Molecular Indices of Apoptosis Activation in Elastase-Induced Aneurysms After Embolization With Platinum Coils

Ramanathan Kadirvel, PhD; Yong-Hong Ding, MD; Daying Dai, MD, PhD; Debra A. Lewis, PhD; Harry J. Cloft, MD, PhD; David F. Kallmes, MD

Background and Purpose—Even though endovascular coils have been widely used for the treatment of intracranial aneurysms, the cellular and molecular responses of aneurysms to the coils after embolization remain poorly understood. The aim of the present study was to understand the mechanism of apoptosis in aneurysms embolized with platinum coils in the rabbit model of elastase-induced aneurysms.

Methods—Elastase-induced saccular aneurysms were created at the origin of the right common carotid artery in 30 rabbits. Aneurysms were allowed to mature for 8 weeks, after which 20 aneurysms were embolized with platinum coils by endovascular means. After 2 (n=10) and 4 (n=10) weeks of implantation, aneurysm samples harboring coils were harvested for apoptotic studies. The remaining 10 uncoiled aneurysms were used as controls; additional controls included the left common carotid artery, which had not undergone any surgical procedure. Control samples were harvested at 12 weeks after aneurysm creation.

Results—Expression of procaspases-3, -8, and -9 was elevated in coiled aneurysms embolized with platinum coils at both time points when compared with uncoiled aneurysms and the left common carotid artery. Cleaved caspases-3, -8, and -9 were found to be expressed only at 4 weeks after embolization. Cells within the aneurysm cavity were terminal dUTP nick end-labeling–positive at 4 weeks only. These apoptotic cells were identified as smooth muscle actin–positive cells. Expression of tumor necrosis factor-α was high in coiled aneurysms when compared with controls. There was no significant difference in the expression of Fas ligand among groups. Decreased expression of antiapoptotic proteins Bcl-2 and phospho-Bad, as well as increased expression of proapoptotic proteins Bax and Bid, was observed in coiled aneurysms at both time points.

Conclusions—Activation of apoptosis in aneurysms after embolization with platinum coils is induced by both tumor necrosis factor-α–mediated extrinsic and Bcl-2-mediated intrinsic pathways. (Stroke. 2007;38:2787-2794.)

Key Words: apoptosis ■ endovascular treatment ■ saccular aneurysms

Endovascular occlusion of saccular intracranial aneurysms with the use of detachable platinum coils has proved to be a safe and durable alternative to traditional surgical clipping.1,2 However, recanalization attributable to coil compaction remains the major limitation of coiling, particularly in wide-necked or large aneurysms.3,4 Several newer coil designs, including platinum coils modified with hydrogel or biodegradable polymeric materials, have been proposed to improve healing and to reduce recanalization of aneurysms after embolization, but clinical series focused on these newer coils have been disappointing.5-7 The continuing frustration with late recanalization reflects the ongoing uncertainty regarding the mechanism of healing within coiled aneurysms. If improved understanding of the biologic mechanisms underlying the tissue response to endovascular coils is achieved, then targeted therapies might be developed to overcome the limitations in endovascular therapy for aneurysms.

Our previous work, performed in the rabbit elastase-induced aneurysm model, demonstrated that aneurysm healing in response to platinum coil embolization appeared to progress through the stages of thrombus formation, granulated tissue organization, and loose connective-tissue formation. Myofibroblasts, the key cellular component involved in healing, appeared early, were progressively reduced with time, and finally disappeared through apoptosis.8

Apoptosis is an energy-dependent form of cell death characterized by distinct morphologic and biochemical changes to the cell, including chromatin condensation, nuclear fragmentation, and cellular shrinkage without loss of membrane integration. This process is followed by the
break-up of nuclear and cellular budding with the formation of apoptotic bodies, which are rapidly phagocytosed by neighboring cells. Apoptosis can be initiated by internal and external triggers, both of which lead to activation of a set of cell-suicide enzymes, the caspases, in mammalian cells. There are 2 classic pathways leading to caspase activation: the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway. Caspases are aspartate-specific cysteine proteases and exist as inactive zymogens that are activated by either autoproteolysis or the proteolytic action of other caspases.

The elastase-induced aneurysm model in rabbits has been widely applied as a preclinical model for testing endovascular devices. This aneurysm model is similar to human aneurysms histologically, morphologically, and hemodynamically. In addition, the rabbit aneurysm model shares some molecular features with human intracranial aneurysms. Histopathologic study has shown that the rabbit aneurysm, after embolization with platinum coils, mimics the healing seen in human aneurysms.

