Atherosclerosis 226 (2013) 74–81

Contents lists available at SciVerse ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Increased expression of fatty acid binding protein 4 and leptin in resident macrophages characterises atherosclerotic plaque rupture

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ARTICLE INFO

Article history:
Received 31 May 2012
Received in revised form
23 September 2012
Accepted 25 September 2012
Available online 5 October 2012

Keywords:
Plaque rupture
Gene expression
Macrophages
Microarray
Laser micro-dissection

ABSTRACT

Objective: Resident macrophages play an important role in atheromatous plaque rupture. The macrophage gene expression signature associated with plaque rupture is incompletely defined due to the complex cellular heterogeneity in the plaque. We aimed to characterise differential gene expression in resident plaque macrophages from ruptured and stable human atheromatous lesions.
Methods and results: We performed genome-wide expression analyses of isolated macrophage-rich regions of stable and ruptured human atherosclerotic plaques. Plaques present in carotid endarterectomy specimens were designated as stable or ruptured using clinical, radiological and histopathological criteria. Macrophage-rich regions were excised from 5 ruptured and 6 stable plaques by laser microdissection. Transcriptional profiling was performed using Affymetrix microarrays. The profiles were characteristic of activated macrophages. At a false discovery rate of 10%, 914 genes were differentially expressed between stable and ruptured plaques. The findings were confirmed in fourteen further stable and ruptured samples for a subset of eleven genes with the highest expression differences ($p < 0.05$). Pathway analysis revealed that components of the PPAR/Adipocytokine signaling pathway were the most significantly upregulated in ruptured compared to stable plaques ($p = 5.4 \times 10^{-7}$). Two key components of the pathway, fatty-acid binding-protein 4 (FABP4) and leptin, showed nine-fold ($p = 0.0086$) and five-fold ($p = 0.0012$) greater expression respectively in macrophages from ruptured plaques.
Conclusions: We found differences in gene expression signatures between macrophages isolated from stable and ruptured human atheromatous plaques. Our findings indicate the involvement of FABP4 and leptin in the progression of atherosclerosis and plaque rupture, and suggest that down-regulation of PPAR/adipocytokine signaling within plaques may have therapeutic potential.

1. Introduction

Atheromatous plaque erosion and rupture leading to atherothrombotic occlusion or distal embolisation is responsible for the majority of the acute morbidity and mortality of atherosclerosis, such as myocardial infarction, unstable angina and thromboembolic stroke [1]. Differences in cellular composition between stable and ruptured plaques are well established. The macrophage is central to the local inflammatory and apoptotic processes leading to plaque instability and rupture, however, the molecular pathways in macrophages that contribute to plaque rupture are incompletely characterized. The presence and character of differences in gene-expression patterns between macrophages in stable and ruptured lesions could identify metabolic and regulatory pathways that influence plaque instability and rupture. Many previous gene expression studies in human samples have compared whole plaques with normal tissue, while fewer have compared gene expression between stable and ruptured plaques [2–10]. The use of whole plaques for gene expression analysis effectively pools the RNA of various cell types in the plaque relative to their abundance, adding a potentially confounding variable to the analysis. A cell-specific approach has the potential to address the question of gene expression differences between particular cell types in stable and unstable plaques with greater precision than approaches based on the study of whole plaques. Using laser micro-dissection, we isolated total RNA from macrophage-rich regions of stable and ruptured human atheromatous plaques derived from carotid endarterectomy

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0021-9150
http://dx.doi.org/10.1016/j.atherosclerosis.2012.09.037

samples which were comprehensively characterized using clinical, radiological and histological criteria, and carried out genome-wide gene expression profiling using microarrays.

2. Materials and methods

2.1. Specimens

Carotid endarterectomy specimens were obtained from patients undergoing surgery for symptomatic or asymptomatic carotid stenoses at the Regional Neurosurgical Centre, Newcastle-upon-Tyne. Magnetic resonance imaging (MRI) of the brain and 3D gadolinium-DTPA contrast-enhanced magnetic resonance angiography (MRA) of the carotid arteries were performed on a 1.5 T scanner (Intera, Philips Medical Systems). Specimens were snap-frozen in liquid nitrogen in the operating theatre immediately upon removal. A portion of each specimen was sent for histopathological analysis, and classified by two independent observers (KL and TP) according to the Virmani scheme [1]. Informed consent was obtained from all patients and Local Research Ethics Committee approval was granted for this study.

We selected contrasting ruptured and stable samples for RNA analysis. The criteria for ruptured samples comprised all three of the following: symptoms consistent with stroke or transient ischaemic event (TIA) within the last 3 months; significant irregularities of plaque surface on 3D MRA (defined as depressions in the plaque surface of at least 2 mm); and histology of a Ruptured Thin Fibrous Cap Atheroma with thrombus present. Conversely, the criteria for stable samples were: no symptoms attributable to CVA/TIA at any time; a smooth plaque surface morphology on 3D MRA and no evidence of cerebral infarction on MRI; and histology of a thick Fibrous Cap Atheroma or Fibro-Calcific Plaque.

