Antirestenotic Effects of a Locally Delivered Caspase Inhibitor in a Balloon Injury Model

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Background—The precise role of arterial barotrauma-mediated apoptosis in causing restenosis is unclear. The purpose of this study was to determine if a link exists between angioplasty-mediated medial smooth muscle cell apoptosis and subsequent neointimal hyperplasia.

Methods and Results—Bilateral iliac artery angioplasty was performed in 25 male New Zealand White rabbits. Simultaneous with balloon injury, each artery was treated locally with either the caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp(Ome)-fluoromethylketone (ZVAD-fmk) or control. In the acute cohort that was survived to 4 hours (n=10, 7 high dose and 3 low dose), an apoptotic index was calculated using the terminal deoxynucleotidyl TUNEL method. In the intermediate cohort that was survived to 2 weeks (n=5), luminal reendothelialization was measured via CD-31 staining. In the chronic cohort that was survived to 4 weeks (n=10), neointimal area was measured. In the acute cohort, there was a 40% reduction in the apoptotic index with high-dose ZVAD-fmk (P=0.008) and a 33% reduction with low-dose ZVAD-fmk (P=0.08). At 2 weeks, there was no significant difference in the degree of luminal reendothelialization. However, at 4 weeks, there was a 33% (0.33±0.23 versus 0.22±0.20 mm²) (P<0.005) reduction in neointimal area in ZVAD-fmk–treated arteries.

Conclusions—The local delivery of ZVAD-fmk during balloon injury inhibits smooth muscle cell apoptosis. This corresponds to a significant reduction in neointimal proliferation seen at 4 weeks without a significant change in the degree of reendothelialization at 2 weeks. (Circulation. 2004;109:108-113.)

Key Words: angioplasty ■ apoptosis ■ restenosis

Restenosis is a limitation of percutaneous coronary interventions.1-3 The precise role of arterial barotrauma-mediated apoptosis in causing restenosis is unclear.

Apoptosis is a noninflammatory mechanism of active, programmed cell death that may play an important role in the pathogenesis and progression of a variety of disease processes, including coronary atherosclerosis and restenosis.4,5 Barotrauma produced by balloon injury during coronary angioplasty causes apoptosis in medial smooth muscle cells (SMCs).6 Previous angioplasty experiments on rabbit and rat arteries have demonstrated that mechanical injury induces apoptosis in up to 70% of medial SMCs within 30 minutes. The medial SMCs lying closest to the arterial lumen are most likely to undergo apoptosis.7 Over a several-week period, a group of surviving medial SMCs migrates toward the arterial lumen to form a neointima.8 It is unknown whether a link exists between balloon injury–mediated SMC apoptosis and neointimal hyperplasia. However, reendothelialization after balloon injury, which occurs within 2 to 4 weeks in rabbits, has been shown to have an inverse relationship with the amount of subsequent neointimal hyperplasia.9,10

The caspases are a family of proteases that play a pivotal role in apoptosis.11 There are naturally occurring as well as synthetic caspase inhibitors, which are capable of preventing apoptosis. N-benzyloxycarbonyl-Val-Ala-Asp (Ome)-fluoromethylketone (ZVAD-fmk) is a synthetic broad-spectrum caspase inhibitor.12,13

In the present study, we sought to establish a causal role for SMC apoptosis in balloon injury–mediated neointimal hyperplasia. The hypothesis was tested by locally delivering ZVAD-fmk to the rabbit arterial wall during balloon injury and quantifying its effect on the degree of SMC apoptosis. In the acute cohort, each artery was treated locally with either the caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp(Ome)-fluoromethylketone (ZVAD-fmk) or control. In the intermediate cohort that was survived to 4 hours (n=10, 7 high dose and 3 low dose), an apoptotic index was calculated using the terminal deoxynucleotidyl TUNEL method. In the intermediate cohort that was survived to 2 weeks (n=5), luminal reendothelialization was measured via CD-31 staining. In the chronic cohort that was survived to 4 weeks (n=10), neointimal area was measured. In the acute cohort, there was a 40% reduction in the apoptotic index with high-dose ZVAD-fmk (P=0.008) and a 33% reduction with low-dose ZVAD-fmk (P=0.08). At 2 weeks, there was no significant difference in the degree of luminal reendothelialization. However, at 4 weeks, there was a 33% (0.33±0.23 versus 0.22±0.20 mm²) (P<0.005) reduction in neointimal area in ZVAD-fmk–treated arteries.

