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ESSENTIALS Apoptosis is the process by which single cells die in the midst of living tissues. It is responsible for most—perhaps all—of the cell death events that occur during the formation of the early embryo and the sculpting of organs. Apoptotic cell death continues to play a critical role in the maintenance of cell numbers in those tissues in which cell turnover persists into adult life, such as the epithelium of the gastro- intestinal tract, the bone marrow, and lymphoid system including both B- and T-cell lineages. Clinical context—apoptosis appears in the reactions of many tissues to injury, including mild degrees of ischaemia, exposure to ionizing and ultraviolet radiation, or treatment with cancer chemotherapeutic drugs. Excessive or too little apoptosis play a significant part in the pathogenesis of autoimmunity, infectious disease, AIDS, stroke, myo- cardial disease, and cancer. When cancers regress, apoptosis is part of the mechanism involved. Mechanism—the process is rapid, taking minutes to hours. (1) Structural changes—dying cells lose contact with their neigh- bours and undergo loss of volume, explosive blebbing from the cell surface and fragmentation into a cluster of membrane-bounded apoptotic bodies, with chromatin condensing in discrete aggregates under the nuclear membrane. The fragments are rapidly phagocyt- osed. (2) Cellular processes—many of the morphological features of apoptosis are attributable to activation of a family of proteases called ‘caspases’, which are activated by two pathways (a) extrinsic—via death-signalling receptors (members of the tumour necrosis factor α (TNFα) receptor family); (b) intrinsic pathway—triggered by many signals from the cell interior including the heat shock response, the unfolded protein response, the stress-activated kinase response, and the DNA damage response. Introduction Apoptosis is the process by which single cells die in the midst of living tissues, playing a crucial role in the formation of the early em- bryo, the sculpting of organs, the maintenance of cell numbers in those tissues in which cell turnover persists into adult life, and the reactions of many tissues to injury. Abnormalities of apoptosis play a significant part in many disease processes. This chapter gives an overview of apoptosis in health and disease. Figure 3.6.1 gives an overview of the process to orient the reader for the account that follows. Structural changes in apoptosis Apoptosis can be recognized because of its characteristic, stereo- typed sequence of structural changes (Fig. 3.6.2). The dying cells lose contact with their neighbours and undergo a rapid loss of volume. There is explosive blebbing from the cell surface, followed by frag-

mentation of the cell into a cluster of subcellular bodies (apoptotic bodies), each membrane-bounded and containing a variety of compacted cytoplasmic organelles. The nucleus undergoes similar shrinkage and fragmentation. Chromatin condenses under the nu- clear membrane in knob-like, hemilunar, or toroidal aggregates. Nuclear membranes overlying residual uncondensed chromatin are rich in pores, but these are absent adjacent to condensed chromatin, suggesting that redistribution takes place. The nucleolus falls apart, its argyrophilic fibrillar centre remaining apparently tethered to the peripheral aggregates of chromatin, while the osmiophilic particles associated with transcription complexes disperse in the central nu- cleoplasm. Eventually the nuclear membrane disappears and the en- tire nuclear remnant fragments into several near-spherical masses of condensed chromatin. Within the cytoplasm, the endoplasmic reticulum dilates. The cell surface loses any pre-existing microvilli or other indices of polarity. The shrunken cell and the apoptotic bodies into which it fragments tend to become spherical. Isolated apoptotic cells lose the ability to maintain ionic homeo- stasis within an hour or so, lose density, swell in volume, and permit the entry of various dyes classically used to mark dead cells (such as trypan blue and propidium iodide). Within tissues, however, this phase is seldom seen, because the apoptotic cell and its fragments undergo rapid phagocytosis. Often this is undertaken by ‘profes-sional’ phagocytes—the resident tissue macrophages—but where unusually large numbers of apoptotic cells are generated, other cell types share in ingesting them, including their viable neighbours. 3.6 Apoptosis in health and disease Mark J. Arends and Christopher D. Gregory

3.6 Apoptosis in health and disease 267 Once within the phagosome of the ingesting cell, the apoptotic cell and its fragments rapidly become indistinguishable from the con- tents of any other large secondary lysosome. For reasons to be expanded later, the process of apoptotic cell phagocytosis inhibits the neutrophil-dominated inflammatory re- action that is often seen when macrophages are activated in other circumstances. Cell loss by apoptosis can therefore be effected with little disruption of the tissue concerned. Moreover, apoptosis, once initiated, is completed swiftly. Although the interval from the ini- tial application of a lethal stimulus to the first manifestations of shrinkage and blebbing can vary from minutes to many hours, phagocytosis may be complete within an hour thereafter. Hence, the evidence for cell loss by apoptosis provided by the ‘snapshot’ of a histological section is often surprisingly scanty relative to the reduc- tion in cell number that occurs. Apoptosis is not the only mode of cell death. In classical necrosis, dying cells show a different pattern of change, dominated by volume overload and, eventually, plasma membrane breakdown and leakage of intracellular contents into the extracellular space. At first, the nu- cleus retains its general structure, although the chromatin patterns coarsen. Later, following equilibration of the cytosol with extracel- lular calcium, and the resultant widespread activation of degradative enzymes such as cathepsins, vestiges of nuclear structure fade away (karyolysis) and only ghost-like cellular outlines remain. Usually there is an associated acute inflammatory reaction. This pattern of death is frequently found when tissues are overwhelmed by high concentrations of toxic substances or in severe ischaemic damage, where vascular perfusion has been arrested. Although apoptosis is the most established and widely studied mode of programmed cell death, it is worth noting other modes that have been described more recently. These in- clude necroptosis, a form of programmed necrosis that occurs when apoptosis is inhibited; pyroptosis, an inflammatory mode of cell death in response to infection; and autophagy-associated cell death, which can occur in response to nutrient deprivation, during involution of tissues or exposure to toxins. In autophagy, Radiation, chemotherapy Growth factor withdrawal Death ligand Death receptor Procaspase 8 Procaspase 9 Active caspase 8 Active caspase 3,7 Active

caspase 9 APAF1 Cytochrome c Mitochondrion APOPTOSOME APOPTOSIS Dismantling of the cell Plasma membrane changes Recognition, response, and removal INTRINSIC PATHWAY EXTRINSIC PATHWAY Bcl-2 Procaspase 3,7 Smac Fig. 3.6.1 Overview of apoptosis: intrinsic and extrinsic pathways. The intrinsic pathway is triggered by many signals from the cell interior, such as genotoxic injury including radiation and chemotherapy, or growth factor withdrawal, leading to release of cytochrome c from the mitochondrial intermembranous space, formation of the apoptosome, and activation of caspase 9. The extrinsic pathway involves ligation of death-signalling receptors by their ligands, recruitment of a cluster of proteins collectively called the death-inducing signalling complex, leading to activation of caspase 8. Caspases from both pathways lead to activation of effector caspases 3 and 7 that bring about dismantling of the cell, plasma membrane changes mediating its recognition and removal by phagocytosis, as well as additional responses involved in tissue repair. See text for details.

