Cardiomyocyte (CM) apoptosis has been reported in a variety of cardiovascular diseases, including myocardial infarction, ischemia/reperfusion, end-stage heart failure, arrhythmogenic right ventricular dysplasia, and adriamycin-induced cardiomyopathy. The role of CM apoptosis in the development and progression of cardiac diseases merits further investigation. Cumulative evidence suggests that reactive oxygen species (ROS), which have been implicated in cardiac pathophysiology, can trigger myocyte apoptosis by up-regulating proapoptotic proteins, such as Bax and caspases, and the mitochondria-dependent pathway. These apoptotic proteins and pathways are inhibited by various antioxidants, as well as by overexpression of the antiapoptotic protein Bcl-2 by way of the antioxidant pathway. Detection of CM apoptosis with the terminal transferase–mediated DNA nick-end labeling assay alone has recently been questioned because of technical concerns regarding its sensitivity and specificity. Because CMs are mononuclear or binuclear, if only one nucleus or a certain percentage of fragmented nuclei is stained with TUNEL assay at the early stage of apoptotic cell death, it remains unknown whether this particular early apoptotic CM is still functionally active. The issue of TUNEL specificity further questions reports of high percentages of apoptotic CM nuclei (0.02%-35%) in the heart. Nevertheless, oxidative stress is a major apoptotic stimulus in many cardiovascular diseases and the process can be inhibited by antioxidants both in vitro and in vivo. (J Lab Clin Med 2003;142:288-97)

Abbreviations: AIF = apoptosis-inducing protein factor; CM = cardiomyocyte; ERK = extracellular signal-regulated protein kinase; ICAD = inhibitor of caspase-activated DNase; iNOS = inducible nitric oxide synthase; JNK = c-jun N-terminal kinase; MAPK = mitogen-activated protein kinase; MCSF = macrophage colony-stimulating factor; NO = nitric oxide; ONOO⁻ = peroxynitrite; PARP = poly (ADP-ribose) polymerase; ROS = reactive oxygen species; SOD = superoxide dismutase; TNF-α = tumor necrosis factor-α; TUNEL = terminal transferase–mediated DNA nick-end labeling.
dows exist for CM apoptosis, and (3) these time windows play an important role in the progression of heart disease and the subsequent development of heart failure. Oxidative stress and apoptosis have been reported to play significant roles in the progression of myocardial infarction, ischemia/reperfusion, hypertension, cardiomyopathies, and atherosclerosis. Apoptosis has been recently reviewed. The purpose of this article is to review the current knowledge about apoptosis in the heart, the role of ROS-dependent apoptotic pathways, and potential therapeutic interventions.

FREE RADICALS

Every atom contains a nucleus, which is surrounded by 1 or more pairs of electrons. Any single electron in an orbit is called an unpaired electron. An unpaired electron around the nucleus constitutes a free radical. Free radicals are highly reactive with radical and non-radical derivatives. ROS include free radicals (superoxide [O$_2^-$] and hydroxyl [OH$^-$] radicals) and non-free radicals (hydrogen peroxide (H$_2$O$_2$) and hypochloride [HOCl]). ROS, highly toxic byproducts of aerobic metabolism, react unfavorably with surrounding macromolecules, resulting in severe cell and tissue damage. Molecular oxygen is a major source of free-radical formation in intracellular and extracellular environments. Intracellular ROS (eg, O$_2^-$, OH$^-$, 1O$_2$, ONOO$^-$, and H$_2$O$_2$) from normal aerobic metabolites or accidental cellular insults (eg, UV radiation, ionization, cigarette smoking) cause a continuing process of cell

Fig 1. A schematic representation of structural and biochemical changes after apoptosis and necrosis in CMs.
OXIDATIVE STRESS AS AN APOPTOTIC STIMULUS

Recent evidence indicates that apoptosis occurs in cardiovascular diseases and may play a significant role in the development of heart failure. Despite intensive investigation of apoptosis in cardiovascular diseases, the exact stimulus of apoptosis remains controversial. The balance between endogenous apoptotic stimuli and inhibitors decide the fate of the cell (ie, death vs survival). Many apoptotic stimuli in the heart have been recognized, including oxidative stress, serum withdrawal, angiotensin II, hyperglycemia, pressure overload, mitochondrial dysfunction, proapoptotic factors such as TNF-α, and loss of CM survival factors.

