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Expression of fibromodulin in carotid atherosclerotic plaques is associated with diabetes and cerebrovascular events

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ABSTRACT

Aims: The small leucine-rich proteoglycans fibromodulin and lumican are functionally related extracellular matrix proteins involved in the regulation of collagen fiber formation. Fibromodulin-deficient apolipoprotein E-null mice have decreased vascular retention of lipids and reduced development of atherosclerosis suggesting that fibromodulin may influence the disease process. The aim of the present study was to investigate if fibromodulin and lumican are expressed in human carotid plaques and to determine if their expression is associated with the occurrence of preoperative symptoms and with risk for postoperative cardiovascular events.

Methods and results: 153 plaques (51% symptomatic) obtained by carotid endarterectomy were included in this study. Plaque content was analyzed by immunohistochemistry and plaque cytokine content by multiplex technology. Fibromodulin and lumican were widely expressed in plaques and fibromodulin expression was significantly higher in symptomatic plaques. Expression of fibromodulin was significantly higher in plaques obtained from patients with diabetes and a high fibromodulin expression was associated with a higher incidence of post-operative cerebrovascular events, whereas no such associations were seen for lumican. Fibromodulin expression also correlated with plaque lipids and several pro-inflammatory cytokines. In addition, fibromodulin expression correlated with low levels of smooth muscle cells and the anti-inflammatory cytokine IL-10.

Conclusions: These observations support previous experimental findings in mice for a role of fibromodulin in atherosclerosis and provide clinical evidence of the involvement of fibromodulin in the inflammatory processes that characterize atherosclerotic plaque vulnerability. They also suggest that this is of particular importance in diabetes.

1. Introduction

The extracellular matrix (ECM) is of key importance for maintaining the stability of atherosclerotic lesions [1,2]. Plaque rupture, due to degradation of the fibrous cap, is today considered to be the main cause of the development of acute myocardial infarction (MI) and stroke [3,4]. The ECM of atherosclerotic plaques is composed of a number of different proteins and glycoproteins, the most abundant being collagen, elastin and proteoglycans [5,6]. ECM components interact to maintain the mechanical stability of the plaque as well as perform several regulatory functions. Binding of LDL to vascular wall proteoglycans is also an initiating factor in plaque development [7].

Fibromodulin is a 59 kDa proteoglycan that binds to collagen type I [8] and its primary structure was determined in 1989 by Oldberg et al. [9] This small leucine-rich repeat proteoglycan (SLRP) is involved in ECM remodeling by regulating collagen fiber assembly [10] and by influencing collagen scaffold formation [11,12] by mechanisms that remain to be fully characterized.

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Fibromodulin shares close homology (~50%) with lumican, another member of the SLRP family [13–15]. Like fibromodulin, lumican binds to collagen type I and contributes to collagen synthesis [11].

We have recently shown that plaque formation is reduced in ApoE-deficient mice lacking fibromodulin and that this is associated with an abnormal formation of collagen fibers in atherosclerotic plaques as well as with decreased lipid retention in the vascular ECM [16]. Reduced lipid uptake was also observed in macrophages cultured on fibromodulin-deficient ECM.

The expression and role of fibromodulin and lumican in symptomatic human atherosclerotic plaques has not been previously investigated. Our previous results suggesting that fibromodulin is involved in plaque development by affecting ECM structure and lipid retention, together with previous publications discussing lumican expression in healthy and pathological vasculature [17–21], raise the question of possible contribution by these SLRPs to atherosclerotic plaque development and its stability. In the present study we have thus for the first time analyzed the expression of fibromodulin and lumican in human carotid plaques in relation to the occurrence of preoperative cerebrovascular symptoms (namely strokes, transient ischemic attacks (TIA) or amaurosis fugax) and postoperative cardiovascular (CV) events. We also assessed the association between the plaque expression of fibromodulin and lumican and the content of plaque lipids, inflammation and indicators of active remodeling.

2. Materials and methods

Additional Materials and Methods may be found in the online data supplement.

2.1. Patients

One hundred and fifty patients (out of which 3 were treated bilaterally) who underwent carotid endarterectomy (CEA) between 2005 and 2011 at the Vascular Department at Skåne University Hospital (Malmö, Sweden) were enrolled in this study after giving written informed consent. The study protocol was approved by the local Regional Ethical Committee (approval reference number 472/2005) and conformed to the principles of the Declaration of Helsinki. Clinical characteristics of the patients are described in Supplemental Tables I and II in the online data supplement.

