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ORIGINAL RESEARCH

Gene expression comparison of flow diversion and coiling in an experimental aneurysm model

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ABSTRACT

Background and purpose Mechanisms of both healing and complications, including spontaneous aneurysm rupture, remain unclear following flow diverter treatment. The aim of this study was to compare gene expression of various key molecules involved in the healing of aneurysms, between aneurysms treated with microcoils and flow diverters.

Methods Saccular aneurysms were created in rabbits. Aneurysms were treated with coils (n=6) or flow diverters (n=6). Aneurysms were harvested at 4 weeks following treatment and used for gene expression and zymography experiments. Genes with a fold change of 1.2 or more were considered upregulated whereas those with a fold change of 0.8 or less were considered downregulated.

Results All coil embolized aneurysms were completely occluded at follow-up. Two aneurysms were occluded and the remaining four samples were incompletely occluded in the flow diverter treated group. The following genes were expressed at lower levels in the flow diverter group compared with the coiled aneurysm group: proteinases (matrix metalloproteinases 2 and 9), cellular markers (endothelial nitric oxide synthase and smooth muscle actin), and structural proteins (collagens and fibronectin). Genes related to inflammation (tumor necrosis factor α and monocyte chemoattractant protein 1) were upregulated in flow diverter treated aneurysms compared with coil embolized aneurysms. Notably, the enzymatic activity of active matrix metalloproteinase 9 was high in aneurysms treated with flow diverters.

Conclusions Our findings may provide improved understanding of rupture risk and healing following aneurysm treatment and inform development of therapies aimed at lowering rupture risk and accelerating healing.

INTRODUCTION

Even after nearly two decades of clinical use, long term occlusion rates with endovascular microcoils in large and broad necked aneurysms remain poor. The introduction of flow diverters in the treatment of aneurysms has alleviated the shortcomings of microcoils and has shown a remarkable rate of complete occlusion of aneurysms, which are difficult to treat using other endovascular techniques.

Both of these approaches primarily elicit thrombus formation in the aneurysm cavity and then promote neointima formation across the neck to seal the aneurysm cavity from the circulation.^{1–4} Coils act within the aneurysm cavity while flow diverters primarily work at the parent artery.^{5,6} Studies aimed at exploring the mechanism of

aneurysm healing following endovascular treatments have mostly focused at the tissue and cellular levels, and a handful of preclinical experiments studied the molecular mechanisms.^{3,7–11} However, the exact biological mechanism of action of these endovascular techniques remains poorly understood. Furthermore, rare but devastating reports of spontaneous aneurysm rupture following aneurysm therapy have diminished enthusiasm for flow diversion devices.^{12,13} The mechanisms underlying the propensity for aneurysm rupture following flow diversion treatment remain enigmatic.

The aim of the current study was to directly compare gene expression of key molecules potentially involved in the healing of aneurysms as well as the activity of proteolytic enzymes, between aneurysms treated with microcoils and flow diverters.

MATERIALS AND METHODS

Aneurysm creation, treatment, and follow-up

The Institutional Animal Care and Use Committee approved all procedures before initiation of the study. Elastase induced saccular aneurysms were created in 12 New Zealand White rabbits (body weight 3–4 kg). Detailed procedures for aneurysm creation have been described in depth elsewhere.¹⁴ Aneurysms were permitted to mature for at least 3 weeks after creation. Rabbits were assigned to either coil or flow diverter treatment groups. Aneurysms were either embolized with platinum coils or treated with flow diverters (Pipeline Embolic Device, Covidien Inc, California, USA) placed in the parent artery, as previously described.^{15,16} Digital subtraction angiography (DSA) of the aortic arch was performed immediately before or after treatment. At 4 weeks following treatment, a follow-up DSA of the aortic arch was performed. The animals were then euthanized by using a lethal injection of pentobarbital. The aneurysms and the parent artery were harvested, longitudinally cut into two halves, and the samples were immediately snap frozen in liquid nitrogen and kept frozen at -70°C until use. One half of the sample was used for gene expression and the other half was used for zymography analysis.

Angiographic evaluation

Images from DSA obtained immediately after device implantation and just before sacrifice were evaluated. Follow-up DSA images were assessed using a trichotomous scale (incomplete occlusion, near complete occlusion, or complete occlusion).



