Apoptosis
Basic Concepts and Implications in Coronary Artery Disease

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Abstract—Apoptosis is an active form of cell death that is intricately regulated and distinct from necrosis. Data suggest that apoptosis may play a role in the pathophysiology of coronary atherosclerotic disease. Anatomic evidence of apoptosis has been observed in coronary atherosclerosis, restenosis, and transplant arteriopathy, accompanied by an increase in biochemical and genetic markers of apoptosis. Vasoactive substances such as nitric oxide and angiotensin II also regulate vascular smooth muscle cell apoptosis; vasodilating factors may induce apoptosis, whereas vasoconstricting factors may inhibit apoptosis. The aim of this article is to review key points regarding the detection of apoptosis, its regulation, and its possible role in the pathogenesis of coronary artery disease. (Arterioscler Thromb Vasc Biol. 1999;19:14-22.)

Key Words: apoptosis ▪ cell death ▪ atherosclerosis ▪ restenosis ▪ coronary disease

Methods to Detect Apoptosis
Detection of apoptosis is challenging owing to the dynamic nature of the process. First, apoptosis and removal of the cellular debris can be completed in just a few hours and therefore, the evidence of apoptosis may go undetected, depending on the sampling time. This may lead to the false conclusion that apoptosis does not occur and thus does not contribute to the pathogenesis of a disease. Moreover, there are technical limitations in the currently applied methods for detecting apoptosis.
**Morphology**

In 1965, Kerr first described the 2 distinct morphological types of cell death, apoptosis and necrosis, based on histochemical changes in the lysosomes of ischemic hepatic tissue (see Table 1). When the cells died via apoptosis they had unique morphological features, typically without associated inflammation; this type of cell death continued over weeks after the insult. Morphology continues to be an important method for the detection of apoptosis. Although light microscopy alone can often detect the features of apoptosis, electron microscopy is often used owing to its improved reliability (see Figure 1). Electron microscopy is essential when evaluating cells with a high nucleus-to-cytoplasm ratio because of the difficulty in distinguishing necrosis from apoptosis in these cells. Morphologically, when a cell undergoes apoptosis, the cell shrinks and the chromatin becomes pyknotic and condensed into sharply delineated masses present at the edge of the nuclear envelope. Simultaneously with these changes, cell volume decreases, cytoplasmic organelles compact, and cell density increases.

Microvilli disappear, cytoplasmic blebs form at the cell surface, and then portions of the cell bud to create apoptotic bodies. These apoptotic bodies, or the remainder of the apoptotic cells, may be phagocytized or may remain free and undergo disruption by secondary necrosis. In this circumstance, apoptosis may be associated with inflammation. The maintenance of the integrity of the cell membrane is quite distinct from necrosis and is essential to prevent the promotion of inflammation. Although inflammation is not typically present, mononuclear cells may be present in tissues where apoptosis is being induced. Morphological evaluation for apoptosis may also be confounded by the occasional confusion of apoptotic bodies with autophagic vacuoles.

In addition to light microscopy and electron microscopy for the morphological evaluation of apoptosis, confocal laser scanning microscopy has become an important adjunct in evaluating nuclear chromatin morphology (see Figure 2). To determine the presence of cell death, this technique uses a fluorescent DNA-binding dye, such as propidium iodide. These dyes stain the DNA in cells with increased permeability of the nuclear envelope. Ultrastructural techniques must then be applied to ensure the differentiation between necrotic and apoptotic cells.

