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Cell death and survival signalling in the cardiovascular system

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TABLE OF CONTENTS

1. ABSTRACT

The loss of cells is an important factor in many diseases, including those of the cardiovascular system. Whereas apoptosis is an essential process in development and tissue homeostasis, its occurrence is often associated with various pathologies. Apoptosis of neurons that fail to make appropriate connections is essential for the selection of correct neural signalling in the developing embryo, but its appearance in adults is often associated with neurodegenerative disease. Similarly, in the cardiovascular system, remodeling of the mammalian outflow tract during the transition from a single to dual series circulation with four chambers is accompanied by a precise pattern of cell death, but apoptosis of cardiomyocytes contributes to ischemia-reperfusion injury in the heart. In many cases, it is unclear whether apoptosis represents a causative association or merely a consequence of the disease itself. There are many excellent reviews on cell death in the cardiovascular system (1-5); in this review we outline the critical signalling pathways that promote the survival of cardiovascular cells, and their relevance to both physiological cell death and disease.

2. INTRODUCTION

2.1. How does cell death occur?

Cell death can occur in a number of ways largely dependent on the nature of the death stimulus. Thus, necrotic cell death is often a consequence of bacterial or viral infection but is also a characteristic of many inflammatory diseases. For example, late stage atherosclerotic plaques contain a lipid-filled core infiltrated with inflammatory cells, many of which undergo necrotic cell death. The loss of membrane integrity in necrotic cells releases cellular contents that act as potent inflammatory signals. Necrotic cell death has (until recently) been regarded as an unregulated process, whereas apoptosis (also referred to as programmed cell death when manifest during development) is a highly regulated, energy-dependent process initiated by a range of extra and intra-cellular triggers (Figure 1). Nonetheless, the eventual fate of an apoptotic cell can be secondary necrosis, whereby a cell undergoing apoptosis loses membrane integrity. Autophagy has also been observed in the cardiovascular system during injury, particularly in the heart (reviewed in (6-8)). Autophagy is involved in the degradation and recycling of the building blocks of organelles, proteins and other

