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Upregulated Expression of Toll-like Receptor 4 in Peripheral Blood of Ischaemic Stroke Patients Correlates with Cyclooxygenase 2 Expression

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Abstract *Objectives:* An inflammatory process following stroke in human brains and systemic inflammatory responses after stroke in humans have been reported by numerous investigators. The aim of the study was to investigate if genes involved in the cyclooxygenase 2 (COX-2) pathway are upregulated at peripheral level in patients after transient ischaemic attack (TIA) and stroke.
Design of Study: Blood samples were obtained from two groups of patients undergoing carotid endarterectomy. The first group included 25 patients who presented TIA or ischaemic stroke. The second group included 35 patients who had an asymptomatic internal carotid artery stenosis. Total RNA was isolated and the expression of Toll-like Receptor 4 (TLR4), COX-2, membrane-associated Prostaglandin E synthase (mPGES-1), Prostaglandin E_2 receptors (EP3 and EP4) was analysed by real time RT-PCR.
Results: Expression of COX-2 and TLR4 were significantly increased in symptomatic patients ($p < 0.001$). Correlation analysis showed that TLR4 expression significantly correlated with COX-2 expression ($R = 0.65$; $p < 0.01$) in ischaemic stroke patients. This correlation was not observed in TIA and asymptomatic patients.
Conclusions: Our results suggest that the peripheral mechanism of inflammatory injury after stroke may be mediated by TLR4 through a COX-2-dependent pathway.

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The risk of ipsilateral ischaemic stroke distal to a carotid stenosis higher than 50% is between 20% and 30% during the first 30 days following a transient ischaemic attack (TIA) or a minor stroke.[1,2] An inflammatory process following stroke is clearly demonstrated in human brains,[3] and systemic inflammatory responses after stroke in humans have been reported by numerous investigators.[4–7] Ischaemic brain tissue induces a response in the peripheral circulation, including an inflammatory response, demonstrated by the upregulation of many inflammatory molecules detected by different methods including genome-wide messenger RNA (mRNA) expression analysis in peripheral blood.[7] Peripheral white blood cell (WBC) count, C-reactive protein (CPR) level and erythrocyte sedimentation rate (ESR) are increased within 24 h after the onset of stroke and can be significantly elevated even 3 months after the event.[3,7] Prostanoids, a family of pleiotropic lipid mediators, exert a variety of actions in several cells and are involved in vascular inflammation.[8,9] In particular, prostaglandin E_2 (PGE_2) plasma levels are augmented in inflammatory processes, including atherosclerosis.[9] The synthesis of PGE_2 depends on the coordinated actions of cyclooxygenase 2 (COX-2) and membrane-bound prostaglandin E synthase-1 (mPGES-1),[10] two inducible enzymes expressed in response to an inflammatory stimulus. PGE_2 exerts its effects through specific G-protein-coupled cell-surface receptors named Prostaglandin E receptors (EPs), and it has been reported that EP3 and EP4 mRNA expression is increased in peripheral blood mononuclear cells (PBMCs) of patients with carotid stenosis.[9] Toll-like receptor 4 (TLR4) is a member of an evolutionarily conserved protein family, which mediates innate immunity.

Both endogenous and exogenous ligands can activate TLR4 on cells, such as endothelial cells, vascular smooth muscle cells, adventitial fibroblasts, dendritic cells and macrophages. Activated TLR4 lead to the activation of nuclear factor-κB (NF-κB)-responsive genes, including COX-2.[11] Activated transcription factors mediate the secretion of pro-inflammatory cytokines and chemokines and also induce the expression of adhesion molecules. Ultimately, these processes might initiate or promote atherosclerotic lesions.[12,13]

Recent evidence suggests that vascular and systemic inflammation induced by TLR4 ligands such as oxidised low-density lipoprotein (oxLDL), pathogenic infections and heat-shock proteins, three key players in human atherogenesis, are mediated in part via the TLR4/NF-κB pathway.[14] Upregulation of TLR4 and downstream inflammatory factors' expression in monocytes from patients with acute cerebral infarction has been reported,[15] and TLR4-deficient mice after ischaemia have significantly lower expression of COX-2 compared with control mice,[11] suggesting that increased expression of TLR4 may mediate brain damage through COX-2-dependent signalling. The aim of the study was to investigate if the genes involved in the COX-2 pathway are upregulated at a peripheral level in patients after TIA and stroke.

