Apoptosis

Lead Article

Programmed cell death: apoptosis and cardiovascular disease
R.L. Engler, R.A. Gottlieb 67

Expert Answers to Three Key Questions

Apoptosis: what prospects for beneficial manipulation? - ***H. Rupp, B. Maisch*** 85

Does apoptosis play a role in the progression of heart failure?
G. Olivetti, R. Maestri, E. Cigola, D. Corradi, F. Quaini 91

Is cardiac apoptosis invariably bad? - ***M.D. Schneider*** 95

Summaries of Ten Seminal Papers - *S. Bialik, R.N. Kitsis* 99

Cell death: the significance of apoptosis - ***A.H. Wyllie and others***

Genetic control of programmed cell death in the nematode *C. elegans* - ***H.M. Ellis and H.R. Horvitz***

Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death - ***D. Hockenbery and others***

Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode
C.E. Murry and others

Reperfusion injury induces apoptosis in rabbit cardiomyocytes
R.A. Gottlieb and others

Preconditioning rabbit cardiomyocytes: role of pH, vacuolar proton ATPase, and apoptosis - ***R.A. Gottlieb and others***

Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death - ***A.M. Chinnaiyan and others***

The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis - ***R.M. Kluck and others***

Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade
P. Li and others

Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked - ***J. Yang and others***

Bibliography of One Hundred Key Papers 111

Programmed cell death: apoptosis and cardiovascular disease

Robert Louis Engler, MD*; Roberta A. Gottlieb, MD*†

**Research Service and Department of Medicine - San Diego VA Health Care System, and University of California, San Diego School of Medicine 3350 La Jolla Village Drive - San Diego - Calif - USA*

†Department of Molecular and Experimental Medicine - The Scripps Research Institute - La Jolla - Calif - USA

Apoptosis occurs in a wide variety of physiological settings and diseases, including cardiovascular diseases. The fact that features of both apoptosis and necrosis are found in cardiomyocyte death following ischemia/reperfusion (I/R) has prompted research into ways of blocking specific events of apoptosis. In isolated cardiomyocytes, reduction of intracellular acidification or calcium accumulation, activation of vacuolar proton ATPase, and inhibition of caspases appear to exert a protective effect, but whether this would translate into preservation of the myocardium after I/R in vivo remains to be determined. The apparent "bottleneck" provided by caspase activation in apoptosis is the current target of antiapoptotic interventions, but other potential targets need to be investigated, such as the Bcl family proteins, or preconditioning. The hypothesis that inhibition of the caspase cascade will reduce ischemic injury remains to be tested in vivo. A direct causal connection between apoptosis and progressive myocyte loss in congestive heart failure has yet to be established. However exciting the prospects of therapeutic interventions based on modulation of apoptosis may be, we should nevertheless be on the lookout for unexpected adverse reactions.

Keywords: apoptosis; cardiomyocyte; ischemia/reperfusion; preconditioning; caspase; mitochondria; ion transport

Address for correspondence: Robert Engler, MD, ACOS Research (151), San Diego VA Health Care System, 3350 La Jolla Village Drive, San Diego CA 92161, USA
(e-mail: rengler@ucsd.edu)

Apoptosis is a term originally coined by Kerr, Wyllie, and Currie[1] to describe a form of cell death characterized by cell shrinkage and nuclear condensation, and derives from the Greek term for the shedding of leaves or petals. Subsequent investigation has shown this form of cell death to be a physiologic, tightly regulated process initiated by a cell in response to internal or external cues. Because of its occurrence at defined times and locations in development, it has also been termed programmed cell death, and its occurrence has been studied in detail in the nematode *Caenorhabditis elegans.* Programmed cell death is also familiar to us as the mechanism by which tadpoles lose their tails and the resorption of interdigital webs in the developing human fetus. It is a critical process during embryogenesis, where remodeling requires highly regulated cell death. Apoptosis occurs in all multicellular organisms as the means to balance cell proliferation in continuously renewing tissues in order to maintain a constant organ size. An excess of proliferation or a decrease in the frequency of apoptosis leads to organ enlargement (hyperplasia), while a decrease in cell proliferation or an increase in apoptosis results in progressive loss of organ mass. In the case of nonrenewing tissues, such as the brain and myocardium, the development of any apoptosis will be problematic. In the heart, as myocytes are lost, an increased load may be placed on the remaining cells; this in turn may promote further cell death, as overstretch has been shown to promote apoptosis.

LESSONS FROM THE WORM

Three of the most important genes that control apoptosis have been identified through studies of

development in *C elegans.* The body plan of the adult nematode calls for 1090 cells to be formed and for 131 cells to die during the course of development. By mutational analysis, two genes were shown to be essential for programmed cell death to occur, designated *ced-3* and *ced-4* (*C elegans* death gene), and one gene was shown to be essential for opposing cell death, *ced-9.*[2] Subsequent investigation has shown that these genes are conserved throughout evolution, being represented—with considerably more complex-ity and diversity—in mammals. The antiapoptosis gene *ced-9* is represented by the mammalian homolog *bcl-2*, which was first identified as an oncogene created by a chromosomal 8;14 translocation in B-cell malignancies. The role of the antiapoptotic protein, Bcl-2, and its relatives—both antiapoptotic and proapoptotic—will be discussed below. CED-3 is a cysteine protease with the unusual characteristic of cleaving peptides after aspartic acid residues. The first mammalian homolog of CED-3 to be identified was interleukin-1β converting enzyme, or ICE. Subsequently, a family of ten related cysteine proteases ("death proteases") have been identified, and have been designated as caspases, for Cysteine ASPartASES.[3] The function of each caspase is still being elucidated, but most interest has been directed to caspase-3 (previously designated as CPP32/Yama/apopain), which is widely expressed and believed to be responsible for cleaving many of the important intracellular protein substrates that are degraded during apoptosis.[4] Most cells express multiple caspases, probably related to the observed redundancy in pathways that initiate cell death; caspases also appear to work in a cascade fashion, perhaps analogous to the amplification seen in the clotting system. The role of caspases will be discussed in more detail below. The second nematode death gene, *ced-4,* has been more mysterious in its function. It interacts, in mammalian systems, with Bcl-2. Apoptotic protease activating factor–1 (Apaf-1), the recently identified mammalian homolog of CED-4, is a cofactor along with cytochrome *c* and caspase-9 for activation of downstream caspases such as caspase-3.[5] It is presumed that CED-4 similarly participates in caspase activation.

FEATURES OF PROGRAMMED CELL DEATH

Upon initiation of the death program, cells undergo a dramatic volume loss, membrane blebbing, and nuclear condensation, followed by chromatin fragmentation. Cell surface markers (phosphatidylserine externalization, adhesion markers) identifying the cell for ingestion are expressed. In the intact organism, apoptotic cells are rapidly ingested by neighboring cells or professional phagocytes before membrane integrity is lost. This prevents spillage of

SELECTED ABBREVIATIONS AND ACRONYMS

Apaf	apoptotic protease activating factor
CED	*Caenorhabditis elegans* death gene
CFTR	cystic fibrosis transmembrane conductance regulator
COS	transformed African green monkey kidney cell line
CrmA	caspase-inhibiting cowpox virus protein
DFF	DNA fragmentation factor
ERK	extracellular signal–regulated kinase
FADD	Fas-associated death domain protein
Fas	APO-1, CD95 (member of the TNF-receptor family)
FLICE	FADD-like interleukin 1β–converting enzyme
G-CSF	granulocyte colony–stimulating factor
HSP	heat shock protein
ICE	interleukin-1β converting enzyme
IF-1	initiation factor–1
JNK	Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MEKK1	mitogen-activated protein kinase /ERK kinase kinase 1
NF-κB	nuclear factor kappa B
PARP	poly(ADP-ribose) polymerase
SAPK	stress-activated protein kinase
SEK-1	stress-activated protein kinase/ERK kinase–1
TdT	terminal deoxynucleotidyl transferase
TNFR	Tumor necrosis factor receptor
TRAIL	TNF (tumor necrosis factor)–related apoptosis-inducing ligand
VPATPase	vacuolar proton ATPase
zVAD-fmk	benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

inflammatory cellular contents (actin, DNA), which is a characteristic of necrosis. Thus, cell elimination by apoptosis is quite a tidy process that is not usually accompanied by inflammation. Programmed cell death is also associated with dramatic cytoskeletal rearrangement and loss of contact with neighboring cells.
In the myocardium, one can envision the consequence for neighboring myocytes, in which cell-cell contact is essential for electrical conduction, force transduction, and distribution of stress. Additional biochemical features of programmed cell death include cytoplasmic acidification, mitochondrial inactivation, and activation of the Jun N-terminal kinase (JNK) pathway.
JNK signaling is associated with induction of a variety of genes, which may be associated with the divide-or-die response. We will consider each of these features in more detail.

The general working model for initiation and completion of apoptosis is depicted in *Figure 1*. A variety of signals including cytokines, loss of survival factors, DNA damage, oxidative stress, and others act through various signaling pathways that may also be countered by simultaneous survival signals (perhaps reflected by the intracellular pH). The "effector phase" is a common pathway that is generally characterized by activation of caspases. Caspase activation may be achieved directly through activation of the upstream caspases -8 and -10, or through an alternate pathway involving mitochondria. One aspect of these processes under study in our laboratories is pH regulation and acidification, which is of particular relevance to myocardial ischemia. The role of cytoplasmic pH in the relative activity of the apoptotic processes is a largely unexplored field.

Membrane blebbing

Membrane blebbing is a characteristic feature of cell death that probably arises as a consequence of the

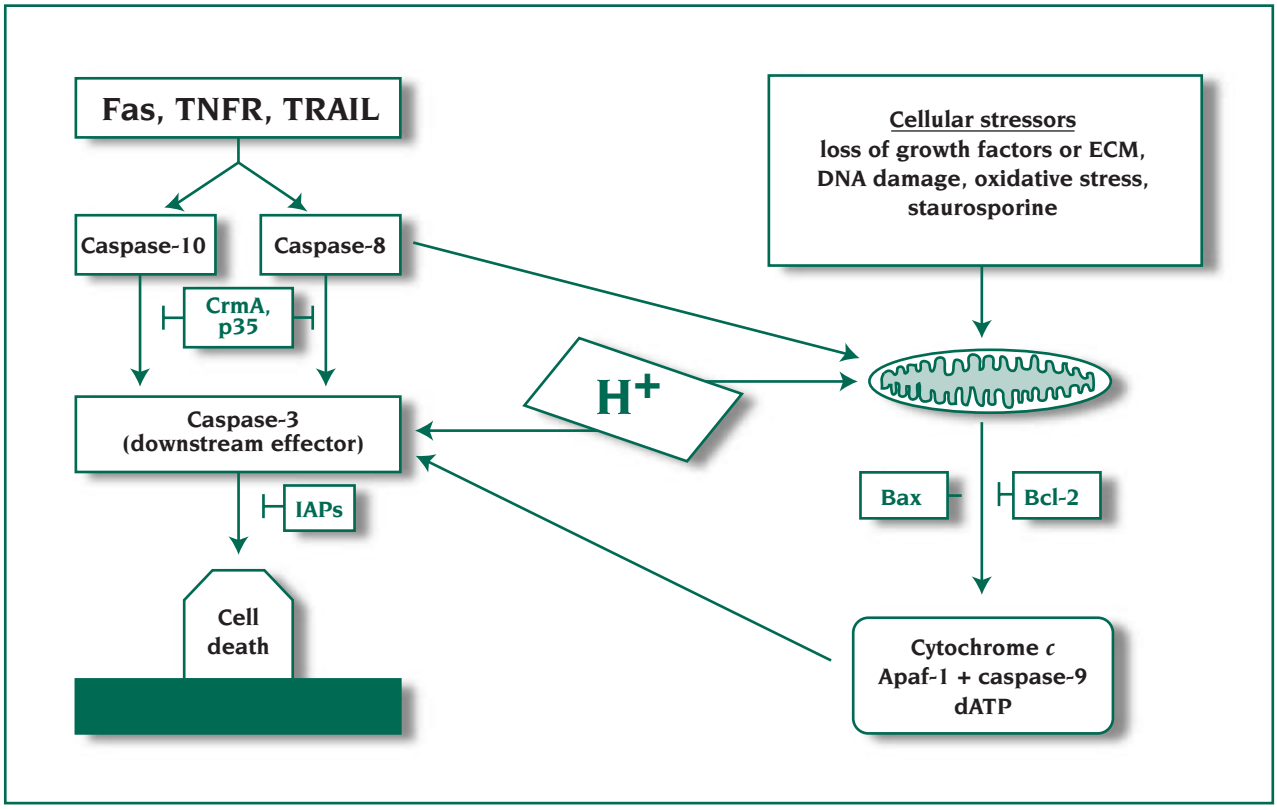

Figure 1. *Two main signaling cascades lead to caspase activation. Extracellular signaling from the death receptors TNFR, Fas, and TRAIL lead to activation of caspase-8 and/or -10, which leads to activation of caspase-3 and other end-effectors of apoptosis. Caspase-8 and -10 are inhibited by CrmA. Cell stressors lead to mitochondrial alterations, resulting in caspase-3 activation through the interaction of Apaf-1, cytochrome c, and caspase-9. Bcl-2 opposes the pathway dependent upon mitochondria. Activation of caspases, cytoplasmic acidification, and mitochondrial dysfunction lead to death of the cell. Apaf-1, apoptosis protease activating factor–1; Bax, proapoptotic regulator protein; CmrA, caspase-inhibiting cowpox virus protein; dATP, deoxyadenosine 5'-triphosphate; ECM, extracellular matrix; IAP, inhibitor of apoptosis protein; TNFR, tumor necrosis factor receptor; TRAIL, TNF-related apoptosis-inducing ligand.*

cytoskeletal rearrangement and the cleavage of a key membrane skeleton protein, fodrin (spectrin). Fodrin, a cytoskeletal protein that underlies and stabilizes the plasma membrane, is cleaved by a caspase during apoptosis. This, coupled with other events, leads to externalization of phosphatidylserine (or "flipping") and the bulging and disorderly ruffling of the plasma membrane that are referred to as blebbing. Eventually, cellular contents, including nuclear remnants, are lost through blebbing as successive bits of the cell are shed in membrane-bound packets.

Cytoplasmic acidification

During the process of apoptosis, intracellular pH_i falls. It is not clear whether this is due to diminished proton elimination or increased proton production. Some evidence suggests that the sodium/hydrogen exchanger is less active in apoptotic cells. Another postulated mechanism is mitochondrial dysfunction, which might increase glycolysis and result in lactate accumulation. Additionally, ATP hydrolysis liberates protons; ATP depletion is a common feature of apoptosis. Acidification may be a general and requisite feature of apoptosis, since cytoplasmic alkalinization has been shown to delay or prevent apoptosis in some settings. However, the protective effect of alkalinization is not observed in every case.

Findings from our laboratory and others have indicated that, under some circumstances, prevention of cytoplasmic acidification delays or prevents apoptosis (reviewed in 6). We found that organic bases delay acidification and apoptosis in Jurkat cells. The characteristic elevated pH_i in cystic fibrosis (CF) cells confers resistance to apoptosis induced by cycloheximide or etoposide. Cells expressing the functional CFTR (cystic fibrosis transmembrane conductance regulator) undergo cytoplasmic acidification in response to an apoptotic stimulus, while those expressing a mutant form of the CFTR do not acidify. In addition, the cells expressing the functional CFTR and treated with cycloheximide or etoposide demonstrate nuclear condensation consistent with apoptosis, while those with the mutated CFTR show no evidence of apoptosis. We also found that overexpression of *bcl-2* or treatment with the caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) blocks acidification and apoptosis. Granulocyte colony–stimulating factor (G-CSF) delays neutrophil acidification and apoptosis by activating a proton pump, which we also found to be expressed and functional in cardiomyocytes subjected to metabolic inhibition.[7]

Regulation of intracellular pH

The general model that we are examining is depicted in *Figure 2*. Although many other signals play a role, pH homeostasis can modulate many of these aspects, with acidification favoring propagation of the death signal.[6] Many survival signals, shown on the left, promote alkalinization and confer protection. Cells possess a number of mechanisms to maintain pH homeostasis. Passive systems that depend upon electrochemical gradients include the sodium/hydrogen exchanger, the sodium/bicarbonate cotransporter, and the anion exchanger. Cells can also eliminate organic acids such as lactate via a coupled transporter. The sodium/hydrogen exchanger usually exchanges extracellular sodium for intracellular protons. However, under conditions of low extracellular pH, the exchanger may run in reverse, or may exchange protons for protons, nullifying its activity. Its activity is increased by phorbol esters via many growth factor receptors as well as integrins. The sodium/bicarbonate cotransporter utilizes the sodium gradient to import bicarbonate and alkalinize the cell. Activity of this cotransporter is upregulated in response to angiotensin II; there are species differences regarding the response of this symporter to purinergic stimulation and α- and β-adrenergic stimulation (reviewed in 8). The anion exchanger, which exchanges chloride and bicarbonate, generally functions to acidify cells, and is most active at pH_i greater than 7.4, serving to counteract alkalinization. A proton-sensing polyhistidine site at the amino terminus is responsible for inactivating the anion exchanger as the pH drops. Any signals or stimuli that alter the function of these pH regulatory mechanisms could affect apoptosis.

In addition to these passive transport systems, cells also possess an energy-dependent proton pump. This proton ATPase is generally used to sequester protons in acidic organelles (hence its name, the vacuolar proton ATPase, or VPATPase), but this multisubunit pump can also be assembled at the plasma membrane to export protons at the expense of ATP. The metabolic consequences of proton elimination via the different pathways vary considerably. For instance, in cells that also possess a Na^+/Ca^{2+} exchanger, proton elimination via the sodium/hydrogen exchanger will lead to sodium elimination via Na^+/Ca^{2+} exchange, with a subsequent increase in intracellular Ca^{2+}. By this mechanism, calcium overload has been implicated in myocyte dysfunction and death after ischemia. The compensatory elimination of

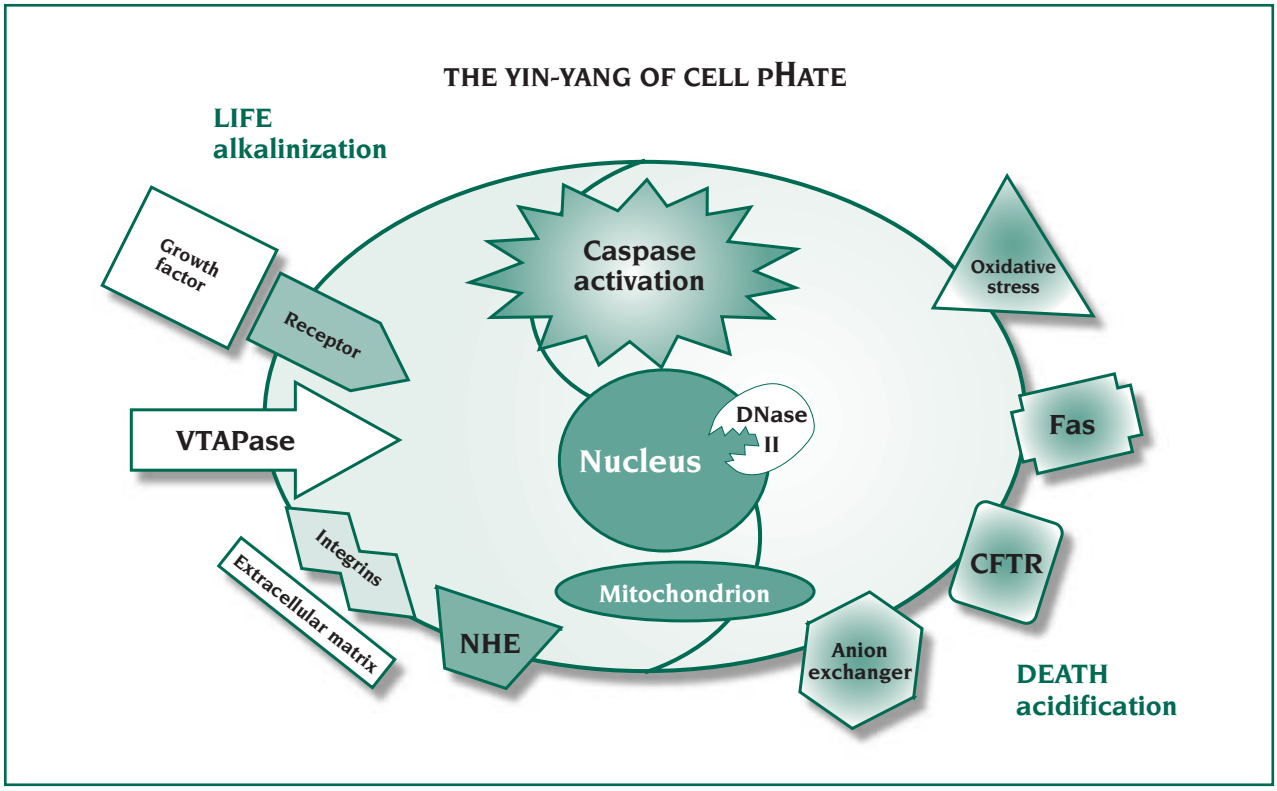

Figure 2. *Yin-yang of cell pHate. Cellular pH homeostasis reflects a balance between survival signals and proapoptotic stimuli. Extracellular signals that act as survival factors promote activation of ion transporters that alkalinize the cell, such as the sodium/hydrogen exchanger or VPATPase. In contrast, apoptotic signals promote cytoplasmic acidification through as yet undefined mechanisms. Cytoplasmic pH shifts may affect the activity of many classes of enzymes and mitochondria. Intracellular pH may represent a mechanism by which the cell integrates conflicting signals (survival signals vs proapoptotic ones) to arrive at a survive-or-die decision. CFTR, cystic fibrosis transmembrane conductance regulator; NHE, sodium/hydrogen exchanger; VPATPase, vacuolar proton ATPase.*

protons by VPATPase will have a high energy cost, but will not result in sodium or calcium influx. Our studies have led us to focus on VPATPase as a protective response in cardiomyocytes, to be discussed below.

VPATPase is a multisubunit enzyme that consists of a complex of transmembrane proteins (V_o), which serve as the proton pore, and a cytosolic complex (V_1), which has ATP hydrolytic activity (reviewed in 9). When the two complexes assemble, transmembrane proton pumping takes place. VPATPase is potently and specifically inhibited by bafilomycin A_1, a macrolide antibiotic, which binds to the 100-kd subunit of V_o. Regulation of VPATPase is still under study, but it has been shown in neutrophils and monocytes that catalytic activity is upregulated by exposure to phorbol esters, and G-protein signaling also activates the pump. The pump may also be regulated by assembly and disassembly of the V_o and V_1 complexes, although the regulation of this assembly and disassembly is unclear.