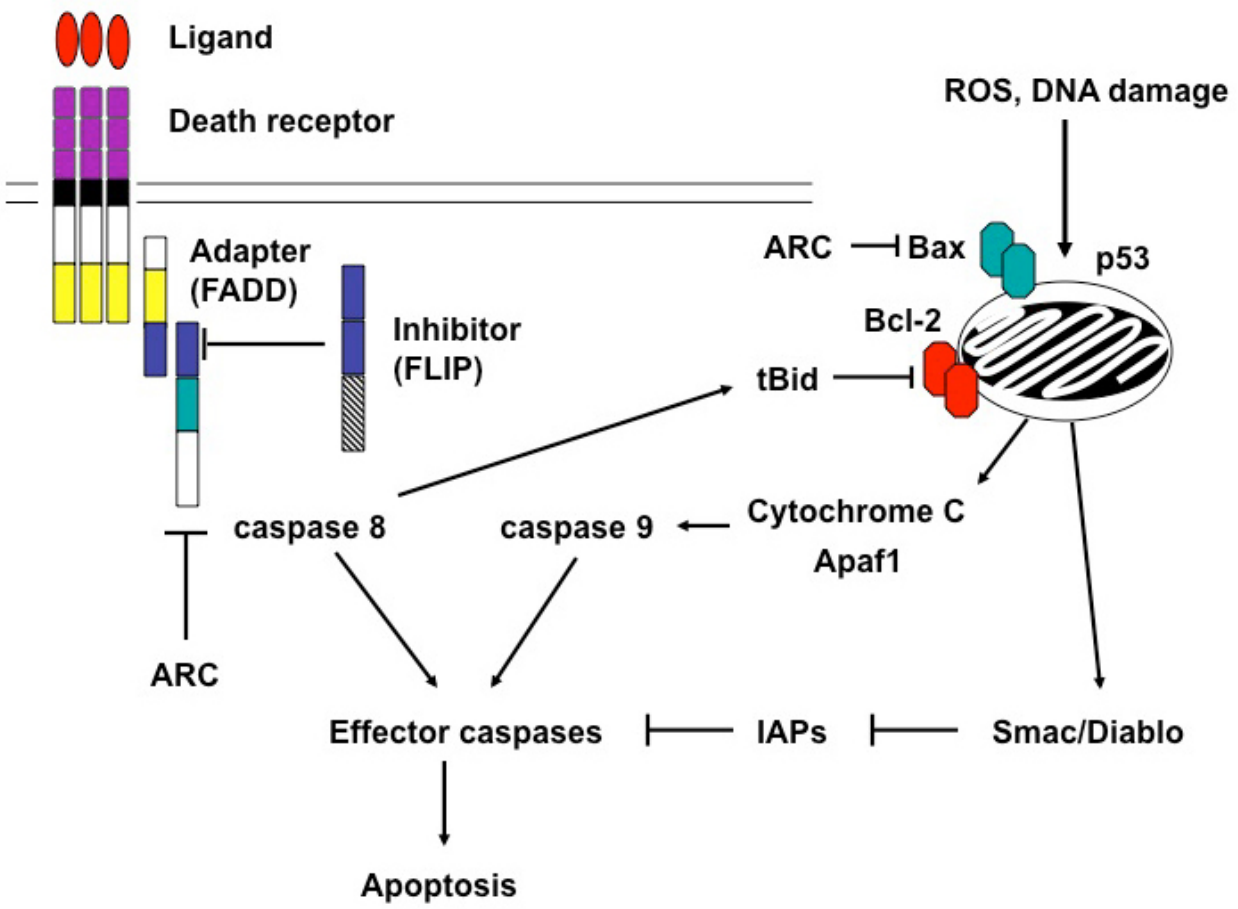

Figure 1. Major signalling pathways involved in apoptosis. Extracellular signals bind to cognate "death receptors" initiating the recruitment of adapter proteins and upstream caspases. In addition, death receptor ligation causes activation of Bid that amplifies the apoptotic response via the mitochondrial pathway. Intracellular signals such as reactive oxygen species (ROS) or DNA damage can, via modulation of the expression and/or activity of members of the Bcl2 family of proteins, cause the release of cytochrome c and Smac/Diablo from the mitochondria. Cytoplasmic cytochrome c causes a conformational change in the Apaf1 adapter that then recruits upstream caspase 9. Activation of the caspase cascade results in apoptosis. Induction of apoptosis is negatively regulated by non-functional adapters such as FLIP, proteins that operate at the mitochondrial membrane such as Bcl2 and those that interfere with caspase activity such as ARC and IAPs (inhibitors of apoptosis)

components of the cytoplasm important for cellular homeostasis (reviewed in (9)). Despite the fact that autophagy can lead to cell survival, recent studies indicate that apoptosis and autophagy involve complementary pathways and that autophagic degeneration may be a part of apoptosis, at least in some cell types (9, 10). There is, in some cases, a temporal blurring of the precise fate by which a cell dies (11). Nonetheless, apoptosis represents the best-documented mode of both physiological and pathological cell death in the cardiovascular system.

2.1.1. Apoptosis

Apoptosis is an ATP-dependent process characterized by chromatin condensation, membrane blebbing and digestion of cellular proteins and DNA (12). Membrane-bound apoptotic bodies are pinched off and the corpses are rapidly and efficiently engulfed by professional phagocytes or neighbouring cells. Thus, deletion of cells by apoptosis generally does not invoke an inflammatory response. Apoptosis is achieved by highly regulated intracellular pathways, the molecular components of which are evolutionarily-conserved (13, 14) (Figure 1).

Signals that lead to apoptosis are diverse. During "developmental programmed cell death" these signals are largely extracellular and dependent on activation of specific receptors on the surface of the target cell (reviewed in (15)). These "death receptors" are members of the tumor necrosis factor receptor (TNFR) family (16). Death receptors can be activated by soluble circulating factors or by interaction with ligands expressed on the surface of other cells. A paradigm is the ligation of the Fas receptor (also known as CD95 or Apo-1) by either soluble Fas ligand (FasL) or FasL expressed on the surface of natural killer cells. Included in the TNF receptor family are "decoy receptors" that bind ligand but fail to transmit an intracellular death signal. In addition to the TNFR family, there are also so-called "dependence receptors" that if not

bound by ligand can elicit apoptosis. For example, the DCC (deleted in colon cancer) receptor triggers apoptosis in the absence of its ligand netrin-1 (17). All of the cell surface death receptors recruit adapter molecules such as Fas-associated death domain-containing (FADD) that, in turn, recruit a distinct class of proteases (caspases) that orchestrate the dismantling of the apoptotic cell. Caspases are expressed as inactive zymogens and need to be cleaved at specific sites to become activated (reviewed in (18)). They fall into two classes: upstream "activator" caspases (for example, caspases 8 and 9) with large prodomains that are recruited by adapter molecules and autocleave as a result of induced proximity, and downstream "effector" caspases that are cleaved into the active form by the upstream enzymes.

Apoptotic signals can also originate from within the cell itself (the "intrinsic" pathway). For example, DNA damage or other stress activates a number of intracellular signalling molecules that initiate apoptosis. Thus, the p53 protein is activated following DNA damage and oxidative stress and induces expression of proteins that act at the mitochondrial membrane to promote the release of cytochrome c ((19), see below). In common with most signalling pathways there is cross-talk between the receptor-mediated "extrinsic" and intrinsic pathways. For example, activated caspase 8 cleaves Bid following death receptor ligation (20). The truncated Bid protein (tBid) acts at the mitochondria to promote cytochrome c release (21). Thus, the extracellular death signal can be amplified by co-opting the intrinsic pathway.

Events at the mitochondria appear to act as a central integrator of apoptotic signalling. There are a number of related proteins (e.g. the Bcl2 family) that are present or recruited to the mitochondrial outer membrane that, in concert, control the release of cytochrome c and other pro-apoptotic proteins such as Smac/Diablo that inhibit Inhibitor of apoptosis (IAP) proteins that, in turn, can inhibit caspases (reviewed in (22). Members of the Bcl2 family either promote or inhibit cytochrome c release. Cytosolic cytochrome c binds to and causes a conformational change in the adapter protein Apaf1 that then recruits caspase 9. This "apoptosome" results in activation of caspase 9 by induced proximity. Thus, the relative expression and activities of the Bcl2 family proteins dictate the threshold at which cytochrome c is released and thus whether an individual cell undergoes apoptosis.