2.2. Postoperative events

The Swedish national inpatient health register was analyzed in order to identify postoperative CV events, with corresponding International Classification of Diseases, Tenth Revision (ICD-10) codes G45, G46, I20 to I25, I60 to I69 and I97 from January 1998 to December 2010. This is a nationwide validated register where more than 99 percent of all somatic (including surgery) and psychiatric hospital discharges are registered [22]. In doubtful cases, information was gained through telephone interviews and medical chart reviews. All causes deaths were verified against the National Population Register. Definition of outcomes is accounted for in detail in the online data supplement.

2.3. Immunohistochemistry

Carotid plaque sections were immunohistochemically stained using primary antibodies against fibromodulin (kindly provided by D. Heinegård, Lund University, Sweden), lumican (ab168348; Abcam, Cambridge, UK), Glycophorin A (M0819, Dako Sweden, Stockholm, Sweden), collagen I and collagen III (ab6308 and ab6310 respectively; Abcam, Cambridge, UK). A biotinylated goat anti-rabbit IgG (Vector BA-1000, Vector Laboratories Inc, Burlingame, CA, USA) or rabbit F(ab')2 anti-mouse IgG (ab98668, Abcam, Cambridge, UK) was used as secondary antibodies. In addition, immunohistochemical stains for smooth muscle α-actin and CD68 were performed as previously described [23,24]. Isotype control antibodies were used in concentrations corresponding to that of each primary antibody (ab27478 and ab81032from rabbit and mouse, respectively; Abcam, Cambridge, UK) – representative images are shown in Supplementary Figure I. Immunoreactivity was quantified blindly using the imaging software program BioPix iQ version 2.3.1 (Biopix Ab, Gothenburg, Sweden). Residual media was not included during image analysis.

2.4. Homogenate analysis

Active Caspase 3 (cleaving at the Asp175/Ser176 site) was measured in carotid plaque homogenate using Human Caspase-3 ELISA (Invitrogen, Life Technologies, Carlsbad, CA). Cytokines were assessed in plaque homogenate (Human Cytokine/Chemokine Immunoassay, Millipore Corporation, MA, USA) and analyzed with Luminex 100 IS 2.3 (Austin, TX). MMPs (1–3, 7, 9, and 12) and tissue inhibitors of MMPs (TIMPs 1–3) were analyzed in plaque homogenate supernatants as previously described [25]. All analyses were performed according to the manufacturer's instructions and results were normalized to plaque wet weight.

2.5. Statistical methods

Continuous variables were not normally distributed and are thus presented as median with interquartile range (IQR; 25th percentile to 75th percentile), while categorical variables are expressed as percentages. During immunohistochemistry and homogenate analyses Mann–Whitney U test and Spearman's rank correlation were used to assess correlations in continuous variables and Chi-square test in categorical variables. Freedom from postoperative events was calculated by life-tables according to Kaplan–Meier survival analysis. Correction for was done through Cox regression analysis. Age, gender, diabetes, hypertension, coronary heart disease, smoking and statin use were used as covariates in the regression model. A P-value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS 22.0 (IBM Corp., Amonk, NY, USA).

3. Results

3.1. Plaque expression of fibromodulin and lumican

Expression of fibromodulin and lumican was found in the fibrous cap and shoulder regions, as well as in the core, though positive staining did not generally appear in all regions in the same plaque (Fig. 1A–D). Fibromodulin and lumican immunoreactivity most often appeared in similar plaque regions when comparing staining patterns from consecutive sections. However, the extent of the positive stain often varied. Only in three plaques was fibromodulin and lumican expression consistently found in completely different regions. Finally, fibromodulin expression was generally absent from the outermost regions of plaques which represent the interface between the plaque and residual media while expression of lumican frequently occurred in this region.

When comparing with a Hematoxylin & Eosin (H&E) stain, plaque regions with positive fibromodulin and lumican immunoreactivity were found in both cellular and acellular regions. Using Masson's trichrome as an overview staining, positive immunoreactivity for fibromodulin and lumican were also detected in regions with a dense ECM such as the cap, as well as in ECM-poor regions