Studies of VPATPase in neutrophils and monocytes have led to a number of insights. Stimulation of human neutrophils results in increased VPATPase activity that was due to fusion of secretory vesicles with the plasma membrane.

Mitochondrial alterations

The current model for caspase activation is based on the observation that certain cellular stressors result in the appearance of cytochrome *c* in the cytosol of apoptotic cells. Cytochrome *c* is postulated to interact with Apaf-1 (the mammalian homolog of CED-4), caspase-9, and deoxyadenosine 5'-triphosphate (dATP), to promote the activation of caspase-3 (see below). Interestingly, it has been shown that ischemia results in the loss of cytochrome *c* from mitochondria. At the time that this observation was made (1985), its significance with respect to the activation of caspases could not have been recognized.[10] Now it is important to reconsider this finding in the context of

commitment to apoptosis. Mitochondrial release of cytochrome *c* is prevented by overexpression of the antiapoptotic protein Bcl-2, although the mechanism is still unclear.[11,12] Alterations in the mitochondrial membrane are likely very important in apoptosis, perhaps permitting caspase entry and activation and/or cytochrome *c* release, where it may activate caspases in the cytosol. This alteration may be pH-dependent, thus allowing the cell a means to integrate information from survival signals (alkalinizing) as well as proapoptotic signals. Loss of cytochrome *c* from its normal association with the electron transport chain prevents delivery of electrons to complex IV, and thence to oxygen. However, it may be possible for electrons to "bleed off" at the upstream site of ubiquinone, leading to superoxide production. This may explain the production of free radicals frequently observed in apoptosis. Effective mitochondrial respiration is shut down and ATP cannot be generated. In the case of transformed cell lines, which have adapted to the somewhat hypoxic conditions of cell culture, decreased mitochondrial ATP production is of marginal significance. However, in the case of cardiomyocytes, whose contractile machinery depends upon massive turnover of ATP, this mechanism may be critical. Interestingly, a compensatory switch to glycolysis may not be possible for cells undergoing apoptosis, as the enzyme poly(ADP-ribose) polymerase (PARP) becomes activated and rapidly consumes nicotinamide adenine dinucleotide (NAD) stores. NAD is required for two steps in glycolysis. Thus, both pathways to ATP generation are shut down during the process of apoptosis. These observations have been made in transformed cell lines; this pathway has not been completely studied in the heart.

JNK signaling

Activation of members of a family of stress- and mitogen-activated protein kinases (SAPK and MAPK) has been noted to occur in response to hypoxia/reoxygenation in cardiac myocytes.[13] Activation is attributed to their regulation by redox events. To date, little evidence in the cardiomyocyte system exists to suggest that activation of a subset of these kinases is required for apoptosis to be initiated. However, several studies from other cell systems have demonstrated that activation of the SAPK and p38 MAPK pathway induces apoptosis, and that expression of dominant negative SEK (stress-activated protein kinase/ERK [extracellular signal-regulated kinase] kinase-1), a kinase that activates SAPK, prevented apoptosis induced by heat shock or treatment with cisplatinum. Additional correlative evidence exists (reviewed in 14); however, stimuli able to activate SAPK do not necessarily induce apoptosis in all cases. Caspase activation is suggested to be linked to SAPK activity. Heat stress and ceramide-induced apoptosis through a SAPK/JNK pathway is inhibited by expression of heat shock protein (HSP) 70. This observation may be very relevant to the heart because of the role of HSPs in delayed onset (the late window) of preconditioning. In the cardiac system of ischemia/reperfusion, a strong correlation has been drawn between the activation of the stress-activated protein kinases and the induction of apoptosis. Whether there is a causal relationship remains to be demonstrated. If these pathways are linked to apoptosis in cardiomyocytes, then this family of kinases represents a therapeutic target for amelioration of cell death following ischemia/reperfusion injury.

Caspase activation

Activation of caspases may be accomplished through either of two general pathways *(Figure 1)*, one involving a cell surface receptor such as Fas, which causes activation of caspase-8 or -10 through association with an intermediate protein. Caspase-8 or -10 may directly activate caspase-3 and related effector caspases. An alternate pathway exists involving alteration of the mitochondria, which makes cytochrome *c* available to interact with Apaf-1, dATP, and caspase-9, to support the activation of caspase-3 through proteolytic processing *(Figure 1)*.[5] Bcl-2 is able to prevent apoptosis initiated through the mitochondria-dependent pathway. Caspases are synthesized as a proenzyme with an amino-terminal prodomain, which is removed by proteolytic processing. The enzyme is further processed into large (~20 kd) and small (~10 kd) fragments, which form a heterodimer. Two heterodimers assemble to form the active tetrameric protease *(Figure 3)*. Caspases are capable of autoprocessing, but the mechanisms leading to caspase activation are still under investigation. Generally, caspases can be grouped into two categories, based on the size of the prodomain. Those with a large prodomain, such as caspase-8 (FLICE [FADD-like interleukin-1β–converting enzyme]), are suggested to respond to signaling events, and may play a key role in transducing apoptotic signals. In contrast, those with a short prodomain (~3 kd), such as caspase-3, are presumed to be effectors, cleaving the majority of intracellular substrates, possibly after one or more amplification steps.

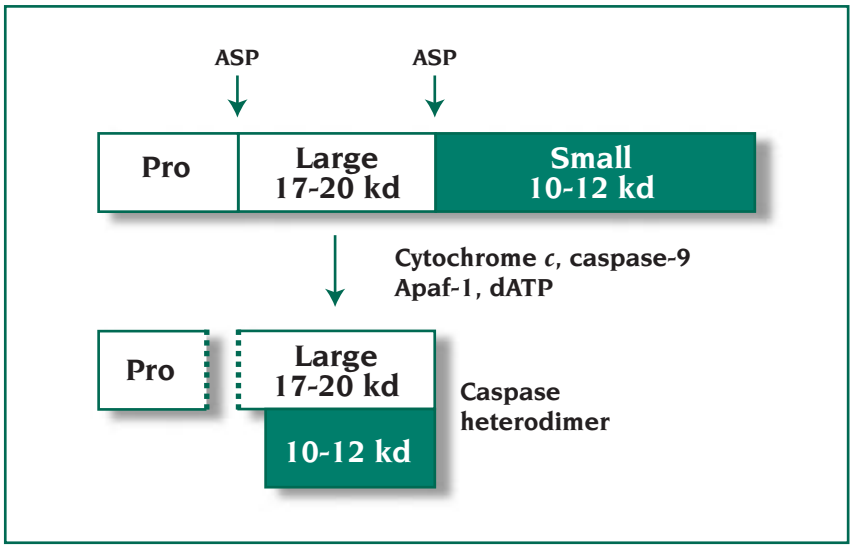

***Figure 3.** Current model for caspase activation. Members of the cysteine aspartate protease (caspase) family are characterized by an amino-terminal prodomain and a catalytic domain, which is processed into a longer (~20 kd) and a shorter (~10 kd) fragment. The two fragments assemble into a heterodimer. Two heterodimers form a tetramer in the active form of the caspase. Caspase-3 is activated through an interaction with caspase-9, Apaf-1, and cytochrome c. The reaction is normally held in check by sequestration of cytochrome c within the mitochondria. However, in response to apoptotic stimuli, cytochrome c becomes accessible to the other components, and activation of caspase-3 results in widespread cleavage of cellular proteins, committing the cells to apoptosis. Apaf-1, apoptotic protease activating factor–1; ASP, aspartate cleavage site; dATP, deoxyadenosine 5'-triphosphate; Pro, prodomain.*

It is generally considered that activation of proteases constitutes an irreversible event and that the caspases are the ultimate effectors of apoptotic cell destruction. There are exceptions, however: recently, it has been shown that CPP32 (caspase-3) is activated in T cells without leading to apoptosis. Inhibition of caspases may not completely prevent cell death, at least in some systems. Gerard Evan's group[15] has shown that inhibition of caspases may prevent certain morphologic features of apoptosis. However, the cell is still unable to replicate, and if observed long enough, falls apart in a necrotic form of death. These findings are particularly relevant to experimental interventions in myocardial ischemia/reperfusion, indicating that long observation intervals are required to evaluate survival end points. Since multiple enzymatic pathways are activated in apoptosis, it is reasonable to think that completion of any subset of processes will result in the death of the cell. Indeed, some of these pathways may be common to both necrotic injury and apoptosis. During physiologic programmed cell death (such as during development) the classic apoptotic morphology is displayed. In contrast, in injured cells, the two processes may compete and lead to evidence of some typical morphology of apoptosis, while other aspects may be absent, or evidence of necrosis may appear. It may be a "race to the finish line"; if caspase cleavage of substrates and chromatin degradation occurs before the cell membrane disintegrates, then morphologically it looks like apoptosis. If, however, ATP stores are depleted rapidly and the cell swells and ruptures before caspases and endonucleases complete their work, the appearance is that of necrosis.

Nuclear alterations

The classic histologic manifestation of apoptosis is the condensation of nuclear chromatin and fragmentation of the nucleus. DNA condensation and classic apoptotic body formation depends on activation of nuclear lamin degrading enzymes.[16] Another characteristic feature is digestion of the DNA by several distinct endonucleases, eventually resulting in fragments representing multiples of the ~200 base pair nucleosome (the so-called nucleosomal ladder). The search for the responsible endonuclease has yielded several candidates, including DNase I (deoxyribonuclease) and DNase II. DNA fragmentation factor (DFF) is cleaved by a caspase to stimulate DNA fragmentation in a cell-free model of apoptosis.[17] In the case of DNase I, it has been shown that the activity can be extracted from thymocytes, and in transfected transformed African green monkey kidney cell line (COS) cells, its overexpression leads to nucleosomal fragmentation. DNase I is a Ca^{2+}- and Mg^{2+}-dependent enzyme, which is inhibited by Zn^{2+}. The addition of zinc ions to cells undergoing apoptosis has been shown to inhibit oligonucleosomal fragmentation, although high-molecular-weight fragments are still generated, implicating an additional endonuclease. However, in other cell types, other nucleases have been implicated: a 15-kd endonuclease has been identified in renal proximal tubules, which is activated by hypoxia/reoxygenation and is Ca^{2+}-dependent. Finally, evidence for an acidic endonuclease (DNase II) has been published by a number of laboratories (reviewed in 6). DNase II is active at pH 6.8 and below, and is independent of divalent cations. Its activity has been demonstrated

in neutrophils and the promyelocytic cell line HL-60, as well as in Chinese hamster ovary cells, lens cells, and cardiomyocytes. Since many cells undergo cytoplasmic acidification during apoptosis or in settings of metabolic stress, DNA degradation by this ubiquitously expressed enzyme may occur readily. One unique feature of DNase II is that it generates 5'-hydroxyl ends (in contrast to the other nucleases, which generate 3'-hydroxyl ends). Such ends would not be efficiently labeled by the widely used assays of apoptosis that rely on terminal deoxynucleotidyl transferase (TdT), unless endogenous phosphatases or exonucleases process the termini of the fragmented DNA to yield the 3'-OH ends necessary for labeling by TdT. Detection assays for DNA fragments with 5'-OH ends are not commercially available, and it is unclear what might be learned from such a labeling system. It has been suggested that the process of DNA fragmentation is not essential for apoptosis, and in *C elegans* the endonuclease is not present in apoptotic cells, but only in the tissue phagocytes that ingest the apoptotic remnants. In addition, it has been shown that DNA fragmentation, and even the nucleus itself, are not required for apoptosis. Thus, efforts aimed at inhibiting the endonuclease as a means of preventing apoptosis have largely been abandoned. The physiologic importance of DNA fragmentation is unclear. It has been suggested that this has evolved as a means to prevent the release of oncogenic fragments of DNA which might be taken up and expressed by neighboring cells. Fragmentation of the DNA may also be important in settings where the apoptotic cells are continuously shed, such as the lumen of the airways and intestines. In these organs, shedding of apoptotic cells with undigested high-molecular-weight DNA, which is highly viscous and inflammatory, may contribute to mucus viscosity and airway inflammation in cystic fibrosis.[18]

APOPTOSIS IN HUMAN DISEASE

The occurrence of apoptosis in pathophysiologic settings, as well as its absence in physiologic settings, results in human disease. More simply, we can group diseases according to whether there is *too much* apoptosis or *too little.*

Too little apoptosis

Since apoptosis must occur at defined times during development, failure for it to occur in the appropriate settings would be expected to give rise to developmental defects. However, genetically defined abnormalities in known elements of the process of apoptosis have not been identified in human developmental disorders. Some insights have been derived from gene knockout studies in mice. Deletion of the gene encoding caspase-3, arguably the most important death protease, results in mice that die in utero or soon after birth with an excess of brain tissue, owing to a failure of programmed cell death during neuronal development. The contribution of apoptosis to other developmental disorders remains to be explored; however, the extensive remodeling required during development of the cardiovascular system suggests that many insights could be gained from investigation of the role of apoptosis in this setting.[19]

In the immune system, deletion of self-reactive T cells is essential to prevent autoimmune disorders. Signaling for lymphocyte deletion is accomplished through engagement of one or more cell surface receptors, including a molecule known as Fas/APO-1/CD95. Engagement of Fas by its ligand results in aggregation of proteins (FADD [Fas-associated death domain protein] and FLICE) through self-association regions known as death domains, culminating in caspase activation and death of the cell. Fas is widely expressed on lymphocytes and is one means by which unwanted T cells are eliminated. Mutation of Fas or its ligand in mice results in a disease that strongly resembles systemic lupus erythematosus; however, a similar defect has not yet been demonstrated in the human disorder. Experimentally, Fas expression is induced in hypoxic rat cardiomyocytes in vitro.[20] However, a mechanistic role of Fas in cardiomyocyte apoptosis has not been demonstrated.

In areas of immune sanctuary, such as the testis, Sertoli cells express high levels of Fas ligand and prevent invading T cells from surviving long enough to mount an immune response against sperm cells (whose haploid nature is somehow recognized as foreign by the body). A similar mechanism of protection is involved in limiting inflammatory responses in viral infections in sensitive organs such as the eye. Immune mediated rejection of transplanted organs rests in part upon the induction of apoptosis in the foreign cells. This mechanism has been exploited by genetic manipulation to express Fas ligand on pancreatic islet cells to prevent induction of apoptosis in the transplanted cells, with resulting prolonged survival of the allograft. This strategy may be useful in cardiac transplants, using, eg, adenovirus to transfect the heart ex vivo with Fas ligand.

A number of viral proteins block apoptosis signaling or effector pathways. Baculovirus p35 and cowpox viral protein CrmA (caspase-inhibiting cowpox virus protein) directly inhibit caspases; adenovirus E1B inhibits caspase-3 activation, and herpesvirus/poxvirus FLICE inhibitor proteins block downstream death domain signaling. The function of these proteins may be critical to viral virulence by blocking the organism's defense against viral replication; infected cells engage the apoptotic machinery and mark themselves for phagocytic ingestion, thus limiting the extent of viral infection. The role of apoptosis in myocarditis has recently been studied in the murine model of encephalomyocarditis virus (EMCV).[21] A ubiquitous signaling pathway through NFκB (nuclear factor kappa B) p65 and p50 prevents apoptosis. Apparently, the EMCV picornavirus induces NFκB p50–dependent signaling to prevent apoptosis sufficient to allow the virus to replicate. Wild type mice have 80% mortality from EMCV, whereas the p50 knockout mice are completely resistant and nearly 100% survive, because p50(-/-) cardiomyocytes can undergo apoptosis to limit the spread of the virus. Isolated murine embryonic p50 (-/-) fibroblasts undergo apoptosis more rapidly than wild type upon exposure to EMCV, and there is a gene/dose-dependent intermediate effect in heterozygotes. Thus, cardiomyocyte apoptosis is a critical defense against this virulent myocarditis; if this observation applies to human viral myocarditis with the related Coxsackie virus or with adenovirus, it has important therapeutic implications. Inhibiting myocyte apoptotic death would be detrimental. The evolutionary survival value of preserving constitutive expression of apoptotic pathways in terminally differentiated cells may lie in limiting the extent of viral infection.

In the field of oncology, it has been useful to view tumor growth as an imbalance between apoptosis and mitosis. For example, the Bcl-2 gene product was discovered because its overexpression prevents the normal death of B cells leading to a leukemia associated with a normal rate of proliferation, but reduced apoptosis. A malignant cell may arise when a cell fails to undergo apoptosis when it should have. Loss of a necessary growth factor or removal from the normal extracellular matrix should trigger a cell to commit suicide. If, however, the cell fails to die, it may survive and proliferate sufficiently for its progeny to acquire other mutations, including loss of p53 and activation of other oncogenes. The p53 gene product is a transcription factor activated by DNA damage to induce a family of p53-dependent genes that regulate the cell cycle and induce apoptosis. Mutations of p53 have been found in many malignant tumors and in some families with hereditary carcinoma. Thus, the first step in oncogenesis may be a failure of apoptosis.

Modulation of apoptosis is widely considered to be a key target for cancer therapy. Although malignant cells are generally considered to be more resistant to the induction of apoptosis, they still possess the necessary cellular machinery, and when exposed to appropriate chemotherapeutic agents (or radiation), usually die by apoptosis, not necrosis. Efforts to decrease their resistance to apoptosis are directed at targets such as Bcl-2. Recently, it has been shown that the clonal disorder paroxysmal nocturnal hemoglobinuria is associated with an increased resistance to apoptosis in the clone of red cell precursors. The red cells produced are unusually sensitive to lysis by complement, resulting in episodes of hemoglobinuria.

In the peripheral vascular system, it may be desirable to increase apoptosis in the setting of preventing restenosis, or preventing or shrinking atherosclerotic plaques. Apoptosis is clearly involved in the regulation of vascular smooth muscle proliferation, interaction with macrophages, and foam cell formation. This area is under active investigation.[22]

One hypothesis about the mechanisms of aging is that too little apoptosis occurs, permitting the survival of cells that have sustained DNA damage. Such damaged cells would function inefficiently at best, owing to the accumulation of mutations in essential genes, and could undergo malignant transformation. Eventually, such marginally functioning and precancerous cells would predominate, with more generalized organ hypofunction as time went on.

Too much apoptosis

Excessive cell death is of particular concern in organs that are populated by terminally differentiated, nondividing cells. Any cells lost, whether by apoptosis or necrosis, are irreplaceable. In settings where cell death is inevitable, inhibiting the enzymatic processes of apoptosis may not salvage the cell, but merely convert its demise to a necrotic form. However, if a cell is damaged beyond repair, a tidy, noninflammatory apoptotic death may still be preferable, avoiding collateral damage from inflammation. Interventions aimed at preventing apoptosis may need to consider this possible adverse outcome.

This cautionary note aside, there are many degenerative diseases in which it would be desirable to prevent cell loss by apoptosis.

Neurodegenerative diseases, including amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease, are all accompanied by neuronal loss through apoptosis. In some cases, overexpression of Bcl-2 or the caspase-inhibiting cowpox virus protein CrmA has been shown to prevent neuronal cell loss in cell culture models. Alzheimer's disease is characterized by the deposition of an abnormal peptide (Aβ) derived from the β-amyloid precursor protein. This peptide aggregates and causes neuronal apoptosis. The mechanism by which cell death is signaled by the Aβ peptide is unclear. Stroke has also been shown to result in neuronal loss through apoptosis (reviewed in 23). The potential to ameliorate these disease processes by preventing apoptosis is under extensive investigation.

Apoptosis in myocardial ischemia/reperfusion

Myocardial ischemia and reperfusion are accompanied by extensive cell death, which includes both apoptosis and necrosis. In 1994, we discovered that in the rabbit, myocardial ischemia and reperfusion were associated with apoptosis.[24] We identified chromatin condensation present in cells which did not show morphologic features of necrosis at the electron microscopic level. The detection of oligonucleosomal DNA fragmentation was consistent with apoptosis. In addition, using the nick translation assay on fixed tissue sections, we found extensive DNA nicking of myocytes in the central ischemic zone as well as in marginal areas of reperfused tissue. The predominant cell type demonstrating DNA nicking was the cardiomyocyte *(Figure 4)*. A number of studies have investigated myocardial apoptosis induced by ischemia and reperfusion. Isolated neonatal cardiomyocytes subjected to hypoxia and reoxygenation upregulate Fas antigen and undergo apoptosis,[20] and tumor necrosis factor has been shown to induce apoptosis in neonatal and adult cardiomyocytes.[25]
In some studies, persistent ischemia is also able to induce cell death with characteristics of apoptosis. Overstretch and congestive heart failure are also accompanied by apoptosis.[26,27] In human hearts, apoptosis has been recognized in cardiac failure and arrhythmogenic right ventricular dysplasia.[28-30]
Thus, terminally differentiated cardiomyocytes undergo apoptosis in response to a variety of stimuli. Mechanisms whereby ischemia induces apoptosis need further study. Free radicals may be implicated, but no specific data are available. Overexpression of p53 in ventricular myocytes can induce apoptosis. However, in an elegant study in transgenic mice lacking p53, the extent of apoptosis after ischemia was the same as in controls.[31] Identification of specific signals for ischemia that induce apoptosis might lead to therapeutic interventions.

Preconditioning is the most effective means of preventing ischemia/reperfusion injury; accordingly, we tested and confirmed that preconditioning isolated myocytes prevents myocyte apoptosis.[7] Preconditioning the heart by a short period of ischemia, pharmacological intervention, or other stress, results in a smaller infarct size, fewer arrhythmias, less myocardial stunning, and diminished coronary vascular injury after a subsequent test of ischemia and reperfusion. In various animal models, preconditioning can be induced with adenosine, phorbol esters, adrenergic stimulation, bradykinin, and heat shock. These interventions produce a short-duration preconditioning effect that lasts about 2 hours, and a delayed or second window of protection after 24 hours that requires synthesis of HSPs. As noted above, HSPs have been found to block apoptosis signals through SAPK/JNK pathways.
One of the most consistent features of ischemic preconditioning is the reduction in cytoplasmic acidification during ischemia. The basis for this effect is unclear, and may run counter to the "pH paradox," which holds that acidosis is protective. This is paradoxical because ample evidence supports the contention that sequentially linked H^+, Na^+, and Ca^{2+} overload results in cell injury or death. Inhibition of the sodium/hydrogen exchanger, which has no effect on intracellular pH during ischemia, or may even exacerbate acidification, is highly protective.[32]
In addition to pH, calcium fluxes, high-energy phosphate metabolism, and mitochondrial substructure are noted to be altered in preconditioned cells during ischemia and reperfusion. Protection by preconditioning has been shown to be effective in every animal model tested and in man.
Preconditioning can also be demonstrated in cell culture with adult rabbit myocytes subjected to metabolic inhibition with deoxyglucose and potassium cyanide.

