

Increased Expression of Cell–Cell Signaling Genes by Stimulated Mononuclear Leukocytes in Patients with Previous Atherothrombotic Stroke

A Whole Genome Expression Profile Study

Caspar Grond-Ginsbach^a Solveig Horstmann^a Manuela Hummel^b Tina Wiest^a
Cornelius Honold^a Karin Pflieger^a Manfred Hergenahn^{†,c} Monika Hollstein^c
Annette Weninger^c Yuri Knyazev^c Ulrich Mansmann^b Simone Wagner^a
Armin J. Grau^d

^aDepartment of Neurology, University of Heidelberg, Heidelberg, ^bIBE, Medical School, LMU Munich, Munich,

^cDepartment of Genetic Alterations in Carcinogenesis, German Cancer Research Center, Heidelberg, and

^dDepartment of Neurology, Klinikum der Stadt Ludwigshafen, Ludwigshafen am Rhein, Germany

Key Words

Stroke · Endotoxin, bacterial · Gene expression · Blood · Mononuclear cells

Abstract

Background/Aims: Inflammation plays an important role in atherosclerosis and stroke. Acute infections are recognized as trigger factors for ischemic stroke. **Methods:** In this whole genome expression profile study of 15 patients and 15 control subjects, we tested the hypothesis that patients with a history of atherothrombotic stroke show enhanced transcription of inflammatory genes in circulating leukocytes. RNA from unstimulated or lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMCs) was analyzed with Affymetrix U133A GeneChips using a pooling design. Expression of single genes and functional groups of genes was analyzed by global statistical tests. **Results:** A total of 10,197 probe sets showed positive calls. After correction for multiple testing no single probe set revealed significant differences either without or with LPS stimulation. However, significant global expression differences were found upon LPS stimulation for the group of genes that are involved in cell–cell signaling. **Conclusion:** LPS stimulation

of PBMCs, a condition mimicking bacterial infection, induces differential expression of a group of cell–cell signaling genes in patients with previous atherothrombotic stroke. This finding can be caused by genetic differences between both groups, but acquired risk factors, medication and technical factors may also have contributed to the result.

Copyright © 2009 S. Karger AG, Basel

Introduction

Several lines of evidence indicate that inflammation plays an important role in stroke and other vascular diseases. Atherosclerosis is now viewed as an inflammatory disease [1, 2] and markers of inflammation including leukocyte counts are predictors of first and recurrent ischemic events [3]. Multiple case-control studies and a large study based on the case series method showed that recent acute infections are triggering factors for stroke; furthermore, chronic infections are discussed as stroke risk factors [4, 5]. There is not one single microbial pathogen or a definable group of microbes that is associated with stroke risk and, most likely, the inflammatory response upon infections leading to a procoagulant state repre-

sents the major pathogenetic mechanism linking infection and stroke [6]. The individual inflammatory response on external stimuli is under strong genetic control and it is an intriguing hypothesis that the strength of this inflammatory response is associated with stroke risk. In fact, a case-control study showed that the odds of stroke increase along the number of proinflammatory polymorphisms in several inflammatory genes [7]. Furthermore, intima-media thickness as a surrogate parameter of stroke risk is associated with the number of proinflammatory genotypes [8].

Based on this background we hypothesized that patients with a history of stroke due to atherosclerotic large artery disease show enhanced expression of inflammatory genes in circulating mononuclear leukocytes upon in vitro stimulation with lipopolysaccharide (LPS), a condition mimicking acute bacterial infections, or possibly already in the unstimulated state. In order to test this hypothesis, we used microarray (GeneChips) technology that allows simultaneous analysis of thousands of transcripts from a tissue sample. Recently, transcriptome analyses of circulating leukocytes have been performed in patients with acute stroke [9–11]. Genes involved in inflammatory response, coagulation and tissue remodeling were found to be significantly upregulated during acute stroke, but study results are at variance. In contrast to these studies, here we investigated patients at least 3 months after atherothrombotic stroke to study whether gene transcription in circulating leukocytes shows increased stimulability or is even chronically upregulated in these patients (unstimulated state).

Patients and Methods

Stroke Survivors and Healthy Referents

Patients recovering from ischemic stroke due to severe intracranial or extracranial (>70%) atherosclerotic stenosis at least 3 months before inclusion were recruited for this study. Ischemic stroke was defined as an acute ischemic lesion on neuroimaging or an acute neurological deficit of vascular origin lasting >24 h. We enrolled unrelated patients and healthy subjects >18 years who were born of German parents. The stringent inclusion and exclusion criteria aimed at the selection of a study sample of patients with highest possible homogeneity and without serious comorbidities or serious post-stroke handicaps that might possibly interfere with the gene expression of the peripheral white blood cells. Excluded from the study were subjects with a history of renal or liver diseases, alcohol abuse, psychiatric disorders, acute or chronic inflammatory diseases, tumor, trauma, surgical treatment, myocardial infarct or other vascular diseases within the last 3 months. None of the analyzed patients was submitted to angioplasty with stenting.

Table 1. Baseline characteristics, vascular risk factors and medication of survivors and referents

	Cases (n = 15)	Referents (n = 15)
Women	3	11
Mean age, years (range)	70.2 (56–83)	65.4 (56–74)
Mean time after stroke months (range)	14 (5–32)	–
Modified Rankin scale <2	15	–
NIHSS at acute stroke ≤3	11	–
Carotid angioplasty or stenting ¹	6	–
Cigarette smoking	4	0
Arterial hypertension	12	0
Diabetes mellitus	4	0
Hypercholesterolemia	7	0
Peripheral occlusive arterial disease	2	0
Coronary artery disease	2	0
ASA	9	0
Clopidogrel	6	0
ACE inhibitor	7	0
Statin	9	0
Diuretics	5	0

ASA = Acetylsalicylic acid; ACE = angiotensin-converting enzyme.

¹ Carotid angioplasty or stenting in the first month following acute stroke.

