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Differential expression of genes in elastase-induced saccular aneurysms with high and low aspect ratios

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Abstract

Background and Objectives—Morphological features are thought to play a critical role in the rupture of intracranial, saccular aneurysms. The objective of the present study was to investigate the gene expression pattern of saccular aneurysms with distinct morphologic patterns.

Materials and Methods—Elastase-induced saccular aneurysms with high (≥ 2.4) and low (≤ 1.6) aspect ratios (AR) (height to neck width) were created in 15 rabbits ($n=9$ for high AR and $n=6$ for low AR). RNA was isolated from the aneurysms and analyzed using a microarray containing 294 rabbit genes of interest. Genes with a statistically significant difference between low and high AR ($p<.05$) and a fold change of ≥ 1.5 and ≤ 0.75 to represent up- and down-regulation in high AR compared to low AR were used to identify pathways for further investigation.

Results—Fourteen genes (4.8%) genes were differentially expressed in the high AR aneurysms compared with the low AR aneurysms. The expression of osteopontin, TIMP, haptoglobin, cathepsin L, collagen VIII, fibronectin, galectin-3, secreted frizzled-related protein-2, CD14, decorin and annexin I were up-regulated, whereas the expression of myosin light chain kinase, Fas antigen and CD34 were down-regulated in the high AR aneurysms.

Conclusion—In a rabbit model of saccular aneurysm, high AR was associated with differential expression of inflammatory/immunomodulatory genes, structural genes, and genes related to proteolytic enzymes, and extracellular matrix-related genes. These findings may focus efforts on specific targets aimed at avoiding spontaneous rupture of intracranial, saccular aneurysms.

Keywords

Saccular aneurysm; gene expression and aspect ratio

INTRODUCTION

The rupture of intracranial aneurysms is a serious clinical event leading to subarachnoid hemorrhage, which is associated with high morbidity and mortality. Geometric features, hemodynamic factors, and arterial wall remodeling have been associated with aneurysm rupture (13,16,25). In addition to these factors, there is a positive correlation between high aspect ratio (AR, or aneurysm height-to-neck width), where correlations have been

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demonstrated between selected populations of ruptured and unruptured aneurysms and AR (28).

The present study was aimed at studying the gene expression pattern of saccular aneurysms with distinct morphologic features (low and high AR) using microarray experiments. We employed the rabbit, elastase-induced saccular aneurysm model, which has been widely applied in preclinical aneurysm research for testing endovascular devices (6,24) and also for studying the mechanism of vascular remodeling of saccular aneurysms (14,15,20). We focused on a panel of genes representing multiple pathways considered relevant in vascular homeostasis as well as dysfunction, to determine if any such genes were differentially expressed in low versus high AR aneurysms. Gene expression patterns correlating with high AR might focus attention on biological pathways causative of aneurysm rupture, thus offering potential for interventions aimed at diminishing rupture risk.

METHODS

Construction of gene chip

Our group has previously applied a rabbit-specific gene chip, which has been updated to include 294 genes (Table - supplement data). We initially identified genes previously identified in the literature as being involved in the pathobiology of intracranial aneurysms and abdominal aortic aneurysms. We identified 209 rabbit genes that had been sequenced and posted on the GenBank data base in 2003 (20), and have since added 85 additional, relevant genes to the gene chip, for a total of 294 genes. Details of gene chip construction have been previously described (20). The list of nucleotide sequences used for the construction of the microarray is available on request.

Aneurysm Creation

The Institutional Animal Care and Use Committee approved all procedures before initiation of the study. Elastase-induced saccular aneurysms with high and low aspect ratios (height to neck width) were created in New Zealand white rabbits (body weight, 3–4 kg) by using the rabbit elastase model. Aneurysms with AR of ≤ 1.6 and ≥ 2.4 were defined as low AR and high AR, respectively. The aspect ratio aneurysms were controlled by adjusting the position of ligation during creation of elastase-induced aneurysms (1,5). Briefly, anesthesia was induced with an intramuscular injection of ketamine, xylazine, and acepromazine (75, 5, and 1 mg/kg, respectively). Using sterile technique, we exposed the right common carotid artery (RCCA) and ligated it distally. A 1 to 2 mm beveled arteriotomy was made, and a 5F vascular sheath was advanced retrograde in the RCCA to a point approximately 3 cm cephalad to the origin of RCCA.