In order to examine the relationship between apoptosis and preconditioning, we established the isolated cardiomyocyte model using adult rabbit cardiomyocytes

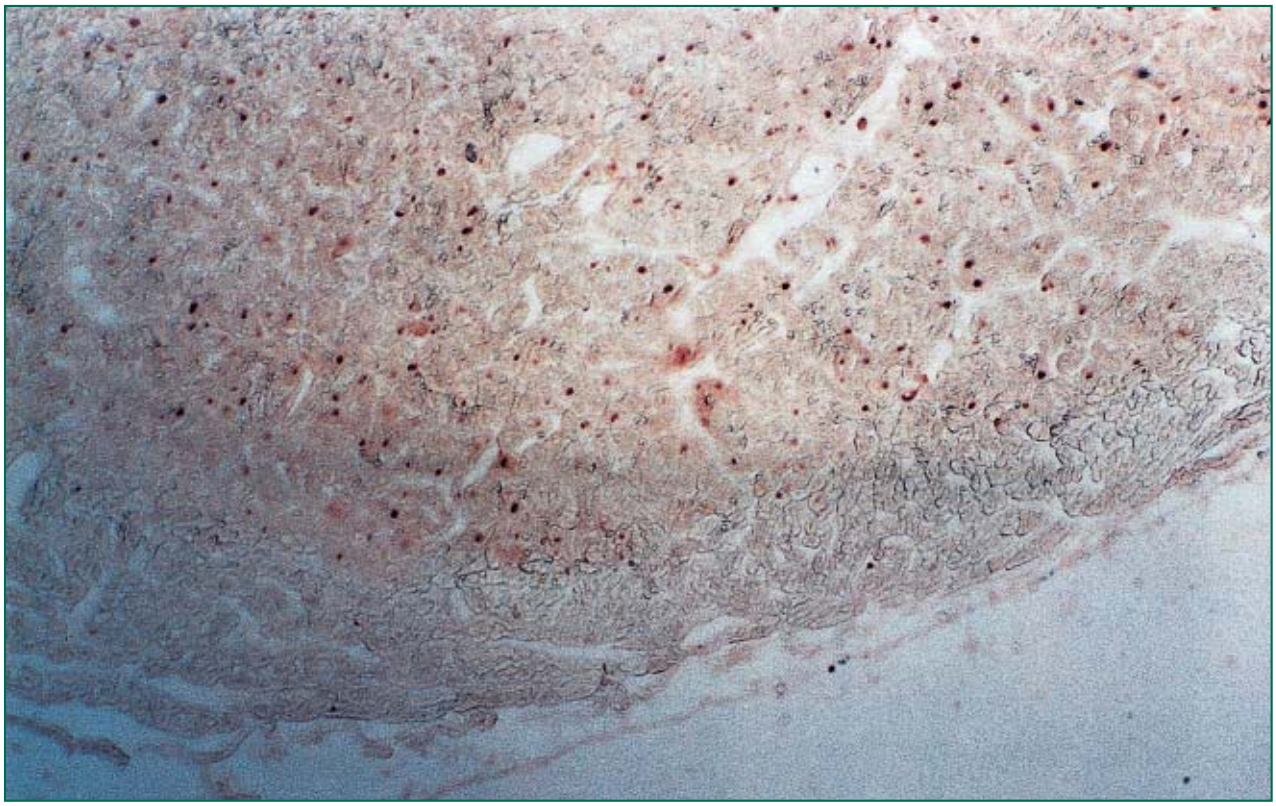

***Figure 4.** Evidence of apoptosis in the myocardium after ischemia/reperfusion. Nuclei containing fragmented DNA are labeled. Scattered nuclei are positive, while striations are preserved, reflecting an apoptotic death with preservation of plasma membranes.*

obtained after collagenase perfusion and Ficoll sedimentation. We simulated ischemia and reperfusion with 2-deoxyglucose and cyanide metabolic inhibition (MI). Preconditioning was induced with a brief (2-minute) period of MI and a 5-minute rest interval, which conferred protection against a 30- to 40-minute period of ischemia and a 4-hour recovery. When we extended the recovery period to 20 hours we saw no increase in cell death; however, functional recovery and longer periods of recovery have not been studied. Both preconditioning and activating protein kinase C (PKC) with phorbol myristate acetate before MI improved survival, and PKC inhibitors prevented preconditioning. We used the nick translation assay to detect DNA fragmentation as an indicator of apoptosis. Positive DNA nicking could be detected in 29% of cells after MI and 20 hours of recovery, compared to 7.5% of control cells not subjected to MI. Preconditioning reduced the DNA nicking after MI and recovery to 17.7%, which was significantly less than MI alone, but still more than control cells ($P<0.05$). DNA nicking was detected in rod-shaped cells as well as in rounded-up cells, and preconditioning reduced the number of cells with DNA nicking in both the rod-shaped and rounded cell populations. Rod-shaped cells exclude trypan blue and are metabolically active, as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. About half of the rounded-up or hypercontracted cells exclude trypan blue and reduce MTT. We suspect that the rod-shaped cells that show DNA nicking will progress to cell death if observed for longer intervals. Prevention of apoptosis by preconditioning has also been confirmed in the intact rat heart.[33]

As noted above, one of the most consistent features of preconditioning is that intracellular acidification is minimized during the subsequent ischemia. Based on the observation in neutrophils that VPATPase can participate in proton export to protect neutrophils against apoptosis, we hypothesized that VPATPase might be active in the heart, and might be upregulated in preconditioned hearts. Accordingly, we examined the ability of bafilomycin, a VPATPase inhibitor, to block the protective effect of preconditioning in cardiomyocytes subjected to MI and recovery. We found that

bafilomycin did not increase the amount of cell death after MI and recovery, but that it completely abrogated the protection conferred by preconditioning.[7] Furthermore, we found that preconditioning attenuated the extent of acidification that occurs with MI, and that bafilomycin actually increased the severity of acidification. These results support the idea that VPATPase plays a role in preconditioning in adult rabbit cardiomyocytes.

Blockade of the sodium/hydrogen exchanger protects cells against injury from MI and recovery by delaying the rise in intracellular calcium. We confirmed this finding, but found that when VPATPase was inhibited simultaneously, the protection conferred by inhibition of Na^+/H^+ exchange was lost. We suggest that acidification and calcium overload can both be lethal insults. The scenario depicted in *Figure 5* describes this dynamic interaction. Proton elimination via Na^+/H^+ exchange leads to Na^+/Ca^{2+} exchange and calcium overload, which would lead to cell death. Blockade of Na^+/H^+ exchange might lead to proton accumulation, but is compensated for by the VPATPase. However, inhibition of this route of proton elimination (by bafilomycin) would lead to cell death with features of apoptosis. Prolonged inhibition with bafilomycin alone leads to apoptosis in neonatal rat cardiomyocytes. This model predicts that VPATPase should diminish calcium accumulation during MI and recovery. We have recently obtained direct evidence of this, using neonatal rat myocytes. Inhibition of VPATPase with bafilomycin resulted in increased calcium accumulation during MI. Inhibition of the sodium/hydrogen exchanger *and* VPATPase resulted in increased apoptosis after MI and recovery.

Since caspases appear to participate in almost every observation of apoptosis to date, we examined whether they might also be involved in the cell death induced by ischemia and reperfusion simulated by MI and recovery. We initially showed that the general caspase inhibitor zVAD-fmk was able to protect cardiomyocytes from injury following MI and recovery, with observations extended out to 24 hours. In subsequent investigations using IDUN#1965 (a peptidomimetic caspase inhibitor from IDUN Inc, San Diego), we were able to show that even if the caspase inhibitor was added at the *end* of MI, or even as long as *15 minutes into recovery*, salvage of a significant number of myocytes was still possible. This suggests that irreversible commitment to apoptosis does not take place until sometime after the beginning of recovery. At this time, attempts to restore ion homeostasis are ongoing, and ATP stores are being regenerated. This may be consistent with recent evidence suggesting that apoptosis involves at least two energy-dependent steps. From a clinical point of view, this implies that it may be possible to salvage additional myocardium after ischemia, by including a caspase inhibitor at the time of reperfusion.

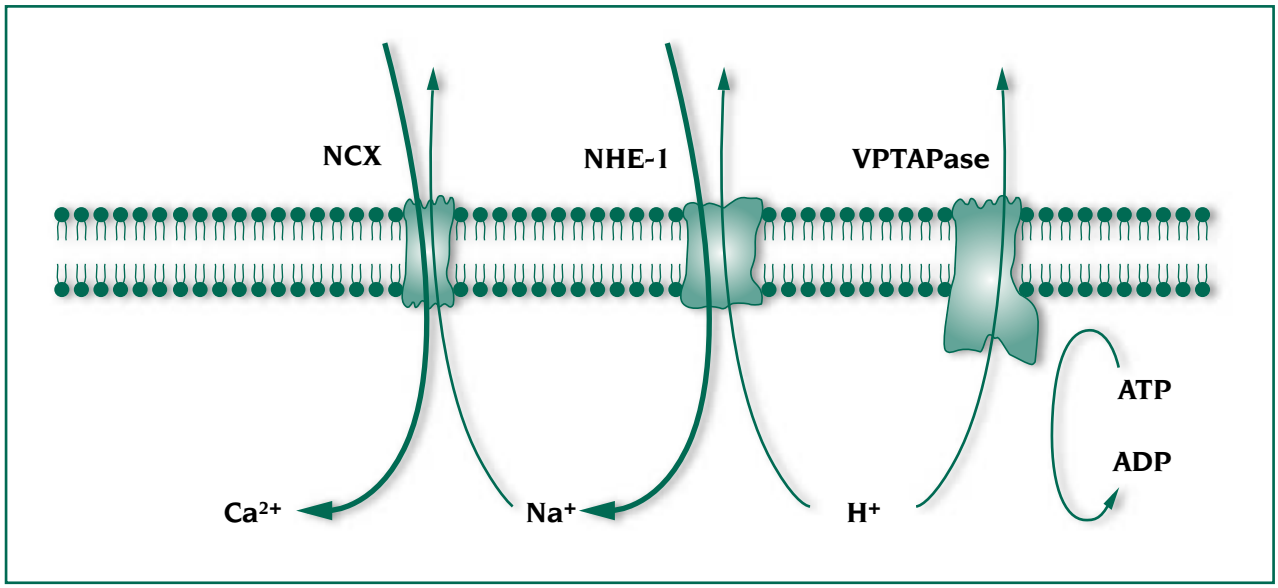

Figure 5. *Proposed interactions of two pathways for proton export in myocytes. Blockade of Na^+/H^+ exchange reduces Ca^{2+}-mediated cell injury, provided other mechanisms of proton elimination are active. One such mechanism is VPATPase. When VPATPase is inhibited, Na^+/H^+ blockade is no longer protective. Preconditioning reduces apoptotic cell death through enhanced VPATPase activity and reduced acidosis. NCX, Na^+/Ca^{2+} exchanger; NHE-1, Na^+/H^+ exchanger–1; VPATPase, vacuolar proton ATPase.*

However, these findings remain to be demonstrated in vivo in an animal model. Evidence from other laboratories is accumulating to support the idea that caspase activation is a feature of cell death following ischemia and reperfusion, and the caspases involved will soon be identified. Other potential approaches to therapeutic intervention in cardiomyocytes include overexpression of Bcl-2 or signaling through initiation factor-1 (IF-1), both of which inhibit cardiomyocyte apoptosis.

Apoptosis in heart failure

Apoptosis contributes to the progressive cell loss in patients with chronic heart failure.[28]
Experimental chronic heart failure caused by coronary microembolization in dogs or hypertension in spontaneously hypertensive (SHR) rats, and myocyte loss from aging in rats, are all associated with myocyte loss and cardiomyocyte apoptosis.[27,34,35]
The failing heart attempts to compensate for an increased load by hypertrophy of myocytes.
A number of years ago, Wyllie et al proposed that activated or injured cells might be faced with a "divide or die" situation; recent data from model systems lend support to this conjecture.[1] In the heart, it is plausible that the same signal transduction pathways that drive hypertrophy also trigger apoptosis. The basis for this contention is taken from the mammalian ultraviolet response, in which the signal transduction pathways activated and the pattern of gene expression induced by exposure to ultraviolet light lead to apoptotic death in some cells and proliferation in the surviving cells.
However, in both cases the signal transduction events are similar, involving activation of the JNK pathway and Jun/Fos transcriptional activation. The parallel may be even closer, since ischemia/reperfusion results in JNK activation in the heart. The significance of JNK activation is made more interesting by the recent report that MEKK1 (mitogen-activated protein kinase/ERK kinase kinase 1), an upstream kinase in the JNK pathway, is cleaved by a caspase, and a cleavage-resistant mutant form of MEKK1 prevents further caspase activation, implying the existence of a positive feedback loop between MEKK1 and caspases. This observation raises the possibility that inhibition of upstream or downstream components of the JNK pathway may also prevent apoptosis, either directly or through inhibition of caspase activation.

CONCLUSIONS

Cardiomyocyte death due to ischemia/reperfusion injury can have features of both apoptosis and necrosis. It appears that many of the biochemical processes are shared (eg, altered ion homeostasis, proteolysis), but that the sequence of events may differ. The potent protective effect of preconditioning suggests that myocardial tissue can be salvaged, at least by interventions *before* ischemia that reduce subsequent apoptosis. Whether interventions that block a specific event in apoptosis will preserve myocardium *after* ischemia and reperfusion in vivo remains to be determined. Our studies in isolated myocytes suggest that a reduced intracellular acidification and diminished calcium accumulation may be essential. In rabbit cardiomyocytes, activation of VPATPase appears to be important to this process. It would appear that inhibition of caspases may also be protective. The apparent "bottleneck" provided by caspase activation in apoptosis is the current target of antiapoptotic interventions, but further work is needed to identify additional targets in the cell death program that could be blocked or augmented (eg, Bcl family proteins) during or even after the injury.

In the Expert Answers section of this issue of *Dialogues,* Heinz Rupp and Bernhard Maisch answer the question

ALTERNATIVE NAMES OF HUMAN CASPASES

Caspase-1	ICE
Caspase-2	Nedd2, ICH-1
Caspase-3	CPP32, Yama, apopain
Caspase-4	ICE_{rel}II, TX,ICH-2
Caspase-5	ICE_{rel}III, TY
Caspase-6	Mch2
Caspase-7	Mch3, ICE-LAP3, CMH-1
Caspase-8	MACH, FLICE, Mch5
Caspase-9	ICE-LAP6, Mch6
Caspase-10	Mch4

"What are the prospects for beneficial manipulation?" One approach to identifying these targets is to recognize those that are modulated by preconditioning. Caspase cascade inhibition is particularly attractive because of its position in the apoptotic process, but the hypothesis that this intervention will reduce ischemic injury remains to be tested in vivo.

Circumstantial evidence implicates a role of apoptosis in progressive myocyte loss in chronic congestive heart failure, but a direct causal connection has not been made, a point gone into by Giorgio Olivetti, Roberta Maestri, Domenico Corradi, and Federico Quaini, who reply to the question **"Does apoptosis play a role in the progression of heart failure?"**

Evidence demonstrating the occurrence of apoptosis in a wide variety of clinical disorders has been presented and is compelling in the heart. As new tools for inhibiting components of the apoptotic pathway are moved from the laboratory to the clinical setting, it will be increasingly important for the clinician to understand the biochemistry of apoptosis in both the pathophysiologic setting and the physiologic setting. Inhibiting apoptosis systemically or as a long-term therapy may have both benefits and undesirable effects: this prompts the question **"Is cardiac apoptosis invariably bad?"** which is answered by Michael Schneider. Inhibition of apoptosis may lead to tumor promotion in other tissues, worsening of viral myocarditis, or may have the undesirable inflammatory consequence of converting apoptosis to necrosis in irreversibly injured tissues. The pressing need for salvaging myocardium in the setting of infarction or heart failure compels us to explore these potential therapies, but we should proceed with our eyes open to the possibility of unexpected adverse consequences.

REFERENCES

1. Wyllie AH, Kerr JFR, Currie AR.
Cell death: the significance of apoptosis.
Int Rev Cytol. 1980;68:251-306.

2. Ellis HM, Horvitz HR.
Genetic control of programmed cell death in the nematode C. elegans.
Cell. 1986;44:817-829.

3. Alnemri ES, Livingston DJ, Nicholson DW, et al.
Human ICE/CED-3 protease nomenclature [Letter to the Editor].
Cell. 1996;87:171.

4. Tewari M, Quan LT, O'Rourke K, et al.
Yama/CPP32β, a mammalian homolog of CED-3, is a Crm-A-inhibitable protease that cleaves the death substrate poly (ADP-ribose) polymerase.
Cell. 1995;81:801-809.

5. Li P, Nijhawan D, Budihardjo I, et al.
Cytochrome c and dATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade.
Cell. 1997;91:479-489.

6. Gottlieb RA.
Cell acidification in apoptosis [Review].
Apoptosis. 1996;1:40-48.

7. Gottlieb RA, Gruol DL, Zhu JY, Engler RL.
Preconditioning in rabbit cardiomyocytes: role of pH, vacuolar proton ATPase, and apoptosis.
J Clin Invest. 1996;97:2391-2398.

8. Puceat M, Vassort G.
Neurohumoral modulation of intracellular pH in the heart.
Cardiovasc Res. 1995;29:178-183.

9. Forgac M.
Structure and properties of the coated vesicle proton pump.
Ann N Y Acad Sci. 1992;671:273-283.

10. Piper HM, Sezer O, Schleyer M, Hutter JF, Spieckermann PG.
Development of ischemia-induced damage in defined mitochondrial subpopulations.
J Mol Cell Cardiol. 1985;17:885-896.

11. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD.
The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis.
Science. 1997;275:1132-1136.

12. Yang J, Liu X, Bhalla K, et al.
Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked.
Science. 1997;275:1129-1132.

13. Laderoute KR, Webster KA.
Hypoxia/reoxygenation stimulates Jun kinase activity through redox signaling in cardiac myocytes.
Circ Res. 1997;80:336-344.

14. Cosulich S, Clarke P.

Apoptosis: does stress kill? [Review].

Curr Biol. 1996;6:1586-1588.

15. McCarthy NJ, Whyte MK, Gilbert CS, Evan GI.

Inhibition of Ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak.

J Cell Biol. 1997;136:215-227.

16. Lazebnik YA, Takahashi A, Moir RD, et al.

Studies of the lamin proteinase reveal multiple parallel biochemical pathways during apoptotic execution.

Proc Natl Acad Sci USA. 1995;92:9042-9046.

17. Liu X, Zou H, Slaughter C, Wang X.

DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis.

Cell. 1997;89:175-184.

18. Gottlieb RA, Dosanjh A.

Mutant cystic fibrosis transmembrane conductance regulator inhibits acidification and apoptosis in C127 cells: possible relevance to cystic fibrosis.

Proc Natl Acad Sci USA. 1996;93:3587-3591.

19. James TN.

Normal and abnormal consequences of apoptosis in the human heart. From postnatal morphogenesis to paroxysmal arrhythmias.

Circ. 1994;90:556-573.

20. Tanaka M, Ito H, Adachi S, et al.

Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes.

Circ Res. 1994;75:426-433.

21. Schwarz EM, Badorff C, Hiura TS, Wessely R,

Knowlton KU. NFκB-mediated inhibition of apoptosis is associated with encephalomyocarditis virus virulence in vivo.

Circulation. 1997;96:I424.

22. Karsan A, Harlan JM.

Modulation of endothelial cell apoptosis: mechanisms and pathophysiological roles.

J Atheroscler Thromb. 1996;3:75-80.

23. Choi DW.

Ischemia-induced neuronal apoptosis. [Review].

Curr Opin Neurobiol. 1996;6:667-672.

24. Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL.

Reperfusion injury induces apoptosis in rabbit cardiomyocytes.

J Clin Invest. 1994;94:1621-1628.

25. Krown KA, Page MT, Nguyen C, et al.

Tumor necrosis factor alpha–induced apoptosis in cardiac myocytes. Involvement of the sphingolipid signaling cascade in cardiac cell death.

J Clin Invest. 1996;98:2854-2865.

26. Cheng W, Li B, Li P.

Stretch-induced programmed myocyte cell death.

J Clin Invest. 1995;96:2247-2259.

27. Sharov VG, Sabbah HN, Shimoyama H, Lesch M, Goldstein S, Goussev AV.

Evidence of cardiocyte apoptosis in myocardium of dogs with chronic heart failure.

Am J Pathol. 1996;148:141-149.

28. Narula J, Haider N, Virmani R, et al.

Apoptosis in myocytes in end-stage heart failure.

N Engl J Med. 1996;335:1182-1189.

29. Olivetti G, Abbi R, Quaini F, et al.

Apoptosis in the failing human heart.

N Engl J Med. 1997;336:1131-1141.

30. Mallat Z, Tedgui A, Fontaliran F, Frank R, Durigon M, Fontaine G.

Evidence of apoptosis in arrhythmogenic right ventricular dysplasia.

N Engl J Med. 1996;335:1190-1196.

31. Bialik S, Geenen DL, Sasson IE.

Myocyte apoptosis during acute myocardial infarction in the mouse localizes to hypoxic regions but occurs independently of p53.

J Clin Invest. 1997;100:1363-1372.

32. Meng H, Pierce GN.

Protective effects of 5-(N,N-dimethyl) amiloride on ischemia-reperfusion injury in hearts.

Am J Physiol. 1990;27:H1615-H1619.

33. Piot CA, Padmanaban D, Ursell PC, Sievers RE, Wolfe CL.

Ischemic preconditioning decreases apoptosis in rat hearts in vivo.

Circulation. 1997;96:1598-1604.

34. Kajstura J, Cheng W, Sarangarajan R, et al.

Necrotic and apoptotic myocyte cell death in the aging heart of Fischer 344 rats.

Am J Physiol. 1996;271(3, pt 2):H1215-H1228.

35. Li Z, Bing OH, Long X, Robinson KG, Lakatta EG.

Increased cardiomyocyte apoptosis during the transition to heart failure in the spontaneously hypertensive rat.

Am J Physiol. 1997;272(5, pt 2):H2313-H2319.

Apoptosis

Expert Answers to Three Key Questions

1

Apoptosis: what prospects for beneficial manipulation?

