

MIT Open Access Articles

Regulation of heparanase expression in coronary artery disease in diabetic, hyperlipidemic swine

The MIT Faculty has made this article openly available. **Please share** how this access benefits you. Your story matters.

Citation: Baker, Aaron B., Yiannis S. Chatzizisis, Roy Beigel, Michael Jonas, Benjamin V. Stone, Ahmet U. Coskun, Charles Maynard, et al. "Regulation of Heparanase Expression in Coronary Artery Disease in Diabetic, Hyperlipidemic Swine." *Atherosclerosis* 213, no. 2 (December 2010): 436–442.

As Published: <http://dx.doi.org/10.1016/j.atherosclerosis.2010.09.003>

Publisher: Elsevier

Persistent URL: <http://hdl.handle.net/1721.1/99184>

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

Terms of use: Creative Commons Attribution-Noncommercial-NoDerivatives





Published in final edited form as:

Atherosclerosis. 2010 December ; 213(2): 436–442. doi:10.1016/j.atherosclerosis.2010.09.003.

Regulation of Heparanase Expression in Coronary Artery Disease in Diabetic, Hyperlipidemic Swine

Aaron B. Baker^{1,*}, Yiannis S. Chatzizisis^{2,*}, Roy Beigel^{2,3}, Michael Jonas^{2,3}, Benjamin V. Stone³, Ahmet U. Coskun⁴, Charles Maynard⁵, Campbell Rogers⁶, Konstantinos C Koskinas², Charles L. Feldman², Peter H. Stone², and Elazer R. Edelman^{2,3}

¹ Department of Biomedical Engineering, University of Texas at Austin, Austin, TX

² Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

³ Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA

⁴ Mechanical and Industrial Engineering, Northeastern University, Boston, MA

⁵ Department of Health Services, University of Washington, Seattle, WA

⁶ Cordis Inc., Warren, NJ

Abstract

Objective—Enzymatic degradation of the extracellular matrix is known to be powerful regulator of atherosclerosis. However, little is known about the enzymatic regulation of heparan sulfate proteoglycans (HSPGs) during the formation and progression of atherosclerotic plaques.

Methods and Results—Swine were rendered diabetic through streptozotocin injection and hyperlipidemic through a high fat diet. Arterial remodeling and local endothelial shear stress (ESS) were assessed using intravascular ultrasound, coronary angiography and computational fluid dynamics at weeks 23 and 30. Coronary arteries were harvested and 142 arterial subsegments were analyzed using histomorphologic staining, immunostaining and real time PCR. Heparanase staining and activity was increased in arterial segments with low ESS, in lesions with thin cap fibroatheroma (TCFA) morphology and in lesions with severely degraded internal elastic laminae. In addition, heparanase staining colocalized with staining for CD45 and MMP-2 within atherosclerotic plaques. Dual staining with gelatinase zymography and heparanase immunohistochemical staining demonstrated colocalization of matrix metalloprotease activity with heparanase staining. A heparanase enzymatic activity assay demonstrated increased activity in TCFA lesions, subsegments with low ESS and in macrophages treated with oxidized LDL or angiotensin II.

Conclusions—Taken together, our results support a critical role for heparanase in the development of vulnerable plaques and suggest a novel therapeutic target for the treatment of atherosclerosis.

Correspondence to: Aaron B. Baker, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Building E25, Room 442, Cambridge, MA 02139, abbaker@mit.edu.

*These authors contributed equally to this work.

Disclosures

Research grants were received from Novartis Pharmaceuticals Inc. and Boston Scientific Inc.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

heparanase; endothelial shear stress; atherosclerosis; thin cap fibroatheromas

1. Introduction

During the course of atherosclerotic disease progression, localized plaques develop throughout the vascular system. While the vast majority of these lesions remain stable, some undergo changes that make them vulnerable to rupture[1]. This subset of vulnerable plaques have morphology in which the atherosclerotic remodeling and inflammatory process has created a thin cap of fibrous tissue over a lipid rich and metabolically active core. These plaques are prone to acute rupture and are primarily responsible for the development of acute coronary syndrome and sudden cardiac death[2]. A question of paramount importance is the specific biological and local vascular factors that cause some atheromas to become prone to rupture while others remain stable. The past decades of intense research into the mechanisms of development of vulnerable atheromas has revealed a complex interplay among multiple aspects of the local vascular environment that ultimately lead to plaque destabilization. While the essential role of matrix metalloproteases (MMPs) in the atherosclerotic process is well recognized [3], little is known about the role of enzymatic regulation of glycosaminoglycans in the formation of atheroma with thin caps.

Proteoglycans are complex molecules consisting of a core protein post-translationally modified with glycosaminoglycan carbohydrate chains. The proteoglycans were one of the earliest class of molecules identified to be associated with atherosclerotic lesions and lipid deposition in the vascular wall [4,5]. Proteoglycans with predominantly chondroitin or dermatan sulfate chains can retain low density lipoprotein (LDL) and are considered atherogenic[6]. However the role of heparan sulfate proteoglycans (HSPGs) in atherogenesis is less clear. Proteoglycans bearing heparan sulfate chains have often been considered anti-atherogenic[7] due to their ability to inhibit monocyte adhesion[8] and vascular smooth muscle cell growth[9,10]. However, recent studies in mice with heparan sulfate deficient perlecan have indicated that heparan sulfate can have pro-atherogenic effects in mouse models of atherosclerosis as well[11,12].