2.1.2. Autophagy

Autophagy (lit. self-eating) is technically a process of cell survival. Prolonged stress, typically nutrient deprivation, initiates a programme of lysosomal-dependent organelle deconstruction within the cell, allowing the building blocks to be reassembled into other molecules. Components of the autophagic pathway are responsible for formation of the autophagosome, a membrane-bound vesicle that carries damaged proteins and organelles to the lysosome (forming the autophagolysosome). Intriguingly, many of the signalling molecules involved in autophagy are regulated by components of apoptotic pathways. For example, the anti-apoptotic protein, Bcl2, also inhibits activity of the Beclin-1 protein, an essential component of autophagy, and extracellular survival factors such as insulin-like growth factor-1 (IGF1) are efficient at inhibiting both apoptosis and autophagy. Nonetheless, inappropriate or excessive activation may lead to apoptosis and is associated with certain neurodegenerative diseases and cardiomyopathies (reviewed in (8, 23-25)). Indeed, there is evidence for autophagy and apoptosis in the same cell.

2.1.3. The other side(s) of death signalling

Most intracellular signalling pathways demonstrate a great degree of overlap and evolution has conferred multiple activities to some components of these pathways. For example, activation of p53 promotes apoptosis, growth arrest and metabolic changes within a given cell, depending on cell type and environment, and contingent on the operation of other signalling pathways. The FoxO proteins induce growth arrest and apoptosis in vascular smooth muscle cells; however, the fate of a particular cell is subject to other, largely unknown, influences. Although elucidation of these pathways has benefited enormously from *in vitro* studies, it is essential that complementary studies in animal models are conducted to understand the roles of these proteins in cardiovascular development and disease.

3. WHERE AND WHEN DOES CELL DEATH OCCUR IN THE CARDIOVASCULAR SYSTEM?

3.1. Heart

Apart from cardiogenic remodeling in the embryo, apoptosis of myocytes of the interventricular septum and right ventricular wall after birth is required for 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.

Cell death is also associated with both dilated and ischemic cardiomyopathy and the transition from compensated to decompensated hypertrophy (see below). Moreover, myocardial cell loss probably leads to the gradual deterioration in cardiac function during both cell and organismal ageing. Whereas the evidence that apoptosis promotes heart failure is persuasive, the direct causative relationship and the nature of the apoptotic triggers is not clear. Mechanical stretch can induce apoptosis, indicating a possible role for volume overload and raised ventricular and diastolic pressures. Others have suggested viral triggers of cardiomyocyte apoptosis.

Although necrosis predominates during myocardial infarction, apoptosis of cardiomyocytes is also observed and it is likely that, at least, some of the necrotic cells are due to secondary necrosis of persistent apoptotic cells. It is possible that myocyte apoptosis initiated by ischemia fails due to the dwindling respiratory capacity of the cells and the cells undergo necrosis. Following reperfusion, ATP, which is required for apoptosis, is generated allowing it to occur.

In addition to cardiac remodeling, physiological apoptosis is also implicated in the remodeling of adult vessels in response to changes in blood flow or injury - for example, changes of lumen diameter in response to changes in blood flow and increasing the number of capillaries (angiogenesis) in response to tissue hypoxia and wound healing. Contraction of the umbilical and uterine vessels following birth involves extensive apoptosis of vascular smooth muscle cells (VSMCs) and death of endothelial cells (ECs) is observed during development and in adult vessels subject to remodeling.

The failure of the heart to pump sufficient blood to meet the systemic needs of the individual results in dyspnoea, oedema, fatigue and, ultimately, heart failure. Although the causes of heart failure are numerous (ranging from inherited congenital malformations to valve disease, persistent hypertension, diabetes and infection), the course of the disease is similar. In pressure overload leading to compensated hypertrophy, cardiomyocytes increase in volume in an attempt to increase cardiac function. At some point the prolonged hypertrophy becomes "decompensated" leading to arrhythmias, heart failure and death. Several studies have observed autophagosomes, characteristic of autophagy, in the cardiomyocytes of failing hearts. The association of autophagy with failing hearts, however, is not evidence for a causative role, although loss of Atg5, a protein essential for autophagosome formation, results in cardiomyocyte hypertrophy, left ventricular enlargement, contractile malfunction and heart failure in experimental animals. Similarly, deficiency of Lamp-2 (a component of the lysosomal membrane) results in abnormal cardiomyocytes and reduced cardiac contractility (26). Although it is unclear why the heart fails when autophagy is reduced, it seems likely that cardiomyocyte autophagy has a protective role during hypertrophy. Interestingly, enhancement of autophagy as a result of increased expression of Atg5, protects against Bnip3 (BCL2 and 19-kDa interacting protein-3)-induced apoptosis following ischemia-reperfusion, probably by removal of damaged mitochondria (27). Bnip3 expression is induced in the heart by hypoxia and interferes with Bcl2-dependent survival signalling at the mitochondrial membrane (28-32).

3.2.Vasculature

As in the heart, apoptosis is also implicated in vessel disease. Arterial aneurysms are sometimes cited as an apoptosis-driven pathology. Although aneurysms are characterized by regions poor in VSMCs, collagen-rich matrix and patent internal elastic laminae (IEL) (33), increased hemodynamic stress can be a sufficient trigger (34). It is thus unclear whether apoptosis of VSMCs alone is sufficient or whether VSMC apoptosis is simply a result of constant or repeated episodes of hemodynamic stress.

There is compelling evidence that loss of VSMCs from the fibrous cap that separates the lipid-rich core of the mature atherosclerotic plaque from the vessel leads to features characteristic of advanced disease such as inflammation, calcification and thrombosis. Death of VSMCs is predicted to weaken the cap and predispose to rupture, thus exposing the underlying thrombogenic collagen and lipid. An increased level of VSMC apoptosis is, indeed, seen in rupture-prone mature plaques associated with unstable versus stable angina (35). Many diverse factors modulate VSMC apoptosis, the details of which are beyond this review (see (1-5) for reviews).

