Determinants of Vascular Smooth Muscle Cell Apoptosis After Balloon Angioplasty Injury
Influence of Redox State and Cell Phenotype
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Abstract—We have observed that acute medial cell loss is an initial event in the response to vascular injury induced by balloon-catheter distention of the rabbit carotid artery. Numerous apoptotic medial cells were observed as early as 30 minutes after balloon inflation, and a 70% loss of cellularity was evident by 90 minutes. Balloon injury was associated with oxidative stress as reflected by a fall in glutathione levels by 63% within 30 minutes after injury. We hypothesized that balloon injury activated a redox-sensitive signaling pathway coupled to the regulation of apoptosis. Indeed, the activity of the proapoptotic signal mediator, stress-activated protein kinase, was increased severalfold within 10 minutes after injury. Moreover, modifying the vascular redox state by the administration of 1 of 2 structurally dissimilar antioxidants, N-acetyl cysteine or pyrrolidine dithiocarbamate, markedly attenuated both stress-activated protein kinase activation and the induction of apoptosis at 30 minutes. We hypothesized further that the induction of vascular smooth muscle cell apoptosis is modulated by phenotype. In contrast to medial cells, we observed that neointimal cells were relatively resistant to apoptotic death induced by angioplasty injury. This resistance to balloon injury–induced death was associated with an upregulation of the antiapoptotic mediator bcl-xL. This study suggests that acute apoptotic cell death after vascular injury is a highly regulated process governed by cellular redox state and the relative expression of antiapoptotic genes. Angioplasty-induced vascular cell apoptosis may be an important determinant of vascular remodeling and restenosis. (Circ Res. 1999;84:113-121.)

Key Words: balloon angioplasty • apoptosis • stress-activated protein kinase • bcl-x • restenosis

Although balloon angioplasty is one of the most common cardiovascular procedures performed in the industrialized world, the cellular mechanisms that govern whether the procedure has long-term success or results in restenosis remain poorly characterized. In previous characterizations of balloon-catheter injury models, it has been well documented that mechanical injury promotes an initial loss of cellularity.1,2 This acute cell loss has been presumed to involve cell death by necrosis as a result of traumatic damage induced by mechanical injury. However, recent studies by our laboratory and others have documented that programmed cell death or apoptosis is observed early after balloon distention injury and may contribute to the early medial smooth muscle cell loss.3 The cellular and molecular mechanisms that govern acute apoptosis after balloon injury remain to be characterized. Although the signal transduction pathways involved in regulating apoptosis are poorly defined, emerging evidence suggests that members of the mitogen-activated protein kinase family of mediators may play an important regulatory role. For example, activation of stress-activated protein kinase (SAPK) occurs in response to a variety of extracellular stimuli associated with apoptosis, such as cytokines, Fas ligand, and biomechanical stretch.4–6 Moreover, an important mediator role is inferred by the demonstration that blockade of SAPK activation prevents cell death.7,8 One potential mechanism by which SAPK activation promotes apoptosis is suggested by recent reports that indicate that SAPK promotes the activation of the caspases that mediate the process of cellular suicide.9

In addition to mediators in the mitogen-activated protein kinase family, an emerging body of evidence indicates that reactive oxygen species may also modulate the cell suicide program.10–12 The potential importance of reactive oxygen species as signaling mediators involved in the response to vascular injury can be inferred from animal studies as well as from clinical trials that have documented modulatory effects of antioxidants on the process of restenosis.13–15 Indeed, a recent study has demonstrated that antioxidant vitamins reduce the levels of superoxide anion produced in vessels after injury.16 Accordingly, our study tests the hypothesis that vascular smooth muscle cell (VSMC) death after balloon injury is governed by intrinsic cellular properties coupled to redox-sensitive regulatory mechanisms.
Materials and Methods