H. Rupp, B. Maisch

2

Does apoptosis play a role in the progression of heart failure?

G. Olivetti, R. Maestri, E. Cigola, D. Corradi, F. Quaini

3

Is cardiac apoptosis invariably bad?

M.D. Schneider

Apoptosis: what prospects for beneficial manipulation?

Heinz Rupp, PhD; Bernhard Maisch, MD

Philipps University of Marburg - Division of Cardiology - Baldingerstrasse 1 - 35033 Marburg - GERMANY

Pharmacological manipulation of programmed cell death could provide a breakthrough in the prevention and treatment of congestive heart failure, inflammatory heart diseases, and vascular lesions. This novel therapeutic approach is based on the inhibition or induction of cell death, depending on the type of disease and cardiovascular cell. Unexplored drug targets should be identified with a view to modulating gene expression in cardiac myocytes. Induction of apoptosis of fibroblasts or myofibroblasts by selective and potent compounds could limit fibrosis. Specific elimination of activated macrophages at the level of vascular lesions might be useful, but selective manipulation of apoptosis or survival of cardiac myocytes, endothelial cells, fibroblasts, and leukocytes should take into account the role of pro- and antiapoptotic factors that are recruited during the progression of a given disease.

Keywords: apoptosis; heart failure; fibrosis; inflammation; restenosis

Address for correspondence:
Heinz Rupp, PhD, Molecular Cardiology Laboratory, Div. of Cardiology, Philipps University of Marburg, Karl-von-Frisch-Straße 1, 35033 Marburg, Germany
(e-mail: rupp@mailer.uni-marburg.de)

Cell death in the cardiovascular system has been associated with necrosis for decades. Such catastrophic death of a multitude of cells is primarily the result of impaired oxygen delivery by the coronary arteries and is associated with inflammatory reactions. Since it was assumed that an energy deficit of the cardiac myocyte was the major cause of necrosis, there were only a few limited possibilities of preventing necrosis. A consequence of the concept of catastrophic cell death was that potential pharmacological interventions (eg, to limit restenosis after balloon angioplasty) only recently began to be explored.

Alongside the pattern of catastrophic cardiac myocyte death characterizing necrosis, an intriguing and unexplained observation had been made, that of the death of scattered myocytes in overloaded or senescent hearts, suggestive of apoptosis. However, studies on apoptosis in the cardiovascular field only started long after "programmed cell death" had been recognized as an important physiological process required for thymic involution, the removal of autoreactive lymphocytes during development, and the removal of excess cells after completion of an immune response, and long after dysregulation of apoptosis had been shown to underly the pathogenesis of autoimmune diseases associated with the survival of abnormal autoreactive lymphocytes. One of the reasons for this long-standing lack of interest in the cardiovascular field can be seen in the nature of the cardiac myocyte. In contrast to lymphocytes or cancer cells, the terminally differentiated cardiac myocyte has only a limited capacity to divide. Thus, therapeutic interventions seeking to act on the homeostatic balance between cell proliferation and cell death are irrelevant, and the only alternative is to prevent all cardiac myocyte death whatsoever.

The inadequacy of focusing solely on necrosis is, however, apparent when one considers the progression of fibrosis during hypertensive heart disease. The concept of necrosis implies a catastrophic death of cardiac myocytes and a corresponding replacement by fibrosis. Another hypothesis postulates excessive stimulation of fibroblasts by angiotensin II (Ang II) or aldosterone, resulting in marked collagen deposition. Both mechanisms suffer, however, from lack of evidence that severe oxygen deprivation persists in hypertensive heart disease. It is also unlikely that, in the absence of left ventricular failure, raised Ang II or aldosterone levels could, by themselves, account for excessive collagen synthesis of fibroblasts.
Necrotic cardiac myocyte death appears thus not to be the main factor responsible for the progres-

sion of hypertensive heart disease or congestive heart failure (CHF). Likewise, at the vascular level, the simplistic concept of cell necrosis with subsequent extracellular remodeling appears inadequate to explain the excessive proliferation of intimal cells after angioplasty.

It is therefore timely to explore the possible benefits of manipulating the death or survival of cardiovascular cells. Despite great efforts, attempts to stem the progression of congestive heart failure, adequately treat various inflammatory heart diseases, and halt restenosis after angioplasty have largely failed. What would the prospects be if apoptosis of cardiovascular cells could be controlled? Would this translate into a benefit for major cardiovascular disorders? This paper seeks to provide evidence in favor of this hypothesis.

CARDIAC MYOCYTES

It has often been argued that CHF in pressure-overloaded hearts was closely linked to the hypertrophy process. Since cardiac myocytes have a limited capacity to divide, hypertrophy remains the only mechanism for coping with an increased workload. Hypertrophied, exercized hearts do not fail, however, and it thus appears that the hypertrophy per se should not necessarily be associated with the onset of failure. The answer seems to lie in the expression of a variety of genes that cause the heart to be unable to cope with the workload. There is increasing evidence that an overloaded heart is insulin resistant[1] and that genes are expressed that are typical of diabetic hearts. Accordingly, interventions that increase glucose oxidation of heart muscle improve the pump performance of pressure-overloaded hearts.[2] One of the key features of pressure-overloaded hearts is diastolic overloading, which represents a crucial trigger for apoptosis.[3] If, in addition, Ang II is raised due to neuroendocrine activation, the threshold for apoptosis of the cardiac myocyte could be reached. Furthermore, an impaired rate of Ca^{2+} sequestration by the sarcoplasmic reticulum is expected to reduce the rate of isovolumic relaxation of the heart and would thus impair coronary perfusion. The fact that the reexpression of fetal genes depends on the wall stress gradient could explain that cardiac myocyte death ensues in an apparently scattered manner. Furthermore, the pleiotropic cytokine tumor necrosis factor–α (TNF-α) is increased in heart failure.[4] TNF-α can be a potent inducer of apoptosis through the TNF receptor–1 (TNFR1), acting probably via a TNFR1-associated "death domain" protein (TRADD).[5]

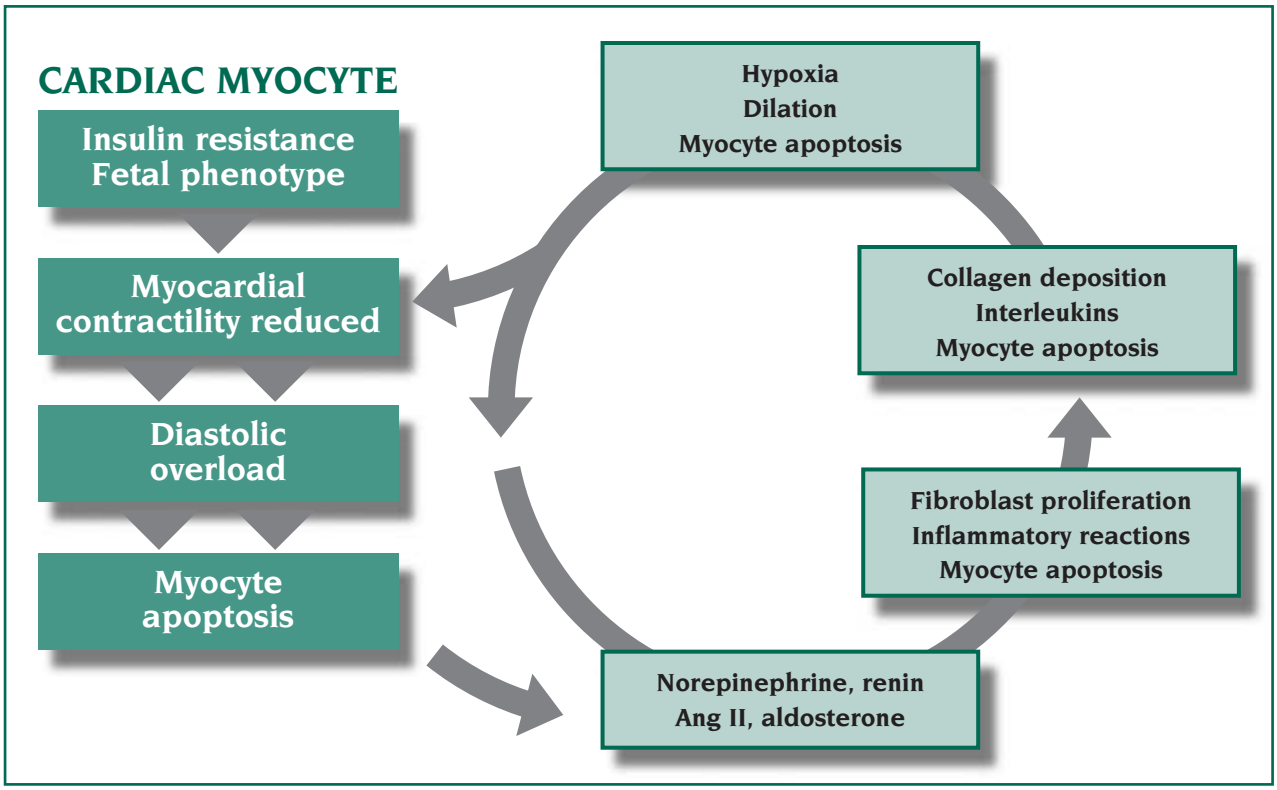

Figure 1. *Detrimental processes occurring during progression of heart failure due to pressure overload.*

This scenario of a borderline depression in pump performance could be sufficient to initiate progression of heart failure. Any loss of cardiac cells would, however, result in reduced performance of the heart muscle. Furthermore, a lost cardiac myocyte would have to be replaced by collagen, leading to diffuse fibrosis and impaired cardiac compliance *(Figure 1).*

What would the prospects be if the progressive scattered loss of cardiac myocytes could be prevented? At the very best, a chronically pressure- or volume-overloaded heart would maintain normal performance. How could this be achieved? In the most straightforward approach, the simultaneous occurrence of various cell death–promoting factors would have to be prevented. Unexplored drug targets of the cardiac myocyte should thus be identified in order to modulate myocyte gene expression. Promising avenues might include insulin-like growth factor–1,[6] cardiotrophin-1,[7] and Bcl-2,[8] which can inhibit apoptosis of cardiac myocytes. Thus, overexpression of insulin-like growth factor–1 was shown to prevent activation of cell death in the viable myocardium after infarction and limited dilation.[6] In favor of the hypothesis that improved heart function is associated with a reduced incidence of apoptotic cardiac myocyte death are recent findings showing that angiotensin-converting enzyme (ACE) inhibitors can reduce apoptosis. Thus, quinapril was found to normalize ACE activity and apoptosis in treated spontaneously hypertensive rats (SHRs) with heart failure associated with increased numbers of apoptotic cells,[9] while captopril ameliorated heart failure and reduced the exaggerated apoptosis.[10] It appears that the major effect of ACE-inhibitor treatment results from a reduction in workload and Ang II influence. Reducing the workload is expected to reduce the levels of catecholamine radicals, and thus the associated apoptosis.

Apoptosis of cardiac myocytes occurring during the transition from a nonfailing to a failing heart with increased wall stress could be one of the long-searched for causes of dilation of the ventricular chambers. Although the phenomenon of fiber slippage has been known for decades, it remains ill-defined and no drug approach is targeted at cardiac dilation. Increased wall stress in response to overload is initially offset by compensatory myocyte hypertrophy. However, if scattered myocyte loss occurs, dilation accompanied by wall thinning ensues. Dilation and increase in wall thickness result in increased oxygen consumption, thus favoring the occurrence of transient ischemic episodes. Pathological dilation of the heart chambers therefore increases the overall incidence of apoptosis of cardiac myocytes. The functional importance of transient ischemic events is also underscored by the fact that rapid ventricular pacing results in a high incidence of apoptosis of cardiac myocytes.[11] Bradycardic agents might thus be expected to have a protective action.

It remains to be shown whether the transduction signal of hypoxia-induced apoptosis can provide a unique drug target. In other cell types, hypoxia-induced apoptosis depends on the ubiquitous DNA-binding protein p53. If p53 is transfected in normal myocytes, they exhibit the morphological changes and genomic DNA fragmentation characteristics of apoptosis.[8] Since DNA damage is typically associated with upregulation of p53, it would contribute to the elimination of injured cells. Although p53 is induced by hypoxia in cardiac myocytes, it appears that a p53-independent pathway exists that mediates myocyte apoptosis during myocardial infarction.[12] The occurrence of apoptosis—particularly in the border zone of an infarcted area—and of reperfusion-related apoptosis would be promising targets for limiting cardiac myocyte loss in myocardial infarction. Of particular interest could be interventions that interfere with the enhanced expression of the death-promoting membrane-bound Fas/Apo-1 receptor, which is in excess when compared with the smaller increase of the apoptosis-inhibiting factor Bcl-2.[13] Alternatively, caspase inhibitors could be used to block the execution of the cell. Thus, Z-Val-Ala-Asp(OMe)-CH_2F (ZVAD-fmk), a tripeptide inhibitor of the caspase interleukin-1β–converting enzyme family of cysteine proteases, was shown to be effective in reducing myocardial reperfusion injury, an action at least partially attributable to the attenuation of cardiac myocyte apoptosis.[14]

FIBROBLASTS

Cardiac fibroblasts differ greatly from cardiac myocytes with respect to apoptosis. Cardiac myocytes are prone to go into apoptosis due to proapoptotic factors or loss of survival factors. By contrast, cardiac fibroblasts appear to be resistant to apoptosis. Hypoxia induced apoptosis in myocytes, but not in nonmyocytes such as fibroblasts.[15-17] In hypoxic conditions, Fas messenger RNA was upregulated twofold compared with controls in cardiac myocytes, whereas that of nonmyocytes was downregulated.[16] This differential response could explain the progression of various

cardiovascular disorders that are characterized by an excessive deposition of collagen. Since fibrosis not only adversely affects the mechanical performance of heart muscle and also severely limits oxygen supply, it is a major contributing factor to the progression of heart failure.

The question obviously arises as to whether the induction of apoptosis of proliferating fibroblasts could be a useful drug target to limit fibrosis. Although specific compounds that induce apoptosis of fibroblasts or activated myofibroblasts could be envisaged, it appears that apoptosis of fibroblasts may also be achieved by withdrawal of growth factors. Deprivation of trophic factors is known to induce apoptosis in various cells that depend on them for survival. In the case of human cardiac fibroblasts, P1, a cyclic peptide analog of platelet-derived growth factor-BB (PDGF-BB), induced apoptosis.[17] It was concluded that this compound might be useful not only for studying the balance of cellular proliferation/apoptosis in wound healing, but might also provide a promising approach to interfering with atherosclerosis and restenosis.

Thus, adventitial myofibroblasts contribute to postangioplasty restenosis by proliferating, forming a fibrotic scar surrounding the injury site, and migrating into the neointima.[18] The induction of apoptosis of fibroblasts or myofibroblasts could, therefore, have important therapeutic implications for limiting fibrosis, and efforts should be made to design compounds with greater potency and selectivity. If differential apoptosis of vascular smooth muscle cells (VSMCs) and fibroblasts could be induced, novel approaches would be available for interfering with vessel injury.

MACROPHAGES AND LYMPHOCYTES

In the initial stage of atherosclerosis during fatty streak formation, monocytes adhere to the endothelium, migrate into the subendothelium, and become transformed into macrophages. Macrophages are transformed into foam cells by uptake of oxidized low-density lipoproteins. Alternatively, macrophages form foam cells subsequent to infection with, for example, *Chlamydia pneumoniae*.[19] Since the accumulation of macrophages is associated with inflammatory reactions arising from the release of cytokines and matrix-degrading enzymes, they appear to have a detrimental influence on the progression of atherosclerosis.

Any intervention which locally interferes with the activation of macrophages could thus be of therapeutic interest. The principal potential of such an approach is shown by sites of "immune privilege" in the body. Since any cellular immune mechanism can damage nearby tissue, the stroma cells of the eye and the Sertoli cells of testes form tissues where dangerous inflammatory reactions cannot occur.[20] This immune privilege does not arise from physical barriers, but rather from an active process involving the expression of the Fas ligand (FasL).[21] FasL was originally thought to occur only in cytotoxic T cells and natural killer cells. FasL has, however, recently been demonstrated in all immunologically privileged sites. By expressing FasL, the eye can kill any infiltrating activated cells by apoptosis, preventing the impairment of vision. The important role of FasL in controlling apoptosis is also demonstrated by those cancer cells that express FasL.[22] Such cells can kill cytotoxic T lymphocytes and other activated T cells, thereby evading the specific immune surveillance and inducing immune suppression in the host.[23]

The crucial role of FasL expression in terminating inflammatory reactions has also been established in models of rheumatoid arthritis. Zhang et al[24] showed that although the expression of Fas was markedly upregulated in activated synovial cells and infiltrating leukocytes at the site of inflammation, the level of FasL was extremely low, and that injection of a recombinant replication-defective adenovirus carrying FasL gene into inflamed joints conferred high levels of FasL expression. Apoptosis of synovial cells was induced and the collagen-induced arthritis was ameliorated. The authors concluded that FasL gene transfer at the site of inflammation effectively ameliorated the autoimmune disease.

Although certain cancer cells express both Fas and FasL, they can escape from self-inflicted injury by mechanisms that make them resistant to Fas-induced apoptosis. One such mechanism involves overexpression of Bcl-2, which can block cell death induced, for example, by chemotherapeutic drugs. The expression of Bcl-2 is repressed by p53. If functional p53 is lost, Bcl-2 levels will be elevated and apoptosis of cancer cells will be inhibited. Overexpression of Bcl-2 has indeed been observed in various human cancers and has been correlated with a poor response to chemotherapy.[25] Compounds that perturb protein-protein interactions of cell death inhibitors (Bcl-2 family) or cell death effectors (Bax family) could, therefore, be useful in tumor therapy.

What can we learn from these studies on lymphocytes and cancer cells? One obvious question is whether the

transient generation of an immune privileged site within a vascular lesion could terminate chronic inflammation. Such an approach could involve expression of FasL or use of antisense oligonucleotides to downregulate members of the Bcl-2 family. Although the selectivity of such interventions cannot be predicted at the current time, they could, in principle, provide a novel regimen for interfering with vascular disorders.

Late vascular lesions could also be of great importance. A particularly detrimental process involves rupture of a destabilized plaque, thrombus formation, and arterial occlusion. Plaque rupture typically occurs in areas where a thinned plaque has a fibrous cap. During this process, macrophages accumulate and become involved in inflammatory reactions. Matrix-degrading enzymes released by macrophages contribute to the destabilization of the fibrous cap. Various cytokines released from macrophages can induce apoptosis of VSMCs that are required for stabilization of the plaque. Accordingly, apoptosis was found greatest in VSMCs subjacent to the endothelium.[26]
Specific elimination of activated macrophages would therefore appear to be a useful approach.

VASCULAR SMOOTH MUSCLE CELLS AND ENDOTHELIAL CELLS

The integrity of the vessel wall depends on a well-balanced equilibrium between proliferation and apoptosis of endothelial cells and VSMCs. After restenosis, both cell proliferation and apoptosis are thought to play a role. A relative decrease in apoptosis of VSMCs may, however, contribute to hyperplastic restenosis by prolonging the life span of intimal cells. Hyperplasia of VSMCs is also the most likely cause of in-stent restenosis, and therapeutic interventions inhibiting VSMC proliferation would greatly enhance the utility of endovascular stents.

The survival factor Bcl-x_L, which inhibits caspase activation and thus cell execution, was found to be more abundantly expressed within intimal cells compared with medial VSMCs.[27] By contrast, the cell death–promoting factor Bax was expressed equally throughout the vessel wall in human atherosclerotic lesions.[27] Downregulation of intimal cell Bcl-x_L with use of antisense oligonucleotides induced apoptosis of intimal VSMCs and acute regression of vascular lesions.[27] It was concluded that targeted apoptosis of VSMCs may be an effective therapy for intimal vascular disease.

FUTURE PROSPECTS

Further progress in the control of apoptotic cell death is expected to initiate novel therapeutic approaches to congestive heart failure, inflammatory heart diseases, and vascular lesions. Control of apoptosis implies the prevention or induction of cell death, depending on the type of cardiovascular cell and the type of disease. Efforts should be directed at selectively manipulating apoptosis or survival of cardiac myocytes, endothelial cells, fibroblasts, and leukocytes. Further work is needed to understand proapoptotic or antiapoptotic factors that become recruited during the progression of a given disease. In spite of the long time it has taken to realize the potential of controlling apoptosis, it appears that pharmacological manipulation of programmed cell death could soon provide the breakthrough in the prevention or treatment of cardiovascular diseases that has been sought for so long.

REFERENCES

1. Paternostro G, Pagano D, Gnecchi-Ruscone T, Bonser RS, Camici PG.
The hypertrophied human heart is insulin resistant.
Circulation. 1997;96(suppl I):I572.

2. Turcani M, Rupp H.
Etomoxir improves left ventricular performance of pressure-overloaded rat heart.
Circulation. 1997;96:3681-3686.

3. Anversa P, Olivetti G, Leri A, Liu Y, Kajstura J.
Myocyte cell death and ventricular remodeling.
Curr Opin Nephrol Hypertens. 1997;6:169-176.

4. Torre-Amione G, Kapadia S, Lee J, et al.
Tumor necrosis factor–alpha and tumor necrosis factor receptors in the failing human heart.
Circulation. 1996;93:704-711.

5. MacLellan WR, Schneider MD.
Death by design. Programmed cell death in cardiovascular biology and disease.
Circ Res. 1997;81:137-144.

6. Li Q, Li B, Wang X, et al.
Overexpression of insulin-like growth factor–1 in mice protects from myocyte death after infarction, attenuating ventricular dilation, wall stress, and cardiac hypertrophy.
J Clin Invest. 1997;100:1991-1999.

7. Sheng Z, Knowlton K, Chen J, Hoshijima M, Brown JH, Chien KR.
Cardiotrophin 1 (CT-1) inhibition of cardiac myocyte apoptosis via a mitogen-activated protein kinase–dependent pathway. Divergence from downstream CT-1 signals for myocardial cell hypertrophy.
J Biol Chem. 1997;272:5783-5791.

8. Kirshenbaum LA, de Moissac D.
The bcl-*2 gene product prevents programmed cell death of ventricular myocytes.*
Circulation. 1997;96:1580-1585.

9. Diez J, Panizo A, Hernandez M, et al.
Cardiomyocyte apoptosis and cardiac angiotensin-converting enzyme in spontaneously hypertensive rats.
Hypertension. 1997;30:1029-1034.

10. Li Z, Bing OH, Long X, Robinson KG, Lakatta EG.
Increased cardiomyocyte apoptosis during the transition to heart failure in the spontaneously hypertensive rat.
Am J Physiol. 1997;272(5, pt 2):H2313-H2319.

