Gene Expression Profiles in Human Ruptured and Unruptured Intracranial Aneurysms
What Is the Role of Inflammation?

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Background and Purpose—Mechanisms underlying development and rupture of intracranial aneurysms (IA) are poorly recognized. The majority of studies on human tissue have focused on predefined pathways. We sought to analyze global gene expression patterns of ruptured IA, unruptured IA, and control vessels.

Methods—Transcription profiles were studied in human ruptured (n=8) and unruptured (n=6) IA, as well as in control intracranial arteries (n=5), using oligonucleotide microarrays. Real-time reverse-transcription polymerase chain reaction was used for confirmation. Functional analysis for determination of over-represented ontological groups among gene expression profiles was also performed.

Results—The expression of 159 genes differed among the studied groups. Compared to the controls, 131 genes showed common directions of change in both IA groups. The most impacted biological processes for IA are: (1) the muscle system; (2) cell adhesion (downregulation); and (3) the immune system and inflammatory response (upregulation). Ruptured and unruptured IA differed in genes involved in immune/inflammatory processes; expression was reduced in ruptured IA.

Conclusions—Decreased expression of genes related to muscle system and cell adhesion is important for the development of IA. The role of immune/inflammatory processes is unclear. Inflammation may participate in the healing process within IA while playing a protective role against IA rupture. (Stroke. 2010;41:00-00.)

Key Words: gene expression • inflammation • intracranial aneurysms • microarray
technique to identify differences in global expression profiles between RA, UA, and control intracranial arteries.

Materials and Methods

Patients and Tissue Samples

Full-thickness vessel wall samples from 8 RA and 6 UA donors were prospectively collected from patients undergoing microsurgical clipping. As a control intracranial artery, 5 middle meningeal artery (MMA) segments were obtained during standard neurosurgical procedures (evacuation after traumatic hematoma, tumor resection, IA clipping). Tissue samples were collected into RNAlater (Qiagen) and stored at −80°C until further processing. Basic demographic and risk factor data were collected. Informed consent was obtained from patients or from their proxies before surgery. Local ethical committee approved the study protocol.

RNA Isolation and Microarray Hybridization

Total RNA was isolated from homogenized specimens using RNeasy Fibrous Tissue Minikit (Qiagen) according to the manufacturer’s protocol and treated with DNase I. RNA concentration was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The quality of RNA was assessed using RNA 6000 Nano LabChip Kit and Agilent Bioanalyzer 2100 (Agilent). Preparation of cDNA and cRNA was performed according to the Affymetrix protocol. A starting amount of 1 μg of high-quality total RNA was used for cDNA reduction. The mRNA (cRNA-reduced) was converted to double-stranded cDNA and further to cRNA using GeneChip WT Sense Target Labeling and Control Reagents (Affymetrix). The fragmented single-stranded DNA was subsequently labeled using recombinant terminal deoxynucleotidyl transferase and hybridized to an Affymetrix GeneChip Human Gene ST 1.0 array at 45°C for 17 hours as recommended by the manufacturer. These arrays contain 768 885 probes representing 28 869 well-annotated genes. Arrays were washed and stained in Fluidic Station 450 (Affymetrix) according to manufacturer’s standard protocol and were scanned using GeneChip Scanner 3000 7G (Affymetrix).

Microarray Data Analysis

Microarray data were initially processed using GeneChip Operating Software. DTT data were transferred by Transfer Tool software (Affymetrix). Chip quality was assessed according to the Affymetrix guidelines. Raw data were processed using model-based expression index implemented in dChip (data built June 10, 2008). After background subtraction (using Perfect Match-only method), the data were normalized using quintile normalization. The signal was taken as the measure of mRNA abundance derived from the level of gene expression. Significance levels (probability values) of differences between the groups were calculated for each probe set using ANOVA. False discovery rates (q values) were calculated for ANOVA significance levels using q-value package for R (q < 0.05). Signal intensity >10 in at least 33% of arrays measured by dChip software was the criteria applied for probe-set detection. Additionally, group-specific differences in the gene expression level were selected by computing the between-group contrast matrix for each probe set using post hoc Tukey tests. In total, 20 185 genes fulfilled the filtering criteria (60.7% of all probe sets on the array). Gene expression values were filtered to remove probe sets with hybridization signal close to the background noise level. Microarray data have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE15629.

Bioinformatic Analysis of Gene Expression Patterns

The functional annotation for each gene was based on the Gene Ontology Consortium. Information was retrieved using WebGestalt (WEB-based GEnie Set AnaLysis Toolkit, http://bioinfo.vanderbilt.edu/webgestalt) with default software parameters. Gene Ontology annotation was available for 138 genes.

