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Gene Expression Changes: Five Years after Creation of Elastase-Induced Aneurysms

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Abstract

Purpose—Intracranial, saccular aneurysms are associated with chronic remodeling of the arterial wall. The pathobiology of aneurysm growth and rupture is poorly understood. The objective of the present study was to study the gene expression patterns in elastase-induced saccular aneurysms in rabbits five years after aneurysm creation as compared to control, unoperated arteries.

Materials and Methods—Elastase-induced saccular aneurysms were created in 25 rabbits and followed up to five years. Thirteen rabbits died during follow-up for reasons unrelated to the aneurysms. RNA was isolated from aneurysm tissue and the control contralateral common carotid artery in 5 of the 12 surviving subjects and analyzed for gene expression using human gene microarrays. Genes with statistical differences between groups ($p < 0.05$ and fold change ≥ 1.5 and ≤ 0.75) were considered differentially expressed. RT-PCR was used for confirmation of gene microarray findings for selected genes.

Results—Fifty (0.33%) of 13,353 genes were differentially expressed in the aneurysms compared with the unoperated control arteries. Molecular and functional pathway analysis revealed that immunoregulatory molecules, growth factors, cell adhesion molecules, and structural molecules were differentially expressed in the aneurysms as compared to controls. RT-PCR results of selected genes confirmed the differential expression identified using the gene chip microarray.

Conclusions—Significant modulation in a variety of biochemical and cellular functions in chronic aneurysms provides molecular insights into the pathophysiology of saccular aneurysms.

Keywords

Genomics; microarray; rabbit; aneurysm; pathophysiology

Introduction

Intracranial, saccular aneurysms are associated with chronic remodeling of the arterial wall. Numerous previous studies using human tissue, animal models, etc. have attempted to probe the mechanism of aneurysm growth and rupture [1-5]. A recent study reported the global gene expression pattern in a small set of human unruptured and ruptured intracranial aneurysms [6]. However, data regarding chronic aneurysms remain elusive. Better

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understanding of the pathobiology of chronic aneurysms could lead to improved therapeutic options for saccular aneurysms.

The rabbit model of elastase-induced experimental aneurysms has been widely used for evaluating endovascular devices as well as studying aneurysm biology [3, 7-9]. This aneurysm model has been shown to remain patent as long as five years [10]. Using the rabbit elastase aneurysms, our group previously reported the differential gene expression pattern between 2 weeks and 12 weeks after aneurysm creation [11]. In this study, we present the gene expression pattern of chronic aneurysms using a human gene microarray.

Materials and Methods

Aneurysm Creation

Elastase-induced, saccular aneurysms were created in 25 New Zealand White rabbits (body weight 3–4 kg) using the rabbit elastase model. Some of these same subjects were reported in previous papers detailing the use of intravenous digital subtraction angiography (IVDSA) [12] as well as the patency rate of aneurysms up to two years [13]. In addition, the angiographic findings from these same subjects were reported separately [10]. The Institutional Animal Care and Use Committee at our institution approved all procedures. Detailed procedures for aneurysm creation have been described [14]. Briefly, using sterile technique, the right common carotid artery (RCCA) was exposed and ligated distally. A 5F vascular sheath (Cordis Endovascular, Miami Lakes, FL) was advanced in a retrograde to the origin of RCCA. A 3F Fogarty balloon (Baxter Healthcare Corporation, Irvine, CA) was advanced through the sheath to the level of the origin of the RCCA under fluoroscopic guidance. Once in place, the balloon was inflated with of iodinated contrast material. Porcine elastase (Worthington Biochemical Corporation, Lakewood, NJ) was incubated within the lumen of the common carotid above the inflated balloon for 20 minutes, after which the catheter, balloon and sheath were removed and the RCCA was ligated below the sheath entry site.

Tissue Harvest

Aneurysm samples were harvested at 5 years (n=5) following aneurysm creation. Follow-up digital subtraction angiography was performed at the time of sacrifice. Then 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. The frozen tissues were stored at -70°C until we were ready to perform tissue RNA extraction. When the aneurysms were relegated to histological analysis, samples were placed in buffered formalin

RNA Extraction

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

Gene Expression Profiling Using Microarrays

We applied Human HT-12 V3 expression BeadChip array (Illumina, San Diego, CA), which can target more than 25000 annotated genes. This gene chip can quantify whole genome expression profile, which includes genes that function cell adhesion, cell differentiation, cell proliferation, maintaining cell structure, immune response, ion transport, growth factors, signaling molecules and metabolic regulators.