11. Leri A, Liu Y, Malhotra A, et al.
Pacing-induced heart failure in dogs enhances the expression of p53 and p53-dependent genes in ventricular myocytes.
Circulation. 1998;97:194-203.

12. Bialik S, Geenen DL, Sasson IE, et al.
Myocyte apoptosis during acute myocardial infarction in the mouse localizes to hypoxic regions but occurs independently of p53.
J Clin Invest. 1997;100:1363-1372.

13. Kajstura J, Cheng W, Reiss K, et al.
Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats.
Lab Invest. 1996;74:86-107.

14. Yaoita H, Ogawa K, Maehara K, Maruyama Y.
Attenuation of ischemia/reperfusion injury in rats by a caspase inhibitor.
Circulation. 1998;97:276-281.

15. Long X, Boluyt MO, Hipolito ML, et al.
p53 and the hypoxia-induced apoptosis of cultured neonatal rat cardiac myocytes.
J Clin Invest. 1997;99:2635-2643.

16. Tanaka M, Ito H, Adachi S, et al.
Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes.
Circ Res. 1994;75:426-433.

17. Brennand DM, Scully MF, Kakkar VV, Patel G.
A cyclic peptide analogue of loop III of PDGF-BB causes apoptosis in human fibroblasts.
FEBS Lett. 1997;419:166-170.

18. Wilcox JN, Waksman R, King SB, Scott NA.
The role of the adventitia in the arterial response to angioplasty: the effect of intravascular radiation.
Int J Radiat Oncol Biol Phys. 1996;36:789-796.

19. Libby P, Egan D, Skarlatos S.
Roles of infectious agents in atherosclerosis and restenosis: an assessment of the evidence and need for future research.
Circulation. 1997;96:4095-4103.

20. Ferguson TA, Griffith TS.
A vision of cell death: insights into immune privilege.
Immunol Rev. 1997;156:167-184.

21. Griffith TS, Brunner T, Fletcher SM, Green DR, Ferguson TA.
Fas ligand-induced apoptosis as a mechanism of immune privilege [see comments].
Science. 1995;270:1189-1192.

22. Shiraki K, Tsuji N, Shioda T, Isselbacher KJ, Takahashi H.
Expression of Fas ligand in liver metastases of human colonic adenocarcinomas.
Proc Natl Acad Sci USA. 1997;94:6420-6425.

23. Zeytun A, Hassuneh M, Nagarkatti M, Nagarkatti PS.
Fas-Fas ligand–based interactions between tumor cells and tumor-specific cytotoxic T lymphocytes: a lethal two-way street.
Blood. 1997;90:1952-1959.

24. Zhang H, Yang Y, Horton JL, et al.
Amelioration of collagen-induced arthritis by CD95 (Apo-1/Fas)-ligand gene transfer.
J Clin Invest. 1997;100:1951-1957.

25. Ghia P, Boussiotis VA, Schultze JL, et al.
Unbalanced expression of Bcl-2 family proteins in follicular lymphoma: contribution of CD40 signaling in promoting survival.
Blood. 1998;91:244-251.

26. Libby P, Geng YJ, Aikawa M, et al.
Macrophages and atherosclerotic plaque stability.
Curr Opin Lipidol. 1996;7:330-335.

27. Pollman MJ, Hall JL, Mann MJ, Zhang L, Gibbons GH.
Inhibition of neointimal cell bcl-x expression induces apoptosis and regression of vascular disease.
Nat Med. 1998;4:222-227.

Does apoptosis play a role in the progression of heart failure ?

Giorgio Olivetti, MD; Roberta Maestri, PhD; Elena Cigola, PhD; Domenico Corradi, MD; Federico Quaini, MD

Department of Pathology - University of Parma - ITALY

The failing heart is characterized by an increase in chamber volume and a reduction in wall thickness, leading to marked reduction in the mass-to-chamber volume ratio. These changes result from extensive myocyte loss, compensatory myocyte hypertrophy, and remodeling of the interstitial compartments. Myocyte loss in heart failure appears to result from the combined effect of myocyte necrosis and apoptosis. DNA strand breaks—the hallmark of apoptosis—have also been recently demonstrated in normal cardiac aging and myocardial infarction. Apoptosis is activated by ischemia and mechanical stress. Reactive oxygen species, atrial natriuretic peptide, angiotensin II, tumor necrosis factor–α, the Fas molecule, and cell-cycle reentry appear to play a role in this process. Intracellular molecular control mechanisms of apoptosis have been evidenced, suggesting therapeutic strategies to prevent apoptosis with a view to increasing survival in patients with cardiac diseases.

Keywords: apoptosis; necrosis; myocyte cell loss; heart failure; gene

Address for correspondence:
Giorgio Olivetti, MD, Dept of Pathology, University of Parma, Via Gramsci 14, 43100 Parma, Italy
(e-mail: olivetti@ipruniv.cce.unipr.it)

Despite the high incidence and ominous outcome of heart failure (HF), very little information is available on the mechanisms by which, in many cardiac diseases, pump dysfunction and overt failure progressively develop over time. It is only recently that quantitative results have been collected on the structural characteristics of the hearts of patients in end stage failure examined at the time of cardiac transplantation.[1,2] These data demonstrate that in ischemic and idiopathic dilated cardiomyopathies, despite extreme degrees of hypertrophic growth of the entire heart (by 80% or more in weight), the ventricles have cavity volumes 4 to 5 times larger than controls. Thus, the myocardial mass–to–chamber volume ratio is approximately 50% below the normal range. The myocardium is composed of myocytes almost double in size with a predominant increase in length (of more than 50%) to accommodate the enlarged chamber volume. Ventricular wall thickness, however, remains within control limits, so that the ratio of wall thickness to chamber volume decreases by almost four. Scars, consisting of segmental, replacement, and interstitial fibrosis, occupy between 13% to 28% of the ventricular myocardium, indicating extensive myocyte cell loss. In essence, these findings are consistent with the notion that myocyte cell loss, myocyte hypertrophy, and remodeling of the interstitial compartment all contribute to the decompensated eccentric ventricular hypertrophy of the failing heart.

Myocyte cell loss in the heart may be attributed to necrosis, apoptosis, or both. Before 1994, only necrosis was described in the myocardium as the outcome of severe and prolonged ischemia. Necrosis is a late morphological event that follows a series of biochemical reactions and functional alterations leading to irreversible myocyte damage. Tiny breaks in the plasmalemmal membrane and amorphous densities in the mitochondria are considered irreversible morphological indexes of this death process. Myocyte necrosis is accompanied by tissue repair involving an inflammatory reaction activated by the release of intracellular materials and the participation of granulocytes, macrophages, and, in later stages, fibroblasts and collagen deposition. The final result is a scar, which may alter the structural and functional properties of the myocardium.

Apoptosis is an earlier event activated by an energy-requiring genetic program in which a Ca^{2+}-dependent endogenous endonuclease cleaves the DNA in regular fragments of 180 to 200 bp. These characteristic DNA double-strand breaks are associated with intact cytoplasm, indicating that DNA damage is the first event of apoptotic cell death. An additional feature of

apoptosis that differs from necrosis is the disappearance of affected cells with residual apoptotic bodies, but no obligatory reparative fibrosis. This property has been considered a suicidal sacrifice of at-risk cells that may protect the remnant tissue from more deleterious and permanent consequences of the necrotic process.

Although difficulties exist in distinguishing between myocyte cell death by necrosis and by apoptosis, techniques have been developed and used in different experimental conditions to measure the number of myocytes involved in both processes.[3] Apoptosis can be confirmed by the presence of DNA strand breaks in agarose gel electrophoresis. However, in most cases, only qualitative observations have been reported, and the amount of myocyte cell death via this mechanism is still controversial. This is a relevant issue because there is evidence that the entire process of death by apoptosis takes minutes or hours, and, since myocyte proliferation is a limited process, the whole heart may disappear within days or months assuming that apoptotic cell death is an ongoing phenomenon.

The adult human heart contains approximately 4 to 6×10^9 myocytes, and aging does not affect myocyte cell numbers in the female heart. In contrast, in the male heart, 64 million myocytes/year are lost and this phenomenon is compensated by hypertrophic growth of the remaining viable cells.[4] Despite this reactive growth, the heart weight progressively decreases with time and this imbalance between loss and growth may explain the higher incidence of HF in elderly male subjects compared with females in whom such a loss is not apparent. In Fischer 344 rats, a well-established rat strain for studies on aging, myocyte cell loss by necrosis and apoptosis has been measured at different ages.[5] In the entire heart, myocyte necrosis precedes apoptosis and involves almost 1500 myocytes at 3 months, increasing to 32 000 myocytes at 24 months. Myocyte apoptosis was restricted to the left ventricle and involved 140 myocytes at 3 months and less than 1000 cells at 24 months. Myocyte cell loss was associated at 16 and 24 months of age with ventricular dysfunction and failure. Thus, in men and rats, a progressive drop-off of cells occurs with aging, explaining at least in part the functional deterioration.

In the terminal stages of failing human hearts, myocyte apoptosis, documented by DNA strand breaks, was found to account for an average of 2318 myocyte nuclei per million, a figure 232 times higher compared to controls. In 77% of apoptotic myocyte nuclei, characteristic morphologic features of apoptosis were found by confocal microscopy.[6] Additionally, DNA laddering was used to document DNA fragmentation biochemically. In several cases, myocardial fibrosis indicative of previous necrotic cell death was present and ranged from 1% to 44%. No difference was found in the amount of apoptosis among ischemic and idiopathic dilated cardiomyopathies and valvular disease.

The circumstantial evidence for the occurrence of apoptosis in cardiac diseases resulting in HF does not explain the stimulus that may trigger myocyte apoptotic cell death. It has been demonstrated that oxygen deprivation, a very well-known cause of necrosis in the myocardium, is able to elicit myocyte apoptosis. Experimentally, apoptosis precedes necrosis after coronary artery ligation in the rat.[3] In the hearts of patients who died shortly after acute myocardial infarction and intractable congestive heart failure, DNA fragmentation was found in 12% of myocyte nuclei in the region bordering the infarcted area, while in only 1% of cells was apoptosis in progress in the remote myocardium.[7] However, following extensive myocardial infarction, in addition to the ischemic event, the remaining viable tissue is exposed to an abrupt elevation in diastolic transmural stress that persists during the healing phases and cannot return to normal in the absence of external intervention. This is because chamber volume increases and ventricular wall thickness decreases. Wall thinning is characterized by a reduced number of cells across the wall, a phenomenon termed side-to-side lateral cell slippage, which is seen in both human and rodent failing hearts. In an attempt to interpret the mechanism of myocyte translation from the inner to the outer layers of the ventricular wall, it has been suggested that stress may be concentrated on a single cell of a hypothetical ring of myocytes, producing irreversible damage and eventually death of the target cell.[8]

More recently, data have accumulated to show that in conditions in which abnormal stress is applied to the myocardium, reactive myocyte growth is associated with apoptotic death signals. Overstretching papillary muscles in vitro, as well as simulating the diastolic overload in vivo, produces myocardial remodeling and apoptotic myocyte cell death.[9] Increased systolic and diastolic wall stress is equally effective in inducing myocyte apoptosis in animals and humans.[10] Terminal stages of HF that are accompanied by severe and prolonged stress are associated with myocyte loss by apoptosis.[6,7] Interestingly, after a period of well-compensated cardiac hypertrophy

in the spontaneously hypertensive rat model, cardiac failure develops only in some animals in which a greater degree of cardiac myocyte apoptotic cell loss can be demonstrated.[11] Reducing the pressure overload on these hearts by angiotensin-converting enzyme inhibition decreases the amount of apoptosis.

Thus, there is no doubt that stress may play a critical role, but very limited information is available on the signal transduction pathway followed by the stimulus from the surface of the cell to the nucleus and on how mechanical, hormonal, or chemical signals can be transformed into intracellular messages. Myocyte apoptosis appears to be modulated by multiple factors promoting or opposing myocyte death. Among others, the members of the Bcl-2 family have been quite extensively evaluated, although their role is still unclear.[3,6,9] Bcl-2, which promotes cell survival, is decreased in HF, whereas Bax, a gene of the same family promoting apoptosis, is unchanged. The precise mechanisms by which the two members of the same family have opposite effects is still unclear. The suggestion has been made that apoptosis is the result of the ratio between proapoptotic and antiapoptotic proteins that probably act as homodimers and heterodimers. Fas antigen, a cell surface molecule that belongs to the tumor necrosis factor and nerve growth factor receptor family, can also stimulate apoptotic myocyte cell death, and Bcl-2 may interfere with this process. Acute coronary occlusion or hypoxia are able to upregulate Fas expression in the ischemic myocytes.[3] However, passive overstretching of isolated papillary muscles produces Fas overexpression, but myocyte apoptosis seems to be dependent upon superoxide anion formation.[9] p53 and Waf-1 may promote myocyte apoptosis directly or through the renin-angiotensin system.[11] The interleukin-1β-converting enzyme family, which, in humans, includes caspase 1 to caspase 10, seems to have a direct role in myocyte cell death.[12] Tumor necrosis factor–α is elevated in patients with HF, is known to signal through functionally active receptors on myocyte sarcolemma, and may induce myocyte apoptosis in vitro by increasing intracellular sphingolipids.[12] Atrial natriuretic peptide provokes apoptosis in neonatal myocytes, suggesting an important role of this molecule alone or in combination with other neuroendocrine effectors.[13] Angiotensin II has been able to increase apoptotic cell death in isolated myocytes through enhanced Ca^{2+} entry into the cytoplasm. This effect was inhibited by specific angiotensin-1 (AT_1) receptor blockade.[14] Angiotensin-converting enzyme inhibition, by reducing the pressure overload on the hypertrophic hypertensive rat heart, seems to be able to protect against apoptosis.[11] Pioneering studies to promote myocyte survival from apoptotic cell death with growth factors are promising.[15]

Finally, the existence of similarities between apoptosis and the different phases of the cell cycle has been noted for some time in several cell systems and, as an extreme view, apoptosis has been considered an unsuccessful mitotic process. Although adult cardiac myocytes have been considered as being postmitotic cells, several studies in humans and animals have demonstrated that HF is associated with enhanced DNA synthesis in myocyte nuclei.[16] This phenomenon is also accompanied by upregulation of molecular markers for cell-cycle progression and mitotic figures. The simultaneous presence of apoptosis and DNA synthesis in HF supports the concept that in order to maintain pump function, extremely stressed, still viable myocytes could be induced to reenter the cell cycle. However, this maneuver may result in myocyte proliferation and/or death depending upon the ability to produce intact or damaged DNA. In this regard, it is well known that cell-cycle progression in the face of DNA damage is, in general, a potent stimulus to apoptotic suicidal induction in order to maintain intact the genomic DNA. In the heart, the suicidal apoptotic program of some myocytes in stressful conditions may be viewed as a positive attempt to avoid more dangerous consequences produced by necrotic cell death.

The hypothesis that apoptosis is involved in the progression of various cardiac diseases leading to irreversible failure is based on several facts: (i) heart failure develops more frequently in patients with hypertension and cardiac hypertrophy and survivors from acute myocardial infarction, conditions in which apoptotic myocyte cell death has been demonstrated; (ii) the presence of programmed myocyte cell death has been found in the myocardium of patients in end-stage cardiac failure; (iii) myocyte apoptosis can be elicited in isolated myocytes exposed to substances present at high levels during the developmental phases of HF; (iv) elevations in mural stress characteristic of HF of various origins may induce myocyte apoptosis; (v) agents that ameliorate survival in humans with HF reduce apoptotic myocyte cell death; and (vi) aging of the heart resulting in HF is accompanied by apoptotic myocyte cell loss.

In the majority of these conditions, however, necrotic myocyte cell death is present, and the final destiny of the failing heart should be attributed

to the cumulative effects of both death processes on the number of dying or surviving cells. The advantage of recognizing that apoptosis is consistently involved in abnormalities underlying heart dysfunction depends on the possible prevention of myocyte loss by pharmacological intervention or gene therapy. Once general and specific mechanisms, gene inducers and protectors, modulators, and other variables involved in apoptosis have been discovered, strategies to promote myocyte rescue will be generated, thereby preventing cell loss by this mechanism. Since the amount of myocyte apoptosis is not trivial in the pathologies where it has been found, patient survival or death may depend on the number of myocytes preserved from dying.

In conclusion, cardiac myocyte cell death by apoptosis is found in different diseases that progress acutely or chronically to HF, decreasing the number of viable myocytes. Thus, the answer to the original question—does apoptosis play a role in the progression to HF?—is yes. On the other hand, apoptosis cannot be considered the sole factor responsible for the progression of cardiac diseases to dysfunction and failure. Necrosis is also present, and the total amount of myocyte cell loss, in addition to the anatomical and structural remodeling of the myocardium, is a prominent factor in pump dysfunction. However, the complexity of events involved in the genesis of HF cannot be attributed to any one process alone, and gene expression, regulation of contractility, changes in the cytoskeleton and extracellular matrix, ion homeostasis, and other unknown factors may all contribute to cardiac failure. In any event, there is still the hope that therapeutic efforts aimed at myocyte preservation from apoptotic cell death may provide a further tool to improve prognosis in HF.

This work was supported in part by CNR and MPI 40%.

REFERENCES

1. Beltrami CA, Finato N, Rocco M, et al.
Structural basis of end-stage failure in ischemic cardiomyopathy in humans.
Circulation. 1994;89:151-163.

2. Beltrami CA, Finato N, Rocco M, et al.
The cellular basis of dilated cardiomyopathy in humans.
J Mol Cell Cardiol. 1995;27:291-305.

3. Kajstura J, Cheng W, Reiss K, et al.
Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats.
Lab Invest. 1996;74:86-107.

4. Olivetti G, Giordano G, Corradi D, et al.
Gender differences and aging: effects on the human heart.
J Am Coll Cardiol. 1995;26:1068-1079.

5. Kajstura J, Cheng W, Sarangarajan R, et al.
Necrotic and apoptotic myocyte cell death in the aging heart of Fischer 344 rats.
Am J Physiol.
1996;271(3, pt 2):H1215-H1228.

6. Olivetti G, Abbi R, Quaini F, et al.
Apoptosis in the failing human heart.
N Engl J Med. 1997;336:1131-1141.

7. Olivetti G, Quaini F, Sala R, et al.
Acute myocardial infarction in humans is associated with activation of programmed myocyte cell death in the surviving portion of the heart.
J Mol Cell Cardiol. 1996;28:2005-2016.

8. Olivetti G, Capasso JM, Sonnenblick EH, Anversa P.
Side-to-side slippage of myocytes participates in ventricular wall remodeling acutely after myocardial infarction in rats.
Circ Res. 1990;67:23-34.

9. Cheng W, Li B, Kajstura J, et al.
Stretch-induced programmed myocyte cell death.
J Clin Invest. 1995;96:2247-2259.

10. Teiger E, Dam TV, Richard L, et al.
Apoptosis in pressure overload induced heart hypertrophy in the rat.
J Clin Invest. 1996;97:2891-2897.

11. Li Z, Bing OHL, Long X, Robinson K, Lakatta EG.
Increased cardiomyocyte apoptosis during the transition to heart failure in the spontaneously hypertensive rat.
Am J Physiol.
1997;272(5, pt 2):H2313-H2319.

12. MacLellan WR, Schneider MD.
Death by design. Programmed cell death in cardiovascular biology and disease.
Circ Res. 1997;81:137-144.

13. Wu CF, Bishopric NH, Pratt RE.
Atrial natriuretic peptide induces apoptosis in neonatal rat cardiac myocytes.
J Biol Chem. 1997;272:14860-14866.

14. Cigola E, Kajstura J, Li B, Meggs LG, Anversa P.
Angiotensin II activates programmed myocyte cell death in vitro.
Exp Cell Res. 1997;231:63-371.

15. Buerke M, Murohara T, Skurk S, Nuss C, Tomaselli K, Lefer AM.
Cardioprotective effect of insulin-like growth factor I in myocardial ischemia followed by reperfusion.
Proc Natl Acad Sci USA.
1995;92:8031-8035.

16. Quaini F, Cigola E, Lagrasta C, et al.
End-stage cardiac failure in humans is coupled with the induction of proliferating cell nuclear antigen and nuclear mitotic division in ventricular myocytes.
Circ Res. 1994;75:1050-1063.

Is cardiac apoptosis invariably bad?

Michael D. Schneider, MD

Molecular Cardiology Unit Departments of Medicine, Cell Biology, and Molecular Physiology & Biophysics, and Graduate Program in Cardiovascular Sciences - Baylor College of Medicine - Division of Cardiology - Houston - Texas - USA

The prevailing assumption that loss of cardiac myocytes via apoptosis would contribute to the loss of cardiac pump function and, therefore, is undesirable, is highly plausible, but untested in mechanistic terms. Four potential counterexamples are discussed: altruistic cell sacrifice, genome surveillance, cell cycle surveillance, and immunological surveillance. Despite the logic underlying the proposed use of antiapoptotic drugs or genes as countermeasures in heart failure, these considerations suggest the need for vigilance to avoid unforeseen complications.

Keywords: apoptosis; cell cycle; gene therapy; heart.

Address for correspondence:
Dr Michael D. Schneider, Molecular Cardiology Unit, One Baylor Plaza, Room 506C, Baylor College of Medicine, Houston, TX 77030, USA (e-mail: michaels@bcm.tmc.edu).

As summarized elsewhere in this issue of *Dialogues in Cardiovascular Medicine* and in other recent reviews,[1,2] apoptosis of cardiac muscle cells (programmed cell death) is a form of cell demise distinct from necrosis, which has been observed to occur in diverse experimental settings of cardiac pathobiology including hypoxia, ischemia, reperfusion injury, myocarditis, aortic banding, spontaneous hypertension, and senescence. Cardiac myocyte apoptosis can be triggered by certain autocrine/paracrine factors that are more abundant in diseased hearts (angiotensin II, atrial natriuretic factor, tumor necrosis factor–α), and, conversely, may be susceptible to inhibition by "survival factors" (insulin-like growth factor–I, cardiotrophin-1) or by the forced expression of antiapoptotic genes (Bcl-2).[1,2] In humans, cardiac apoptosis has been noted in myocardial infarction, dilated cardiomyopathy, arrhythmogenic right ventricular dysplasia, myocarditis, and familial heart block (in the conduction system).[1,2] Together, these findings suggest the highly plausible premise that pharmacological or genetic countermeasures to interrupt the molecular cascade for apoptosis merit investigation, the 1990s version, perhaps, of salvaging jeopardized myocardium.