268 SECTION 3 Cell biology which is an overt cell survival response, cells typically show a set of structural changes characterized by portions of cyto- plasm, including mitochondria and other organelles (but not the nucleus), becoming enveloped by their own cell's endosomal membranes and undergoing destruction through fusion with lysosomes. It is one of the principal mechanisms responsible for cell atrophy (the organized reduction in volume and com- plexity of cytoplasm), but is probably not intrinsic to the pro- cess of death. Studies of gene expression show many differences between autophagy and apoptosis, and autophagy can occur without cell death. Although both may occur in parallel within involuting tissue, autophagy appears to be an adaptive response, effected by cells living through adverse conditions, but apop- tosis always implies cell death. Caspases: Effectors of apoptosis with other functions Many of the morphological features of apoptosis are attributable to activation of a family of proteases known as caspases (so called be- cause of the presence of the amino acid cysteine (c) in their cata- lytic site, and their preferential cleavage of peptides immediately C-terminal to aspartate (asp) residues). There are at least 18 mammalian caspases (Table 3.6.1). All are ini- tially synthesized as inactive proenzymes and undergo proteolysis to generate two fragments of around 10- and 20-kDa molecular weight (p10 and p20), together with a fragment of variable length from the original N terminus and a linker fragment (Fig. 3.6.3). These p10 and p20 subunits oligomerize in pairs to form a tetramer, which is the active enzyme. Long N-terminal sequences provide the op- portunity for regulation through interaction with various binding proteins. Caspases recognize motifs of four amino acids that are present in many proteins. Significantly, such caspase target sites are often highly conserved between species, and frequently occur in strategic intramolecular locations, such that caspase cleavage would radically alter the function of the substrate protein. In particular, the cleavage of caspase substrates accounts for many of the structural changes of apoptosis already described. Particularly interesting substrates in- clude proteases, kinases, cytoskeletal proteins, proteins involved in DNA damage and repair, and cell-cycle regulatory proteins. Caspases and proteases The cleavage sites involved in the processing of caspases to their ac- tive form are themselves typical caspase target sequences. Hence, caspase activation can occur either by autocatalysis or through a sequential cascade-like process in which initiator caspases with (c) (b) (a) (d) (e)
Fig. 3.6.2 The structure of apoptosis. (a) Scanning electron micrograph of a normal macrophage shows its surface sprouting many pseudopodia. In (b) the cell has been injured (in this case by oxidized lipid of the type often present in high concentration in atheromatous plaques) and is throwing out and retracting multiple surface blebs (explosive surface blebbing). In (c) the whole cell is fragmenting into roughly spherical apoptotic bodies. Some of these are cratered by the

orifices of the dilated endoplasmic reticulum fusing with the membrane. (d) Transmission electron micrograph (TEM) of an ultrathin section of a normal macrophage. (e) An apoptotic macrophage (TEM) shows the condensed chromatin (arrowhead), nucleolar remnant (arrow), and convoluted surface with dilated endoplasmic reticulum. Micrographs by courtesy of Dr Jeremy Skepper and Dr Jing Xia, Cambridge School of Biology Multi-imaging Centre.

3.6 Apoptosis in health and disease 269 long N-terminal sequences (caspases 8, 9, 10, and 2) are activated first and then activate, by cleavage, the short effector or executioner caspases (3 and 7). Caspases can also activate other proteases. Thus, the calpain-inhibitor protein calpastatin is inactivated by caspase cleavage, so turning on calpain digestion within the dying cell. Caspases and protein kinases The small G-protein rho regulates the mobility of the cell surface. Two rho-dependent kinases, PAK2 and ROCK-1, are rendered constitutively active by caspase cleavage, through excision of their negative regulatory domains. PAK2 activity is a factor in the early retraction of the apoptotic cell from its neighbours or from sub- strate attachment, while ROCK-1 activity is responsible for the enhanced action of a myosin light-chain kinase that drives the cell-membrane blebbing immediately preceding fragmentation of the apoptotic cell. FAKp125 is the kinase associated with focal adhesion plaques. It is a critical element in the signalling pathway that links cellular aware- ness of substrate attachment (through integrins) to other cellular functions, including movement, attachment, and new transcription. FAKp125 is cleaved and inactivated by caspases, hence isolating the cell from such signals, many of which would normally promote sur-vival. Somewhat similarly, the adenomatous polyposis coli protein and β-catenin are cleaved by caspases, at molecular sites that en- sure loss of their function. Both are members of the Wnt-1 signal- ling pathway, connecting cell-to-cell signals with regulation of cell function. Caspases and cytoskeletal proteins Actin (the major protein of the cytoskeleton), fodrin (which pro- vides the deformable shell underlying the plasma membrane), vimentin (an intermediate filament protein of the cytoskeleton), and the lamins (which form a major component of the nuclear en- velope) are all caspase substrates. Caspase cleavage of these large polymeric proteins ensures they are rapidly disassembled to mono- mers. Gelsolin, a further caspase substrate, is an actin-binding pro- tein that cleaves actin filaments in a calcium-dependent manner. Caspase cleavage of gelsolin separates the calcium-sensitive nega- tive regulatory domain from the protease domain, and hence actin- filament cleavage is effected under normal intracellular calcium Table 3.6.1 Caspases Function Caspase Domain structure Size (AAs) Species Comment Apoptosis—initiator Caspase-2 CARD-L-S 452 h,m Nedd2. CARD domain for activation Caspase-8 DED-DED-L-S 479 h,m DED domains for activation Caspase-9 CARD-L-S 416 h,m CARD domain for activation Caspase-10 DED-DED-L-S 521 h DED domains for activation Apoptosis—executioner Caspase-3 L-S 277 h,m Lack N-terminal interaction domains Caspase-6 L-S 293 h,m Lack N-terminal interaction domains Caspase-7 L-S 303 h,m Lack N-terminal interaction domains Inflammation Caspase-1 CARD-L-S 404 h,m ICE Caspase-4 CARD-L-S 377 h Caspase-5 CARD-L-S 434 h Caspase-11 CARD-L-S 373 m Caspase-12-L* CARD-L-S 419 h,m Caspase-12-S* CARD-L 231 h Other Caspase-13 CARD-L-S 377 b Caspase-14 L-S 242 h,m Skin epidermis—cornification Caspase-16 L-S 183 h,m Caspases, cysteine-ASPartic proteases; CARD, caspase recruitment domain; DED, death effector domain; L, large subunit; S, small subunit; L*, long form; S*, short form; AAs, number of amino acids; ICE, interleukin-1-β-converting enzyme was the early name (later renamed) for caspase-1; Nedd2 was later renamed caspase-2. 18 mammalian caspases are known, but recently identified caspases-15, -17, and -18 are absent from placental mammals; caspase-5 is not present in mice; caspase-11 and -13 are murine (m) and bovine (b) orthologues of human (h) caspase-4, respectively. Caspase-12 has two

forms: long (L*) and short (S*) forms in humans, but only long in rodents. Caspase-14 is expressed in skin epidermis and has a role in cornification. Apoptosis executioner caspases-3, -6, and -7 lack N-terminal interaction domains, whereas apoptosis initiator caspases possess N-terminal interaction domains of either DED (Caspase-8 or -10) or CARD (caspase-2 or -9) types, to mediate dimerization and/or recruitment into larger complexes for their activation. Caspase-8 mainly mediates the extrinsic pathway (at the cell membrane) and caspase-9 the intrinsic pathway (at the mitochondrion, involving mitochondrial outer membrane permeabilization [MOMP]) of activation of apoptosis. Caspase-2 can be activated upstream (CARD-mediated) or downstream of MOMP and can be recruited to the PIDDosome multiprotein complex (including RAIDD & PIDD). As well as apoptosis, some caspases can be activated during pyroptosis (macrophage death after shigella or salmonella infection, involving caspase-1 or -11) and autophagy-associated cell death (involving ATG proteins, beclin-1, and several caspases -2, -3, -6, -8). N-terminal sequence p20 Linker p10 Active sites Fig. 3.6.3 Schematic diagram of caspase activation. The proenzyme on the left is processed by cleavage to the active form shown on the right: the N-terminal sequence and the linker are lost and two pairs of p10 and p20 subunits combine, each contributing to the active sites of the enzyme.