Materials and Methods

Study patients

Consecutive, eligible patients with internal carotid artery stenosis undergoing carotid endarterectomy at GB Rossi University Hospital and Civile Maggiore Hospital (Verona) were included in this study. Blood samples were obtained for each patient immediately prior to endarterectomy. A total of 25 symptomatic patients and 35 asymptomatic patients were selected for the gene expression analysis. The first group included patients, who presented TIA or ischaemic stroke within the previous 23 days. The atherothrombotic nature of stroke was established by: (1) neuroradiologic aspect of a non-lacunar lesion in the carotid artery territory; (2) evidence of ipsilateral cerebral embolisation on transcranial Doppler monitoring; (3) no embolic cardiac sources as assessed by echocardiography; and (4) ipsilateral carotid plaque, which could benefit from carotid endarterectomy according to North American Symptomatic Carotid Endarterectomy Trial (NASCET).[16] The second group included patients with asymptomatic carotid plaques causing carotid stenosis of 60% or more, not associated with any neurological symptom.[17]Asymptomatic patients were enrolled by the Vascular Surgery Department of the University Hospital of Verona for carotid endarterectomy, usually after a carotid Duplex scan and either carotid magnetic resonance (MR) or computed tomography (CT) or angiography to confirm carotid stenosis.

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

Quantitative real-time-RT-PCR

Whole blood samples rather than a specific leucocyte subgroup, were analysed from each patient. Blood samples (2.5 ml) were collected in PAXgene™ Blood RNA tubes and total RNA was isolated using the standardised RNA Kit (PreAnalytiX, Qiagen). The RNA isolated with this protocol comes from all blood cells, including polymorphonuclear (PMN) (neutrophil, basophil and eosinophil), mononuclear cells (lymphocytes and macrophage/monocytes), platelets and red blood cells. Total RNA was then quantified by absorbance at A_{260} nm and the purity was estimated by the ratio A_{260} nm/A_{280} nm. RNA integrity was confirmed by non-denaturing agarose gel electrophoresis. RNA was stored at −80 °C for later use. RNA samples were reverse transcribed using a complementary DNA (cDNA) synthesis kit (Invitrogen), according to the supplier's instructions.

TLR4, COX-2, mPGES-1, EP3 and EP4 gene expression was determined by real time RT-PCR using Sybr Green I. The specificity of the Sybr green fluorescence was tested by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon. RT-PCR reactions were performed in 10 μl, containing 5 μl of 2 × Hot-Rescue Real Time master mix (Diatheva), 15 ng of cDNA template and 200 nM of forward and reverse primer. The condition for PCR was set up as follows: a initial activation step at 95 °C for 10 min, followed by 40 cycles of denaturing at

Table 1 Primers used for Real Time RT-PCR.

Genes	Forward primer (5′—3′)	Reverse primer (5′—3′)
TLR4	AAATTTCCGCTTCCTGGTCT	TCAGCCCATATGTTTCTGGA
COX-2	TGAAACCCACTCCAAACACA	AGGAGAGGTTAGAGAAGGCT
mPGES-1	CATCAAGARGTACGTGGT	GGAGTAGACGAAGCCC
EP3	GTGCTGTCGGTCTGCTG	CTTTCTGCTTCTCCGTGTG
EP4	CATCTGCTCCATCCCGCT	GGATGGCCTGCAAATCTGG
GAPDH	TCCTGTTCGACAGTCAGC	ACGACCAAATCCGTTGAC
TBP	TGTATCCACAGTGAATCTTGG	ATGATTACCGCAGCAAACC

95 °C for 15 s, annealing at 60 °C for 15 s and extension at 72 °C for 20 s. Amplifications were performed using an Eppendorf Mastercycler® *realplex*. Each sample was amplified in triplicate and, for each procedure, negative controls without template were included. Potential genomic DNA contamination was excluded by using intron-encompassing primers and DNase treatment of the RNA samples. Amplification efficiency was calculated for each assay by a standard curve made out of four serial dilutions of a pool of cDNA of symptomatic and asymptomatic patients. The experiment was conducted twice. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TATA box-binding protein (TBP) genes were chosen as the endogenous controls to normalise target genes. Normalisation of target gene-expression levels was performed to compensate for intra- and inter-kinetic Real Time RT-PCR variations (sample-to-sample cell concentration and run-to-run variations). Primers used for amplification are described in Table 1. The comparative quantification of gene expression was determined using Pfaffl's efficiency corrected calculation. The Pfaffl mathematical model is based on the correction for exact PCR efficiencies and the mean crossing point deviation between sample group(s) and control group(s). This method takes into account the fact that small efficiency differences between target and reference gene amplifications generate a false expression ratio, and the researcher over- or underestimates the 'real' initial mRNA amount.

