

Correlations between gene expression highlight a different activation of ACE/TLR4/PTGS2 signaling in symptomatic and asymptomatic plaques in atherosclerotic patients

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ABSTRACT

Aiming to look into the elements that induce the development of a vulnerable or stable atherosclerotic plaque, and considering that inflammation has a central role in its progression of lesions, we analyzed the expression of genes involved in the ACE/TLR4/PTGS2 signaling in carotid plaques of symptomatic and asymptomatic patients.

Patients with internal carotid artery stenosis undergoing carotid endarterectomy at Verona University Hospital were included in this study. A total of 71 patients was considered for gene expression analysis (29 atherothrombotic stroke patients and 42 asymptomatic patients).

Total RNA was extracted from the excised plaques and expression of PTGS2, ACE, TLR4, PTGER4, PTGER3, EPRAP and ACSL4 genes was analyzed by Real Time PCR. The correlation between pair of genes was studied by Spearman coefficient.

From the analyzed genes, we did not observe differences in gene expression but the assumed gene expression networks deduced from the correlation coefficient, suggest a different activation of pathways in the two groups of carotid plaques.

INTRODUCTION

Atherosclerosis is the underlying cause of the majority of clinical cardiovascular events, such as stroke and myocardial infarction (Glass and Witztum 2001; Roger et al. 2012). Atherogenesis is described as an active process that involves altered cellular behavior in response to defined molecular signals. The detailed information now available of the interactions between blood components, including cells, proteins, and small molecules, and the intrinsic cells that populate the normal artery wall contribute to the understanding of the pathogenesis of this disease (Libby and Ridker 2006).

Carotid atherosclerotic plaques can be present with stable or unstable atheromatous lesions. Atherosclerotic plaques prone to rupture (unstable plaque) due to their composition such as a large lipid core, thin fibrous cap and intraplaque hemorrhage are associated with subsequent thromboembolic ischemic events (Schaar et al. 2004).

A unifying view of the pathophysiology of atherosclerosis is that inflammation has a key role and translates the effects of many known risk factors for the disease in vulnerable plaques. Inflammatory signaling alters the behavior of the intrinsic cells of the artery wall (endothelium and smooth muscle cells), and recruits further inflammatory cells that interact to promote lesion formation and complications (Libby et al. 2011). Characterization of plaques may enhance the understanding of natural history and ultimately the treatment of atherosclerotic disease.

In this study, we selected six genes involved in ACE/TLR4/PTGE2 signaling that have been previously associated with the atherogenesis processes [i.e. Prostaglandin-Endoperoxide Synthase 2 (PTGS2), Toll like receptor 4 (TLR4), Angiotensin I converting enzyme (ACE), Fatty Acid CoA Ligase 4 (ACSL4), EP4 receptor-associated protein (EPRAP), and prostaglandin E receptors 3 and 4 (PTGER 3, and PTGER 4)], to be analyzed in plaques of symptomatic and asymptomatic atherosclerotic patients.

Many studies have demonstrated the presence of Cox2 in atherosclerotic lesions and in fatty streaks (Burleigh et al. 2002, 2005). Cox-2 produced by activated macrophages, is the key enzyme in the production of prostaglandins (Gómez-Hernández et al. 2006). In particular, prostaglandin E2 (PGE2) has been implicated in the development of atherosclerotic plaques promoting the activation of chemotaxis, vascular permeability, propagation of the inflammatory cytokines cascade and stimulation of SMC migration (Cipollone et al. 2008; Schober et al. 2011). PGE2 interacts with four G protein-coupled cell surface receptors, EP1-4. EP3 and EP4 have been reported to be increased in peripheral blood mononuclear cells and in the inflammatory region of atherosclerotic plaques from