Injury Models

Balloon Distention Injury

Normal Carotid Artery
Experiments were performed on 87 male New Zealand White rabbits weighing 3 to 4 kg. All experiments were performed in accordance with a protocol approved by the Standing Committee on Animals, Harvard Medical School. Rabbits were anesthetized with an intramuscular injection of 0.25 mL xylazine (20 mg/mL) and 2.25 mL ketamine hydrochloride (100 mg/mL). The left common carotid artery was exposed by a 6-cm midline cervical incision. Proximal and distal blood flow was occluded by clamping. Polyethylene 10 tubing was inserted retrograde into the internal carotid artery and advanced into the left common carotid artery. After gentle flushing of the artery with normal saline, the tubing was removed and a 2-French (F) Fogarty embolectomy balloon catheter was inserted. Balloon inflation to 1.5 to 1.8 times the external diameter of the artery was achieved by caliper measurement under stereomicroscopy. After holding the inflation for 30 s, the catheter was removed.

Model of Reinjury
Initial Injury. The left common carotid artery was exposed, and a 2-F Fogarty embolectomy balloon catheter was inserted retrograde through the internal carotid artery. The balloon was inflated until moderate resistance to catheter withdrawal was felt. The catheter was withdrawn to the carotid bifurcation, deflated, and then readvanced. The procedure was repeated for a total of 3 times before catheter removal, ligation of the internal carotid artery, and closure of the incision. After a 4-week period to allow formation of a neointima, the left common carotid artery was reexplored, and a 2-F embolectomy catheter was inserted retrograde through the facial artery. A balloon distention injury to 1.5 to 1.8 times the vessel diameter was performed as described above, the facial artery was ligated, and the incision was closed.

Endothelial Removal
The left carotid artery was exposed, flushed with normal saline, and cannulated retrograde via the internal carotid artery with a 2-F Fogarty embolectomy balloon catheter as described above. Under stereomicroscopic visualization, the balloon was inflated until minimal resistance to catheter withdrawal was felt without affecting the external diameter of the vessel. The balloon catheter was withdrawn to the carotid bifurcation, deflated, and readvanced. The procedure was repeated for a total of 3 times before removing the catheter. The extent of denudation achieved by this method (>80%) was assessed in separate animals by staining with Evans blue.

Local Antioxidant Administration
The left carotid artery was exposed, cannulated, and flushed with normal saline as described above. After performing the balloon distention injury described above, polyethylene 10 tubing was advanced 1 cm into the common carotid artery. The artery was gently flushed and incubated at a nonstressing pressure for 30 minutes with either PBS (vehicle), 100 μmol/L pyrrolidine dithiocarbamate (Sigma Chemical), or 50 mmol/L N-acetylcyesteine (NAC) (Sigma). After incubation, the tubing was removed and blood flow was restored.

Tissue Analyses

Tissue Preparation
At the end of each experimental protocol, animals were euthanized by intravenous pentobarbital overdose. Carotid vessels were quickly harvested, pressure fixed for 15 minutes at 100 mm Hg in 10% neutral buffered formalin, and immersed in the same fixative for ≥24 hours.

Analysis of Apoptosis

DNA Chromatin Morphology
After fixation, the vessels were stained for 2 hours at 37°C with Hoechst 33342 (5 μg/mL in PBS) (Molecular Probes), and the luminal surface was viewed en face under UV microscopy. Vessels were analyzed in a blinded fashion for normal versus condensed and coalesced (apoptotic) nuclear chromatin morphology.17

DNA Laddering
At the time of vessel harvest, total cellular DNA was extracted from unfixed vessels, as previously described.17 Five-microgram samples were size fractionated by 2% agarose gel electrophoresis and stained for 30 minutes with SYBR Green I (1:10 000 dilution in Tris acetate EDTA) (Molecular Probes) before UV visualization.

Assessment of Cellularity
After fixation, vessels were stained with Hoechst 33342 as described above, and the luminal surface was viewed en face under UV microscopy (×100). As an assessment of cellularity, a total fluorescence index (nuclear staining index) was obtained by computer-assisted analysis (Sony charge-coupled device camera and NIH Image software) and averaged over 5 fields of view. The total fluorescence index represents nuclear staining in the 2 most luminal layers of medial smooth muscle cells. In pilot experiments, this index correlates with en face manual cell counts.

