HEART REVIEW

Cell death in the cardiovascular system

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Cell death is important for both development and tissue homeostasis in the adult. As such, it is tightly controlled and deregulation is associated with diverse pathologies; for example, regulated cell death is involved in vessel remodelling during development or following injury, but deregulated death is implicated in pathologies such as atherosclerosis, aneurysm formation, ischaemic and dilated cardiomyopathies and infarction. We describe the mechanisms of cell death and its role in the normal physiology and various pathologies of the cardiovascular system.

Cell death is essential for embryonic development and tissue homeostasis in adults. Abnormalities in the control of cell death contribute to a variety of diseases. A number of different cell types undergo cell death in the cardiovascular system. For example, the death of endothelial and vascular smooth muscle cells is implicated in vessel remodelling and injury and in several vascular pathologies such as atherosclerosis and formation of aneurysm (box 1). Loss of cardiomyocytes is associated with ischaemic and dilated cardiomyopathies, with myocardial infarction (MI) and with injury due to ischaemia/reperfusion. Here, we review the evidence for, and mechanisms of, cell death within the cardiovascular system.

Mechanisms of cell death

The death of individual cells within a multicellular organism involves both physiological and pathological processes and can occur by a number of distinct mechanisms, including apoptosis, autophagy and necrosis. Apoptosis is involved in the developmental remodelling of many tissues, in tissue homeostasis in the adult and in response to stress (for a review, see Reeve *et al.*¹). Autophagy is involved in the degradation and recycling of the building blocks of organelles, proteins and other components of the cytoplasm important for cellular homeostasis (for a review, see Baehrecke²). Finally, necrotic cell death is generally restricted to pathological processes such as inflammation after tissue damage. These three forms of cell death have all been observed in the cardiovascular system.

Apoptosis, autophagy and necrosis are defined on the basis of morphology, tissue location and the dependence on lysosomes (figs 1 and 2). Apoptosis is an energy-dependent process characterised by cytoplasmic and nuclear condensation, DNA fragmentation, membrane blebbing, and the engulfment and lysosomal degradation of apoptotic

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bodies by phagocytes. The apoptotic pathway, many of the components of which are conserved from yeast to mammals, leads to the activation of a class of proteases known as caspases that are responsible for the ordered dismantling of the cell (fig 3). The rapid engulfment of apoptotic cells without the loss of cellular material fails to elicit an inflammatory response, and apoptosis is an important mode of physiological cell death. In contrast, autophagy (which is involved in cell survival as well as cell death) is not associated with phagocytosis, and is characterised by the presence of autophagic vacuoles that fuse with lysosomes to form autophagolysosomes within the dying cells that are responsible for self-degradation (fig 2). It is an evolutionarily conserved mechanism that, in addition to its role in the normal turnover of proteins and organelles, is involved in the cell's response to stress and starvation. In contrast to apoptosis and autophagy, the swelling of organelles and rupture of the plasma membrane during necrosis result in the release of many inflammatory molecules, leading to an inflammatory response.

Although the classification into different modes of death is useful, there is considerable overlap between the different mechanisms, particularly in vivo. For example, not all the features of apoptosis may be observed in all cell types and, in some instances, the apoptotic cell may undergo secondary necrosis. In addition, recent studies indicate that apoptosis and autophagy may involve complementary pathways and that autophagic degeneration may be a part of apoptosis, at least in some cell types^{2–3} (fig 3). Alternatively, the presence of both active caspases and autophagy in the same cell may simply indicate concurrent death mechanisms initiated by a common trigger.

Physiological cell death

Cell death occurs in both physiological and pathological contexts in the cardiovascular system. Physiological cell death is responsible for the sculpting and remodelling of the heart and blood vessels in response to the changing requirements of the tissues they supply.