Increased EC turnover, most likely due to increased apoptosis, is also observed in atherosclerotic lesions. However, over 40% of myocardial infarctions are associated with plaques that do not show classical features of plaque rupture (36, 37). Erosion may be associated with heavy inflammatory cell infiltration (37), although in many cases inflammation is absent (36). The endothelium is often absent (36, 37) and recent studies indicate that experimental induction of EC apoptosis induces thrombus formation with appearances similar to eroded vessels (38, 39).

In advanced atherosclerotic plaques, up to 50% of the apoptotic cells are macrophages (40). Although the direct consequences of macrophage apoptosis are unknown, its localisation to both the necrotic core (41) and sites of plaque rupture (42), suggests that macrophage death may promote core expansion and plaque instability, respectively.

These observations raise the critical question of the nature of the apoptotic triggers that are essential for correct physiological remodeling on one hand, and the induction of apoptosis that is characteristic of disease on the other. Are the triggers the same or different in each case? Is the response to these triggers modified by temporal or spatial factors or dependent on the local microenvironment? Although there is great value in identifying the triggers and apoptotic pathways that occur in cardiovascular disease, it is equally important to understand the nature of signalling pathways that confer cell survival and to elucidate how attenuation of these pathways may contribute to the phenotype.

4. SURVIVAL PATHWAYS

Like death signalling, intracellular signalling pathways that promote cell survival are also elicited by a range of factors. In parallel with extracellular "death signals" there are also a range of extracellular survival signals. These include circulating factors such as insulin-like growth factor-I (IGF1) and platelet-derived growth factor (PDGF), as well as survival signals elicited by cell-cell contact and from the extracellular matrix. In addition, cells of the cardiovascular system are subject to mechanical signals such as stretch and shear stress from blood flow.

5. EXTRACELLULAR SURVIVAL SIGNALS

There is a diverse range of external factors that can promote survival of many cell types, including those of the cardiovascular system. For example, many circulating soluble factors such as hormones, cytokines, chemokines and peptide growth hormones (e.g. IGF1, gas-6 and Thymosin b4), cell-cell contacts and biomechanical forces