Heparanase is an endo- β -D-glucuronidase that cleaves a specific motif in heparan sulfate to create fragments that are 10–20 sugar units long and still biologically active[13]. In this work, we provide in-vivo evidence that the expression of heparanase is highly regulated in atherosclerosis and development of vulnerable plaques. Here we demonstrate that heparanase protein, mRNA and activity are enhanced in regions of low endothelial shear stress (ESS) and during the progression of atheromas from intermediate lesions to advanced thin cap fibroatheromas (TCFAs).

2. Methods

2.1. Diabetic, hyperlipidemic swine model of atherosclerosis

All experimental protocols were approved by the Harvard University Animal Care and Use Committee. Twelve male Yorkshire swine were treated with intravenous infusion of 50 mg/kg streptozotocin once a day for three days to induce diabetes[14]. The animals were fed a high fat and cholesterol diet supplemented with sucrose in quantities to maintain total serum cholesterol between 500–700 mg/dL and blood glucose between 150–350 mg/dL[14]. Twelve animals were assigned to three different treatment groups (n=4 for each group) including placebo, Valsartan (320 mg per day) or Valsartan in combination with Simvastatin (40 mg per day). These treatments were added to ensure variation in plaques within the

segments examined included lesions from all the categories of severity. For analysis the groups were pooled and analyzed as described below and in the supplemental methods section. After 23 weeks, the animals underwent vascular profiling using intravascular ultrasound and coronary angiography for all of the major epicardial coronary arteries as described previously[15]. At week 30, vascular profiling was repeated and the animals were euthanized. The mean weight of pigs on the day of euthanasia was 66 ± 4 kg. The mean total cholesterol during the follow-up period was 611 ± 28 mg/dL, whereas the mean blood glucose was 230 ± 26 mg/dL. Coronary arteries were harvested, frozen in isopentane cooled with liquid N_2 and stored at $-80^\circ C$ until further analysis. One pig died prior to the second vascular profiling session and was excluded from the study. Consequently, a total of 31 coronary arteries from 11 pigs were analyzed. The total cholesterol levels among the treatment groups were placebo (610 ± 41 mg/dL), Simvastatin and Valsartan (564 ± 12 mg/dL) and Vasartan alone (647 ± 65 mg/dL). The blood glucose levels among the treatment groups were placebo (246 ± 53 mg/dL), Simvastatin and Valsartan (241 ± 60 mg/dL) and Vasartan alone (207 ± 42 mg/dL).

The lesions were classified into three categories of plaque progression as proposed by Virmani et al[1]: minimal (MIN) lesions with minimal lipid deposition and inflammatory cells; intermediate (INT) lesions with increased lipid accumulation, intima-media ratio of less than 0.15 and no fibrous cap; and TCFAs with inflamed fibrous cap overlying a necrotic lipid core. To be considered in the analysis, each subsegment of interest was required to be free of apparent atherosclerotic plaque at baseline, defined by IVUS as maximum intima-media thickness (IMT) ≤ 0.5 mm[15]. We defined 4–5 subsegments per artery to contain a range of segments with low and high endothelial shear stress. Each of these subsegments was 3 mm in length. Lesions with necrosis but without thin cap morphology were included in the intermediate category. Lesions were also classified for degradation of the inner elastin lamina (IEL) based on the following criteria: grade 0, intact IEL; grade 1, few breaks in IEL (10 or less breaks per field of view); grade 2, many breaks in IEL but with intact intima media layer (greater than 10 breaks per field of view); grade 3, severely fragmented IEL with degraded media[15]. Vascular profiling and computational analysis was also used to determine local ESS levels and classify the lesions in ESS categories (see supplemental section for details). From the available coronary arteries, 142 subsegments were analyzed with real time PCR, immunohistochemical staining, histochemical staining, zymography and a heparanase activity assay as described in the supplemental methods section.

2.2. Cell Culture and Western Blotting

A human monocyte cell line (THP-1, ATCC) was maintained in RPMI media with 10% Fetal Bovine Serum and mercaptoethanol. The cells were induced with Phorbol Myristate Acetate (PMA) for five days to induce differentiation into macrophages. The cells were treated with 50 μ g/ml LDL, 50 μ g/ml copper oxidized LDL or 10 μ M angiotensin II (Sigma) overnight. The cells were then washed twice with PBS and harvested in the extraction buffer containing 20 mM Tris (pH = 7.0), 0.5% Triton X-100 and protease inhibitors. The extracts were sonicated for 10 minutes. Cell debris was removed by centrifugation at 12,000g for 10 minutes at $4^\circ C$.

2.3 Measurement of Heparanase Activity

The cell or tissue samples were lysed in lysis buffer containing 0.5% triton X-100 and protease inhibitors and assayed according to the manufacture's instructions (CisBio, Bedford, MA). Briefly, 3 μ l of lysates in dilution buffer was added into microtubes. After 10 min pre-incubation at $37^\circ C$, an enzyme reaction was initiated by adding 6.0 μ l of Bio-HS-Eu(K) (4.2 ng in 0.2 M $NaCH_3CO_2$ pH 5.5), and the microtubes were incubated for 30 min at $37^\circ C$. To stop the enzyme reaction and detect the remaining substrate, 12 μ l of a 1.0 μ g/

ml SA-XLent solution was added into the microtubes. After a 15 min incubation at RT, 20 μ l of the reaction mixture was transferred to a 384 microplate and read using excitation at 337 nm with readings taken at emissions 620 nm and 665 nm.