Husbands or wives of the patients were asked to participate in the study as healthy controls, in order to match both study groups as to lifestyle, social environment and diet. However, due to the fact that most patients were men, this selection of control subjects resulted in different ages and gender percentages among the study groups. Histories of vascular diseases or vascular risk factors were additional exclusion criteria for the control group (table 1). All patients were independent and had recovered well from stroke (modified Rankin scale 0 or 1). Blood sampling of all patients and control persons was performed between 10 and 12 a.m. All samples were handled by the same technician in the same laboratory. The study protocol was approved by the local ethics committee and all participants gave written informed consent.

Preparation of Peripheral Blood Mononuclear Cells and Isolation of RNA

Peripheral blood mononuclear cell (PBMC) transcriptomes were studied from 15 stroke survivors (survivors) and 15 healthy control subjects (referents). RNA was prepared from PBMCs either immediately after blood sampling (resting cells) or after 2-hour incubation of isolated PBMCs with LPS. 20 ml free-flowing peripheral venous blood was taken into pyrogen-free syringes containing EDTA and cooled on ice. PBMCs were isolated immediately by 12-min density-gradient centrifugation at 4°C using pre-cooled Ficoll-Paque (Amersham Biosciences, Uppsala, Swe-

Table 2. Composition of PBMC isolate after density gradient centrifugation

	Cases			Referents			P
	P1	P2	P3	P1	P2	P3	
Lymphocytes, %	77	84	80	76	82	81	n.s.
Monocytes, %	17	14	13	17	16	12	n.s.
Granulocytes, %	6	2	7	7	2	8	n.s.
Platelets, × 1,000/ml	119	119	149	126	122	147	n.s.

The proportion of lymphocytes, monocytes and granulocytes within the total amount of leukocytes was measured for each PBMC isolate. Mean values were calculated for each pool of 5 individuals. The number of platelets was standardized for a PBMC isolate volume containing 10^7 leukocytes.

den). An aliquot of each isolate was analyzed in a cell counter (table 2). After being washed in PBS at 4°C half of the leukocyte suspension was diluted in 10 ml RPMI-1640 to which LPS from *Salmonella abortus equi* (500 pg/ml final concentration, Biermann, Bad Nauheim, Germany) had been added. After incubation at 37°C for 120 min the cells were spun down, washed in ice-cold PBS and passed to 1.0 ml Trizol reagent. The second half of the leukocytes was processed without delay for RNA isolation after density gradient centrifugation. For cells that were not treated with LPS the whole procedure from blood sampling onto the lysis of the leukocytes in Trizol reagent was performed within 37 min and under continuous cooling at 4°C. For the isolation of the RNA by Trizol we followed the instructions of the provider. After isopropanol precipitation and 2 washes in 70% ethanol the RNA pellet was dissolved in RNase-free water and subsequently purified on an RNeasy MinElute Cleanup column (Qiagen, Valencia, Calif., USA). The 14- μ l eluate from the Cleanup column was analyzed by standard photometry (for RNA quantification) and with an Agilent 2100 Caliper LabChip Bioanalyzer (Agilent Technologies, Palo Alto, Calif., USA) to test for the integrity of the RNA.

Affymetrix GeneChip U133A Microarray Studies

RNA from the PBMCs of 5 subjects of each group was pooled at equal amounts for GeneChip analysis and analyzed on a single U133A GeneChip. Twelve array hybridization reactions were performed (three pools of LPS-stimulated cells and three pools of resting cells from stroke survivors, and three corresponding pools of material from referents with and without LPS), resulting in 12 readings for each probe set.

During the whole procedure of cRNA labeling and array hybridization, we followed the protocols from Affymetrix Inc. (Santa Clara, Calif., USA). Two micrograms of RNA from each of 5 different samples were pooled for the synthesis of cDNA. This 10- μ g mRNA sample of total cellular RNA was converted to double-stranded cDNA using Superscript (Gibco-Invitrogen, Karlsruhe, Germany) with a T7-(dT)24 primer containing the T7 RNA polymerase promoter. The cDNA was in vitro transcribed to biotinylated complementary RNA (cRNA) by incorporating biotin-CTP and biotin-UTP using the Enzo BioArray High Yield RNA labeling kit (Enzo Diagnostics, Farmingdale, N.Y., USA). Biotinylated cRNA from each sample was fragmented and 15 μ g of the fragmented cRNA were used for the preparation of 300 μ l hybridiza-

tion mix (80 μ l were probed on a Test 3 Array and 200 μ l on a U133A Array). Arrays were hybridized for 16 h at 45°C with constant rotation at 60 rpm. Following washes, arrays were sequentially stained with streptavidin R-phycoerythrin (Molecular Probes, Eugene, Oreg., USA), biotinylated goat anti-streptavidin (Vector Laboratories, Burlingame, Calif., USA) and another streptavidin-phycoerythrin for signal amplification. After a series of washes the GeneChips were scanned with an argon-ion laser microscope (Hewlett-Packard, Palo Alto, Calif., USA) for fluorescence signal detection. All washes and staining procedures were performed on an Affymetrix Fluidics station. The raw expression data derived from Affymetrix Micro-array Suite 4.0.1 software gave each transcript an absolute expression level (signal intensity) and a 'present' or 'absent' call based on the signal/noise ratio.

Real-Time Reverse-Transcribed Polymerase Chain Reaction Analysis

As an independent validation of the Affymetrix results, the transcripts from 3 different genes were quantified by reverse-transcribed polymerase chain reaction (RT-PCR). In these experiments we did not use pooled RNA samples, but analyzed 10 individual RNA specimens from patients and 10 samples from referent subjects. Five of the RNA samples were from stroke survivors and healthy subjects that were previously studied by Affymetrix Arrays, five further RNA samples were taken from additional control subjects and additional stroke survivors with baseline characteristics similar to the other patients and referents.