“Oxidative stress” refers to the cytopathologic consequences of an imbalance between the production of free radicals and the defense system, the antioxidants. Recent data from in vitro and experimental studies in the heart suggest that oxidative stress plays an important role in CM cell death by way of apoptosis or necrosis. Exposure to UV radiation and ionization, which generate such ROS as H2O2 and OH•, are known to cause apoptosis. High doses of H2O2 induce necrosis, whereas low doses cause apoptosis in a variety of cell types, including CM. The latter occurrence confirms that oxidative stress is a mediator of apoptosis. Another oxidant, NO•, has also been found to act as an inducer of apoptosis in monocytes, macrophages, and CM. The anticancer drug adriamycin, although not a free radical, is also a strong inducer of oxidative stress and apoptosis. TNF-α also induces CM apoptosis in the heart; this effect is mediated by oxidative stress.
extent of apoptosis. Myocardial apoptosis has been suggested as the initiating factor in postinfarction left-ventricular remodeling.\textsuperscript{24}

Apoptosis and necrosis have been examined at intervals ranging from 12 hours to 2 weeks in explanted hearts.\textsuperscript{20} Apoptosis was present in early stages of myocardial infarction and was the first and predominant form of CM cell death; no apoptosis was found between 5 days and 2 weeks. This study suggests that apoptosis in human myocardium is an early form of CM cell death in myocardial infarction and may give way to necrosis in the older infarct.\textsuperscript{20} There appears to be a time window for apoptosis after myocardial infarction; this has also been documented in the pathogenesis of heart failure caused by adriamycin.\textsuperscript{25}

In atherosclerosis, the exact mechanism of cell death vis-à-vis apoptosis versus necrosis is far from clear. In human atheroma, macrophages, endothelial cells, and smooth-muscle cells have been reported to be positive for apoptosis, whereas no apoptosis was noted in non-atherosclerotic regions. The apoptotic range in atherosclerotic plaques is highly variable (1\%–30\%)\textsuperscript{26,27} and varies with the stage of the atherosclerotic plaque, so that the apoptotic cell count is directly related to the stage of the atherosclerotic plaque. Specimens of peripheral arteries from patients with repeat stenosis showed a higher incidence of apoptosis compared with that in primary lesions.\textsuperscript{26} In another study of 35 patients, 25 atherosclerotic samples showed apoptosis.\textsuperscript{27}

In atherosclerosis, the activation of apoptosis in endothelial cells, macrophages, and smooth-muscle cells could be a result of increased oxidative stress, TNF-\(\alpha\), and oxidized low-density lipoprotein.\textsuperscript{1,2,28,29}

Oxidative stress has been reported to play a key role in adriamycin-induced heart failure,\textsuperscript{11} but the dominant mechanism of myocyte cell death in this syndrome (apoptosis, necrosis, or both) remains a matter of controversy. Adriamycin has been suggested to cause apoptosis in variety of tissues, including kidney, hair follicles, and intestine.\textsuperscript{30–32} Adriamycin has also been reported to cause apoptosis in the interstitial cells and macrophages in the heart.\textsuperscript{30} Adriamycin-induced apoptosis in vitro\textsuperscript{33} and in vivo is mediated by oxidative stress.\textsuperscript{25} The amount of apoptosis in the standard model of adriamycin cardiotoxicity, examined at 4, 10, 16, and 21 days after adriamycin administration, was highest at 4 days, had decreased significantly at 10 days, and again showed an increasing trend between 16 and 21 days.\textsuperscript{25} This biphasic response of apoptosis in adriamycin toxicity is difficult to explain, but this study also suggests the existence of an apoptotic time window, which merits further investigation to define its precise role in cardiac dysfunction.\textsuperscript{25} Taken together, these findings suggest that apoptosis plays an important role in the pathogenesis of adriamycin cardiomyopathy, which is mediated by oxidative stress and leads to congestive heart failure.