The aim of the present study was to understand the mechanism of apoptosis in aneurysms embolized with platinum coils in the elastase-induced aneurysm model in rabbits. An improved understanding of the cellular and molecular responses of the aneurysm to platinum coils after embolization will allow rational design of next-generation microcoils.

Materials and Methods

Aneurysm Creation
The institutional animal care and use committee approved all procedures before initiation of the study. Elastase-induced saccular aneurysms were created in 30 New Zealand White rabbits (body weight, 3 to 4 kg). Detailed procedures for aneurysm creation have been described in depth elsewhere. In brief, anesthesia was induced with an intramuscular injection of ketamine, xylazine, and acepromazine (75, 5, and 1 mg/kg, respectively). With sterile techniques, the right common carotid artery (RCCA) was exposed and ligated distally. A 1- to 2-mm beveled arteriotomy was made, and a 5F vascular sheath was advanced retrograde in the RCCA to a point approximately 3 cm cephalad to the origin of the RCCA. A 3F Fogarty balloon inflated with iodinated contrast material was advanced into the artery. A guidewire was passed through the angioplasty catheter, balloon, and sheath were removed. The RCCA was ligated below the arteriotomy and the incision was closed. The rabbits were randomly assigned to 1 of 3 groups: (1) untreated (aneurysm not embolized) control group; (2) 2-week survival group after embolization with platinum coils as previously described. Aneurysm cavities were densely packed in all cases. Where appropriate, a microballoon was placed and inflated across the aneurysm neck to facilitate coil placement. After embolization, a final control DSA was performed. The catheters and sheath were removed, the femoral artery was ligated, and the incision was closed.

Tissue Harvest
Aneurysm samples were harvested at either 2 or 4 weeks after embolization. Untreated aneurysms and control LCCAs were harvested at 12 weeks after aneurysm creation. Under general anesthesia, the left common femoral artery was accessed by percutaneous techniques. A 5F sheath was placed, a 5F diagnostic catheter was placed into the embolized vessel, and DSA was performed. The angiographic occlusion scores were calculated on the basis of the percentage of iodinated contrast medium inside the aneurysm cavity. All sacrifice angiograms were compared with postembolization angiograms and assessed for changes in coil configuration or aneurysm filling. Aneurysms were assigned to 1 of 3 result categories: stable, progressive occlusion, or coil compaction/recanalization compared with the posttreatment angiograms.

After DSA and immediately before sacrifice, 2 mL heparin (1000 U/mL) was injected into each animal to prevent blood clotting. Then the animals were euthanized, and the aneurysm and unoperated LCCA were harvested. The samples were placed in buffered formalin for histopathologic experiments and terminal dUTP nick end-labeling (TUNEL) staining. When the aneurysms were relegated to molecular biology experiments, the aneurysm samples were dissected into 2 samples, including the neck and dome. Coils from the embolized vessels were removed carefully, and extreme care was taken to minimize cell loss. These samples were kept frozen at −70°C.

Histopathology
Tissues were fixed in formalin for 24 hours, embedded in paraffin, and sectioned as described previously. Sections were stained with hematoxylin and eosin for conventional histopathologic evaluation.

TUNEL Staining
TUNEL staining was performed according to the manufacturer’s protocol in tissue sections to identify apoptotic cells according to the DeadEnd colorimetric TUNEL system (Promega, Madison, Wis). Two experienced observers using an Olympus BH2 microscope (Olympus America, Melville, NY) independently reviewed all slides. Images were recorded with a Spot RT digital camera (Diagnostic Instruments, Sterling Heights, Minn). Five random, high-power organized areas in both the aneurysm dome and neck were imaged. TUNEL-stained cells were recorded.

Immunofluorescence
To determine which cells were undergoing apoptosis, tissue sections were subjected to a fluorescence TUNEL assay (Promega, Madison, Wis). Then the slides were incubated with either a monoclonal mouse anti-human smooth muscle actin (SMA, 1:200; Dako, Denmark) antibody or a monoclonal mouse anti-CD 31 antibody (1:30; Dako, Denmark). After the primary antibody incubation, slides were washed in phosphate-buffered saline and incubated with Cy3-conjugated donkey anti-mouse IgG (Jackson Immuno Research Laboratories Inc, West Grove, Pa) at 37°C for 1 hour. After being washed, the slides were viewed and imaged by fluorescence confocal microscopy.