Real-time RT-PCRs of ATF3, NF κ B2 and IL1F9 were carried out in 96-well PCR plates with a SYBR-Green PCR kit (Applied Biosystems, Weiterstadt, Germany) in an ABI PRISM 5700 Sequence Detector (Applied Biosystems) following standard procedures. As housekeeping gene we used glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following primers were used: GGTCTCTGCCTCGGAAGTGA and TGCTTGTCTGGATCCGAAA for ATF3; CAGTGAGAAGGGCCGAAAGA and AGC-ACGAGGTGGTCACTGT for NF κ B2; GCACTCCAGGAGACGCTGAT and ACTTCGTGGAAGTCCACAAG for IL1F9, and TGGGTGTGAACCATGAGAAG and GCTAAGCAGTTGTGGTGC for GAPDH. The amplification reactions were performed in duplicate. For relative quantification of the transcripts, a standard curve was calculated by measuring serially diluted amounts of cDNA.

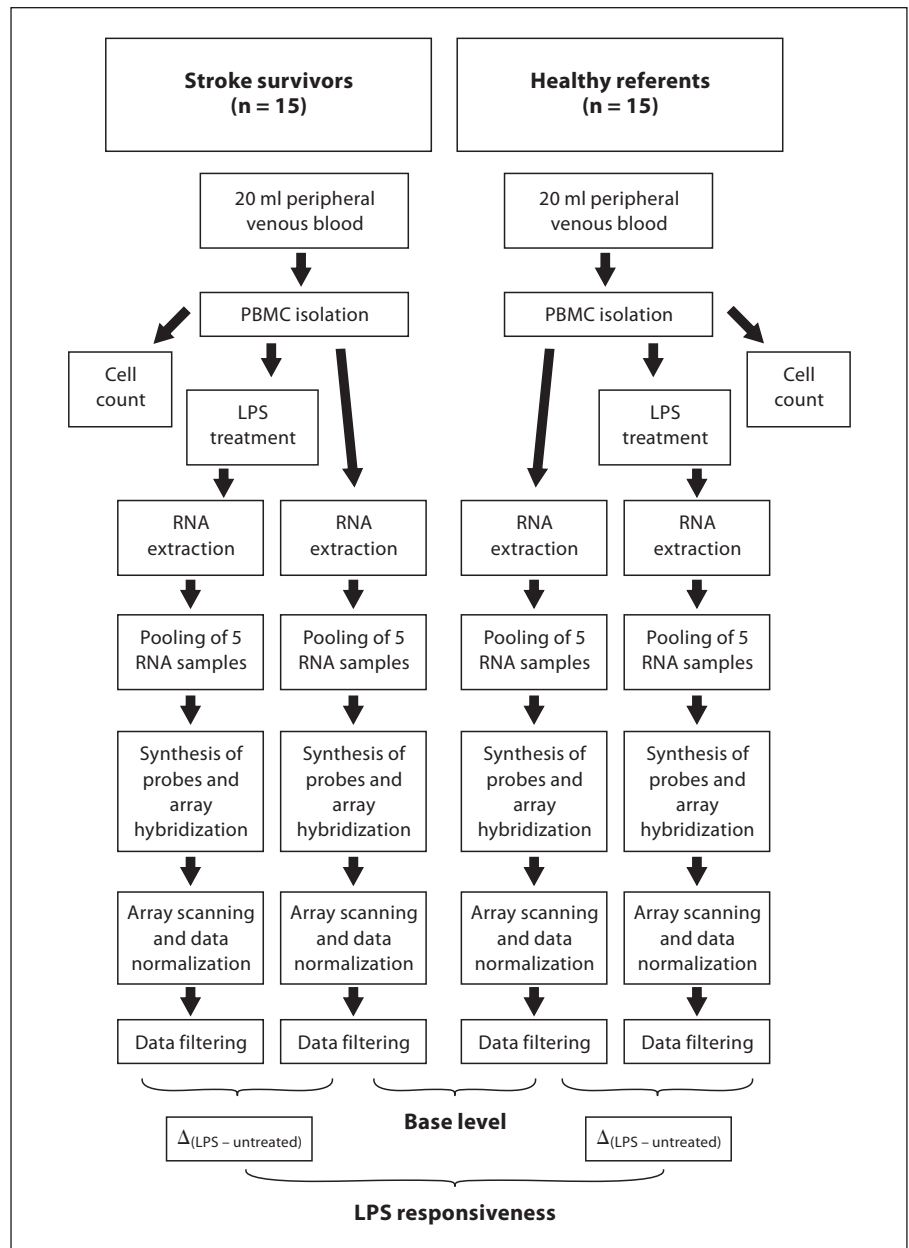


Fig. 1. Flowchart showing the design of the study. PBMCs from patients with previous atherothrombotic stroke (stroke survivors) and from controls (healthy referents) were analyzed under two conditions (base level gene expression and stimulated gene expression after in vitro LPS treatment).

Evaluation of GeneChip Data

The statistical analysis was carried out with the open source software R (<http://www.r-project.org/>, version 2.3.0) and Bioconductor (<http://www.bioconductor.org/>, version 1.8) [12]. The U133A raw data were analyzed after GC-RMA (robust multi-array) normalization [13]. Hybridization intensities were compared between pooled samples of survivors and referents for each probe set. Log₂ ratios (= log-fold changes, logFC) were calculated. Gene expression of resting PBMCs (called 'base level' gene expression) was compared between survivors and referents. We also compared survivors and referents as to the difference in gene expression between LPS-treated and resting cells (LPS responsiveness). Correc-

tion for multiple testing was carried out by controlling the false discovery rate according to Benjamini and Hochberg [14] or by the Bonferroni method. Moreover, predefined groups of genes were analyzed with a global ANCOVA test for each group or with non-parametric Kolmogorov-Smirnov rank statistics [15, 16]. The global ANCOVA approach is a test for the association between expression values and clinical entities within a well-defined group of genes. If the mean expression level for at least one of these genes differs between phenotypes, the global null hypothesis is violated. Significance is assessed by a permutation approach (permutation of phenotype labels). This global test is freely available as Bioconductor package (Global ANCOVA). The Kolmogorov-Smirnov ap-

Table 3. Tests of groups of genes for differences between survivors and referents