Glutathione Assay
Total cellular glutathione levels were analyzed with an enzymatic recycling assay in which glutathione was oxidized by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of glutathione reductase, as previously described.18 Carotid artery segments were immediately frozen in liquid nitrogen, pulverized, homogenized in lysis buffer (10 mmol/L Tris, 1% SDS), and centrifuged at 10 000 rpm at 4°C for 15 minutes. A small aliquot of the supernatant was removed for protein determination with a bicinchoninic acid (BCA) protein assay (Pierce Chemical Co). To assay for total glutathione, 700 μL of 0.3 mmol/L NADPH (Sigma), 100 μL of 6 mmol/L DTNB (Sigma), and 200 μL of sample homogenate were warmed to 30°C. Glutathione reductase (50 U) (Sigma) was then added, and the rate of 2-nitro-5-thiobenzoic acid formation was monitored over a linear time frame at 412 nm. Glutathione concentration was determined by comparison with a standard curve generated with glutathione standards. Total cellular glutathione content was expressed as μg of glutathione/mg protein.

SAPK Activity Assay
Rabbit carotid arteries were rinsed with PBS and snap frozen in liquid nitrogen. Samples were homogenized in buffer C (10 mmol/L Tris HCl, 5 mmol/L EDTA, 50 mmol/L NaF, 50 mmol/L NaCl, 1% v/v Triton X-100, 0.1% wt/vol fatty acid-free BSA, 20 μg/mL aprotinin, and 2 mmol/L Na3VO4) and centrifuged at 14 000 rpm at 4°C. Samples were then repeatedly washed with buffer C, followed by buffer D (50 mmol/L Tris HCl, 0.1 mmol/L EGTA, 0.5 mmol/L Na3VO4, and 0.1% β-mercaptoethanol). The pellets were resuspended in kinase buffer (20 μmol/L ATP, 20 mmol/L HEPES, 20 mmol/L MgCl2, 20 mmol/L β-glycerophosphate, 2 mmol/L DTT, and 0.1 mmol/L Na3VO4), 1 μCi of [γ-32P]ATP, and 2 μg of glutathione 3-transferase c-Jun (Upstate Biotechnology), and incubated at 30°C for 20 minutes. Laemmli sample buffer was added, and samples were boiled for 3 minutes before being loaded on a 12% SDS gel. Following SDS-PAGE, the gel was stained overnight, fixed, dried, and analyzed by autoradiography.
Immunohistochemistry

We assessed immunohistochemical localization of bcl-x within samples obtained from rabbit carotid artery neointimal hyperplasia lesions (4 weeks after balloon injury). Rabbit polyclonal anti-bcl-x antibody (Transduction Laboratories) and a control rabbit IgG (Sigma) were biotinylated (HTI Bio-Products) and used as primary antibodies. Acetone-fixed 6-μm cryosections were treated with peroxidase block (Dako Corp), 10% goat serum and 2% BSA. Samples were sequentially incubated with primary antibody (25 μg/mL), streptavidin peroxidase (Dako Corp), and 3-amino-9-ethylcarbazol and photographed under light microscopy (×200).

Immunoblot

For immunoblot analysis, segments of normal rabbit carotid arteries or the neointima of balloon injury–induced lesions were selectively removed by fine dissection, pulverized in liquid nitrogen, and homogenized in lysis buffer (10 mmol/L Tris and 1% SDS solution). Protein concentrations were determined by a BCA assay (Pierce Chemical). Cell lysates (50 μg) were loaded on a 12% SDS-polyacrylamide gel, electrophoretically transferred to a nitrocellulose membranes (Hybond enhanced chemiluminescence, Amersham), and the membrane was stained with india ink to verify equal loading and transfer efficiency. The membrane was blocked in PBS, 0.1% Tween, and 5% nonfat dry milk and probed with a biotinylated polyclonal bcl-x antibody (1 μg/mL) (Transduction Laboratories), followed by a streptavidin-biotinylated horseradish peroxidase–linked secondary antibody (1:100 dilution) (Dako Corp), and detected with electrochemical luminescence detection reagents (Amer- sham) by autoradiography.