**NITRIC OXIDE AND APOPTOSIS IN THE HEART**

The free-radical gas NO is generated from L-arginine by way of an enzymatic reaction of a family of enzymes, including neuronal NO synthase and endothelial NO synthase, that are known as constitutively active isoforms, as well as a third isoform known as inducible nitric oxide synthase (iNOS).\textsuperscript{34} NO plays a physiologic as well as a pathologic role in vascular and cardiac diseases.\textsuperscript{34} The exact role of NO and under what conditions NO shows its dual action, cytoproteective and toxic, is not clear. The toxicity of NO is significantly increased when ONOO\(^{−}\) is yielded as a result of chemical reaction between NO and superoxide (O\(_{2}^{−}\)) in the cell.\textsuperscript{35} It has been reported that NO is proapoptotic in many cell types, including CMs.\textsuperscript{35} Recently Arstall et al\textsuperscript{35} reported that NO produced by iNOS induces apoptotic cell death in neonatal and adult CMs. CM apoptosis induced by NO alone has been shown to be significantly less than that induced by ONOO\(^{−}\).\textsuperscript{35} NO alone or ONOO\(^{−}\) have also been reported to cause a decrease in myocardial function\textsuperscript{36} and to induce CM apoptosis.\textsuperscript{37} Transgenic animal studies have shown that iNOS is not responsible for early left-ventricular remodeling 1 month after myocardial infarction but that it contributes significantly to left-ventricular remodeling 4 months after infarction by increasing apoptosis and decreasing myocardial function.\textsuperscript{38} In contrast, NO has also been shown to exert cytoprotective effects in vascular and cardiac tissues.\textsuperscript{34} The antiapoptotic effects of NO have also been reported in different cell lines, including CM after ischemia/reperfusion.\textsuperscript{39} Overall, the mode of action of NO is complex and the controversy may be a result of the short half-life of NO, the formation of ONOO\(^{−}\), the use of different animal models, and the source of NO formation from the different isoforms.

**INHIBITION OF APOPTOSIS BY ANTIOXIDANTS**

Free radicals generated by aerobic mechanisms in normal life are counterbalanced by endogenous enzymatic SOD, catalase, glutathione peroxidase, and non-enzymatic antioxidants such as vitamins A, E, and C. Apoptosis induced by TNF-\(\alpha\) is mediated by oxidative stress and inhibited by antioxidants such as thioredoxin and N-acetylcysteine.\textsuperscript{7} SOD and vitamin E also inhibit apoptosis.\textsuperscript{18} Recently the powerful antioxidants trolox and probucol were shown to modulate adriamycin-induced apoptosis in myocyte culture and in vivo rat hearts, respectively.\textsuperscript{25,33} Another antioxidant, carvedilol, was shown to attenuate apoptosis induced by
ischemia-reperfusion in rabbit hearts. The broad-spectrum caspase inhibitor zVAD-fmk was shown to limit CM apoptosis after ischemia/reperfusion in rat hearts.

Mitochondria-initiated apoptosis is inhibited by the antiapoptotic proteins Bcl-2 and Bcl-xl in different cell types and CMs. Overexpression of Bcl-2 decreases apoptosis, as well as ROS species and lipid peroxidation, and is believed to act as an antioxidant. Similarly, other oxidant scavengers, such as SOD-1 and glutathione, have been shown to reduce ROS and inhibit apoptosis. Taken together, these findings suggest that apoptosis triggered either directly or indirectly by oxidative stress is inhibited by antioxidants (Fig 2).

REGULATION OF APOPTOTIC MECHANISMS IN THE HEART

Bcl-2 family. The Bcl-2 gene family consists of 12 different gene products with pro- and antiapoptotic
mechanisms. These pro- and antiapoptotic proteins are structurally different, have different tissue distribution, and exert different functional effects. The antiapoptotic protein Bcl-2 is localized mainly in the outer mitochondrial membrane. It is also present in the nuclear membrane and the endoplasmic reticulum. In contrast, the proapoptotic proteins Bax and Bad reside mainly in the cytoplasm and are activated by various apoptotic stimuli. Bax translocates to the mitochondria, where it forms a complex with Bcl-2. An increased ratio of Bax/Bcl-2 leads to the formation of pores in the mitochondria, release of cytochrome c, and activation of the apoptotic pathway (Fig 2).1,2,43 The exact mechanism of action of Bcl-2 remains poorly defined. It has been suggested that apoptosis is caused by the generation of oxygen free radicals and inhibited by Bcl-2 through the antioxidant pathway,42 which could have application in myocardial infarction, ischemia/reperfusion injury, and adriamycin cardiotoxicity.