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Methods

Animal Care and Surgical Procedure

This study conforms to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (publication No. 85-23, revised 1985). Twenty-five male New...
Zealand White rabbits (Covance, Denver, Pa) weighing 2.5 to 3.0 kg were used for the study. Animals were anesthetized by intramuscular injections of ketamine (35 to 40 mg/kg) and xylazine (5 to 7 mg/kg), endotracheally intubated, and ventilated as previously described.14

Before angioplasty, the left common carotid artery was cannulated with a 22-gauge intravenous cannula, and a 0.014-inch guidewire was passed down the descending aorta into the appropriate common iliac artery. The bifurcation of the aorta into the common iliac arteries was used as a landmark. The intravenous cannula was removed, and the Remedy balloon catheter (3.5/20 mm) (Boston Scientific) was advanced over the guidewire into the proximal common iliac artery under fluoroscopic guidance.

The Remedy catheter is a noncompliant angioplasty balloon with intramural infusion channels that allow controlled, site-specific, targeted drug delivery independent of the inner dilation balloon pressure. This local delivery approach minimizes systemic toxicity while allowing high intramural drug concentration in the arterial wall at the site of balloon injury. Each animal received 500 U of heparin intravenously before balloon inflation. The balloon injury was achieved by 3 balloon inflations each of 2-minute duration to obtain a maximal balloon to artery ratio of 1.5:1. The balloon was deflated for 1 minute between inflations.

ZVAD-fmk (Enzyme Systems Products) was reconstituted in 0.2% dimethylsulfoxide. In each animal, the control artery was dilated and treated with 0.2% dimethylsulfoxide in saline (control vehicle). After this, the contralateral artery was dilated and treated with ZVAD-fmk. Delivery into the arterial wall with each of the 3 balloon inflations occurred at a constant rate of 0.5 mL/min for 2 minutes via the infusion port of the Remedy balloon using a Harvard pump.

Acute Cohort

Bilateral iliac artery balloon angioplasty was performed in 10 rabbits. Acute animals were treated with high-dose (45 μg, n = 7) or low-dose (4.5 μg, n = 3) ZVAD-fmk. Similar doses of ZVAD-fmk were used in a rat stroke model.15 The acute cohort was euthanized 4 hours after balloon angioplasty. The injured segments of the common iliac arteries were surgically removed and washed in PBS and immersion fixed in 10% (vol/vol) neutral buffered formalin.

Intermediate and Chronic Cohorts

The intermediate and chronic cohorts underwent the same procedure as the acute cohort except that they were survived to 14 and 28 days, respectively. All intermediate (n = 5) and chronic (n = 10) animals received locally delivered high-dose ZVAD-fmk (45 μg) in 1 artery and control vehicle in the contralateral artery. After euthanasia, the descending aorta was cannulated and the arteries were pressure fixed with 10% neutral buffered formalin at 80 mm Hg for 30 minutes. Injured segments of the common iliac arteries were surgically removed and sectioned for analysis.

Acute Cohort: Quantification of Apoptosis

Quantification of the degree of apoptosis was accomplished by the terminal deoxynucleotidyl nick-end labeling (TUNEL) method. Injured segments of the common iliac arteries were harvested and then embedded in paraffin and subsequently cut into 5-μm longitudinal sections.

The ApoTag Plus Peroxidase In Situ Apoptosis detection kit for immunoperoxidase staining was used (S7101, Intergen). Paraffin-embedded tissue was deparaffinized using xylene and absolute alcohol and then treated with proteinase K. Endogenous peroxidase was then blocked with a 3% hydrogen peroxide solution (5 minutes at room temperature). The TUNEL assay was performed using TdT enzyme followed by treatment with antidigoxigenin peroxidase conjugate. Counterstaining was performed with methyl green. TUNEL-positive SMC nuclei were detected by brown coloration. Appropriate positive and negative controls were used. Additionally, cell shrinkage and a small rounded nucleus served as supporting evidence for apoptosis under ×100 objective.