3. Results

3.1. Heparanase protein and mRNA levels are enhanced in TCFA lesions

We examined atherosclerotic lesions formed in pigs after 30 weeks post-induction of diabetes and hyperlipidemia. Atherosclerotic lesions were histopathologically classified into minimal (MIN), intermediate (INT) and thin cap fibroatheroma (TCFA) lesions. We found that heparanase staining and gene expression were increased in TCFA lesions when compared to minimal or intermediate lesions (Figure 1a–c). Immunohistochemical staining for heparanase revealed a similar pattern of heparanase staining to immunohistochemical detection of inflammatory cells by CD45 staining (Figure 1a).

3.2. Heparanase staining co-localizes with inflammatory cells, gelatinase zymography and MMP-2 Immunostaining

Matrix metalloproteases degrade extracellular matrix and are established markers of atherosclerosis and contributors to the formation of vulnerable plaques[16]. We hypothesized that heparanase expression was enhanced due to the increased number of inflammatory cells in severe plaques and, thus, may be expressed in a similar distribution to inflammatory cells and, consequently matrix metalloproteases. Dual immunostaining of arteries for heparanase and CD45 demonstrated colocalization between heparanase and inflammatory cells within atherosclerotic plaques (Figure 2a). We visualized gelatinase activity within the tissues by treating sections with quenched fluorescein-labeled gelatin followed by immunohistochemical staining for heparanase. This analysis revealed that in-situ gelatinase activity was colocalized with heparanase immunostaining in intermediate and TCFA plaques (Figure 2b). We also co-immunostained for heparanase and MMP-2 and found these molecules to be colocalized in the inflamed regions of the atheromas (Figure 2c).

3.3. Heparanase protein and gene expression were increased in plaques under low ESS

Endothelial shear stress (ESS) is a crucial factor in the development of TCFA morphology and an important predictor of vulnerable plaque development[15,17–20]. We performed vascular profiling at weeks 23 and 30 with intravascular ultrasound and used these results to calculate local shear stress with a computational model of fluid flow. An example of a calculate shear stress map for an artery is shown in Figure 3a. For analysis, we categorized arterial subsegments into those with ESS greater than or equal to 1.0 Pa and those less than 1.0 Pa at week 23. Heparanase expression and staining was 3.5 fold greater in subsegments with low ESS (Figure 3b–d).

3.4. Association of heparanase and proteoglycan expression with fragmentation of internal elastic lamina (IEL)

Extracellular matrix protease activity contributes to degradation of the IEL, compromising IEL integrity and facilitating macrophage and vascular smooth muscle cell migration[21,22]. We examined the heparanase expression in lesions with varying levels of IEL fragmentation as quantified by Verhoeff's staining and histomorphometric quantification. This analysis revealed a significant increase in heparanase in plaques with severe versus minimal IEL fragmentation (Figure 4a and 4b). The overall magnitude of heparanase mRNA expression did not show statistically significant differences (data not shown).

3.5. Low density lipoprotein (LDL), oxidized LDL and angiotensin II increase heparanase activity in human macrophages

To better understand if the alterations in heparanase expression led to higher levels of increased arterial heparanase enzymatic activity, we lysed cryosections from arterial subsegments and measured heparanase activity relative to total protein content. A two-fold increase in heparanase activity was found in TCFA lesions in comparison to MIN and INT lesions (Figure 5a). Similarly, arteries with low ESS had an increase in heparanase activity (Figure 5b). From our immunostaining analysis it appears that macrophages may be the primary source of heparanase within the atherosclerotic plaque. We hypothesized that increased LDL or angiotensin II might be responsible for the increased heparanase expression observed in the arteries. To examine the potential mechanisms of increased heparanase activity in atherosclerotic plaques we exposed human macrophages in culture to LDL, oxidized LDL or angiotensin II. Exposure to these factors increased the activity of heparanase in the cells by 20–50% (Figure 5c).

4. Discussion

One intriguing aspect of fibroatheromatous plaque stabilization has been the emergence of enzymatic regulation as a dominant force in atherosclerotic arterial remodeling. Early work on atherosclerosis suggested that there was an upregulation of matrix metalloproteases in severe atherosclerotic plaques[23,24]. This observation has led to nearly two decades of scientific inquiry identifying a complex cascade of enzymatic regulation of atheroma development and maturation[25]. The balance of collagen synthesis and degradation is critical to determining risk of plaque rupture and is under the relative control of the MMPs and their endogenous inhibitors[23,24]. A major advancement in the understanding of atherosclerosis was the advent of the “vulnerable plaque” concept, which asserts that among the many plaques that form in an individual’s life only a select few go on to develop a specific morphology and composition that makes them prone to acute rupture and thrombosis[1,2]. In the continuum of atheroma morphology, vulnerable plaques are thought to be those with lowered collagen content and a thin, acellular cap over a lipid-rich core region. Thus, the regulation of the extracellular matrix is a key aspect to stabilizing these plaques by preventing plaque cap thinning and eventual rupture. Thin cap fibroatheroma morphology in swine has been found to associate with plaque rupture[26]. Swine with thin cap lesions also exhibit sudden coronary death when under stress[27]. In addition, similar morphology in humans has been extensively linked with plaque rupture[28–31]. In this work we sought to examine the enzymatic regulation of heparan sulfate by heparanase during the natural history of atherosclerotic plaque development in the coronary arteries of hyperlipidemic, diabetic swine. Our results demonstrate that heparanase accumulation and activity is strongly increased in TCFA and in arteries with risk factors that enhance TCFA formation including low ESS and fragmented IEL. Together these results suggest a role for heparanase in atherogenesis and progression of plaque severity.