patients with carotid stenosis (Gómez-Hernández et al. 2006; Cipollone et al. 2008; Schober et al. 2011). EP4 may exert two opposite roles in the regulation of inflammation. It may exert anti-inflammatory effects when it is bound to EP4 by interfering with activation of *nuclear factor- κ B* (NF- κ B) and consequently inhibiting the release of chemokines (Minami et al. 2008; Tang et al. 2012) or it can exert pro-inflammatory effects, being involved in the biosynthesis of metalloproteinases from plaque macrophages (Cipollone et al. 2005b; Pavlovic et al. 2006). ACSL4 which codify for the fatty acid CoA ligase 4 (FACL4), is a member of the long-chain acyl-CoA synthetize (ACSL) family that has a marked preference for arachidonic acid (AA). It is a competitor of Cox-2 for AA and may play an anti-inflammatory role in stable carotid plaques. FACL4 converts arachidonic acid into arachidonoyl CoA ester, recycling it into membrane phospholipids, thus making it unavailable to metabolism by other competitive pathways. A strong difference in its expression between stable and unstable carotid plaques has been reported (Cipollone et al. 2005a). TLR4 gene is a key component of pathogen-associated molecular pattern recognition machinery, which mediates innate immunity (Medzhitov 2001). The involvement of TLR4 in atherosclerotic stroke is demonstrated by several studies, which reported an overexpression of this gene in human symptomatic atherosclerotic lesions and in 'unstable' - 'more prone to rupture' carotid plaques (Xu et al. 2001; Katsargyris et al. 2010, 2011; Malgor et al. 2014). Engagement of TLR4 with its ligands triggers a cascade of cellular signals, leading to the activation of NF- κ B and *mitogen-activated protein kinase* [MAPK] signal transduction pathways, inducing expression of inflammatory genes (Tobias and Curtiss 2005; Rifkin et al. 2005; Lu et al. 2008), including PTGS2, (Akira and Takeda 2004; El-Achkar et al. 2007; Park et al. 2007; Küper et al. 2012).

The proatherogenic actions of *Angiotensin II* (AngII) have been extensively studied (Brasier et al. 2002; Cheng et al. 2005). AngII via the type 1 (AT1) receptors can induce various transcription factors as Activating Protein-1 [AP-1], the Signal Transducers and Activators of Transcription (STATs), and NF- κ B, upregulating many proinflammatory molecules as adhesion molecules, cytokines, and chemokines. Ang II, via involvement of nuclear factor- κ B, has been implicated in the regulation of PTGS2 (Ohnaka et al. 2000; Derbyshire et al. 2005; Ji et al. 2009; Morinelli et al. 2009), and the activation of several inflammatory chronic disease processes mediated by Cox-2, including atherosclerosis (Das 2005; Gómez-Hernández et al. 2006). Ang II can induce inflammatory response involved in pathogenesis of atherosclerosis partly via TLR4-dependent signaling pathway (Derbyshire et al. 2005; Das 2005; Wolf et al. 2006; Ji et al. 2009; Nakashima et al. 2015).

Aiming to look into the elements that induce the development of a vulnerable or stable plaque, this

study analyzes the expression of various genes known to be involved in Cox2 signaling, in symptomatic and asymptomatic plaques of atherosclerotic patients.

MATERIALS AND METHODS

Patients

Consecutive eligible patients with internal carotid artery stenosis undergoing carotid endarterectomy at Verona University Hospital were included in this study. A total of 71 patients was selected for gene expression analysis. For gene expression analysis in carotid plaques, 29 atherothrombotic stroke patients and 42 asymptomatic patients were selected. The athero-thrombotic nature of the event was established as described by Ferronato et al. (2011).

Clinical information and characteristics of patients, vascular risk factors and current therapy were collected by study neurologists. Exclusion criteria included other inflammatory pathologies, and patients ongoing non-steroidal or glucocorticoid anti-inflammatory therapy. The study was approved by the Ethical Committee of the Hospital and informed written consent was obtained from all patients before enrollment.

Quantitative Real Time-PCR

Carotid plaques were retrieved from patients during carotid endarterectomy and immediately placed in RNAlater (Ambion) solution. Total RNA from carotid plaques was purified by ultracentrifugation in cesium chloride gradient after homogenization in a 4M solution of guanidinium thiocyanate/ β mercaptoethanol. RNA samples were reverse transcribed using a complementary DNA (cDNA) synthesis kit [Invitrogen], according to the supplier's instructions.

ACE, TLR4, PTGS2, ACSL4, EPRAP, PTGER3 and PTGER4 gene expression was determined by real time RT-PCR using Sybr Green I as described by Ferronato et al. (2011). PCR reactions were performed in triplicate for each gene, using two independent cDNAs. Primers used for amplification are described in Table 1.

Statistical analysis

Differences in gene expression levels were analyzed by Student's *t* test. Pearson correlation and linear regression were conducted to investigate the association between pairs of genes in both symptomatic and asymptomatic groups. Significance of single analyses was adjusted for multiple tests for the genes investigated ($P < 0.01$). The Shapiro-Wilk test was applied to check for Gaussian distribution. Data were log transformed when not normally distributed.

For risk factors and drug therapy, associations between variables were evaluated by Fisher's exact test.

RESULTS

Demographic and clinical data of subjects are summarized in **Table 2**.