Statistical Analysis

Tests for significance of differences were made by Student’s t test using the program StatView. Significance was determined as P<0.05.

Results

Acute Induction of Apoptosis After Vascular Injury

In our initial studies, we performed a time course analysis to define the onset and mode of cell death after injury. As shown in Figure 1, we observed evidence of apoptotic nuclei within the media as early as 30 minutes after injury and ongoing cell death over the ensuing 24 hours. This process of cell death was most readily apparent at the early time points within the innermost layers of the media (M) after staining with Hoechst 33342 (×300 UV photomicroscopy). L indicates lumen (n=12; representative experiment). It is noteworthy that substantial cell death occurs without a significant inflammatory cell influx into the vessel wall.

The medial smooth muscle cells exhibited the characteristic condensed and fragmented nucleus typical of apoptotic cell death. To further verify that the mode of cell deletion...
involves an apoptotic process, we analyzed extracted DNA from the injured vessels to determine the pattern of DNA fragmentation by gel electrophoresis. These studies confirmed that DNA harvested from balloon-injured vessels is cleaved into the classical internucleosomal pattern typical of cell death by apoptosis (Figure 1). Taken together, these data indicate that acute cell death after balloon injury involves the activation of an intrinsic cell death program.

We postulated that the induction of apoptosis may be determined by either the mechanical stimulus of overdistention or blood-borne humoral mediators that come in close proximity to medial cells after denudation of the endothelium. To address this question, we initially examined the effect of gentle denudation of the endothelium in a manner that avoids significant distention injury to the medial layer using methods similar to those previously described. Although gentle endothelium denudation results in the exposure of medial cells to blood flow and blood-borne elements, we did not see evidence of medial smooth muscle cell death (data not shown). Thus exposure to blood-borne elements after denudation of the endothelium is not a sufficient stimulus to induce apoptotic cell death of medial smooth muscle cells.

On the basis of these findings, we hypothesized that the mechanical distention of the vessel wall may be a critical determinant of cell death after balloon inflation injury. To further test this hypothesis, we performed experiments in which we induced balloon injury in the usual manner but prevented subsequent contact with blood-borne elements by ligating the vessel to prevent blood flow into the carotid artery after injury. Assessment of vessel cellularity at 24 hours after injury in the absence of blood flow revealed significant apoptotic cell death and acellular regions within the medial layer (Figure 2). In contrast, uninjured control vessels subjected to a similar occlusion of blood flow manifested preserved medial smooth muscle cell viability during this time interval. Thus, distention injury created by balloon inflation appears to be a sufficient stimulus for the induction of cell death.

Although we established that endothelial denudation and exposure to blood-borne elements is not sufficient to induce medial cell death, we hypothesized that exposure to blood-borne elements may modulate the kinetics of the response to the mechanical distention. Indeed, in a time-course analysis, we observed that the induction of cell death after vascular injury was substantially delayed in the absence of blood flow. As shown in Figure 2, medial cell viability is maintained at 2 hours after vascular injury in the absence of flow, whereas there is substantial cell death.