In his role as *agent provocateur* for this issue, Bob Engler has posed the question: is cardiac apoptosis necessarily and invariably a bad thing? For clinical adult cardiology, much of which comprises a race to avert the death of ischemic muscle cells, this question might seem needlessly contrarian. Indeed, our own research has come to view infarction and heart failure each as a "myocyte-deficiency disease." From this perspective, clinical recovery is seen as confounded by three intrinsic biological limitations in the adult heart: first, the postmitotic phenotype (a virtually complete loss of the ability of ventricular muscle to generate new daughter cells and restore pump function through an increase in cell number); second, the so-called "fetal" phenotype during compensatory hypertrophy of surviving myocytes (quantitative and qualitative changes in cardiac gene expression, which are nominally adaptive, but whose eventual effect may be maladaptive and contribute to heart failure); and, third, the lack of an obvious stem cell population, equivalent to satellite cells in skeletal muscle, which might be recruited into a cardiac differentiation pathway as an alternative source of cardiac myocytes in response to cardiac injury. Thus, the single most obvious expectation regarding cardiac apoptosis is its presumptive role as a contributor to impaired

pump function, whether in the context of acute myocyte loss in the first hours of infarction, the setting of iatrogenic cell loss upon successful reperfusion, or the more chronic situation of established compensatory hypertrophy and an inexorable progression to heart failure. These consequences would encompass not only the direct effects of cell loss on net mechanical performance, but also the confounding effects of wall thinning, increased wall stress, and adverse remodeling.

A pathogenic role of apoptosis likewise would seem straightforward, when this form of cell death is seen in cardiac conduction tissue. Recently, apoptosis has also been associated with a number of teratogenic interventions that induce abnormal cardiac morphogenesis, affecting both intrinsic myocardial cells themselves and cells of the cranial neural crest, which migrate to cardiac structures.[3] Cardiac myocyte apoptosis can be provoked in P19 cells, a pluripotent embryonal carcinoma cell line, when cardiogenesis in vitro is blocked using antisense methods to interfere with the transcription factor GATA-4,[4] a zinc finger protein that is needed for normal heart tube formation.[5] Although little or no evidence thus far links apoptosis to congenital heart disease in humans, it is reasonable to anticipate this association at least for a subset of congenital heart disorders.

In short, it is attractive, plausible, and uncomplicated to reason that cell death is bad for the heart, that apoptosis is a cause of cardiac cell death, and that apoptosis, in turn, likewise is bad. *Quod erat demonstrandum.* Is there even a need for a counterhypothesis? As formal possibilities, two scenarios seem especially germane: first, the circumstances (if any) in which cardiac apoptosis might ordinarily exert a beneficial effect, and, second, the related circumstances in which efforts to prevent cardiac apoptosis might therefore have unforeseen adverse effects. The potential salutary functions that relate to cardiac disease in adults can be summarized succinctly as cell sacrifice, genome surveillance, cell cycle surveillance, and immunological surveillance, in addition to potential roles of apoptosis in normal cardiac morphogenesis that will not be discussed here.

CELL SACRIFICE

By cell sacrifice, I allude to the postulate that regulated cell death might even be an altruistic adaptation in underperfused myocardium, sacrificing a portion of jeopardized cells to the benefit of others.[1] Although this concept might be an example of teleological and anthropomorphic reasoning, one of the strengths of this notion is to alert investigators to the possibility, even if remote, that antiapoptotic drugs and genes could therefore prove to be another proverbial "two-edged sword."

GENOME SURVEILLANCE

A stronger foundation exists for the concept of genome surveillance—that apoptotic pathways become activated upon oxidative DNA damage or other genotoxic stress, to prevent the survival (and expansion) of cells whose DNA has become defective.[6] In postmitotic ventricular muscle, the molecular sensors of DNA damage and their resulting effectors have not been characterized thoroughly and might differ from those that have been studied in proliferating cells.

CELL CYCLE SURVEILLANCE

Cell cycle surveillance refers to the cardiac myocyte's "irreversible" exit from the cell cycle under normal conditions, and to the compelling association between apoptosis and forced cell cycle reentry in many settings. In our own experiments, we have shown that reinitiation of DNA synthesis can be efficiently provoked in cardiac myocytes (even adult ventricular muscle cells, in vitro and in vivo) by forced expression of the transcription factor E2F-1 after adenoviral gene delivery.[7] In cells that are quiescent with regard to proliferative growth, E2F-1 resides in the "pocket" of tumor suppressor pocket proteins, of which the archetype is the retinoblastoma gene product Rb. By binding simultaneously to Rb and to the promoters of certain genes controlling DNA synthesis, E2F-1 can tether Rb to these promoters and keep the genes repressed. Mitogenic signaling cascades induce cell cycle–dependent proteins known as cyclins, which then activate cyclin-dependent protein kinases that phosphorylate Rb (and other essential targets). Rb phosphorylation releases E2F-1 from the "pocket," allowing the E2F-dependent genes to be induced, and thus enabling DNA synthesis. The fact that cell cycle reentry provoked by E2F-1 is lethal in the absence of *bcl-2* or a related antiapoptotic gene suggests there is additional information during normal mitogenic signaling that is absent when Rb is bypassed in

this way. Similarly, apoptosis is prevalent during G1 exit induced by viral proteins that bind the "pocket" and inactivate Rb (E1A, SV40 large T antigen). Although increased DNA synthesis and even sporadic mitoses have been reported in heart failure, the exact signal and mechanisms for G1 exit in this setting are unknown. Consequently, the tight association between apoptosis and cardiac pathologies that are permissive for G1 exit is consistent with this biochemical explanation (protection from incomplete or abortive cell cycle reentry), as well as the more conventional explanation that cycling is not "meant" to occur in adult myocardium (eg, given the complex constraints of sarcomere disassembly and reassembly, or more macroscopic considerations such as the mechanical integrity of the ventricle).

IMMUNOLOGICAL SURVEILLANCE

Beneficial effects of apoptosis also appear to exist in connection with immunological surveillance in the myocardium, during myocarditis or allograft acceptance. To illustrate, apoptosis was not seen following cardiac isografts in mice (DBA/2 to DBA/2) and was sporadic (<1% of cells) even in rejecting cardiac allografts (DBA/2 to C57BL/6). By contrast, apoptosis was prevalent (≈20%) in the periarterial cell infiltrate of accepted cardiac allografts (DBA/2 to C57BL/6, treated with anti-CD4 monoclonal antibody or gallium nitrate).[8] Analogously, it has been proposed that the longevity of graft-infiltrating myocytes seen in certain human cardiac allografts ("Quilty effect") results from the failure of normal apoptosis to occur and is associated with high-level expression of Bcl-2 in the lymphocytes.[9] As in the murine study cited above, apoptosis of ventricular myocytes was not seen even during acute rejection in humans.[9] Together, these experimental and clinical studies suggest the inference that the principal target of apoptosis in cardiac transplantation is the infiltrating lymphocyte, under conditions that are permissive for graft survival.
An especially striking cardioprotective effect of apoptosis was described following experimental infection of mice with a picornavirus, the principal agent for viral myocarditis. The mortality following encephalomyocarditis virus infection was 80% in wild-type mice, but 0% in genetically engineered mice lacking the p50 subunit of nuclear factor kappa B (NFκB), a transcription factor that suppresses certain forms of apoptosis.[10] Although virus replication was equivalent in both genetic backgrounds, early apoptosis occurred in the p50-deficient cells, hours before the viral "burst."[10] Hence, the improved survival in p50-deficient mice was attributed to rapid apoptosis (allowing early phagocytosis) of the infected cells.

Notwithstanding these specific latter counterexamples, it appears appropriate for now to regard the premise that apoptosis contributes to the disease phenotype as the default hypothesis in the more general settings of ischemic heart disease and congestive heart failure. As a cautionary note, however, it is vital to stress the null hypothesis (that apoptosis does not measurably alter cardiac function in these settings), given that the available information is largely confined to observational studies, and Koch's postulates have yet to be tested systematically here, eg, through overexpression or deletion of apoptotic and antiapoptotic genes, or through the use of existing pharmacological inhibitors.

One recently published study reports the reduction of myocardial reperfusion injury by a caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk)[11]; however, more extensive work of this kind is clearly needed to substantiate these conclusions, to address other models and conditions, and to permit an extrapolation to clinical benefit.

REFERENCES

1. Bromme HJ, Holtz J.
Apoptosis in the heart: when and why?
Mol Cell Biochem. 1996;164:261-275.

2. MacLellan WR, Schneider MD.
Death by design. Programmed cell death in cardiovascular biology and disease.
Circ Res. 1997;81:137-144.

3. Choi DS, Ward SJ, Messaddeq N, Launay JM, Maroteaux L.
5-HT_{2B} receptor–mediated serotonin morphogenetic functions in mouse cranial neural crest and myocardiac cells.
Development. 1997;124:745-1755.

4. Grepin C, Nemer G, Nemer M.
Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor.
Development. 1997;124:2387-2395.

5. Kuo CT, Morrisey EE, Anandappa R, et al.
GATA-4 transcription factor is required for ventral morphogenesis and heart tube formation.
Genes Dev. 1997;11:1048-1060.

6. Kharbanda S, Pandey P, Schofield L, et al.

Role for Bcl-xL as an inhibitor of cytosolic cytochrome c accumulation in DNA damage–induced apoptosis.

Proc Natl Acad Sci USA. 1997;94:6939-6942.

---

7. Agah R, Kirshenbaum LA, Truong LD, et al.

Adenoviral delivery of E2F-1 directs cell cycle re-entry and p53-independent apoptosis in post-mitotic adult myocardium in vivo.

J Clin Invest. 1997;100:2722-2728.

---

8. Bergese SD, Klenotic SM, Wakely ME, Sedmak DD, Orosz CG.

Apoptosis in murine cardiac grafts.

Transplantation. 1997;63:320-325.

---

9. Dong C, Winters GL, Wilson JE, McManus BM.

Enhanced lymphocyte longevity and absence of proliferation and lymphocyte apoptosis in Quilty effects of human heart allografts.

Am J Pathol. 1997;151:121-130.

---

10. Badorff C, Schwarz EM, Hiura T, Wessely R, Knowlton KU.

NFκB-mediated inhibition of apoptosis is associated with encephalomyocarditis virus virulence in vivo.

Circulation. 1997;96:I424.

---

11. Yaoita H, Ogawa K, Maehara K, Maruyama Y.

Attenuation of ischemia/reperfusion injury in rats by a caspase inhibitor.

Circulation. 1998;97:276-281.

Apoptosis

Summaries of Ten Seminal Papers

1

Cell death:
the significance of apoptosis

A.H. Wyllie and others. *Int Rev Cytol.* 1980

2

Genetic control of programmed cell death
in the nematode *C. elegans*

H.M. Ellis and H.R. Horvitz. *Cell.* 1986

3

Bcl-2 is an inner mitochondrial membrane protein
that blocks programmed cell death

D. Hockenbery and others. *Nature.* 1990

4

Ischemic preconditioning slows energy metabolism
and delays ultrastructural damage during
a sustained ischemic episode

C.E. Murry and others. *Circ Res.* 1990

5

Reperfusion injury induces apoptosis
in rabbit cardiomyocytes

R.A. Gottlieb and others. *J Clin Invest.* 1994

6

Preconditioning rabbit cardiomyocytes:
role of pH, vacuolar proton ATPase, and apoptosis

R.A. Gottlieb and others. *J Clin Invest.* 1996

7

Interaction of CED-4 with CED-3 and CED-9:
a molecular framework for cell death

A.M. Chinnaiyan and others. *Science.* 1997

8

The release of cytochrome *c* from mitochondria:
a primary site for Bcl-2 regulation of apoptosis

R.M. Kluck and others. *Science.* 1997

9

Cytochrome *c* and dATP-dependent formation of
Apaf-1/caspase-9 complex initiates an apoptotic
protease cascade.

P. Li and others. *Cell.* 1997

10

Prevention of apoptosis by Bcl-2: release of
cytochrome *c* from mitochondria blocked

J. Yang and others. *Science.* 1997

Selection of seminal papers by
R.L. Engler, MD - ACOS Research (151) San Diego VA
Health Care System - 3350 La Jolla Village Drive
San Diego CA 92161 - USA

***Prepared by S. Bialik, PhD and* R.N. Kitsis, MD**
Departments of Medicine and Cell Biology
Albert Einstein College of Medicine
1300 Morris Park Ave - Bronx, NY 10461 - USA

Highlights of the years by **Dr P.B. Garlick**
Division of Radiological Sciences - Guy's Hospital
London SE1 9RT - UK

Cell death: the significance of apoptosis

A.H. Wyllie, J.F. Kerr, A.R. Currie

Int Rev Cytol. 1980;68:251-306

This classic review by the initial describers of apoptosis is of relevance today primarily for its superb discussion of the morphology of apoptosis. Specifics of the light and electron microscopic characteristics of apoptosis are presented in a wide variety of cell types. In addition, appropriate credit is given to the unifying features that define this process in most cell types.

In this discussion, the review takes considerable care to differentiate the morphological aspects of apoptosis from those of necrosis. This approach has been important over the years in the recognition of the existence of apoptosis as an entity and has served as a useful paradigm in efforts to understand its mechanism. It should be pointed out, however, that, in more recent years, questions have been raised as to whether the dichotomy between apoptosis and necrosis is absolute and (blasphemous as it may sound) and whether these two processes may represent different phenotypic expressions of the same or similar molecular death mechanisms.

Although additional work will be needed to sort these issues out, it is conceivable that, in the future, cellular death will be described primarily by the molecular pathways involved (eg, caspase-dependent vs caspase-independent cell death).

In addition to morphology, the review provides a survey of situations in fetal and adult life in which apoptosis occurs and may play important roles. One interesting aspect of the discussion relating to the maintenance of homeostasis in adult tissues concerned estimates that rates of apoptosis balance those of proliferation in some normal tissues.

Several pathological situations were also discussed. Of particular relevance, given the currently hypothesized role of cardiac myocyte apoptosis in ischemic heart disease, is the authors' belief that ischemia-induced apoptosis results from only mild degrees of ischemia. For example, the authors cite their early description of apoptosis in a liver model in which the portal vein was occluded with maintenance of hepatic artery flow. The observation that myocyte apoptosis can result from marked decreases in myocardial perfusion suggests, however, that the general applicability of this maxim requires reexamination.

As can be reasonably expected from a review written almost two decades ago, the portions dealing with molecular mechanisms are somewhat limited. Even here, however, the authors showed foresight in anticipating that apoptosis would be regulated at both the posttranscriptional and transcriptional levels.

1980

Ronald Reagan wins a landslide victory to become the 40th president of the USA;
John Lennon is shot dead outside his apartment in New York;
and Sir Alfred Hitchcock, British film director, dies, aged 80

Genetic control of programmed cell death in the nematode *C elegans*

H.M. Ellis, H.R. Horvitz

Cell. 1986;44:817-829

Programmed cell death—or apoptosis—was first identified as a morphologically distinct mode of cell death in the 1970s. In the 1980s, Robert Horvitz catapulted the field of cell death into the realms of molecular and cellular biology. The nematode *Caenorhabditis elegans.* proved to be a particularly useful organism in which to study apoptosis, as 131 out of its original 1090 cells are genetically programmed to die during development. Furthermore, cell lineage is fixed and well documented, making it relatively easy to track the fate of individual cells. Lastly, dead cells can easily be identified in the transparent organism by differential interference contrast microscopy.

In this paper, the authors identify the genes that regulate programmed cell death. Dying cells are normally removed by phagocytosis within 1 hour of the first signs of cell death. In the *ced-1* mutant, however, engulfment is delayed for many hours. A second mutant, *egl-1*, was also utilized. This worm is defective in egg laying because the hermaphrodite-specific neurons (HSNs) required for proper egg laying are removed inappropriately by programmed cell death. These mutants were subjected to mutagenic screens to uncover additional genes that affect cell death. In this manner, *ced-3* and *ced-4* were identified. Two recessive mutations in the *ced-3* gene were found in the original screen. While the phenotype of these worms was normal, embryonic and postembryonic cell death was not observed. Furthermore, *ced-3* mutations rescued the *egl-1* phenotype; HSNs survived in the double mutant *ced-3*; *egl-1* hermaphrodites and egg laying was normal. *Ced-3* is therefore required for programmed cell death to occur, in all cell types. The cell death survivors did not divide, and did not affect the cell lineage of other cells. Some, however, were capable of differentiating and adopting the functional capabilities of their normal, surviving sister cells.

Mutations in the second gene, *ced-4,* showed a similar phenotype as those in the *ced-3* mutants. Recessive mutations were also identified that resulted in survival of cells that would normally be deleted by programmed cell death. *Ced-4* mutations also suppressed the *egl-1* defect, in a recessive manner. Both *ced-3* and *ced-4* are epistatic to *ced-1* and two other mutations that affect disposal of the dying cells, *ced-2* and *nuc-1*.

The following pathway was proposed: *ced-3* and *ced-4* are necessary for the death process itself. In the absence of functional gene products, the 131 cells that are normally fated to die survive. Downstream of these genes are *ced-1* and *ced-2,* which are required for engulfment of cells that have exhibited death features. Finally, *nuc-1* is responsible for degradation of the DNA of the engulfed cells.

The *C elegans* death pathway is now known to be much larger. The death process can be divided into three stages. 1. Cell-specific genes determine a cell's individual decision to die. 2. Execution of death is controlled by *ced-3, ced-4,* and *ced-9. Ced-9* lies upstream of *ced-3* and *ced-4* and antagonizes their activities. *Ced-4,* in turn, lies upstream of, and activates, *ced-3. Egl-1* has recently been placed upstream of all three of these genes, and negatively regulates *ced-9.* (The *egl-1* mutation described here was a gain of function mutation.) The last phase of death involves engulfment and breakdown of the dying cell. This is controlled by c*ed-1, ced-2, ced-5, ced-6, ced-7,* and *ced-10,* and *nuc-1*. The function of these genes has been and continues to be an area of much interest. The discovery of mammalian homologs of *ced-3* (caspases), *ced-4* (Apaf-1), and *ced-9* (Bcl-2) gave further insight into the actions of these regulators and the central apoptotic machinery. Recent cloning of *egl-1, ced-5,* and *ced-7* revealed that these three also have mammalian counterparts. The field of apoptosis research owes not only its humble beginnings to the nematode, but also its continuing progress.

1986

Imelda Marcos leaves 1060 pairs of shoes in the presidential palace in the Philippines; William Hurt wins an Oscar (Best Actor) for his role in "Kiss of the Spider Woman"; and Christopher Isherwood, British novelist, dies, aged 81

Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death

D. Hockenbery, G. Nuñez, C. Milliman, R.D. Schreiber, S.J. Korsmeyer

Nature. 1990;348:334-335

In the late 1980s, an interesting oncogene, the product of a translocation between chromosomes 14 and 18, was described and named *bcl-2.* The genetic rearrangement inserted *bcl-2* in the immunoglobulin locus, resulting in the ectopic expression of the gene in B cells. This in turn led to B cell lymphoma. It turned out that, unlike other known oncogenes, Bcl-2 did not affect the rate of proliferation. Rather, it affected the rate of cell survival.

In this short but seminal paper, the authors demonstrated that overexpression of Bcl-2 in FL5.12 cells, an interleukin-3– (IL-3) dependent pro-B lymphocyte cell line, blocked apoptosis induced by IL-3 withdrawal. This was the first demonstration that Bcl-2's oncogenic properties derived from its ability to block cell death.

Furthermore, the authors performed cell fractionation and indirect immunofluorescence studies to determine the localization of Bcl-2 in both the overexpressing FL5.12 cells and in RL-7 cells that bear the t(14, 18) translocation. Immunofluorescence staining for Bcl-2 revealed a punctate, cytoplasmic distribution that coincided with markers of the mitochondria, but not other organelles. Bcl-2 contains a hydrophobic segment in its carboxy terminus that is likely to target it for mitochondrial membrane insertion. In fact, upon cell fractionation, Bcl-2 was localized to the heavy membrane fraction, which contains mitochondrial membranes. Further fractionation revealed that it was localized to the inner mitochondrial membrane.

Interestingly, this observation was contradicted by a later paper that demonstrated that Bcl-2 is associated with the outer mitochondrial membrane, with all but the carboxy terminus of the protein exposed to the cytoplasm. The discrepancy may be explained by the possibility that Bcl-2 lies in regions of the outer mitochondrial membrane that maintain direct contact with the inner membrane. It is now accepted that Bcl-2 is localized to the outer mitochondrial, endoplasmic reticular, and nuclear membranes.

Thus, while erroneous in one of its conclusions, this was a landmark paper in that it established the first clue as to the mechanism of Bcl-2 action. It not only showed that Bcl-2 is an antiapoptosis factor, but it also suggested that its localization may facilitate this activity. In fact, work in the next decade demonstrated the close fundamental link between Bcl-2, mitochondria, and apoptosis.

1990

Popeye celebrates his 60th birthday;
Jessica Tandy wins an Oscar (Best Actress)
for her role in "Driving Miss Daisy";
and US composer and conductor
Leonard Bernstein dies, aged 72

Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode

C.E. Murry, V.J. Richard, K.A. Reimer, R.B. Jennings

Circ Res. 1990;66:913-931

The objectives of this study were to determine the effect of preconditioning on the ultrastructural and metabolic changes that characterize ischemia. To do this, dogs were subjected to 4 episodes of ischemia (5 minutes) followed by reperfusion (5 minutes). This was immediately followed by an additional 5, 10, 20, or 40 minutes of sustained ischemia. The major observation was that preconditioning slows down—but does not ultimately change—the evolution of these parameters. Controls underwent identical periods of sustained ischemia but without prior preconditioning.

After 5 minutes of ischemia, some control hearts showed mild, reversible ultrastructural abnormalities including myofibrillar stretching and mitochondrial swelling. These changes became more marked and uniform by 10 minutes and were joined by condensation and margination of chromatin. After 20 minutes of ischemia, amorphous matrix densities, a marker of irreversible injury, could be detected in some mitochondria. By 40 minutes, all of these changes were more severe and uniform and, in addition, discontinuities in, and detachment of, the sarcolemma from Z disks—further evidence of irreversible damage—was present. In contrast, the kinetics of these ultrastructural abnormalities was retarded in preconditioned hearts. Immediately following preconditioning itself, no ultrastructural abnormalities were evident, and only mild reversible changes were present after 5 to 10 minutes of ischemia. Notably, even after 20 minutes of sustained ischemia, evidence of injury in preconditioned hearts was still quite heterogeneous and did not include irreversible changes. Only after 40 minutes of ischemia did preconditioned hearts exhibit irreversible changes; even here, however, the pathology was less uniform than in control hearts. Thus, in agreement with this group's prior measurements of infarct size and histology, the onset of ultrastructural abnormalities characteristic of ischemia is slowed by preconditioning.

