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Altered expression of inflammation-related genes in human carotid atherosclerotic plaques

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ABSTRACT

Objective: Inflammation is a pivotal process in atherosclerosis development and progression, but the underlying molecular mechanisms remain largely obscure. We have conducted an extensive expression study of atherosclerotic plaques to identify the inflammatory pathways involved in atherosclerosis.
Methods: We studied 11 human carotid plaques, their respective adjacent regions and 7 control arteries from different subjects. Expression of 92 genes was studied by TaqMan low-density array human inflammation panel. Human aortic endothelial and smooth muscle cells were used for *in vitro* experiments.
Results: The mRNA levels of 44/92 genes (48%) differed significantly between the tissues examined (13 up-regulated and 31 down-regulated). Dysregulated genes encode molecules belonging to different functional classes although most of them encode enzymes involved in the eicosanoid synthesis pathway. The expression of PTGIS and PTGIR genes was decreased in human aortic endothelial and smooth muscle cells stimulated with oxLDL and TNF-α.
Conclusions: This study not only reveals several dysregulated genes in human lesions but also focuses the role played by the genes involved in the eicosanoid synthesis pathway during atherosclerotic development. The decrease of PTGIS and PTGIR expression after oxLDL treatment mirrors the decreased mRNA levels in atherosclerotic lesions versus control arteries, which suggests that oxidation is important for PTGIS and PTGIR regulation in human vessel cells during atherosclerosis development.

1. Introduction

Atherosclerosis is a multi-factorial disease associated with an oxidative and inflammatory status whose molecular mechanisms are not yet completely known [1,2]. Oxidized low-density lipoproteins (oxLDL) are a main component of atherosclerotic plaques and are also present in the peripheral plasma of atherosclerotic patients. Oxidized products give rise to inflammatory events that, in turn, lead to the progression of plaques [1,2]. Inflammation is involved in the atherosclerotic process by recruiting leucocytes, promoting plaque growth and inducing plaque destabilization. In fact, pro-inflammatory cytokines promote the infiltration of leucocytes through the endothelial expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [3,4]. Other molecules, such as integrins [5] CD40/CD40 ligand [6] and metalloproteinases [7], seem to contribute to lesion progression and morphologic changes including plaque degradation and destabilization. The COX and 5-LO pathways respectively catalyze the synthesis of

Abbreviations: AHA, American Heart Association; ALOX5, arachidonate 5-lipoxygenase; HAEC, human aortic endothelial cells; HASMC, human aortic smooth muscle cells; IL1R1, interleukin 1 receptor, type I; IL2RB, interleukin 2 receptor, beta; IL2RG, interleukin 2 receptor, gamma; ITGAL, integrin, alpha L; ITGAM, integrin, alpha M; ITGB1, integrin, beta 1; ITGB2, integrin, beta 2; LFA-1, lymphocyte function-associated antigen-1; LTA4H, leukotriene A4 hydrolase; LTC4S, leukotriene C4 synthase; MAC-1, macrophage antigen-1; oxLDL, oxidized low-density lipoprotein; PGD2, prostaglandin D2; PGF, prostaglandin F; PTGER2, prostaglandin E receptor 2; PTGER3, prostaglandin E receptor 3; PTGFR, prostaglandin F receptor; PTGIR, prostaglandin I2 (prostacyclin) receptor; PTGIS, prostaglandin I2 (prostacyclin) synthase; PTGS1, prostaglandin-endoperoxide synthase 1; TBXA2R, thromboxane A2 receptor; TBXAS1, thromboxane A synthase 1; TNF-a, tumor necrosis factor a; VCAM1, vascular cell adhesion molecule 1.

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Table 1
Characteristics of the studied population ($n = 11$).

Parameters	Total	Males $n = 8$	Females $n = 3$
Age (years)	69.2 ± 8.3	67.8 ± 8.2	73.0 ± 8.7
BMI (kg/m^2)	25.8 ± 3.1	25.9 ± 2.2	25.3 ± 5.6
Smokers *n* (%)	4 (36.4)	3 (37.5)	1 (33.3)
Symptomatics *n* (%)	9 (81.8)	7 (87.5)	2 (66.7)
Plaques in other sites *n* (%)	11 (100)	8 (100)	3 (100.0)
History of stroke *n* (%)	5 (45.5)	3 (37.5)	2 (66.7)
History of heart event *n* (%)	4 (36.4)	3 (37.5)	1 (33.3)
Diabetes *n* (%)	5 (45.5)	4 (50.0)	1 (33.3)
Cholesterol (mmol/L)	4.77 ± 0.81	4.59 ± 0.79	5.27 ± 0.77
LDL (mmol/L)	3.04 ± 0.71	2.85 ± 0.69	3.71 ± 0.17
HDL (mmol/L)	1.17 ± 0.27	1.12 ± 0.25	1.34 ± 0.37
Triglyceride (mmol/L)	1.61 ± 0.57	1.57 ± 0.57	1.71 ± 0.66
Glucose (mmol/L)	6.16 ± 2.63	6.30 ± 3.04	5.77 ± 1.35
C-reactive protein (mg/L)	2.68 ± 2.75	2.41 ± 2.29	3.39 ± 4.29

Values are reported as mean ± SD. Distributions are not significantly different between males and females.

prostaglandins and leukotrienes, which produce many pro-inflammatory or anti-inflammatory effects that probably modulate the atherosclerotic process [8,9]. Some genes involved in the production of eicosanoids were found to be up-regulated in initial and advanced plaques, which indicates an active inflammation process in atherosclerosis development and progression [10–12].