Heparanase protein levels and activity increased with progression of atherosclerotic lesions from minimal lesions to TCFA. A critical comparison is that of the intermediate and TCFA lesions as this represents the transition from a stable to potentially unstable lesion. A greater than three-fold increase in heparanase between these two groups suggests that heparanase may be involved not only in initial lesion formation but in the progression of stable lesions to vulnerable plaques. Immunohistochemical analysis of heparanase revealed a pattern of heparanase accumulation that was similar to lipid deposition and inflammatory cell distribution suggesting that the major source of heparanase was the inflammatory cells. Immunohistochemical staining for heparanase in human coronary arteries found it to be increased in arteries with atherosclerosis versus non-diseased arteries[32], consistent with our findings here. Lipids have been shown to regulate heparanase expression in some cell

types[33]. Our in-vitro studies on human macrophages demonstrated that heparanase activity can be increased by oxidized LDL and angiotensin II. Thus, oxidized lipid or angiotensin-induced expression of heparanase in macrophages may be a primary mechanism increasing heparanase in these plaques.

Both collagen and elastin play an integral role in the atherosclerotic plaque development process. Collagen is thought to be the major structural protein that maintains plaque stability. Destruction of this extracellular matrix molecule is therefore key to facilitating plaque thinning and rupture. Similarly, elastin acts as a barrier to migration of macrophages and vascular smooth muscle cells. Destruction of the elastic lamina is a critical part of atherosclerotic plaque progression. In this context, heparanase could act as a facilitator of the MMP and cathepsin-mediated destruction of extracellular matrix molecules. Heparan sulfate interacts with fibrillin-1 and is required for effective assembly of elastin fibrils[34]. Thus, removal of these chains may destabilize and prevent repair of elastin fibrils. Several other synergistic activities of heparanase and MMPs are suggested by previous work. We recently showed an increase in the expression and activity of MMP-2 and MMP-9 in a porcine model of atherosclerosis[35]. Removal of heparan sulfate from the cell surface has been shown to increase activation of MMP-2[36]. Heparanase and MMP-9 can also work synergistically to enhance the differentiation of vascular smooth muscle cells[37]. We used gelatinase zymography and heparanase immunostaining to demonstrate co-localization between MMP activity and heparanase localization. Heparanase and MMP-2 were also co-localized in the lesions in this study. Together, these results support a mechanism in which heparanase may act in concert with MMP activity to remodel the atherosclerotic lesions.

A major risk factor for local TCFA formation is the presence of low ESS[15,17]. Low ESS regulates a number of pro-atherogenic molecules in endothelial cells including the expression of adhesion molecules such as P-selectin and VCAM-1 as well as paracrine vascular regulators like nitric oxide and HSPGs[17]. During plaque progression, arterial cells produce increased amounts of extracellular matrix and matrix degrading enzymes. These processes facilitate atherosclerotic plaque development and progression to unstable TCFA morphology. Endothelial HSPGs are known inhibitors of vascular smooth muscle cell proliferation and migration[10]. A recent paper by van den Berg et al demonstrated that the dimension and composition of the glycocalyx was altered in high risk atherogenic regions of the mouse carotid artery [38]. Specifically, the thickness and amount of heparan sulfate in the glycocalyx was reduced in the carotid sinus versus that in the common carotid. In addition they demonstrated that the thinner, less heparan sulfate-rich portion of the artery had increased LDL “leakage” into the intimal layers of the artery. These results imply that loss of the glycocalyx layer, by disturbed flow and/or hyperlipidemia, may lead to enhanced arterial accumulation of lipids. Our studies are consistent with these results and demonstrated that heparanase is increased in arterial regions with low ESS. In this context, heparanase activity would lead to loss of heparan sulfate from the glycocalyx and enhanced lipid permeability to lipoproteins. Thus, heparanase may have an important role in the well-known atherogenic effects of low or disturbed flow within the artery through altering the glycocalyx.

Taken together, our results suggest a role for heparanase as an enzymatic regulator of the progression and development of atherosclerotic plaques. Our experiments were performed in an advanced model of atherosclerosis that demonstrates human-like atheroma development allowing the study of the stable to unstable plaque transition. In this context, heparanase was associated with proatherogenic risk factors and severe plaque remodeling. Further, co-localization of heparanase with macrophages and MMP activity suggests that heparanase may work in concert with proteases to enhance TCFA formation. These results suggest a

critical role for heparanase as an enzymatic regulator of vulnerable lesion development and potential target for future therapeutic treatment of atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to acknowledge the essential scientific contributions of Dr. Ross Gerrity who passed away during completion of this work. The authors gratefully acknowledge Philip Seifert and Adam Groothuis for their technical assistance. This work was supported by grants from Novartis Pharmaceuticals Inc., Boston Scientific Inc, the George D. Behrakis Research Fellowship (to Y.S.C.), the Hellenic Heart Foundation (to Y.S.C.), the Hellenic Atherosclerosis Society (to Y.S.C.), the Alexander S. Onassis Public Benefit Foundation (to Y.S.C.), postdoctoral fellowship from the Philip Morris External Research Program (to A.B.B.) and a Scientist Development Grant from the American Heart Association (to A.B.B.).

