Supplemental material

Material and Methods

Human material

Human endarterectomy samples from 360 patients undergoing surgery for asymptomatic (as) or symptomatic (s) carotid stenosis were part of the Biobank of Karolinska Endarterectomies (BiKE). Majority of patients’ were Caucasian. Symptoms of plaque instability were defined as transitory ischemic attack (TIA), minor stroke (MS) and *amaurosis fugax* (AF). Among these samples 127 (40 as/87 s) were analyzed by gene microarrays (patient demographics described in previous publications from BiKE \(^1,^2\)) and 233 additional (74 as/159 s) plaques by quantitative real-time PCR (qRT-PCR). Ten control iliac and radial arteries were obtained from organ donors without any history of cardiovascular disease. One control internal carotid artery sample was obtained from a 61 year old male patient treated for a neck tumor. Thirty-four separate carotid plaques (28 s/6 as) were used for construction of TMAs (patients’ characteristics and clinical variables given in Supplementary Table I), as well as control kidney and spleen tissues. All samples were collected with informed consent from patients, organ donors or their guardians. The study was approved by the Ethical Committee of Northern Stockholm.

Antibodies

A summary of primary antibodies provided by the Human Protein Atlas (HPA) project is given in Supplementary Table II. A polyclonal antibody against the N-terminal domain of PCSK6, recognizing all identified isoforms of the protein, was used for IHC. For colocalizations, antibodies for cell-specific markers were used: anti-CD137 (ab64836, Abcam, Cambridge, UK, characterized by IHC in Supplementary Figure I), von Willebrand factor (vWF, M0616, DAKO, Glostrup, Denmark,) CD4 (NCL-L-CD4368, Novocastra, Newcastle, UK) CD163 (ab74604, Abcam), CD8 (NCL-L-CD8295, Novocastra), and smooth muscle α-actin (M0851, DAKO). Extracellular matrix components and matrix-degrading enzymes were identified with antibodies against: collagen I (SAB1402151, Sigma), collagen IV (2150-0121, Serotec), fibronectin (FN, ms167-pl, Neomarker), heparan sulfate (HS, 370255-1, Amsbio), Heparanase (HPSE, INS-26-1-000-12, Cell Sciences, Canton, MA), and MMP9 (MAB911, R&D Systems). Secondary antibodies were conjugated to Alexa Fluor 488 or 568 dyes (Invitrogen).

RNA extraction

Human tissue samples were frozen at -80°C immediately after surgery. RNA was prepared using Qiazol Lysis Reagent (Qiagen, Hilden, Germany) and purified by the RNeasy Mini kit (74106, Qiagen), including DNase digestion. The concentration was measured using Nanodrop ND-1000 (Thermo Scientific, Waltham, MA) and quality estimated by a Bioanalyzer capillary electrophoresis system (Agilent Technologies, Santa Clara, CA). For *in vitro* studies, cells were also collected in Qiazol, RNA isolated by chlorophorm extraction and purified following the same procedure used for tissues.
Microarray analysis

For gene expression analysis by microarrays, RNA extracted from 127 endarterectomy and 10 control iliac artery specimens, was analyzed by Affymetrix HG-U133 plus 2.0 Genechip arrays (Affymetrix, Santa Clara, CA). Robust multi-array average normalization was performed and processed gene expression data was returned in log2-scale as previously described. Full data set is available from Gene Expression Omnibus (accession number GSE21545). Microarray data was filtered by first omitting the 33888 probesets (of 54675) with lowest expression value, and then further subset to leave only probesets which had gene IDs corresponding to the 7853 genes present in the Human Protein Atlas (version 5.0 2009).

Real-time PCR

For qRT-PCR total RNA was reverse-transcribed using High Capacity RNA-to-cDNA kit (4387406, Applied Biosystems, Life Technologies Corporation, Carlsbad, CA). PCR amplification was done in 96-well plates in 7900 HT real-time PCR system (Applied Biosystems), using TaqMan® Universal PCR Master Mix (Applied Biosystems) and TaqMan® Gene Expression Assays (PCSK6: Hs01060077_m1, Applied Biosystems). All samples were measured in triplicates. Results were normalized to the equal mass of total RNA as well as the Ct values of RPLPO or TBP housekeeping controls (probes Hs99999902_m1 and Hs99999910_m1, respectively). The relative amount of target gene mRNA was calculated by 2^-ΔΔCt method.

Construction of tissue microarrays

Plaques used for the generation of TMAs were fixed in 4 % formalin for 24 hours, paraffin embedded and assembled as described previously (www.proteinatlas.org). Briefly, cross-sections through each plaque were made to construct two donor blocks, at the middle level and 10 mm adjacent (Figure 1). Tissue quality was assessed by Hematoxylin and Eosin staining, and areas with the least calcification were chosen. Six cores (1 mm x 4 mm), three from each of the donor blocks, were punched to compensate for the tissue heterogeneity. The individual cores were arrayed into two recipient multicore TMAs and used for immunohistochemical stainings.

