Differential Gene Expression in Well-healed and Poorly Healed Experimental Aneurysms after Coil Treatment¹

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Purpose:

To compare gene expression patterns between well-healed and poorly healed aneurysms following coil embolization in a rabbit model.

Materials and Methods:

The Institutional Animal Care and Use Committee approved all procedures before initiation of the study. Elastase-induced, saccular aneurysms were created in rabbits and embolized by using platinum microcoils. Group 1 aneurysms were densely packed (volumetric packing density, >30%) to achieve good healing, whereas group 2 aneurysms were loosely packed (volumetric packing density, <20%), which yields poor healing. At 2 or 4 weeks after implantation, samples were harvested. RNA was isolated separately from the necks and domes of the aneurysms and analyzed by using a microarray containing 294 rabbit genes. Genes with significant differences between groups ($P < .05$; false discovery rate, <0.1; fold change, ≥ 1.2 and ≤ 0.8) were considered differentially expressed.

Results:

At 2 weeks, of 294 genes, 22 (7.5%) genes in the neck and 14 (4.8%) genes in the dome were differentially expressed between groups; at 4 weeks, of 294 genes, 25 (8.5%) genes in the neck and 17 (5.8%) genes in the dome were differentially expressed between groups. Genes overexpressed in group 1 as compared with group 2 aneurysms included those encoding proteases, adhesion molecules, and chemoattractant molecules. Conversely, group 2 aneurysms had increased expression of genes encoding structural molecules, including collagens, as compared with expression in group 1 aneurysms.

Conclusion:

Robust healing after coil embolization is associated with substantial biological activity, as evidenced by overexpression of proteases, adhesion molecules, and chemoattractants. However, contrary to prior hypotheses, structural molecules such as collagen were not associated with the healing response in the rabbit model.

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Endovascular treatment of aneurysms with platinum microcoils has been in routine clinical use for more than a decade (1). However, long-term occlusion rates with these microcoils in large and broad-necked aneurysms remain poor (2,3). Even after nearly 2 decades of clinical use, insight remains limited into the molecular pathways responsible for poor long-term occlusion rates.

Numerous previous investigators have proposed modification of endovascular coils aimed at improving the “biological activity” of the device, with resultant improved healing (4–7). The exact meaning of this activity remains poorly defined but has included increases in inflammation and collagen synthesis (8,9). These research hypotheses about biological activity have led to development and marketing of various second-generation coils, with varying effects on angiographic outcome (10–12). Gaining a better understanding of exactly what type of biological activity leads to durable aneurysm occlusion would speed development of future endovascular devices.

In animal models (13) and in clinical practice (14), the durability of aneurysm occlusion is superior in densely packed aneurysms, as compared with loosely packed aneurysms. We hypothesized that experimental aneurysms could be used to probe, by using gene microarrays focused on proteins and other factors

relevant to vascular homeostasis and healing (15,16), the differences in gene expression between densely packed well-healed aneurysms and loosely packed poorly healed aneurysms. To improve our understanding of the biological mechanisms associated with aneurysmal healing following embolization, we compared gene expression patterns between well-healed and poorly healed aneurysms following coil embolization in a rabbit model.

Materials and Methods

One author (D.F.K.) receives research support from several vendors of endovascular devices, including MicroVention (Tustin, Calif), Sequent Medical (Aliso Viejo, Calif), NFocus Neuromedical (Palo Alto, Calif), Micrus (San Jose, Calif), and eV3 (Plymouth, Minn). The current study had no direct ties to any of these vendors. The experiment was focused on the biology of the aneurysms with use of home-made devices and was funded by the National Institutes of Health. At some point in the future, it is conceivable that the advances made in this research will be used by device manufacturers to design future devices, but there is no implicit or explicit plan with any vendor at this time.

Aneurysm Creation

The Institutional Animal Care and Use Committee approved all procedures before initiation of the study. Elastase-induced, saccular aneurysms were created at the origin of the right carotid artery in 80 female New Zealand white rabbits (body weight, 3–4 kg) by one author (Y.H.D., with 10 years of experience) (17).