Synthesis of double-stranded cDNA followed by synthesis of biotin-labeled cRNA was carried out according to manufacturer's instructions in the Mayo Clinic Advanced Genomics Technology Center core facility. Biotin-labeled cRNA was hybridized to Human HT-12 V3 expression BeadChip array (Illumina, San Diego, CA), then washed with buffers of varying stringencies, stained with streptavidin-Cy3, and scanned with an Illumina BeadArray reader following the manufacturer's protocols. The Illumina BeadStudio software program was then used to generate data for subsequent analysis.

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 for Apo E, HLA-DRB6, LECT1, smoothelin and FEZ1 with an iCycler (Bio-Rad, CA). The specific primers were designed from corresponding sequences obtained from GenBank using Primer 3 software [15].

Statistical Analysis

The 't' test statistics and corresponding p-values were used as a measure of the mean change in expression between the aneurysm and control groups relative to the variability. As compared to control, genes in aneurysms with statistically significant differences at $p < 0.05$ and a fold change of 1.5 or above were considered upregulated, whereas those with $p < 0.05$ with a fold change of 0.75 or below were considered downregulated. The t test-based P values were adjusted for multiple comparisons by using the false discovery rate (FDR) multiple correction approach ($FDR < 0.1$) [16].

Results

Rabbit elastase-induced aneurysms are remained fully patent until 5 years. Figure 1 shows the images of aneurysm at different time points after aneurysm creation.

Molecular Findings

Our preliminary quality control analysis of hybridizing rabbit RNA with human Human HT-12 V3 expression BeadChip array showed that human array can be able to detect 15,353 genes. In the gene expression experiments, using both FDR multiple corrections and magnitude of fold change criteria, 50 (0.33%) of 15,353 genes were found to be differentially expressed in aneurysm tissue compared to unoperated control left carotid artery tissue (Table 1). Out of these 56 genes, 19 (34%) genes were down-regulated whereas 37 (66%) genes were up-regulated in aneurysms as compared to the control artery.

Cell adhesion molecules

Cell adhesion molecules are proteins expressed on the cell surface and play bind with other cells and extracellular matrix components. In the aneurysm tissue, expression of procadherin 17 (1.62 fold, $p=0.01$), cell adhesion molecule-4 (1.88 fold, $p=0.049$) and aggrecan (1.67 fold, $p=0.012$) were upregulated compared to control arterial tissue.

Immuno modulatory molecules

Role of inflammation in the pathobiology of aneurysm formation and rupture is well established [17, 18]. Among genes related to immune response, the levels of interleukin-17 (0.58 fold, $p=0.016$) and leukocyte cell derived chemotaxin 1 (0.42 fold, $p=0.019$) were significantly decreased. The following genes were upregulated compared to control tissue: HLA-DQB1 (5.09 fold, $p=0.0048$), HLA-DQA1 (3.78 fold, $p=0.0112$), allograft anti-inflammatory factor-1 (3.32 fold, $p=0.018$) and complement 1 R subunit (1.27 fold, 0.0034).

Structural molecules

Structural molecules are responsible for cell movement and organization of the organelles within the cell. Among structural molecules, the expression of smoothelin (0.54 fold), fibulin 1 (0.64 fold) and tropomyosin 2 (0.66 fold, FDR=0.056) were downregulated and the expression of TRAF3 interacting protein 3 (1.59 fold) neuron navigator 3 (1.77 fold), myosin (1.83 fold) and actin (1.83 fold) were elevated in aneurysm tissue.

Growth, transcription and translation molecules

The function of cell proliferation and differentiation and biochemical signaling pathways are regulated by growth factors, transcription factors and translation factors. Compared to controls, aneurysm samples demonstrated increased expression of transcription factors, growth factors and other transcriptional and translational regulatory molecules which includes nuclear receptor interacting protein 1 (1.5 fold, $p=0.0047$), e26 oncogene (1.58 fold, $p=0.00017$), c-fos protooncogene (1.65 fold, $p=0.041$), early growth response 1 factor (1.77 fold, $p=0.019$) and IGF 2 mRNA binding protein (1.53 fold, $p=0.0041$). Levels of chromobox protein homolog 6 (0.66 fold, $p=0.0096$), leucine zipper putative tumor suppressor 1 (0.54 fold, $p=0.005$) and odd-skipped relate 1 gene (0.64 fold, $p=0.026$) were diminished in aneurysm tissue compared to control arteries.