Cell death in the development of the cardiovascular system

The development of the embryonic endocardial cushion into valves and septa is a critical stage in cardiogenesis, initiating the development of the

Abbreviations: EC, endothelial cell; ECM, extracellular matrix; MI, myocardial infarction; NO, nitric oxide; oxLDL, oxidised low-density lipoprotein; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell

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Box 1: Cardiovascular diseases in which cell death has been implicated

Cardiac (myocyte)

- Idiopathic dilated cardiomyopathy
- Ischaemic cardiomyopathy
- Acute myocardial infarction
- Arrhythmogenic right ventricular dysplasia
- Myocarditis

Cardiac (conducting tissues)

- Pre-excitation syndromes
- Heart block, congenital complete atrioventricular heart block, long QT syndromes

Vascular

- Atherosclerosis
- Restenosis after angioplasty/stenting
- Vascular graft rejection
- Arterial aneurysm formation

mammalian four-chambered heart. Remodelling of the outflow tract during the transition from a single-to-dual series circulation with four chambers is accompanied by a precise pattern of cell death. Coincident with septation of the heart, cardiomyocytes are lost from the outflow tract, which shortens and rotates, allowing the aorta and pulmonary artery to connect to the left and right ventricles, respectively. Apoptosis occurs at regions of fusion of the atrioventricular or bulbar cushions, and both aortic and pulmonary valves in non-myocytes. The critical role of programmed cell death in cardiogenesis is illustrated in experiments wherein death is either blocked or augmented. Inhibition of caspases in the embryonic avian heart attenuates shortening and rotation of the outflow tract, resulting in defects reminiscent of congenital conotruncal heart defects.^{4,5} Moreover, promotion of cardiomyocyte apoptosis in the outflow

tract by targeted delivery of the Fas ligand results in a similar phenotype,⁶ suggesting that any perturbation in the precise control of cell death can cause cardiac abnormalities. Apoptosis of myocytes also occurs in the interventricular septum and right ventricular wall after birth, during the transition from fetal to adult circulations. The conducting tissue also undergoes apoptosis, and aberrant apoptosis is implicated in congenital heart block and long QT syndrome or the persistence of accessory pathways.

Although the physiological triggers for apoptosis are poorly characterised, there is some evidence to support the notion that local hypoxia is the stimulus for both recruitment of pro-endothelial cells into the developing vascular network and apoptosis-mediated remodelling in the outflow tract.⁴ Co-ordination of these two events may result from hypoxia-inducible factor 1 α -dependent expression of vascular endothelial growth factor (VEGF) receptor 2, such that VEGF signalling through the serine/threonine kinase Akt (a central modulator of diverse intracellular signalling pathways, including the inhibition of apoptosis) regulates cardiomyocyte death as well as recruits endothelial progenitor cells within the outflow tract in response to hypoxia.

Cell death in the remodelling of the adult cardiovascular system

Cell death is also implicated in the remodelling of adult vessels in response to changes in blood flow or injury. Vascular remodelling occurs in two ways. First, alteration in the lumen diameter of the larger vessels occurs in response to changes in blood flow. Second, an increase in the number of capillaries (angiogenesis) occurs in response to tissue hypoxia and wound healing. Cell death has an important role in both these processes. For example, contraction of the umbilical and uterine vessels after birth is accompanied by extensive apoptosis of the vascular smooth muscle cells (VSMCs). Remodelling of the rabbit and mouse carotid artery in response to experimental ligation is accompanied by the proliferation and apoptosis of VSMCs.^{7,8} Angiogenic remodelling in the adult also involves cell death. Chronic exposure to a hypoxic environment leads to a near doubling of the capillary density in the rat brain. The hypoxia-induced angiogenesis is mediated by hypoxia-inducible factor 1 α , angiopoietin 2 and capillary regression, after normoxia is accomplished by apoptosis.⁹

Figure 1 Autophagy, apoptosis and necrosis have all been observed in the cardiovascular system. They are characterised by different morphology, the involvement of phagocytes and tissue location. Whereas necrosis is generally restricted to pathological situations, apoptosis and autophagy are, in addition, involved in physiological processes such as vascular remodelling.

Although apoptosis of endothelial cells (ECs) is difficult to detect in most adult vessels, it is readily detected during development and in dynamic adult vessels that are subject to remodelling, such as the ovary, endometrium and mammary gland. ECs are exposed to a range of proapoptotic signals such as transforming growth factor β , lipopolysaccharide, endostatin, thrombospondin 1, angiotensin II, high D-glucose concentration and changes in cytoskeletal organisation. A number of survival factors such as VEGF-A, angiopoietin-1, erythropoietin, interleukin 8, hepatocyte growth factor and fibroblast growth factors have been identified *in vitro*, many of which involve signalling through Akt. In addition, integrin-mediated attachment of EC to the extracellular matrix (ECM) elicits prosurvival pathways. Detachment from the basement membrane may render ECs refractory to VEGF-A-mediated survival signalling and induce a form of apoptosis known as anoikis. Blood flow and shear stress also regulate EC survival via activation of the endothelial nitric oxide synthase, although the precise role of nitric oxide (NO) production in the fate of ECs is not clear.