Embolization Procedure

Aneurysms were permitted to mature for 3 weeks after creation prior to em-

bolization. Embolization of aneurysms was performed with platinum coils, as previously described, by the same author who created them (18).

High rates of volumetric occlusion, typically defined as greater than 25%, have been associated not only with low recurrence rates clinically (19) but also with robust histologic healing in experimental aneurysm models (13). Conversely, low volumetric occlusion rates, less than 20%, are associated with frequent recanalization and poor healing. In clinical practice, high packing density is achieved easily in relatively small aneurysms, whereas such high packing density is difficult or impossible to achieve in large aneurysms (14).

To simulate the clinical environment, we prospectively identified relatively small experimental aneurysms, with maximum aneurysm volume of 50 mm³ or less, to target for high-packing-density experiments, with volumetric occlusion rates of greater than 25% (group 1). We targeted relatively large aneurysms, with minimum volume of 70 mm³, for low-packing-density experiments, with volumetric occlusion rates of less than 20% (group 2). From a total of 80 consecutive aneurysm creation surgical procedures, subjects with small aneurysm volume (<50 mm³) and large aneurysm

Advances in Knowledge

- Comparison of the gene expression pattern in well-healed and poorly healed aneurysms following platinum coil embolization has not been previously well documented in the literature.
- Expression of structural molecules is downregulated in the densely packed well-healed aneurysms compared with expression in loosely packed poorly healed aneurysms in rabbits.
- Increased gene expression in densely packed aneurysms was associated with adhesion molecules, proteases, and cytokines in the rabbit model of saccular aneurysms.

Implication for Patient Care

- These findings have the potential to offer specific targeted treatments aimed at improving the long-term healing of intracranial, saccular aneurysms if the data can be validated in aneurysms in humans.

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Abbreviations:

DSA = digital subtraction angiography
FDR = false discovery rate
MMP = matrix metalloproteinase
PCR = polymerase chain reaction

Author contributions:

Guarantors of integrity of entire study, R.K., D.F.K.; study concepts/study design or data acquisition or data analysis/interpretation, all authors; manuscript drafting or manuscript revision for important intellectual content, all authors; approval of final version of submitted manuscript, all authors; literature research, R.K.; experimental studies, all authors; statistical analysis, R.K.; and manuscript editing, R.K., D.A.L., D.F.K.

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volume ($>70 \text{ mm}^3$) were selected for dense packing and loose packing, respectively. The volumetric occlusion rate was calculated as follows: $V = \pi (D^2 \cdot L)/6$, where V is volume, D is dome, and L is length. The value of the aneurysm dome in this formula was equal to that of the aneurysm width. Considering the aneurysm as a two-dimensional ellipsoid, the volumetric occlusion was calculated in real time, during aneurysm embolization, by using a tool for that purpose (AngiCalc tool; <http://www.angiocalc.com/>) by one author (R.K., with 8 years of experience). Platinum microcoils were placed in the aneurysm as in typical practice. Before and after each successive coil was placed, volumetric occlusion was calculated. After embolization, final control digital subtraction angiography (DSA) was performed. The rabbits in each group were randomly assigned to either 2-week ($n = 6$ for both groups) or 4-week ($n = 10$ for both groups) survival groups after embolization with platinum coils. On the basis of the power calculation by using our preliminary data for 80% power with $P < .05$ (two sided), six animals per group were chosen for this study.

Tissue Harvest

Animals were deeply anesthetized (ketamine hydrochloride, Fort Dodge Health, Fort Dodge, Iowa; xylazine, Lloyd Laboratories, Shenandoah, Iowa; acepromazine maleate, Boehringer Ingelheim, St Joseph, Miss). DSA was performed, followed by euthanasia by using a lethal injection of pentobarbital (Fort Dodge Health). For histopathologic studies, aneurysms were immediately fixed in 10% neutral buffered formalin. The degree of aneurysm neck tissue coverage was evaluated, as previously described (20). For aneurysms assigned to microarray experiments, samples were dissected into two samples, including the neck and dome. These samples were kept frozen at -70°C .

