

**ORIGINAL
RESEARCH**

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Proteomic Analysis of Aneurysm Healing Mechanism after Coil Embolization: Comparison of Dense Packing with Loose Packing

BACKGROUND AND PURPOSE: In clinical practice, durability of occlusion following coil embolization is superior in densely packed, compared with loosely packed, aneurysms. In a rabbit model, we probed, by using proteomics tools, the biologic mechanisms associated with densely packed and completely occluded aneurysms, compared with loosely packed and incompletely occluded aneurysms, to explore the biologic mechanisms of intra-aneurysmal healing following embolization.

MATERIALS AND METHODS: Elastase-induced, saccular aneurysms were created in 24 rabbits. Aneurysms were allowed to mature, after which aneurysms were either densely (packing attenuation >25%) or loosely (packing attenuation <20%) packed with platinum coils by endovascular means. After 2 weeks ($n = 6$ for both groups) and 4 weeks ($n = 6$ for both groups) of implantation, aneurysm samples harboring coils were harvested. Soluble proteins were extracted from the necks and domes of aneurysms, and proteins were studied using proteomics and bioinformatics tools.

RESULTS: In dome tissue, 128 proteins at 2 weeks, and 8 proteins at 4 weeks, were differentially expressed in densely packed, compared with loosely packed, aneurysms. In the neck tissue, 2 proteins at 4 weeks were differentially expressed in densely packed aneurysms. Specific pathway analysis revealed that compared with loosely packed aneurysms, densely packed aneurysms were associated with up-regulation of cell-to-cell signaling and cell adhesion at 2 weeks. Conversely, at 4 weeks, densely packed aneurysms showed a decrease in the expression of structural proteins compared with loosely packed aneurysms.

CONCLUSIONS: These findings may focus efforts on specific targets aimed at improving the long-term healing of intracranial, saccular aneurysms.

ABBREVIATIONS: FDR = false discovery rate; GO = gene ontology; KEGG = Kyoto Encyclopedia of Genes and Genomes

Cerebral, saccular aneurysms are routinely treated with platinum coils. However, the rate of recanalization after endovascular treatment of aneurysms is high, especially in larger aneurysms.^{1,2} A better understanding of molecular events related to healing or, conversely, to recanalization might lead to interventions designed to prevent aneurysm regrowth or recanalization following endosaccular embolization.

Both clinical and preclinical data have shown that packing attenuation in aneurysms predicts the degree and extent of healing, with loosely packed, wide-neck experimental aneurysms showing features of poor histologic healing, and densely packed, narrow-neck aneurysms demonstrating features of good histologic healing.^{3,4} Clinical findings have also confirmed that angiographic recanalization is related to packing attenuation, with higher rates of recanalization noted with loosely packed aneurysms.⁵⁻⁷

Our group previously explored the differential gene expression patterns between densely and loosely packed aneurysms using gene microarrays in a rabbit model.⁸ In the current study, we extend our previous work of profiling transcription level analysis to focus on translational level analysis.⁹ Proteins represent the functional end production of gene expression, performing functions essential for most cellular processes.¹⁰ We probed, by using proteomics^{11,12} and bioinformatics tools,^{13,14} the biologic mechanisms associated with densely packed aneurysms, compared with loosely packed aneurysms, to explore biologic mechanisms of intra-aneurysmal healing following embolization in a rabbit, elastase-induced aneurysm model.

Materials and Methods

Aneurysm Creation and Embolization

The Institutional Animal Care and Use Committee (Mayo Clinic, Rochester, Minnesota) approved all procedures before initiation of the study. Aneurysms were created in 24 female New Zealand white rabbits (body weight 3–4 kg) using the elastase-induced aneurysm model in rabbits.¹⁵ Aneurysms were permitted to mature for 3 weeks after aneurysm creation before embolization. All subjects were embolized with standard platinum coils by endovascular means.¹⁶

In clinical practice, high packing attenuation is easily achieved in relatively small aneurysms but is difficult or impossible in large aneu-

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rysms. 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 attenuation” experiments, with target volumetric occlusion rates of >25% (41.1 ± 14.1%). Conversely, we prospectively identified relatively large aneurysms, with minimum volume of 60 mm³, for “low packing attenuation” experiments, with target volumetric occlusion rates of <20% (13.0 ± 4.1%).