**In Situ Hybridization**

Two procedures have been developed that allow in situ detection of apoptosis in formalin-fixed or paraffin-

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**TABLE 1. Key Features Distinguishing Apoptosis From Necrosis**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Apoptosis</th>
<th>Necrosis</th>
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<tbody>
<tr>
<td>Morphology</td>
<td>Volume loss, chromatin condensation, intact organelles</td>
<td>Increased volume, nucleus intact until late, disruption of organelles</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>Remains intact</td>
<td>Early disruption</td>
</tr>
<tr>
<td>DNA breakage</td>
<td>Early, internucleosomal pattern</td>
<td>Later, random DNA breakdown</td>
</tr>
<tr>
<td>Mechanism of DNA breakage</td>
<td>Gene activated, endonucleases</td>
<td>Random injury, ATP depleted</td>
</tr>
<tr>
<td>Tissue reaction</td>
<td>No inflammation, phagocytosis</td>
<td>Inflammatory response</td>
</tr>
<tr>
<td>Calcium</td>
<td>Moderate influx</td>
<td>Massive influx</td>
</tr>
</tbody>
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*Figure 1. Electron microscopy image of an apoptotic cell in the adventitia of the coronary artery from a diet-induced hypercholesterolemic pig. Note the scant amount of cytoplasm. Additionally, the nuclear membrane appears to be intact (arrow), which is distinct from necrosis. Apoptotic bodies (asterisk) are also visible.*

*Figure 2. Confocal microscopy image using propidium iodide in the adventitial layer of a coronary artery from a diet-induced hypercholesterolemic pig. Note the characteristic morphological features of chromatin condensation and nuclear blebbing.*
embedded tissue: terminal deoxynucleotidyl transferase (TdT)–mediated dUTP-biotin nick end labeling (TUNEL) and in situ end labeling (ISEL). Both of these techniques rely on the occurrence of internucleosomal DNA fragmentation by an endonuclease into characteristic 180- to 200-bp segments. Staining with TUNEL is the classic in situ technique for detecting apoptosis (see Figure 3). DNA breaks are labeled within individual nuclei at the 3′-OH end with biotinylated deoxyuridine. This signal is then amplified by avidin-peroxidase. The ISEL technique utilizes DNA polymerase to incorporate biotinylated nucleotides at the DNA strand breaks. The benefits of these techniques are the ability to evaluate a whole section of tissue. The specificity of TUNEL staining has been questioned in recent investigations, since necrotic cells have also been shown at times to stain with this technique. Therefore, studies using TUNEL staining as their only method of detecting apoptosis should be considered cautiously. An additional difficulty with using TUNEL staining in atherosclerotic tissue has been labeling of matrix vesicles by this technique.

Agarose Gel Electrophoresis (DNA Laddering)

The regular cleavage pattern of 180 to 200 bp of DNA created by endonucleases as part of apoptosis creates the classic pattern of DNA laddering when the DNA is evaluated by gel electrophoresis (see Figure 4). This is quite different from necrosis, wherein the DNA breakdown is random and leads to irregular-length DNA fragments and an indistinct pattern on gel electrophoresis. However, apoptosis may occur even without the presence of a DNA ladder pattern on gel electrophoresis in certain cell types. Nevertheless, apoptosis can be incited by adding endonuclease into cells and inhibited by antibody directed toward endonuclease, further supporting the role of this enzyme in apoptosis. Thus, the finding of a regular pattern of DNA fragmentation on gel electrophoresis is still suggestive of apoptosis, but its absence does not exclude apoptosis.

Flow Cytometry

This technique utilizes the flow cytometer to rapidly analyze large numbers of single cells in suspension from either homogeneous or heterogeneous cell populations. With the use of fluorescent dyes, flow cytometry can quantitatively measure the amount of immunofluorescence of individual cells, and the cells can then be classified based on the immunofluorescent intensity. Although flow cytometry has been used mainly to characterize peripheral blood cells, this technique may also be used to characterize other cell types. However, this technique is limited to cells that can be placed into single-cell suspensions and may not be used for tissues. An additional advantage of this method is that in heterogeneous populations, immunophenotyping can be added to characterize the cell types.

To evaluate apoptosis by flow cytometry, multiple known parameters of apoptosis have been employed. These include DNA degradation; reduction in cell volume; and structural changes such as increased plasma membrane permeability, altered intracellular ions, enhanced production of specific gene products associated with apoptosis, and altered plasma and mitochondrial membrane polarity.