Angiographic Evaluation

All angiograms obtained at sacrifice were compared with postembolization angiograms and were assessed for changes in coil configuration or aneurysm filling.

Aneurysms on angiograms at sacrifice were compared with those on the post-treatment angiograms by one author (Y.H.D.) and were assigned to one of three categories: stable, progressive occlusion, or coil compaction (21).

Histopathologic Examination

Samples ($n = 4$ each for both groups at 4 weeks) were embedded in paraffin and sectioned at $1000\text{-}\mu\text{m}$ intervals in a coronal orientation (20). Coil fragments were then carefully removed. Sections were reembedded in paraffin and sectioned at $5\text{-}\mu\text{m}$ intervals. Sections were stained with hematoxylin-eosin and Masson trichrome stains for collagen staining. Sections were viewed by one experienced, unblinded reviewer (D.D., with 9 years of experience), paying particular attention to the neointima coverage and endothelialization at the aneurysm neck and the degree of thrombus organization within the dome. An ordinal grading system was used to evaluate histologic response following coil embolization (22).

Immunohistochemical Analysis

Tissue sections were immunostained with rabbit antimacrophage 11, CD31, type I collagen, matrix metalloproteinase (MMP) 2 (MMP-2) and MMP 9 (MMP-9), cathepsin D, and cathepsin L. Five random, high-power organized areas in the aneurysm cavity were selected for counting immunostained cells by one author (D.D.).

Microarray Analysis

RNA was isolated from frozen tissue ($n = 6$ for both groups at 2 weeks and $n = 6$ for both groups at 4 weeks) by using an extraction kit (RNeasy Fibrous Tissue Mini Kit; Qiagen, Valencia, Calif) by one author (R.K.). Microarray slides had been printed with the oligonucleotide set (Operon, Huntsville, Ala). Each probe was spotted in triplicate. Details of microarray analysis have been described previously (16).

Quantitative Real-time Polymerase Chain Reaction Analysis

First-strand complementary DNAs were synthesized from 500 ng of total RNA by using a synthesis system (SuperScript

III First-Strand Synthesis System; Invitrogen, Carlsbad, Calif) by one author (R.K.). Real-time polymerase chain reaction (PCR) assays were performed for osteopontin, vascular cell adhesion molecule-1, type VIII collagen, MMP-9, and vascular endothelial growth factor 1 with a cycler (iCycler; Bio-Rad, Hercules, Calif). The specific primers were designed from corresponding sequences obtained from GenBank (the genetic sequence database of the National Institutes of Health, National Center for Biotechnology Information) by using a Web tool (Primer 3; <http://frodo.wi.mit.edu/primer3/>), developed by Rozen and Skaletsky (23).

Statistical Analysis

Differences between groups were assessed with the Student t test for angiographic and histologic findings by one author (R.K.). A difference with $P < .05$ was considered significant. For microarray experiments, analyses were performed by using the base-2 logarithm transform of the median signal intensity and all analyses. The data were normalized by using two-channel Fastlo (24). The t test statistics and corresponding P values were used as a measure of the mean change in expression between the aneurysm groups relative to the variability. The t test-based P values were adjusted for multiple comparisons by using the false discovery rate (FDR) approach (25). As compared with group 1, genes in group 2 with significant differences at an FDR of less than 0.1 and a fold change of 1.2 or greater were considered upregulated, whereas those with an FDR of less than 0.1 with a fold change of 0.8 or less were considered downregulated.