Real-Time Reverse-Transcription Polymerase Chain Reaction

Selected differentially expressed genes were validated by reverse-transcription polymerase chain reaction using TaqMan Gene Expression Assays and TaqMan Universal polymerase chain reaction Master Mix. Reverse-transcription was performed with Omniscript RT enzyme (Qiagen) at 37°C for 60 minutes. Reaction was performed in the presence of RNase inhibitor (rRNAin; Promega) with oligo(dT)6 primer (Qiagen). Quantitative reverse-transcription polymerase chain reaction reactions were performed on the same samples as in the microarray study using Assay-On-Demand Tagman probes according to the manufacturer’s protocol (Applied Biosoys) and run on an iCycler device (BioRad) with the 3.0a version of software. For each reaction, ∼50 ng of cDNA synthesized from the total RNA template of an individual donor was used. To minimize the contribution of contaminating genomic DNA, primers were designed to span exon junctions. Controls reactions without reverse-transcription enzyme were also performed. Amplification efficiency was determined by running a standard dilution curve. Expressions of glyceraldehyde-3-phosphate dehydrogenase and hypoxanthine guanine phosphoribosyl transferase 1 genes were quantified to control for variation in cDNA amounts. The cycle threshold values were calculated automatically by iCycler IQ 3.0a software with default parameters. The abundance of RNA was calculated as 2−Ct (threshold cycle).

Immunohistochemistry

To get some insight into cellular composition of the vessels wall, immunohistochemistry was performed on samples obtained from patients who were not included into the microarray study (3 RA, 3 UA, and 2 MMA donors). Their clinical characteristics were similar to the “microarray subjects”; all RA underwent operation on day 0 to 1 after bleeding. Samples were fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 4 μm. We used Masson trichrome for collagen fibers and anti-human antibodies (dilution) from DAKO (CD31 [endothelial cells marker, 1:40]; smooth muscle actin [smooth muscle cells (SMC), 1:50]; leukocyte common antigen, 1:100; and CD68 [macrophages, 1:50]). For visualization, EnVision System (DAKO) was used.

Statistical Analysis

Statistical comparison of expression levels between groups was performed using ANOVA followed by post hoc Tukey tests. P<0.05 was considered statistically significant.

Results

Samples were obtained from 8 RA patients (55.3±11.7 years), 6 UA patients (54.5±12.3 years), and 5 MMA donors (53.4±11.2 years). All subjects were white. The clinical characteristics are summarized in Table I.

Comparison between studied groups revealed significantly differential expression of 172 probes representing 159 identified genes (supplemental Table I, available at http://stroke.ahajournals.org), because some genes were represented by multiple probes on the array. When compared with controls, there were 131 genes common for RA and UA with the same direction of changes in transcription (8 were upregulated and 123 downregulated), 2 RA-specific genes (downregulated), and 26 UA-specific genes (upregulated; Table 2).

A comparison between RA and UA transcription profiles revealed 32 differentially expressed genes; 28 genes showed RA-specific or UA-specific expression different from that of the control, and 4 genes were from the overlapping file. All but 1 showed significantly higher expression levels in UA (Table 3).
A summary of the functional classification of the differentially expressed genes is presented in Table 4. The top 5 of the significantly enriched classes of upregulated and downregulated genes in both IA groups as compared to controls are listed. In both IA groups, genes related to inflammatory/immune processes were upregulated, whereas genes related to muscle system, cell adhesion, and cell membrane were downregulated. Comparing RA with UA, we found the same 5 top Gene Ontology categories, which were upregulated in IA in relation to controls; genes involved in inflammatory/immune processes presented significantly higher expression levels in UA.

Differential expression of selected genes was validated by real-time reverse-transcription polymerase chain reaction. Selection was based on the degree of difference in expression, contribution to Gene Ontology processes, and the potential contribution to aneurysm rupture/growth. The analysis included 5 genes related to muscle system (CASQ2, DES, MYOM1, RYR2, SMTN), 5 genes related to cell adhesion (CLSTN3, AOC3, CDH5, ITGA7, MMRN1), and 2 genes involved in the complement pathway (C4A, CFB). The probes used are described in supplemental Table II. Results were consistent with microarray analyses (Figure 1).

Because 2 RA patients underwent delayed surgery (RA01 and RA04), we performed additional analyses after exclusion of their data. This did not influence the results significantly. Exclusion of the youngest patient (RA06) also did not affect the results (data not shown).

Figure 2 presents representative results of immunohistochemistry. When compared to MMA, in IA vascular structure was disorganized (Masson trichrome, CD31, smooth muscle actin), and inflammatory infiltration was more visible within the vessel wall (leukocyte common antigen, CD68). Lack of specific immunostainings for differentially expressed genes did not allow us to assess whether those differences also are present on the protein level.