The size of the aneurysm cavity was assessed by direct comparison with radiopaque sizing devices during DSA. The volumetric occlusion was calculated in real time, during aneurysm embolization, using the AngiCalc tool (<http://www.angiocalc.com/index.aspx>). Appropriately sized coils were placed into the aneurysm as in typical practice. After embolization, a final control DSA was performed. The rabbits in each group were randomly assigned to either 2-week ($n = 6$ for both groups) or 4-week ($n = 6$ for each group) survival groups after embolization with platinum coils. These time points were chosen based on predicate data indicating the cellular responses to therapy are metabolically active at earlier time points.¹⁷

Tissue Harvest

At the time of euthanasia, animals were deeply anesthetized. DSA was performed, followed by euthanasia using a lethal injection of pentobarbital. The aneurysm sac was horizontally dissected in the middle into 2 parts, including the neck (lower sac) and dome (upper sac). These samples were kept frozen at -70°C.

Protein Extraction

Frozen aneurysm samples were pulverized in liquid nitrogen, and soluble proteins were extracted by homogenizing samples in lysis buffer containing 50 mmol/L Tris-HCl, pH 7.4, 0.1% sodium dodecyl sulfate, and protease inhibitors. After centrifugation at 10,000 g for 20 minutes at 4°C, the protein concentration of the supernatant was determined (Pierce Biotechnology, Rockford, Illinois).

Proteomic Analysis

One-Dimensional Gel Electrophoresis. Equal amounts of proteins were separated by Criterion XT gels (Bio-Rad Labs, Hercules, California) and stained with BioSafe colloidal blue stain (Bio-Rad Labs). Each lane of the gel was excised from the gel and cut into 5 fractions, based on molecular mass. These gel fractions were subsequently reduced and alkylated, and in gel digestion performed using trypsin. Tryptic peptide extracts were stored frozen at -80°C until analysis by mass spectrometry.

Protein Identification by Mass Spectrometry. The trypsin-generated digests were separated by nanoscale liquid chromatography, coupled with high accuracy mass spectrometry and data-dependent tandem mass spectrometry (nLC-MS/MS), coupled to a nanoLC-2D HPLC system (Eksigent, Dublin, California). Relative protein quantification between the groups was performed on the Orbitrap survey scan data using the Elucidator software package (Rosetta Biosoftware, Seattle, Washington).^{18,19} Peptide sequences were assigned to MS/MS spectra by using the Mascot data base search engine (Ver. 2.2.04, <http://www.matrixscience.com>). Due to the incomplete representation of rabbit proteins in protein databases, we used a human, mouse, rat, and rabbit subset of the UniProt data base (<http://www.uniprot.org>), using the homologs from other species as surrogates for the rabbit version of the protein. Validation of the data base search results was done by using the Elucidator implementation of peptide and protein teller algorithms,^{20,21} with an estimated false discovery rate of 1%, esti-

mated from reversed sequence protein entries appended to the data base as decoys.²² Validated peptides were annotated to their molecular signals across the aligned data from each sample.