Results

Angiographic Findings

Angiographic data are summarized in Table 1, and representative angiographic images are presented in Figure 1. As expected, the mean aneurysm volume was significantly smaller in group 1 relative to group 2. Also, as expected, the volumetric packing density values were

Figure 1

Figure 1: Representative angiograms of aneurysms that were densely and loosely packed with platinum coils. **(a)** Relatively smaller aneurysm (volume, $< 50 \text{ mm}^3$) cavity before embolization. **(b)** Aneurysm densely packed with platinum coils. Aneurysm cavity is completely occluded immediately after embolization. **(c)** Follow-up image at 4 weeks after embolization shows stable occlusion of the aneurysm from the parental artery. **(d)** Relatively larger aneurysm (volume, $> 70 \text{ mm}^3$) cavity before embolization. **(e)** Aneurysm loosely packed with platinum coils. Aneurysm cavity is not completely occluded immediately after embolization. **(f)** Follow-up image at 4 weeks after embolization shows coil compaction.

Table 1

Angiographic Findings in Densely and Loosely Packed Aneurysms

Finding	Group 1 (n = 16)	Group 2 (n = 16)
Aneurysm neck (mm)	2.1 ± 0.7	5 ± 1.5
Aneurysm width (mm)	3.1 ± 0.7	4.9 ± 1.8
Aneurysm height (mm)	7.5 ± 2.2	9.1 ± 1.7
Aneurysm volume (mm^3)	35 ± 16	127 ± 104
Volumetric packing density (%)	45 ± 11	12 ± 5
Percentage of stable occlusion	100	25
Percentage of progressive occlusion	0	0
Percentage of coil compaction	0	75

Note.—Data are the mean $\pm$ standard deviation except where otherwise indicated.

high in group 1 compared with group 2. In group 2, three (25%) of 12 aneurysms showed stable occlusion. The remaining nine (75%) aneurysms showed coil compaction. Eleven (92%) of 12 aneurysms in group 1 showed stable occlusion. There were no instances of progressive occlusion in group 1 or 2 aneurysms, as observed by one author (Y.H.D.).

Histologic Findings

All gross and microscopic histologic grading scores (22) were higher for group 1 than for group 2, including mean gross neck score (2.5 ± 1.0 vs 0 , $P = .018$), mean microscopic neck score (3.1 ± 0.3 vs 0.1 ± 0.3 , $P = .029$), mean

Figure 2

Figure 2: Photomicrographs show cells with positive immunohistochemical staining (brown). (a) Rabbit antimacrophage 11. (Original magnification, $\times 400$.) (b) MMP-2. (Original magnification, $\times 400$.) (c) Cathepsin L. (Original magnification, $\times 200$.)

microcompaction score (2.0 ± 0.0 vs 0.5 ± 1.0 , $P = .013$), mean dome healing score (2.6 ± 1.1 vs 0 , $P = .02$), and mean total score (7.5 ± 1.0 vs 0.6 ± 1.2 , $P = .027$), as observed by one author (D.D.).

Group 1 (densely packed aneurysms).—At gross examination, three of four aneurysms showed complete coverage across the neck by thin, translucent, and membranous tissue. A single aneurysm showed partial neck coverage by a thin membrane. At microscopy, aneurysms had loose, organized tissue that filled most of the aneurysm dome, with only small areas of poorly organized thrombus. All four aneurysms in the group had neointima that covered completely across the entire neck. The neointima was lined with a single cell layer that was continuous with the endothelial cell layer of the parent artery. Two of the four samples had inflammatory cells.

Group 2 (loosely packed aneurysms).—All four samples showed that the aneurysm cavity was completely open to the parent artery. There was no membranous tissue across the neck

orifice. Microscopy demonstrated that unorganized thrombus filled the aneurysm cavity in one of four aneurysms. In the remaining three samples in this group, one had less than one-third of the cavity filled with poorly organized thrombus, with the proximal part of the dome empty and open to the parent artery. The other two samples had very small amounts of organized connective tissue, along with the coil loops, in the distal area of the lumen. Inflammatory cell infiltration was observed in two of the four aneurysms in this group.