Thus, EC survival is regulated by the integration of growth factor-dependent and integrin-dependent signalling, which are in turn governed by the composition of the ECM and by the haemodynamic properties of the vessel.¹⁰ Experimental inactivation of genes involved in survival also suggests a role for EC apoptosis in vessel remodelling. For example, inactivation of VEGF-A or angiopoietin-1 in mice results in severe vascular abnormalities. However, many of the genes involved in survival are also involved in pathways that regulate cell proliferation and migration, so it is difficult to assign a precise role for EC apoptosis in remodelling *in isolation*. Similarly, experiments demonstrating the requirement for EC apoptosis in an *in vitro* model of angiogenesis may not represent what happens *in vivo*, since factors such as shear stress, cell-cell signalling and apoptotic body clearance are absent or are reduced *in vitro*.

Pathological cell death

Heart failure

The ability of cardiomyocytes to divide or to differentiate *in situ* from either circulating or resident precursors is currently a focus of intense research. Despite the potential for cardiac

repair, cell death in the heart is implicated in a variety of different pathologies. Autophagy may be triggered by the accumulation of damaged organelles as a result of ageing or following external stress such as hypoxia, and irreversibly damaged or redundant cells undergo apoptosis.

Cell death is associated with both dilated and ischaemic cardiomyopathy. Cardiomyocyte apoptosis occurs in end-stage heart failure and may contribute to heart failure in a variety of situations.^{11,12} Ageing is associated with myocardial cell loss, and cardiomyocyte apoptosis may result in the gradual deterioration in cardiac function. Apoptosis is observed during transplantation, with some studies suggesting higher levels in ischaemic versus idiopathic dilated cardiomyopathy. The transition from compensated to decompensated hypertrophy is also associated with myocyte apoptosis, and high levels of apoptosis are seen in arrhythmogenic right ventricular dysplasia, a condition characterised by myocardial replacement with fibrofatty material. There is also increasing evidence that toxic cardiomyopathies, such as that induced by doxorubicin (adriamycin), are associated with cardiomyocyte apoptosis.

Although the evidence that apoptosis promotes heart failure is persuasive, the extent of cell death is controversial. Vastly different rates of apoptosis have been reported in both human and animal heart failure, with rates of up to 35.5%. Given that apoptosis takes <24 h to complete, such high rates, even in very localised areas, would result in rapid involution of the heart. Recently, more realistic rates of <0.5% cell death have been reported in end-stage heart failure. In addition, in end-stage heart failure, necrosis is still more frequent (up to 7 times) than apoptosis.

The increase in lysosomal activity observed during hypoxic stress in the heart suggests that autophagy also plays a role. Within the myocardium of patients with dilated cardiomyopathy, many of the cardiomyocytes show prominent cytoplasmic vacuoles characteristic of autophagolysosomes.¹³ These vacuoles contain intracellular organelles such as mitochondria and myofibrils, and are associated with markers of lysosomal markers (cathepsin D and lysosome-associated membrane protein 1). However, cathepsin D can also act as a proapoptotic

Figure 2 Electron microscopic appearance of a vascular smooth muscle cell undergoing apoptosis *in vitro* (A), showing peripheral condensation of nuclear chromatin and intense membrane blebbing and vesicle formation, or undergoing autophagy *in vivo* (B), demonstrating typical lamellar body formation.