Statistical and Bioinformatic Analysis

Peptide data from 5 gel sections for each comparison results from Elucidator were first combined. Proteins with a statistically significant difference between densely and loosely packed aneurysms ($P < .05$ and FDR $\leq 0.1^{23}$), and a fold change ≥ 1.2 and ≥ 0.8 to represent up- and down-regulation in densely versus loosely packed aneurysms, were used to identify pathways. Redundant proteins from several sections were removed by keeping the one with the lowest P value. Network pathways were reconstructed utilizing the Ingenuity Pathway Analysis tool (<http://www.ingenuity.com>), and GO and biologic processes were derived by using DAVID Bioinformatics Resources 6.7 (<http://david.abcc.ncifcrf.gov>).²⁴

Results

Angiographic Findings

The mean aneurysm volume was statistically smaller in densely packed aneurysms (44.3 ± 23.5 mm³) compared with loosely packed aneurysms (103.1 ± 53.7 mm³, $P = .003$). The volumetric packing attenuation values were higher in densely packed aneurysms (41.1 ± 14.1%) than in the loosely packed aneurysms (13.0 ± 4.1%, $P = 6.4 \times 10^{-7}$). Representative angiographic images are presented in Fig 1.

Molecular Findings

In dome tissue, 128 proteins at 2 weeks, and 8 proteins at 4 weeks, were differentially expressed in densely packed, compared with loosely packed, aneurysms. In the neck tissue, 2 proteins at 4 weeks were differentially expressed in densely packed aneurysms. No protein demonstrated statically significant differences between the attenuated packing and loose packing groups at 2 weeks postembolization along the neck.

Proteins Differentially Expressed at 2 Weeks Postembolization

We performed ontologic enrichment analysis with particular attention to KEGG pathways and GO biologic processes for the proteins that were differentially expressed at the dome of 2-week aneurysms. This bioinformatics analysis revealed protein up-regulation in the dome of densely packed aneurysms in several broad biologic processes, including proteins involved in energy production, cell adhesion, protein transport, and response to injury (Table 1). Specific up-regulated pathways included the gap junction, citrate cycle, vascular smooth muscle contraction, lysosome, and fatty acid metabolism (Table 2). Our Ingenuity pathway analysis revealed that networks of genetic disorder, metabolic disease, and posttranslational modification were highly up-regulated in the dome of densely packed aneurysms compared with loosely packed aneurysms (Table 3). Proteins exhibiting the highest relative expression in the dome of densely packed compared with loosely packed aneurysms were C-reactive protein (5.7-fold up-regulation) and creatine kinase M-type (3.6-fold).

The expression of plasminogen (0.44-fold), interferon-induced very large GTPase 1 (0.48-fold), and inter-alpha-trypsin inhibitor (0.5-fold) were markedly down-regulated in

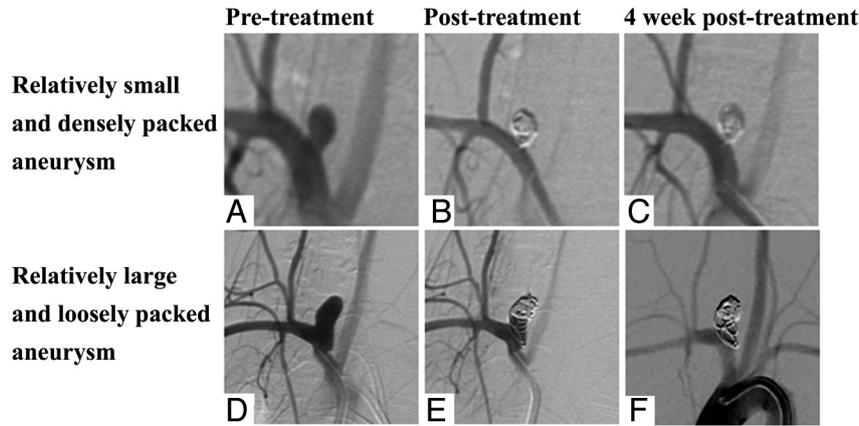


Fig 1. Representative angiograms of densely and loosely packed aneurysms with platinum coils. *A*, A relatively small aneurysm (volume $<50 \text{ mm}^3$) cavity before embolization. *B*, The aneurysm was densely packed with platinum coils and the cavity was completely occluded immediately after embolization. *C*, Follow-up image at 4 weeks postembolization shows stable occlusion of the aneurysm from the parental artery. *D*, A relatively large aneurysm (volume $>70 \text{ mm}^3$) cavity before embolization. *E*, The aneurysm was loosely packed with platinum coils and the cavity did not completely occlude immediately. *F*, Follow-up image at 4 weeks postembolization shows coil compaction.