Statistical analysis

The Shapiro—Wilk test was applied to check for normal distribution. Statistical analysis was done with logarithmically transformed values when they were not normally distributed. Differences in gene expression were analysed by Student's *t* test. We tested the significance of the Pearson correlation coefficient to evaluate the relations between the target genes. Significance was assumed with a p value of 0.05. For risk factors and drug therapy, variables were compared by the use of Fisher's exact test.

Results

Patient demographics

Demographic and clinical data of subjects are summarised in Table 2. The asymptomatic group was represented by 23 men and 12 women with a median age of 72.6 years (range 50—87) while the symptomatic group was represented by 23

Table 2 Characteristics of the Study Patients. There were no significant differences between groups ($p > 0.05$ for all groups).

Variable	Asymptomatic	Symptomatic
	$N = 35$	$N = 25$
Age, *y* (mean)	72.6 ± 8.1	70.8 ± 9.5
Sex		
Male (%)	23 (66)	23 (92)
Female (%)	12 (34)	2 (8)
TIA patients (%)	0	8 (32)
Stroke patients (%)	0	17 (68)
Risk factors		
Hypertension (%)	30 (86)	17 (68)
Diabetes (%)	6 (17)	6 (24)
Smoking (%)	16 (46)	12 (48)
Dyslipidemia (%)	26 (74)	12 (48)
NSAID or glucocorticoid treatment (%)	0	0
Acetylsalicylic acid (%)	23 (66)	19 (76)
Statin (%)	19 (54)	13 (52)
ACE-inhibitor (%)	20 (57)	12 (48)

NSAID, non-steroidal anti-inflammatory drug; TIA, transient ischaemic attack.

men and two women with a median age of 70.8 years (range 50–85). Fisher's exact test showed no significant differences between sex, cardiovascular risk factors and the current drug therapy between the two groups of patients.

TLR4, COX-2, mPGES-1, EP3 and EP4 gene expression

Real Time RT-PCR was used to determine gene expression on total RNA from peripheral blood. A significant increase of COX-2 and TLR4 gene expression was observed in symptomatic compared with asymptomatic patients (1.51 ± 0.36 vs. 0.81 ± 0.63; $p < 0.001$; 1.54 ± 0.94 vs. 0.84 ± 0.56; $p < 0.001$, respectively) (Fig. 1(A) and (B)). The expression of both genes was independent of the time from ischaemic event at which blood samples were collected. (Pearson correlation for COX-2, TLR4 and EP4: $R = -0.04$, -0.05 and 0.21, respectively; figures not shown). The EP4 gene did not show differential expression between symptomatic and asymptomatic patients (Fig. 1(C)). The method was not sensitive enough to consistently detect mPGES-1 and EP3 gene expression, even using different sets of primers and amplification conditions (data not shown).