The asymptomatic group was represented by 27 men and 15 women with a mean age of 72.05 ± 8.33 . The symptomatic group was represented by 27 men and 2 women with a mean age of 70 ± 10.3 . The two groups of patients showed no statistical differences in risk factors, and currently drug therapy. Sex was the only variable significantly different between groups ($P < 0.05$), being women less represented in the symptomatic group of patients.

Gene expression analysis in carotid plaques

PTGS2, ACE, TLR4, ACSL4, PTGER3, PTGER4 and EPRAP gene expression of the same set of samples was measured for each gene by RealTime PCR, in a total of 42 asymptomatic and 29 symptomatic carotid plaques. Gene expression values are shown in **Table 3**. Student's t test showed no differences in gene expression between the two groups of atherosclerotic patients when analyzing ACE, TLR4, COX-2, FACL4, EPRAP, PTGER3 and PTGR4. Only the expression of PTGER3 receptor gene resulted higher in asymptomatic carotid plaques (0.98 ± 0.55 vs 1.55 ± 1.2 , $P < 0.02$) compared to the symptomatic group. This value lost significance after correcting by multiple testing.

Correlations between expression of selected genes in carotid plaques

Based on the concept that genes that are involved in a particular pathway or that respond similarly to experimental conditions could be co-expressed and show similar patterns of expression, we next analyzed pairwise correlation coefficients between PTGS2, ACE, TLR4, ACSL4, PTGER4, PTGER3 and EPRAP gene expression through Spearman coefficient (r) calculation. We observed that almost all pairs of genes were in some way positively correlated in both types of plaques, but the correlation coefficient appeared different when comparing symptomatic to asymptomatic plaques (Figure 1 and 2). Analysis of R value, reported in **Table 4**, showed that the correlation between ACE and TLR4 ($R = 0.70$; $P < 0.0001$), ACE and PTGS2 ($R = 0.79$; $P < 0.0001$), TLR4 and PTGS2 ($R = 0.57$; $P = 0.001$), TLR4 and PTGER3 ($R = 0.57$; $P = 0.001$), TLR4 and ACSL4 ($R = 0.66$; $P < 0.0001$), ACSL4 and EP4 ($R = 0.59$; $P = 0.0008$), PTGER4 and PTGER3 ($R = 0.70$; $P < 0.0001$) was stronger in symptomatic, than in asymptomatic plaques ACSL4 and PTGER3, and PTGS2 and PTGRE3 were only correlated in symptomatic plaques ($R = 0.48$; $P = 0.008$; $R = 0.37$; $P = 0.05$) although after correcting by multiple test, the

P values lost significance. TLR4 and PTGER4 ($R=0.75$; $P<0.0001$), and ACE and PTGER4 ($R=0.49$; $P=0.001$) were stronger correlated in asymptomatic plaques than in symptomatic plaques. All other gene correlations demonstrated no significance (Table 4).

DISCUSSION

Cardiovascular disease, currently the leading cause of death and disease in developed countries, is one of the major health problem worldwide (Libby 2002; Anrather and Iadecola 2016).

Although the contribution of genetics to atherosclerosis has been demonstrated in several studies, detailed involvement of the individual causative genes has not yet been clarified.

Differential gene expression analyses are a common method actually used to identify genes involved in a pathology. Nevertheless, such methods may be biased by selecting those genes or pathways that display the most pronounced differential expression. In other words, these methods may not capture genes with moderate expression differences that may have important functional roles. Moreover, genes can interact with each other, and even a very small change in expression of a particular gene may have dramatic physiological consequences if the protein encoded by this gene plays an important role in a specific cell function. Recent reports have suggested the utility of measuring changes in correlation may be an important complement to measuring differential gene expression. Changes in correlation can be indicative of differential wiring of regulatory networks (Jupiter and VanBuren 2008; Hu et al. 2009), or can have the potential to detect more subtle signals, such as local variabilities within a particular pathway. Moreover, the interaction among genes may add important information in the way to interpret gene expression analysis, describing which genes are closely connected within a given pathway.

This is the first study that assesses the correlation between genes involved in the ACE/TLR4/PTGS2 pathway as determinants of plaque vulnerability.

No differences were observed in the expression of the genes analyzed (Table 4), but the correlation between pairs of genes was different between plaques.

Based on the present correlation results, we hypothesize a different pattern of activation in symptomatic and asymptomatic carotid plaques, as presented in Figure 3.

ACE expression correlates with TLR4, PTGS2, and ACSL4 in both types of plaques, but downstream signaling appear different.

