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MICROARRAY BASED IDENTIFICATION REVEALS ARTERIAL ANGIOTENSIN SYSTEM EXPRESSION IN HUMAN

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ABSTRACT: Purpose: We studied by microarray analysis the tissue angiotensin system organization. We elucidated the expression of chymase, cathepsins D, G and angiotensin-converting enzyme (ACE), potentially involved in intraparietal angiotensin II formation and atheroma. **Methods:** mRNA gene expression was measured by an Affymetrix Gene Chip Human Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA) using RNA prepared from 68 specimens of endarteriectomy from 34 patients. **Results:** The studied mRNAs could be measured in all patients. ACE mRNA was increased 1.2 fold (p=1.21E-07) in atheroma. A 1.4 fold increase in cathepsin D mRNA (p=2.53E-07) was observed in atheroma plaque. Concerning chymase and cathepsin G, 1.22 and 1.24 fold change (p=0.001) were observed respectively. Angiotensin type 1 receptor (AT1R) mRNA was decreased 0.7 fold (p=2.74E-06) in atheroma compared to intact tissue. **Conclusion:** All components required for angiotensin II formation are expressed locally in the arterial wall. The genes expression showed clear changes in ATH compared to MIT by enhancing the involvement of genes associated with Ang II production. Over expression of ACE and cathepsin D may lead to angiotensin II overproduction and contribute, to the lower amount of AT1R in atheroma. Although further evidence is needed, our results support previous data.

INTRODUCTION: Atherosclerosis is the main cause of cardiovascular diseases. It was demonstrated a major beneficial effect of the angiotensin-converting enzyme (ACE) inhibitor ramipril in primary prevention of cardiovascular diseases in high-risk patients [1]. Besides, the renin-angiotensin system (RAS) blockade with ACE inhibitors decreased both mortality and frequency of recurrent ischemic events after myocardial infarction, suggesting an effect on the atherosclerotic process [2].

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It has been shown that the amount of immunoreactive-ACE and angiotensin II are significantly increased in atherosclerotic regions of coronary arteries compared to intact ones [3, 4]. Angiotensin II administration accelerates the development of the atherosclerotic lesions in apoE-deficient mice [5]. It should be noted that the efficacy of ACE inhibitors in attenuating the progression of atherosclerosis has also been demonstrated in animal models [6, 7].

The administration of an angiotensin II receptor type 1 (AT1R) antagonist weakly slowed the progression of the disease, suggesting that other pathways may be involved [8, 9]. The importance of the role of the angiotensin system in vascular remodelling comes from two types of arguments: the beneficial effect of RAS blockade in clinical

and experimental settings and potentiation of vascular remodelling by angiotensin II in animal models. Angiotensin II production pathways within the arteriel wall might be different from the systemic plasma angiotensin II pathway [10].

There are two types of enzymes in tissues leading to angiotensin II formation: Cathepsin D, G and ACE [10, 11]. Since the potential of cathepsins D, G and ACE as angiotensin II-forming enzymes has been explored in the arterial wall, the aim of the present work was to investigate the RAS components in human carotid artery with or without atherosclerotic lesions using microarray analysis, in an attempt to shed more light on the possible molecular mechanism involved in the arterial production of angiotensin II.

MATERIALS AND METHODS:

Patients and Tissue Sampling: The investigation conforms to the principles outlined in the declaration of Helsinki [12], all procedures were approved by the local ethical committee and the patients gave informed consent. Thirty-four patients who had undergone 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 macroscopically intact tissue (MIT). Each fragment was further divided: one part was immediately frozen in liquid nitrogen (LN2) for RNA analysis, whereas the other was used for histological examination. To avoid the inherent problems of control tissue collection, we made intra-patient comparison of the transcript profiles.

Histological Analysis: Fragments from endarterectomy tissue were fixed in 4% para-formaldehyde 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.

Total mRNA Isolation: mRNA were extracted from the tissue using Trizol (Invitrogen, USA) according to 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).

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.

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.

RESULTS:

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**.

Histological Control of Tissue Sampling: Endarterectomy specimens were characterized

histologically according to the classification proposed by the Nomenclature Committee of the American Heart Association [13]. Analyzed sections consisted of the intima and a majority of the tunica media, the adventitia was excluded, the fragment considered as atheroma plaque presented mostly stage IV and/or V lesions. What was considered macroscopically as 'intact tissue' was almost exclusively composed of stage I and II lesions.

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.

Microarray Analysis: There were no significant correlations between genes expression and calcification degree, 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 plaque compared to MIT. From the data obtained with microarray analysis, we focused our attention on the expression of 8 genes. The selection was based on two criteria: microarray results of differential expression and the potential role/current knowledge of the gene product in RAS expression. For comparison purpose, we then examined levels of differential expression of interest genes (**Table 2**). Results shown in **Fig. 1** indicate gene expression (log 2) in ATH compared to MIT. We used fold change as cut-off criterion.

This genome-wide microarray expression study of carotid plaques and intact tissue yield several potential regulators of RAS expression. In this approach, we discovered that the atheroma plaque formation coincided with a marked increase in cathepsin D and ACE expression: alternatives enzymes generating angiotensin II. On the other hand, we showed a significant negative correlation between AT1R and cathepsin D mRNA levels ($r = -0.67$, $p < 0.05$) and AT1R and ACE mRNA levels ($r = -0.59$, $p < 0.05$) within the arterial tissue.