Figure 2. Determinants of balloon injury–induced apoptosis. 1, Mechanical distention without blood reflow is sufficient to induce medial cell loss. A, En face view of the media of a rabbit carotid artery after staining with Hoechst 33342. Twenty-four hours after balloon distention injury and ligation to prevent blood reflow, significant cell loss is evident by UV microscopy (×200). B, In contrast, normal cellularity is seen in control ligated uninjured artery (n=3; representative experiment). 2, Exposure to blood-borne elements accelerates the induction of apoptosis after balloon distention injury; DNA laddering. In contrast to the DNA fragmentation seen in balloon-injured vessels 30 minutes after blood reflow (lane 2), injured vessels in which blood reflow was prevented showed no evidence of acute apoptosis (lane 1). Lane 3, 100-bp size marker (n=3; representative experiment).
in injured vessels exposed to blood flow. Hence, blood flow and exposure to blood-borne elements markedly accelerated the time-course kinetics of apoptotic cell death induced by balloon distention injury.

Molecular Modulators of Acute Apoptosis: Role of Cellular Redox State

To further elucidate the regulatory mechanisms that may lead to acute cell death after balloon distention injury, we examined the role of SAPK. Activation of SAPK occurs in response to a variety of extracellular stimuli associated with apoptosis such as cytokines, Fas ligand, and biomechanical stretch. It has recently been established that stimulation of SAPK activation promotes activation of the caspases that mediate apoptotic cell death. Moreover, blockade of SAPK activation results in the prevention of cell death. We postulated that SAPK activation may be associated with cell death after balloon injury. As shown in Figure 3, a normal, uninjured vessel exhibits low levels of SAPK activity. In contrast, there is a dramatic increase in SAPK activity within 10 minutes after balloon distention injury and blood reflow.

We were intrigued by the observation that blood flow appears to accelerate the induction of apoptosis after vascular injury. Accordingly, we hypothesized that exposure to blood flow and blood-borne elements may promote vascular injury and subsequent cell death by promoting oxidative stress. To test this hypothesis, we measured vascular tissue levels of glutathione, an endogenous antioxidant that serves as a marker of the cellular redox state. Glutathione levels in normal vessels were compared with vessels harvested 30 minutes after balloon distention injury. As shown in Figure 4, normal vessels had glutathione levels of 7.0 ± 1.0 μg glutathione/mg protein. However, in response to vascular injury, glutathione levels fell rapidly by 63% to a mean value of 2.6 ± 0.4 μg glutathione/mg protein (n = 6; P < 0.001). Thus, balloon distention injury and the subsequent induction of cell death were associated with oxidative stress and a depletion of vascular glutathione content.

An emerging body of evidence indicates that reactive oxygen species may modulate the cell fate programs regulating cell growth as well as apoptosis. We hypothesized that reactive oxygen species generated in response to balloon distention and blood-borne elements may activate a redox-sensitive pathway linked to SAPK activity and the induction of apoptosis. To test this postulate, we examined the effect of treating vessels immediately following balloon distention and before blood reflow with the antioxidant NAC. As shown in Figure 5, NAC treatment significantly reduced SAPK activation in response to balloon distention injury. These findings suggest that SAPK is part of a redox-sensitive pathway associated with the acute induction of medial smooth muscle cell apoptosis in response to balloon injury. To further confirm this working hypothesis, we examined whether blockade of oxidative stress and inhibition of SAPK activity in response to antioxidants would also prevent apoptotic cell death after balloon injury. In vessels treated with vehicle, we observed cell loss of 70 ± 9% 90 minutes after injury (Figure 6). In contrast, treatment of the vessel immediately following balloon distention and before blood reflow with the antioxidant NAC markedly reduced the induction of cell loss from 70 ± 9% to 15 ± 5%. To further verify the specificity of this response, we assessed the effect of a mechanistically distinct antioxidant, pyrrolidine dithiocarbamate (PDTC). Indeed, treatment of the vessel with PDTC also resulted in a similar reduction in the incidence of cell death in response to balloon distention vascular injury (70 ± 9% to 23 ± 7%). The results obtained by computer-assisted analysis of total fluorescence indices (nuclear staining indices) correlated with manual cell counts (615 ± 27 cells/field [control uninjured], 158 ± 17 cells/field [injured], 512 ± 27 cells/field [NAC], and 457 ± 29 cells/field [PDTC]).
field [PDTC]; n=3; P<0.001 for NAC and PDTC, compared with injured vessels). These findings indicate that the process of cell death after vascular injury is regulated by a redox-sensitive signaling pathway associated with SAPK activation.