 concentrations. These cytoskeletal proteolytic events probably contribute to the rounded shape of apoptotic bodies and to the eventual dissolution of the nuclear envelope. Caspases and DNA damage and repair ICAD (inhibitor of caspase-activated DNase) is a cytoplasmic chap- erone that binds a double-strand DNase, CAD (caspase-activated DNase). The ICAD–CAD complex is normally cytoplasmic. ICAD, however, is a caspase substrate and once cleaved ceases to chaperone CAD, which unfolds, displaying a nuclear localizing signal. Once within the nucleus, CAD initiates the digestion of DNA, first to large fragments of around 50 kilobase pairs and eventually—through cleavage of chromatin at internucleosomal sites—to a series of fragments that are multiples of the 180- to 200-base pair unit wrapped around each nucleosome. The genesis of these DNA fragments is ex- ploited in several cytological and electrophoretic methods for iden- tifying apoptosis. DNA-PK, ATM, PARP, and Rad51 are all DNA repair proteins concerned with the recognition and response to double-strand DNA breaks. Significantly, all are cleaved in apoptosis at sites that separate their DNA-binding and catalytic domains, thus removing their ability to repair DNA. This may be important in preventing re-ligation of the heavily digested DNA of the apoptotic nucleus, so avoiding the generation of large numbers of undesirable recom- binant DNA molecules. Caspases and cell-cycle proteins Unexpectedly, several proteins that normally inhibit movement around the cell cycle are targets for caspase cleavage. These include p21CIP1 and p27KIP1 (inhibitors of cyclin-dependent kinases that catalyse movement through the G1-S and G2 phases of the cell cycle), WEE-1 (which blocks movement from G2 to mitosis), and CDC27 (which inhibits entry into mitosis). The purpose of this potential reactivation of cell-cycle activity during the process of death is ob- scure. It occurs during the apoptosis of cells such as neurons that have long since ceased movement around the cycle. Nonapoptotic roles of caspases It is becoming clear that caspases play a multitude of roles in addition to those in apoptosis. Caspase 1, for example, is key to processing interleukin-1β in inflammatory responses, and several others are involved in inflammation (Table 3.6.1). Even the executioner caspases of apoptosis have additional nonapoptotic roles. Thus, caspases 3 and 8 play important, nonapoptotic roles in immune regulation. It seems that caspases are, perhaps unsurprisingly, highly pleiotropic proteins playing roles in cell proliferation, differentiation in a range of developmental and adult tissue contexts. In this way they may be regarded as important cell-fate decision-making enzymes con- tributing to

the fundamental processes of cell population expansion, specialization, and death. The activation of apoptosis Two pathways converge on and activate the effector or executioner caspases. One connects extracellular cytokine-based stimuli to the caspase cascade, through death-signalling receptors on the cell sur- face, and is often referred to as the extrinsic pathway. The other, termed the intrinsic pathway, links the caspase cascade to a great variety of signals from the cell interior, reflecting dysfunction in me- tabolism, genotoxic injury, hypoxia, and the status of the cytoskel- eton. Both pathways may be triggered by physiological as well as pathological stimuli. Death-signalling receptors coupled to apoptosis The death-signalling receptors are all members of the tumour ne- crosis factor alpha (TNFα) receptor family. They are type 1 mem- brane receptors (that is, with the N terminus on the external surface), containing a series of cysteine-rich incomplete repeats in the ligand-binding domain, a single transmembrane domain, and a cytoplasmic moiety with one or more signalling domains (Fig. 3.6.4). Their ligands are homologues of the cytokine TNFα. The prototype death-signalling receptor is Fas (also called CD95 or Apo-1). On binding its ligand, FasL, this receptor trimerizes and immediately recruits to its cytoplasmic moiety a cluster of proteins collectively called the death-inducing signalling complex (DISC). The aggregation of DISC proteins is the result of protein–protein interaction at an α-helical region called the death domain (DD). Through their DDs, Fas interacts with an adapter protein called FADD (Fas-associated protein with death domain) that contains a further interactive region called DED (for death effector domain). Through DED, FADD recruits procaspase 8 to the DISC, an initi- ator caspase with two DEDs in its N-terminal sequence. Because they are at high local concentration in the DISC, the procaspase 8 molecules can catalyse their own activation, and so initiate the proteolytic cascade that ultimately turns on the effector caspases. While Fas is widely expressed in many tissues, FasL expression is largely restricted to cytotoxic lymphocytes and to cells in immuno- logically privileged sites. In this way, the Fas/FasL system plays a major role in cell killing by cytotoxic T lymphocytes (CTLs) but can repulse CTLs at immunologically privileged sites. TNFR1, the high-affinity TNFα receptor, also trimerizes on binding its ligand TNFα, but the downstream pathways are more di- verse than those of Fas. Three types of protein complex form around the cytoplasmic moiety of the activated TNF receptor. Each initially comprises a basic DISC that includes a DD-containing adapter pro- tein called TRADD (for TNF receptor-associated death domain protein), a threonine kinase called RIP (for receptor interacting protein), and a third protein, TRAF-2 (for TNF receptor associated factor). From this common origin, three types of protein complex develop, each responsible for a different pattern of signal transduc- tion (Fig. 3.6.4 and Box 3.6.1). DR3 is a receptor closely similar in structure to TNFR1 but with a narrower tissue distribution. Whereas TNFR1 is ubiquitous, DR3 is expressed predominantly in the lymphocytes of spleen, thymus, and peripheral blood. Interestingly, the expression of the ligands ap- pears to adopt the opposite pattern, with TNFα being a product pre- dominantly of activated macrophages and lymphocytes, whereas the DR3 ligand (variously also called Apo3L and TWEAK) is expressed in many tissue types. DR4 and DR5 are similar receptors that bind a ligand called TRAIL (TNF-related apoptosis-inducing ligand). The downstream signalling appears to involve both FADD and caspase 8. Both TRAIL and its receptors are expressed in many tissue types. TRAIL has excited attention as a potential therapeutic agent because it is

 frequently cytotoxic to tumour cells under conditions in which normal cells are unharmed. Variant receptors that lack the cyto- plasmic signalling moieties (e.g. DcR1, Fig. 3.6.4) are expressed in many normal tissues and appear to act as inhibitory decoys for TRAIL. Mitochondrial signals coupled to apoptosis The mitochondrial pathway depends upon the

