Identification of two genes potentially associated in iron-heme homeostasis in human carotid plaque using microarray analysis

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Classic characteristics are poor predictors of the risk of thromboembolism. Thus, better markers for the carotid atheroma plaque formation and symptom causing are needed. Our objective was to study by microarray analysis gene expression of genes involved in homeostasis of iron and heme in carotid atheroma plaque from the same patient. mRNA gene expression was measured by an Affymetrix GeneChip Human Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA) using RNA prepared from 68 specimens of endarteriectomy from 34 patients. Two genes involved in iron-heme homeostasis, CD163 and heme oxygenase (HO-1), were analysed in 34 plaques. CD163 (2.18, p=1.45E−08) and HO-1 (fold-change 2.67, p=2.07E−09) mRNAs were induced. We suggest that atheroma plaques show a more pronounced induction of CD163 and HO-1. Although further evidence is needed, our results support previous data. To our knowledge, this is the first report comparing gene expression between intact arterial tissue and carotid plaque using microarray analysis.

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1. Introduction

Features associated with a symptomatic atheroma plaque include the degree of vessel stenosis, ulceration, inflammatory cell infiltration and a thin fibrous cap (Barnett *et al.* 1998; Golledge *et al.* 2000; Takaya *et al.* 2006). However, some classic characteristics are poor predictors of the risk of thromboembolism and, as a result, 80% of patients undergoing carotid endarterectomy are needlessly exposed to surgical risks (Kappelle 2002; Ijäs *et al.* 2007). Thus better markers for the carotid atheroma plaque formation and symptom causing are needed. Gene microarray technology can be used to investigate global mRNA expression to identify mRNA populations that exhibit differential regulation in disease processes, thus providing important clues to the underlying molecular pathology. This led us to a microarray study on patients operated due to significant carotid stenosis. Genes involved in the metabolism of iron and heme were studied. CD163 and heme oxygenase (HO-1) were further analysed at mRNA level in 34 independent carotid plaques. The aim of our study was to verify gene expression of CD163 and HO-1 because the induction of these two genes would fit with more frequent degradation of haemoglobin and intraplaque hemorrhages in atheroma plaques.

2. Materials and methods

2.1 *Patients and tissue sampling*

The investigation conforms to the principles outlined in the declaration of Helsinki (World Physician Organization 1997); all procedures were approved by the local ethical committee and the patients gave informed consent. Thirty-four patients who underwent carotid endarterectomy at the university hospital of Lyon (Hôpital Edouard Herriot) were included in the study. The carotid endarterectomy samples were collected in the surgery room and immediately dissected in two fragments: the atheroma plaque (ATH) and the macrospically intact tissue (MIT). Each fragment was further divided: one part was immediately frozen in liquid nitrogen (LN2) for RNA analysis and the other was used for histological examination. To avoid the inherent problems of

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control tissue collection, we made intra-patient comparison of the transcript profiles.

2.2 *Histological analysis*

Fragments from endarterectomy tissue were fixed in 4% paraformaldehyde in phosphate buffer saline and subsequently paraffin-embedded. Samples of endarterectomy tissue from 34 patients, separated as described above, were numbered and given blind to the pathologist for conventional processing and analysis, to determine the stage of the lesion. Each sample was graded for smoothness, ulceration, hemorrhage, necrosis, calcification, inflammation, media thickness, fibrosis, spumous cells and intramural thrombus.

2.3 *Total mRNA isolation*

mRNA were extracted from the tissue using Trizol (Invitrogen, USA) following the manufacturer's instructions, then treated with DNAse (Qiagen, FRANCE), and purified using the RNeasy MinElute TM clean up kit (Qiagen, FRANCE) according to the manufacturer's instructions. Quantification and estimation of RNA purity was performed using NanoDrop (Nanodrop, USA). Finally, RNA integrity was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, USA) in order to measure RNA integrity number (RIN).

2.4 *Microarray experiments*

Samples of high quality were transferred to the platform of the Strasbourg Genopole for labeling and hybridization with Affymetrix Human GeneChip Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol. Each mRNA sample was hybridized to its own microarray resulting in 68 arrays from 34 patients.

2.5 *Statistical analysis of expression data*

Multiple probe set IDs for a given gene were averaged to obtain a representative expression value for the gene. Transcripts were considered to be present in the data set if at least one detectable probe set was detected by the detection above background (DABG) method with a probability of 0.05. Expression values were determined for entire transcripts thereby averaging over multiple probe sets and were scaled logarithmically. Fold changes are reported as an absolute value. Comparisons between MIT and atheroma plaques gene expression level were performed. Differential expression was tested by paired *t*-test. Benjamini and Hochberg multiple testing corrections were applied to obtain the false discovery rates using the significance analysis of microarrays.