The overexpression of Bcl-2 in mice significantly inhibits apoptosis and decreases myocardial-infarct size after ischemia/reperfusion.44 With the use of immunohistochemical methods, expression of Bcl-2 was demonstrated in salvaged myocytes of human myocardial infarction, whereas overexpression of Bax was demonstrated in old infarcts, suggesting their respective anti- and proapoptotic roles in myocardial infarction.1,2 Increased expression of antiapoptotic proteins Bcl-2 and Bcl-xL was demonstrated in patients with end-stage heart failure.45 In the rat model of adriamycin cardiotoxicity, the degree of apoptosis correlated with the duration of treatment and expression of the proapoptotic protein Bax and the anti-apoptotic protein Bcl-2.25 At 4 days, increased Bax and decreased Bcl-2 expression were associated with marked apoptosis.25 In contrast, at 10 days, when the magnitude of apoptosis was low, the expression of Bax was decreased and that of Bcl-2 increased. These findings suggest that adriamycin-induced CM apoptosis in vivo is modulated by the Bax/Bcl-2 ratio.

**Mitochondria-dependent pathway.** Recent evidence indicates that the mitochondria play a pivotal role in cell death and cell survival. Mitochondria are a source of high-energy ATP through mitochondrial oxidative phosphorylation. Mitochondrial dysfunction results in decreased ATP production, which is associated with increased ROS.46 The mitochondria are also a main source of the production of the superoxide radical, which leads to the formation of H$_2$O$_2$, OH$^-$, and ONOO$^-$.47,48 Increased oxidative stress in the presence of mitochondrial calcium influx can induce apoptosis49 and opens permeability transition pores.50 The opening of the permeability transition pore is not directly related to cytochrome c release and apoptosis; apoptosis can occur in a cellular system devoid of mitochondrial DNA.51 Cytochrome c is localized to the intermembrane space and to the surface of the inner mitochondrial membrane,52 which translocates to the cytosol on mitochondrial alterations and induces apoptosis. Release of cytochrome c from mitochondria has been observed in staurosporin-induced52 and ionization-induced53 apoptosis in vitro and with ischemic cardiomyopathy in vivo.54 The mitochondrial released cytochrome c forms a complex with Apaf-1, procaspase 9 and dATP, cellular energy and starts the mitochondrial-dependent apoptotic pathway (Fig 2).55

The concept that ATP directs the injured cell to go through apoptosis or necrosis is still not well developed. Because ATP is required to start the apoptotic machinery, the intracellular ATP depletion in injured cells causes a shift from apoptosis to necrosis. This appears to be the case in the posts ischemic reperfused heart, in which both apoptosis and necrosis occur. The transition from apoptosis to necrosis or vice versa may be a result of changes in intracellular energy stores. The available evidence suggests a significant role of mitochondria in CM apoptosis.54 In CMs, the release of cytochrome c from mitochondria into cytosol has been reported in association with normal structural appearance and without DNA degradation.54 It is possible that myocytes require a certain threshold for cytochrome c release in the cytosol to start the apoptotic machinery or an alternative protein released from the mitochondria, such as AIF.55 The release of AIF from the mitochondria also activates caspase-3 for the initiation of the apoptotic pathway (Fig 2).46

**Caspases.** The caspase family consists of more than a dozen caspases (caspase 1 through caspase 14) required for the regulation of apoptosis and inflammation.56 Caspases have a common structure and are present in zymogen form, with an N-terminal prodomain large subunit and a C-terminal small subunit. The inactive zymogen is cleaved enzymatically2 to form active tetramers, consisting of 2 large/small subunit heterodimers.2 Functionally, caspases are divided into 3 different types: apoptotic initiators (caspases 2, 8, 9, and 10), executioners (caspases 3, 6, and 7), and cytokine precursors (caspases 1, 4, 5, 11, 12, 13, and 14). In this review, we focus on oxidative stress-related caspase activation in the heart (Fig 2).