An investigator blinded to the treatment given at time of vessel injury counted 200 medial SMC nuclei per longitudinal section of the common iliac artery. Five such sections were counted for each artery (for a total of 1000 SMC nuclei per artery). Of the 1000 medial SMC nuclei counted, the number positive for apoptosis by the TUNEL assay was recorded. The apoptotic index was then calculated as a percentage (medial SMC cell nuclei positive for apoptosis/100 medial SMC nuclei counted).

Several additional histological methods were performed on samples treated with high-dose ZVAD-fmk and the control vehicle. For these experiments, arteries were snap frozen in liquid nitrogen after immersion in optimal cutting temperature compound. The arteries were then transversely cut into 5-μm sections. 4′,6-diamidino-2-phenylindole (DAPI) stain was used to identify SMC nuclei in each group.16 Sections were also stained with rabbit C92-605 monoclonal antibody (mAb) (BD Biosciences, San Diego, Calif), which specifically recognizes the active form of caspase-3 and identifies cells that may undergo apoptosis.17 These arteries were also compared for the degree of apoptosis using the TUNEL assay with an antidigoxigenin-rhodamine second antibody.

Intermediate Cohort: Anti–CD-31 Antibody Staining

Injured sections of the common iliac arteries were embedded in paraffin and sectioned transversely into 5-μm cross-sections. Murine anti–CD-31 mAb (clone JC/70A, DAKO Corp, Carpinteria, Calif) staining was used to measure the amount of reendothelialization at 14 days after injury.9 The technique has been previously described.18 The anti–CD-31 antibody–stained sections were photographed and digitized. Using the Scion image program, the percent reendothelialization was calculated as the proportion of the cross-sectional circumference stained with the anti–CD-31 antibody stain.

Chronic Cohort: Histology

Sections were prepared as described above. Elastin staining was performed. Area of the arterial media and intima was calculated using the following formulas: medial area (mm²) = (area bounded by the external elastic lamina)− (area bounded by the internal elastic lamina) and neointimal area (mm²) = (area bounded by the internal elastic lamina)− (area bounded by the lumen). Average medial and neointimal areas were calculated. Arterial cross sections stained for elastin were photographed and digitized. The medial and neointimal areas were calculated using the Scion image program (Scion Corporation).

Statistical Analysis

Data are presented as mean ± SEM. The 2-tailed paired Student’s t test was used to compare group means. For comparison of the apoptotic index, the Kendall’s W nonparametric test was performed. P < 0.05 was considered significant.

Results

Reduction in Medial SMC Apoptosis With ZVAD-fmk

Four hours after balloon injury, in the high-dose ZVAD-fmk group (45 μg, n = 7), the mean apoptotic index was 24±5% versus 40±7% (control arteries) for a percent reduction in the apoptotic index of 40% (P = 0.008). For the low-dose ZVAD-fmk group (4.5 μg, n = 3), the mean apoptotic index was 23±13% versus 34±19% for the control group. The percent reduction in the apoptotic index was 33% (P = 0.08) (Figure 1).

Figure 2 depicts qualitative differences in the number of SMC nuclei (DAPI stain), the amount of apoptosis (Rhodamine fluorescence-based TUNEL assay), and caspase-3 activation (C92-605 mAb label) between arteries treated with
high-dose ZVAD-fmk and corresponding controls. A higher degree of caspase-3 activation (green fluorescence), corresponding to a greater degree of apoptotic nuclei (red fluorescence), was seen in the control arteries compared with the high-dose ZVAD-fmk–treated arteries.

**Effects of ZVAD-fmk on Reendothelialization, Medial Area, and Neointimal Area**

The intermediate and chronic cohort results are summarized in the Table. In the intermediate group, at 2 weeks, there was no statistically significant difference observed in the degree of reendothelialization between the ZVAD-fmk–treated arteries and controls (90.0% versus 81.4%, \( P = 0.52 \)) (Figure 3).