Survivors vs. controls Gene group	n	Base level expression		LPS responsiveness	
		ANCOVA p value	K-S p value	ANCOVA p value	K-S p value
GO:6954 inflammation	119	0.65	0.93	0.20	0.24
GO:6917 apoptosis	98	0.85	0.97	<0.001	0.06
GO:6940 stress response	82	0.85	0.95	0.65	0.97
GO:7596 coagulation	40	0.95	0.64	0.10	0.52
GO:7155 cell adhesion	162	1.00	0.76	0.35	0.19
GO:7267 cell-cell signaling	117	0.75	0.22	<0.001	0.04
GO:6955 immune response	298	0.65	0.85	0.25	0.29
GO:6629 lipid metabolism	140	0.55	0.29	0.35	0.92
hsa00590 prostaglandin	24	0.85	0.60	0.25	0.50
hsa04350 TGF- β receptor	16	0.15	0.39	0.25	0.38
hsa04620 TLR signaling pathway	96	0.30	0.20	0.15	0.24
LPS-activated genes [18]					
Early LPS response	77	0.45	0.91	0.20	0.0001
Late LPS response	67	0.60	0.52	0.20	0.04
Acute stroke [10]					
Bonferroni list	198	0.60	1.00	0.40	0.92
Westfall and Young list	85	0.80	0.99	0.55	0.49
Acute stroke [11]					
3 h	38	0.35	0.23	0.70	0.95
5 h	431	0.45	0.97	0.40	0.54
24 h	345	0.45	0.58	0.55	0.43

Functional GO (gene ontology) and hsa-KEGG (Kyoto Encyclopedia of Genes and Genomes) groups as well as gene groups found in expression profiling studies of acute stroke patients were studied. n refers to the number of probe sets among the 10,197 present cells analyzed here that identify genes belonging to the group. For each gene group the H_0 (no difference between survivors and referents) was tested with ANCOVA and Kolmogorov-Smirnov-like rank tests. Bold p values indicate p values of <0.05.

proach assesses whether the global level of differential expression within a group of genes is outstanding compared to the global level of differential expression in the remaining genes. In the procedure all genes on the array are ranked due to a score for differential expression. While exploring the ranks of genes, the method answers the question whether the difference in expression for a given set of genes is more pronounced compared with the differential expression in the remaining genes. A p value for each gene group is computed by a permutation approach (permutation of genes).

Definition of Gene Groups

Predefined functional gene groups were selected chosen from the Gene Ontology (<http://www.geneontology.org/>) and Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.jp/kegg/kegg2.html>) databases [17]. Gene Ontology groups 0006954 (inflammatory response), 0006917 (induction of apoptosis), 0006950 (response to stress), 0007596 (blood coagulation), 0007155 (cell adhesion), 0007267 (cell-cell signaling), 0006955 (immune response), 0006629 (lipid metabolism) and KEGG groups hsa00590 (prostaglandin and leukotriene metabolism), hsa04350 (TGF- β -signaling pathway) and hsa0462 (TLR-signaling pathway) were selected. We furthermore analyzed some

groups of genes identified in GeneScan studies of circulating cells from acute stroke patients [10, 11] as well as a group of genes that was reported to be activated by LPS in macrophages [18].

Results

PBMCs from 15 stroke survivors and 15 healthy control subjects (referents) were studied without LPS stimulation as well as upon LPS stimulation (fig. 1). After normalization of the data with GC-RMA, we filtered probe sets with present calls in all 12 analyzed RNA pools or at least in all 6 LPS-induced pools. Thus, 10,197 probe sets (of all 22,263 probe sets of the U133A array) were selected and analyzed further. For each probe set logFCs were calculated for base level gene expression as well as for LPS responsiveness. LPS stimulation induced dramatic differences in the gene expression profiles of PBMCs (online

Table 4. Probe sets with highest contribution to the significant test results (top ten in both cases)

<i>Cell-cell signaling (global ANCOVA test)</i>			
211122_s_at	CXCL11	Hs.518814	Chemokine (C-X-C motif) ligand 11
207442_at	G-CSF	Hs.2233	Colony-stimulating factor 3 (granulocyte)
210163_s_at	CXCL11	Hs.518814	Chemokine (C-X-C motif) ligand 11
220322_at	IL1F9	Hs.211238	Interleukin-1 family member 9
206295_at	IL18	Hs.83077	Interleukin-18 (interferon- γ -inducing factor)
206620_at	GRAP	Hs.546336	GRB2-related adaptor protein
204533_at	CXCL10	Hs.413924	Chemokine (C-X-C motif) ligand 10
207691_x_at	CD39	Hs.550467	Ectonucleoside triphosphate diphosphohydrolase 1
205767_at	EREG	Hs.115263	Epiregulin
207426_s_at	TNFSF4	Hs.181097	Tumor necrosis factor (ligand) superfamily member 4
<i>Cell-cell signaling (Kolmogorov-Smirnov-like test)</i>			
205560_at	PCSK5	Hs.368542	Proprotein convertase subtilisin/kexin type 5
211122_s_at	CXCL11	Hs.518814	Chemokine (C-X-C motif) ligand 11
207442_at	G-CSF	Hs.2233	Colony-stimulating factor 3 (granulocyte)
220558_x_at	TSPAN32	Hs.271954	Tetraspanin 32
206582_s_at	GPR56	Hs.513633	G-protein-coupled receptor 56
210354_at	IFNG	Hs.856	Interferon- γ
217496_s_at	IDE	Hs.500546	Insulin-degrading enzyme
32137_at	JAG2	Hs.433445	Jagged 2
206295_at	IL18	Hs.83077	Interleukin-18 (interferon- γ -inducing factor)
211506_s_at	IL8	Hs.624	Interleukin-8

suppl. fig. 1a–d, for all supplementary material, see www.karger.com/doi/10.1159/000215878). However, the variation among the microarray data between patients and referents remained low. 9,715 probe sets (95.3%) had LogFCs between -0.4 and $+0.4$ for base level gene expression, which corresponds to nominal fold changes between 0.76 and 1.32. Only 23 probe sets had $\log_{2}FC > 1$ (online suppl. fig. 2a–d). Variation of LPS responsiveness was slightly larger (85.4% of the probe sets had $\log_{2}FC$ s between -0.4 and 0.4). We found 124 probe sets with $\log_{2}FC > 1$ after LPS stimulation. In none of the 10,197 probe sets analyzed did the difference between patients and the reference group remain significant after correction for multiple testing (Bonferroni or Benjamini and Hochberg), either in base level expressions or in LPS responsiveness. The use of less conservative methods for correction (like procedures that control the false discovery rate) did not result in the detection of significant differences either.