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Quantitative real time PCR analysis

The mRNA expression of selected genes that have been associated with vascular remodeling of aneurysms was assessed by quantitative real time PCR. These selected genes were categorized into cell adhesion (vascular cell adhesion molecule 1 (VCAM-1) and selectin E), structural molecules (type VIII collagen, type I collagen, and fibronectin), growth factors and cytokines (vascular endothelial growth factor 1 (VEGF-1), tumor necrosis factor α (TNF α), osteopontin, and monocyte chemo-attractant protein 1 (MCP-1)), proteinases and their inhibitors (matrix metalloproteinase (MMP) 2 and 9, tissue inhibitor of MMP-1 and MMP-2 (TIMP-1 and TIMP-2), and cathepsin L), and cellular and oxidative stress markers (hypoxia inducible factor 1 (HIF-1), heme oxygenase 1, endothelial nitric oxide synthase, von Willebrand factor, and smooth muscle actin). Briefly, total RNA was extracted from aneurysm tissue with RNeasy kit (Qiagen). First strand complementary DNAs were synthesized from 500 ng of total RNA using a synthesis system (SuperScript III First-Strand Synthesis System; Invitrogen, Carlsbad, California, USA). Then real time PCR was performed with a cycler (iCycler; Bio-Rad, Hercules, California, USA) using SYBR Green PCR kit (Invitrogen). 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) using a Web tool (Primer 3; <http://frodo.wi.mit.edu/primer3/>).¹⁷

Gelatin zymography for MMPs

Soluble proteins were extracted from frozen samples.⁹ Protein samples were separated by 10% zymogram gel (Bio-Rad). Gels were washed with renaturation buffer (Bio-Rad) for 1 h and then incubated for 48 h at 37°C in development buffer (Bio-Rad). The gels were stained with 0.5% Coomassie blue R-250. The gelatinolytic bands, representing the activities of MMPs, were scanned and analyzed densitometrically using Licor software.

Statistical analysis

Genes with a fold change of 1.2 or more were considered upregulated whereas those with a fold change of 0.8 or less were considered downregulated.¹⁰

RESULTS

Angiographic results

Angiographic data are summarized in [table 1](#), and representative angiographic images are presented in [figure 1](#). There were no statistically significant differences in mean neck, width, and height between coil and flow diverter treated groups. Of the six aneurysms treated with flow diverter devices, at 4 weeks post-treatment, two aneurysms showed complete occlusion, two aneurysms showed near complete occlusion, and the remaining two aneurysms were patent, whereas all six aneurysms treated with platinum coils showed stable occlusion at 4 weeks post-

treatment. Representative angiographic images are presented in [figure 1](#).

Gene and protein expression results

Cell adhesion and structural components

None of the adhesion molecules (VCAM and selectin E) showed differential expression between the flow diverter and coil treated aneurysms. Among the structural molecules studied, both collagen I (0.18-fold) and fibronectin (0.32-fold) showed remarkably decreased expression in the flow diverter treated aneurysms compared with the coiled aneurysms, whereas collagen VIII (0.82-fold) exhibited no significant change between the flow diverter and coil groups ([table 2](#)).

Growth factors, cytokines, and inflammatory molecules

The expression profiles of osteopontin (2.4-fold) and the proinflammatory genes TNF- α (1.3-fold) and MCP-1 (1.4-fold) were increased in aneurysms treated with flow diverters compared with aneurysms treated with platinum coils. VEGF expression (0.37-fold) was significantly less in the flow diverter treated samples.

Cellular and stress markers

Both the hypoxia marker, HIF-1 (0.42-fold), and the oxidative stress marker, heme oxygenase 1 (0.7-fold), were downregulated in flow diverted treated aneurysms compared with coiled

