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Increased expression of heparanase in symptomatic carotid atherosclerosis

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ABSTRACT

Objective: Proliferation of smooth muscle cells (SMCs) can stabilize atherosclerotic lesions but the molecular mechanisms that regulate this process in humans are largely unknown. We have previously shown that heparan sulfate proteoglycans (HSPGs), such as perlecan, regulate SMC growth in animal models by modulating heparin-binding mitogens. Since perlecan is expressed at low levels in human atherosclerosis, we speculated that the effect of heparan sulfate (HS) in human disease was rather influenced by HS degradation and investigated the expression of heparanase (HPSE) in human carotid endarterectomies.

Methods and results: Gene expression analysis from 127 endarterectomies in the BiKE database revealed increased expression of *HPSE* in carotid plaques compared with normal arteries, and a further elevation in symptomatic lesions. Increased HPSE protein expression in symptomatic plaque tissue was verified by tissue microarrays. HPSE mRNA levels correlated positively with expression of inflammatory markers IL-18, RANTES and IL-1β, and also T-cell co-stimulatory molecules, such as B7.2, CD28, LFA-1 and 4-1BB. Previously reported single nucleotide polymorphisms within HPSE were associated with differential mRNA expression in plaques. Immunohistochemistry revealed that inflammatory cells were major producers of HPSE in plaque tissue. HPSE immunoreactivity was also observed in SMCs adjacent to the necrotic core and was co-localized to deposits of fibrin.

Conclusions: This study demonstrates increased expression of HPSE in human atherosclerosis associated with inflammation, coagulation and plaque instability. Since HS can regulate SMC proliferation and influence plaque stability, the findings suggest that HPSE degradation of HS take part in the regulation of SMC function in human atherosclerosis.

1. Introduction

Atherosclerotic plaque instability with plaque rupture and thromboembolism is the major cause of death and disability in the Western world. Whereas the vulnerability of an atherosclerotic lesion has largely been attributed to inflammation [1], lesions are stabilized when smooth muscle cells (SMCs) proliferate and deposit collagen [2]. Elucidating mechanisms that regulate SMC proliferation in the progression of atherosclerosis is thus essential in order to develop therapies to control plaque stability and prevent atherothrombotic events such as myocardial infarction and stroke.

Heparin and heparan sulfate proteoglycans (HSPGs) are important regulators of cellular and molecular processes in atherogenesis such as in cell signaling and adhesion, lipoprotein clearance, recruitment of inflammatory cells and are essential modulators of SMC function [3–6]. We have previously demonstrated that the vessel wall extracellular matrix (ECM) plays a major role in the regulation of SMC proliferation. In particular HSPGs, such as perlecan in the SMC basement membrane, may function as a reservoir for heparin-binding mitogens and control interactions with cell surface growth factor receptors. In mice expressing HS-deficient perlecan, a reduced HS content in the vessel wall was accompanied by increased SMC proliferation and intimal hyperplasia after vascular injury. The animals developed lesions with more SMCs and more prominent fibrous caps when bred against an Apo $E^{-/-}$ background [7].

While perlecan is prominently expressed in mouse atherosclerosis, we observed a significant down-regulation of perlecan in human carotid lesions [8]. Hence, it is possible that the ability of HS

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to influence SMC proliferation in the diseased human vessel wall may instead depend on post-synthesis processes such as structural modification by HS degradation, consequently releasing a spectrum of HS-bound mitogens [8,9].

Synthesis and modification of HS chains require the activity of an array of enzymes whereas degradation of mammalian HS is primarily carried out by one enzyme, heparanase (HPSE), which cleaves the HS side chains of HSPGs into fragments of 10–20 sugar units (5–10 kDa) [10]. Under physiological conditions, HPSE is expressed at low levels; increased HPSE expression has been associated with inflammation and is also considered a marker of poor prognosis in cancers [11].

Although HPSE has been implicated in SMC proliferation and development of intimal hyperplasia in experimental models [12,13], the expression and function of HPSE in human vascular disease are largely unknown. Here, we investigated expression of HPSE gene and protein in a Biobank of human carotid endarterecomy samples obtained from patients undergoing surgery for symptomatic and asymptomatic carotid disease [14]. HPSE was overexpressed both at the mRNA and protein level in symptomatic carotid lesions, and was mainly observed in inflammatory cells, an expression pattern influenced by genotype. Given a role for HS in the regulation of SMC proliferation, these findings are of importance for elucidating the mechanisms involved in the control of plaque stability.

2. Methods

2.1. Human material

Human carotid endarterectomy samples from 127 patients undergoing surgery for asymptomatic or symptomatic carotid stenosis were part of the Biobank of Karolinska Endarterectomies [14]. Control samples from individuals without any history of cardiovascular disease were obtained from normal iliac arteries of organ donors (29–61 years old) and one internal carotid artery sample from a 61 year old male patient. All samples were collected with consent from patients, organ donors or organ donors' guardians. The Ethical Committee of Northern Stockholm approved the study.

