in the plaque showing cholesterol clefts

Inflammatory cells

Fibrin

Blich et al,
Suppl. Fig. V
Blich et al
Suppl. Fig. VI
### Suppl. Table I. Figure 3B and Suppl. Figure IIIC, D summary of statistics

<table>
<thead>
<tr>
<th></th>
<th>p</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TNF-α</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con vs. Hepa</td>
<td>0.0001</td>
<td>14</td>
</tr>
<tr>
<td>Con vs. 8c</td>
<td>0.0001</td>
<td>5</td>
</tr>
<tr>
<td>PD+Hepa vs. Hepa</td>
<td>0.005</td>
<td>3</td>
</tr>
<tr>
<td>PD+8c vs. 8c</td>
<td>0.005</td>
<td>3</td>
</tr>
<tr>
<td>LY+Hepa vs. Hepa</td>
<td>0.03</td>
<td>3</td>
</tr>
<tr>
<td>LY+8c vs. 8c</td>
<td>0.03</td>
<td>3</td>
</tr>
<tr>
<td><strong>MCP-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con vs. Hepa</td>
<td>0.05</td>
<td>3</td>
</tr>
<tr>
<td>Con vs. 8c</td>
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<td>3</td>
</tr>
<tr>
<td>PD+Hepa vs. Hepa</td>
<td>0.07</td>
<td>3</td>
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<tr>
<td>PD+8c vs. 8c</td>
<td>0.05</td>
<td>3</td>
</tr>
<tr>
<td>LY+Hepa vs. Hepa</td>
<td>0.05</td>
<td>3</td>
</tr>
<tr>
<td>LY+8c vs. 8c</td>
<td>0.02</td>
<td>3</td>
</tr>
<tr>
<td><strong>IL-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con vs. Hepa</td>
<td>0.0001</td>
<td>5</td>
</tr>
<tr>
<td>PD+Hepa vs. Hepa</td>
<td>0.09</td>
<td>4</td>
</tr>
<tr>
<td>LY+Hepa vs. Hepa</td>
<td>0.03</td>
<td>4</td>
</tr>
<tr>
<td><strong>MMP-9</strong></td>
<td></td>
<td></td>
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<tr>
<td>Con vs. Hepa</td>
<td>0.0001</td>
<td>6</td>
</tr>
<tr>
<td>PD+Hepa vs. Hepa</td>
<td>0.01</td>
<td>4</td>
</tr>
<tr>
<td>LY+Hepa vs. Hepa</td>
<td>0.01</td>
<td>4</td>
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</table>

See figure legend for more details.
**Suppl. Table II.** Figure 4A summary of statistics

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<tr>
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</thead>
<tbody>
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<td><strong>TNF-α</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con vs. Hepa</td>
<td>0.0005</td>
<td>6</td>
</tr>
<tr>
<td>Con vs. Hepa+bay</td>
<td>N/S</td>
<td>6</td>
</tr>
<tr>
<td>Hepa vs. Hepa+bay</td>
<td>0.0005</td>
<td>6</td>
</tr>
<tr>
<td><strong>MMP-9</strong></td>
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<td></td>
</tr>
<tr>
<td>Con vs. Hepa</td>
<td>0.005</td>
<td>3</td>
</tr>
<tr>
<td>Con vs. Hepa+bay</td>
<td>N/S</td>
<td>3</td>
</tr>
<tr>
<td>Hepa vs.Hepa+bay</td>
<td>0.007</td>
<td>3</td>
</tr>
<tr>
<td><strong>IL-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con vs. Hepa</td>
<td>0.003</td>
<td>3</td>
</tr>
<tr>
<td>Con vs. Hepa+bay</td>
<td>0.004</td>
<td>3</td>
</tr>
<tr>
<td>Hepa vs. Hepa+bay</td>
<td>0.006</td>
<td>3</td>
</tr>
<tr>
<td><strong>MCP-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con vs. Hepa</td>
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<td>3</td>
</tr>
<tr>
<td>Con vs. Hepa+bay</td>
<td>0.002</td>
<td>3</td>
</tr>
<tr>
<td>Hepa vs. Hepa+bay</td>
<td>0.0001</td>
<td>3</td>
</tr>
</tbody>
</table>

N/S-non significant

See figure legend for more details.
**Suppl. Table III.** Figure 4D summary of statistics