**Discussion**

We found 3 major functional groups of genes to be differentially expressed in aneurysmal tissue compared to nonaneurysmal tissue. These genes are related to: (1) the muscle system; (2) cell adhesion; and (3) the immune/inflammatory system. The first 2 groups were significantly downregulated, whereas the third was upregulated in RA and UA. Interestingly, genes related to the immune/inflammatory system were overexpressed in UA compared to RA.

These results are in line with the current knowledge about mechanisms implicated in the pathogenesis of IA, including a local inflammatory reaction and degeneration of the aneurysmal wall with extracellular matrix (ECM) remodeling and decellularization. The significant downregulation of genes related to the muscle system and cell adhesion is in agreement with histological analyses of human IA showing a decreased number of endothelial cells (EC) and SMC in the IA wall that result, at least partially, from apoptosis. There is also strong evidence that the ECM plays an essential role in the pathophysiology of IA. Some inherited conditions affecting ECM increase the risk of harboring IA. Elevated expression of matrix metalloproteinases cleaving ECM components were demonstrated in aneurysmal walls. In some animal models, IA are created using elastase. Finally, Peters et al investigated an IA in a 3-year-old girl with a SAGE-lite method
### Table 2. Differentially Expressed Genes in IA Compared With Controls

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**Downregulated**

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**(Continued)**
In underlined cells are shown genes common for RA and UA groups in relation to MMA.

*ANOVA followed by post hoc Tukey test, nominal P value.

and found an overexpression of genes involved in ECM remodeling.

Simultaneously decreased expression of genes related to the muscles and genes related to cell adhesion seems to be understandable. Cells of the vessel wall produce ECM ele-

ments. However, the primary event remains unclear; it could be loss of the cells within the vessel wall and decreased ECM production or ECM degradation that lead to cellular death. For adherent cells, anchorage to the ECM is necessary for survival. Loss of focal adhesion can be caused by ECM disintegration or decreased mechanical stress on the vascular wall.15,16 Low wall shear stress seems to facilitate IA growth and rupture.17 Mechanical signals conveyed by the ECM influence the activity of transcription factors and vascular remodeling.18 EC subjected to low wall shear stress are more susceptible to apoptotic stimuli and show increased expression of adhesion and inflammatory molecules.19

Caderhin 5 participates in the transduction of mechanical stretch and wall shear stress into proliferative signals of EC

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and SMC. Deficiency in mice results in vascular malformations. Here, we found a significant downregulation of cadherin 5 in IA.

Integrins are other molecules involved in cellular adhesion engaged in control of cell survival and proliferation. Cell–matrix adhesion via integrins protects from apoptosis and is required for a proliferative response to mitogens. We noticed lower expression of integrins α6 and α7, which are expressed by EC and SMC. Blocking of the α6 integrin inhibits vascular endothelial growth factor-induced adhesion and migration of brain microvascular EC. Embryonic loss of α7 integrin results in reduced cerebral vascularization and decreased number of SMC concomitant with cerebral hemorrhaging.

One of the crucial players in the pathophysiology of IA is inflammation. In rats, progression of the inflammatory zone and IA formation were in parallel. Analysis of genes downregulated in RA when compared with UA revealed the same 5 top GO categories, genes, and P values for them as in the case of genes upregulated in IA when compared with MMA. Genes in bold is validated in RT-PCR.

Table 4. Functional Analysis of the Gene Ontology Biological Processes Associated With Differentially Expressed Genes (IA vs MMA): Top 5 for Every Direction of Changes

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Gene Ontology (GO) level indicates GO annotation level.

Figure 1. Results of the real-time reverse-transcription polymerase chain reaction analyses. *P<0.05; †P<0.01; ‡P<0.001.
analyses of human IA revealed macrophage and lymphocyte infiltration, with increased expression of proinflammatory molecules. Interestingly, the degree of inflammatory response in RA did not correlate with the time elapsed between rupture of aneurysm and the onset of fixation of the specimens, suggesting that inflammation is not only a response to bleeding but also could also have existed before rupture.

In the context of our findings, previous observations of an extensive inflammatory reaction in unruptured aneurysms is intriguing. In the present study, genes related to the inflammatory/immune response were not only overexpressed in IA compared to controls; in UA, their expression was higher than in RA.

Among this group of genes, some complement components were present (C2, C4A, CFB). These are involved in all 3 complement activation pathways and are upstream to the membrane attack complex, a final product of complement activation. Tulamo et al detected higher expression of membrane attack complex in RA than UA; such expression was positively correlated with wall degeneration, de-endothelialization, and inflammation. However, the role of complement may be more complex than only weakening of IA walls. At sublytic doses, membrane attack complex protects SMC from apoptosis, can induce proliferation of EC and SMC, and increases synthesis of IL-8 and monocyte chemoattractant protein-1.