However, when the expression of predefined functional groups of genes were analyzed, significant differences between patients and controls could be observed after LPS stimulation.

For the analysis of gene groups we chose predefined gene ontology groups with possible involvement in stroke and some published gene groups with possible relevance

to stroke or genes responding to LPS (table 3). Two statistical approaches were used to evaluate the differences in expression. An analysis of variance-like approach (global ANCOVA test) examines differential gene expression within a group of genes. A global difference may be found if expression values for some of the genes in the group are strongly different, or alternatively if many genes show slight differences in expression. The Kolmogorov-Smirnov rank test asks whether the differential expression for a group of genes is more pronounced compared to all the remaining genes. If many probe sets in the group of interest have extreme t values, the test considers this group as important. As shown in table 3, differences between gene groups were not significant for base level gene expression. However, in the cell–cell signaling gene group, stroke survivors showed a stronger gene expression difference upon LPS than the referents (significant results according to both tests procedures). The significant result of the Kolmogorov-Smirnov test suggests that many genes involved in cell–cell signaling are strongly differentially expressed. Such strong differential expression in most cases also provokes a significant result for the global ANCOVA test. Regarding genes involved in the LPS response, small p values are observed with the Kolmogorov-Smirnov procedure but not with global ANCOVA. This may be

due to correlations between genes which are not captured with the former approach in contrast to the latter one. The significant global ANCOVA result for genes involved in apoptosis (and also the relatively small Kolmogorov-Smirnov *p* value) suggests that in this group there are many moderately differentially expressed genes.

We further analyzed the group of genes involved in cell-cell signaling in order to identify which genes contributed most strongly to the differences found between survivors and referents (table 4). This group (GO:0007267) is represented by 421 probe sets on the U133A array. 117 of these 421 probe sets (identifying 83 different genes) show present calls in our material. Probe sets identifying transcripts of CXCL11, IL18 and granulocyte colony-stimulating factor (G-CSF) contributed in both statistical test procedures most strongly to the significant difference. The upregulation of these genes upon LPS was more dramatic in stroke survivors than in referents (fig. 2).

We studied the expression of three genes (ATF3, NFκB2 and IL1F9) also by real-time RT-PCR in LPS-treated PBMCs from 5 of the survivors and referents. These analyses were moreover repeated in newly isolated PBMCs from 5 additional stroke survivors and 5 additional referents. We chose these three genes as examples of genes that are strongly affected by LPS treatment (ATF3; see online suppl. fig. 1c), that are moderately up-regulated by LPS (NFκB2), and that belong to the cell-cell signaling proteins encoding genes (IL1F9). The expression values measured by the Affymetrix GeneScan experiments were confirmed by real-time RT-PCR analysis. Significant gene expression differences upon LPS were assessed by both methods, but also small and non-significant expression differences between survivors and referents (online suppl. fig. 3).

Discussion

We analyzed circulating PBMCs from patients in the chronic stage after stroke and healthy control subjects to test the hypothesis that gene expression profiles with and without LPS stimulation are persistently different between both groups. No significant differences in gene expression were found between resting PBMCs from stroke survivors and control samples. Acute infection is a stroke risk factor [5]. Therefore, we stimulated cells with LPS to test whether such stimulation increases or evokes gene expression differences between both groups possibly indicating that differential responsiveness may be associ-

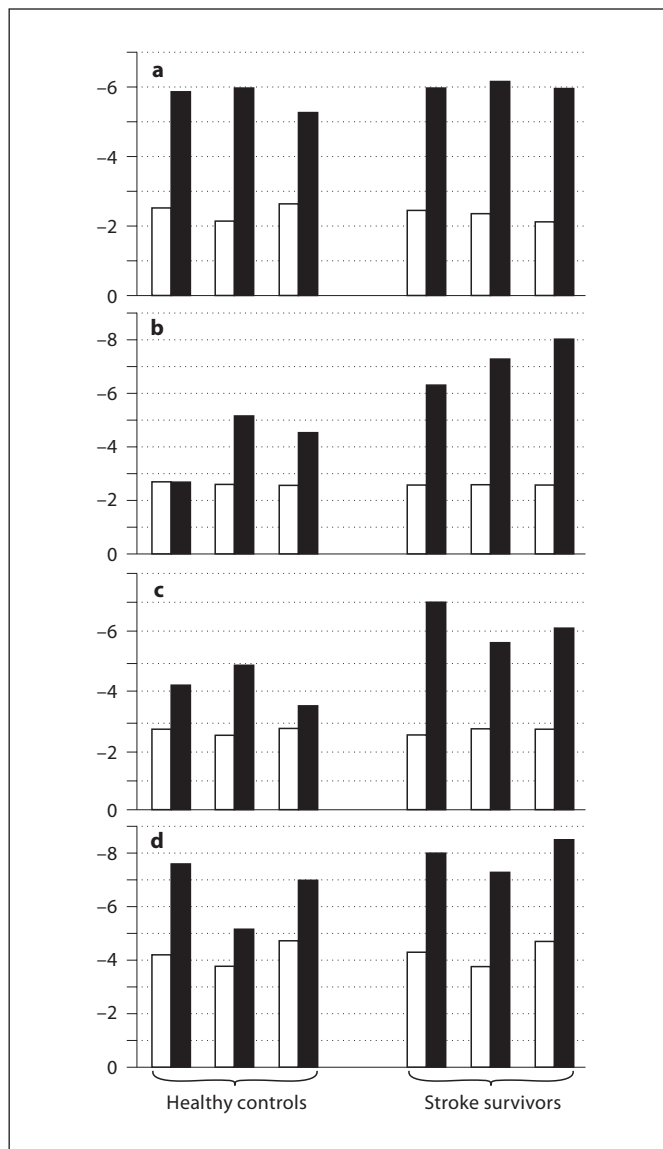


Fig. 2. Base level gene expression (□) and gene expression upon LPS (■) for single probe sets. **a** COX2. **b** CXCL11. **c** G-CSF. **d** IL18. Three pools of RNA were analyzed in each experiment. Y-axis shows log₂-transformed normalized expression values.

ated with the proneness to stroke. The group of genes involved in cell-cell signaling showed global expression differences between groups after LPS stimulation.