Mitochondrial dysfunction causes release of cytochrome c which binds to Apaf-1 in the presence of ATP and further promotes activation of procaspase 9 and then to caspase 3.2 Activated caspase 8 promotes apoptosis by acting on Bcl-2-interacting domain. Bcl-2 family protein (s) and activating caspase 3, which starts apoptosis by way of cleavage of ICAD, PARP, protein...
kinase C-δ, and gelsolin. These cleaved proteins induce both cytoplasmic and nuclear apoptosis, including DNA fragmentation. Activated caspase 3 has been detected in CMs with the use of immunohistochemical methods. Recently translocation of cytochrome c and cleavage of PARP and protein kinase C-δ leading to apoptosis was detected in human cardiomyopathic and ischemic hearts. Activated caspase 3 was also detected in adriamycin cardiomyopathy and in postischemic reperfused hearts. In addition, the broad-spectrum caspase inhibitor zVAD-fmk has been shown to inhibit apoptosis in an in vivo rat model of cardiac ischemia/reperfusion injury.

**Oxidative stress-induced signal-transduction pathways and apoptosis.** MAPKs, which include ERK-1 (p44) and ERK-2 (p42), JNK, and the p38 kinases, are activated by phosphorylation of serine/threonine and tyrosine residues. On being activated, MAPKs regulate various cellular proteins, including growth-factor receptors, transcriptional factors, and other protein kinases. The MAPKs, ERK, JNK, and p38 are activated by different extracellular stimuli, such as hypoxia/reoxygenation, ROS, and ischemia-reperfusion. The activation of ERK, JNK, and p38 is stimulus-specific. For example, serum withdrawal from PC-12 cells causes suppression of ERK activity and increases in JNK and p38 activities, whereas H2O2 treatment in HeLa cells results in an increase in ERK and JNK/SAPK activities. The individual activation of MAPK and crosstalk among MAPK family members may also decide whether the cell goes through proliferation, differentiation, or cell death. The role of MAPK activation in apoptosis or cell survival is not clearly understood. The suppression of ERK and activation of JNK and p38 MAPKs play a role in apoptosis in different cell lines. The activation of JNKs and p38 MAPKs has been demonstrated in perfused rat hearts and in ischemia/reperfusion, which is mediated by oxidative stress.

Daunomycin, an analog of adriamycin, triggers the MAPK-family protein p38 and induces CM apoptosis mediated by ROS. Doxorubicin (adriamycin) also activates p38 and induces CM apoptosis. The activation of JNKs in cultured CMs subjected to hypoxia/reoxygenation and SAPKs in ischemia/reperfusion is inhibited by antioxidants. Both apoptosis and the expression of p38 induced by doxorubicin were suppressed with metallothionein protein, which acts as an antioxidant. Taken together, these findings suggest that ROS are responsible for the activation of MAPK-protein subfamilies JNK and p38 after ischemia/reperfusion and adriamycin cardiomyopathy and that this process is inhibited by antioxidants.

**IDENTIFICATION OF APOPTOSIS**

Two main types of myocyte cell death are generally recognized: apoptosis and necrosis. Apoptosis is a genetically controlled form of cell death that involves specific structural and biochemical changes that differ from those in necrosis (Fig 1). Apoptosis can be divided into 3 different phases (initiation, intermediate, and final) on the basis of cellular structural and biochemical changes. The initiation phase can be induced by apoptotic stimuli such as ischemia/reperfusion, myocardial remodeling, ROS, and adriamycin (Fig 2). In this phase, the early signs of membrane blebbing are clearly observed in CM culture after exposure to adriamycin. This phase generally gives way to an intermediate phase, which involves the release of cytochrome c and activation of caspases (Fig 2). The final stage of apoptosis involves further activation of downstream caspases and inactivation of PARP, which eventually leads to DNA fragmentation (Fig 2) and formation of apoptotic bodies. These 3 phases of apoptosis have been characterized on electron and light microscopy as well as with biochemical assays.