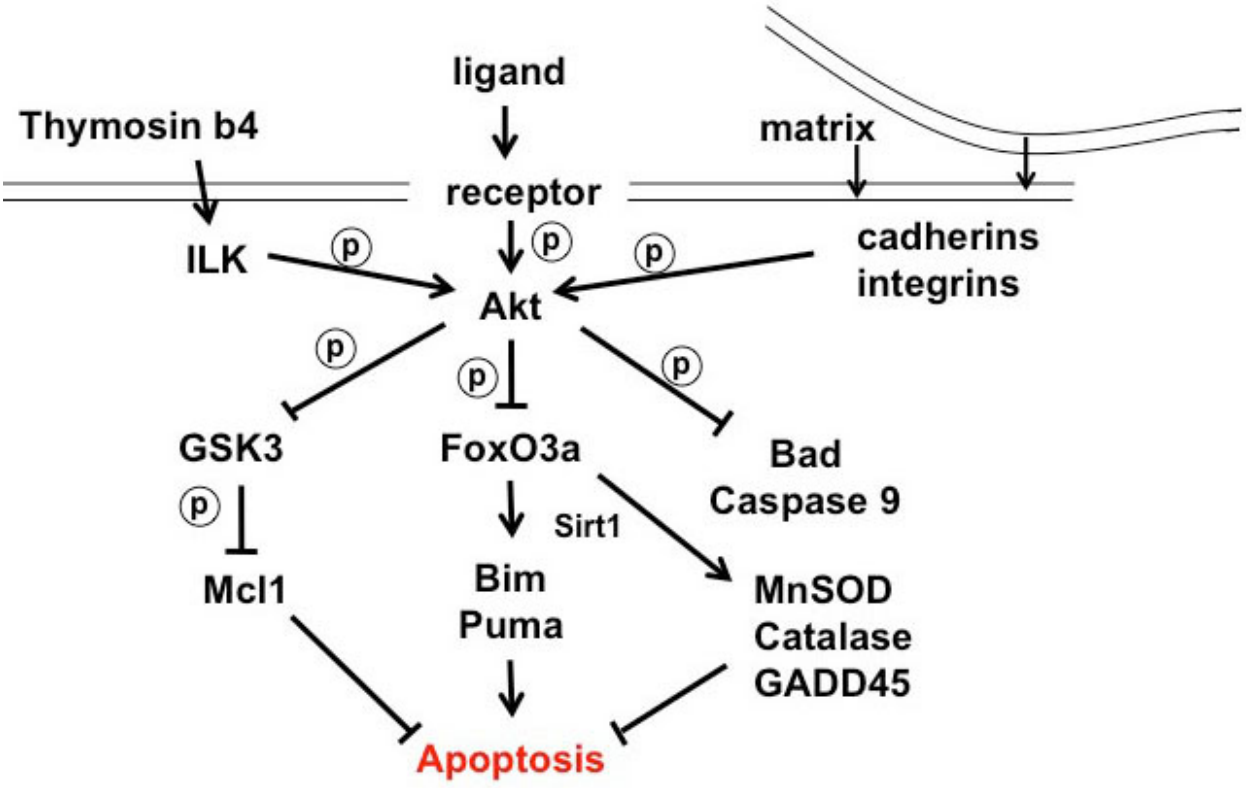

Figure 2. The Akt-dependent survival signaling pathway. Akt appears to be a central integrator of survival signaling by attenuating the activity of many downstream pro-apoptotic targets in response to a variety of upstream signals. Activation of a range of growth factor/cytokine receptors (such as IGF1R, FGFR, IL-8R) by their cognate ligands or cell-cell and cell-matrix signals stimulates the kinase activity of Akt. Akt phosphorylated targets include several proteins with pro-apoptotic activity such as GSK3, FoxO3a, Bad. Amongst these, Foxo proteins are intimately involved in both cell death and survival of cells of the cardiovascular system depending on which transcriptional targets are activated. represents phosphorylation.

such as stretch and shear stress all influence cell survival (Figure 2). Although many of the extracellular survival signals are common to several cell types there are, not surprisingly, some signals that are particularly relevant for specific cardiovascular cells.

5.1. Vascular smooth muscle cells

Arguably, the best-documented and ubiquitous soluble survival factor in the cardiovascular system is insulin-like growth factor-1 (IGF1), a polypeptide protein hormone that binds to the insulin-like growth factor receptor (IGF1R). Binding to the IGF1R, a receptor tyrosine kinase, triggers intracellular signalling pathways involved in cell growth and survival including insulin receptor substrate-1 (IRS-1), phosphotidylinositol 3-linase (PI3K) and mitogen-activated protein kinase (MAPK). There is considerable evidence for the importance of IGF1 signalling in the cardiovascular system (reviewed in (43) and IGF1 is a potent survival factor for VSMCs (44). There is also evidence that VSMCs of atherosclerotic lesions exhibit reduced IGF1R expression (45, 46) consistent with their greater sensitivity to apoptosis (46, 47). The reduction of IGF1R expression noted in plaque-derived VSMCs (compared to VSMCs from healthy tissue) results from oxidative stress, at least *in vitro* (48). Consistent with IGF1 acting as a survival factor for VSMCs, ectopic expression of IGF1R in plaque-derived cells enhances their survival in response to IGF1 (49). Interestingly, Shai *et al* (50) report that increased IGF1 expression in VSMCs results in advanced atherosclerotic plaques with features of plaque stability in fat-fed *ApoE*$^{-/-}$ animals, consistent with an anti-apoptotic effect of IGF1 *in vivo*. Activation of the IGF1R elicits intracellular kinase cascades common to many cell types, including those of the cardiovascular system. The Ras/Raf1/Mek/ERK and PI3K/Akt pathways regulate a wide range of biological effects including cell growth, proliferation, migration, metabolism and survival. These signalling pathways are described below.

The growth arrest-specific gene 6 encodes a secreted protein (Gas6) that is also an effective suppressor of VSMC apoptosis (51). Gas6 acts as a ligand for the receptor tyrosine kinase, Axl, and activation of Axl leads to PI3K-dependent activation of Akt but not ERK (51, 52). Studies in balloon-injured rat carotid arteries suggest that Gas6/Axl may be involved in VSMC survival in response to vascular injury, probably via the Akt pathway (51).

There is considerable circumstantial evidence that, in many cell types, apoptosis is the default pathway

and needs to be constantly inhibited in order for a cell to survive. Although soluble survival signals are well documented, cell-matrix and cell-cell contacts are important promoters of cell survival, with loss of these contacts leading to anoikis (detachment-induced apoptosis). Cadherins are transmembrane proteins that regulate calcium-dependent cell-cell contacts that affect a number of cell fates, including differentiation, migration, proliferation and survival. For example, disruption of N-cadherin, that mediates cell-cell and cell-matrix contacts, results in increased apoptosis of VSMCs in culture (53, 54). In agreement with this, an increase in N-cadherin expression significantly decreases VSMC apoptosis (55). Survival signalling downstream of cadherins again appears to invoke the serine/threonine kinase Akt/PKB (55).

5.2. Cardiomyocytes

In addition to its effects on VSMCs, IGF1 also suppresses the apoptosis of cultured cardiomyocytes (56) and *in vivo* after myocardial infarction (57) and ischemia-reperfusion injury (58). Indeed, several studies have noted the correlation between low IGF1 levels and an increase in the risk of heart failure. Recovery following MI appears to be improved in patients with higher serum IGF1 leading to the suggestion that stem cell recruitment to the recovering cardiac tissue is enhanced by IGF1 as described for skeletal muscle (59), partially by promoting cell proliferation and preserving a reservoir of competent cardiac cells (60).

Other secreted factors such as thymosin b4 have been shown to enhance the survival of cardiac muscle cells (61), reviewed by (62). Thymosin b4 is one of several related proteins that sequester actin monomers. Thymosin b4 promotes the migration of cells during cardiac development and promotes both wound healing and neovascularization. Addition of thymosin b4 to isolated rat cardiac muscle cells in culture enhances their survival. Moreover, administration of thymosin b4 to animals subjected to coronary occlusion reduces scar volume and inhibits ventricular dilatation, resulting in improved heart function. Thymosin b4 binds to and is internalized by target cells, and Akt acts as a common node of intracellular signalling. Bock-Marquette *et al* (61) showed that thymosin b4 activates the integrin-linked kinase (ILK) via association with the cytoskeletal protein PINCH and that this activates Akt (see below). Akt activation correlates with resistance to oxygen depletion (63), probably by promoting survival of cardiac cells, consistent with the ability of thymosin b4 to improve heart function after injury.