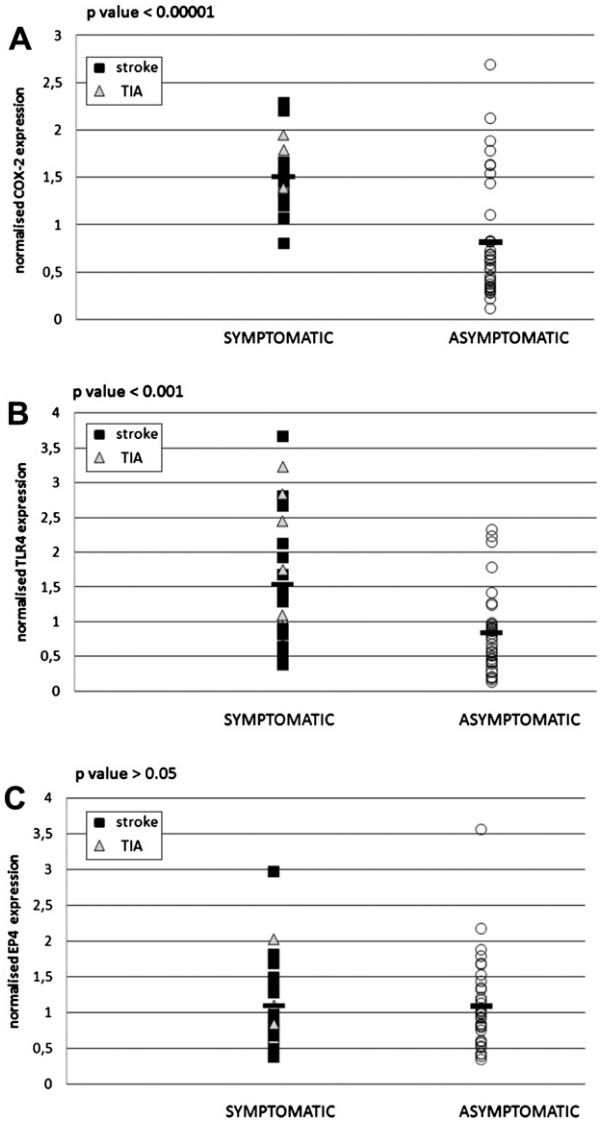

Figure 1 Gene expression of COX-2 (A), TLR4 (B) and EP4 (C) in symptomatic and asymptomatic patients. Real time RT-PCR was performed on total RNA extracted from whole peripheral blood from 25 symptomatic (stroke and TIA) patients and 35 asymptomatic patients. Gene expression was normalised to the GAPDH and TBP housekeeping genes. Bars indicate mean values of each group. *P* values are indicated in each graph.

Correlation between genes analysed

Stratifying the symptomatic group in TIA and stroke, we could observe a correlation between TLR4 and COX-2 gene expression ($R = 0.65$; $p < 0.01$) (Fig. 2). This correlation was not observed in TIA ($R = 0.01$; $p > 0.05$) and asymptomatic patients ($R = 0.03$; $p > 0.05$) (data not shown).

Discussion

Immediately after cerebral infarction, intense peripheral inflammatory reactions may occur, including activation of inflammatory cells and production of high levels of inflammatory cytokines. Because of injury to the blood–brain barrier after cerebral infarction, activated mononuclear macrophages enter the brain tissue, causing inflammatory and intensified cerebral injury.[15] Previous studies have demonstrated that markers of inflammation including interleukins (IL1, IL-8, IL-10 and IL-17), interferon gamma and macrophage inflammatory protein (MIP) mRNAs are elevated in PBMCs in patients and animals with ischaemic stroke. Similarly, leucocyte proteinases, interleukins, matrix metalloproteinases (MMPs) and integrins are elevated in patients after both acute stroke and TIAs,[6] and can be significantly elevated even 3 months after the event.[5] The first studies of whole genome expression in human blood after ischaemic stroke were reported by Tang et al. and Moore et al.[6,18] They provide the proof-of-principle studies in humans that changes of gene expression occur at very early times in the peripheral blood of patients after ischaemic stroke. Peripheral blood has become an attractive prime tissue for biomarker detection because of its critical role in immune response, metabolism, communication with cells and the extracellular matrix in almost all tissues and organs in the human body. The dynamic and interactive

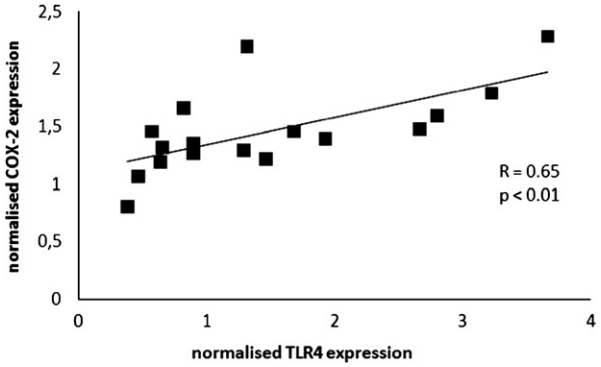

Figure 2 Association between TLR4 and COX-2 mRNA values in peripheral blood of 17 stroke patients. TLR4 and COX-2 was measured by real-time-RT-PCR and normalised to GAPDH and TBP.