Funding Sources

Research funding was received from Novartis Pharmaceuticals Inc. and Boston Scientific Inc.

References

1. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 2000;205:1262–75. [PubMed: 10807742]
2. Virmani R, Burke AP, Farb A, Kolodgie FD. Pathology of the vulnerable plaque. *J Am Coll Cardiol* 2006;478 (Suppl):C13–8. [PubMed: 16631505]
3. Hansson GK, Libby P, Schonbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res* 2002;914:281–91. [PubMed: 12193460]
4. Camejo G, Hurt-Camejo E, Wiklund O, Bondjers G. Association of apo B lipoproteins with arterial proteoglycans: pathological significance and molecular basis. *Atherosclerosis* 1998;1392:205–22. [PubMed: 9712326]
5. Williams KJ, Tabas I. The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol* 1995;155:551–61. [PubMed: 7749869]
6. Wight TN, Merrilees MJ. Proteoglycans in atherosclerosis and restenosis: key roles for versican. *Circ Res* 2004;949:1158–67. [PubMed: 15142969]
7. Pillarisetti S. Lipoprotein modulation of subendothelial heparan sulfate proteoglycans (perlecan) and atherogenicity. *Trends Cardiovasc Med* 2000;102:60–5. [PubMed: 11150731]
8. Sivaram P, Obunike JC, Goldberg IJ. Lysolecithin-induced alteration of subendothelial heparan sulfate proteoglycans increases monocyte binding to matrix. *J Biol Chem* 1995;27050:29760–5. [PubMed: 8530367]
9. Clowes AW, Karnowsky MJ. Suppression by heparin of smooth muscle cell proliferation in injured arteries. *Nature* 1977;2655595:625–6. [PubMed: 859561]
10. Nugent MA, Nugent HM, Iozzo RV, Sanchack K, Edelman ER. Perlecan is required to inhibit thrombosis after deep vascular injury and contributes to endothelial cell-mediated inhibition of intimal hyperplasia. *Proc Natl Acad Sci U S A* 2000;9712:6722–7. [PubMed: 10841569]
11. Tran-Lundmark K, Tran PK, Paulsson-Berne G, Friden V, Soininen R, Tryggvason K, Wight TN, Kinsella MG, Boren J, Hedin U. Heparan Sulfate in Perlecan Promotes Mouse Atherosclerosis. Roles in Lipid Permeability, Lipid Retention, and Smooth Muscle Cell Proliferation. *Circ Res*. 2008
12. Vikramadithyan RK, Kako Y, Chen G, Hu Y, Arikawa-Hirasawa E, Yamada Y, Goldberg IJ. Atherosclerosis in perlecan heterozygous mice. *J Lipid Res* 2004;4510:1806–12. [PubMed: 15258195]