release of cytochrome c, together with deoxyATP (dATP), from the intermembranous space of mitochondria. Cytochrome c and dATP bind to and ef- fect a conformational change in a protein of the outer mitochon- drial membrane, APAF-1 (for apoptotic protease activating factor), so that it exposes a protein-binding domain (generically called a CARD, for caspase-activating recruitment domain) capable of re- cruiting and activating procaspase 9. This molecular assembly has been called the apoptosome. Caspase 9 then activates the execu- tioner caspases. Triggers for the release of cytochrome c include reactive oxygen species, cellular redox stress, and proteins of the BCL-2 family (Fig. 3.6.5). BCL-2 is a protein with a C-terminal hydrophobic domain that allows it to anchor to the outer mitochondrial membrane. It was first identified because of its consistent activation (through a chromo- some translocation) in follicular B-cell lymphoma. Its major physio- logical role is that of a survival factor, and thus it can cooperate with other oncogenes during carcinogenesis to sustain the life of clones of cells that otherwise might be deleted by apoptosis. The mammalian BCL-2 family contains at least 15 members in three major branches, distinguished on the basis of their function, which may facilitate ei- ther survival or apoptosis, and the presence or absence of certain conserved BCL-2 homology domains, called BH1 to BH4 (Fig. 3.6.6). Among the prosurvival molecules are BCL-2 itself, BCL-xL, RIP TNFα TNFR1 D C B A TNFR1 TRADD FADD NFκB JNK/p38 life death FasL TRAIL DcR1 FADD TRADD TRAF 2 D C B A E TNFR1 TRADD FADD NFκB JNK/p38 death life AIP ASK1 ASK1 AIP RIP caspase 8 caspase 8 death Fas Fig. 3.6.4 Death-signalling receptors. The Fas receptor, with its ligand (FasL) and DISC, signalling exclusively to death, is shown in (A). The more complex TNFα receptor 1 (TNFR1) is shown in (B), (C), and (D). One outcome of activation of this receptor (shown diagrammatically in B) signals for survival through the transcription factor NF-κB, by a RIP kinase dependent pathway. Another, (C), dependent upon recruitment of ASK-1 to the DISC, activates the Jun kinase/p38 pathway and can support either survival or apoptosis, depending on cell type and conditions. The third (D) requires internalization of the receptor, and activates caspase 8 through protein–protein interaction between TRADD and FADD. (E) shows DcR1, one of the decoy receptors for TRAIL (TNF-related apoptosis-inducing ligand). This receptor has no membrane anchor and so competes for TRAIL with the death-signalling membrane receptors, DR4 and DR5. Box 3.6.1 TNFα signalling When TNFα binds to its receptor, three pathways with different out- comes may be activated. • First, the basic DISC—comprising TRADD, RIP, and TRAF2—may dir- ectly recruit regulatory elements of the MAP kinase pathway, leading to activation of the transcription factor NF-κB and a set of pro-survival, NF-κB-dependent events. • Second, and apparently following internalization of the receptor and its DISC, TRADD can recruit FADD and hence procaspase 8 or 10, so providing the means of activating apoptosis. • Third, activation of the TNF receptor can lead to the dissociation from it of a protein called AIP (for ASK-interacting protein). While it is bound to the TNF receptor AIP is in an inactive, folded form, but on release it unfolds, becomes phosphorylated by RIP, and contributes to a new signalling complex comprising TRAF-2, RIP, AIP, and ASK-1. ASK-1 (for apoptosis signal-regulating kinase, also called MAP3K5) is an upstream kinase in the MAP kinase cascade and ultimately directs the activation of JNK and p38 kinase, as will be discussed later. Thus, activation of the TNF receptor may induce survival or apoptosis, depending on the cell type and local environmental conditions.

 BCL-w, MCL-1, and A1, all of which share all four BH homology do- mains. In contrast, BAX and BAK form a branch of the BCL-2 family that possesses BH3, BH2, and BH1 domains but exerts pro-death functions The third—and still expanding—family branch consists of proapoptotic proteins whose sole region of homology with the others is a single BH3 domain

(amounting to no more than 9–16 amino acids): BID, BAD, BIK, BIM, BNIP3, NOXA, PUMA, BMF, HRK, and Mule/ARF-BP1, as well as others. The BH1, BH2, and BH3 domains of the pro-survival family members together form a hydrophobic groove into which BH3 do- mains of the BH3-only proteins and the multidomain proapoptotic proteins can fit, in much the same way as a ligand binds to its re- ceptor. Such binding prevents the oligomerization of BAX and BAK and in so doing neutralizes their proapoptotic functions. However, in the presence of the 'BH3-only' family members, most of which bind to the hydrophobic cleft with high affinity, BAX and BAK are displaced from the groove to form homo-oligomeric permeability pore structures that lodge in the outer mitochondrial membrane, creating there the conditions that permit efflux of cytochrome c and dATP (see Box 3.6.2) and hence procaspase 9 activation, as previ- ously described. An alternative scenario suggests that the BH3-only proteins may bind to the hydrophobic groove of BAX or BAK, so catalysing their oligomerization directly. The BH3-only, proapoptotic proteins play important roles in coupling the powerful mitochondrial pathway to a broad variety of stimuli—physiological and pathological—in the cellular envir- onment (Fig. 3.6.5). Notably, BID is activated through cleavage by caspase 8 of a small peptide from its N terminus. The truncated BID, now activated, translocates from the cytosol to mitochondria and effects the mitochondrial release of cytochrome c. In this way, stimuli emanating from cytokine receptors but too small to activate the effector caspases directly can be amplified by recruitment of the mitochondrial pathway. Put another way, activation of BID lowers the threshold at which cytokines trigger apoptosis. Somewhat simi- larly, BAD is involved in a mechanism to raise the threshold at which apoptosis is engaged, depending on the availability of cyto- kine growth factors. BAD is phosphorylated by the kinases AKT (protein kinase B) and RSK, both in turn dependent on PI3 kinase and the growth factors responsible for its activation. Normally, phosphorylated BAD is sequestered in the cytoplasm by the chap- erone 14-3-3. In conditions of growth factor deprivation, however, unphosphorylated BAD becomes available, translocates to the mito- chondria, and activates cytochrome c release. BNIP3 is a mitochon- drial protein that accumulates under conditions of hypoxia. It may thus provide a trigger linking hypoxia to apoptosis. Normally, BIM binds to the light chain of dynein and BMF to myosin V, cytoskeletal inactive BAX BCL2 activated BAX BAX complex with BCL2 APAF-1 caspase 9 silenced by IAP APAF-1 caspase 9 active cytochrome c dATP BCL2 BH3-only complex with BCL2 SMAC Loss of contact DNA damage Oncogene overdrive BMF Ca flux UV Cytokine deprivation Fas/TNFαR stimulation BID tBID BIM ARF-BP1 ARF p53 NOXA PUMA BAD BAX oligomer permeability pore hypoxia BCL2 BCL2
Fig. 3.6.5 A summary of the processes involved in intrinsic pathway activation involving caspase 9, and the BCL-2 family proteins. BAX, activated in the cytoplasm, translocates to the surface of mitochondria where it initially binds to BCL-2 (or combinations of BAX or BAK with BCL-2 or BCL-xL). Excess BAX forms BAX-BAX homo-oligomers that generate a permeability pore and are responsible for the release of cytochrome c and dATP. Alternatively, BAX may be displaced from BCL-2 by competition with the BH3-only proteins. Caspase 9 complexed with APAF-1 is activated by cytochrome c and dATP released from the intermembranous space of the mitochondrion, but this activation can be inhibited by IAPs. Release of SMAC from the intermembranous space blocks this inhibition. The roles of several BH3-only proteins (purple boxes) in connecting the apoptotic machinery to a variety of stimuli are also shown, including the caspase-8-mediated cleavage of BID to truncated BID (tBID) which links extrinsic pathway activation involving Fas/TNFR1 to intrinsic pathway activation.