A 3F Fogarty balloon was advanced through the sheath to the level of the origin of the RCCA with fluoroscopic guidance and was inflated with iodinated contrast material. Porcine elastase (Worthington Biochemical, Lakewood, NJ) was incubated within the lumen of the common carotid artery above the inflated balloon for 20 minutes, after which the catheter, balloon, and sheath were removed. The RCCA was ligated below the sheath entry site, and the incision was closed.

Tissue Harvest

Aneurysm samples were harvested at 12 weeks (n=15) following aneurysm creation. Under general anesthesia, animals were euthanized by a lethal injection of sodium pentobarbital. The entire aneurysm was dissected free from the surrounding tissues and was then immediately frozen in liquid nitrogen or immersed in 10% buffered formalin for histology.

The frozen tissues were stored at -70°C until we were ready to perform tissue RNA extraction.

Histopathology

After fixing the tissue for at least 24 h, samples were embedded in paraffin, then cut in cross-sections and stained with hematoxylin and eosin (H&E) to assess the damage to elastic lamina, inflammation, thrombosis and damage to endothelium. Verhoeff-van Gieson (VVG) staining was also performed to specifically stain elastin.

Immunohistochemistry

Tissue sections were immunostained with anti-smooth muscle actin (SMA) and anti-CD31 antibodies for smooth muscle cells and endothelial cells, respectively.

RNA Extraction

RNA was isolated from frozen tissue using RNeasy Fibrous Tissue Mini Kit (Qiagen). The quantity of the RNA was measured using spectrophotometry and the integrity of the RNA was confirmed by electrophoretic separation using Agilent 2100 Bioanalyzer (Palo Alto, CA).

Microarray Analysis

Microarray slides had been printed with the oligo nucleotide set (Operon, Huntsville, AL). Each probe was spotted in triplicate.

Details of microarray analysis have been described previously (20). Briefly, total RNA (1 μg) was amplified and synthesized into complementary RNA (cRNA). Cy3 and Cy5 labeling of cRNA was carried out with Agilent Low RNA Input Fluorescent RNA Amplification kit (Palo Alto, CA). Equal amount of Cy3-labeled low AR aneurysm cRNA and Cy5-labeled high AR aneurysm cRNA were then mixed and hybridized to the microarray. The Cy3/Cy5 labeling of cRNA was swapped and the hybridization was repeated to control the Cy3/Cy5 fluorescent effect on hybridization. The microarray slides were then scanned and intensity of Cy3 and Cy5 fluorescence signals were quantified for each spot on the microarray (Axon GenePix 4000B, Axon, Union City, CA).

Quantitative Real-Time PCR Analysis

First-strand cDNAs were synthesized from 500 ng of total RNA using Superscript III first strand synthesis (Invitrogen, CA). Real-time PCR assays were performed with an iCycler (Bio-Rad, CA). The specific primers were designed from corresponding sequences obtained from GenBank using Primer 3 software(23). The sequences of primers are given in Table 1.

Statistical Analysis

Analyses were performed using the base-2 logarithm transform of the median signal intensity and all analyses were conducted using SAS[®] Version 9 statistical software. The data were normalized using two-channel fastlo, a semi-parametric approach that corrects for intensity-dependent effects, developed by Eckel et al.(7). The parametric component consists of an additive main effect for the gene. The non-parametric component consists of a set of non-parametric loess smoothers, one for each array, dye and block combination. The normalized signal intensity for each observation was estimated by subtracting the predicted value obtained from the non-parametric component, which was the original base-2 logarithm transformation of the median signal. To test for differential expression between groups, a mixed-effects linear model was fit obtained for each gene. The normalized expression values were the dependent variable in a mixed-effects linear model, array and dye were fit as fixed

effect co-variants and each rabbit was included as a random effect. The “t” test statistics and corresponding p-values, calculated from a linear contrast, were used as a measure of the mean change in expression between the aneurysm groups relative to the variability. Genes with a statistically significant difference between low and high AR ($p < .05$) and a fold change ≥ 1.5 and ≤ 0.75 to represent up- and down-regulation in high AR compared to low AR were used to identify pathways for further investigation.

RESULTS

Geometry

From a total of 60 consecutive aneurysm creation surgeries, we selected subjects demonstrating low (AR < 1.6 ; $n=6$) and high AR (AR > 2.4 ; $n=9$). Mean values for low AR and high AR were 1.57 ± 0.10 and 3.62 ± 1.19 , respectively. There was a statistically significant difference between AR groups ($p=0.00079$). Subjects were divided into those used for histopathology ($n=2$ and 5 for low AR and high AR, respectively) and those used for gene chip analysis ($n=4$ and 4 for low AR and high AR, respectively).