The peripheral inflammatory response during the acute phase of stroke was analyzed in earlier studies with Affymetrix U133 arrays [9–11]. Here we studied for the first time the gene expression patterns of blood samples from patients who have recovered from stroke. We cannot rule out that some effects of the brain ischemia re-

main traceable in the PBMC transcriptome even after at least 3 months. However, we suspect that our findings rather reflect the chronic condition of our patients.

The high similarity of the expression profiles of the survivors and the referents made the discrimination between statistical noise and real differences between groups difficult. To face this difficulty we not only focused on the study of single genes, but studied genetic programs as well. Our data suggest that the LPS responsiveness of a group of genes involved in cell–cell signaling differs significantly between patients and referents. This difference can be regarded as important since differential expression was detected using two statistical methods (the global ANCOVA approach and the Kolmogorov-Smirnov test). However, both methods have their limitations. A limitation of global ANCOVA in this case is the small sample size which restrains the number of possible permutations of the phenotype labels. The Kolmogorov-Smirnov test has the drawback of assuming the independence of variables, a condition that is not fulfilled in expression profiling studies since gene expression varies for many genes in a coordinated way.

We tried to reduce the heterogeneity within the groups by selecting referents without vascular risk factors and patients with a good outcome after a mild ischemic stroke at least 3 months before inclusion. Moreover, we enrolled stroke patients from a single etiologic subgroup – large artery atherosclerosis. To avoid methodological variation we followed a very strict protocol. Blood sampling of patients and referents was performed in exactly the same way between 10 a.m. and noon. All samples were handled by the same technician in the same laboratory. Mononuclear cells were isolated immediately after blood sampling by density gradient centrifugation at 4°C. The study of PMBCs after LPS stimulation and the comparison of resting and stimulated cells from the same subjects are a further strength of this study. Limitations of our study were the restricted number of subjects and the requirement of pooling, which reduced the power of statistical tests. Therefore, some minor gene expression differences may have been missed in our study. Nevertheless, pooling does not affect the estimates of absolute expression values or calculated folds (expression ratios of patients and controls). Our observation that not a single gene is strongly up- or downregulated is not compromised by pooling.

The observed differences in PBMC transcriptomes may partially reflect inherited differences in gene regulation between stroke survivors and control subjects. Hence, genes from the group of cell–cell signaling genes

might be promising candidates to search for inherited variants associated with stroke risk. In fact, genes coding for interleukins and cytokines were considered interesting candidates in several genetic stroke association studies [3, 9, 19, 20].

However, other factors such as vascular risk factors and differences in lifestyle as well as medication might have contributed to the observed differences in gene expression.

It is a limitation of our study that a second control group with vascular risk factors was not investigated. This analysis of such a second control group would have enabled us to eliminate the potential influence of medications and vascular risk factors. Further limitations were disparities in age and sex between groups and the stimulation of the cells with only a single LPS dose at a single time point.

All survivors were treated with acetylsalicylic acid (ASA) or clopidogrel for secondary stroke prevention. Moreover, many patients took statins, ACE inhibitors and diuretics. Apart from its inhibiting effect on platelet aggregation, ASA is an inflammation-modulating drug that interferes with cell–cell signaling. High doses of ASA inhibited proinflammatory cytokine production in a human whole blood assay. However, LPS in combination with low to moderate doses of ASA modestly enhanced the production of proinflammatory cytokines [21]. Inhibition experiments suggested that the induction of proinflammatory cytokines by ASA was mediated by COX2 inhibition [22]. Since COX2 was not downregulated in our patients (fig. 2), the increased LPS responsiveness of cell–cell signaling genes in the survivors is probably not explained by their ASA medication. Clopidogrel treatment is unlikely to explain the increased LPS responsiveness of this gene group in our patients, as clopidogrel reduces platelet-monocyte aggregation and probably has anti-inflammatory effects [23].

Differences in the PBMC transcriptomes from stroke survivors and healthy control subjects must not necessarily reflect differential gene activity. Peripheral blood contains a heterogeneous mixture of cells and PBMC isolates might differ in their cellular composition. Stroke survivors have increased leukocyte counts [24]. Flow cytometry of whole blood from stroke patients in the convalescent phase (3–14 months after acute stroke or transient ischemic attack) showed increased platelet activation in patients and increased leukocyte-platelet complexes despite antithrombotic therapy [21]. We characterized each PBMC isolate immediately after density gradient centrifugation and did not find significant differences. How-

ever, minor differences between PBMC composition of stroke survivors and referents may exist. However, our finding that resting cells of survivors and referents have similar transcriptomes, but that differences arise upon LPS stimulation, is not in favor of differences in PBMC composition explaining our findings.

Stroke is a complex disease. Many studies suggest that single genetic variants probably do not confer a large effect on risk of ischemic stroke [for recent discussion, see 25]. Since many interacting genetic (and environmental) factors seem to modulate the risk of stroke, it is of interest to consider functional groups of genes as candidates that play a role in the etiology of stroke.

Most known human genes belong to several gene ontology groups. Moreover, the gene ontology groups are still under construction and must not be considered as complete and definite yet [26]. The positive result for the particular group of cell–cell signaling genes therefore suggests, but does not prove, that variation in cell–cell signaling plays a role in the pathogenesis of stroke: other genetic programs which share many genes with this gene ontology group might be involved in this association.