 proteins that appear to generate signals relating to microtubule in- tegrity and cell attachment, respectively. The transcription of PUMA and NOXA is

directly dependent on p53, hence providing a link be- tween nuclear DNA damage and apoptosis. Mule/ARF BP1 is a ubi- quitin ligase that targets for proteasomal destruction the cell-cycle regulator CDC6. This has the effect of arresting the cell cycle, but as discussed next, can also initiate apoptosis. There is also specifi- city as to which members of the pro-survival BCL-2 family proteins are targeted by individual BH3-only proteins: whereas tBID, BIM, and PUMA bind to all five pro-survival BCL-2 family proteins, the others have more limited affinities. In this way the BH3-only pro- teins provide a summation of injury and physiological death signals from all over the cell, and translate that, in a cell-type-dependent manner, to the final decision between life and death. Mitochondria are not unique among cellular organelles in pro- viding the location for procaspase-containing protein complexes whose activation is affected by BCL-2 family members. Procaspase 2 can be found in the nucleus and Golgi apparatus of some cells. BCL- 2 is present on nuclear and endoplasmic reticulum membranes. Activated BAX locates to endoplasmic reticulum as well as to mito- chondria. Hence, multiple organelles may contribute to the execu- tion of apoptosis as well as the audit of its initiating stimuli. Apoptosis and cell stress The question arises how apoptosis relates to the other molecular mechanisms whereby cells respond to stresses of various kinds. Injured cells activate stereotyped reactions, of which the heat shock response, the unfolded protein response, the stress-activated kinase response, the metabolic response, and the DNA damage response are of particular relevance here. BCL-2 BCL-XL BCL-w BAX MCL-1 BAK BID BAD BIK BIM BNIP3 NOXA PUMA ARF-BP1 (a) (b) (c) TM BH2 BH1 BH3 BH4 Fig. 3.6.6 Examples of the human BCL-2 family, showing schematically the relative positions in the unfolded protein of the BCL-2 homology domains (BH1–4), and the transmembrane domain (TM). (a) Four pro-survival members. The orientation of the BH domains in A1 is similar, but this protein lacks a transmembrane domain. (b) The two major multidomain proapoptotic proteins, BAX and BAK. (c) Some BH3-only proteins. Note that although some of these possess transmembrane domains, the great majority have only the BH3-homology domain in common, and they differ greatly in total size. Thus, PUMAα (the longest splice-variant isoform of the PUMA gene) has 193 amino acids, while ARF-BP1 has 4374, and hence is not drawn to scale relative to the others. Box 3.6.2 Mitochondrial outer membrane permeabilization There has been controversy over the precise mode of action of BAX and BAK in effecting the release of cytochrome c and dATP from mitochon- dria. Under normal conditions, there is an electrical potential across the mitochondrial membrane (ΔΨm) sustained by proton pumping. Immediately prior to apoptosis, ΔΨm dissipates abruptly, associated with mitochondrial outer membrane permeabilization (MOMP) or de- polarization, suggesting unselective passage of ions. MOMP has been described as the point of no return or commitment to execution of apop- tosis. One possibility is that osmotic expansion of the inner mitochon- drial compartment could lead to rupture of the outer membrane and hence escape into the cytoplasm of cytochrome c and dATP. However, direct experiments with artificial reconstructions of lipid bilayers and super resolution microscopy show that homo-oligomers of BAX can insert directly into such membranes, creating grommet-like channels, rings, and arcs, through which large molecules can move and the col- lapse of ΔΨm is secondary to the appearance of such channels. These permeability pores permit release of cytochrome c and dATP from the mitochondrial intermembranous space, with binding to the apoptosome and activation of caspase 9.

 The heat shock response Heat shock proteins (HSPs) are molecular chaperones of diverse molecular weight that share the property of greatly enhanced tran- scription following cell stress. Thermal, osmotic, and redox stress, ultraviolet and ionizing radiation all may induce HSP transcription. The heat shock response sustains cell survival under adverse circum-

stances and inhibits apoptosis in several different ways: Hsp27 in- hibits caspase 8 cleavage of BID, and hence the release of cytochrome c from mitochondria; Hsp40 and Hsp70 inhibit BAX translocation to mitochondrial membranes; Hsp70 and Hsp90 may dissociate the components of the apoptosome. Presumably, each cell has a threshold at which full activation of caspases and entry to apoptosis become inevitable. The HSPs raise that threshold, but little is known of how the threshold itself is defined in the first place. The unfolded protein response (UPR) This regulates the rate of protein synthesis so that correct folding and export from the endoplasmic reticulum occur. Without the UPR, in- soluble aggregates of misfolded protein begin to accumulate in the ER, a manifestation of 'ER stress'. In summary, the UPR is initiated by three receptor proteins—PERK, ATF6, and IRE-1. PERK is a kinase, and responds to the presence of misfolded protein by inhibiting (by phosphorylation) the translation initiation function of eIF2. ATF6 is a transcription factor, and migrates to the nucleus where it stimulates the transcription of chaperone proteins GRP78, GRP94, and XBP1. IRE-1 is a dual function serine-threonine kinase and ribonuclease. It splices XBP1 mRNA to generate a further transcription factor for chaperones. However, the UPR has a clearly recognizable boundary at which its function changes from cytoprotective to proapoptotic. On prolonged stimulation, certain specific proteins are translated at high abundance, despite the general inhibition of eIF2. Among them is a protein called CHOP that lowers the cell's apoptosis threshold by inhibiting transcription of BCL-2. Further, IRE-1 forms an acti- vating complex with TRAF-2 and ASK-1, proapoptotic elements of the JNK/p38 kinase pathway to be described next. The stress-activated kinase response The MAP kinases are serine-threonine protein kinase cascades, described in detail elsewhere in this textbook (see Chapter 3.5). Activation of these cascades is initiated by phosphorylation of regu- latory upstream members, MAP kinase kinases (MAP3Ks), and leads ultimately to activation of transcription factors. Two of the three major sets of mammalian MAP kinase cascades are directly involved in transduction of stimuli that lead to apoptosis: the p38 kinases often being part of a stress-related proapoptotic process, the JNK kinases sometimes, depending on local circumstances. That these kinases have a role in the activation of apoptosis is clearly demonstrated by the attenuation of apoptosis in cells from appropriate knockout ani- mals, but how and why these roles are played have proved more dif- ficult to define. The stress kinase cascades appear to engage with the apoptosis effectors in several different ways. Thus, they activate (by phosphorylation) p53, CHOP, and several BH3-only proapoptotic BCL-2 family members, inactivate (again by phosphorylation) BCL-2 and BCL-XL, and activate the transcription of Fas ligand, all processes that lower the threshold for apoptosis. By stimulating cell- cycle movement through transcriptional activation of c-MYC, under conditions in which cycle movement is blocked (e.g. by p53) they also promote apoptosis, as described next. ASK-1 is a significant up- stream MAP3K connecting the relevant environmental stimulus to the stress kinase cascades. ASK-1 is itself activated by reactive oxygen species, as it is normally bound in inactive conformation by the redox sensor thioredoxin. In the presence of a strong oxidative environ- ment, thioredoxin dissociates, ASK-1 is activated, and the JNK/p38 cascades are stimulated. ASK-1 is also activated as part of a complex with TRAF-2 following TNF receptor stimulation and in the UPR. The metabolic response The close association between the mitochondrion and the regulation of apoptosis, along with the long-standing supposition that meta- bolic stresses are signalling routes to apoptosis, indicate a close as- sociation between apoptotic and metabolic circuitries. Perhaps the best example of the closeness of this association at the molecular level is cytochrome c, whose prototypic function is in the production of mitochondrial ATP during oxidative phosphorylation. As just de- scribed, cytochrome c is also essential for the initiation of apoptosis via the intrinsic pathway. Additional links between metabolic inter- mediates and regulated cell death processes