Figure 3 Pathways of apoptosis and autophagy. Members of the tumour necrosis factor death receptor family (eg, Fas) bind their cognate ligand. The activated receptor recruits specific adapter proteins that activate either upstream “initiator” caspases in the apoptotic pathway or autophagy-specific genes (Atg) such as Beclin 1, and BNIP3 via the Janus kinase (JNK) pathway. The activated upstream caspases (8 and 10) cleave and activate downstream “effector” caspases that digest cell components. Caspase 8 also cleaves Bid, and the active truncated protein (tBid) interacts with, and inhibits, Bcl-2, the prototypic member of a family of antiapoptotic proteins that inhibit the release of cytochrome C from the mitochondria. Cytoplasmic cytochrome C stimulates recruitment of the Apaf1 adapter and another upstream caspase (caspase 9) in the apoptosome complex. Activated caspase 9 can cleave and activate effector caspases. Thus, apoptosis in response to death receptor ligation may be amplified via the “intrinsic” mitochondrial pathway. Bcl-2 also inhibits autophagy by binding and inhibiting Beclin 1. Survival factors such as insulin-like growth factor 1 activate the phosphoinositide kinase (PI3K)/Akt pathway, and Akt is able to inhibit both autophagy (via the mammalian target of rapamycin (mTOR), a sensor of nutrient status) and apoptosis. PDK, phosphoinositide-dependent kinase; PIP₂, phosphatidylinositol bisphosphate; PIP₃, phosphatidylinositol 3, 4, 6-triphosphate.

mediator in some cells, and its inhibition reduces free radical-induced apoptosis of rat cardiomyocytes. Although nuclear condensation is observed, it is not typical of apoptosis, and the relative contribution of autophagy and apoptosis in cardiomyocyte death is unclear (fig 3).

In heart failure, a huge variety of apoptotic stimuli have been suggested. *In vitro*, mechanical stretch can induce apoptosis, indicating a possible role for volume overload and raised ventricular and diastolic pressure. Pressure overload after aortic banding also induces early myocyte apoptosis, prior to significant hypertrophy. Both 4 weeks of rapid ventricular pacing and catecholamines induce myocyte apoptosis and heart failure in dogs, suggesting that catecholamine responses may be directly toxic to myocytes.

Myocardial infarction

MI has been considered to be a *prima facie* example of necrotic cell death due to the breakdown of cellular energy metabolism. However, the apoptosis of cardiomyocytes also occurs in a temporally and spatially specific manner. After about 20 min of ischaemia, myocytes begin to undergo necrosis, with spillage of intracellular components and subsequent inflammation, resulting in cell loss and extensive infarction. Pharmacological or invasive intervention to re-establish blood flow (reperfusion) significantly reduces infarction size and survival, possibly owing to the re-direction of cell death from necrosis to apoptosis.¹⁴ However, assessing the contribution of either ischaemia or reperfusion in determining the fate of myocyte *in vivo* is difficult. The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling staining that

measures fragmented DNA, a terminal stage of apoptosis, is seen to increase only after reperfusion, whereas staining for active caspase-3, an early marker of apoptosis, is detected after ischaemia alone.¹⁵ This suggests that myocytes initiate apoptosis in response to ischaemia, but, owing to the energy demands of the programme and the dwindling respiratory capacity of the cells, apoptosis is stalled. Therefore, during prolonged ischaemia, myocytes shift from an initial apoptotic to a necrotic fate, while following reperfusion oxygen and thus energy is restored, allowing apoptosis to complete. The key to decide the fate of myocyte is the level of ATP, a factor required for completion of apoptosis in most cells. Thus, acute MI manifests both forms of cell death, with apoptosis occurring predominantly at the hypoperfused border zones between a central area of necrosis and viable myocardium. The central, unperfused region also manifests apoptosis particularly within the first 6 h, although necrosis is more common between 6–24 h. If necrosis prevails, large areas of the infarct are infiltrated by inflammatory neutrophils and macrophages. In addition, release of proteolytic enzymes from leucocytes can lead to the direct disruption of connective tissue, potentially leading to infarct expansion. In contrast, apoptotic cells are rapidly phagocytosed before secondary necrosis occurs, thus causing minimal inflammatory response.¹⁶