Cross-sections of rabbit common iliac arteries stained for elastin, harvested at 4 weeks after balloon injury, are shown in Figure 4. The 3 layers of the arterial wall are demarcated by the internal and external elastic laminae. In the chronic group, at 4 weeks, there was no statistically significant difference in the medial area in the ZVAD-fmk–treated arteries compared with control arteries (0.44 versus 0.40 mm², \( P < 0.08 \)). However, ZVAD-fmk–treated arteries had 33% less neointimal growth compared with controls (0.22 versus 0.33 mm², \( P < 0.005 \)) (Figure 5). Furthermore, the neointimal area to medial area ratio was 45% less in the ZVAD-fmk–treated arteries (0.44 versus 0.80, \( P < 0.001 \)).

**Discussion**

The occurrence of SMC apoptosis after balloon angioplasty has been well established in both animal models and humans.\(^4\)–\(^7\),\(^14\)–\(^21\) However, the precise role of apoptosis in the restenosis process is unclear. Many studies have quantified the degree of apoptosis and the amount of neointimal hyperplasia at various intervals after balloon injury.\(^7\),\(^14\),\(^19\)–\(^21\) However, none have differentiated whether apoptosis is a bystander or a participant in restenosis. In the present study, we demonstrate that the inhibition of apoptosis at the time of balloon injury leads to a significant reduction in the amount of neointimal hyperplasia observed at 4 weeks in a rabbit model.

**Caspase Inhibition**

The caspases are a family of intracellular cysteine proteases that mediate apoptosis activation.\(^{11,22}\) Caspase-3 is believed to be the final common component of the caspase cascade resulting in apoptosis. Enhanced expression of activated caspase-3 in hypertrophied scars and keloid has been shown to induce apoptosis in fibroblasts.\(^{23}\) Naturally occurring viral caspase inhibitors have the ability to inhibit caspase activation and activity, thus averting apoptotic cell death. Synthetic caspase inhibitors, such as ZVAD-fmk, applied directly to

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**Figure 1.** Acute cohort, quantitative reduction in apoptotic index. High-dose ZVAD-fmk (45 µg/animal) vs control. Each line represents 1 animal. Apoptotic index (%) = TUNEL-positive medial SMC nuclei/100 medial SMC nuclei counted.

**Figure 2.** Representative samples comparing control (upper panels) vs high-dose ZVAD-fmk–treated (lower panels) arteries. DAPI stains all SMC nuclei blue. B, TUNEL assay (apoptotic SMC nuclei, indicated by red fluorescence) demonstrates more apoptosis in control vs treated arteries. C, Caspase-3 activation (green, C92-605 mAb) was greater in controls vs treated arteries.
infarcting rat myocardium have been shown to limit infarct size. By directly delivering ZVAD-fmk to the arterial wall during balloon injury, we achieved substantial early inhibition of SMC apoptosis. Although an intact endothelium may be an important regulator for proliferation control, ZVAD-fmk administration did not significantly alter reendothelialization at 2 weeks in the present study. However, ZVAD-fmk did suppress the amount of detectable caspase-3 activity. These results indicate that the primary effect of ZVAD-fmk was via inhibition of SMC apoptosis.

Apoptosis and Neointimal Hyperplasia

Apoptosis occurs in rabbit iliac arteries after balloon angioplasty within 30 minutes, resulting in a substantial loss of medial SMCs within 4 hours. SMCs closest to the lumen undergo the greatest degree of apoptosis. In one rabbit model, the degree of intimal SMC apoptosis and proliferation peaked at 7 days, with the amount of intimal hyperplasia greatest at 28 days. In a rabbit model comparing balloon angioplasty with stent implantation, a greater degree of early SMC apoptosis was seen in stented arteries, and this corresponded to an increase in the amount of neointima formation at 4 weeks compared with balloon-only injured arteries.