Biochemical and Genetic Controls of Apoptosis

There are 4 stages of apoptosis: the initiation or stimulus for cell death, the active programmed cell death when the events become irreversible, phagocytosis of the dead cellular material, and inhibitory mechanisms of apoptosis. Some of the major stimuli and inhibitors of apoptosis are summarized in Table 2 and diagrammed in Figure 5. Initiation of apoptosis can result from multiple stimuli, including heat, toxins, free radicals, growth factor withdrawal, cytokines such as transforming growth factor-β, loss of matrix attachment, glucocorticoids, nitric oxide, and radiation. These stimuli work in conjunction with other intrinsic factors that determine the cell’s potential to undergo apoptosis. For example, smooth muscle cells from a normal blood vessel undergo a high rate of apoptosis with the withdrawal of growth factors such as insulin-like growth factor and platelet-derived growth factor, whereas plaque-derived smooth muscle cells proceed to apoptosis at a high rate even in the presence of these
important growth factors and at an even higher rate without them. Cell type and basal conditions of the cell, including cell cycle and differentiation, markedly influence the likelihood of whether a cell will undergo apoptosis. Moreover, many genes in the complex system of apoptosis regulation also control the cell cycle and cell differentiation, further demonstrating a complex interaction between these 2 processes.

Fas is a cell surface molecule that typically induces apoptosis. This mediator is important in controlling cell death in transformed cells, regulating normal immune responses, and ensuring self-recognition. In certain conditions, Fas activation also regulates T-cell activation and proliferation. Fas-induced apoptosis may be mediated through the sphingomyelin pathway, which leads to ceramide accumulation. Ceramide is an important mediator of apoptosis induced by both the sphingomyelin pathway and via activation of ceramide synthase. Another well-described regulator of apoptosis is c-myc, an example of how the regulation of cellular proliferation and apoptosis can be coupled. This proto-oncogene encodes for a DNA-binding protein that modulates transcription and can act as both a potent inducer of proliferation and a promoter of apoptosis. Evidence from increased levels of c-myc in many tumors emphasizes the importance of this gene in proliferation, but cytokines or oncogenes are required to block the apoptotic effects of c-myc. Thus, the regulation of cellular proliferation and apoptosis by c-myc is linked and modified through the cellular environment.

The interleukin-converting enzyme (ICE) family of proteases, more recently renamed the caspases, is a novel family of cytoplasmic cysteine proteases related to interleukin-1β-converting enzyme (caspase-1). Caspases are believed to play a central role in the apoptosis cell signaling pathway. Inhibitors of caspases cause cells to be resistant to apoptosis when stimulated by multiple mechanisms. This finding supports the theory that multiple stimuli favoring apoptosis converge on 1 or a few central pathways from which the action of cell death proceeds. Although there is supporting evidence for the role of the caspases in apoptosis, it is yet unclear what the exact role this enzyme family has and how the different family members interact.

The bcl-2 protein, which is a membrane-associated protein of the mitochondria, nuclear envelope, and endoplasmic reticulum, is a potent inhibitor of apoptotic cell death. In gene transfer experiments, elevated levels of bcl-2 protein inhibited a number of apoptosis-promoting stimuli, including radiation, antineoplastic agents that cause nuclear damage, viral infection, growth factor withdrawal, and cytotoxic lymphokines. Excess bcl-2 expression in various cell types can lead to inhibition of apoptosis and is an important oncogenic factor in follicular lymphoma. Although this protein is important in the suppression of cell death, other members of the bcl family may activate cell death.