Histopathology

High aspect ratio samples (n=5)—In high AR aneurysms, the distal wall appeared to be distinct morphologically from the proximal wall (Figure 1A). The distal wall showed marked thinning of the wall and the structure of the three layers (intima, media and adventitia) was nearly absent and replaced with collagenized tissue (Figure 1B). Along the proximal wall, the neointimal hyperplasia was seen and the three mural layers were easily recognized (Figure 1C). VVG staining showed complete absence of the internal and external elastic lamellae. SMA staining showed only a few SMA positive cells along the distal wall, with other areas devoid of SMA positive cells. CD31 staining was negative throughout the aneurysm, indicating absence of endothelial cells along the wall of the aneurysm domes in these 12-week aneurysms.

Low aspect ratio samples (n=2)—In low AR aneurysms, both proximal and distal walls were collagenized; there were no notable differences between distal and proximal walls. CD31 and VVG staining results were similar to that of high AR aneurysms. SMA staining showed the numbers of SMA positive cells were decreased compared with the normal parent artery wall.

Microarray Analysis

Fourteen (4.8%) of 294 genes in the microarray were differentially expressed in the high AR aneurysm compared with the low AR aneurysm, using our definition above. Expression of eleven genes was up-regulated, while three genes were down-regulated when the high AR were compared to low AR aneurysms (Table 2). In high AR aneurysms, elevated expression was noted among inflammatory/immunomodulatory genes such as osteopontin, haptoglobin, galactin-3, annexin-1 and CD14, structural genes such as collagen VIII and decorin, and extracellular matrix-related genes such as TIMP and fibronectin. Additional up-regulated genes in high compared to low AR specimens included the proteolytic enzyme cathepsin L and cell survival factor secreted frizzled-related protein 2. Genes with diminished expression in high compared to low AR aneurysms included CD38, Fas antigen, and myosin heavy chain kinase.

Validation of microarray by qRT-PCR

We selected all 14 genes which showed differential expression between groups to validate the microarray results by qRT-PCR. Quantitative PCR results correlated well with the microarray data (Table 3).

DISCUSSION

In this study, we applied gene microarray technology to better understand the perturbations in gene expression in aneurysms with different aspect ratios. AR is considered relevant in aneurysm pathology; previous authors have noted a correlation between high AR and risk for spontaneous rupture (25,28). We demonstrated differential expression among a number of genes that might be important in aneurysm progression and rupture. Specifically, we identified disturbed expression of genes important in inflammation, vessel wall remodeling, structural integrity, and proteolysis. Our findings suggest not only that variations of AR are associated with specific biological changes in aneurysms, but also that targeted therapies based on identified, biological disturbances might be developed.

The precise mechanisms underlying differential gene expression remain unknown. One potential factor is that of different hemodynamic features between low- and high AR aneurysms. Ujiie et al., (2001) reported not only that AR was a reliable index for predicting imminent aneurysmal rupture but also that within domes of aneurysms with high AR (>1.6) there were extremely low flow conditions as compared to lower AR aneurysms (28). These low flow areas may be subject to diminished shear stress, which has been shown in previous studies to be associated with altered expression of various groups of genes (1,2,10). Many of the genes identified in our study are in concurrence with investigations into related vascular pathobiology, such as atherosclerosis, including cathepsin L, fibronectin and CD38 (9,18,30).

The pathophysiology of saccular aneurysms is complex and may relate to congenital factors, hemodynamics, and inflammation. In our model, high AR aneurysms showed up-regulation in the expression of genes involved in inflammation. Increased expression of osteopontin and haptoglobin, as seen in our study, has been reported in the abdominal aortic aneurysms (11,29). Osteopontin functions as a cytokine and is expressed by a wide variety of inflammatory cells. The diverse biological actions of osteopontin could potentially regulate processes pertinent to vascular diseases, including cell adhesion, inflammation and calcification (17). Haptoglobin is an acute phase reactant during inflammation, infection and traumatic damage. It binds to CD11b receptors to exert its immuno-modulatory function (8). All of these gene interactions could lead to alterations within the aneurysm wall that might lead to rupture.