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</tr>
</thead>
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<td><strong>TNF-α</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepa TLR2/- vs. WT Hepa</td>
<td>0.0001</td>
<td>4</td>
</tr>
<tr>
<td>DM TLR2/- vs. WT DM</td>
<td>0.002</td>
<td>3</td>
</tr>
<tr>
<td>Hepa TLR4/- vs. WT Hepa</td>
<td>0.0001</td>
<td>4</td>
</tr>
<tr>
<td>DM TLR4/- vs. WT DM</td>
<td>0.001</td>
<td>3</td>
</tr>
<tr>
<td>Hepa TLR2,4/- vs. WT Hepa</td>
<td>0.0001</td>
<td>4</td>
</tr>
<tr>
<td>DM TLR2,4/- vs. WT DM</td>
<td>0.001</td>
<td>3</td>
</tr>
<tr>
<td><strong>MMP-9</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepa TLR2/- vs. WT Hepa</td>
<td>0.0001</td>
<td>4</td>
</tr>
<tr>
<td>DM TLR2/- vs. WT DM</td>
<td>0.02</td>
<td>3</td>
</tr>
<tr>
<td>Hepa TLR4/- vs. WT Hepa</td>
<td>0.003</td>
<td>4</td>
</tr>
<tr>
<td>DM TLR4/- vs. WT DM</td>
<td>N/S</td>
<td>3</td>
</tr>
<tr>
<td>Hepa TLR2,4/- vs. WT Hepa</td>
<td>0.004</td>
<td>4</td>
</tr>
<tr>
<td>DM TLR2,4/- vs. WT DM</td>
<td>0.02</td>
<td>3</td>
</tr>
<tr>
<td><strong>MCP-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepa TLR2/- vs. WT Hepa</td>
<td>0.0001</td>
<td>4</td>
</tr>
<tr>
<td>DM TLR2/- vs. WT DM</td>
<td>0.009</td>
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<tr>
<td>Hepa TLR4/- vs. WT Hepa</td>
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</tr>
<tr>
<td>DM TLR4/- vs. WT DM</td>
<td>N/S</td>
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</tr>
<tr>
<td>Hepa TLR2,4/- vs. WT Hepa</td>
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<td>4</td>
</tr>
<tr>
<td>DM TLR2,4/- vs. WT DM</td>
<td>0.008</td>
<td>3</td>
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</tbody>
</table>

N/S-non significant

See figure legend for more details
**Suppl. Table IV.** Morphometric analysis of heparanase staining in specimens of VP compared to specimens of SP and control (N)

<table>
<thead>
<tr>
<th></th>
<th>VP (n= 10)</th>
<th>SP (n= 4)</th>
<th>N (n= 6)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining Percent (mean± SD)</td>
<td>3.7 ± 2.5</td>
<td>0.6 ± 0.4</td>
<td>1.2 ± 1.8</td>
<td>*p = 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>p = 0.04</strong></td>
</tr>
<tr>
<td>Optical density (mean ± SD)</td>
<td>156 ± 15.5</td>
<td>131 ± 18.8</td>
<td>153 ± 12.04</td>
<td>*p = 0.02</td>
</tr>
</tbody>
</table>

VP- vulnerable plaque; SP- stable plaque, N- normal coronaries. *p= VP vs. SP  
**p= VP vs. N


Supplementary Figure Legends

Suppl. Figure I. Heparanase purification. Latent 65 kDa heparanase was purified from serum-free medium conditioned by heparanase-transfected CHO cells. Cells were grown to confluence and were then cultured in serum-free medium for 24 h. Medium was collected, filtered (0.45 micron) and applied onto a HiTrap heparin column (Pharmacia) equilibrated with 20 mM phosphate buffer, pH 6.0. Following washes (15 column volumes), bound material was eluted with a linear gradient of NaCl in 20 mM phosphate buffer (pH 6.0). Eluted fractions were analyzed by gradient SDS-PAGE followed by Gelcode (Pierce, Rockford, IL, USA) staining. A single, highly purified protein band was obtained in fractions eluted with 0.7-1 M NaCl (A7-A12) and used for all subsequent experiments.

Suppl. Figure II. Dose response. J774 cells (A, B) or human monocytes isolated from peripheral blood of healthy volunteers (C) were left untreated (Con) or were incubated with the indicated concentration of heparanase. Medium was collected after 24 h and the levels of TNFα (A, C) and IL-1 (B) were quantified by ELISA.

Suppl. Figure III. A, B. Heparin treatment. J774 cells were left untreated (Con) or were incubated with heparanase (5 µg/ml) in the absence (Hepa) or presence of heparin (50 µg/ml; Hepa+heparin). Medium was collected after 24 h and the levels of TNFα (A) and IL-1 (B) was quantified by ELISA. C, D. MAPK and PI3-K inhibitors. J774 cells were pretreated with LY 294002 (Ly; 20 μM) or PD 98059 (PD; 30 μM), selective inhibitors of PI 3-kinase and MAPK, respectively, for 30 min prior to the addition of heparanase C-domain (8C). Vehicle (DMSO) or medium alone (Con) were included as controls. Culture medium was collected after 20 h and TNFα (C) and MCP-1 (D) levels were quantified by ELISA.

Suppl. Figure IV. TLR2- and 4-deficient cells still respond to appropriate TLR ligands. Macrophages isolated from thioglycolate-treated wild type (WT, blue), TLR2−/− (red), and TLR4−/− (green) mice were incubated (37°C, 24 h) with the indicated concentrations of LPS and TNFα levels were quantified by ELISA (A). Cells were similarly treated with CpG (10 µg/ml) or PAM (2.5 µg/ml) and TNFα levels were quantified as above (B). Note marked increase in TNFα in TLR2−/−, but not in TLR4−/− cells, by LPS and an opposite effect following PAM treatment.
Suppl. Figure V. Histological feature of VP. A-C, Hematoxilin and Eosin staining. Shown are representative photomicrographs of VP obtained post mortem from patients who died from acute MI. Original magnification A x4; B x20; C x10.

Suppl. Figure VI. Macrophages exhibit heparanase activity. Macrophages were isolated from wild type mice and left untreated (Con; ■) or were stimulated with PMA (PMA; 100 ng/ml, ▲) for 24 h. Cell lysate (A) and medium (B) samples were then incubated (20 h, 37°C) with 35S-labeled ECM and labeled degradation products released into the incubation medium were analyzed by gel filtration, as described under 'Materials and Methods'. Note increased heparanase activity (higher amounts and small size of HS degradation fragments) in the cell lysate and conditioned medium following PMA stimulation.