### Acute Induction of Apoptosis: Influence of Vascular Cell Phenotype

An abundance of experimental evidence indicates that VSMCs that accumulate within the neointima after vascular injury are phenotypically distinct from medial smooth muscle cells.21 We postulated that the susceptibility to the induction of apoptosis may be modified by the intrinsic properties of neointimal cells that are distinct from medial cells. To test this hypothesis, we examined the effect of balloon injury in vessels with preexistent neointimal lesions. In the single-injury model described above, we noted that the innermost layer of medial vascular cells appeared to be the most vulnerable population of cells to undergo acute apoptotic cell death after balloon injury. We predicted that intimal cells would experience the greatest mechanical stress, as well as the highest exposure to blood-borne elements, and would therefore exhibit a more rapid and marked induction of cell death in response to vascular injury.

As shown in Figure 7, the response of medial smooth muscle cells to balloon distention in vessels with preexistent neointimal lesions was remarkably similar to the response in normal vessels. Balloon distention injury induced medial smooth muscle cell apoptosis within hours after vascular injury. However, in contrast to the response of medial smooth muscle cells, neointimal smooth muscle cells proved to be remarkably resistant to the induction of cell death by distention injury. At 24 hours after vascular injury, large acellular regions were clearly evident within the medial layer, whereas the cellularity of the neointima remained intact. Apoptotic cells were readily apparent within the medial layer but were rarely observed within the neointima in response to acute injury. These data indicate that a component of the distinctive intimal VSMC phenotype appears to involve an intrinsic antiapoptotic mechanism that renders them resistant to the proapoptotic effects of balloon distention injury.

Previous studies in nonvascular cells have indicated that the regulation of apoptosis involves the balance in expression of proapoptotic mediators such as bax and antiapoptotic mediators such as bcl-2 or bcl-x.22 Accordingly, we postulated that the relative resistance of intimal smooth muscle cells to the activation of apoptosis after vascular injury may involve an alteration in the expression of antiapoptotic genes as a component of the intimal cell phenotype. In our initial pilot studies, we detected very little expression of bcl-2 in the medial VSMCs (data not shown). Similarly, we observed relatively low levels of the antiapoptotic mediator bcl-x in the medial layer of normal uninjured vessels. However, immunohistochemical analysis of vessels at 4 weeks after vessel injury documented a relative increase in the expression of bcl-x within neointimal smooth muscle cells as compared with the low levels observed in the medial smooth muscle cells (Figure 8). Furthermore, this differential pattern of expression was confirmed by immunoblot analysis of normal medial cells compared with neointimal cells isolated from vascular lesions (Figure 8). Additionally, through immunoblot analysis we confirmed that it is the long form splice variant, bcl-xL, that is predominantly upregulated within neointimal cells. It is the bcl-xL splice variant that is an effective endogenous inhibitor of apoptosis. Overall, these observations are consistent with the hypothesis that the upregulation of an antiapoptotic gene such as bcl-xL by intimal smooth muscle cells may confer a relative resistance to the induction of cell death by balloon injury.

![Figure 6](http://circres.ahajournals.org/)

**Figure 6.** Cell death after vascular injury is regulated by a redox-sensitive mechanism. Shown is a histogram of computer-assisted quantification of cellularity after balloon distention injury. Balloon-injured vessels treated with vehicle alone exhibit a dramatic cell loss 90 minutes after injury (control). In contrast, cell loss is markedly reduced in injured vessels treated with the antioxidant NAC (50 mmol/L) or PDTC (100 μmol/L). *P<0.001; n=6.