properties of blood give rise to the possibility that subtle changes occurring within the body, such as changes in association with a disease process or in response to an injury, may leave 'footprints' in blood. Therefore, transcriptional profiling from whole blood cells might provide an alternative to tissue biopsy in the search for biomarker genes of cardiovascular disease.[19] RNA extracted from peripheral blood cells is easily accessible and can be routinely used for diagnostic laboratory analysis, and, thus, it is a good resource for additional tests that might define the extent of vascular artery disease. It must be remembered that detection of the presence of an mRNA provides no information on whether that mRNA will be translated into a protein. Immunohistochemistry, Western blotting or other protein-quantification methods would be necessary to determine if protein levels correspond with mRNA expression.

In this study, we provide evidence of an augmented TLR4 and COX-2 gene expression in peripheral whole blood of patients, who have had a stroke or TIA, compared with patients with asymptomatic stenosis. Upregulation of TLR4 has been previously observed[15] on peripheral blood monocytes from patients with acute cerebral infarct within 24 h after the onset of the symptoms as compared with TIA and healthy control groups. An increase of COX-2 expression in the inflammatory region of atherosclerotic plaques,[9,20] and in PBMCs of patients with carotid stenosis as compared with healthy controls[9] have been reported. Our results show an upregulation of TLR4 and COX-2 in TIA and stroke patients in peripheral blood up to 23 days (10.9 ± 7.2 days) after the ischaemic event. Time from appearance of the symptoms to blood collecting ranged from 0 to 23 days. No correlation was found between gene expression level and time from stroke/TIA to blood collection. Analysis of whole blood RNA expression does not exclude the possibility that percentages of specific blood subtypes differ between each sample, and hence that different numbers of subtypes are responsible for the observed effect. On the other hand, the isolation process of subtypes of leucocytes could, in itself, affect the gene expression pattern.

To the best of our knowledge, this is the first time that differential expression of TLR4 and COX-2 has been demonstrated in whole peripheral blood of symptomatic carotid patients compared with patients with asymptomatic plaques. To determine if these changes represent a result of stroke or a contribution to plaque destabilisation as a precursor to stroke, we have ongoing gene-expression studies on whole peripheral blood of cardioembolic stroke patients and on vulnerable and stable plaques. A positive correlation between TLR4 and COX-2 expression was observed in patients who presented ischaemic stroke, suggesting that TLR4 may participate in the peripheral inflammatory mechanism after stroke through a COX-2-dependent pathway. This hypothesis is in part supported by studies on TLR4-deficient mice suggesting that brain damage may be mediated by an increased expression of TLR4 through a COX-2-dependent signalling.[11] Further *in vivo* animal studies on peripheral blood and *in vitro* cell culture experiments are necessary to confirm these observations. The correlation between TLR4 and COX-2 was not observed in TIA patients. The TIA sample size is rather small and this may significantly compromise the statistical power; hence, the lack of correlation between genes observed may be due because the statistic power is not greater enough to identify the significance. However, recent histological studies in carotid plaques have reported that inflammatory components in TIA are different from those in stroke plaques suggesting that molecular mechanisms underlying TIA and stroke are different,[21] with TIA a more chronic and stroke a more acute process.[3] The analysis of COX-2 downstream-related genes demonstrated little or no expression of mPGES and EP3, and no differential expression of EP4. This fact suggests that stimulation of these downstream COX-2 related genes, as seen in the shoulder region of human atherosclerotic plaques,[9] or in rat brain after cerebral ischaemia,[22,23] may occur after activated monocytes have infiltrated the target tissue. In conclusion, the present study indicates that upregulation of TLR4 expression on whole blood from symptomatic patients positively correlates with the upregulated expression of the pro-inflammatory gene COX-2. These results suggest that TLR4 may participate in peripheral mechanisms of inflammatory injury after stroke through a COX-2-dependent pathway, and support other studies that encourage the development of TLR4 inhibitors as an alternative to COX-2 inhibitors (coxibs) in the treatment of the acute phase of ischaemic stroke.[24,25]

Conflict of Interest

None.

Acknowledgements

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