5.3. Endothelial cells

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 apoptosis induced by various stimuli, and a lack of hemodynamic 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 pro-apoptotic signalling 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 nitric oxide (NO) that inhibits the caspase cascade via S-nitrosylation of the essential cysteine residue in the caspase active site. In addition, an enhanced anti-oxidative capacity of ECs induced by shear stress may contribute to the anti-apoptotic effect (64).

Endothelial cells are exposed to a range of pro-apoptotic signals such as transforming growth factor beta (TGFbeta1), 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 (Ang-1), erythropoietin, interleukin–8 (IL-8), hepatocyte growth factor (HGF), and fibroblast growth factors (FGF) have been identified *in vitro*, many of which involve signalling through Akt. In addition, integrin-mediated attachment of EC to the extra-cellular matrix (ECM) elicits pro-survival pathways. Detachment from the basement membrane may render endothelial cells refractory to VEGF-A-mediated survival signalling and induce anoikis. Blood flow and shear stress also regulate EC survival via activation of the endothelial nitric oxide synthase (eNOS), although the precise role of NO production in the fate of endothelial cells is not clear.

Thus, EC survival is regulated by the integration of growth factor- and integrin-dependent signalling, which are in turn governed by the composition of the ECM, and by the hemodynamic properties of the vessel (65). Experimental inactivation of genes involved in survival also suggests a role for EC apoptosis in vessel remodelling. For example, inactivation of VEGF-A or Ang-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 remodeling 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 reduced *in vitro*.

6. INTRACELLULAR SURVIVAL SIGNALLING PATHWAYS

Despite a wide range of external stimuli, survival signalling is channelled through a limited number of common pathways and effectors.

6.1. The Akt/PKB pathway

The serine/threonine protein kinase Akt (also known as PKB) is a key mediator of signal transduction pathways implicated in cell growth, insulin signalling and cell survival. The kinase was discovered as the cellular counterpart of the transduced oncogene, *v-akt,* found in the acutely transforming retrovirus, AKT8, that causes T-cell lymphoma in rodents (reviewed in (66)). All three isoforms of Akt have extensive homology to protein kinases A, G, and C within their kinase domains (67). Although mammalian isoforms of Akt (Akt1/PKBalpha,

Akt2/PKBbeta and Akt3/PKBgamma) are similar in structure, containing an amino-terminal pleckstrin homology (PH) domain and a carboxy-terminal catalytic domain (67), they do not share identical activities. Mice lacking Akt1 show neonatal mortality and growth impairment, those lacking Akt2 show type-II-diabetes, and studies in Akt3-deficient mice suggest that it is important for post-natal brain development (68). Growth factors, cytokines, insulin and IGF1 all induce Akt/PKB activation. Activated receptor tyrosine kinases (such as the IGF1R) and G-protein coupled receptors promote phosphatidylinositol 3-kinase (PI3K) activity leading to an increase in two lipids, phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P_2) and phosphatidyliositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3). These products have high affinity towards the PH domain of Akt and stimulate PDK1 (PH-domain-containing protein kinase)-dependent phosphorylation and activation of Akt (69, 70). Phosphorylation of Akt at specific sites (T308 and S473 in human Akt1) correlates with its activity. Consistent with this, expression of an Akt protein lacking its PH domain but containing a myristoylation (membrane targeting) signal from the Src protein is correctly phosphorylated and results in full Akt activity. Thus, the PH domain is required for recruitment and phosphorylation-dependent activation of Akt but not for its kinase activity. Akt phosphorylates a large number of substrates impinging on a range of biological process such as cell growth, proliferation, migration, metabolism and inhibition of cell death. Akt-dependent phosphorylation can result in activation or inhibition of the target protein's activity. Here, we will focus on Akt-mediated cell survival.

Akt1 has multiple roles in the cardiovascular system including protection from oxidative stress-induced apoptosis, regulation of cardiac hypertrophy, angiogenesis and cell migration and proliferation (71, 72). Akt1 is both necessary (as an effector of IGF1) and sufficient for the survival of cultured rat VSMCs following oxidative stress (49). Expression of a dominant negative molecule that inhibits endogenous Akt activation suppresses IGF1-dependent survival in VSMCs (49, 73), suggesting that Akt is a major downstream target of IGF1R. In contrast, expression of an ectopically regulated Akt protein inhibits oxidative stress-induced VSMC apoptosis at least as effectively as IGF1. There is evidence that IGF1 can also protect VSMCs via MAPK pathways and protein kinase C-epsilon (73, 74). Consistent with the reduced expression of IGF1 (45) and IGF1R (46, 49) in human plaques, there is a reduction in active phosphorylated Akt (but not total Akt) in plaque-derived VSMCs *in vitro* (46) and in plaque intimal VSMCs (compared to medial VSMCs) in intact plaques (49). As is often the case, it is difficult to determine whether the reduction in Akt contributes to atherosclerosis or is simply a consequence of the disease process. Fortunately, in this case we have some direct evidence for the involvement of Akt. Stress-induced apoptosis is enhanced and proliferation and migration reduced in *Akt1*-deficient VSMCs *in vitro* (71). Importantly, high fat-fed *Akt1*$^{-/-}$; *ApoE*$^{-/-}$ mice develop atherosclerotic lesions that display a large necrotic core, and reduced fibrous cap and collagen content (71), characteristics of plaques thought prone to rupture. Although the reduction in proliferation and migration in *Akt1*$^{-/-}$ VSMCs may contribute to this phenotype, the striking resemblance to plaques of *ApoE*$^{-/-}$ mice following targeted deletion of VSMCs (75, 76) suggests that the primary mediator of this phenotype may be loss of Akt1-dependent survival.