are provided by ATP, acetyl-CoA, NAD+, NADP+ and reactive oxygen species. Although the details are not yet clear, it seems that ‘metabolic checkpoints’ exist in order to determine whether a cell responds to metabolic im- balances by eliciting an appropriate adaptive response or, alterna- tively, by signalling its own demise. The DNA damage response Damage to nuclear DNA is a particularly important source of injury- related stimuli for caspase activation. Separate molecular mechan- isms exist for responding to the presence of inappropriately inserted bases (base excision repair), nucleotides that have become modified through intrastrand cross-linking or the formation of covalently bound adducts (nucleotide excision repair), nucleotide mismatch, insertion-deletion loops, or abnormal methylation (mismatch re- pair), interstrand cross-links (Fanconi repair) and double-strand breaks (homologous recombination or nonhomologous end- joining). In mismatch repair, MSH2 and MLH1 are recruited sequen- tially into a molecular complex at the injury site, which activates p53, effects cell-cycle arrest, and, in the meantime, initiates repair at the site of damage. Similarly, among the first molecules to bind to DNA double-strand breaks in nonhomologous end-joining are the DNA kinases ATM, ATR, and DNA-PK. In turn, these recruit and activate p53 and other molecules (e.g. CHK-1 and CHK-2). In surviving cells, these effect arrest at a variety of points around the cell cycle, so en- suring that there is opportunity to load the repair machinery on to the damaged DNA template before this is further altered by DNA replication (in S-phase) or chromatid separation (in mitosis). A profoundly different means of limiting the effect of genome damage, however, is to commit the damaged cell to apoptosis. Elements such as p53 within the repair complex in both mismatch repair and nonhomologous end-joining can do this. The molecular basis for the decision between apoptosis or survival with repair is still largely unknown. Activation of p53 is common to both outcomes, and it is therefore reasonable to search in and around this molecule for clues to the nature of the life or death decision. Activated p53 alters the transcription of a large number of genes. Some are well- known inhibitors of cell-cycle progression, such as p21CIP1, but others (e.g. BAX, Fas, a membrane protein called PERP, and the BH3-only

 proapoptotic molecules NOXA and PUMA) are associated almost exclusively with apoptosis. A further transcriptional target of p53 is the nontranslated microRNA miR-34 (see Box 3.6.3), activation of which is associated with both cell-cycle arrest and apoptosis. The situation is further complicated by the fact that p53 also signals to the apoptosis effector process by nontranscriptional means, via an N-terminal sequence that does not appear to be instrumental in ef- fecting cell-cycle arrest. Phosphorylation provides one of the critical signals for p53 activation, and there are several different phos- phorylation sites that respond preferentially to the various kinases (including Jun kinase, as mentioned earlier). Thus, the precise phos- phorylation status of p53 could provide a molecular signature indi- cative of the nature, and perhaps the outcome, of the DNA damage. Another potential factor in controlling the outcome of DNA injury is a kinase (called DAP kinase because it was originally discovered as a death-associated protein) that influences the selection of p14ARF rather than p16INK4A—alternative splice forms from the same gene. Whereas p16INK4A is a cell-cycle regulator, inhibiting the cyclin-dependent kinases, p14ARF displaces p53 from its inhibitor, MDM2, so generating a sustained p53 signal that may favour apoptosis. Further evidence for integration of multiple factors in the response to double-strand DNA breaks comes from detailed study of the in- jured nucleus. Within an hour of DNA damage, large complexes of phosphorylated proteins form around the damaged site, including ATM, p53, many repair proteins and the phosphorylated histone γH2AX. Within a few hours, these foci come to lie in close juxtapos- ition with pre-existing intranuclear bodies called PML bodies, into which p53 and many other proteins in the DNA damage response are recruited. Nuclei

without PML mount only an attenuated version of the expected p53-dependent apoptotic response to DNA damage, even though p53 itself is available. One explanation for this is that the PML body is an intracellular location for the activation of p53 protein by acetylation. The PML body may thus form the local envir- onment in which the state of the injured DNA is evaluated and final decisions made regarding the ultimate fate of the cell. The replicative status of the cell is a further important deter- minant of its sensitivity to apoptosis following DNA injury. The proto-oncogene c-MYC is normally among the earliest genes to be expressed when cells are stimulated by growth factors to leave quiescence and enter the replicative cycle. Paradoxically, however, c-MYC expression is also a powerful factor lowering the threshold for apoptosis. In particular, c-MYC expression without concurrent molecular evidence of external growth factor stimulation (such as phosphatidylinositol-3 (PI3) kinase and AKT activation) is inter- preted as a death signal. Similarly, other early regulators of cell-cycle entry, including inhibition of function of the retinoblastoma protein and the release of the transcription factor E2F-1 from its binding pocket, also trigger apoptosis in the absence of concurrent evi- dence of external mitogenic stimulation. Perhaps this represents a means whereby tissues are protected from autonomous cell replica- tion: survival of replicating cells is made conditional on the presence of appropriate stimuli in the cellular environment. The benefits of re- moving cells that show a tendency for such replicative autonomy are obvious, but the precise mechanism that couples replication to death except in acceptable circumstances is far less clear. It seems probable that the cell cycle itself includes checkpoints at which the decision to engage the apoptosis machinery can be taken should any of the appropriate conditions for replication be absent. Indeed, it is pos- sible that injured cells may force the activation of such checkpoints as one way to access their apoptosis programme. This might explain the paradoxical activation of cyclin-dependent kinases by caspases in cells such as neurons that normally do not engage in replicative cycles at all, as mentioned earlier. Inhibitors of caspase activation The role of the BCL-2 family proteins in the activation and inhib- ition of apoptosis has been described, but there are other powerful endogenous inhibitors of caspase-associated cell death. One is FLIP, a DED-containing version of procaspase 8 that lacks caspase activity. High local concentrations of FLIP compete with procaspase 8 for re- cruitment into the DISC and so inhibit further propagation of death signals originating from the TNF family of receptors. IAPs (inhibitors of apoptosis proteins) inhibit caspase activity after autocatalytic processing of the procaspase has begun. All con- tain an element called a BIR domain, which binds to the N-termini of the short fragment of partially processed caspases in such a way that adjacent elements of the IAP molecule drape across the caspase active site and sterically hinder substrate attachment. There are sev- eral such proteins—IAP1 and IAP2, ILP, the neuronal NAIP, and an X-linked family member X-IAP, all of which possess several BIR do- mains, and LIVIN and SURVIVIN, which contain a single BIR do- main. One manifestation of the importance of IAPs is the presence of an IAP inhibitor, variously called SMAC or DIABLO, which is re- leased from mitochondria along with cytochrome c during caspase activation by the mitochondrial pathway. The inhibitor SMAC has an N-terminal sequence that competes with partially processed caspase for the binding site in the BIR domain, and so allows the caspase to escape from the inhibitory embrace of the IAPs. The IAPs provide a further example of the interconnections be- tween the cell cycle and cell death. SURVIVIN, apparently associ- ated with caspase 9, forms a complex with and is phosphorylated by active CDK1 (cyclin-dependent kinase-1) during mitosis. Loss of phosphorylation leads to dissociation of the SURVIVIN–caspase 9 heterodimer, activation of caspase 9, and apoptosis. As normal mi- tosis proceeds, SURVIVIN associates with kinetochore proteins, the spindle microtubules, and finally, at cytokinesis, with the mid-body. Complexes of SURVIVIN with cyclin-dependent kinases active earlier in the cycle (e.g. CDK4) have also been

identified and promote transit through G1. Thus, SURVIVIN may form part of a regulatory network, providing a means whereby the threshold for apoptosis is varied through the cell cycle. Finally, IAPs are multifunctional Box 3.6.3 MicroRNA MicroRNAs (miRNA) are a family of short RNA species that are not trans- lated but exert profound influence over the patterns of translation. They bind to regions of sequence homology in the 3' untranslated regions of messenger RNA, inhibiting translation and creating double-stranded RNA that becomes a target for digestion by double-strand RNA spe- cific ribonucleases called argonaute proteins. There appear to be only a few hundred distinct types of miRNA, each capable of inhibiting its own spectrum of specific messenger RNA types. Hence altered patterns of miRNA transcription can swiftly alter the overall pattern of messenger RNA that is available for translation. Certain miRNA profiles are charac- teristic of some types of cancer.