TABLE 2: DIFFERENTIALLY EXPRESSED GENES FROM THE MICROARRAY ANALYSIS IN THE ARTERIAL WALL

Gene	Title Gene	Symbol Probe	Accession No.	Fold Change	p-value†	FDR‡
AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	7924987	NM_000029	0.84	0.06	0.38
AT1R	angiotensin II receptor, type 1	8083240	NM_031850	0.71	8.74E-07	0
Cath G	cathepsin G	7978351	NM_001911	1.24	0.001	0.06
Cath D	cathepsin D	7945666	NM_001909	1.46	2.53E-07	0
Renin	renin	7923608	NM_000537	0.98	0.38	42.86
Chymase 1	chymase 1, mast cell	7978343	NM_001836	1.22	0.001	0.08
ACE	angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	8009096	NM_000789	1.21	1.21E-06	0

† Paired t-test, unadjusted p-values. ‡ FDR= the false discovery rate, p-values adjusted by Benjamin-Hoechberg multiple testing corrections.

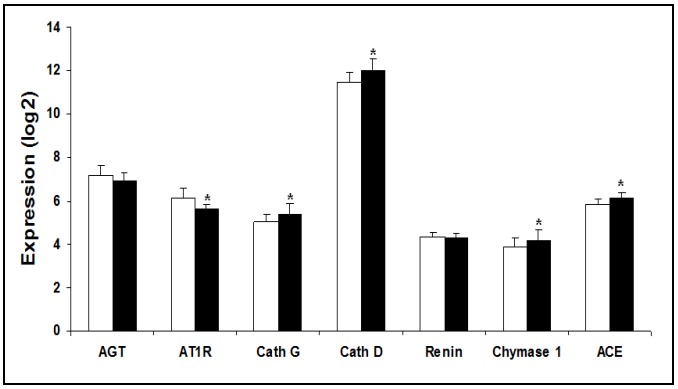

FIG. 1: GENE EXPRESSION IN ATHEOMA PLAQUE COMPARED TO ADJACENT MACROSCOPICALLY INTACT TISSUE

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 macroscopically intact tissue, ATH: atheroma plaque, AGT: angiotensinogen, AT1R: angiotensin II receptor type 1, Cath G: cathepsin G, Cath D: cathepsin D, ACE: angiotensin I converting enzyme.

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 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 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 RAS activation have been less in focus. The present study started from a large-scale microarray analysis in 34 patients to screen RAS gene expressions between MIT and arheroma 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 [14-16] but some groups have used only specific areas of plaque activity in their analysis [17, 18]. 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 remodeled 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.

In this work we have: (1) showed the expression of components of the angiotensin system (angiotensinogen, chymase ACE, Cathepsin D, G and AT1R) in the human carotid artery, (2) demonstrated, at the mRNA level, that the increase of angiotensin II production enzymes (ACE and Cathepsin D, G and Chymase) in the arterial tissue is associated with a decrease in AT1R mRNA content (**Fig. 1**).

Previous works have also demonstrated the presence of the mRNAs of angiotensinogen and ACE in human vascular wall [19]. Renin gene transcription in the arterial wall is controversial in human tissue [20, 21]. The presence of angiotensinogen mRNA in the carotid artery suggests the presence of a local paracrine or autocrine RAS.

Other enzymes such as cathepsins D, G and ACE, are expressed in the arterial wall as shown in our study. These findings suggest that the generation of

angiotensin II is possible from angiotensinogen within the arterial wall [11]. All components required for angiotensin II formation are expressed locally in the arterial wall, in the absence of renin, cathepsin G could be a major angiotensin-generating enzyme. Overexpression of ACE and cathepsin G may lead to angiotensin II overproduction and contribute, with decreased number of differentiated smooth muscle cells, to the lower amount of AT1R in atheroma.

In our study, a decrease in AT1R mRNA observed in the atheroma plaque could thus be indicative of a local increase in angiotensin II. Although the mechanisms of the increased mRNA levels observed in this study have not yet been characterized, the modulation of tissue angiotensin system gene transcription in the arterial wall in atheroma provides a molecular explanation for the benefit of ACE inhibitors [22-24].

Of note, immunohistochemical staining has already demonstrated the presence of ACE and AT1R in the arterial wall [3]. ACE, AT1R and angiotensin II have been demonstrated in endothelial, smooth muscle cells and mainly in macrophages in human atherosclerosis using immune-histochemical techniques [25].

CONCLUSION: We employed a genome-wide gene expression approach in order to discover novel genes involved in expression, an alternative enzyme generating angiotensin II. RNA microarray results revealing differential expression of the cathepsin D and ACE in atheroma plaque comparing to MIT.

The study we have established in this study provides the basis for further more elaborate studies that would take into account the variability in each tissue, due to age, gender or ethnicity. Molecular mechanisms within this organization need to be elucidated, as well as enzymatic activity, peptide production and signalling. Our results led us to conduct more detailed morphological studies of their expression in carotid atherosclerosis and to examine their correlation with the carotid plaque phenotype.

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, reverse transcriptase polymerase chain reaction and Western blot confirmation of the changes found in the microarray study. Inclusion of this data and histological immuno-histochemistry to show localization of the proteins within the lesions is required to prove our hypothesis.

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CONFLICT OF INTEREST: None declared.

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