Immunohistochemical Findings

In group 1 aneurysms, the cells lining the neointima were positive for CD31 immunohistochemical staining, indicating that they were endothelial cells. The presence of cells stained positive for MMP-9 and cathepsin D, in both well-healed and poorly healed aneurysms, was not evident. Collagen deposition was minimal in all samples on the basis of Masson trichrome staining. In group 2, there was no indication of endothelial cell coverage along the neck in any

of the aneurysms. Collagen deposition was minimal within the organized tissue. The mean number of cells staining positive for rabbit antimacrophage 11, a marker for the monocyte or macrophage line, was 103.75 ± 41.45 and 43.5 ± 25.7 for groups 1 and 2, respectively ($P = .048$) (Fig 2a). The mean number of cells staining positive for MMP-2 was 123.25 ± 23.66 and 32.25 ± 16.88 for groups 1 and 2, respectively ($P < .001$) (Fig 2b). The mean number of cells staining positive for cathepsin L, a lysosomal protease, was 180.2 ± 38.0 and 57.00 ± 65.84 for groups 1 and 2, respectively ($P = .017$) (Fig 2c), as observed by one author (D.D.).

Molecular Findings

With the use of FDR multiple corrections and magnitude of fold-change criteria, of 294 genes, 22 (7.5%) genes in the neck and 14 (4.8%) genes in the dome of 2-week aneurysms and 25 (8.5%) genes in the neck and 17 (5.8%) genes in the dome of 4-week aneurysms were found to be differentially expressed (Table 2), as observed by one author (R.K.).

Two-week samples.—In both the neck and dome tissue, genes that were upregulated in group 1 as compared with group 2 included those associated with cell adhesion (osteopontin and vascular cell adhesion molecule-1), oxidative stress, heme oxygenase 1, as well as carrier or transport molecules (apolipoprotein D, haptoglobin, and hemoglobin beta). In contrast, expression of structural molecules (types I and VIII collagen, fibronectin) and the transcription regulatory molecule, galactin-3, were downregulated in both the neck and dome tissue of group 1, as compared with group 2. In tissue along the neck only, genes that were upregulated in group 1 compared with group 2 included growth factors (transforming growth factor-induced protein, vascular endothelial growth factor 1) and the protease cathepsin L; conversely, the gene encoding the structural molecule type III collagen was downregulated along the neck tissue in group 1 compared with group 2. In dome tissue only, increased expression of the inflammatory cytokine

monocyte chemoattractant protein-1 and decreased expression of the structural molecule type V collagen were noted in group 1, as compared with group 2.

Four-week samples.—Both the neck and dome tissue exhibited increased expression of the cell adhesion molecule osteopontin, proteases (cathepsin E and cathepsin L, MMP-9, MMP-12, and metalloelastase), and the inflammatory molecule monocyte chemoattractant protein-1 for group 1 compared with group 2. Conversely, decreased expression in group 1, as compared with group 2, was observed for type VIII collagen and tissue inhibitor of MMP 3 (TIMP3). In the dome tissue alone, we noted downregulation in the expression of structural molecules (types I and V collagen) and MMP-2 (group 1 compared with group 2). In the neck tissue alone, for group 1 compared with group 2, we noted upregulation of genes associated with oxidative stress (glutathione peroxidase and 12-lipoxygenase activating protein) and inflammation (CD14), whereas calcium-binding molecules (annexin I and osteonectin) and growth factors (osteoglycin and connective tissue growth factor) were down-regulated.

Confirmation of Microarray with Real-time-PCR

We examined the accuracy of the microarray analysis by choosing five genes for real-time-PCR analysis that encompassed a wide range of expression ratios, as observed by one author (R.K.). All genes tested were confirmed to be in accord with the microarray data (Table 3).