Immunoblotting
Frozen samples were pulverized under LN₂ and extracted in ice-cold lysis buffer (10 mmol/L sodium phosphate, pH 7.2, 150 mmol/L NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, and 0.2% NaN₃, with a protease inhibitor cocktail).
After centrifugation at 10 000 g for 20 minutes at 4°C, the protein concentration of the supernatant was determined (Pierce Biotechnology, Rockford, Ill).

Total protein was fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, Calif). Membranes were incubated with monoclonal anti-Bcl-2, Bax, Bid, Fas ligand (BD Biosciences, San Jose, Calif), tumor necrosis factor (TNF)-α, phospho-Bad, and caspase-8 (Cell Signaling Technology, Danvers, Mass), and rabbit polyclonal anti-caspase-3, and caspase-9 (Cell Signaling Technology, Danvers, Mass) overnight at 4°C. The membranes were incubated with the appropriate secondary antibody, either a diluted sheep anti-rabbit or goat anti-mouse IgG (Bio-Rad, Hercules, Calif) conjugated to horseradish peroxidase. Immune complexes were visualized with an enhanced chemiluminescence detection system (Amersham Biosciences, now GE Healthcare Bio-Sciences Corp, Piscataway, NJ), and bands were measured by densitometric analysis of autoradiograph films (UN-SCANIT software, Silk Scientific, Orem, Utah).

Statistical Analyses

Results are expressed as mean±SD. Uncoiled aneurysms and unoperated LCCAs were used as controls. Differences between groups were assessed by ANOVA. Significant ANOVAs were followed by Tukey’s post hoc test. Statistical significance was set at P<0.05.

Results

Angiographic Findings

All aneurysms were embolized without any technical complications and without acute or chronic morbidity or mortality. In both the 2- and 4-week groups, all aneurysms were as tightly packed as possible with platinum coils. Representative angiograms are shown in Figure 1. Based on DSA studies, the mean aneurysm neck diameter, width, and height were 4.37±1.34, 3.87±1.08, and 8.50±3.21 mm, respectively, in the control group; 2.54±1.01, 2.98±1.08, and 6.17±3.04 mm, respectively, in the 2-week group; and 3.53±1.70, 3.63±1.15, and 7.77±2.09 mm, respectively, in the 4-week group. The neck diameter in the control, uncoiled aneurysm group was significantly larger than that in the coiled aneurysm group at 2 weeks (P<0.01). There were no other differences in geometry. At 2 weeks, there were 7 stable aneurysms, no progressive occlusions, and 3 coil compactions, and at 4 weeks, there were 10 stable aneurysms and no progressive occlusions or coil compactions.

Histologic Findings

Untreated control aneurysms were characterized by marked elastin degradation, thinning of the arterial wall media, scattered loss of endothelial cells, and the absence of inflammatory cells (Figure 2A). Aneurysms embolized with platinum coils at 2 weeks showed unorganized thrombus in the aneurysm cavity, with rare endothelial cells along the neck of the aneurysm (Figure 2B). After 4 weeks, the treated aneurysm domes demonstrated loose connective tissue along the unorganized thrombus, whereas endothelial cells lining the thin neointima were seen at the neck (Figure 2C).

Molecular Biology Findings

TUNEL Staining

A TUNEL assay was performed to identify apoptotic cells. Apoptotic cells were absent in both uncoiled control aneurysms and in treated 2-week aneurysms, whereas TUNEL-positive cells were found in treated 4-week aneurysms. No TUNEL-positive cells were observed in the intimal or medial layer of these aneurysms, but cells within the treated 4-week
aneurysm cavities were positive for TUNEL staining. The mean number of apoptotic cells per high-power field was significantly higher in the dome compared with the neck of the coiled-aneurysm group \((P < 0.05; \text{Figure 3A})\). To identify the types of cells that were apoptotic, immunofluorescence studies by confocal microscopy were performed. TUNEL-positive cells were also positive for SMA and negative for CD-31, indicating that smooth muscle–derived cells were undergoing apoptosis after coil embolization (Figure 3B).

**Activation of Caspases**

Because caspases are the prime enzymes responsible for apoptosis, we analyzed the activation of caspase-8 (receptor-mediated initiator), caspase-9 (mitochondrially mediated initiator), and executioner caspase-3 in control aneurysms, control arteries, and aneurysms embolized with platinum coils by Western blot assay. Representative images and densitometric quantification of the expression of caspases are shown in Figure 4. There was a significant induction in the expression of procaspases-3, -8, and -9 in coiled aneurysms at 2 and 4 weeks \((P < 0.001 \text{ at both time points})\) compared with controls. The activated cleaved caspase-3 (17 kD), caspase-8 (43 kD), and caspase-9 (17 kD) were detected at 4 weeks only. These aneurysm domes showed higher expression of cleaved caspases than did the neck region \((P < 0.05)\).