13. Vlodavsky I, Goldshmidt O, Zcharia E, Atzmon R, Rangini-Guatta Z, Elkin M, Peretz T, Friedmann Y. Mammalian heparanase: involvement in cancer metastasis, angiogenesis and normal development. *Semin Cancer Biol* 2002;122:121–9. [PubMed: 12027584]
14. Gerrity RG, Natarajan R, Nadler JL, Kimsey T. Diabetes-induced accelerated atherosclerosis in swine. *Diabetes* 2001;507:1654–65. [PubMed: 11423488]
15. Chatzizisis YS, Jonas M, Coskun AU, Beigel R, Stone BV, Maynard C, Gerrity RG, Daley W, Rogers C, Edelman ER, Feldman CL, Stone PH. Prediction of the localization of high-risk coronary atherosclerotic plaques on the basis of low endothelial shear stress: an intravascular ultrasound and histopathology natural history study. *Circulation* 2008;1178:993–1002. [PubMed: 18250270]
16. Libby P. Atherosclerosis: disease biology affecting the coronary vasculature. *Am J Cardiol* 2006;9812A:3Q–9Q.
17. Chatzizisis YS, Coskun AU, Jonas M, Edelman ER, Feldman CL, Stone PH. Role of endothelial shear stress in the natural history of coronary atherosclerosis and vascular remodeling: molecular, cellular, and vascular behavior. *J Am Coll Cardiol* 2007;4925:2379–93. [PubMed: 17599600]
18. Cheng C, Tempel D, van Haperen R, van der Baan A, Grosveld F, Daemen MJ, Krams R, de Crom R. Atherosclerotic lesion size and vulnerability are determined by patterns of fluid shear stress. *Circulation* 2006;11323:2744–53. [PubMed: 16754802]
19. Gimbrone MA Jr, Topper JN, Nagel T, Anderson KR, Garcia-Cardena G. Endothelial dysfunction, hemodynamic forces, and atherogenesis. *Ann N Y Acad Sci* 2000;902230–9. discussion 9–40.
20. Stone PH, Coskun AU, Yeghiazarians Y, Kinlay S, Popma JJ, Kuntz RE, Feldman CL. Prediction of sites of coronary atherosclerosis progression: In vivo profiling of endothelial shear stress, lumen, and outer vessel wall characteristics to predict vascular behavior. *Curr Opin Cardiol* 2003;186:458–70. [PubMed: 14597887]
21. Bentzon JF, Weile C, Sondergaard CS, Hindkjaer J, Kassem M, Falk E. Smooth muscle cells in atherosclerosis originate from the local vessel wall and not circulating progenitor cells in ApoE knockout mice. *Arterioscler Thromb Vasc Biol* 2006;2612:2696–702. [PubMed: 17008593]
22. Sukhova GK, Wang B, Libby P, Pan JH, Zhang Y, Grubb A, Fang K, Chapman HA, Shi GP. Cystatin C deficiency increases elastic lamina degradation and aortic dilatation in apolipoprotein E-null mice. *Circ Res* 2005;963:368–75. [PubMed: 15653570]
23. Henney AM, Wakeley PR, Davies MJ, Foster K, Hembry R, Murphy G, Humphries S. Localization of stromelysin gene expression in atherosclerotic plaques by in situ hybridization. *Proc Natl Acad Sci U S A* 1991;8818:8154–8. [PubMed: 1896464]
24. Vine N, Powell JT. Metalloproteinases in degenerative aortic disease. *Clin Sci (Lond)* 1991;812:233–9. [PubMed: 1653668]
25. Dollery CM, Libby P. Atherosclerosis and proteinase activation. *Cardiovasc Res* 2006;693:625–35. [PubMed: 16376322]
26. Shi ZS, Feng L, He X, Ishii A, Goldstine J, Vinters HV, Vinuela F. Vulnerable plaque in a Swine model of carotid atherosclerosis. *AJNR Am J Neuroradiol* 2009;303:469–72. [PubMed: 19147719]
27. Badimon L. Atherosclerosis and thrombosis: lessons from animal models. *Thromb Haemost* 2001;861:356–65. [PubMed: 11487025]
28. Cheruvu PK, Finn AV, Gardner C, Caplan J, Goldstein J, Stone GW, Virmani R, Muller JE. Frequency and distribution of thin-cap fibroatheroma and ruptured plaques in human coronary arteries: a pathologic study. *J Am Coll Cardiol* 2007;5010:940–9. [PubMed: 17765120]
29. Naghavi M, Libby P, Falk E, Casscells SW, Litovsky S, Rumberger J, Badimon JJ, Stefanadis C, Moreno P, Pasterkamp G, Fayad Z, Stone PH, Waxman S, Raggi P, Madjid M, Zarrabi A, Burke A, Yuan C, Fitzgerald PJ, Siscovick DS, de Korte CL, Aikawa M, Airaksinen KE, Assmann G, Becker CR, Chesebro JH, Farb A, Galis ZS, Jackson C, Jang IK, Koenig W, Lodder RA, March K, Demirovic J, Navab M, Priori SG, Reikter MD, Bahr R, Grundy SM, Mehran R, Colombo A, Boerwinkle E, Ballantyne C, Insull W Jr, Schwartz RS, Vogel R, Serruys PW, Hansson GK, Faxon DP, Kaul S, Drexler H, Greenland P, Muller JE, Virmani R, Ridker PM, Zipes DP, Shah PK, Willerson JT. From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part II. *Circulation* 2003;10815:1772–8. [PubMed: 14557340]

30. Naghavi M, Libby P, Falk E, Casscells SW, Litovsky S, Rumberger J, Badimon JJ, Stefanadis C, Moreno P, Pasterkamp G, Fayad Z, Stone PH, Waxman S, Raggi P, Madjid M, Zarrabi A, Burke A, Yuan C, Fitzgerald PJ, Siscovick DS, de Korte CL, Aikawa M, Juhani Airaksinen KE, Assmann G, Becker CR, Chesebro JH, Farb A, Galis ZS, Jackson C, Jang IK, Koenig W, Lodder RA, March K, Demirovic J, Navab M, Priori SG, Rekhater MD, Bahr R, Grundy SM, Mehran R, Colombo A, Boerwinkle E, Ballantyne C, Insull W Jr, Schwartz RS, Vogel R, Serruys PW, Hansson GK, Faxon DP, Kaul S, Drexler H, Greenland P, Muller JE, Virmani R, Ridker PM, Zipes DP, Shah PK, Willerson JT. From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part I. *Circulation* 2003;10814:1664–72. [PubMed: 14530185]
31. Redgrave JN, Gallagher P, Lovett JK, Rothwell PM. Critical cap thickness and rupture in symptomatic carotid plaques: the oxford plaque study. *Stroke* 2008;396:1722–9. [PubMed: 18403733]
32. Cohen-Mazor M, Sela S, Mazor R, Ilan N, Vlodaysky 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* 2008;2942:H651–8. [PubMed: 18032524]
33. Chen G, Wang D, Vikramadithyan R, Yagyu H, Saxena U, Pillarisetti S, Goldberg IJ. Inflammatory cytokines and fatty acids regulate endothelial cell heparanase expression. *Biochemistry* 2004;4317:4971–7. [PubMed: 15109255]
34. Cain SA, Baldock C, Gallagher J, Morgan A, Bax DV, Weiss AS, Shuttleworth CA, Kielty CM. Fibrillin-1 interactions with heparin. Implications for microfibril and elastic fiber assembly. *J Biol Chem* 2005;28034:30526–37. [PubMed: 15980072]
35. Chatzizisis YS, Jonas M, Beigel R, Coskun AU, Baker AB, Stone BV, Maynard C, Gerrity RG, Daley W, Edelman ER, Feldman CL, Stone PH. Attenuation of inflammation and expansive remodeling by Valsartan alone or in combination with Simvastatin in high-risk coronary atherosclerotic plaques. *Atherosclerosis* 2009;2032:387–94. [PubMed: 18786669]
36. Munesue S, Yoshitomi Y, Kusano Y, Koyama Y, Nishiyama A, Nakanishi H, Miyazaki K, Ishimaru T, Miyaura S, Okayama M, Oguri K. A novel function of syndecan-2, suppression of matrix metalloproteinase-2 activation, which causes suppression of metastasis. *J Biol Chem* 2007;28238:28164–74. [PubMed: 17623663]
37. Fitzgerald M, Hayward IP, Thomas AC, Campbell GR, Campbell JH. Matrix metalloproteinase can facilitate the heparanase-induced promotion of phenotype change in vascular smooth muscle cells. *Atherosclerosis* 1999;1451:97–106. [PubMed: 10428300]
38. van den Berg BM, Spaan JA, Vink H. Impaired glycocalyx barrier properties contribute to enhanced intimal low-density lipoprotein accumulation at the carotid artery bifurcation in mice. *Pflugers Arch* 2009;4576:1199–206. [PubMed: 18839207]