**TABLE 2. Some of the Major Genetic and Biochemical Regulators of Apoptosis**

<table>
<thead>
<tr>
<th>Apoptosis promoters</th>
<th>Apoptosis inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fas</td>
<td>bcl-2</td>
</tr>
<tr>
<td>Caspases (the interleukin-converting enzyme family of proteases)</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>p53</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>Bax</td>
<td></td>
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<tr>
<td>Ceramide</td>
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<tr>
<td>Nitric oxide</td>
<td></td>
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<tr>
<td>Atrial natriuretic peptide</td>
<td></td>
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<tr>
<td>C-type natriuretic peptide</td>
<td></td>
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<tr>
<td>Increased intracellular calcium</td>
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</tbody>
</table>

**Figure 4.** Electrophoretic pattern of DNA fragments in myocytes extracted from the two control hearts (top panel, lanes 1 and 2), from the heart of a patient with idiopathic dilated cardiomyopathy (top panel, lane 3), and from the hearts of three patients with ischemic cardiomyopathy (bottom panel, lanes 1 through 3). A combination of DNA laddering and diffusion is apparent in the bottom panel, lane 2. MW denotes markers of molecular weight. The arrows indicate multiples of 180 bp. Reproduced from Olivetti G, Abbi R, Quaini F, Kajstura J, Cheng W, Nitihara JA, Quaini E, Di Loreto C, Meltrami CA, Krajewski S, Reed JC, Anversa P. Apoptosis in the failing human heart. *N Engl J Med*. 1997;336:1138–1141. Copyright 1997 Massachusetts Medical Society. All rights reserved.
again emphasizes that apoptosis and cellular growth may be regulated in a coordinate fashion.

**Apoptosis in Coronary Diseases**

Cell loss in atherosclerosis has been known since 1858, when Virchow described atherosclerosis as a process of replication of cells within the plaque followed by the death of these cells. More recently, with the further understanding of the mechanisms of apoptosis, there has been a resurgence in the interest in the mechanisms governing cell death associated with atherosclerosis.

Multiple studies in both animals and humans have found apoptosis in atherosclerotic coronary, carotid, and aortic arteries. The major studies are summarized in Table 3. These studies have shown that smooth muscle cells principally located in the intimal fibrotic portion of the atherosclerotic plaque and macrophages located in the intima, especially the lipid-laden core of the atheroma, show increased evidence of apoptosis compared with normal vessels. Apoptosis is also found in smooth muscle cells of the media underlying atherosclerotic lesions and in conjunction with the vasa vasorum and perivascular cells of the adventitia in human atherosclerotic tissue. Furthermore, in atherosclerotic tissue apoptosis is associated with the formation of matrix vesicles rich in calcium and has led to the proposal that apoptosis may be important in the calcification of atherosclerotic tissue. It is yet unclear whether apoptosis is a late finding as part of the end stage of this disease or whether increased apoptosis is associated with the early stages of atherogenesis. In atherosclerosis, evidence supports the role of apoptosis in vascular remodeling; apoptosis may be beneficial by preventing excessive cellular proliferation.

Transplant coronary arteriopathy is a form of accelerated atherosclerosis. In transplant coronary arteriopathy nearly 100% of all endothelial cells and one third of T lymphocytes and macrophages express the Fas receptor. Whereas, there is virtually no expression of the Fas receptor in typical atherosclerotic coronary disease and in normal controls. Additionally, endothelial cells, T lymphocytes, and macrophages are positive for TUNEL staining, and nearly all TUNEL-positive cells are Fas-positive in the tissue from the transplant patients. Although Fas positivity in smooth muscle cells and immune cells is associated with induction of apoptosis, Fas may not trigger apoptosis in endothelial cells. Thus, the significance of Fas on endothelial cells in transplant arteriopathy is speculative. However, the observation that apoptosis as well as cell proliferation is prominent in transplant arteriopathy supports the hypothesis that apoptosis is involved in the early stages of this accelerated form of coronary artery disease.
TABLE 3. Summary of the Major Articles Relevant to Apoptosis in Coronary Diseases