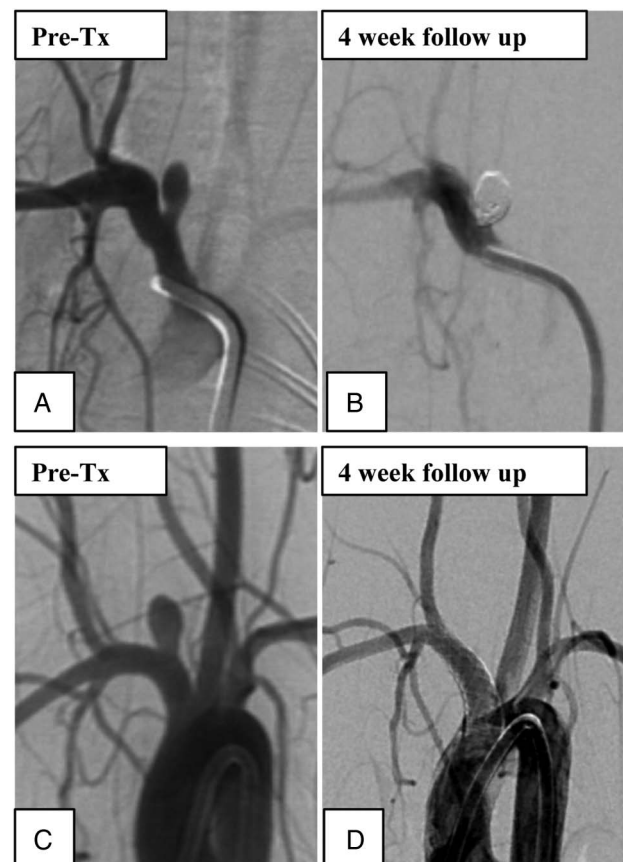


Figure 1 Representative angiograms of treated aneurysms. (A) Digital subtraction angiography (DSA) image showing the aneurysm cavity prior to coil treatment (Tx). (B) Follow-up DSA image showing obliteration of the aneurysm. (C) DSA image showing the aneurysm cavity prior to flow diverter placement. (D) Follow-up DSA image showing complete occlusion of the aneurysm.

Table 1 Aneurysm geometry in the two treatment groups

	Flow diverter	Coil
Neck width (mm)	3.7 \pm 0.9	3.3 \pm 1.6
Width (mm)	4.5 \pm 1.4	3.7 \pm 0.6
Height (mm)	9.4 \pm 2.5	8.0 \pm 1.3

Table 2 Relative expression of genes in flow diverter treated aneurysms compared with coiled aneurysms

Gene name	Median (range)	Expression changes
Adhesion molecules		
VCAM	0.94 (0.11–661.7)	No change
Selectin E	0.91 (0.19–1.54)	No change
Structural molecules		
Collagen VIII	0.82 (0.07–180.39)	No change
Collagen I	0.18 (0.06–1.38)	↓
Fibronectin	0.32 (0.08–0.36)	↓
Vascular remodeling		
MMP-2	0.43 (0.09–2.42)	↓
MMP-9	0.36 (0.02–94.03)	↓
Cathepsin L	0.25 (0.07–1.1)	↓
TIMP-1	2.4 (0.11–1.13)	↑
TIMP-2	0.18 (0.02–2.51)	↓
Growth factors/cytokines/inflammatory molecules		
Osteopontin	2.4 (0.11–133.9)	↑
Monocyte chemoattractant protein	1.29 (0.37–5.35)	↑
TNF α	1.37 (0.63–27.19)	↑
VEGF	0.37 (0.28–1.13)	↓
Oxidative stress		
HIF	0.42 (0.06–251.6)	↓
HO-1	0.69 (0.15–1.84)	↓
Cellular markers		
vWF	0.35 (0.12–0.14)	↓
eNOS	0.08 (0.02–0.14)	↓
SMA	0.69 (0.34–0.72)	↓

eNOS, endothelial nitric oxide synthase; HIF, hypoxia inducible factor; HO-1, heme oxygenase 1; MMP, matrix metalloproteinase; SMA, smooth muscle actin; TIMP, tissue inhibitor of MMP; TNF α , tumor necrosis factor α ; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

aneurysms. Significantly decreased expression of both endothelial cell makers, von Willebrand factor (0.35-fold) and endothelial nitric oxide synthase (0.08), and the smooth muscle cell marker, smooth muscle actin (0.69-fold), was observed in the aneurysms of flow diverted treated animals.

Proteases and their inhibitors

Compared with flow diverter treated aneurysms, expression of MMP-2, MMP-9, and cathepsin L was upregulated over twofold in coil embolized aneurysms. TIMP-1 expression (2.4-fold) was elevated and TIMP-2 expression (0.2-fold) was diminished in the flow diverted treated group compared with the coiled group.