**TNF-α– and Bcl2-Mediated Activation of Caspases**

Because increased expression of caspase-8 and -9 was observed in embolized aneurysm samples, we also measured the expression of other relevant molecules in the activation of both the extrinsic and intrinsic pathways. Expression of TNF-α was decreased in untreated aneurysm segments when compared with control arteries \((P < 0.001)\); whereas in treated aneurysms at both 2 and 4 weeks, there was a significant \((P < 0.001)\) elevation compared with that of LCCA controls (Figure 5A). There were no significant changes among groups in the expression of Fas ligand (Figure 5B).

Changes in the expression of Bcl-2, Bax, Bid, and phospho-Bad were also measured. Expression of Bcl-2, Bax, Bid, and phospho-Bad were not different in untreated aneurysms compared with LCCAs. Two- and 4-week coiled aneurysms showed a decrease in the expression of Bcl-2 and phospho-Bad and an increase in the expression of Bax and Bid when compared with controls \((P < 0.001, \text{Figures 5C through 5F})\). The differential expression of Bcl-2 proteins was
Figure 4. Expression of caspase family proteins. A, Representative Western blots and densitometric analysis of the expression of procaspase-3 among control and platinum coil-embolized aneurysms. B, Representative Western blots and densitometric analysis of the expression of active caspase-3 among control and platinum coil-embolized aneurysms. C, Representative Western blots and densitometric analysis of the expression of procaspase-8 among control and platinum coil-embolized aneurysms. D, Representative Western blots and densitometric analysis of the expression of active caspase-8 among control and platinum coil-embolized aneurysms. E, Representative Western blots and densitometric analysis of the expression of procaspase-9 among control and platinum coil-embolized aneurysms. F, Representative Western blots and densitometric analysis of the expression of active caspase-9 among control and platinum coil-embolized aneurysms. Abbreviations are as described in the legend to Figure 3.
Figure 5. Expression of TNF and Bcl-2 family proteins. A, Representative Western blots and densitometric analysis of the expression of TNF-α among control and platinum coil-embolized aneurysms. B, Representative Western blots and densitometric analysis of the expression of Fas ligand among control and platinum coil-embolized aneurysms. C, Representative Western blots and densitometric analysis of the expression of Bcl-2 among control and platinum coil-embolized aneurysms. D, Representative Western blots and densitometric analysis of the expression of phospho-Bad among control and platinum coil-embolized aneurysms. E, Representative Western blots and densitometric analysis of the expression of Bax among control and platinum coil-embolized aneurysms. F, Representative Western blots and densitometric analysis of the expression of Bid among control and platinum coil-embolized aneurysms. Abbreviations are as defined in the legend to Figure 3.
Table. Overview of the Parameters Analyzed and Results of the Present Study

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<th>Parameters</th>
<th>LCCA</th>
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Symbols ↑ and ↓ represent increased and decreased expression, respectively.

UCAN indicates uncoiled aneurysm neck; CAN-2, coiled aneurysm neck at 2 weeks after embolization; CAN-4, coiled aneurysm neck at 4 weeks after embolization; UCAD, uncoiled aneurysm dome; CAD-2, coiled aneurysm dome at 2 weeks after embolization; and CAD-4, coiled aneurysm dome at 4 weeks after embolization.

UCAN and UCAD were compared with LCCA. CAN-2 and CAN-4 were compared with LCCA and UCAN. CAD-2 and CAD-4 were compared with LCCA and UCAD.

Discussion

In this study, we used experimental aneurysms in rabbits to study the pathways leading to apoptosis after embolization of saccular aneurysms with platinum coils. This study is the first to provide insight into the molecular mechanisms underlying apoptosis in aneurysms embolized with platinum coils. We report that both the extrinsic death receptor-mediated and the intrinsic mitochondrial pathways lead to the activation of caspase-3 for the execution of apoptosis in this model.

Coil Embolization and Recanalization

Although endovascular platinum coils have been used for the treatment of intracranial aneurysms for more than a decade, there has been little progress toward understanding the biologic response to embolization and to either the healing or recanalization phenomenon. One of the major limitations of endovascular treatment is that, over time, blood flow compresses the coils within the aneurysm, resulting in recurrence. It remains likely, based on observational studies, that recurrence often occurs early after treatment. Raymond et al. reported that nearly 50% of recurrences were present by 6 months after coiling in humans. Consequently, studying the biologic aspects of aneurysms early after embolization, as done in the current study, may be helpful in understanding the cellular responses to endovascular coils.