<table>
<thead>
<tr>
<th>Type of Study</th>
<th>Cell Type and Location</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo endothelial denudation of rat aorta</td>
<td>Superficial intimal smooth muscle cells; absence in media</td>
<td>15</td>
</tr>
<tr>
<td>Human thoracic aorta and coronary arteries from autopsies</td>
<td>Smooth muscle cells, macrophages, and T cells in the intima near atherosclerotic lesions; medial smooth muscle cells, medial cells in conjunction with the vasa vasorum and perivascular cells of the adventitia</td>
<td>63</td>
</tr>
<tr>
<td>Human coronary tissue at transplantation and carotid endarterectomy specimens</td>
<td>Smooth muscle cells of the intima in atherosclerotic vessels; few apoptotic cells in the media</td>
<td>14</td>
</tr>
<tr>
<td>Human atherectomy specimens</td>
<td>Smooth muscle cell apoptosis in macrophage-enriched areas of the intima, little apoptosis in the media</td>
<td>65</td>
</tr>
<tr>
<td>Rat vascular balloon injury model</td>
<td>Smooth muscle cells in the neointima</td>
<td>69</td>
</tr>
<tr>
<td>Human saphenous vein grafts</td>
<td>Smooth muscle cells in close proximity to luminal foam cell accumulation</td>
<td>71</td>
</tr>
<tr>
<td>Human atherectomy specimens</td>
<td>Smooth muscle cell apoptosis varies from localized in restenotic tissue to diffuse in de novo atherosclerosis.</td>
<td>69</td>
</tr>
<tr>
<td>Rat and rabbit in vivo balloon injury model of carotid and internal iliac arteries</td>
<td>Medial smooth muscle cell apoptosis, none in the adventitia</td>
<td>64</td>
</tr>
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</table>

Restenosis is also a process of excess cellular proliferation in atherosclerotic vessels. Restenotic lesions have a higher degree of medial smooth muscle cell apoptosis compared with native atherosclerotic lesions. Apoptosis in atherectomy specimens from restenotic tissue shows a different pattern compared with atherosclerotic tissue, with restenotic tissue displaying foci of increased apoptosis compared with a lower incidence of apoptosis in a more diffuse pattern in the atherosclerotic tissue. An important mitogen, basic fibroblast growth factor (FGF), is prominently expressed in restenosis as well as in intimal hyperplasia after endothelial denudation and in unstable coronary plaques. Inhibition of basic FGF induces apoptosis. Therefore, 1 mechanism by which restenosis occurs may be via FGF inhibition of apoptosis, thus favoring the balance toward cellular proliferation. Additionally, in animal studies, apoptosis is prominent in the vascular smooth muscle cells within 30 minutes after balloon injury. Furthermore, apoptosis remains increased in the neointima at least 1 month after injury. When stent implantation was compared with balloon injury, both medial smooth muscle cell proliferation and apoptosis were increased. Additionally, despite an equal rate of cellular proliferation in the neointima, stenting was associated with increased neointimal apoptosis, macrophage accumulation, and increased extracellular matrix secretion. Thus, regulation of the balance between cellular proliferation and apoptosis appears to be a major determinant of restenosis.

In human saphenous vein grafts stenosis is secondary to myointimal thickening and an associated luminal accumulation of foam cells with overlying thrombus. The foam cells prominently stain for markers of proliferation (Ki-67 and proliferating cell nuclear antigen), but in adjacent areas of the vessel there was progression to hypocellular regions of smooth muscle cells with increased apoptosis. The spatial association of foam cells with smooth muscle cell loss suggests that these foam cells may mediate smooth muscle cell apoptosis. Therefore, this study supports the hypothesis that the apoptotic process is intimately associated with the proliferative process.