Akt attenuates cell death by regulating the activities of components of apoptotic signalling at various levels. First, Akt phosphorylates and inhibits proteins such as FoxO3a that transcriptionally activate pro-apoptotic members of the Bcl2 family such as Puma and Bim. Second, it can directly phosphorylate and inhibit some of these pro-apoptotic proteins. For example, Akt directly phosphorylates the Bcl2 homology domain pro-apoptotic protein BAD thereby inhibiting its activity (see above). Third, Akt can phosphorylate human caspase 9, a downstream effector of apoptotic signalling. However, the lack of a conserved Akt consensus phosphorylation site (RXRXX(S/T)) in other mammalian caspase 9 proteins has cast some doubt on this (77, 78). Moreover, because of the interaction between multiple pathways within an individual cell, it is difficult to determine the importance of Akt-dependent phosphorylation on individual Akt substrates in survival signalling. For example, at least part of Akt cardioprotective function derives from activation of its target, Pim-1 (79-82). The Pim-1 kinase increases the levels of the anti-apoptotic proteins, Bcl2 and Bcl-x_L (83). In contrast, Akt-dependent inhibition of FoxO3a is important in the survival of cultured VSMCs subjected to oxidative stress (see below).

Although Akt is sufficient to suppress oxidative stress-induced apoptosis of VSMCs, in the presence of a non (Akt)- phosphorylatable FoxO3a protein (in which all three Akt consensus phosphorylation sites, T32, S253 and S315 are changed to alanine residues) this is attenuated (49). This suggests that survival of VSMCs is critically dependent on inhibition of FoxO3a activity by Akt signalling. Consistent with this, FoxO3a is sufficient to induce apoptosis in VSMCs (84). Whereas phosphorylated FoxO3a is translocated to the cytosol where it is targeted for ubiquitination and proteasomal degradation(85), dephosphorylated nuclear FoxO3a activates genes involved in apoptosis, DNA repair, cell cycle progression, differentiation, oxidative stress, longevity and glucose metabolism. Among FoxO3a's pro-apoptotic targets are Puma (86), Bim (87-90), FasL (85), and IGFBP1 (91). In addition, FoxO proteins can down-regulate proteins that suppress apoptosis such as the caspase 8 inhibitor FLIP (92). Indeed, expression cDNA arrays indicate that there are in excess of 1600 genes that might be transcriptionally regulated by FoxO3a in VSMCs (J. Tucka and T. D. Littlewood, unpublished data). In addition, there is evidence that FoxO proteins can operate in a negative feed-back loop by enhancing Akt activity and attenuating the insulin response in cardiomyocytes (93).

In contrast to their pro-apoptotic activity, FoxO proteins can also mediate cell survival in response to oxidative stress and DNA damage (reviewed in (94). For example, mice deficient in FoxO1 and FoxO3a exhibit

increased ROS following myocardial infarction or acute ischemia/reperfusion injury consistent with reduced expression of the antioxidant proteins catalase and manganese superoxide dismutase (MnSOD) (95), known transcriptional targets of FoxO proteins (96, 97); reviewed in (98). FoxO3a can also promote survival in response to hypoxia by inducing transcription of CITED2, a transcriptional cofactor that functions in a negative feedback loop inhibiting HIF-1 induced apoptosis (99). In addition, FoxO transcription factors can activate genes involved in autophagy in response to starvation and oxidative damage (100). Finally, FoxO proteins also promote expression of genes involved in the DNA damage repair such as GADD45 (101) and recent evidence suggests that FoxO4 interacts with Ku70 (102).

So, how are these contrasting activities of FoxO proteins manifest? The obvious answer is that FoxO pro-apoptotic and pro-survival activities are differentially regulated in different cell types, partly in response to their environmental milieu. For example, is the FoxO – dependent read out dictated by the strength or persistence of the potential apoptotic signal? But then how is the protein activity regulated at the molecular level? One possibility comes from the observation that FoxO proteins are also phosphorylated by other kinases. For example, serum and glucocorticoid-inducible kinase (SGK), I kappa kinase (IKK) and cyclin-dependent kinase 2 (CDK2) also target FoxO and inhibit its activity. In contrast, Jun N-terminal kinase (JNK) and macrophage stimulating 1 (MST1, also known as hepatocyte growth factor-like 1) proteins activate FoxO3a in response to oxidative stress (103). Even in the presence of phosphorylation by Akt and SGK, JNK and MST1-phosphorylated FoxO is shuttled to the nucleus. This hierarchy is crucial to the oxidative stress resistance (104). A second mechanism involves a different post-translational modification. For example, hydrogen peroxide (but not UV irradiation) promotes the deacetylation of FoxO3a leading to cell cycle arrest in mammalian cells (105). Sirtuins are NAD^+-dependent class III histone deacetylases that have been shown to protect cells from oxidative stress. Acetylation of FoxO3a promotes its apoptotic activity and its deacetylation by Sirt1 promotes stress adaptation and cell cycle arrest (103, 105, 106). Therefore, Sirt1-dependent deacetylation of FoxO3a alters its function from cell death to stress resistance (105). Given the evidence that FoxO3a transcriptionally activates pro-apoptotic targets in response to oxidative stress, it seems counter-intuitive that others (107) have reported that acetylation suppresses FoxO3a transcriptional activity.