Two independent statistical evaluations suggested that IL18, CXCL11 and G-CSF are among the genes that contribute most strongly to the significant difference in the cell–cell signaling gene group. Interleukin-18 (alias IL1 γ) is a potent proinflammatory cytokine belonging to the IL1 family. In diabetic patients elevated plasma IL18 is associated with carotid intima-media wall thickness, an early marker of atherosclerosis [27]. IL18 was found to be highly expressed in human atherosclerotic plaques. Significantly higher levels of IL18 mRNA were found in symptomatic (unstable) plaques than asymptomatic (stable) plaques in a real-time PCR study [28]. Studies in mice have shown that exogenously administered IL18 accelerates the rate of atherosclerotic lesion development and increases plaque size and inflammatory cell content. Conversely, the IL18-binding protein (IL18BP), a natural antagonist of IL18, decreases inflammatory cell infiltrate and generates a stable plaque phenotype [29]. Therefore, the elevated LPS-responsiveness of the IL18-gene in circulating cells of our patients with atherothrombotic stroke may have contributed to atherogenesis but beyond that IL18 may also play a role in brain injury. Elevated levels of IL18 were detected in the brains of mice 7 days after (traumatic) injury and in human CSF up to 10 days after brain trauma. Mice that were treated with IL18BP, a specific inhibitor of IL18, 1 h after trauma, showed a significantly improved neurological recovery [30].

The chemokine CXCL11 (alias interferon-inducible T-cell- α chemoattractant) is one of the most potent chemoattractants of CD4(+) and CD8 T(+) cell transendothelial migration [31]. The increased LPS responsiveness of this gene in stroke patients might suggest that stroke survivors display stronger lymphocyte migration upon infection or other insults. Moreover, I-TAC/CXCL11 as well as MIG/CXCL9 and IP-10/CXCL10 are small peptides with direct antimicrobial activity, similar to human defensins [32]. The cytokine CSF3 (G-CSF) was initially described as a hematopoietic growth factor. It is currently studied as a promising candidate for the treatment of neurodegenerative conditions because of its anti-inflammatory and neurotrophic effects [33, 34]. It might be speculated that the elevated LPS responsiveness of this gene in stroke survivors represents a long-term response to brain injury. This would imply that the differential expression of G-CSF might be a late consequence of the ischemic stroke event, rather than a preexistent condition. Indeed only a prospective study could unequivocally identify a gene expression profile associated with an increased stroke risk. In such an observational study whole genome gene expression profiles must be analyzed in a cohort of healthy individuals and associated with future ischemic stroke events. The current pilot study was merely designed to get a first insight into gene expression differences in the peripheral circulation related to stroke risk.

This gene expression analysis of circulating mononuclear leukocytes suggests that the transcriptomes of stroke survivors and healthy control subjects are very similar under non-stimulated conditions. Whereas resting PBMCs do not show a pattern of increased expression of proinflammatory genes, LPS treatment uncovers different induction of some cell-signaling proteins. G-CSF, IL18 and CXCL11 genes mainly contributed to these differences in the group of cell–cell signaling genes and functional SNPs in these genes may be interesting candidates for genetic association studies with stroke patients.

Acknowledgements

The work of Tina Wiest and Karin Pflieger was supported by NGFN grant 01GI9908. The work of Manuela Hummel was supported by NGFN grant 01GR0459.