**Structure.** The main physiologic advantage of apoptotic cell death in injured tissue is that it avoids the indiscriminate release of the inflammatory immune response (which is normally associated with necrosis) on other cells. Structurally, the characteristic features of apoptosis include formation of membrane blebbing; chromatin condensation, which is reflected in the half-moon or horseshoe appearance of the nucleus; internucleosomal DNA fragmentation of 180 to 200 base pairs; proteolytic degradation by cysteine proteinases; and formation of apoptotic bodies. These apoptotic bodies consist of cellular organelles, fragments of nucleus, and mitochondria, and are easily subjected to phagocytosis by neighboring cells and eventually degraded inside the cell. CM apoptosis in the heart has been characterized structurally with the use of electron microscopy.

**Biochemical methods.** The appearance of phosphatidylserine on the outer surface of the plasma membrane of a cell is recognized as an early sign of apoptosis. In contrast, phosphatidylserine is located on the inner leaflet in normal viable cells. The translocation of phosphatidylserine has been identified at the light microscopic level with the use of annexin V staining. The DNA fragments formed by endogenous DNases generate blunt ends and single-strand 3' overhangs. The biochemical methods most commonly used to detect apoptosis are based on DNA fragmentation. The DNA fragments are detected as a ladder pattern of fragments, with multiples of 180 to 200 base pairs, in various apoptotic cells, including CMs.
detected at the microscopic level and visualized with the TUNEL assay.

**Technical concerns.** Apoptosis and its role in the progression of heart failure are intriguing for cardiovascular basic scientists and clinicians. However, the topic of apoptosis is surrounded by various questions about the reliability of the apoptotic myocyte cell count resulting from limitations of small sample size, sensitivity, and specificity of detection techniques. Most of the quantitative and qualitative reports on apoptosis in various heart diseases are based on 3 different techniques, such as the TUNEL assay, the DNA ladder, and electron microscopy. However, these techniques used to detect myocyte apoptosis are not satisfactory in providing a clear picture of the quantitative count of apoptotic cells.

First, the TUNEL assay identifies single-strand DNA breaks as well as double-strand DNA breaks with free 3′-OH terminals. The specificity of TUNEL staining has recently become doubtful; it also detects necrotic cells and healthy myocytes going through the normal cellular process of DNA repair. Moreover, the criteria used to measure the percentage of apoptotic myocyte cell death varies among investigators. Collective evidence suggests that the use of the TUNEL assay is useful in identifying apoptosis but should be complemented by additional evidence of apoptosis, such as the up-regulation of pro- or antiapoptotic gene products or structural criteria.

Second, the DNA ladder, which reflects DNA fragmentation as analyzed on gel electrophoresis, is sensitive. However, DNA gels available from adriamycin treated CMs (postischemic or postadriamycin) show a mixture of DNA laddering and smear, indicative of apoptosis and necrosis. Moreover, it is a qualitative method, and its use is limited to in vitro cell culture. This method can be applied to cells from in vivo studies, as has been done by many researchers. However, the DNA preparation from the heart homogenates is not pure; rather, it consists of CMs plus other cell types that may also undergo apoptosis, such as non-CM interstitial cells, fibroblasts, vascular cells, Purkinje cells, and inflammatory cells.

Third, electron microscopy is often used to detect apoptosis on the basis of the findings of DNA fragmentation, crescent chromatin condensation, and typical apoptotic bodies. The sensitivity of electron microscopy in detecting apoptosis is very high compared with that of the TUNEL assay. However, it is a laborious qualitative technique, and the tissue sample used is small compared with that examined with the TUNEL assay.

**CONCLUSIONS**

There has been tremendous progress in the CM apoptosis literature over the last few years. CM apoptosis has been suggested as an active player in the development of cardiac dysfunction and remodeling. However, the implication of the broad range of CM apoptosis (apoptotic cell counts from 0.02% to 35%), the significance of apoptosis in different cell types in the myocardium, the relative roles of different apoptotic pathways causing CM apoptosis, and their role in the pathogenesis of cardiovascular diseases demonstrate the need for further classification. The discrepancies regarding the incidence of apoptosis in different reports suggests that more careful investigation in well-designed studies is needed. Special attention should also be given to windows of apoptosis, both in evaluations of apoptosis and in the testing of potential antiapoptotic therapies. Overall, oxidative stress seems to play a significant role in the initiation as well as the regulation of CM apoptosis in different cardiac diseases. Because this form of cell death can be significantly inhibited by antioxidants, these agents should be studied as potential therapeutic interventions for limiting apoptosis in cardiac diseases.

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