MMP activities

MMP zymography was performed to assess the activities of both inactive precursor and active functional forms of MMPs. Pro-MMP-2 expression was not different between the coil and flow diverted treated samples whereas active MMP-2 expression was 52% higher in coiled aneurysms. Pro-MMP-9 expression was diminished (66%) while active MMP-9 expression was increased twofold in flow diverter treated aneurysms compared with coiled control aneurysm tissues (figure 2A, B).

DISCUSSION

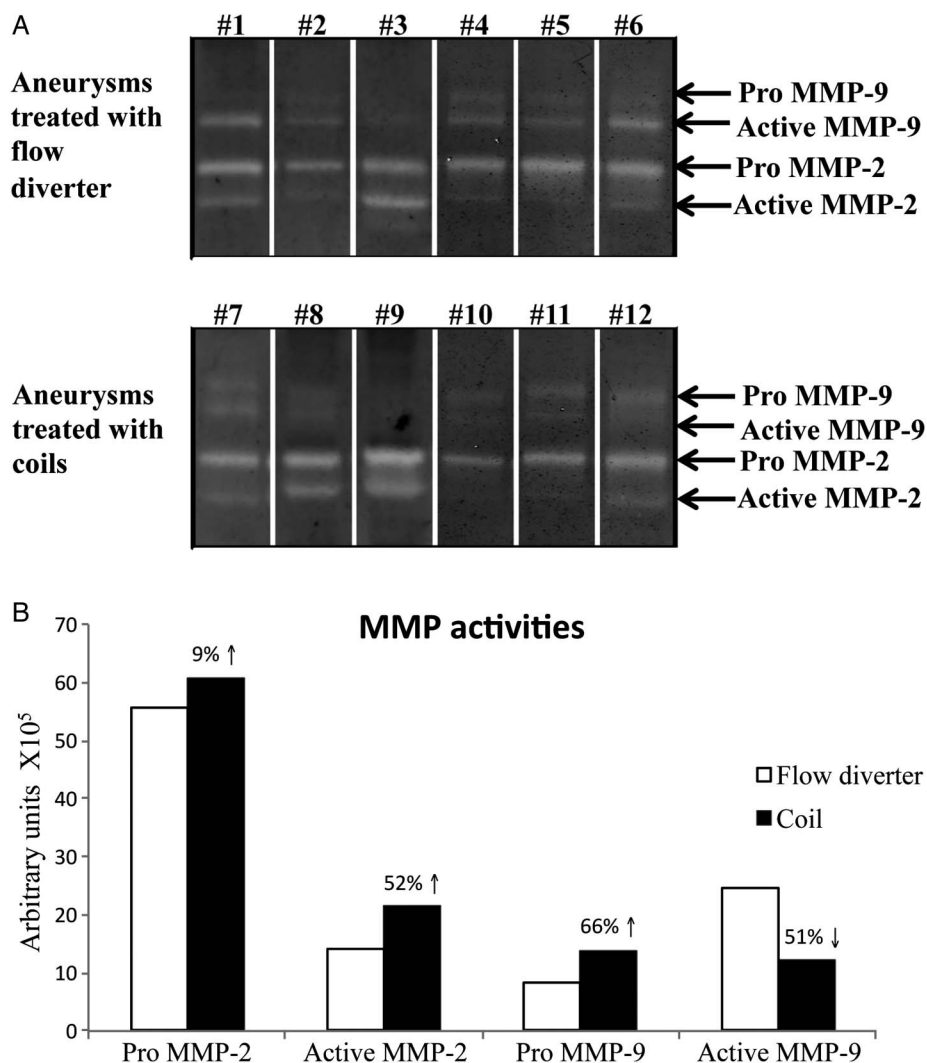
In this study, we compared the gene expression pattern between aneurysms treated with diverters and aneurysms embolized with

coils to probe the mechanism of aneurysm healing following two different interventional approaches. The major finding of this study was that active MMP-9 was involved in the remodeling of flow diverter treated aneurysms, as evidenced by an increase in the enzymatic activity of active MMP-9, despite a decrease in the level of mRNA. Our findings also demonstrated over expression of proinflammatory markers and under expression of structural genes and cellular markers in flow diverter treated aneurysms. There were no observed trends in the expression of VCAM-1 or selection E to suggest a role for adhesion molecules to account for flow diverter related remodeling.