276 SECTION 3 Cell biology proteins: they are themselves potential substrates of caspase attack, activators of the survival factor NF-κB, and downstream products of NFκB-directed transcription. They thus form part of positive- feedback systems for both survival and death. Recognition of apoptotic cells The rapid clearance of apoptotic cells requires that they are efficiently sensed by phagocytes at an early stage. Engulfment by juxtaposed neighbours (including ‘nonprofessional’ phagocytes) does not re- quire a migratory response on the part of the phagocyte. By con- trast, ‘find me’ signals released by apoptotic cells induce chemotactic migratory responses in mononuclear (‘professional’) phagocytes. These chemoattractant molecules encompass lipid, protein, and nu- cleotide moieties (Fig. 3.6.7) and are released from apoptotic cells via cleavage and channel-activating events, at least some of which are caspase-dependent. For example, extracellular ATP acts as a po- tent ‘find me’ signal following release from apoptotic cells through caspase-3-activated pannexin 1 channels. Phagocyte Chemotaxis “Find me” signals released CD31 CD31 CD47 ‘Don’t eat me’ signals lost Phosphatidylserine exposure MFG-E8 Gas6 or Protein S Exposed sugars Phagocyte lectins ANTI-INFLAMMATORY & REPARATORY RESPONSES ENGULFMENT BAI1 Tim-4 Stablin-2 Int egrins TAM-RTK SCARF1 LRP1 CD14 Integrins CD36 SIRP1 Phagocyte Apoptotic Cell Apoptotic cell TSP CRT C1q Fig. 3.6.7 Clearance of apoptotic cells by phagocytes. Apoptosis elicits plasma membrane changes—‘eat me’ signals—notably exposure of phosphatidylserine, that permit interaction with multiple receptors of phagocytes either directly (e.g. TIM-4) or via bridging molecules (e.g. Gas6 or Protein S bridging to the Tyro-Axl-Mer receptor tyrosine kinases, TAM-RTK). Receptor-ligand interactions lead to engulfment as well as anti-inflammatory and repair mechanisms. Interaction is also dependent upon inhibition of ‘don’t eat me’ signals. In the case of professional phagocytes, engulfment is preceded by sensing of chemotactic molecules (‘find me’ signals), released by apoptotic cells—see inset.

3.6 Apoptosis in health and disease 277 Macrophages subsequently recognize and bind to the surface of apoptotic cells by virtue of multiple molecular ‘eat me’ signals (Fig. 3.6.7). The disposition of phosphatidylserine (PS) residues on the apoptotic cell surface is one of the most characteristic of these. Normally PS appears only on the inner leaflet of the cell membrane, but this strict polarity is lost very early in apoptosis: around the time of rounding up, substantially earlier than chromatin condensation and DNA cleavage. PS exposure requires the caspase-mediated in- activation of ATP11C, a member of the P4-ATPase family, which, in viable cells, acts as a PS ‘flippase’, maintaining the phospholipid’s asymmetric distribution on the inner plasma membrane leaflet. In concert, the PS scramblase, Xkr8 is activated by caspase cleavage during apoptosis to promote PS externalisation. Macrophages pos- sess multiple receptors that bind to the exposed PS

residues. The ex- posed PS may also bind to ‘bridging’ molecules in the extracellular environment that then form linkers to receptors on macrophage sur- faces. Thus MFG-E8 helps bind PS on the apoptotic cell surface to β3 and β5 integrins on the macrophage surface. Gas6 and protein S similarly bridge PS to the TAM (Tyro-Axl-Mer) family of receptor tyrosine kinases (TAM-RTK). Other bridging molecules include the complement fragment iC3b, which links to macrophage β2 integrins, thrombospondin 1, which links to β3 integrins and CD36, whereas the near-ubiquitous extracellular molecule β2 glycoprotein-1 links to a macrophage receptor specific for it. In the same way, extracel- lular complement component C1q links specific binding sites on the apoptotic cell surface to receptors on the macrophages. Along with the macrophage tethering receptor CD14 (whose binding moieties have not been clearly established), a group of scavenger receptors (SRA, CD36, CD68, LOX-1) may tether directly to poorly defined oxidized lipid groups (similar to those in oxidized low-density lipo- proteins) exposed on the surfaces of apoptotic cells. Endogenous macrophage surface lectins also bind to sugars (such as N-acetyl glu- cosamine) selectively exposed on apoptotic cell membranes. These multiple mechanisms that facilitate macrophage phagocyt- osis of apoptotic cells ensure that degradation of dying cells does not usually occur before they are securely engulfed within the phago- somes of the ingesting cells. Presumably this forestalls innate and acquired immune reactions to intracellular proteins, or the voiding into extracellular space of potentially recombinogenic and immuno- genic fragments of genomic DNA. A distinctive feature of macrophage binding to apoptotic cells is the concurrent effect on macrophage function. Macrophages that phagocytose particles opsonized by immunoglobulin or comple- ment component C3b effect a sharp increase in oxygen usage (the respiratory burst), generate reactive oxygen species and nitric oxide, and release of inflammatory cytokines such as TNFα. These recruit other acute inflammatory cells to the site. In contrast, macrophages that ingest apoptotic bodies show suppression of pro-inflammatory responses, mediated through the release of different cytokines, such as TGFβ. The basis of these contrasting effector responses appears to be the different signalling pathways that are activated by the macro- phage receptors engaged by apoptotic bodies as opposed to opson- ized particles. Responses to apoptotic cells are not limited either to those of macrophages or to anti-inflammatory effects (although the latter are the most renowned). In certain situations, such as during de- velopment or as a consequence of wounding or radiotherapy, apop- tosis elicits compensatory proliferative responses of neighbouring cells. Apoptotic cells can also engender angiogenic responses. These effects suggest that apoptosis can promote tissue repair and regen- eration. Furthermore, although apoptosis is generally regarded as a tolerogenic process, chemotherapy-induced apoptosis by anthracyclines and oxaliplatin can be immunogenic, an effect that is mediated by dendritic cells which engulf apoptotic tumour cells and activate T cells to stimulate adaptive antitumour immunity. Are caspases necessary and sufficient for cell death? Although caspase activation plays a dominant role in the effector phase of apoptosis, it is not responsible for all the phenomena of apoptosis. For example, developmentally programmed cell death can sometimes occur on schedule in embryonic tissues in which caspases have been inhibited, or key members of the caspase activation system (such as APAF-1) rendered deficient through germline gene knockout. In all these circumstances, the morphology of the caspase- free death is not that of apoptosis. The nuclei swell rather than under- going chromatin condensation. The cytoplasm shows signs of fluid overload, sometimes with the formation of conspicuous fluid-filled vacuoles. Some of these changes are reminiscent of necrosis rather than apoptosis. Rather similar changes take place during the devel- opmental death of phylogenetically ancient multicellular organisms that do not possess recognizable close homologues to the caspases, such as the slime mould Dictyostelium discoides. These observations suggest that