Table 1: Geneontology analysis of proteins up-regulated in the dome of densely packed aneurysms compared with loosely packed aneurysms at 2 weeks following coil embolization

Biologic Process	Number of Proteins Up-Regulated	Protein Symbol
Generation of precursor metabolites and energy	17	ALDOA, ALDOC, ATP5A1, ETFA, GNAS, IDH3B, IDH1, MDH2, NDUFA10, PDHA1, PPP1CA, PYGL, UQCRC2, SUCLG1, SUCLG2, ACAA1, ATP6V1C1
Oxidation reduction	15	ACADM, AKR1B10, AKR1C4, CBR1, ETFA, GPD1, IDH3B, IDH1, IVD, KCNB2, MDH2, NDUFA10, PDHA1, UQCRC2
Response to organic substance	14	GOT2, ALDOC, CA2, CTSC, CORO1A, CRYAB, AHSG, GNAS, IDH1, PRKACA, MAPK1, PPP2CA, RBM4, THBS1,
Protein localization	13	GNAS, LMAN2, MAPK1, MVP, MYO1C, HSPG2, PPT1, SEC61A1, SELENBP1, SCAMP2, SNX5, TOMM40, VPS26A
Response to wounding	12	SERPINC1, CRP, VCAN, F13A1, AHSG, FN1, GNA13, GNAQ, ITGB2, MGLL, PLG, THBS1
Cell adhesion	12	AEBP1, TGFBI, CORO1A, VCAN, ECM2, FN1, ITGB2, HSPG2, PGM5, RPSA, SPON1, THBS1
Protein transport	12	GNAS, LMAN2, MAPK1, MVP, MYO1C, PPT1, SEC61A1, SELENBP1, SCAMP2, SNX5, TOMM40, VPS26A
Response to endogenous stimulus	10	ALDOC, CA2, CRYAB, AHSG, GNAS, IDH1, PRKACA, MAPK1, RBM4, THBS1
Glucose metabolic process	9	ALDOA, ALDOC, CRYAB, GPD1, MDH2, PDHA1, PGM5, PPP1CA, PYGL
Response to hormone stimulus	9	CA2, CRYAB, AHSG, GNAS, IDH1, PRKACA, MAPK1, RBM4, THBS1

Note:—Top 10 biologic process with descending protein count and Expression Analysis Systematic Explorer (EASE) score, $P < 0.05$.

Table 2: Pathway analysis of proteins up-regulated in the dome of densely packed aneurysms compared with loosely packed aneurysms at 2 weeks following coil embolization

Biologic Pathways	Number of Proteins Up-Regulated	Protein Symbol
Gap junction	7	GNAI1, GNAI2, GNAI3, GNAQ, GNAS, PRKACA, MAPK1
Citrate cycle	6	IDH3B, IDH1, MDH2, PDHA1, SUCLG1, SUCLG2
Vascular smooth muscle contraction	6	GNA13, GNAQ, GNAS, PRKACA, MAPK1, PPP1CA
Lysosome	6	CTSC, CTSD, CTSK, CD63, M6PR, PPT1
Tight junction	6	EPB41, ACTG1, GNAI1, GNAI2, GNAI3, PPP2CA
Fatty acid metabolism	5	ACADL, ACADM, ACAA1, ACAT1, ACAA2
Valine, leucine, and isoleucine degradation	5	ACADM, IVD, ACAA1, ACAT1, ACAA2

Note:—Top 10 biologic process with descending protein count and EASE score, $P < 0.05$.

the dome of densely packed compared with loosely packed aneurysms (On-line Table 1).

Proteins Differentially Expressed at 4 Weeks Postembolization

Densely packed aneurysm dome tissue, compared with loosely packed dome tissue, demonstrated down-regulation in the expression of structural proteins (cysteine-glycine rich protein-1,

transgelin, smooth muscle actin, and actin-alpha 1) and up-regulation in the expression of cell signaling proteins (myosine heavy chain-9 and cullin-1) (Table 4).