3. Results

3.1 *Patients*

Patients were under various therapies and type 2 diabetes mellitus (T2D) patients were defined since they received an anti-diabetic treatment. The included patients were found to be hypertensive, since they received antihypertensive drug treatment. Clinical parameters of the patients included in this study are given in table 1.

3.2 *Histological control of tissue sampling*

Endarterectomy specimens were characterized histologically according to the classification proposed by the Nomenclature Committee of the American Heart Association (Stary *et al.* 1995). Analysed sections consisted of the intima and a majority of the tunica media, the adventitia was excluded, the fragment considered as atheroma plaque presented

Table 1. Clinical parameters from 34 patients included in this study

Clinical parameters	
Age, years	70±10
Gender, F/M	5/29
Hypertensive, %	76
Diabetic,%	38
Symptomatic, %	26
Lipidemic, %	77
BMI, kg/m^2	26±4
SBP, mmHg	156±23
DBP, mmHg	83±11
Plasma glucose, mmol/L	6±1
LDL, mmol/L	2.7±1
HDL, mmol/L	1±0.3
Triglycerides, mmol/L	1.8±1
ApoB, g/L	0.9±0.2
HbA1c, %	6.5±1
CRP, mg/L	16±24
Statin, %	62
ACEI/ARB, %	50

Results are the mean ± SD. BMI indicates body mass index, SBP : systolic blood pressure, DBP : diastolic blood pressure, LDL : low-density lipoprotein, HDL : high-density lipoprotein, ApoB : apolipoprotein B, HbA1c : glycosylated hemoglobin, CRP : C-reactive protein. ACEI/ARB : angiotensin I converting enzyme inhibitors/ angiotensin II receptor blockers.

mostly stage IV and/or V lesions. What was considered macroscopically as 'intact tissue' was almost exclusively composed of stage I and II lesions.

3.3 *Microarray analysis*

There were no significant correlations between genes expression and clinical parameters or history of cerebral ischemia. We could not detect any relationship between mRNA expression for any of the genes and any of the various drugs or drug associations taken by the patients. No effect from the patients' treatment was detected on gene mRNA expression, probably because of the large panel of drugs used by patients and the absence of untreated patients. The difference between T2D and control patients in all mRNA did not reach statistical significance.

The present results show that it is possible to identify genes that are differentially expressed in human atheroma plaques compared to MIT. Genes involved in iron-heme production within the arterial wall were analyzed. We used fold change as cut-off criterion.

From the data obtained with microarray analysis, we focused our attention on the expression of 2 genes. The selection was based on two criteria: microarray results of differential expression and the potential role/current knowledge of the gene product in homeostasis of iron and heme metabolism. For comparison purpose, w e then examined levels of differential expression of interest genes (table 2). Results shown in figure 1 indicate gene expression (log 2) in ATH compared to MIT.

This genome-wide microarray expression study of carotid plaques and intact tissue yield several potential regulators of plaque destabilization. A spectrum of factors underlying iron-heme production has been studied, but distinguishing between high-risk and low-risk plaques is still an enigma. In this approach, we discovered that the atheroma plaque formation coincided with increased expression of CD136 and HO1, iron-heme markers.

4. Discussion

Microarray technology provides a rapid means to screen gene expression in the tissues of interest. Transcriptional profiling was based on Affymetrix Human GeneChip Gene 1.0 ST

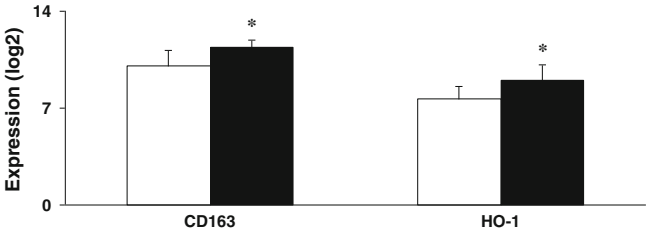

Figure 1. Gene expression in atheoma plaque compared to adjacent macroscopically intact tissue. Comparison of mRNA concentrations in atherosclerotic plaques (ATH) and in adjacent macroscopically intact tissue (MIT) from 34 patients. Black and empty and bars represent measurements in atheroma plaque and in nearby macroscopically intact tissue respectively. Results are mean ± SEM. *$p<0.05$, MIT cells vs ATH. MIT indicates macrospically intact tissue, ATH : atheroma plaque, HO1 : heme oxygenase.

microarray (Affymetrix, Santa Clara, CA, USA) that is a whole transcript-based array for gene expression profiling. An important feature of this array is that, as for the Human Exon 1.0 ST array, it queries the entire transcript in contrast to most older Affymetrix arrays that query the 3′ end of transcripts.