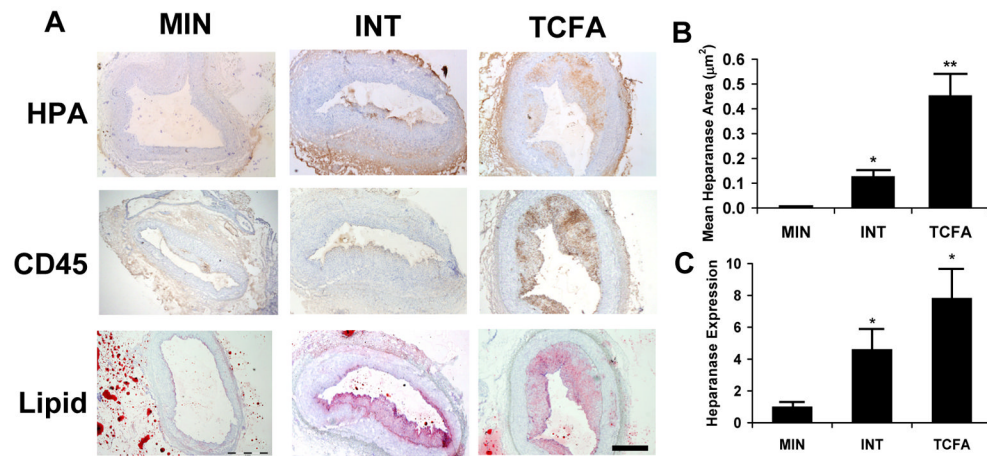


Figure 1. Heparanase staining, mRNA and activity are increased in severe atheromas
 Lesions were histopathologically classified as minimal lesions (MIN, n=26), intermediate lesions (INT, n=56) or thin cap fibroatheromas (TCFAs, n=60) based on morphologic and oil O red staining. (A) Immunohistochemical staining for heparanase was increased with progression from MIN to TCFA lesions. Bar = 500 µm. (B) Quantitative analysis of heparanase staining within arteries. (C) Gene expression analysis by real time PCR revealed increased expression of heparanase. *Statistically different from MIN group with $p < 0.05$. **Statistically different from MIN and INT groups with $p < 0.05$.

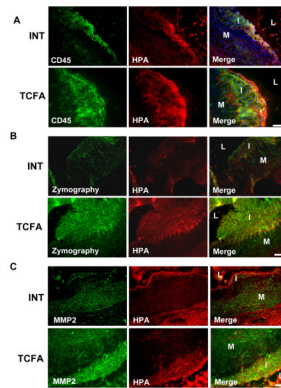


Figure 2. Heparanase protein is co-localized with CD45, gelatinase activity and MMP-2 immunostaining

(A) Immunofluorescence staining demonstrates co-localization between heparanase and CD45 positive cells. (B) Gelatinase zymography and immunostaining for heparanase (HPA) were performed on tissue sections. Colocalization was found between gelatinase activity and heparanase immunostaining. Representative samples are shown for intermediate lesions (INT) or thin cap fibroatheroma (TCFA) lesions. (C) Immunofluorescence staining demonstrated co-localization between heparanase and MMP-2. Bar = 50 μ m. L = Lumen. I = Intima. M = Media.

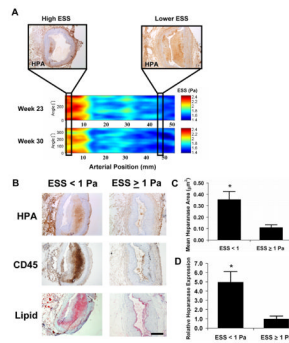


Figure 3. Local heparanase expression is increased in arterial segments with low endothelial shear stress

Arterial segment morphology was measured using intravascular ultrasound and computational modeling used to calculate ESS. The segments were separated into groups of those with ESS less than 1.0 Pa (n=76) or with greater than or equal to 1.0 Pa (n=66) for analysis. (A) Shear stress maps calculated from intravascular ultrasound. Shown are histological slides from two regions of the same artery. The box shows the region from which the histological sections were taken. (B and C) Heparanase protein levels were increased with low ESS as measured by immunohistochemical staining. Bar = 500 μ m. (D) Gene expression analysis by real time PCR revealed increased expression of heparanase in arteries with low ESS. *p < 0.05 versus high ESS group.