Remodeling of aneurysm wall structure, induced by enzymatic digestion, has been reported to be involved in the mechanism of aneurysm growth and eventual rupture. We noted elevations in expression of a cysteine and aspartate protease, cathepsin L, in high AR aneurysms. In addition, osteopontin was demonstrated to up-regulate the activity of both MMP-2 and MMP-9 both *in vitro* and *in vivo* (12,26). The matrix metalloproteinases (MMPs) most strongly implicated in development and growth of cerebral aneurysms are MMP-2 and MMP-9, and osteopontin was demonstrated to up-regulate the activity of both MMP-2 and MMP-9 both *in vitro* and *in vivo* (12,26). In the current study, statistically significant increases in the gene expression level of MMP-2 and MMP-9 were observed in the high AR, but these ratios (1.17 and 1.23 for MMP-2 and -9, respectively) did not reach the threshold of 1.5 to make our definition of a “substantial increase”. However, we did find 1.47 and 4.87 fold increases in the expression of MMP-2 and MMP-9, respectively by real-time PCR (Data not shown). A 2.1-fold elevation in the tissue inhibitor of MMP (TIMP)

expression was seen in the high AR as compared to low AR aneurysms. MMPs activities are regulated by the level of TIMPs. Expression MMPs and TIMPs are controlled in tight feedback loops, so levels of TIMP expression might have diminished the MMPs levels in our experimental model (22) after 12 weeks.

In addition to inflammation and vessel wall remodeling, we also noted differences in genes related to cell growth and apoptosis. Increased expression of secreted frizzled-related protein-2 (SFRP-2) and decreased expression of Fas antigen were noted in the high AR aneurysms compared to low AR aneurysms. SFRP-2 is an Akt-mesenchymal stem cell-released paracrine factor attributed to the cell survival and repair (21). In contrast, Fas antigen is known to induce receptor-mediated apoptosis of arterial cells (19). The observed up-regulation of SFRP-2 and down-regulation of Fas antigen in aneurysms with high AR can be correlated with imbalance in the cell survival environment inside the aneurysms.

The animal model applied in the present study, the elastase-induced saccular aneurysm in rabbit, is similar to human intracranial saccular aneurysms histologically, morphologically, and hemodynamically (3,15,27). The rabbit elastase model has achieved widespread application and has added advantages, beyond the histologic homology with humans, including: 1) location along a curved vessel with anatomy that simulates human aneurysms such as those in the ophthalmic region; 2) demonstrated long term patency (4); 3) aneurysms of sizes similar to the mean size of human cerebral aneurysms (27). In addition, the rabbit aneurysm model shares some molecular features with human intracranial aneurysms (20). Histopathologic studies have shown that the rabbit aneurysm, after embolization with platinum coils, mimics the healing seen in human aneurysms (3).

There are several limitations to the current study. We collected the whole aneurysm sac for the gene expression analysis, rather than selectively targeting specific regions within the aneurysm cavity. Previous authors have shown substantial variation in blood flow and wall shear stress in different areas of high AR aneurysms. It remains possible that only portions of the high AR aneurysm sacs were exposed to hemodynamics distinct from those of low AR, and thus our experimental design may have diluted actual differences between groups. Our group is in the process of detailed computational fluid dynamics studies of the elastase-induced aneurysm model, which may permit improved experimental designs in the future. We are also embarking on a study employing laser capture microdissection in order to enhance the spatial resolution of our gene expression studies. Other substantial limitations of this study include the use of an artificial aneurysm model and use of only a single time point following aneurysm creation for study. As we have previously noted changes in gene expression patterns over time, we plan to study time-dependent differences in low and high AR aneurysms. Finally, we used a custom rabbit array with a relatively small number of selected, relevant genes. Further studies are underway in our laboratory using pan-genome arrays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Photomicrographs of a high AR aneurysm sample

A. A low magnification view of a high aspect ratio aneurysm. Boxes indicate where higher magnification photomicrographs (Figures B and C) have been taken (H&E), magnification = 12.5X)

B. High magnification of Figure A The collagenized (arrow) distal wall of the high AR aneurysm (H&E, magnification = 200X)