In addition to direct effects on components of apoptotic signalling, there is evidence that Akt also exercises its pro-survival role through cross-talk with pathways that have other roles in other Akt-dependent effects, such as metabolism. Glycogen synthase kinase-3beta (GSK3beta) is a ubiquitously expressed serine/threonine kinase that regulates a variety of cellular functions, notably cell metabolism. GSK3beta-dependent phosphorylation of Mcl1, an anti-apoptotic member of the Bcl2 family, targets it for rapid proteasomal degradation and Akt-dependent inhibition of GSK3beta results in stabilization of Mcl1 and enhanced survival (108). Moreover, phosphorylation of GSK3beta correlates with cardioprotection (109) and it has been suggested as a therapeutic target (110).

Akt also inhibits Ask1 (Apoptosis signal-regulating kinase 1) that activates MAPKK (mitogen-activated protein (MAP) kinase kinase) and in turn activates the c-Jun N-terminal kinase (JNK) signalling pathway (111). JNKs are stress-activated protein kinases (SAPKs) that have important roles in cellular responses to environmental stresses. JNKs are activated by upstream kinases, MEK kinase (MEKK), JNK kinase (JNKK)/stress-activated Erk kinase (SEK-1) and once phosphorylated activate transcription factors such as c-Jun, ATF-2, and Elk-1. There are three mammalian JNK isoforms JNK1, JNK2, and JNK3 (also termed stress-activated protein kinase (SAPK)-gamma, SAPK-alpha and SAPK-beta, respectively). $JNK1^{-/-}JNK2^{-/-}$ knockout animals resulted in embryonic lethality and neuroprotection in $JNK3^{-/-}$ animals. One potential function of JNK may be the initiation of programmed cell death possibly by activating expression of pro-apoptotic proteins (112, 113).

In addition, Akt activates the anti-apoptotic nuclear factor-kappa B (NF-kappa B) (114, 115). NF-kappaB can act as an inhibitor of apoptosis and as a protective factor in cardiovascular injury (reviewed in (113), possibly by direct effects on HIF-1alpha transcription (116). Consequently, HIF-1alpha-mediated protection can be elicited not only by hypoxia, but also cytokine activators of Akt activity such as IGF1 and PDGF.

6.2. Non-Akt dependent pathways

Many other intracellular molecular pathways modulate cell death of cardiovascular cells. The Apoptosis repressor with caspase recruitment domain (ARC) protein is highly expressed in both striated and heart muscle (117). As implied by its name, ARC possesses a caspase recruitment domain (CARD) that allows it to interact with and inhibit the initiator caspases 2 and 8 thus suppressing death receptor signalling (Figure 1; (117). Interestingly, ARC also interacts and interferes with Bax activation suppressing the release of cytochrome c (Figure 1; (118)). Although ARC-deficient mice develop normally and exhibit normal cardiac function at rest, they show accelerated cardiomyopathy following aortic constriction and increased myocardial infarction size after ischemia/reperfusion injury (119). Consistent with this, increased ARC expression is cardioprotective following ischemia/reperfusion in isolated rat hearts (120). Furthermore, cells of failing human hearts show a decreased expression of ARC indicating that its anti-apoptotic effects might be relevant in cardiac disease (119).

There is also considerable evidence that Hypoxia inducible factor (HIF) has an important protective role in response to oxygen deprivation, such as during experimental or pathological cardiac ischemia (reviewed in (121). It is possible that HIF modulates reperfusion injury by regulating genes that affect cell survival pathways. For

example, increased expression of the HIF-regulated heme oxygenase-1 (HO-1) gene reduces infarct size (122) possibly by regulating cell survival (123). HO-1 degrades heme generating carbon monoxide and bilirubin, both of which might act as antioxidants. Carbon monoxide suppresses apoptosis of endothelial cells, thereby contributing to protection against vascular injury. The anti-apoptotic effects of carbon monoxide depends on the MAPK pathway that leads to activation of Signal transducer and activator of transcription-3 (STAT-3) resulting in attenuation of Fas expression and caspase 3 activity (124) and increased expression of the anti-apoptotic proteins, Bcl2 and BclX. The STAT family of transcription factors mediate both cell death and survival (reviewed in (125). Thus, stimulation (by phosphorylation) of STAT-1 activity following ischemia/reperfusion transcriptionally activates Fas and FasL in cardiomyocytes and reduces the basal activity of the Bcl2 and BclX promoters, promoting cell death (126).

7. CONCLUSIONS

Apoptosis is common to many pathologies of the cardiovascular system, including in vascular smooth muscle cells and macrophages in atherosclerosis and cardiomyocytes in cardiac hypertrophy, ischemia, infarct and failure. Although intracellular signalling pathways leading to apoptosis have been extensively characterized in many cell types, less is known about the pathways that counteract these apoptotic tendencies. Nonetheless, it is clear that components of survival signalling are evolutionarily conserved and we are beginning to elucidate their mechanisms of action. At least in the cardiovascular system, it seems as though the pleiotropic effects of Akt signalling are responsible for much of the protective signalling following tissue injury.

8. ACKNOWLEDGEMENTS

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