Several metabolic parameters were also studied. Steady state levels of ATP decreased in response to ischemia in both preconditioned and control groups. The kinetics of this change differed significantly, however. First, even prior to the onset of sustained ischemia, ATP levels in preconditioned hearts were 71% of those found in control hearts. Despite this lower starting point, the amount of ATP in preconditioned hearts exceeded that in control hearts subjected to 10 minutes of ischemia (50% vs. 37% of normal ATP levels respectively). Following 20 to 40 minutes of ischemia, however, preconditioned and control hearts both contained similar amounts of ATP (≈11% of normal ATP levels). Lactate levels were similar in preconditioned and control hearts during the first 5 minutes of sustained ischemia. In contrast, lactate levels were significantly lower in preconditioned as compared with control hearts subjected to 10, 20, and 40 minutes of ischemia. The authors believed the increased ATP levels in preconditioned hearts subjected to 10 minutes of ischemia to result from decreased ATP utilization rather than increased ATP production. This opinion was based on the fact that: (i) differences in aerobic ATP production were unlikely given similar collateral blood flows in preconditioned and control groups; and (ii) differences in anaerobic production could not explain the higher ATP levels in preconditioned hearts as these hearts had lower lactate levels than controls.

A model is proposed in which preconditioning reduces myocardial energy demand during ischemia. The biochemical mechanisms by which this occurs are not known. The consequences of such a scenario would be higher ATP levels and reduced concentrations of catabolites. The second part of the model posits that either or both of these metabolic changes results in increased myocardial viability.

1990

Michaelangelo, Raphael, Leonardo, and Donatello eat pizza and fight crime in the sewers; "Sex, Lies, and Videotape" wins the Palme d'Or at Cannes; and Susan Butcher wins the Alaskan Iditarod Trail dogsled race for the fourth time

Reperfusion injury induces apoptosis in rabbit cardiomyocytes

R.A. Gottlieb, K.O. Burleson, R.A. Kloner, B.M. Babior, R.L. Engler

J Clin Invest. 1994;94:1621-1628

Ischemia is a complex process characterized by, among other things, an insufficient supply of oxygen and other nutrients relative to tissue demands. It has long been known that myocardial ischemia is accompanied by death of cardiac myocytes, and that the most effective treatment for acute ischemia is restoration of blood flow. Reperfusion, however, is a double-edged sword that can in itself lead to tissue injury. This may be due to the production of reactive oxygen species or inflammation. In this paper, the authors demonstrate a novel mechanism for myocardial reperfusion injury. They show that cardiac myocytes undergo apoptosis in response to ischemia/reperfusion.

The authors subjected rabbits to ischemia/reperfusion injury by transient occlusion of the left coronary artery followed by subsequent restoration of blood flow. Thirty minutes of ischemia followed by 4 hours of reperfusion induced apoptosis in 7/7 rabbit hearts. This conclusion was supported by the presence of internucleosomal DNA fragmentation of total genomic DNA isolated from the ischemic zones. Such fragmentation is considered to be a distinguishing hallmark of apoptosis. The authors then investigated the identity of the particular cell type that was undergoing apoptosis. Because polymorphonuclear monocytes are known to undergo apoptosis, they represented a potential contributor to the apoptotic phenotype. DNA cleavage was still observed, however, in rabbits made granulocytopenic by treatment with nitrogen mustard. This result excluded polymorphonuclear monocytes as the sole cell type undergoing apoptosis. In contrast, cardiac myocytes were shown to be the main contributors to DNA fragmentation in the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay, an in situ technique, which, although not as specific an apoptotic marker as nucleosome laddering, allows the involved cell type to be identified. TUNEL positivity occurred more frequently towards the endocardium of the reperfused hearts. No DNA fragmentation or myocyte TUNEL staining was observed in nonischemic myocardium, or in hearts reperfused after only 5 minutes of ischemia.

Permanent occlusion for 30 minutes or 4.5 hours did not reveal any internucleosomal DNA fragmentation, indicating that the apoptotic stimulus resulted from reperfusion. TUNEL-positive staining was observed, however, after 4.5 hours of continuous ischemia. By electron microscopic analysis, the abnormal nuclei following this stimulus had a distinct appearance from those seen following ischemia/reperfusion. Following reperfusion, 97% of abnormal nuclei exhibited variable density and a diffuse pattern of condensation. In contrast, in 72% of abnormal nuclei in the permanently occluded hearts, chromatin condensation was homogeneously dense with a central clearing. The remainder appeared similar to the reperfused nuclei. Interestingly, these were located in regions of the myocardium that exhibited cytoplasmic changes that are typical of reperfusion (eg, contraction bands), suggesting that even under permanent occlusion, some unintentional reperfusion might occur. The authors failed to see any apoptotic bodies in either ischemic or reperfused tissue.

This groundbreaking paper was the first documentation of cardiac myocyte apoptosis in an in vivo model of ischemic heart disease. It would be followed soon after by reports demonstrating cardiac myocyte apoptosis in numerous animal models of heart disease, such as long periods of continuous ischemia, chronic heart failure, and hemodynamic overload, as well as in human patients. Researchers are currently concentrating on understanding the molecular regulation of myocyte apoptosis in response to these stimuli and evaluating the significance of apoptosis to the pathogenesis of the various syndromes in which it occurs. This paper thus ushered in a new era in cardiovascular research, with hopes of producing novel approaches to the treatment of heart disease.

1994

Phoolan Devi, Indian's legendary
"Bandit Queen" is released from prison;
a mortar bomb attack on the central market in
Sarajevo kills 68 civilians;
and North Korean President Kim Il Sung,
the longest-ruling dictator, dies, aged 82

Preconditioning rabbit cardiomyocytes: role of pH, vacuolar proton ATPase, and apoptosis

R.A. Gottlieb, D.L. Gruol, J.Y. Zhu, R.L. Engler

J Clin Invest. 1996;97:2391-2398

Both ischemic preconditioning and some agents that protect cells against apoptosis are characterized by diminished intracellular acidification. Based on these observations, the authors hypothesized that preconditioning may block intracellular acidification, thereby, decreasing myocyte apoptosis, and that these effects may be modulated through specific ion pumps or channels. The vacuolar proton ATPase (VPATPase) is a good candidate for these functions because it pumps protons across the sarcolemma from the cytoplasm to the extracellular space.

This study showed that: (i) VPATPase does indeed play an important role in the effects of preconditioning on the preservation of intracellular pH and myocyte survival; and (ii) ischemia/reperfusion-related increases in apoptotic markers such as terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining are ablated both by preconditioning and caspase inhibitors, suggesting that preconditioning protects myocytes, at least in part, by inhibiting apoptosis.

As a surrogate for ischemia in this study, cultures of adult rabbit cardiac myocytes were subjected to 1 mM sodium cyanide and 20 mM 2-deoxyglucose (this cocktail is referred to as metabolic inhibition [MI]) for 30 minutes. Reperfusion was modeled by adding back control media for 4 or 24 hours. In some cases, cells were preconditioned by being subjected to 2 minutes of MI followed by 2 solution changes of control media. After a 5-minute rest period, they were then subjected to 30 minutes of MI followed by 4 or 24 hours of reperfusion (MI/R). End points included the percentages of rod shaped cells, TUNEL-positive cells, and changes in intracellular pH.

The first set of experiments demonstrated that MI/R—without preconditioning—resulted in a 30% loss of viability following both 4 and 24 hours of reperfusion. Loss of viability induced by MI/R could be largely ablated by preconditioning. The protective effect of preconditioning could be mimicked by pretreatment with phorbol ester, an activator of protein kinase C (PKC). Conversely, chelerythrine, a PKC blocker, inhibited the protective effects of preconditioning. The role of VPATPase in modulating the effect of preconditioning on MI/R-induced loss of myocyte viability, was explored with the specific VPATPase inhibitor bafilomycin A1 (BAF). BAF completely abrogated the protective effects of both preconditioning and phorbol ester pretreatment on MI/R-induced myocyte death. These experiments showed that VPATPase plays an important role in the mechanism by which preconditioning protects myocytes against MI/R, and that VPATPase may be activated through a PKC-dependent pathway, perhaps PKC itself, during preconditioning.

The investigators then assessed the role of VPATPase in modulating intracellular pH during preconditioning. As expected, preconditioning diminished intracellular acidification in response to MI. Inhibition of VPATPase with BAF ablated this effect completely and resulted in a pH even lower than that of MI alone. This experiment shows that VPATPase is important in modulating the alkalinizing effects of preconditioning on myocytes and in preserving myocyte pH during ischemia without preconditioning.

Finally, preconditioning was shown to decrease myocyte apoptosis by two criteria. First, it decreased the number of TUNEL-positive myocytes. Second, zVAD-fmk, a broad-spectrum caspase inhibitor, was shown to mimic the effects of preconditioning on TUNEL. Although these data are correlative, they indicate that myocytes are dying by a reversible process and suggests that this process is apoptosis.

The major limitations of the study include the extent to which the in vitro model of I/R is a valid model of ischemia in vivo and potential, unanticipated effects of the inhibitors employed in other processes. Nevertheless, this work provides a potentially important molecular hook that might be used to identify a downstream pathway of PKC in preconditioning.

1996

World War I veterans remember the 80th anniversary of the Battle of the Somme; Sheikha Hasina Wajed becomes Bangladesh's first female prime minister; and Ella Fitzgerald, American jazz singer, dies, aged 79

Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death

A.M. Chinnaiyan, K. O'Rourke, B.R. Lane, V.M. Dixit

Science. 1997;275:1122-1126

Genetic analysis of developmental programmed cell death in the nematode *Caenorhabditis elegans* identified three factors that regulate the execution of cell death in an individual cell: *ced-3*, *ced-4*, and *ced-9.* The first two promote apoptosis, while *ced-9,* which lies genetically upstream of *ced-3* and *ced-4,* blocks cell death.
The mammalian homologs of *ced-9* and *ced-3* are the *bcl-2* and caspase families, respectively. In mammals, the Bcl-2 family can be subdivided into those members, that, like CED-9, inhibit apoptosis, and, conversely, those that induce apoptosis. The former group includes Bcl-2 and Bcl-x_L, while the latter includes Bax and Bad.

This paper was one of three published within a week of one other in *Science* and *Nature* that examined the physical and functional relationships between CED-4 and CED-9. In the paper discussed here, the authors further investigated the relationship between these two proteins, CED-3, and their mammalian counterparts.
Their approach involved overexpression of these proteins in mammalian cells.

CED-4 expression induced apoptosis in the heterologous systems. CED-4–induced killing was blocked by coexpression of CED-9 and Bcl-x_L. The authors proposed that the mechanism of this inhibition was direct interaction between CED-4 and CED-9/Bcl-x_L, since they were able to co-immunoprecipitate these complexes.
In contrast, mutant CED-9/Bcl-x_L constructs that were not capable of inhibiting apoptosis did not interact with CED-4. Bax and other proapoptotic family members did not bind CED-4, but rather blocked the interaction between CED-4 and CED-9/Bcl-x_L. This may be a result of the ability of the Bcl-2 family members to heterodimerize.

The authors also observed that an active site dominant negative mutant of CED-3, as well as caspase inhibitors p35, CrmA, and benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk), blocked CED-4 killing, indicating a requirement for caspase activity. This is consistent with previous reports that place *ced-4* genetically upstream of *ced-3*. In fact, CED-4 co-immunoprecipitated with CED-3 and long prodomain caspases-1 and -8, but not short prodomain caspases-3 and -6. Furthermore, CED-4 biochemically linked CED-9 and CED-3; when coexpressed, a ternary complex of CED-3, CED-4, and CED-9 was formed.

A similar scenario was predicted for the mammalian equivalents: caspase-1 and caspase-8 were capable of indirectly interacting with Bcl-x_L, an interaction that was inhibited by expression of mutant CED-4 that was incapable of binding CED-9.

The authors proposed that an unidentified mammalian homolog of CED-4 mediated this interaction between the long prodomain caspases and the apoptosis-inhibiting Bcl-2 family members. This model suggests a mechanism by which the Bcl-2 family might regulate apoptosis. Interaction between the putative CED-4 homolog and the long prodomain caspases would lead to the latter's activation. Bcl-2/Bcl-x_L, by simultaneously binding mammalian CED-4, would block this activation.
On the other hand, Bax and other proapoptotic family members would induce apoptosis by sequestering Bcl-2/Bcl-x_L away from this complex. The authors' predictions proved quite prescient, for, within the year, the mammalian homolog of CED-4 was identified, and the model proposed here proved true.

1997

Andy Green breaks the sound barrier on land in Black Rock Desert, Nevada;
Geoffrey Rush wins an Oscar (Best Actor) for his portrayal of David Helfgott in "Shine";
and Mother Teresa dies, aged 87

The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis

R.M. Kluck, E. Bossy-Wetzel, D.R. Green, D.D. Newmeyer

Science. 1997;275:1132-1136

This paper and that of Yang et al (reviewed on page 109) were published back-to-back in *Science* and proposed the same solution to the long unanswered puzzle concerning (at least one) of the biochemical functions of the antiapoptotic protein Bcl-2. Both built on the previous observation that cytochrome *c* was necessary to induce apoptosis in a cell-free system.

In this article, the authors used their own cell-free system that consisted of *Xenopus* egg extract. The extract is capable of spontaneously inducing apoptotic-like changes in exogenous nuclei when the heavy membrane fraction, which contains the mitochondria, is included. In this system, cytochrome *c* is released from the mitochondria. This release corresponds with the induction of caspase activity in the cytosolic fractions.

The authors proved that the induction of apoptosis was caused by the release of cytochrome *c* from the mitochondria, as opposed to the accumulation of newly synthesized cytochrome *c* that was not imported into the mitochondria, by verifying that the cytochrome *c* contained its heme group, which is only added in the mitochondria, and by including cycloheximide in the extract, which precluded de novo synthesis. The addition of Bcl-2 to this system blocked the release of cytochrome *c*, as well as subsequent caspase activity and nuclear apoptosis. It was not capable, however, of blocking apoptosis if cytochrome *c* was directly added to the cytosol. Thus it seems that mitochondrial-associated Bcl-2 blocks the proapoptotic activity of cytochrome *c* only indirectly by preventing its release from the mitochondria. It had previously been suggested that a decrease in mitochondrial membrane potential was an important regulatory event in inducing apoptosis, which was blocked by Bcl-2. However, the release of cytochrome *c* from the mitochondria was unaccompanied by any changes in mitochondrial potential, dissociating these events.

These observations were also corroborated in intact cells. Cytochrome *c* release from the mitochondria was observed in CEM and HeLa cells following diverse apoptotic stimuli, including UV irradiation, peroxide, staurosporine, and actinomycin D. This release coincided with the activation of caspase-3 and cleavage of caspase substrates. Overexpression of Bcl-2 blocked cytochrome *c* release and subsequent apoptosis. In contrast, caspase inhibitors blocked caspase-3 activation, substrate cleavage, and apoptosis, but not cytochrome *c* release. These experiments suggest that one of the functions of Bcl-2 in vivo is to block release of cytochrome *c* upstream of caspase activation.

1997

The comet Hale Bopp brightens the night skies;
"Tamagotchi," or virtual pets,
are cared for by children worldwide;
and Communist leader
Deng Xiaoping dies, aged 92

Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade

P. Li, D. Nijhawan, I. Budihardjo, S.M. Srinivasula, M. Ahmad, E.S. Alnemri, X. Wang

Cell. 1997;91:479-489

This is the third paper in a series from Xudong Wang and colleagues describing the results of biochemical analysis of a cell-free apoptosis system. Three major factors involved in apoptosis had been identified: apoptotic protease activating factors (Apaf) -1, -2, and -3. Apaf-2, the first factor identified, is cytochrome *c*, a peripheral inner mitochondrial membrane protein located in the intermembrane space that serves to shuttle electrons between components of the electron transport chain. Apaf-1 turned out to share homology in its central domain to *Caenorhabditis elegans* CED-4. This paper identifies the third factor, Apaf-3, as caspase-9, and in doing so, proposes a mechanism by which caspases are activated by the mitochondria.

Like caspase-3, caspase-9 is a cysteine protease that cleaves peptide bonds following aspartic acid residues. Both are synthesized as inactive proenzymes that contain a prodomain, a large ~20-kd subunit and a small ~10-kd subunit. The prodomain of caspase-9 is a long one that contains a motif known as the caspase recruitment domain (CARD). Activation of the caspases requires proteolytic processing to separate the two active subunits from the prodomain and from each other. Such processing of caspase-9 was observed upon coincubation of cytochrome *c*, Apaf-1, and Apaf-3/caspase-9 in the presence of hydrolyzable dATP or ATP. This same mixture was also capable of processing caspase-3. The authors demonstrated that caspase-9 was necessary for activation of caspase-3 by immunodepleting caspase-9 from the cell extracts. In the absence of caspase-9, the cytosolic extract failed to cleave caspase-3; this activity was rescued by restoration of purified Apaf-3. Furthermore, they showed that recombinant caspase-9 was capable of cleaving caspase-3 in vitro. Immunoprecipitation experiments revealed that caspase-9 interacts with Apaf-1 only in the presence of cytochrome *c* and dATP. This interaction occurs via the CARD domain of caspase-9 with a homologous CARD domain found in the amino terminus of Apaf-1. The authors briefly mention here, and confirm in more detail in a subsequent paper, that an Apaf-1 construct missing its carboxy terminus can bind caspase-9 even in the absence of cytochrome *c* and dATP. Thus, they propose the following model: cytochrome *c* is released from the mitochondria into the cytoplasm of cells that have received an apoptotic stimulus, whereby it binds Apaf-1. In the presence of dATP/ATP, this relieves the inhibition of the C-terminus of the protein, enabling it to recruit caspase-9. This recruitment in turn leads to auto-activation of caspase-9, which then cleaves caspase-3, initiating the proteolytic cascade that will lead to death of the cell. In support for the involvement of this pathway in regulating apoptosis in intact cells, an active site mutant of caspase-9 blocked caspase-3 processing and apoptosis in intact cells in which Apaf-1 or the apoptosis promoter Bax had been overexpressed.

This paper completes the Apaf story by identifying the last factor necessary for inducing cell-free apoptosis. It is important in that it provides only the second mechanistic explanation for how caspases may be activated during apoptosis (the first being the formation of death receptor complexes at the cell surface). Much of the data in support of the model have been derived from systems, albeit elegant, that are far removed form the intact cell. Subsequent studies from this group and others, however, have demonstrated the importance of the mitochondrial/Apaf/caspase-9 signaling pathway in regulating apoptosis in response to a variety of stimuli and circumstances. An important remaining challenge is to understand how apoptotic signals originating elsewhere in the cell activate the mitochondrial pathway. Significant efforts will be directed at the unraveling of this black box over the next few years. It is likely that the biochemical approach illustrated in this paper will contribute to these answers.

1997

NASA's Pathfinder probe explores Mars;
"The English Patient" wins
the Best Picture Oscar and eight others;
and Jeanne Calment, the oldest person in the world,
dies, aged 122

Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked

J. Yang, X. Liu, K. Bhalla, C.N. Kim, A.M. Ibrado, J. Cai, T.I. Peng, D.P. Jones, X. Wang

Science. 1997;275:1129-1132

In this companion paper to Kluck et al's (reviewed on page 107), it is shown that one mechanism by which Bcl-2 blocks apoptosis is to prevent the release of cytochrome *c* from the mitochondria.

HL-60 cells overexpressing Bcl-2 were induced to undergo apoptosis by treatment with staurosporine or etoposide. Unlike controls, cells expressing Bcl-2 failed to activate caspase-3, as indicated by the lack of cleavage of the caspase-3 substrate poly (ADP-ribose) polymerase (PARP), and did not undergo apoptosis. Furthermore, cytochrome *c* was released from the mitochondria of control cells in a time-dependent manner that preceded caspase activation. Bcl-2, however, blocked this release. Similar results were observed with Bcl-x_L.

A decrease in mitochondrial membrane potential followed induction of apoptosis, and Bcl-2 was capable of blocking this change. However, the drop in membrane potential was observed long after cytochrome *c* was released in control cells, ruling it out as the mechanism of cytochrome *c* release and Bcl-2 protection.

Bcl-2 also prevented the nonspecific release of cytochrome *c* from mitochondrial membranes during cell fractionation. Incubation of the supernatants of normal mitochondria with HeLa cell extracts immunodepleted of cytochrome *c* resulted in cleavage of exogenous caspase-3, presumably due to the nonspecific release of cytochrome *c* from the ruptured membranes. However, when mitochondria expressing Bcl-2 were used instead, this was prevented. The authors also showed that the active cytochrome *c* is the holo (with heme group) form, and not the apo (without heme group) form of the protein. This suggests that Bcl-2 blocks release of cytochrome *c* from the mitochondria, where it acquires its heme group, as opposed to preventing a pool of cytochrome *c* from being imported into the mitochondria.

It is still not known how cytochrome *c* is released from the mitochondria, or how Bcl-2 blocks this. It has been recently demonstrated that Bcl-2, Bcl-x_L, and Bax can form channels in membranes; this channel activity may regulate cytochrome *c* release. Although questions remain, the article reviewed here, as well as that by Kluck et al, seem to be pointing researchers in the right direction to search for the answers to these questions.

1997

World Chess Champion Gary Kasparov
is beaten by a computer;
earthquakes severely damage the 13th century
basilica of Saint Francis at Assisi, in Italy;
and Bo Widerberg, Swedish film director,
dies, aged 66

Apoptosis

Bibliography of One Hundred Key Papers

Adachi S, Cross AR, Babior BM, Gottlieb RA.
Bcl-2 and the outer mitochondrial membrane in the inactivation of cytochrome c during Fas-mediated apoptosis.
J Biol Chem. 1997;272:21878-21882.

Alnemri ES, Livingston DJ, Nicholson DW, et al.
Human ICE/CED-3 protease nomenclature [Letter to the Editor].
Cell. 1996;87:171.

Armstrong S, Downey JM, Ganote CE.
Preconditioning of isolated rabbit cardiomyocytes: induction by metabolic stress and blockade by the adenosine antagonist SPT and calphostin C, a protein kinase C inhibitor.
Cardiovasc Res. 1994;28:72-77.