Monocyte chemoattractant protein-1 plays an important role in IA development. Thus, the complement system might be involved in maintenance of tissue homeostasis and regulation of regeneration processes. Interestingly, Krischek et al found a significant overexpression of major histocompatibility complex class II and class I genes in human IA. These genes are located on chromosome 6p21.3 in the same region as complement components overexpressed in our IA samples, particularly in UA in comparison with RA. In animals, anti-inflammatory strategies efficiently decelerate IA growth. Thus, the question of “friend or foe?” in relation to the role of the immune/inflammatory system in the pathogenesis of IA growth and rupture remains open.

Among our UA patients, 4 of 6 were hypertensive. Because hypertension is related to wall shear stress and gene expression, this could influence our results. However, 2 patients were untreated, and 2 were treated (angiotensin-converting enzyme-I and calcium-channel blockers). Considering the number of subjects, this relative overrepresentation of hypertensive patients probably did not significantly influence the final results.

There are some findings common to our study and recently published articles on microarray analysis of human IA. Generally, differential expression was observed in genes related to antigen presentation, the complement system, inflammation, focal adhesion, and ECM receptors. Clearly, differences between these studies with respect to single genes are unavoidable. In every study, different platforms were used. Different controls were also used in the previous studies. Weinsheimer et al used intracranial arteries from autopsies. Krischek et al used feeders of the arteriovenous malformations. Although these were extranidal fragments, altered blood flow conditions could result in adaptive changes in the vessel wall, including gene expression. Shi et al used the superficial temporal artery, which is an extracranial vessel. Thus, it is uncertain whether the observed differences in transcription are related only to the presence of IA or instead mirror differences in location and function of investigated vessels. We used MMA (an intracranial vessel) as a control. Another important point is the number of analyzed samples and statistical methods used. In the current study, we decided a priori to analyze RA and UA separately. Thus, we directly compared all three groups: RA vs UA vs MMA using ANOVA. It remains unclear why only a minority of IA ruptures. The differences in pathobiology between RA and UA also remain unclear. Second, the number of samples was sufficient to use ANOVA. Finally, the ethnicity of patients...
might influence the final results of the studies (Finnish and black,\textsuperscript{2} Japanese,\textsuperscript{3} heterogeneous group of Asians, white, Hispanic,\textsuperscript{4} and Polish [present report]).

Our study has some potential limitations. One is related to the microarray technique itself, which is essentially a screening method. However, considering the difficulties of obtaining human samples, the microarray is a very useful tool. The other drawback is lack of confirmation of the obtained results.

Some differences in clinical characteristics could influence the obtained results.

A strong point of the study is the relatively large number of analyzed samples derived from RA, UA, and control intracranial arteries. This allowed us to find differences between RA and UA apart from differences between IA and controls.

Moreover, all of the patients were ethnically homogenous.

CRANIAL ARTERIES. This allowed us to find differences between IA and controls.

Moreover, all of the patients were ethnically homogenous.

Summary

In the current study, we found that in both ruptured and unruptured human IA the expression of genes related to the immune/inflammatory system is unclear. An upregulation was found in IA with respect to the controls, but in the unruptured IA the expression levels were significantly higher than in ruptured ones.

Sources of Funding

This work was supported by the Polish Ministry of Science (grant N402 091 31/2893 to J.P.).

Disclosure

None.

References


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*p*ANOVA followed by post hoc Tukey test.
ANOVA (p) indicates nominal p value; ANOVA (q), p value corrected for multiple testing (false discovery rate).
Table II. Characteristics of TaqMan Probes Used in the Study for Real-Time Reverse-Transcription Polymerase Chain Reaction

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<td>CASQ2</td>
<td>Calsequestrin 2 (cardiac muscle)</td>
<td>Hs00154286_m1</td>
<td>5–6</td>
<td>867</td>
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<td>Hs00174344_m1</td>
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<td>CFB</td>
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<td>RYR2</td>
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<td>Hs00181461_m1</td>
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<td>Smoothelin</td>
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<td>Hypoxanthine guanine phosphoribosyl transferase 1</td>
<td>Hs99999999_m1</td>
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The assay location is the location of the assay on the corresponding RefSeq.

The amplification efficiency of a quantitative polymerase chain reaction was based on the slope of the standard curve. The standard curve was generated from a dilution series of template (pooled cDNA from all the samples). The quantitative polymerase chain reactions with total RNA and water as a template were used as negative controls. The calculations were performed automatically using Q-Gene Core Module file qgene96.xls (Muller PY, Janovjak H, Miserez AR, Dobbie Z. Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques. 2002;32:1372–1374).
Gene Expression Profiles in Human Ruptured and Unruptured Intracranial Aneurysms. What Is the Role of Inflammation?
Joanna Pera, Michal Korostynski, Tadeusz Krzyszkowski, Jacek Czopek, Agnieszka Slowik, Tomasz Dziedzic, Marcin Piechota, Krzysztof Stachura, Marek Moskala, Ryszard Przewlocki and Andrzej Szczudlik

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