Other molecules

Several intracellular signaling molecules, ion transporters and enzymes involved in metabolic and biochemical functions were differentially expressed in aneurysms. A 3.7 fold elevation in the expression of serine protease inhibitor serpine 2 and a 0.67 fold change in the expression of matrix metalloproteinase 23B were observed in aneurysms.

Validation of microarray data

Verification of differential gene expression in the aneurysm and control artery was examined in 5 selected genes. Microarray gene expression levels were in comparable with results obtained by RT-PCR (Figure 2).

Discussion

In the present study we analyzed the differential expression of a wide array of genes in tissue from chronic, experimental aneurysms as compared to normal arteries. 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 and structural integrity. Our findings may provide insight into the biology of unruptured, intracranial, saccular aneurysms.

Structural molecules demonstrated differential expression in aneurysmal tissue, including decreased expression of cytoskeleton proteins such as smoothelin, fibulin 1 and tropomyosin 2. Smoothelin is essential for functional contractility of vascular smooth muscle. Expression of smoothelin is rapidly decreased under pathologic conditions associated with impaired function of smooth muscle, such as abdominal aneurysms to restenosis [19, 20]. Fibulin 1 is an extracellular matrix protein that regulates cell adhesion and migration along protein fibers within the extracellular matrix. It is important for various developmental processes and contributes to the supramolecular organization of ECM architecture, in particular to those of basement membranes[21, 22]. Our finding suggests that the structure and function of the vascular wall changes over time in experimental aneurysms.

Our previous report showed that expression of genes related to cell adhesion and inflammation pathways was under regulated at 2 weeks but was not significantly different

from that of controls at 12 weeks[11]. However, in this study, increased expression of cell adhesion molecules, growth factors suggests that metabolic activity of cells in saccular aneurysms is deranged.

Our experimental technique circumvents the difficulty presented by obtaining clinical tissue and our primary findings regarding the expression of immuno modulatory molecules have been previously reported in human aneurysm tissue [5, 6]. Several previous studies reported the presence of inflammatory cells in the wall of intracranial aneurysms[23, 24]. Histologic analysis of human, intracranial aneurysms using electron microscopy identified the presence of extracellular lysosome-like granules and leukocytes near disorganized elastic lamina, suggesting the presence of degradation of elastic lamina by lytic enzymes secreted by leukocytes in the vascular wall [24, 25]. Ryba et al., reported a link between human leukocyte antigens (HLA) and subarachnoid hemorrhage following aneurysm rupture [26]. Thus, the role of inflammation in aneurysm rupture appears prominent and is buttressed by our experimental findings.

There are several limitations of the study. Previously we reported the gene expression pattern of aneurysm growth using rabbit specific microarray [11, 27]. As rabbit specific arrays have the ability to detect only 400 genes, as a pilot study, to explore the use of human microarrays for rabbit genes. Our quality control analysis of hybridization of rabbit genes with human probes confirmed that 15,353 (68.3%) out of 22,493 human genes are homologously similar with rabbit. Now that rabbit specific microarrays are commercially available we have undertaken steps to use commercial rabbit arrays in our future studies.

In this study we used the contralateral carotid artery as control tissue for aneurysms since the propensity of thrombus formation in sham-operated arteries (saline incubation instead of elastase). Despite increased expression of inflammatory molecules, we did not find inflammatory cells in our previous work[28]. Even though this rabbit model of elastase-induced aneurysms mimics the hemodynamics and geometric features of human intracranial aneurysms, it is not a natural pathologic aneurysm. In addition, these experimental aneurysms do not rupture. Even within these limitations, the model may have value in understanding the expression of vital biomolecules at chronic stages of aneurysms.

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Figure 1.

Representative images of serial DSA in an elastase-induced aneurysm:

- A.** Intra arterial DSA image of an aneurysm obtained 1 month after creation demonstrates an aneurysm cavity along the brachiocephalic artery, at the origin of the ligated RCCA.
- B.** Intravenous DSA image of the same aneurysm 2 years after creation
- C.** Intra arterial DSA image of the same aneurysm 5 years after creation. Aneurysm dimensions remain constant throughout follow-up.

Figure 2.
Validation of microarray results by RT-PCR assay.