Immunohistochemistry

Paraffin blocks of TMAs were sectioned at 4 µm thickness, deparaffinized, hydrated, and exposed to high-temperature antigen retrieval. Staining of TMA slides was performed in an automated histostaining instrument (Autostainer XL, Leica). Slides were blocked with 0.3 % H2O2 in 95 % ethanol for 5 minutes to quench endogenous peroxidase, incubated with primary antibodies, washed and then incubated with peroxidase-conjugated secondary antibody. After final washing, the signal was developed with chromogen for immunoperoxidase staining (DAB Plus). All slides for quantification of the staining intensities were scanned with equal settings using an automated Aperio XT (Aperio Technologies) system.

For individual staining of plaques and control tissue, all IHC reagents were from Biocare Medical (Concord, CA). Isotype rabbit and mouse IgG were used as negative controls. In brief, 5 µm sections were deparaffinized in Tissue Clear and rehydrated in ethanol. For antigen retrieval, slides were subjected to high-pressure boiling in DIVA buffer (pH 6.0) or TE buffer (pH 9.0). After blocking with Background Sniper, primary antibodies diluted in Da Vinci Green solution were applied and incubated at
room temperature for 1 hour. A probe-polymer containing alkaline phosphatase or a double-stain probe-polymer detection kit containing both alkaline phosphatase and horseradish peroxidase was applied, with subsequent detection using Warp Red and Vina Green. All slides were counterstained with Hematoxylin QS (Vector Laboratories, Burlingame, CA), dehydrated and mounted in Pertex (Histolab, Gothenburg, Sweden). Images were taken using a Nikon OPTIPHOT-2 microscope equipped with digital camera and NIS-Elements software.

**Immunofluorescence**

For immunofluorescence (IFL), paraffin sections (4 µm) were pretreated as for IHC and exposed to blocking with 5 % normal goat serum. Cells grown on glass-coverslips were fixed in 3 % paraformaldehyde for 20 minutes at room temperature, permeabilized with 0.1 % Triton X-100/PBS for 5 min, followed by blocking with 2 % bovine serum albumin/PBS for 1 hour. Both tissue and cell samples were incubated with primary antibodies diluted in the blocking solution for 1 hour at room temperature, washed with PBS and counterstained with Alexa Fluor 488 or 568-conjugated secondary antibodies. Nuclei were stained with diamidino-2-phenylindole (DAPI). For double labeling, incubations were performed sequentially to prevent cross-reactions. Photos were taken using Zeiss LSM510 confocal microscope, with 20x, 40x or 63x objectives.

**Cell culture**

Human carotid artery SMCs (3014-05a, Cell Applications), were grown in 5 % CO₂ humidified environment at 37°C, in complete medium (311-500, Cell Applications). Cells were maintained in conditions shown to promote a synthetic and dedifferentiated cellular phenotype. SMCs at passages 5-10 were plated on fibronectin (PHE0023, Invitrogen) coated 6-well plates and left to adhere. After overnight serum-starvation, cells were separately treated with a panel of cytokines and growth factors (PDGFBB, PHG0044, Gibco, 50 ng/ml; TGFβ1, T7039, Sigma, 20 ng/ml; TNFα, PeproTech, Rocky Hill, NJ, 20 ng/ml; IFNγ, 285-IF-100, R&D Systems, 20 ng/ml) and collected at several time-points (2h, 4h, 8h and 24h) for RNA isolation and qRT-PCR analyses. All samples were measured in triplicates.

**Data handling and statistical analyses**

Student’s T-test comparing symptomatic and asymptomatic patients was used for statistical analyses of microarray data. Because of the different gender-ratios in each group, we repeated the analysis using a linear model of gene expression and symptom-status, including gender as covariate. All genes selected for further analysis were also significant using this approach. Correction for multiple comparisons was not performed, instead all p-values were reported. Blinded semi quantitative evaluation of the TMA staining intensity was performed according to a four-grade scale: 0 - no staining signal, 1 - weak signal or a few cells stained, 2 - medium or strong signal localized in a certain area, 3 - strong staining of the whole core section area. Scoring values were compared between groups by Mann-Whitney test. Cronbach’s reliability analysis was performed with a randomly chosen antibody staining to test for the validity of the grading results. Spearman correlations were calculated to determine association between expression of PCSK6 and different genes from microarrays. For genes with multiple probe sets, probes with the highest sensitivity and specificity according to GeneAnnot were chosen. Statistical analyses
were done with the GraphPad Prism 6 software and p-value <0.05 was considered to indicate significance.
References