Apoptosis in Endovascular Treatment

Involvement of apoptosis in the pathophysiology of aneurysms and restenosis has been well documented. Moreover, the occurrence of smooth muscle cell apoptosis has been established in interventional procedures such as balloon angioplasty and stent implantation. Unlike those previous studies, our report has shown that cells in the rabbit model undergo apoptosis within the aneurysm cavity, whereas cells in the wall of the aneurysm do not.

In this study, we have shown that increased expression of procaspases was present at both 2 and 4 weeks. However, activated cleaved caspases were detected at 4 weeks only. Activation of caspase-8 and -9 suggests that apoptosis in aneurysms embolized with platinum coils is triggered by both death receptor- and mitochondrially dependent apoptosis. This finding was accompanied by the identification TUNEL-positive cells at 4 weeks, suggesting that apoptosis mechanisms activate later than 2 weeks but earlier than 4 weeks after embolization. Morphologic and immunohistochemical studies have shown that myofibroblasts are the primary cell type within the aneurysm cavity 4 weeks after embolization; at later time points, these myofibroblasts disappeared. In the present study, the apoptotic cells were positive for SMA, suggesting that myofibroblasts are the predominant cell type undergoing apoptosis after coil embolization.

We surmise that programmed cell death in the aneurysm cavity occurs because there is no physiologic stimulation of the cells surrounding the coils. Cells in the artery wall are exposed to pulsatile flow, but such pulsatility may be diminished or absent in the dome of an aneurysms harboring coils. The indices of apoptosis were more prevalent in the dome than in the neck of the coiled aneurysms. It may be that there is some physiologic stress still present along the cells near the aneurysm neck, whereas physiologic stress at the dome is muted or obliterated by the coils.

The Bcl-2 family of proteins regulates apoptosis by controlling the permeability of mitochondria and the release of cytochrome c. Antiapoptotic Bcl-2 resides in the outer mitochondrial wall. Bax and Bad, the proapoptotic Bcl-2 proteins, reside in the cytosol but translocate to the mitochondria, form a pro-apoptotic complex with Bcl-2, and release cytochrome c to the cytosol, where cytochrome c activates caspase-9 by binding its regulator subunit Apaf-1. Phosphorylation of Bad inhibits the translocation of Bad and thus prevents apoptosis. The observed decrease in the levels of Bcl-2 and phospho-Bad and the increase in the level of Bax are associated with the activation of apoptosis in the aneurysms embolized with platinum coils.

In contrast to the mitochondrial pathway, in the death receptor pathway, ligation of a receptor belonging to the TNF receptor family, such as Fas, TNF-α, and TRAIL receptors, leads to the induction of apoptosis through activation of caspase-8. These experiments did not demonstrate significant changes in the expression of Fas, although an increase in the expression of TNF-α, indicating that the death receptor pathway was mediated by TNF-α, was observed.
expression of TNF-α in neointimal cells after embolization has been demonstrated in a beagle aneurysm model. Cleavage of Bid by activated caspase-9 can activate caspase-8 by releasing cytochrome c into the cytosol. Despite an increase in the level of Bid, we did not see cleaved Bid in coiled aneurysms, suggesting that the 2 apoptotic pathways may not be coupled.

Limitations of the Study
There are numerous signal transduction pathways leading to apoptosis. The present study examined what we consider to be the most important molecules involved in apoptosis. Recanalization was not observed in this study, indicating that there was no correlation between recurrence and apoptosis in elastase-induced aneurysms at the time points studied. The significance of apoptosis after coiling is not well known. Long-term studies are needed to explore the role of apoptosis in the healing or recanalization of aneurysms. Perhaps supplementing antiapoptotic molecules in subjects after aneurysm embolization or the use of coils coated with cells expressing antiapoptotic proteins for embolization might be helpful in understanding the role of apoptosis in the healing of aneurysms.

Conclusions
Both the TNF-α–mediated extrinsic and the Bcl-2–mediated intrinsic pathways are involved in the apoptosis of myofibroblasts located within aneurysm cavities after embolization with platinum coils. These findings may point toward improved therapeutic designs that would enhance aneurysm healing after endovascular coil embolization.

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Disclosures
None.

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