2.2. Genotyping and gene expression analysis

RNA, extracted from endarterectomy and control specimens, was analyzed by Affymetrix HG-U133 plus 2.0 Genechip arrays. Robust multi-array average (RMA) normalization was performed and processed gene expression data was returned in a $\log_2$-scale. Blood DNA ($n = 96$) was obtained for high-coverage, high-density genotyping using Illumina 610w-Quad BeadArrays. EQTL analysis was performed with imputed genotypes (generated by use of MACH 1.0.16 and the 1000 Genomes Caucasian (CEU) reference panel).

2.3. Tissue microarrays (TMA)

Generation of TMAs was performed in accordance to strategies used in the Human Protein Atlas (www.proteinatlas.org; [15]). In brief, formalin-fixed, paraffin embedded endarterectomies from patients with symptomatic or asymptomatic carotid stenosis obtained from the BiKE Biobank were used as donor blocks. To construct the TMAs, tissue cores with a diameter of 1 mm were punched from 3 different regions of the endarterectomy blocks in order to compensate for tissue heterogeneity. The tissue cores were assembled in a recipient paraffin block, and sections were cut at two different levels with a distance of 10 mm in between, and used for immunohistochemical staining. Thirty endarterectomy samples from symptomatic ($n = 24$) and asymptomatic ($n = 6$) patients were used for the TMAs and in total 170 tissue core sections analyzed separately.

2.4. Immunohistochemistry

All immunohistochemistry reagents were from Biocare Medical (Concord, CA). Isotype rabbit and mouse IgG were used as negative controls. In brief, sections (5 μm) were deparaffinized in Tissue Clear and rehydrated in a 99–96–70% ethanol series. For antigen retrieval, slides were subjected to high-pressure boiling in DIVA buffer (HPSE and α-smooth muscle actin, fibrin, pH 6.0) or TE buffer (CD3, CD68 and CD163, pH 9.0). After blocking with Background Sniper solution, primary antibodies diluted in Da Vinci Green solution were applied to the sections and incubated at room temperature for 1 h. The primary antibodies were; rabbit polyclonal antibody against CD3 (DAKO, Glostrup, Denmark) and mouse monoclonal antibodies against CD163 (clone 10D6, Abcam, Cambridge, UK), CD68 (clone 514H12, Novocastra, Newcastle upon Tyne, UK), human HPSE (Clone HP3/17, Insight, Rehovot, Israel) and α-smooth muscle actin (clone M0851, DAKO) and CD61 (clone M0753, DAKO). For specific detection of fibrin, the monoclonal antibody 59D8 [16] was used. A probe–polymer system containing alkaline phosphatase was applied to the sections, with subsequent detection using Vulcan Fast Red. All slides, except TMAs, were counterstained with Mayer's hematoxylin (Vector Laboratories, Burlingame, CA), and finally mounted in Pertex (Histolab, Gothenburg, Sweden).

2.5. Statistics

The Students *t*-test was used to identify gene expression differences between control arteries and carotid plaques and differences in protein expression between plaque tissue from symptomatic and asymptomatic patients obtained by blinded semiquantitative grading of tissue core sections on TMAs, respectively. Pearson correlations were calculated to determine correlation between expressions of different genes, and correlation between staining intensities of different proteins on TMAs. *p* values were corrected for multiple comparisons according to the method of Bonferroni and $p < 0.05$ was considered to indicate statistical significance. Analysis of expression quantitative locus (eQTL) effects was performed by grouping the genotypes as previously described in Ref. [17]. Low (LR): GG/GG, GG/AG, GG/AA, or AG/GG. Intermediate (MR): AG/AG or AG/AA. High (HR): AA/AG, AA/AA – for the genotypes rs4693608/rs4364254, respectively. Statistical significance was calculated by encoding the LR, MR, and HR groups as 0, 1 and 2 and fitting an additive linear regression model to the expression level of HPSE.

3. Results

3.1. Elevated HPSE mRNA and protein expression in symptomatic carotid endarterectomies

Microarray analysis of RNA extracted from 127 human carotid plaques showed a 6.6-fold increase in levels of HPSE mRNA in comparison to control tissue from non-atherosclerotic iliac arteries ($p < 0.0001$; Fig. 1A). Symptomatic lesions from patients presenting with ischemic stroke, TIA or amaurosis fugax showed a 1.3-fold further elevation of HPSE mRNA levels as compared to asymptomatic plaques ($p < 0.05$). In addition, HPSE mRNA expression correlated with increased serum creatinine levels ($p < 0.05$) and a reduced glomerular filtration rate (GFR) ($p < 0.05$), whereas no correlations were found between HPSE mRNA levels and age, gender, CRP levels, HbA1c, or plaque morphology as assessed by preoperative duplex examination. Blinded semiquantitative grading of 170 endarterectomy tissue core sections on TMAs