Asimakis GK, Inners-McBride K, Medellin G, Conti VR.
Ischemic preconditioning attenuates acidosis and postischemic dysfunction in isolated rat heart.
Am J Physiol. 1992;263(3, pt 2):H887-H894.

Badorff D, Schwarz EM, Hiura T, Wessely R, Knowlton KU.
NFκB-mediated inhibition of apoptosis is associated with encephalomyocarditis virus virulence in vivo.
Circulation. 1997;96:I424.

Barry MA, Eastman A.
Endonuclease activation during apoptosis: the role of cytosolic Ca^{2+} and pH.
Biochem Biophys Res Commun. 1992;186:782-789.

Barry MA, Eastman A.
Identification of deoxyribonuclease II as an endonuclease involved in apoptosis.
Arch Biochem Biophys. 1993;300:440-450.

Belgrau D, Gold D, Selawry H, Moore JJ, Franzusoff A, Duke RC.
A role for CD95 ligand in preventing graft rejection.
Nature. 1995;377:630-632.

Bertin J, Armstrong RC, Ottilie S, et al.
Death effector domain–containing herpesvirus and poxvirus proteins inhibit both Fas- and TNFR1-induced apoptosis.
Proc Natl Acad Sci USA. 1997;94:1172-1176.

Bialik S, Geenen DL, Sasson IE, et al.
Myocyte apoptosis during acute myocardial infarction in the mouse localizes to hypoxic regions but occurs independently of p53.
J Clin Invest. 1997;100:1363-1372.

Bielowska AE, Shapiro JP, Jiang L, et al.
Ceramide is involved in triggering of cardiomyocyte apoptosis induced by ischemia and reperfusion.
Am J Pathol. 1997;151:1257-1263.

Brown DG, Sun XM, Cohen GM.

Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation.
J Biol Chem. 1993;268:3037-3039.

Brunskill NJ, Morrissey JJ, Klahr S.

Association and interactions of GTP-binding proteins with rat medullary H^+-ATPase.
Am J Physiol. 1994;267(6, pt 2):F944-F951.

Bump NJ, Hackett M, Hugunin M, et al.

Inhibition of ICE family proteases by baculovirus antiapoptotic protein p35.
Science. 1995;269:1885-1888.

Cardone MH, Salvesen GS, Widmann C, Johnson G, Frisch SM.

The regulation of anoikis: MEKK-1 activation requires cleavage by caspases.
Cell. 1997;90:315-323.

Cheng W, Li B, Li P, et al.

Stretch-induced programmed myocyte cell death.
J Clin Invest. 1995;96:2247-2259.

Chinnaiyan AM, O'Rourke K, Lane BR, Dixit VM.

Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death.
Science. 1997;275:1122-1126.

Choi DW.

Ischemia-induced neuronal apoptosis. [Review].
Curr Opin Neurobiol. 1996;6:667-672.

Chuang TH, Hahn KM, Lee JD, Danley DE, Bokoch GM.

The small GTPase Cdc42 initiates an apoptotic signaling pathway in Jurkat T lymphocytes.
J Biol Chem. 1997;8:1687-1698.

Cosulich S, Clarke P.

Apoptosis: does stress kill? [Review].
Curr Biol. 1996;6:1586-1588.

Eguchi Y, Shimizu S, Tsujimoto Y.

Intracellular ATP levels determine cell death fate by apoptosis or necrosis.
Cancer Res. 1997;57:1835-1840.

Ellerby HM, Martin SJ, Ellerby LM, et al.

Establishment of a cell-free system of neuronal apoptosis: comparison of premitochondrial, mitochondrial, and postmitochondrial phases.
J Neurosci. 1997;17:6165-6178.

Ellis HM, Horvitz HR.

Genetic control of programmed cell death in the nematode C. elegans.
Cell. 1986;44:817-829.

Engler RL, Gottlieb R.

Myocyte Apoptosis. In: Svendsen JH, Aldershvile J, Haunso S, eds.
Coronary Microcirculation During Ischaemia and Reperfusion. Copenhagen, Sweden: Munksgaard; 1997.

Evan GI, Wyllie AH, Gilbert CS, et al.

*Induction of apoptosis in fibroblasts by c-*myc *protein.*
Cell. 1992;69:119-128.

Fernandes-Alnemri T, Litwack G, Alnemri ES.
CPP32, a novel human apoptotic protein with homology to Caenorhabditis elegans *cell death protein Ced-3 and mammalian interleukin-1-beta-converting enzyme.*
J Biol Chem. 1994;269:30761-30764.

Forgac M.
Structure and properties of the coated vesicle proton pump.
Ann NY Acad Sci. 1992;671:273-283.

Gagliardini V, Fernandez PA, Lee RK, et al.
Prevention of vertebrate neuronal death by the crmA gene.
Science. 1994;263:826-828.

Gottlieb RA.
Cell acidification in apoptosis [Review].
Apoptosis. 1996;1:40-48.

Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL.
Reperfusion injury induces apoptosis in rabbit cardiomyocytes.
J Clin Invest. 1994;94:1621-1628.

Gottlieb RA, Dosanjh A.
Mutant cystic fibrosis transmembrane conductance regulator inhibits acidification and apoptosis in C127 cells: possible relevance to cystic fibrosis.
Proc Natl Acad Sci USA. 1996;93:3587-3591.

Gottlieb RA, Giesing H, Engler RL, Babior BM.
The acid deoxyribonuclease of neutrophils: a possible participant in apoptosis-associated genome destruction.
Blood. 1995;86:2414-2418.

Gottlieb RA, Giesing H, Zhu JY, Engler RL, Babior BM.
Cell acidification in apoptosis: granulocyte colony–stimulating factor delays programmed cell death in neutrophils by up-regulating the vacuolar H^+-ATPase.
Proc Natl Acad Sci USA. 1995;92:5965-5968.

Gottlieb RA, Gruol DL, Zhu JY, Engler RL.
Preconditioning in rabbit cardiomyocytes: role of pH, vacuolar proton ATPase, and apoptosis.
J Clin Invest. 1996;97:2391-2398.

Gottlieb RA, Nordberg JA, Skowronski E, Babior BM.
Apoptosis induced in Jurkat cells by several agents is preceded by intracellular acidification.
Proc Natl Acad Sci USA. 1996;93:654-658.

Griffith TS, Brunner T, Fletcher SM, Green DR, Ferguson TA.
Fas ligand-induced apoptosis as a mechanism of immune privilege.
Science. 1995;270:1189-1192.

Gu Y, Wu J, Faucheu C, et al.
Interleukin-1-beta converting enzyme requires oligomerization for activity of processed forms in vivo.
EMBO J. 1995;14:1923-1931.

Haldar S, Beatty C, Tsujimoto Y, Croce CM.
The bcl-2 *gene encodes a novel G protein.*
Nature. 1989;342:195-198.

Hockenberry D, Nu—ez G, Milliman C, Schreiber RD, Korsmeyer SJ
Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death.
Nature. 1990;348:334-336.

James TN.

Normal and abnormal consequences of apoptosis in the human heart. From postnatal morphogenesis to paroxysmal arrhythmias.
Circulation. 1994;90:556-573.

Johnson LN, Gardner AM, Diener KM, et al.

Signal transduction pathways regulated by mitogen-activated/extracellular response kinase kinase kinase induce cell death.
J Biol Chem. 1996;271:3229-3237.

Juarez-Salinas H, Sims JL, Jacobson MK.

Poly(ADP-ribose) levels in carcinogen-treated cells.
Nature. 1979;282:740-741.

Kajstura J, Cheng W, Sarangarajan R, et al.

Necrotic and apoptotic myocyte cell death in the aging heart of Fischer 344 rats.
Am J Physiol. 1996;271(3, pt 2):H1215-H1228.

Kane DJ, Sarafian TA, Anton R, et al.

Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species.
Science. 1993;262:1274-1277.

Karsan A, Harlan JM.

Modulation of endothelial cell apoptosis: mechanisms and pathophysiological roles.
J Atheroscler Thromb. 1996;3:75-80.

Kirshenbaum LA, de Moissac D.

The bcl-2 *gene product prevents programmed cell death of ventricular myocytes.*
Circulation. 1997;96:1580-1585.

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD.

The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis.
Science. 1997;275:1132-1136.

Krippner A, Yagi A, Gottlieb RA, Babior BM.

Loss of function of cytochrome c in Jurkat cells undergoing Fas-mediated apoptosis.
J Biol Chem. 1996;271:21629-21636.

Krown KA, Page MT, Nguyen C, et al.

Tumor necrosis factor alpha–induced apoptosis in cardiac myocytes. Involvement of the sphingolipid signaling cascade in cardiac cell death.
J Clin Invest. 1996;98:2854-2865.

Laderoute KR, Webster KA.

Hypoxia/reoxygenation stimulates Jun kinase activity through redox signaling in cardiac myocytes.
Circ Res. 1997;80:336-344.

Lazebnik YA, Takahashi A, Moir RD, et al.

Studies of the lamin proteinase reveal multiple parallel biochemical pathways during apoptotic execution.
Proc Natl Acad Sci USA. 1995;92:9042-9046.

Li J, Eastman A.

Apoptosis in an interleukin-2-dependent cytotoxic T lymphocyte cell line is associated with intracellular acidification.
J Biol Chem. 1995;270:3203-3211.

Li P, Nijhawan D, Budihardjo I, et al.

Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade.
Cell. 1997;91:479-489.

Li Q, Li B, Wang X, et al.

Overexpression of insulin-like growth factor–1 in mice protects from myocyte death after infarction, attenuating ventricular dilation, wall stress, and cardiac hypertrophy.

J Clin Invest. 1997;100:1991-1999.

Li Z, Bing OH, Long X, Robinson KG, Lakatta EG.

Increased cardiomyocyte apoptosis during the transition to heart failure in the spontaneously hypertensive rat.

Am J Physiol. 1997;272(5, pt 2):H2313-H2319.

Liu GS, Thornton J, Van Winkle DM, Stanley AWH, Olsson RA, Downey JM.

Protection against infarction afforded by preconditioning is mediated by adenosine A_1 receptors in rabbit heart.

Circulation. 1991;84:350-356.

Liu X, Zou H, Slaughter C, Wang X.

DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis.

Cell. 1997;89:175-184.

Liu Y, Cigola E, Cheng W, et al.

Myocyte nuclear mitotic division and programmed myocyte cell death characterize the cardiac myopathy induced by rapid ventricular pacing in dogs.

Lab Invest. 1995;73:771-787.

Long X, Crow MT, Sollott SJ, et al.

Enhanced expression of p53 and apoptosis induced by blockade of the vacuolar proton ATPase in cardiomyocytes.

J Clin Invest. 1998;101:1453-1461.

Mallat Z, Tedgui A, Fontaliran F, Frank R, Durigon M, Fontaine G.

Evidence of apoptosis in arrhythmogenic right ventricular dysplasia.

N Engl J Med. 1996;335:1190-1196.

Marber MS, Latchman DS, Walker JM, Yellon DM.

Cardiac stress protein elevation 24 hours following brief ischemia or heat stress is associated with resistance to infarction.

Circulation. 1993;88:1264-1272.

Martin SJ, O'Brien GA, Nishioka WK, et al.

Proteolysis of fodrin (non-erythroid spectrin) during apoptosis.

J Biol Chem. 1995;270:6425-6428.

McCarthy NJ, Whyte MK, Gilbert CS, Evan GI.

Inhibition of Ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak.

J Cell Biol. 1997;136:215-227.

Meisenholder GW, Martin SJ, Green DR, Nordberg J, Babior BM, Gottlieb RA.

Events in apoptosis: acidification is downstream of protease activation and Bcl-2 protection.

J Biol Chem. 1996;271:16260-16262.

Meng H, Pierce GN.

Protective effects of 5-(N,N-dimethyl) amiloride on ischemia-reperfusion injury in hearts.

Am J Physiol. 1990;258(5, pt 2):H1615-H1619.

Miossec C, Dutilleul V, Fassy F, Diu-Hercend A.

Evidence for CPP32 activation in the absence of apoptosis during T lymphocyte stimulation.

J Biol Chem. 1997;272:13459-13462.

Mosser DD, Caron AW, Bourget L, Denis-Larose C, Massie B.
Role of the human heat shock protein hsp70 in protection against stress-induced apoptosis.
Mol Cell Biol. 1997;17:5317-5327.

Murphy E, Perlman M, London RE, Steenbergen C.
Amiloride delays the ischemia-induced rise in cytosolic free calcium.
Circ Res. 1991;68:1250-1258.

Murry DE, Richard VJ, Reimer KA, Jennings RB.
Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode.
Circ Res. 1990;66:913-931.

Nanda A, Brumell JH, Nordstrom T, et al.
Activation of proton pumping in human neutrophils occurs by exocytosis of vesicles bearing vacuolar-type H^+-ATPases.
J Biol Chem. 1996;271:15963-15970.

Narula J, Haider N, Virmani R, et al.
Apoptosis in myocytes in end-stage heart failure.
N Engl J Med. 1996;335:1182-1189.

Nicholson DW, Ali A, Thornberry NA, et al.
Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis.
Nature. 1995;376:37-43.

Olivetti G, Abbi R, Quaini F, et al.
Apoptosis in the failing human heart.
N Engl J Med.1997;336:1131-1141.

Peitsch MC, Polzar B, Stephan H, et al.
Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death).
EMBO J. 1993;12:371-377.

Piot CA, Padmanaban D, Ursell PC, Sievers RE, Wolfe CL.
Ischemic preconditioning decreases apoptosis in rat hearts in vivo.
Circulation. 1997;96:1598-1604.

Piper HM, Sezer O, Schleyer M, Hutter JF, Spieckermann PG.
Development of ischemia-induced damage in defined mitochondrial subpopulations.
J Mol Cell Cardiol. 1985;17:885-896.

Puceat M, Vassort G.
Neurohumoral modulation of intracellular pH in the heart.
Cardiovasc Res. 1995;29:178-183.

Rao L, Debbas M, Sabbatini P, Hockenbery D, Korsmeyer S, White E.
The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins.
Proc Natl Acad Sci USA. 1992;89:7742-7746.

Ray CA, Black RA, Kronheim SR, et al.
Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1 beta converting enzyme.
Cell. 1992;69:597-604.

Reed JC.
Cytochrome c: can't live with it—can't live without it.
Cell. 1997;91:559-562.

Schulze-Osthoff K, Walczak H, Droge W, Krammer PH.
Cell nucleus and DNA fragmentation are not required for apoptosis.
J Cell Biol. 1994;127:15-20.

Seimiya H, Mashima T, Toho M, Tsuruo T.

c-Jun NH2-terminal kinase-mediated activation of interleukin-1β converting enzyme/CED-3-like protease during anticancer drug-induced apoptosis.

J Biol Chem. 1997;272:4631-4636.

Sekler I, Kobayashi S, Kopito RR.

A cluster of cytoplasmic histidine residues specifies pH dependence of the AE2 plasma membrane anion exchanger.

Cell. 1996;86:929-935.

Sharov VG, Sabbah HN, Shimoyama H, Lesch M, Goldstein S, Goussev AV.

Evidence of cardiocyte apoptosis in myocardium of dogs with chronic heart failure.

Am J Pathol. 1996;148:141-149.

Steenbergen C, Perlman ME, London RE, Murphy E.

Mechanism of preconditioning: ionic alterations.

Circ Res. 1993;72:112-125.

Sulston JE, Horvitz HR.

Post-embryonic cell lineages of the nematode Caenorhabditis elegans.

Dev Biol. 1977;56:110-156.

Sulston JE, Schierenberg E, White JG, Thomson JN.

The embryonic cell lineage of the nematode Caenorhabditis elegans.

Dev Biol. 1983;100:64-119.

Tanaka M, Ito H, Adachi S, et al.

Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes.

Circ Res. 1994;75:426-433.

Tewari M, Quan LT, O'Rourke K, et al.

Yama/CPP32β, a mammalian homolog of CED-3, is a Crm-A-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase.

Cell. 1995;81:801-809.

Thome M, Schneider P, Hofmann K, et al.

Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors.

Nature. 1997;386:517-521.

Torriglia A, Chaudun E, Chany-Fournier F, Jeanny JC, Courtois Y, Counis MF.

Involvement of DNase II in nuclear degeneration during lens cell differentiation.

J Biol Chem. 1995;270:28579-28585.

Wu D, Wallen HD, Nu—ez G.

Interaction and regulation of subcellular localization of CED-4 by CED-9.

Science. 1997;275:1126-1129.

Wyllie AH, Kerr JFR, Currie AR.

Cell death: the significance of apoptosis.

Int Rev Cytol. 1980;68:251-306.

Xue D, Horvitz HR.

Inhibition of the Caenorhabditis elegans. *cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein.*

Nature. 1995;377:248-251.

Yang J, Liu X, Bhalla K, et al.

Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked.

Science. 1997;275:1129-1132.

Yin T, Sandhu G, Wolfgang CD, et al.

Tissue-specific pattern of stress kinase activation in ischemic/reperfused heart and kidney.
J Biol Chem. 1997;272:19943-19950.

Ytrehus K, Liu Y, Downey JM.

Preconditioning protects ischemic rabbit heart by protein kinase C activation.
Am J Physiol. 1994;35(3, pt 2):H1145-H1152.

Zanke BW, Boudreau K, Rubie E, et al.

The stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum, UV irradiation or heat.
Curr Biol. 1996;6:606-613.

Zhang J, Feng Y, Forgac M.

Proton conduction and bafilomycin-binding by the V_o domain of the coated vesicle V-ATPase.
J Biol Chem. 1994;269:23518-23523.

Zou H, Henzel WJ, Liu X, Lutschg A, Wang X.

Apaf-1, a human protein homologous to C. elegans *CED-4, participates in cytochrome c–dependent activation of caspase-3.*
Cell. 1997;90:405-413.

Instructions for authors

GENERAL INSTRUCTIONS

- **Manuscripts** should be provided on **word-processor disks** (3.5-in, for IBM, IBM-compatible, or Apple computers) with **three hard copies (text and figures)** printed on one side of **standard-sized white bond paper, double-spaced, with 2.5-cm margins.** Pages must be numbered. **Standard typed page = 25 lines of 75 characters (including spaces) double-spaced, 2.5-cm margins = a total of 275 words per page.**

- **All texts should be submitted in English.** In the case of translations, the text in the original language should be included.

- On the **title page,** provide **title of manuscript** (title should be concise, not exceeding 120 characters, including spaces), **short running title, keywords,** and **acknowledgments,** as well as **full names** (first name, middle name(s), and last name) with **highest academic degrees** (in country-of-origin language), affiliations/address, telephone No., fax No., and E-mail address.

- **Illustrations** (photographs, tables, graphs, figures–hard copies and on disk, where possible) should be of good quality or professionally prepared, numbered according to their order, with proper orientation indicated (eg, "top," or "left"), and **SHORT legends** provided, not repetitive of text. As figures and graphs may need to be reduced or enlarged, all absolute values and statistics should be provided. All **illustrations should be cited** in the text, with distinct numbering for figures and tables. Illustrations will be reproduced in full color only when clearly necessary, eg, images from nuclear medicine or histology.

- Include **HEADINGS** using a consistent style for the various levels of headings, to highlight key points and facilitate comprehension of the text. The Publisher reserves the right to add or delete headings when necessary.

- **Indent first line of each new paragraph.**

- **Abbreviations** should be used sparingly and expanded at first mention.

- Use **Systeme International** (SI) units.

- Use **generic names of drugs.**

- All **references** should be cited in the text and **numbered consecutively using superscript arabic numerals.** The author-date system of citation is **NOT** acceptable. "In press" references are to be avoided. In the **bibliography,** titles of journals **should be abbreviated according to the Index Medicus.** All authors should be listed up to six; if there are more, only the first three should be listed, followed by "et al" *(Uniform requirements for manuscripts submitted to biomedical journals "the Vancouver style." Br Med J Clin Res. 1982;284:1766-1770).* Where necessary, references will be styled to *Dialogues in Cardiovascular Medicine* copyediting requirements. Authors bear total responsibility for the accuracy and completeness of all references and for correct text citation. Example of style for references:

1. Ouriel K, Geary K, Green RM, Geary JE, DeWeese JA. Factors determining survival after ruptured abdominal aneurysm. *J Vasc Surg.* 1990;11:493-496.

2. Darling RC, Brewster DC, Ottinger LW. Autopsy study of unoperated abdominal aortic aneurysms: the case for early resection. *Circulation.* 1977;56(suppl II):II161-II164.

3. Schulman JL. Immunology of influenza. In: Kilbourne ED, Alfade RT, eds. *The Influenza Viruses and Influenza.* Orlando, Fla: Academic Press Inc; 1975:373-393.

- **Copyediting:** all contributions to *Dialogues in Cardiovascular Medicine* will be styled by the Publisher's editorial dept according to the specifications of the current edition of the **American Medical Association Manual of Style, Williams & Wilkins.** Page proofs will be sent to authors for approval and should be returned within 5 days. If this time is exceeded, changes made by the editorial dept will be assumed to be accepted by the author. Authors are responsible for all statements made in their work, including changes made by the editorial dept and authorized by the author. The Publisher will edit Editorials, Abstracts, and Seminal Paper Summaries to required size if their length does not comply with specific requirements.

- **Copyright** of articles will be transferred to the Publisher of *Dialogues in Cardiovascular Medicine.* For reproduction of existing work, it is the author's responsibility to obtain copyright from the author(s) (including self) and the publisher(s) and provide copies of these authorizations with the manuscript.

EDITORIAL

The editorial should not exceed **2 standard typed pages** (maximum **600 words**).

LEAD ARTICLE

The lead article should not exceed **25 standard typed pages** (maximum **8000 words**), including an **abstract of no more than 200 words, no more than 20 references,** and a **maximum of 10 illustrations (figures and tables).** Illustrations correspond to approximately 2/3 of a standard typed page or 200 words and are counted as part of the maximum allowed 25 standard typed pages. A maximum of **5-10 keywords** should be included.

RESPONDENT ARTICLES

Respondent articles should not exceed **10 standard typed pages (maximum 2500 words),** including an **abstract of no more than 125 words, no more than 10 references,** and a **maximum of 3 illustrations (figures and tables).** Illustrations correspond to approximately 2/3 of a standard typed page or 200 words and are counted as part of the maximum allowed 10 standard typed pages. A maximum of **5-10 keywords** should be included.

SEMINAL PAPER SUMMARIES

Seminal paper summaries take up one page of *Dialogues in Cardiovascular Medicine:* the length of each summary should IMPERATIVELY be comprised between 500 and 600 words, ie, **not exceed 3000 characters.** Summaries that are too short or too long will be edited by the Publisher. No figures, tables or references should be included in seminal paper summaries.