References

- 1 Libby P, Ridker PM, Maseri A: Inflammation and atherosclerosis. *Circulation* 2002;105:1135–1143.
- 2 Ross R: Atherosclerosis – an inflammatory disease. *N Engl J Med* 1999;340:115–126.
- 3 Grau AJ, Aulmann M, Lichy C, Meiser H, Bugge F, Brandt T, Grond-Ginsbach C: Increased cytokine release by leucocytes in survivors of stroke at young age. *Eur J Clin Invest* 2001;31:999–1006.
- 4 Elkind MS: Inflammation, atherosclerosis, and stroke. *Neurologist* 2006;12:140–148.
- 5 Smeeth L, Thomas SL, Hall AJ, Hubbard R, Farrington P, Vallance P: Risk of myocardial infarction and stroke after acute infection or vaccination. *N Engl J Med* 2004;351:2611–2618.
- 6 Lindsberg PJ, Grau AJ: Inflammation and infections as risk factors for ischemic stroke. *Stroke* 2003;34:2518–2532.
- 7 Flex A, Gaetani E, Papaleo P, Straface G, Proia AS, Pecorini G, Tondi P, Pola P, Pola R: Proinflammatory genetic profiles in subjects with history of ischemic stroke. *Stroke* 2004;35:2270–2275.
- 8 Markus HS, Labrum R, Bevan S, Reindl M, Egger G, Wiedermann CJ, Xu O, Kiechl S, Willeit J: Genetic and acquired inflammatory conditions are synergistically associated with early carotid atherosclerosis. *Stroke* 2006;37:2253–2259.
- 9 Grond-Ginsbach C, Hummel M, Wiest T, Horstmann S, Pflieger K, Hergenahn M, Hollstein M, Mansmann U, Grau AJ, Wagner S: Gene expression in human peripheral blood mononuclear cells upon acute ischemic stroke. *J Neurol* 2008;255:723–731.
- 10 Moore DF, Li H, Jeffries N, Wright V, Cooper RA, Elkahoulou A, Gelderman MP, Zudaire E, Blevins G, Yu H, Goldin E, Baird AE: Using peripheral blood mononuclear cells to determine a gene expression profile of acute ischemic stroke: a pilot investigation. *Circulation* 2005;111:212–221.
- 11 Tang Y, Xu H, Du X, Lit L, Walker W, Lu A, Ran R, Gregg JP, Reilly M, Pancioli A, Khoury JC, Sauerbeck LR, Carrozzella JA, Spilker J, Clark J, Wagner KR, Jauch EC, Chang DJ, Vero P, Broderick JP, Sharp FR: Gene expression in blood changes rapidly in neutrophils and monocytes after ischemic stroke in humans: a microarray study. *J Cereb Blood Flow Metab* 2006;26:1089–1102.
- 12 Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J: Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 2004;5:R80.
- 13 Wu Z, Irizarry RA, Gentleman R, Martinez Murillo F, Spencer F: A model-based background adjustment for oligonucleotide expression arrays. *J Am Statist Assoc* 2004;99:909–917.
- 14 Benjamini Y, Hochberg Y: Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc B* 1995;57:289–300.
- 15 Lamb J, Ramaswamy S, Ford HL, Contreras B, Martinez MV, Kitrell FS, Zahnow CA, Patterson N, Golub TR, Ewen ME: A mechanism of cyclin D1 action encoded in the patterns of gene expression in human cancer. *Cell* 2003;114:323–334.
- 16 Mansmann U, Meister R: Testing differential gene expression in functional groups. Goeman's global test versus an ANCOVA approach. *Methods Inf Med* 2005;44:449–453.
- 17 Yue L, Reisdorf WC: Pathway and ontology analysis: emerging approaches connecting transcriptome data and clinical endpoints. *Curr Mol Med* 2005;5:11–21.
- 18 Stolovitzky GA, Kundaje A, Held GA, Duggar KH, Haudenschild CD, Zhou D, Vasicek TJ, Smith KD, Aderem A, Roach JC: Statistical analysis of MPSS measurements: application to the study of LPS-activated macrophage gene expression. *Proc Nat Acad Sci USA* 2005;102:1402–1407.
- 19 Brenner D, Labreuche J, Touboul PJ, Schmidt-Petersen K, Poirier O, Perret C, Schönfelder J, Combadière C, Lathrop M, Cambien F, Brand-Herrmann SM, Amarenco P: Cytokine polymorphisms associated with carotid intima-media thickness in stroke patients. *Stroke* 2006;37:1691–1696.
- 20 Worrall BB, Brott TG, Brown RD, Brown WM, Rich SS, Arepalli S, Wavrant-De Vrièze F, Duckworth J, Singleton AB, Hardy J, Meschia JF: IL1RN VNTR polymorphism in ischemic stroke: analysis in 3 populations. *Stroke* 2007;38:1189–1196.
- 21 McCabe DJH, Harrison P, Mackie IJ, Sidhu PS, Purdy G, Lawrie AS, Watt H, Brown MM, Martin M, Machin SJ: Platelet degranulation and monocyte-platelet complex formation are increased in the acute and convalescent phases after ischaemic stroke or transient ischaemic attack. *Br J Haematol* 2004;125:777–787.
- 22 Härtel C, von Puttkamer J, Gallner F, Strunk T, Schultz C: Dose-dependent immunomodulatory effects of acetylsalicylic acid and indomethacin in human whole blood: potential role of cyclooxygenase-2 inhibition. *Scand J Immunol* 2004;60:412–420.
- 23 Storey RF, Judge HM, Wilcox RG, Heptinstall S: Inhibition of ADP-induced P-selectin expression and platelet-leukocyte conjugate formation by clopidogrel and the P2Y12 receptor antagonist AR-C6931MX but not aspirin. *Thromb Haemost* 2002;88:488–494.
- 24 Grau AJ, Boddy AW, Dukovic DA, Bugge F, Lichy C, Brandt T, Hacke W: Leukocyte count as an independent predictor of recurrent ischemic events. *Stroke* 2004;35:1147–1152.
- 25 Matarin M, Brown WM, Scholz S, Simon-Sanchez J, Fung HC, Hernandez D, Gibbs JR, De Vrièze FW, Crews C, Britton A, Langefeld CD, Brott TG, Brown RD, Worrall BB, Frankel M, Sillimar S, Case LD, Singleton A, Hardy JA, Rich SS, Meschia JF: A genome-wide genotyping study in patients with ischaemic stroke: initial analysis and data release. *Lancet Neurol* 2007;6:414–420.
- 26 Gant TW: Inflammatory genomics. *Environ Health Perspect* 2005;113:A794–A795.
- 27 Aso Y, Okumura K, Takebayashi K, Wakabayashi S, Inukai T: Relationships of plasma interleukin-18 concentrations to hyperhomocysteinemia and carotid intimal-media wall thickness in patients with type 2 diabetes. *Diabetes Care* 2003;26:2622–2627.
- 28 Mallat Z, Corbaz A, Scoazec A, Besnard S, Leseche G, Chvatchko Y, Tedgui A: Expression of interleukin-18 in human atherosclerotic plaques and relation to plaque instability. *Circulation* 2001;104:1598–1603.
- 29 Anderson JL, Carlquist JF: Cytokines, interleukin-18, and the genetic determinants of vascular inflammation. *Circulation* 2005;112:620–623.
- 30 Yatsiv I, Morganti-Kossmann MC, Perez D, Dinarello CA, Novick D, Rubinstein M, Otto VI, Rancan M, Kossmann T, Redaelli CA, Trentz O, Shohami E, Stahel PF: Elevated intracranial IL-18 in humans and mice after traumatic brain injury and evidence of neuroprotective effects of IL-18-binding protein after experimental closed head injury. *J Cereb Blood Flow Metab* 2002;22:971–978.
- 31 Mohan K, Ding Z, Hanly J, Issekutz TB: IFN-gamma-inducible T cell alpha chemoattractant is a potent stimulator of normal human blood T lymphocyte transendothelial migration: differential regulation by IFN-gamma and TNF-alpha. *J Immunol* 2002;168:6420–6428.
- 32 Cole AM, Ganz T, Liese AM, Burdick MD, Liu L, Strieter RM: Cutting edge: IFN-inducible ELR-CXC chemokines display defensin-like antimicrobial activity. *J Immunol* 2001;167:623–627.
- 33 Schäbitz WR, Kollmar R, Schwaninger M, Juettler E, Bardutzky J, Scholzke MN, Sommer C, Schwab S: Neuroprotective effect of granulocyte colony-stimulating factor after focal cerebral ischemia. *Stroke* 2003;34:745–751.
- 34 Schneider A, Kuhn HG, Schäbitz WR: A role for G-CSF (granulocyte-colony stimulating factor) in the central nervous system. *Cell Cycle* 2005;4:1753–1757.