Discussion

In our study, we used a rabbit-specific, custom-microarray-expression profiling approach to identify genes and pathways that might contribute to the healing of saccular aneurysms treated with embolization with platinum coils. With the use of this approach, we identified numerous genes that were significantly and differentially expressed in densely packed and well-healed, as compared

Table 2

Genes Differentially Expressed in Group 1 with Densely Packed Aneurysms Compared with Group 2 with Loosely Packed Aneurysms

Gene Name	GenBank Accession No.	Fold Change at 2 Weeks		Fold Change at 4 Weeks	
		Neck	Dome	Neck	Dome
Adhesion molecules					
Osteopontin	D16544	3.06*	3.12*	2.77*	2.69*
Vascular cell adhesion molecule 1	AY212510	1.23 [†]	1.24 [†]	...	...
Extracellular matrix remodeling					
Collagen					
Type I alpha 2	D49399	0.79 [†]	0.78 [†]	...	0.78 [†]
Type III pro-alpha 1	S83371	0.77 [†]	...	...	...
Type V alpha 1	AF451331	...	0.75 [†]	...	0.77 [†]
Type VIII alpha 1	J05042	0.56*	0.78 [†]	0.53*	0.59*
Fibronectin	S80206	0.72 [†]	0.75 [†]	...	...
Growth factors/cytokines					
Connective tissue growth factor	AB217855	...	...	0.75*	...
Osteoglycin	AF487889	0.75 [†]	...	0.70 [‡]	...
Transforming growth factor- induced protein	U66205	1.23 [‡]	...	...	...
Vascular endothelial growth factor	AY196796	1.3 [†]	...	...	...
Proteases and their inhibitors					
Cathepsin D	EF472890	...	...	1.26 [‡]	...
Procathepsin E	L08418	...	...	1.65*	1.51*
Cathepsin L	AF458078	1.29 [†]	...	1.43 [†]	1.4 [†]
HTRA	AF097708	0.75 [‡]	0.77 [†]	0.66*	...
MMP-2	D63579	...	...	...	0.78 [†]
MMP-9	D26514	...	...	1.35 [†]	1.44 [†]
MMP12	AB006779	...	...	1.64 [‡]	1.4 [†]
Metalloelastase	R86534	...	...	1.34 [†]	1.27 [†]
<i>TIMP</i> , hypoxia induced	J04712	1.75*	1.34 [†]	1.33 [†]	...
<i>TIMP3</i>	AF069714	...	...	0.68*	0.73 [‡]
Oxidative stress					
5-lipoxygenase-activating protein	M96556	...	...	1.2 [†]	...
Cyclooxygenase-2	NM_001082388	1.24 [‡]	...	...	...
Glutathione peroxidase	X13837	...	...	1.42 [‡]	...
Heme oxygenase 1	AY421756	1.52 [†]	1.2 [†]	...	...
Transcription regulation					
Elongation factor 1-beta	X74728	1.24 [‡]	...	1.32 [‡]	...
Galectin-3	U06470	0.62 [‡]	0.61 [‡]	1.3 [†]	...
Particle major protein 50	U16821	...	...	1.26 [‡]	1.24 [†]
Transport or carrier					
Apolipoprotein D	L42979	1.79*	1.53 [‡]	1.48 [‡]	1.51 [‡]
Apolipoprotein J/clusterin	AF118852	1.24 [‡]	...	...	...
Haptoglobin	AF372520	2.36*	2.42*	...	...
Hemoglobin					
Alpha	NM_001082389	1.57 [‡]	...	...	1.29 [†]
Beta	M24885	1.72 [‡]	1.37 [†]	1.31 [†]	1.59 [‡]
Miscellaneous					
Annexin I	U24656	...	...	0.78 [‡]	...
Osteonectin	AF247647	...	...	0.78 [‡]	...
Secreted frizzled-related protein 2	AF323272	0.57 [‡]	...	0.52 [‡]	0.5 [†]

Table 2 (continued)

Table 2 (continued)

Genes Differentially Expressed in Group 1 with Densely Packed Aneurysms Compared with Group 2 with Loosely Packed Aneurysms

Gene Name	GenBank Accession No.	Fold Change at 2 Weeks		Fold Change at 4 Weeks	
		Neck	Dome	Neck	Dome
Inflammation					
CD14	NM_001082195	...	...	1.22 [†]	...
Monocyte chemoattractant protein-1	M57440	...	1.39 [‡]	1.22 [†]	1.38 [‡]

Note.—Numbers represent fold change of group 1 compared with group 2. Fold change of 1.2 or greater indicates overexpression, whereas fold change of 0.8 or less represents diminished expression.