Several efforts have been made to study large-scale gene expression in human atherosclerosis, for example by comparing gene expression in normal and atherosclerotic arteries. Changes involved in homeostasis of iron and heme of the atherosclerotic plaque have been less in focus. The present study started from a large-scale microarray analysis in 34 patients to screen gene expression of iron-heme synthesis that are differentially expressed between MIT and atheroma plaque within the same individual. To our knowledge, this is the first report comparing gene expression between MIT and atheroma carotid plaques. Our cohort of 34 patients included all consecutive patients admitted to university hospital of Lyon for carotid endarterectomy throughout 2009. Consequently, the microarray study has enough power to provide significant results at the genome-wide level.

In this study, we used all available plaque tissue for mRNA quantification. An alternative would be to use only tissue from carefully characterized areas of plaque morphology. Similarly, we and several others have adopted microarray analysis to the whole plaque (Faber *et al.* 2001; Randi *et al.* 2003; Adams *et al.* 2006) but some groups have used

Table 2. Differentially expressed genes from the microarray analysis in the arterial wall

Gene	Title gene*	Accession no.	Symbol probe	Fold change	*p*-value†	FDR‡
CD163	CD163 molecule	NM_004244	7960794	2.18	1.45E-08	0
HO-1	heme oxygenase (decycling) 1	NM_002133	8072678	2.67	2.07E-09	0

*Genes previously suggested to have a role in the arterial iron-heme metabolism are screened and the appropriate reference is given.

† Paired *t*-test, unadjusted *p*-values.

‡ FDR is the false discovery rate, *p*-values adjusted by Benjamini and Hochberg multiple testing correction.

only specific areas of plaque activity in their analysis (Papaspyridonos *et al.* 2006; Dhore *et al.* 1998). Interestingly, despite the different approaches used, the results shared considerable similarity. This suggests that both approaches yield meaningful information and can be used to complement each other.

Additionally, the Gene 1.0 ST Array uses a subset of probes from the Human Exon 1.0 ST Array and covers only well-annotated content. Each gene is represented on the array by approximately 26 probes spread across the full length of the gene, providing a more complete and more accurate picture of gene expression than the 3′ based expression array designs.

Concerning results interpretation, we have to keep in mind that atherosclerosis is a general disease and thus what we called 'intact tissue' is, in fact, already remodelled tissue. However, in human studies it is almost impossible to obtain real normal human tissue suited for gene expression analysis. Nevertheless, the intra-patient comparison allows us to draw conclusions about the atherogenic process per se.

We could show that two genes involved in the homeostasis of iron and heme, CD163 and HO-1, were induced at the mRNA level in a larger set of carotid plaques (n=34). It could be argued that overexpression of CD163 in atheroma plaques reflect differences in the cellular composition. Still, it is possible that overexpression of CD163 indicates over-representation of a certain macrophage subtype in atheroma plaques. Ijäs *et al.* (2007) showed that HO-1 stained macrophages that had endocytosed red blood cells or hemosiderin and were surrounded by cholesterol crystals.

Especially HO-1 is regarded as an anti-atherogenic agent during the early phases of atherosclerosis (Siow *et al.* 1999). HO-1 is known to prevent proliferation of vascular smooth muscle cells and endothelial cells after vascular injury (Duckers *et al.* 2001), which can be important to sustain plaque integrity and stability. Yet, both HO-1 and CD163 have characteristics that might paradoxically turn harmful in advanced atheroma. CD163, again, is linked to an increased risk of vascular complications via its different efficiencies to remove hemoglobin, depending on haptoglobin genotypes (Schaer 2002).

Our study revealed two genes that are potentially important in the destabilization of atheroma plaque. Especially, CD163 and HO-1, known to be involved in the degradation of haemoglobin, were induced in atheroma plaques. The idea is novel; however, we think that our data are preliminary. The limits of the study are that it is an isolated microarray study, without validation of the gene expression finding or any mechanism of action analysis to assess the relevance of the finding. The work requires, as a minimum, RT-PCR and Western blot confirmation of the changes found in the microarray study. Inclusion of this data and histological immuno-histo-chemistry to show localization of the proteins within the lesions is required to prove our hypothesis. However, another work showed that both mRNA and protein levels of CD163 and HO-1 correlated strongly with iron deposits (Faber *et al.* 2001). In line, their expression correlated with traditional markers of unstable carotid disease, the degree of carotid stenosis and plaque ulcerations. Intuitively, the induction of CD163 and HO-1 would fit with more frequent intraplaque hemorrhages in atheroma plaques. This suggests that intraplaque hemorrhages might be one important incidental factor causing plaque destabilization. On the other hand, a recent work of Ijäs *et al.* showed that HO-1 protein was overexpressed in symptomatic plaques. The expression of HO-1 and CD163 correlated with tissue iron content (Ijäs *et al.* 2007). These data corroborate our gene expression results.

In conclusion, the mechanisms underlying our observation warrant further research, the morphological study of the expression of CD163 and HO-1 in carotid atherosclerosis, will hopefully reveal new molecular targets for therapeutic applications stabilizing atherosclerotic plaques and preventing ischemic thromboembolic strokes.

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