Compared with loosely packed aneurysms, the neck region of densely packed aneurysms exhibited decreased expression of cytoskeletal adapter protein sorbin and SH3 domain-containing protein 2 (0.55-fold reduction) and leukotriene prostaglandin reductase-1 (0.53-fold reduction) (Table 5).

Table 3: Network of proteins up-regulated in the dome of densely packed aneurysms compared with loosely packed aneurysms at 2 weeks following coil embolization

Biologic Process	Number of Proteins Up-Regulated	Protein Symbol
Genetic disorder, metabolic disease, posttranslational modification	25	ACAA1, ACADL, ACADM, ACAT1, ACTG1, AEBP1, ALDOA, ALDOC, CAPZA2, CLEC11A, CORO1A, CTSK, ECM2, EMD, ETFA, GNA13, GNAS, GOT2, IVD, MYO1C, PPP1CA, RPSA, TMOD3, TPM2, TWF2
Cell morphology, cellular development, visual system development and function	21	CD63, CRP, CTSK, ELAVL1, F13A1, FN1, GNAI1, GNAI2, GNAI3, HNRNPA1, HNRNPA2B1, HNRNPUL2, HSPG2, HTRA1, PCBP1, PCOLCE, SLC3A2, TAGLN, TGFB1, TGM2, THBS1
Carbohydrate metabolism, cell cycle, cellular growth and proliferation	16	ACAA2, ACY1, BUB3, CAB39, EHD3, PPT1, PYGL, RBM4, SNX5, STOML2, SUCLG1, SUCLG2, SULT1A1, TTC35, TUFM, VPS26A
Energy production, small molecule biochemistry, cell morphology	14	ALDOA, ATP5A1, DNAJB11, EIF3H, FMNL1, MDH2, OGN, PRKCDBP, SCAMP2, SELENBP1, SFXN3, SLC3A2, TCEA1, TGFB1
Carbohydrate metabolism, small molecule biochemistry, cell-to-cell signaling and interaction	14	AEBP1, AKR1B10, CKM, CLEC3B, ERLIN2, HLA-A, IDH1, IDH3B, KCNAB2, LMAN2, MGLL, MOBKL1A, PLD3, SERPINB1

Table 4: Proteins that are differentially expressed in the dome of densely packed aneurysms compared with loosely packed aneurysms at 4 weeks following coil embolization

Protein Symbol	Protein Name	UniProt Accession ID	Fold Change	P Value	FDR (q Value)	Protein Classification
CSRP1	Cysteine and glycine-rich protein-1	P21291	0.27	1.07E-05	0.007	Other
HSPA1A/HSPA1B	Heat shock 70kDa protein-1A	Q61696	0.38	1.15E-04	0.028	Other
TAGLN	Transgelin	Q01995	0.39	1.00E-03	0.1	Other
ACTA2	Actin, alpha 2, smooth muscle, aorta	P62736	0.46	7.77E-05	0.024	Other
ACTC1	Actin, alpha, cardiac muscle 1	P68032	0.57	3.40E-05	0.014	Enzyme
EIF3A	Eukaryotic translation initiation factor 3, subunit A	Q14152	1.46	4.95E-04	0.067	Translation regulator
CUL1	Cullin-1	Q13616	1.6	0.001	0.1	Cell cycle regulator
MYH9	Myosin, heavy chain 9, nonmuscle	P35579	1.9	2.46E-04	0.038	Enzyme

Table 5: Proteins that are differentially expressed in the neck of densely packed aneurysms compared with loosely packed aneurysms at 4 weeks following coil embolization

Protein Symbol	Protein Name	UniProt Accession ID	Fold Change	P Value	FDR (q Value)	Protein Classification
PTGR1	Prostaglandin reductase 1	Q14914	0.53	1.50E-05	0.010	Enzyme
SORBS2	Sorbin and SH3 domain containing 2	Q94875	0.55	1.69E-04	0.082	Other

Discussion

Our group has previously studied the role of a limited number of genes by using transcription-level analysis to probe the healing mechanism in aneurysms treated with platinum coils.⁸ In the present study, we have extended our previous work through the use of proteomic analysis, which not only provides a substantially greater number of genes and pathways that can be studied but also focuses on the translational, rather than transcriptional, level processes as seen with gene chips.