* FDR < 0.001.

[†] FDR < 0.1.

[‡] FDR < 0.01.

Table 3

Quantitative Real-time PCR Confirmation of Differential Gene Expression in Densely Packed Aneurysms versus Loosely Packed Aneurysms

Gene	Fold Change at 2 Weeks		Fold Change at 4 Weeks	
	Neck	Dome	Neck	Dome
Osteopontin	8.65	9.13	6.74	6.52
Vascular cell adhesion molecule-1	2.78	3.05	...	...
Type VIII alpha 1 collagen	0.51	0.52	0.42	0.38
MMP-9	...	...	3.51	3.07
Vascular endothelial growth factor 1	2.58	...	...	...

Note.—Genes were identified by using microarray analysis.

with loosely packed and poorly healed, experimental aneurysms. Specific pathway analysis revealed that increased gene expression in densely packed aneurysms was associated with adhesion molecules, proteases, and cytokines in the rabbit aneurysm model. Conversely, loosely packed aneurysms showed increased expression of multiple structural molecules, including various types of collagen. These findings may finally allow the precise definition of *improved biological activity* that has been proposed for many years as a method for improving aneurysm healing after coil embolization. Focusing on these specific pathways might facilitate rapid screening of candidate materials aimed at enhancing device effectiveness by using either in vitro or in vivo models.

Our experimental technique circumvents the difficulty presented by obtaining clinical aneurysm tissue. Our primary

findings, that expression of structural molecules is downregulated in the densely packed aneurysms, are in direct contrast to the leading hypotheses in regard to how aneurysms heal. Numerous investigators have proposed augmentation of collagen delivery or synthesis of collagen as a technique to improve long-term occlusion rates (7,26,27). Previous histologic studies in both preclinical and human aneurysms treated with platinum coils show a dearth of collagen synthesis within the aneurysm cavity and along the neck-parent artery interface (28). Collagen synthesis is a central part of wound healing, providing both a structural matrix for the healing wound, as well as a surface or substrate for adhesion of cells to the injured area (29,30). As such, it seems logical that collagen synthesis would be associated with robust healing in aneurysms. However, despite increased gene expression of types

I, III, V, and VIII collagen in the poorly healed cohort in our study, histologic findings revealed that collagen deposition was minimal in both experimental groups, irrespective of healing. This lack of collagen formation, even within the setting of collagen synthesizing gene up-regulation, may be attributed to ongoing remodeling and continued collagen turnover in poorly healed aneurysms. Potentially more important, however, is the finding that aneurysms considered to be well healed are not forming collagen. Thus, it appears that collagen should not be a primary focus for therapies aimed at improved healing. As noted above, cytokines appear to be important in differential healing in aneurysms and, therefore, may be even more relevant than structural molecules in the healing of aneurysms following embolization.

On the basis of fold-change values, the single most markedly upregulated gene in well-healed, as compared with poorly healed aneurysms, in our study was osteopontin, a cell adhesion molecule. Osteopontin is a glycoprotein that is expressed in a range of cells, including macrophages, endothelial cells, and smooth muscle cells. It functions in chemotaxis and wound healing (31). Osteopontin interacts with multiple cell surface receptors, including integrins, to mediate cell adhesion and cell migration (32). It also inhibits apoptosis and enhances cell proliferation in many pathologic conditions (33,34). Our current data suggest that local delivery of osteopontin may have a beneficial effect in the treatment of cerebral aneurysms.