Aneurysm formation

Arterial aneurysms are often cited as a classic apoptosis-driven cardiovascular pathology, and are typified by VSMC-poor medial regions that display evidence of degradation and fragmentation of the internal elastic lamina and collagen-rich matrix. Although increased haemodynamic stress propagates dilatation and eventual rupture of such weakened regions, it remains controversial whether apoptosis of VSMCs alone is sufficient to instigate the atrophy. Animal models of aneurysm typically use vessel ligation and hypertension to instigate the lesion. Although these conditions increase VSMC apoptosis, it is always in the presence of altered haemodynamics, and, thus, whether VSMC apoptosis drives pathology or is just an unfortunate bystander event is unproven. Arterial aneurysm tissue *ex vivo* contains high levels of apoptotic VSMCs and increased expression of proapoptotic factors.¹⁷ However, it is hard to determine whether progressive cell loss causes the acute dilatation and rupture. Indeed, there is evidence to suggest that aneurysms can be instigated, propagated and fatally concluded through little more than repeated haemodynamic stress-induced bioengineering fatigue of the internal elastic lamina.¹⁸

Atherosclerosis

The mature atherosclerotic plaque consists of a lipid-rich core separated from the vessel lumen by a fibrous cap composed of VSMCs, collagen and ECM. Rupture of the fibrous cap exposes the underlying collagen, resulting in glycoprotein VI-mediated platelet activation, thrombosis and potential occlusion. The significance of apoptosis of both VSMCs and infiltrating macrophages in the mature plaque is discussed below.

Vascular smooth muscle cells

Stability of the cap is dependent on its structural properties, determined primarily by the number of VSMCs and the collagen and ECM they synthesise. Thus, loss of VSMCs by apoptosis would be expected to weaken the cap and predispose to rupture. Indeed, increased levels of VSMC apoptosis are seen in mature plaques compared with control vessels,^{19–21} and increased levels are seen in unstable angina compared with stable angina.²² Many diverse factors modulate VSMC apoptosis, the details of which are beyond the scope of this review (for a detailed review, see McCarthy and Bennett²³), but include

proapoptotic stimuli such as macrophage-directed killing via tumour necrosis factor (TNF) α , Fas-L, or NO, oxidised low-density lipoprotein (oxLDL), angiotensin II type 2 receptor activation, increased p53 expression and altered mechanical stress. Conversely, antiapoptotic regulators include insulin-like growth factor I-mediated activation of the PI3 kinase/Akt pathway,²⁴ platelet-derived growth factor and ECM-derived survival signals. Experimental mouse models that use adenovirus-mediated expression of proapoptotic factors such as p53²⁵ or Fas ligand²⁶ show VSMC loss from the fibrous cap and some features of vulnerable human plaques. However, the core pathways regulating cell death are conserved in virtually every mammalian cell type, and thus expression of such factors is not VSMC-specific. In addition, adenovirus induces a prolific innate immune response, making interpretation of the effect of VSMC apoptosis difficult.

Endothelial cells

ECs show a low mitotic rate in areas that do not develop atherosclerosis, and may remain viable for 20 years. In contrast, EC in lesion-prone regions are characterised by increased turnover, suggesting a mechanistic link between cell turnover and susceptibility to atherosclerosis. The enhanced EC turnover is most probably owing to increased EC apoptosis. A number of pro-atherosclerotic agents, including high glucose concentrations, oxLDL and reactive oxygen species, induce EC apoptosis (reviewed in Dimmeler and Zeiher²⁷), suggesting that apoptosis may be a common pathway by which risk factors affect the vasculature. The bacterial toxin lipopolysaccharide also triggers EC apoptosis suggesting a link between infections and EC injury.²⁸ EC apoptosis can also be induced by a variety of proinflammatory cytokines that are present in atherosclerotic plaques—for example, the inflammatory cytokine TNF α .²⁹

Laminar blood flow is a potent endogenous anti-atherosclerotic factor, as demonstrated by the focal nature of atherosclerotic lesion development in areas with turbulent or low blood flow, such as bifurcations. Laminar shear stress can completely prevent the induction of apoptosis by various stimuli, and a lack of haemodynamic force triggers apoptosis of ECs. The effectiveness of shear stress in inhibiting a number of diverse apoptotic triggers suggests that it interferes with a common proapoptotic signaling pathway. Indeed, exposure of human ECs to laminar flow inhibits the activation of caspase-3. This occurs, at least in part, via the shear stress-stimulated release of NO, which inhibits the caspase cascade via S-nitrosylation of the essential cysteine residue in the caspase active site. In addition, an enhanced antioxidative capacity of ECs induced by shear stress may contribute to the antiapoptotic effect.³⁰

Plaque erosion

Between 40% and 65% of MIs are caused by plaques that do not show classical features of plaque rupture.^{31–32} Erosion may be associated with heavy inflammatory cell infiltration,³¹ although in many cases inflammation is absent.³² Endothelium is typically absent in these lesions, and the superficial tissue consists of VSMCs embedded in a proteoglycan-rich matrix.^{31–32} Recent studies indicating that massive induction of EC apoptosis in manipulated arteries can induce the formation of thrombus with appearances similar to eroded vessels³³ suggests that EC apoptosis may trigger thrombosis.