![Figure 7](http://circres.ahajournals.org/)

**Figure 7.** Neointimal cells are resistant to apoptosis after balloon distention injury: morphological evidence in a rabbit double-injury model. Shown are UV photomicrographs of rabbit carotid artery cross-sections stained with Hoechst 33342. A, Uniform cellularity is seen within both the intima (I) and media (M) of a nonreinjured control artery (×200). B, In contrast, while a marked cell loss occurs within the media of a reinjured artery 24 hours after balloon distention injury, intimal cellularity is preserved (×250). C, Condensed and coalesced nuclei (arrow) characteristic of apoptosis are seen within the media but not within the intima 24 hours after reinjury (×400) (n=6; representative experiment).
The process of restenosis after balloon angioplasty is a complex and vexing problem. The relative role of pathobiological processes such as cell growth, cell migration, matrix modification, thrombus formation, and vascular remodeling remain areas of controversy and ongoing investigation. The characterization of the initiating factors involved in the response to balloon angioplasty injury has been the focus of drug-discovery programs designed to prevent restenosis. It has been postulated that the process of vascular lesion formation and vascular remodeling involve the parallel activation of cell growth and apoptotic cell death. Recent reports suggest that cell death is observed during the latter stages of vascular lesion formation in animal models of vascular injury as well as human restenotic specimens. During the preparation of the manuscript for this article, Perlman et al reported that the induction of apoptosis is one of the first cellular events induced by balloon angioplasty injury. However, the cellular signaling events regulating cell fate after vascular injury remained to be further defined.

The present study extends the observations by Perlman et al by indicating that the acute induction of apoptotic death in response to balloon distention injury appears to involve a redox-sensitive signaling pathway associated with SAPK activation that is modulated by the expression of endogenous antiapoptotic genes such as bcl-xL.

In addition to providing several lines of evidence that indicate that balloon injury induces acute cell death by apoptosis (nuclear chromatin morphology and DNA fragmentation), we were intrigued by our observations that the process of cell loss does not reflect a passive response to a noxious stimulus typical of necrotic cell death. Indeed, the induction of cell death is subject to regulatory processes and could be modified by interventions that influence cellular behavior. For example, the observation that balloon distention injury in the absence of blood flow failed to induce significant cell death within 2 hours suggests that the mechanical trauma alone is not sufficient to promote the acute cell death and that other cellular signals derived from blood-borne elements must be integrated by the cell to acutely activate the cell death machinery. It is conceivable that blood reflow may be necessary to supply the cellular energy needs (ie, ATP production) to undergo the energy-dependent process of apoptosis. However, our observation that distention injury is sufficient to induce apoptosis in the absence of blood reflow argues against this hypothesis. Similarly, the finding that the induction of cell death could be modified by altering the cellular redox state or inhibited by alterations in vascular injury accelerated the induction of cell death. Although it is likely that cell necrosis also occurs after injury, several lines of evidence indicate that the acute induction of apoptosis is a prominent initial feature of the process of lesion formation and remodeling after vascular injury.

Although the cellular mediators that govern cell fate remain to be further elucidated, a growing body of evidence indicates that reactive oxygen species may be important signaling molecules in the regulation of cell growth as well as cell death. The observation that exposure of medial smooth muscle cells to blood flow and blood-borne elements after vascular injury accelerated the induction of cell death suggested that alterations in the cellular milieu such as the redox state may be a critical determinant of cell fate in this context. Indeed, we documented that balloon injury is associated with a marked reduction in glutathione levels, a marker of oxidative stress within tissue. These findings are consistent with recent reports that have documented increased generation of reactive oxygen species in response to vascular injury or mechanical stretch. Furthermore, we demonstrated that treatment with antioxidants such as NAC and PDTC inhibits the acute induction of cell death. Taken together, these findings suggest that balloon injury induces acute cell death via a redox-sensitive mechanism. This observation is consistent with a number of reports involving nonvascular cells that have indicated that the cellular redox state is an important determinant in the activation of the cell death program. The focus of the current study was on the effect of acute administration of antioxidants on the initial wave of cell death induced by vascular injury. It remains to be determined whether chronic administration of potent antioxidants that modulate vascular redox state exert a long-term influence on subsequent waves of cell death that occur after vascular injury. It is intriguing that studies in large-animal
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models, as well as recent clinical trials, indicate that antioxidant drugs may have particular efficacy in preventing restenosis in response to balloon injury. Further studies are needed to determine whether the salutary effects of antioxidants are mediated by alterations in cell growth, cell death, or matrix modulation.