Apo E : Apolipoprotein E
 HLA-DRB6 : Human Major histocompatibility complex, class II, DR beta 6
 LECT1 : Leukocyte cell derived chemotaxin 1
 SMTN : Smoothelin
 FEZ1 : Leucine zipper putative tumor suppressor 1

Table 1

Differentially expressed gene in the aneurysm five years after aneurysm creation. Genes with statistical differences between groups ($p < 0.05$, $FDR < 0.1$ and fold change ≥ 1.5 and ≤ 0.75) were considered differentially expressed.

Gene Symbol	Gene Name	Fold Change	p-value
Cell adhesion molecules			
PCDH17	Protocadherin 17	1.62	0.01010
CADM4	Cell adhesion molecule 4	1.88	0.04920
ACAN	Aggrecan	1.67	0.01211
RSPRY1	Ring finger and SPRY domain containing 1	0.61	0.00149
TIPARP	TCDD-inducible poly(ADP-ribose) polymerase	1.66	0.01190
FST	Follistatin	2.80	0.00678
NAV3	Neuron navigator 3	1.77	0.00966
Immuno modulatory molecules:			
LYZ	Lysozyme	2.28	0.01470
LECT1	Leukocyte cell derived chemotaxin 1	0.42	0.01880
IL17B	Interleukin 17B	0.58	0.01620
C1R	Complement component 1, r subcomponent	1.57	0.01740
HLA-DRB6	Major histocompatibility complex, class II, DR beta 6	1.80	0.01790
AIF1	Allograft inflammatory factor 1	3.32	0.01830
HLA-DQA1	Major histocompatibility complex, class II, DQ alpha 1	3.78	0.01120
HLA-DQB1	Major histocompatibility complex, class II, DQ beta 1	5.09	0.00480
Structural molecules			
SMTN	Smoothelin	0.54	0.01140
MAP7D1	MAP7 domain containing 1	0.63	0.00019
FBLN1	Fibulin 1	0.64	0.02470
TPM2	Tropomyosin 2 (beta)	0.65	0.00262
TRAF3IP3	TRAF3 interacting protein 3	1.59	0.00060
ARPC1B	Actin related protein 2/3 complex, subunit 1B, 41kDa	1.83	0.02670
SCIN	Scinderin	1.90	0.00406

Gene Symbol	Gene Name	Fold Change	p-value
MYH9	Myosin, heavy chain 9, non-muscle	1.83	0.01470
Growth factors and transcription molecules:			
CLK1	Dual specificity protein kinase CLK1	1.62	0.00087
MARCKSL1	MARCKS-like 1	1.68	0.00557
MARCKS	Myristoylated alanine-rich protein kinase C substrate	2.15	0.00735
NRIP1	Nuclear receptor interacting protein 1	1.52	0.00465
TSC22D1	TSC22 domain family, member 1	1.56	0.03670
ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1	1.58	0.00017
FOS	Proto-oncogene c-Fos	1.65	0.04090
EGR1	Early growth response 1	1.77	0.01880
FEZ1	Leucine zipper putative tumor suppressor 1	0.54	0.00498
CBX6	Chromobox protein homolog 6	0.66	0.00956
OSR1	Odd-skipped related 1	0.64	0.02690
GADD45A	Growth arrest and DNA-damage-inducible, alpha	1.52	0.00751
IGF2BP2	Insulin-like growth factor 2 mRNA binding protein 2	1.53	0.00408
AFF2	AF4/FMR2 family, member 2	1.53	0.00509
PLA2R1	Phospholipase A2 receptor 1, 180kDa	1.61	0.02040
Other molecules:			
APOE	Apolipoprotein E	3.01	0.03300
KCNMA1	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	0.59	0.00286
SCN5A	Sodium channel protein type 5 subunit alpha	0.65	0.00556
KCNB2	Potassium voltage-gated channel, Shab-related subfamily, member 2	0.66	0.00512
TMEM160	Transmembrane protein 160	0.66	0.00483
ITM2A	Integral membrane protein 2A	2.85	0.00751
MT1A	Metallothionein 1A	2.06	0.03490
PCSK5	Proprotein convertase subtilisin/kexin type 5	0.65	0.03460
MMP23B	Matrix Metalloproteinase 23B	0.66	0.01560
SERPINE2	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	3.68	0.00209
GALNTL4	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 4	0.62	0.00282
DPYSL3	Dihydropyrimidinase-like 3	1.51	0.02410
HNMT	Histamine N-methyltransferase	1.63	0.03900
PLA2G7	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	2.36	0.03440

Gene Symbol	Gene Name	Fold Change	p-value
VNN3	Vanin 3	4.97	0.00630