In an attempt to identify inflammatory pathways that play a major role in atherosclerosis, we performed an expression study of 92 inflammation-related genes in human atherosclerotic plaques. We also investigated the role of oxLDL and inflammatory (TNF-α) stimuli in the expression of a subset of dysregulated genes by *in vitro* experiments on human aortic endothelial cells (HAEC) and on human aortic smooth muscle cells (HASMC) co-cultured with the human macrophage cell line THP-1.

2. Methods

2.1. Subjects and specimens

Carotid plaque samples were obtained from 11 patients undergoing carotid endarterectomy for stenosis ≥70% or stenosis ranging from 50% to 70% associated to clinical symptoms according to American Heart Association (AHA) guidelines [13]. The patients were enrolled at the Dipartimento di Chirurgia Vascolare e Endovascolare. Symptomatic patients were defined subjects with a history of transient attacks, stroke or *amaurosis fugax*. The study was performed according to the current version of the Helsinki Declaration and informed consent was obtained for each patient. The demographic and biochemical features of the population studied are reported in Table 1.

The plaque area and tissue from the region immediately adjacent to the plaque were collected from our patients as previously described [14]. We used the region immediately adjacent to the plaque as control because of the difficulty in obtaining healthy carotid arteries—a strategy used in previous studies [15–17]. As a control of vascular tissue, we used 7 healthy arteries (mesenteric and iliac arteries) obtained from patients (4 males and 3 females) undergoing gastro-intestinal tract surgery, collected at the Dipartimento di Scienze Biomorfologiche e Funzionali-Sezione Anatomia Patologica e Citopatologica. The control arteries were obtained from subjects without a history of stroke or heart events (average age, 60 years).

2.2. Lesion characterization

Atherosclerotic plaques and their adjacent regions were classified based on histology hematoxylin–eosin stain according to AHA guidelines [18,19]. The group of 11 advanced plaques was constituted by 2 type V lesions (16.7%), 5 type VI, complicated, lesions (41.7%), 4 type VII, calcified, lesions (33.3%) and 1 type VIII, fibrotic, lesion (8.3%). Their respective adjacent regions were classified as follows: 6 type II lesions (54.5%) and 5 type III lesions (45.5%). All control arteries showed stage I thickening (physiological thickening).

2.3. In vitro experiments

Human aortic endothelial cells (HAEC) and human aortic smooth muscle cells (HASMC) (Lonza) were cultured according to the supplier's instructions and used between passages 4 and 6 at 30,000 cells/cm^2 on 6-well plates. The next day, a 0.4-μm pore Transwell support (Corning) was inserted in each well, and 300,000 THP-1 macrophage cells were added to each well. Each treatment was also performed without macrophage cells. The optimal concentrations and exposure times were determined for each treatment by dose–response and time course experiments (data not shown). Experiments were performed using oxLDL at 100 μg/ml and TNF-α at 20 ng/ml both for 48 h.

3. mRNA expression evaluation

3.1. RNA extraction and cDNA preparation

Total RNA was isolated from tissues homogenized in guanidinium thiocyanate/β-mercaptoethanol as previously described [14]. Total RNA was extracted from cells with Tripure Isolation Reagent (Roche) according to the manufacturer's instructions. RNA integrity was verified by electrophoretic agarose gel. cDNA was synthesized from 2.5 μg total RNA by using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems).

3.2. Real-time quantitative PCR by TaqMan low-density array human inflammation panel

cDNA from tissues was amplified on an ABI Prism 7900HT Sequence Detection System (SDS 2.3 software, Applied Biosystems) using the TaqMan low-density array human inflammation panel (Applied Biosystems). Each card contains 384 wells pre-assembled with 96 TaqMan gene expression assays for the simultaneous mRNA expression of 92 inflammation-related genes and of 4 housekeeping genes (endogenous controls). The complete list of genes is reported at http://www.appliedbiosystems.com/support/human_inflammation.xls. To determine which of the 4 housekeeping genes (rRNA 18S, beta-actin, GAPDH and β-2-microglobulin) to use as endogenous control, we performed a preliminary analysis to find the gene whose expression, in our experimental conditions, varied least among the samples analyzed. This analysis showed that β-2-microglobulin gene expression differed the least among samples; hence, β-2-microglobulin served as our endogenous control. The real-time PCR was performed in duplicate with the TaqMan gene expression master mix (Applied Biosystems) in a reaction volume of 2 μl containing 0.1 ng/μl cDNA according to TaqMan low-density array instructions. One sample was used as calibrator for data normalization.

3.3. Real-time quantitative PCR by single assay

Single pre-developed TaqMan assays (PTGIS: Hs00919949_m1; PTGIR: Hs00168765_m1) and the beta-glucuronidase as