To further elucidate the regulatory mechanisms that may lead to acute cell death after balloon distention injury, we examined the role of SAPK activation. Previous studies have determined that SAPK activation occurs in response to stimuli associated with apoptosis such as Fas and tissue necrosis factor, as well as biomechanical forces such as stretch. It is also noteworthy that SAPK activation appears to be a necessary condition for apoptotic cell death associated with the disruption of integrin-mediated cell-matrix interactions or anoikis. Furthermore, SAPK activation is a prerequisite for cell death under various conditions, and blockade of SAPK activation can abort apoptotic cell death. A potential central role for SAPK as a mediator of apoptosis can also be inferred from recent studies that link SAPK stimulation with activation of the caspase proteolytic cascade involved in cell execution. Taken together, these findings suggest that SAPK may play an important mediator role as an intracellular signaling pathway leading to cell death. We were intrigued by the possibility that balloon distention injury may involve a similar disruption of integrin-mediated cell matrix interactions. Indeed, we documented for the first time that balloon injury is associated with a marked SAPK activation in association with medial cell death. Furthermore, we demonstrated that treatment with antioxidants such as NAC and PDTC inhibits both SAPK activation and the acute induction of cell death. Overall, these data are consistent with the hypothesis that SAPK activation is part of the redox-sensitive pathway regulating the acute induction of medial smooth muscle cell apoptosis in response to balloon angioplasty injury. However, it is important to note that SAPK activation is neither necessary nor sufficient for apoptosis in some cell types in response to certain stimuli. Clearly, further studies need to be performed to establish a direct causal link between SAPK activation and medial smooth muscle cell apoptosis following balloon angioplasty injury.

On the basis of our observations that alterations in the cellular redox state modulate the activation of the cell death program, we postulated that alterations in vascular cell phenotype may also regulate cell fate in response to vascular injury. A wealth of experimental data has established that the intimal cell phenotype may involve the upregulation of the antiapoptotic mediator bcl-xL compared with medial cells. These data suggest that a novel property of the intimal cell phenotype may involve the upregulation of antiapoptotic genes to enhance cell viability in the context of lesion formation. Although there may be multiple mechanisms by which bcl-x prevents cell death, it is noteworthy that expression of related genes is associated with a resistance to oxidative stress and the inhibition of SAPK activation. We postulate that the induction of intrinsic mechanisms (e.g., upregulated bcl-x expression) that prevent activation of the apoptotic death program may be a necessary condition for the accumulation of viable cells within the neointima. Indeed, recent studies from our laboratory support the notion that the upregulation of the antiapoptotic mediator bcl-x may be a necessary condition for neointima formation.

It has become clear that balloon injury is a complex stimulus capable of activating a variety of cellular functions. It remains to be determined whether the induction of acute apoptosis promotes deleterious sequelae such as neointima formation and constrictive remodeling or whether the induction of vascular cell death ameliorates the course of disease by reducing cellularity of the lesion and promoting enlarge ment remodeling. The recent clinical trials indicating that antioxidant drugs may have particular efficacy in preventing restenosis are intriguing in light of our findings. Future studies are necessary to determine whether there is therapeutic value in either preventing or enhancing this process of cell death after injury. Nevertheless, the findings of this study are
consistent with the postulate that acute cell death after vascular injury is a regulated process that is governed by the intrinsic properties of VSMCs such as the cellular redox state and the relative expression of antiapoptotic genes. These results have important implications for understanding the process of lesion formation after vascular injury in animal models as well as the causes of restenosis after balloon angioplasty in the clinical context.

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