Inflammation

EC death has a number of direct consequences in the vasculature, including coagulation, plaque erosion and inflammation. For example, apoptotic ECs become procoagulant,³⁴ promoting platelet and neutrophil aggregation, and thereby amplify the inflammatory response. Apoptosis induces

upregulation of a number of inflammatory genes and may be one method of releasing biologically active cytokines such as IL1 β . Apoptotic cells also release oxidised membrane vesicles and blebs that contain biologically active oxidised phospholipids capable of inducing monocyte–endothelial interactions. These studies suggest that EC apoptosis is directly proinflammatory. In contrast, apoptosis can also shed proinflammatory receptors from the cell surface, such as TNF-R1, preventing activation of inflammatory genes.³⁵ Although these studies have been performed *in vitro*, the effect of EC apoptosis on inflammation in the intact vessel wall, either in normal vessels or in plaques, is unknown.

Macrophages

In advanced atherosclerotic plaques, up to 50% of the apoptotic cells are macrophages.³⁶ Although the direct consequences of macrophage apoptosis are unknown, its localisation to both the necrotic core³⁷ and sites of plaque rupture³⁸ suggests that macrophage death may promote core expansion and plaque instability, respectively. The inducers and consequences of macrophage death are likely to be different between early and late lesions. For example, proapoptotic factors derived from activated dysfunctional ECs overlying the fatty streak, such as TNF α , Fas ligand and NO, may be more important in early lesions, whereas oxidised LDL, oxysterols, hypoxia/ATP depletion and the intracellular accumulation of unesterified or free cholesterol may be relevant in the more mature lesions. Incorporation of free cholesterol into endoplasmic reticulum membranes results in stiffening of the normally fluid bilayer, leading to activation of the unfolded protein response and the cell death effector CHOP.³⁹ CHOP in concert with cholesterol loading-dependent JNK2 activation and ligation of scavenger receptor A results in apoptosis of macrophages.

Removing the corpse

Although much has been made of the direct effects of cell death in vascular pathologies, the importance of efficient phagocytosis has been somewhat overlooked. To avoid the release of potentially deleterious intracellular components such as proteases, apoptotic cells must be recognised and phagocytosed before undergoing lysis. A number of receptors on the phagocyte surface are responsible for binding and uptake of apoptotic cells. Of these, SR-A, CD36 and LOX-1 mediate uptake of apoptotic cells and modified lipoproteins by macrophages and foam cells. Indeed, oxLDL delays engulfment (but not binding) of apoptotic cells by competing for scavenger receptors, and subsequently promotes release of proinflammatory molecules. Consistent with this, a higher level of uneaten apoptotic cells is seen in human plaques than in normal tonsil.⁴⁰ Therefore, in the presence of oxLDL, both the secondary necrosis of uneaten apoptotic cells and increase in release of proinflammatory cytokines from phagocytes may promote inflammation. Given the complex microenvironment of the plaque, it will be difficult to determine the relative contribution of phagocytosis or, more importantly, reduced phagocytosis in the persistence of inflammation and thus the pathogenesis of atherosclerosis.

CONCLUSIONS

It is clear that tightly regulated apoptosis is essential for the development of the cardiovascular system and its maintenance in the adult. It is also evident that cell death (apoptosis, autophagy and necrosis) has a significant role in a number of pathologies of both the heart and the vascular system. What is not so clear is the nature of the triggers of physiological cell death and how these triggers are implicated in disease states. Nonetheless, the role of neighbouring and infiltrating cells on regulating cell death, the clearance of apoptotic bodies and how

the multiple proapoptotic and antiapoptotic influences are integrated *in vivo* are now being elucidated. Since apoptosis is implicated in cardiovascular diseases, the future identification of apoptotic regulators may lead to the development of effective treatments.

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