Our experimental approach also offers insight into the role of proteases in aneurysm healing. MMPs represent the most intensely studied family of proteases in regard to many types of aneurysms (35). This protease family cleaves most of the constituents of the extracellular matrix and is involved in the breakdown and remodeling of many tissues and organs. Bouzeghrane et al (36) reported that MMP-9 plays an important role in recanalization and recurrence in a canine aneurysm model, as well as in a murine carotid occlusion model. In contrast, our experimental animal model

showed that the healing of an aneurysm is associated with increased expression of MMP-9. This observation in regard to MMP-9 may relate to the complete neointimal coverage that was observed along the necks of well-healed aneurysms in our study. Previous studies have shown that MMP-9 regulates the migration of smooth muscle cells from media to intima, which is a critical step in neointima formation (37,38). Furthermore, *TIMP3*, an important inhibitor of MMPs, was shown to be downregulated in well-healed aneurysms. Multiple investigators have implicated MMPs, both MMP-2 and MMP-9, as important in formation and progression of both saccular, intracranial aneurysms as well as abdominal aortic aneurysms. Indeed, trials have been performed by using MMP inhibitors such as doxycycline to stabilize aneurysms (39,40). However, our data suggest that it may be counterproductive to inhibit MMP-9 after coil treatment of an aneurysm, if such inhibition were to impede neointimal growth over the neck of the aneurysm.

Our study had several limitations. We applied the rabbit elastase model for understanding the healing mechanism of aneurysms of the human brain. We acknowledge that animal models are imperfect predictors of the human response. At present, given the extreme dearth of available human histologic findings, animal models represent the best methods for studying the basic biological mechanism. It remains possible that the extracranial, extraluminal environment of aneurysms in the rabbit may have a profound effect on the biological response. Observed findings in our study may not be directly applicable to the clinical system, considering the differences between the human and the rabbit genome. Neither densely packed nor loosely packed aneurysms were compared with untreated aneurysms. We did not identify any specific marker for aneurysm healing or recanalization after coil treatment. We simply studied biological differences between two sets of aneurysms. Further, we selected relatively smaller aneurysms for dense packing and larger aneurysms for loose packing instead of choosing the same size of aneurysms for both

experiments, which was done to mimic the clinical scenario and may have induced bias. We chose only two times for comparison. Additional times may have been useful in understanding the long-term healing mechanism.

Implications in regard to our results and their generalizability to humans should be drawn with caution from this exploratory model and could, in part, be attributable to a high degree of homogeneity among the small number of test animals; our conclusions will require further validation. By using an animal model and controlling environmental variables, there was a high degree of homogeneity among the small number of test animals, and this homogeneity reduced the variance and allowed us to detect with significance small differences in fold changes between the groups. For example, to detect a significant fold change of 1.2 or 0.8 (with a fold change of ≥ 1.2 indicating overexpression and a fold change of ≤ 0.8 representing diminished expression), the standard error of the model needed to be 0.12 or less, which we achieved. In addition, we present both the *P* value and the FDR, which provides an estimate of the proportion of observations that are incorrectly called significant owing to multiple testing, to allow the readers to draw their own conclusions in regard to the genes presented. As with most gene expression studies, we recognize that any results obtained are exploratory in nature and need to be explored further; to that end, we did validate several results with real-time-PCR and will continue to explore these results further in other models. Likewise, because of normal variations, there likely are genes for which our threshold levels were not achieved that may have an effect in humans; because a gene is not significantly up- or downregulated, that does not necessarily imply that it is not relevant.

Our study serves as a guide to multiple, ongoing studies in our laboratory. To address the limitations listed above for our study, we undertook several new initiatives. First, we are now using laser capture microdissection, which can resolve gene expression at the level of the cell. Results from these laser capture

microdissection experiments may allow us to identify the exact cell type responsible for differential gene expression between groups. Second, we are performing proteomics experiments to confirm and expand understanding of the biological processes in healing aneurysms.

In conclusion, specific pathway analysis revealed that increased gene expression in densely packed aneurysms was associated with adhesion molecules, proteases, and cytokines in the rabbit aneurysm model. Conversely, loosely packed aneurysms showed elevated expression of multiple structural molecules, including various types of collagen.

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