The role of MMPs in the pathophysiology of cerebral aneurysms is well characterized, and they have also been reported in the recanalization and recurrence of canine aneurysms embolized with gelatin sponges.⁸ Similarly, high expression of MMP-9 has been associated with the healing of aneurysms following stent placement in the parent artery of a swine aneurysm model.⁷ Diminished expression of pro-MMP-2 and pro-MMP-9, seen in the zymogram, was correlated with decreased gene expression of the respective genes in the flow diverter treated group. Interestingly, profound elevation of active MMP-9 in the flow diverter treated aneurysms indicates a major role for MMP-9 in the remodeling of the aneurysm wall, which may be a possible explanation for the spontaneous rupture of aneurysms treated with flow diverters in patients.^{12 13} Moreover, the activity of MMP-9 is important in cell migration of neointimal cells, and occlusion of aneurysms following flow diverters is thought to be derived from neointimal cell growth originating from the vessel wall.³ The observed increase in the activity of active MMP-9 in the flow diverter treated group could also be related to the ongoing vascular remodeling in the aneurysm cavity. The activity is more important in predicting the function of an enzyme than the level of mRNA.^{18 19} Our previous study comparing the gene expression pattern between aneurysms with a high aspect (height to neck width) revealed high expression of hypoxia induced TIMP in high aspect ratio aneurysms.²⁰ Similarly, in the present study, an increase in expression of TIMP-1 was observed in the flow diverter treated aneurysms. This could be related to active extracellular matrix turnover and vascular remodeling, which may lead to aneurysm rupture.²¹

TNF α is a proinflammatory cytokine with strong necrotic activity and is secreted by a variety of cells, including macrophages, endothelial cells, and smooth muscle cells. The chemokine MCP-1 recruits monocytes and neutrophils to the site of injury and promotes chemotaxis and inflammation. Deposition of inflammatory cells was noted in the struts of the flow diverters at the neck of the aneurysms. High levels of detected MCP-1 and TNF α could be related to the inflammatory events in the aneurysm following flow diverter placement.³ Chow *et al*¹² showed inflammatory infiltrates, mural thinning, and necrosis within the aneurysm of a delayed spontaneous rupture of a posterior inferior cerebellar artery aneurysm following treatment with flow diversion. An increase in the level of TNF α detected in this study may be associated with the inflammation and necrotic cell death of spontaneous rupture of flow diverter treated aneurysms. TNF has also been shown to increase MMP-9 activity.^{22 23} Moreover, high levels of both TNF α and MMPs were reported in ruptured human cerebral aneurysms,²⁴ and inhibition of TNF α has been shown to decrease the incidence of aneurysm rupture in a mouse model.²⁵ The observed elevated levels of HIF and VEGF in coiled aneurysms could be related to the neovascularization of thrombus in the aneurysm^{26 27} as the geometry of untreated rabbit aneurysms remains stable for up to 5 years.²⁸

Figure 2 (A) Matrix metalloproteinase (MMP)-2 and MMP-9 enzymatic activities of aneurysm samples at 4 weeks following flow diverter and coil embolization on gelatin zymography. (B) Densitometry analysis of activities of pro- and active MMP-2 and MMP-9.



Our study had several limitations. The flow diverter and coil treated groups were not compared with untreated aneurysms or normal control arteries. We assessed gene expression of completely occluded coiled aneurysms against incompletely occluded flow diverter treated aneurysms. In addition, we compared differences between complete occlusion, near complete occlusion, and patent groups. Even though the rabbit aneurysm model employed in this study is similar to human aneurysms histologically,²⁹ morphologically,³⁰ biologically,³¹ and hemodynamically,³² further studies on human samples will be necessary to validate our findings. We only studied one time point following embolization, and gene expression may change over time.

CONCLUSION

In the rabbit saccular aneurysm model, active MMP-9 activity and genes involved in the production of proteins that function under inflammation were upregulated whereas genes coding for cellular markers and structural proteins were downregulated. These findings may be helpful towards further understanding of the molecular pathways involved in the healing of aneurysms treated with either flow diverter devices or platinum coils.

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Contributors CP: gene expression experiments, statistical analysis, and manuscript drafting. DD and Y-HD: animal experiments, including aneurysm creation,

embolization, and follow-up, and tissue harvest. JC, DK, and RK: study design, data analysis, and manuscript editing. All of the authors have read and approved submission of the manuscript.

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Competing interests None.

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