C. Intimal hyperplasia along the proximal wall of the high AR aneurysm (arrow) (H&E, magnification = 100X)

Table 1

List of primers used for the real-time PCR experiments

	Gene name	GenBank ID	Primer sequences
1	Osteopontin	D16544	5'-CAGCTCCGAGGAAAAGCAG-3' 5'-ACTCGGAGGGTCTCTTGT-3'
2	TIMP	J04712	5'-GTCATCAGGGCCAAGTTTGT-3' 5'-GGCGTCAAATCCTTTGAACA-3'
3	Haptoglobin	AF372520	5'-GTGGAGCACTTGGTCCGTTA-3' 5'-CGGCTTCCACATACTGCTT-3'
4	Cathepsin L	AF458078	5'-AATGGTGGCCTCATGGACTA-3' 5'-TACAGGTTCCATCCATTCCTTC-3'
5	Collagen VIII	J05042	5'-GAGTACCCACACCTCCCTCA-3' 5'-CTGGCTAATGGTATTTCTTTGC-3'
6	Fibronectin	S80206	5'-GCTCTACCGGCCACTCCTAC-3' 5'-ACGTCTAGAGGCATGAAGCA-3'
7	Galectin-3	U06470	5'-ACAGACTCGCTTTGGATTCA-3' 5'-CAAATGGGAAAGTCGTCTGC-3'
8	Secreted frizzled-related protein 2	AF323272	5'-CCACGAGACCATGAAAGAGG-3' 5'-GAAGAGCGAGCACAGGAAGT-3'
9	CD14	NM_001082195	5'-CTTGTGCTGCTGCTCCTG-3' 5'-GGATCCGAGAAGTTGCAGA-3'
10	Decorin	S76584	5'-GACACGACGCTACTGGACCT-3' 5'-TTGTTGACGAGGATCAATGC-3'
11	Annexin I	U24656	5'-AGCAGGCCTGGTTTATTGAC-3' 5'-GAAGGCAGGATAGGGACTCA-3'
12	CD38	NM_001082683	5'-TGAACACCTTCACAAGTGCAT-3' 5'-TGCAAGGAACAGTTTGAGTGA-3'
13	Fas antigen spliced variant	AB021297	5'-CACCAAATGCAAAGAAAAAGG-3' 5'-AGCTGATGCATCTGTCAACAC-3'
14	Myosin light chain kinase	M76233	5'-CAAGGACCGCATGAAGAAGT-3' 5'-TCGGTTCCAGTCTCTCTGC-3'

Table 2

Genes that are differentially expressed in high AR compared to low AR

	Gene name	GenBank ID	Fold change	P value	Function
1	Osteopontin	D16544	4.20	1.73108E-36	Cell adhesion and cytokine
2	TIMP	J04712	2.10	2.99033E-24	Regulators of extracellular matrix turnover and vascular remodeling.
3	Haptoglobin	AF372520	2.00	2.41213E-11	Acute phase protein with immunomodulatory properties; Binds with CD11
4	Cathepsin L	AF458078	1.71	3.80234E-23	Lysosomal cysteine protease
5	Collagen VIII	J05042	1.65	3.11765E-16	Structural component
6	Fibronectin	S80206	1.65	2.27769E-06	Cell signaling
7	Galectin-3	U06470	1.61	9.01596E-06	Inflammation; Anti-apoptotic
8	Secreted frizzled-related protein 2	AF323272	1.59	5.43362E-09	Promote cell survival
9	CD14	NM_001082195	1.54	1.30083E-20	Monocytic signal transduction
10	Decorin	S76584	1.52	9.08682E-16	Fibrillogenesis
11	Annexin I	U24656	1.50	6.71221E-19	Inflammation
12	CD38	NM_001082683	0.75	4.21704E-13	Cell adhesion
13	Fas antigen spliced variant	AB021297	0.74	0.001181687	Receptor-mediated apoptosis
14	Myosin light chain kinase	M76233	0.58	2.51204E-18	Regulates phosphorylation of myosin II

Table 3

Quantitative real-time PCR confirmation of differential expression in high AR aneurysm vs low AR aneurysms for all 14 genes identified by the microarray analysis.

Name of the gene	Fold change
Osteopontin	21.25 $\pm$ 6.25
TIMP	4.67 $\pm$ 5.12
Haptoglobin	1.64 $\pm$ 3.65
Cathepsin L	2.02 $\pm$ 0.46
Collagen VIII	2.09 $\pm$ 0.21
Fibronectin	2.8 $\pm$ 0.46
Galectin-3	3.86 $\pm$ 0.74
Secreted frizzled-related protein 2	3.74 $\pm$ 0.36
CD14	4.36 $\pm$ 0.85
Decorin	4.3 $\pm$ 0.94
Annexin I	1.36 $\pm$ 0.41
CD38	0.31 $\pm$ 0.083
Fas antigen spliced variant	0.5 $\pm$ 0.092
Myosin light chain kinase	0.48 $\pm$ 0.076