Apoptosis is also associated with the atherosclerosis risk factors of hypertension and hypercholesterolemia. With the use of explant techniques, cultured aortic vascular smooth muscle cells from spontaneously hypertensive animals showed increased proliferation characterized by increased growth rates with increased [3H]leucine and [14C]leucine incorporation even after multiple passages. Additionally, cultured vascular smooth muscle cells from the aortas of hypertensive rats had a greater apoptotic response to apoptosis inducers such as transforming growth factor-β and tumor necrosis factor-α. Thus, the enhanced cell proliferation in hypertensive animals is paralleled by increased susceptibility to apoptosis. Additionally, decreases in intracellular pH are an important step in apoptosis, and intracellular alkalization is 1 mechanism to prevent apoptosis. Thus, increased Na+/H+ antiporter activity in hypertension may lead to alterations in proliferation and apoptosis regulation. Therefore, hypertrophy of target organs and proliferation of the vascular wall may be followed by atrophy induced by excessive apoptosis of tissues in hypertensive states. Treatment of this complex systemic disease with agents like angiotensin-converting enzyme inhibitors that interfere with the proliferative pathway may help to restore the balance between proliferation and apoptosis. Furthermore, different antihypertensive agents have diverse effects on the rate of apoptosis. Controlling abnormal regulation of both proliferation and apoptosis may therefore be an important treatment strategy.

In coronary atherosclerosis, cholesterol may be important in the induction of apoptosis. Although cholesterol itself has no direct angiotoxicity, it forms cholesterol oxides that have multiple toxic effects on the vasculature. Cholesterol oxides, including 7β-hydroxycholesterol, 7-ketocholesterol, 19-hydroxycholesterol, cholesterol 5α, 6α-epoxide, and 25-hydroxycholesterol all promote the loss of cell adhesion and increase the rate of apoptosis in cultured endothelial cells. Cholesterol oxides promote disruption of actin microfilaments, most notably with the disappearance of stress fibers within the cell body.
Additionally, oxidized LDL increases apoptosis in vitro in a dose-dependent fashion and is associated with increased caspase-3, 1 of the ICE-like proteases also known as CPP32.81 Caspase-3 cleaves actin in cell-free extracts, which may be 1 mechanism by which cholesterol oxides can cause apoptosis.82 However, the role of apoptosis in hypercholesterolemia and early atherosclerosis in vivo remains to be proven.

Vasoactive substances that are often altered in atherosclerosis are regulators of apoptosis. Nitric oxide, an important mediator of vasodilatation, platelet inhibition, and suppression of smooth muscle proliferation, upregulates Fas and induces apoptosis in vascular smooth muscle cells.18,19,83 Other vasodilators such as atrial natriuretic peptide and C-type natriuretic peptide also induce apoptosis in vascular smooth muscle cells.84 Additionally, angiotensin II, a mitogen and vasoconstricting peptide, antagonizes nitric oxide–induced apoptosis.19 Another important vasoconstricting peptide, endothelin-1, also counterbalances apoptotic promoters, and endothelin receptor antagonism is associated with increased apoptosis.20,22 It may be speculated that the critical balance between vasodilators that are growth inhibitors and vasoconstrictors that are growth promoters may involve the apoptotic process. Abnormalities in this balance associated with atherosclerosis may be 1 mechanism of atherosclerosis progression.

Thus, it has been demonstrated that apoptosis is present in atherosclerotic and postangioplasty lesions and confirmed by the additional findings of increases in other known markers of apoptosis. Apoptosis may be enhanced through multiple mechanisms including those associated with hypertension and hypercholesterolemia. The essential regulatory system of cell proliferation and apoptosis is fundamentally important to mediate responses to injury and the subsequent pathological processes in the blood vessels.

In summary, dysregulation of cell proliferation and apoptosis are clearly seen in multiple forms of vascular disease, including hypertension, transplant arteriopathy, and atherosclerosis. Apoptosis may play a significant role in the pathogenesis of coronary atherosclerosis and may be initiated by atherosclerotic risk factors. Previous studies support the hypothesis that a central balance between vasodilators with antimitogenic properties and vasoconstrictors with growth-promoting abilities is a major determinant of the response to injury and the effects of remodeling on the vessel wall. It may be speculated that one of the mechanisms by which this balance contributes to progression or repair in atherosclerosis is through regulation of cell apoptosis. A better understanding of the mediation of these events and a better knowledge of the role of proliferation and apoptosis in the pathophysiology of these disorders will further our understanding of cardiovascular disease and will help us to tailor therapeutic options to some of these important regulatory mechanisms.

Acknowledgments

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