Our model targets the early inhibition of SMC apoptosis to decrease subsequent neointimal hyperplasia. Apoptosis may play a critical signaling role in the induction of SMC proliferation and migration, and, consequently, its inhibition may prevent these processes. This may be triggered by alteration in the interaction between SMCs and their extracellular matrix in response to stretch injury. Stretch injury and

### Analysis of Control Versus ZVAD-fmk–Treated Arteries

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<th>Control</th>
<th>Treated</th>
<th>P Value</th>
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<td>Intermediate cohort (2 weeks), percentage reendothelialization</td>
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<td>Chronic cohort (4 weeks), neointimal area</td>
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<td>0.22±0.20 mm²</td>
<td>&lt;0.005</td>
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<tr>
<td>Chronic cohort (4 weeks), ratio of neointimal area to medial area</td>
<td>0.80±0.23</td>
<td>0.44±0.19</td>
<td>&lt;0.001</td>
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![Figure 3](http://circ.ahajournals.org/)

Figure 3. Intermediate-cohort (2 weeks) arteries stained with anti–CD-31 for endothelial cells (brown, arrows). There was no statistically significant difference in the degree of re-endothelialization between control (A) and ZVAD-fmk–treated (B) arteries.
arterial barotrauma have been implicated in the initiation of apoptosis.\textsuperscript{19,25} The disruption of the extracellular matrix has also been shown to facilitate apoptosis as well as SMC phenotype modulation.\textsuperscript{26,27} Those SMCs that do not undergo apoptosis after balloon injury are phenotypically altered to proliferate and migrate to form the neointima.\textsuperscript{28} This phenotypic change may represent a survival mechanism for SMCs in the face of apoptosis by neighboring cells, and the inhibition of apoptosis could abort this process.

Stretch injury may also induce apoptosis by stimulating mitogen-activated protein kinases (MAPKs), a family of serine/threonine protein kinases that phosphorylate transcription factors necessary for the expression of genes involved in SMC growth and proliferation.\textsuperscript{29–31} MAPK activity has been shown to markedly increase within 5 minutes after balloon injury in porcine carotid and coronary arteries.\textsuperscript{29} Similarly, within 10 minutes after balloon injury, rabbit carotid arteries showed a severalfold increase in the activation of stress-activated protein kinase (SAPK), an MAPK subtype.\textsuperscript{30} SAPK activation has been shown to promote caspase activation. Within 30 minutes of balloon injury, these arteries exhibited SMC apoptosis. However, arterial segments treated locally with the antioxidant N-acetylcysteine immediately after balloon injury demonstrated less SAPK activation and a substantial reduction in SMC apoptosis. Lastly, rabbit carotid arteries dilated by balloons coated with ceramide, a sphingolipid-derived second messenger, exhibited immediate inhibition of extracellular signal-regulated kinases, another MAPK subtype.\textsuperscript{31} There was also less SMC apoptosis observed in the ceramide-treated arteries. At 2 weeks, these arteries showed a dramatic reduction in neointimal hyperplasia. Together, these experiments establish a potential link between stretch injury, MAPK activation, apoptosis, and neointimal hyperplasia. Our results also support this sequence of events as a possible pathway for balloon injury–mediated neointimal hyperplasia.

Existing strategies to prevent neointimal hyperplasia primarily target SMC cell replication. Paclitaxel inhibits SMC proliferation and migration via microtubule polymerization. In a rabbit carotid artery restenosis model, locally delivered paclitaxel led to a reduction in neointimal area similar to that observed in the present study (0.36 to 0.26 mm\textsuperscript{2}, 28%, versus 0.33 to 0.22 mm\textsuperscript{2}, 33%).\textsuperscript{24} Rapamycin inhibits cyclin-dependent kinases, blocking cell cycle progression at the G1/S transition.\textsuperscript{32} It also inhibits SMC migration. In a rabbit iliac artery model, rapamycin-coated stents achieved a significant reduction (23% low dose, 45% high dose) in neointimal area compared with uncoated stents at 4 weeks.\textsuperscript{33} The degree of neointimal inhibition with rapamycin is similar to that observed in the present study. However, when applied clinically, rapamycin-eluting stents were shown to dramati-
cally reduce clinical restenosis rates at 1 year in coronary arteries. This suggests that this degree of reduction in neointimal hyperplasia observed in an animal model can signify a substantial improvement in patient outcomes after percutaneous coronary intervention.

Conclusions
The present study shows a link between early-onset apoptosis after balloon injury and neointimal hyperplasia. The successful result obtained with the local delivery of ZVAD-fmk lends promise for a potential clinical application of this antirestenotic strategy.

Acknowledgments
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References
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