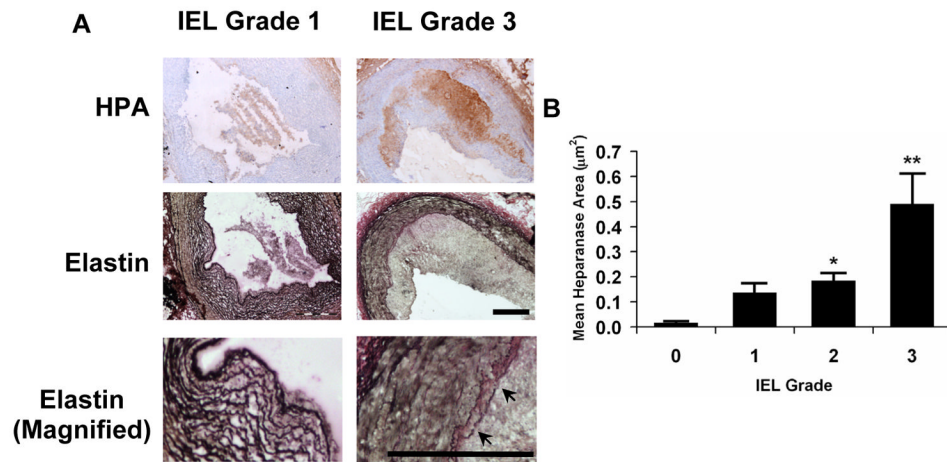


Figure 4. Heparanase is increased in arteries with degraded internal elastic laminae (IEL)
 Based on histomorphology following elastic fiber staining, arterial subsegments were categorized into the following groups: group 0, no IEL breaks (n=22); group 1, minimal IEL breaks (n=22); group 2, numerous IEL breaks (n=56); and group 3, severe IEL and medial fragmentation (n=42). (A and B) Staining for heparanase is increased in arteries with severe IEL fragmentation. Bar = 500 µm. *p<0.05 versus IEL grade 0 group. **p < 0.05 versus all other groups.

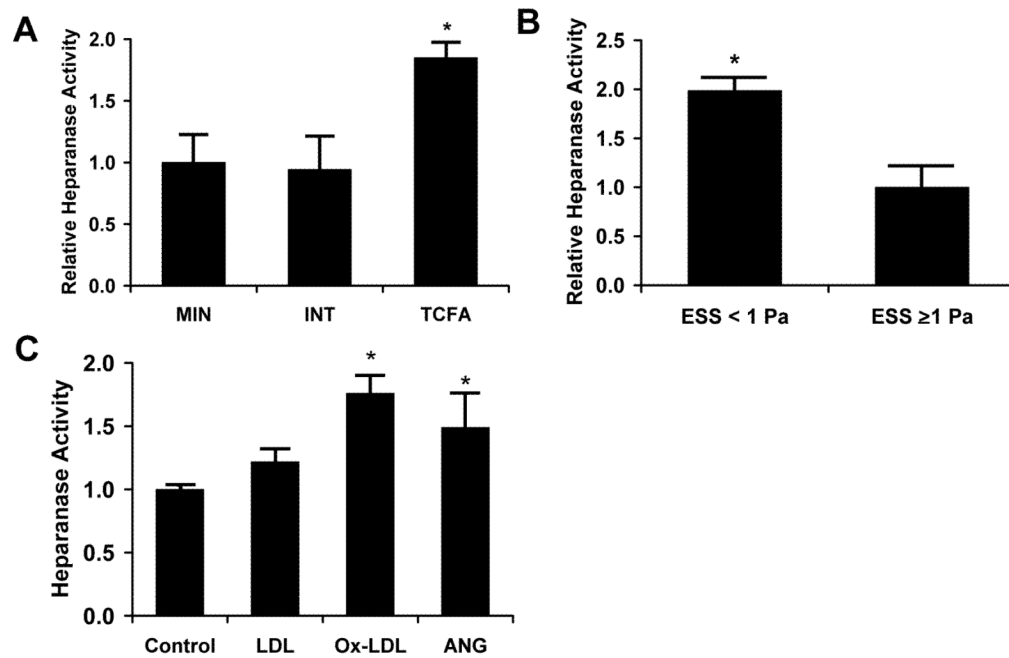


Figure 5. Heparanase enzymatic activity is increased in TCFA lesions, subsegments with low ESS and in macrophages treated with oxidized LDL and angiotensin

A FRET-based heparanase was used to measure heparanase activity in lysed arterial sections. (A) Lesions with TCFA morphology have a two-fold increase in heparanase activity in comparison with intermediate and minimal lesions. * $p < 0.05$ versus MIN and INT groups. (B) Subsegments with low ESS have increased heparanase activity. * $p < 0.05$ versus high ESS group. (C) Human macrophages in culture were treated with LDL (50 $\mu\text{g}/\text{ml}$) or angiotensin II (10 $\mu\text{g}/\text{ml}$) for 24 hours. The cells were lysed and heparanase activity was measured. * $p < 0.05$ versus control group.