In symptomatic plaques, TLR4 correlates with PTGS2, and PTGS2 with EP3 supporting *in vitro* studies in cellular models indicating that Ang II activation of NF- κ B and PTGS2 (Küper et al. 2012), may be mediated by TLR4 (Ohnaka et al. 2000; Derbyshire et al. 2005; Das 2005; Cheng et al. 2005; Wolf et al. 2006; Ji et al. 2009; Hu et al. 2009; Morinelli et al. 2009; Ferronato et al. 2011; Nakashima et al. 2015; Han et al. 2017).

In asymptomatic plaques, ACE correlates with PTGS2 but since TLR4 does not correlate with PTGS2, the effect of Ang II upon PTGS2 seems not to be mediated by TLR4 in this case. It has been reported that Ang II can affect PTGS2 at the transcriptional level and mRNA stabilization, (Hu et al. 2002; Wong et al. 2011).

The correlation of PTGS2 with EP3, shows a positive trend only in symptomatic plaques which corresponds to the pro-inflammatory role of EP3 in the destabilization of atherosclerotic plaques (Schober et al. 2011).

ACSL4 is an arachidonic acid (20:4)-preferring ACSL isoform, and is therefore a feasible regulator of lipid mediator production in cells. Expression of ACSL4 gene, did not show differences in gene expression between symptomatic and asymptomatic plaques as reported by Cipollone et al. (2005a), but its stronger correlation in symptomatic plaques with TLR4, PTGS2, PTGER3 and PTGER4, all genes associated with plaque vulnerability, suggests its participation in the activation of the destabilization process as suggested by Golej et al. (2011). In their study, Golej and colleagues demonstrated that human arterial smooth muscle cells (SMCs) express ACSL4 and allow an important incorporation of 20:4 into phospholipids and, in turn, modulates PGE2 synthesis and secretion. That ACSL4 regulates PTGS2 expression and the production of prostaglandin has been also demonstrated in breast cancer cells (Maloberti et al. 2010).

The strong correlation between PTGER4 and ACSL4 may be due to the production of cAMP by EP4 signaling (Alfranca et al. 2006; Konya et al. 2013; Yokoyama et al. 2013) that in turn may induce ACSL4 expression (Mele et al. 2012).

The strong correlation between EP3 and EP4 only in symptomatic plaques indicates a possible synergic effect of both receptors upon plaque instability, probably by different signaling as indicated by their correlations with PTGS2 and ACSL4, respectively.

In asymptomatic carotid plaques, we could not observe significant correlations between TLR4 and PTGS2 nor between PTGS2 and PTGER3 or PTGER4. Whether PGH2 is metabolized to prostacyclin or other prostaglandins which may have a compensatory anti-inflammatory effect in this kind of plaques (Alfranca et al. 2006; Yokoyama et al. 2013), could be of interest in future studies. The result showing that TLR4 expression is strongly correlated with PTGER4 expression, but not with PTGS2, suggests that EP4 in this case may have a compensatory anti-inflammation effect mediated via ligand-independent activation (Hiken et al. 2017). The inhibition of NF- κ B by EP4, can have an anti-inflammatory action, triggering a compensative anti-inflammatory process increasing anti-inflammatory cytokines and TLR4 effectors through cAMP (Takayama et al. 2002; Minami et al. 2008; Wall et al. 2009; Gerlo et al. 2011; Mele et al. 2012; Konya et al. 2013; Yokoyama et al. 2013; Birrell et al. 2015; Hiken et al. 2017). In addition, the fact that PTGS2 is not correlated with PTGER4 or PTGER3 indicates that in this case PGE2 may exerts its action through other EP receptors that may have anti-inflammatory effects (Ricciotti and FitzGerald 2011).

CONCLUSION

The assumed gene expression networks deduced from the correlation coefficients identified in this study, indicates a different activation of the pathway in the two groups of carotid plaques. These results show that correlation analysis may highlight biological processes otherwise not detectable by means of solely gene expression analysis.

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Fig. 1 Correlation between gene expression in symptomatic carotid plaques. In each graph the Spearman correlation [R] and the P value are indicated. Only relations with an $R > 0.50$ are represented

Fig. 2 Correlation between gene expression values in asymptomatic carotid plaques. In each graph the Pearson correlation [R] and the P value are indicated. Only relations with an $R > 0.50$ are represented.

Fig. 3 Schematic representation of proposed models of activation of ACE/TLR4/Cox-2 dependent pathway in asymptomatic [A] and symptomatic [B] carotid plaques.

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