2.2. Laser micro-dissection (LMD) and microarray analysis

Cryosections of 10 μm thickness were mounted on RNase-free treated Leica thermoplastic membrane slides, then fixed in 75% ethanol, stained with haematoxylin, dehydrated in increasing gradients of ethanol and rendered RNA stable for laser micro-dissection. At every 10 sections, 3 additional 'scout' sections were stained with haematoxylin and eosin to identify anatomical features; these sections were immuno-stained using antibodies to smooth muscle actin (Dako, 1A4, 1:400) to identify regions rich in vascular smooth muscle cells, and CD68 (Dako, PG-M1, 1:125) to identify regions rich in macrophages, using the Vectastain Elite ABC Kit. These sections were used to guide laser micro-dissection of macrophage-rich regions performed on the Leica AS LMD instrument. Macrophage-rich CD68+ regions underlying thick fibrous caps and overlying atheromatous cores of stable lesions, and macrophage-rich CD68+ regions underlying thin fibrous caps, overlying atheromatous cores and adjacent to the defect/rupture of unstable lesions were microdissected. Cellularly mixed regions containing both macrophage and SMCs were avoided.

RNA was isolated using the Qiagen RNeasy Micro kit. RNA quality and quantity were assessed by RIN score using the RNA6000 Pico Labchip (Agilent Bioanalyser 2100, Agilent). Only samples with RIN scores of 6 and above were considered suitable for microarray analysis. For microarray analyses, 20–60 ng of total RNA were subjected to two cycles of linear amplification using the Affymetrix GeneChip Two-Cycle Target Labeling Kit and hybridized to Affymetrix U133plus2 chips. Five ruptured and six stable samples underwent microarray analysis. To replicate the results of the microarray analysis for the most significantly differentially expressed genes, an additional seven stable and seven ruptured samples underwent laser microdissection, and RNA extraction and quality assessment, using the same protocol.

2.3. Quantitative PCR

The most significantly differentially expressed genes from the microarray experiment were confirmed using qPCR both in the samples from the microarray experiment and the replication set. Unamplified total-RNA was reverse transcribed to cDNA by random hexamer primers using the SuperScript3 First Strand Synthesis System for RTPCR (Invitrogen). Quantitative real-time PCR was performed using Taqman Gene Expression Assay primer-probes with Taqman Universal PCR Master Mix on the ABI 7900HT platform (Applied Biosystems). Expression levels of succinate dehydrogenase complex subunit-A (SDHA) and peptydylprolyl isomerase-A (PPIA) were used as references. These 2 control genes were selected and validated from 7 candidate genes (details in Supplemental materials). The analysis was performed using the relative quantitation method with PCR efficiency corrections on QBase [11].

2.4. Immunohistochemistry

Sections of 5 μm thickness from formalin-fixed paraffin-embedded carotid atheromatous plaque were immunostained with anti-leptin mouse monoclonal antibodies (ABCAM, BDI142, 1:500) and anti-fatty-acid binding-protein 4 (FABP4) rabbit polyclonal antibodies (ABCAM, 1:150) using the Vectastain Elite ABC Kit and visualized using Di-Amino Benzidine, to confirm the expression domains of Leptin and FABP4.

2.5. Statistical analysis

The microarray expression profiles were analysed using the GeneSpring GX 7.3.1 analysis package. Raw microarray signal data were pre-processed and normalized using GCRMA. Probesets that were called absent in more than 6 of the 11 samples were excluded from subsequent analyses. To confirm that our LMD protocol had successfully isolated RNA from macrophage-rich regions, we compared the microarray data with expression profiles from the NCBI Gene Expression Omnibus (GEO) repository (www.ncbi.nlm.nih.gov/geo/). These included data from a panel of 79 different cell and tissue types [12] ($n = 158$), from adipocytes [13] ($n = 24$) and from 4 separate macrophage experiments [14–17] ($n = 106$). Relationships were visualized by Condition Tree clustering (using Spearman correlation, as the similarity measure and confidence levels were assessed using 100 bootstraps), and Principal Component Analysis (PCA). Additionally, a list of genes differentially expressed during macrophage activation was obtained from the paper by Cho et al. (3815 genes) [14]. The expression profiles of the micro-dissected specimens for this subset of genes were compared with repository samples of blood cell origin as well as activated and unactivated macrophages.

Differentially expressed probesets were identified using ANOVA. We made allowance for multiple testing using the false discovery rate [18], adopting a threshold FDR of 10%. The BiNGO plug-in (Biological Network Gene Ontology tool) for the open-source Java platform Cytoscape [19] was used to identify significantly over-represented Gene Ontology Biological Processes among the differentially expressed genes. We used WebGestalt [20] (WEB-based Gene SeT AnaLysis Toolkit) to identify among the pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database those that were significantly over-represented among the set of differentially expressed genes. The significance of over-representation was calculated using the hypergeometric test.