In the current study, we identified several biologic pathways differentially expressed between groups in distinct areas of the aneurysm cavity. Early after aneurysm embolization, the dome of densely packed aneurysms showed increased expression of cell-signaling molecules, cell-adhesion molecules, and acute-phase proteins compared with loosely packed aneurysms. However, we later noted decreased expression of cytoskeleton molecules in densely packed compared with loosely packed aneurysms. This constellation of findings suggest that modifications aimed at improved long-term outcomes likely need to act at early time points following implantation and

logically would target adhesion molecules and related acute-phase proteins.

The most markedly up-regulated protein was C-reactive protein in the dome of densely packed aneurysms at 2 weeks. C-reactive protein is an acute-phase protein and is a marker of inflammation.²⁵ Its level increases in blood in response to tissue injury.^{26,27} This observation may lend credence to the “inflammatory” hypothesis put forth by numerous investigators who have proposed the use of bioactive materials to improve aneurysm healing. Importantly, the expression of proteins associated with cell adhesion and wound-healing mechanisms (fibronectin, thrombospondin, and versican) were elevated in the dome of densely packed aneurysms at 2 weeks. Fibronectin plays a crucial role in wound healing, as it is deposited at the site of injury, where it binds with fibroblasts and induces the differentiation of fibroblast, thereby promoting wound repair.²⁸ In the current study, we noted that the increase in the energy production mechanism was highly active, as evidenced by the elevation in proteins participating in the carbohydrate and fatty acid metabolic pathways of densely packed aneurysms.

Our data further demonstrated that the expression of cysteine and glycine-rich protein-1 is dramatically increased in the dome of loosely packed aneurysms at 4 weeks after coil embolization. Cysteine and glycine-rich protein-1 is involved in regulatory processes important for development and cellular differentiation, particularly in actin-cytoskeleton organization.²⁹ Increased expression of this protein in loosely packed aneurysms suggests ongoing cell differentiation in the loosely packed aneurysm with platinum coils.

Increased levels of cell adhesion molecules and decreased levels of structural molecules in the dome of densely packed aneurysms, compared with loosely packed aneurysms, at 2 weeks are in accordance with our previous study,⁸ which focused on gene expression profiling between densely and loosely packed aneurysms. However, in the current study, we observed that only 2 proteins (sorbin and SH3 domain-containing protein 2 and leukotriene prostaglandin reductase-1) are differentially expressed at the neck of 4-week densely packed aneurysms, compared with 25 genes noted in our previous gene expression study.⁸ The difference in the expression of number of proteins or genes may be attributed to a mass spectrometry analysis limitation, which is more sensitive in analyzing the highly abundant peptide fragments that mask the less abundant peptides. In addition, not all messenger RNA transcripts are necessarily translated into proteins.

The present study suffers some limitations. Neither densely nor loosely packed aneurysms were compared with untreated aneurysms. Cell-specific protein expression analysis would offer additional information in understanding the mechanism of healing or recurrence of treated aneurysms. Because the availability of the rabbit-specific protein data base is limited, we retrieved protein data from available animal and human data bases. We did not perform detailed pathway analysis for 4-week aneurysms, as the number of differentially expressed proteins was low. We acknowledge that this was an exploratory and preliminary study with only 2 time points.

Conclusions

In the rabbit saccular aneurysm model, increased protein expression in the densely packed aneurysms was associated with cell-signaling molecules. Loosely packed aneurysms showed an elevated expression of structural molecules.

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