caspase activation, although in- trinsic to the subtle and highly coordinated death process recognized as apoptosis, may not be the only event that commits cells to death. The existence of at least one caspase-independent death pathway is highlighted by a flavoprotein released from the mitochondria of in- jured cells called AIF (apoptosis-inducing factor). AIF translocates to the nucleus, where it can effect chromatin cleavage to large frag- ments, but not the extreme condensation observed in apoptosis. It also appears to reproduce the cellular volume overload described earlier, even in the presence of caspase inhibition. Phylogenetically close homologues of AIF are found in bacteria and plants as well as invertebrate and vertebrate animals. Apoptosis and disease There are few disease processes in which apoptosis does not feature, but the examples that follow are chosen because they exemplify how various steps in the apoptosis pathways may be critical for, or are subverted in, the course of disease pathogenesis. Immunity and its disorders Apoptosis is used extensively in the normal function of the immune system to facilitate the process of clonal selection. Antigen stimu- lation of T-cell proliferation is usually followed by expression of both Fas and FasL, a recipe for apoptosis on a grand scale (called activation-induced cell death, AICD) unless there is rescue by a sur- vival stimulus. This can be provided by costimulation from the im- mediate environment—adhesion molecules or cytokine receptors. A particularly important route for costimulation is through CD28, a receptor on T cells for signals transmitted from antigen-presenting

278 SECTION 3 Cell biology cells, which increases the expression of several cytokines and their receptors. Similarly, clonally expanded populations of stimulated B cells in the bone marrow or those undergoing affinity maturation in lymph-node follicle centres are deleted by Fas signalling, but can be selectively rescued by costimulation through CD40. Cytotoxic T lymphocytes kill their targets by delivering to them the contents of their granules. Among these are perforin, which cre- ates regions in the target-cell membrane of enhanced permeability at the points of contact with the CTL, and granzyme B, a protease that directly activates the caspases of the target cell. In this way, CTLs induce target-cell apoptosis. The main effect of PD-1 signalling in T cells upon PD-L1/2 binding is usually functional inactivation rather than programmed cell death. The importance of apoptosis for the normal function of the im- mune system is underscored by the effects of genetic defects. Strains of mice with loss-of-function mutations in the genes encoding fas or fas ligand (called lpr and gld, respectively) show similar immuno- logical phenotypes, characterized by massive lymphoproliferation and autoimmune disorders. The human homologue is the rare condition of Canale–Smith syndrome (childhood autoimmune lymphoproliferative syndrome or ALPS) in which there is a muta- tion in the DD of Fas. Inherited deficiency in C1q also leads to an autoimmunity syndrome: affected individuals almost always de- velop systemic lupus erythematosus. The pathogenesis here appears to be ineffective recognition and phagocytosis of endogenous apop- totic cells, so that their intracellular antigens are inappropriately processed. In particular, the persistence of nondegraded DNA that results from failed clearance of apoptotic cells has fundamental im- portance for the development of autoimmune disease. Infective disorders Shigella dysentery is due to pathogenic strains of Shigella flex- neri. Pathogenicity is conferred by plasmid-borne genes that neu- tralize the primary host defence: phagocytosis and destruction of the bacteria by macrophages in the intestinal lamina propria. The plasmid-encoded protein Ipa B activates macrophage caspase 1, so annihilating the defence by inducing macrophage apoptosis. This strategy appears to be successful, because the bacterium that would normally be destroyed if it persisted within the phagosome of the ingesting macrophage can escape from the cytoplasm of macro- phages that undergo apoptosis. The initial response to Trypanosoma cruzi, the parasite

respon- sible for Chagas' disease, is dominated by T-lymphocyte activation. The resultant AICD generates a population of apoptotic lympho- cytes. These impinge upon the macrophages that, suitably armed by pro-inflammatory cytokine stimulation, would be one of the most effective elements in the host defence against the parasite. As de- scribed earlier, sustained macrophage phagocytosis of these large numbers of apoptotic cells leads to suppression of pro-inflammatory cytokine release. The parasite subverts this aspect of the physiology of apoptosis into a source of protection from the host-defence reaction. The intracellular parasite chlamydia makes a protein (CPAF) that comprehensively targets BH3-only proapoptotic molecules for proteasomal destruction. This illustrates the value to the organism of keeping a live cell environment around it, but also provides vivid affirmation of the key role played by BH3-only proteins in activating apoptosis. Viruses engage with the machinery of apoptosis in many ways. Even lytic viruses have strategies designed to conserve the life of their host cells for some time. DNA viruses, in particular, require means to abort apoptosis, as they must activate the cellular DNA synthesis machinery in order to replicate their own genomes, yet must then avoid the apoptosis that would otherwise follow DNA synthesis unaccompanied by commensurate external stimuli. The E6 gene of high-risk human papillomaviruses (HPV) 16 and 18 en- codes a protein that targets p53 for ubiquitination and subsequent degradation, and so permits cellular survival as the viral E7 protein binds Rb and initiates entry into S-phase. The transforming genes of adenoviruses pair up to effect rather similar outcomes: E1A binds Rb and initiates DNA synthesis, the 55-kDa subunit of E1B binds and inhibits p53, and the 19-kDa subunit neutralizes proapoptotic members of the BCL-2 family. Human herpesviruses such as HHV8 encode their own version of FLIP (v-FLIP). They also have their own pro-survival BCL-2 family members, such as BHRF1 in the Epstein– Barr virus (EBV) and KS-BCL2 in HHV8. The HHV8 strategy is particularly subtle, because the virus also destroys the endogenous BCL-2. Unlike endogenous BCL-2, this viral surrogate lacks an in- ternal caspase site, and cannot be converted into a killer peptide by caspase cleavage. Baculovirus encodes a 35-kDa protein with BIR domains that is a prototypical IAP. Apoptosis plays a key role in the pathogenesis of AIDS. The pro- gressive loss of circulating CD4+ T cells, by which the course of HIV- 1 infection to clinical AIDS can be charted, involves loss of numbers of cells that are several orders of magnitude greater than the numbers that ever carry the virus. It is therefore clear that the overwhelming majority of the dying cells must be bystanders, sensitized to apop- tosis by the presence of infection but not infected themselves. Viral proteins released from infected cells effect this sensitization by sev- eral parallel routes. The HIV proteins Tat and Nef induce Fas, FasL, and TRAIL. Tat alters the cellular redox equilibrium in a manner that may activate ASK-1. Vpr binding protein modulates p53 in- duced apoptosis. A type of AICD may be induced by stimulation of CD4 and the cytokine receptor CXCR4 (both of which bind HIV epitopes). In infected cells, however, Nef inhibits ASK-1, and so may selectively protect these from apoptosis. Rather similar mechanisms underlie the deletion of neurons in HIV-associated dementia. Cardiovascular disease Pathogenetic mechanisms that interface with apoptosis are relatively poorly understood in cardiovascular disease, but there are several observations of potential relevance. Laminar flow inhibits ASK-1 in endothelium, while the generation of reactive oxygen species in- duces the p38 and JNK stress kinase pathways. Thus, turbulence and the presence of generators of reactive oxygen species such as oxi- dized low-density lipoproteins—both known risk factors in the gen- esis of atheroma—are liable to promote apoptosis in endothelium. Other elements of the vascular wall are also abnormal in atheroma. Vascular smooth muscle cells from atheromatous vessels express p53, induce Fas, and undergo apoptosis in increased numbers, par- ticularly in the shoulders of the plaque, thus weakening attachment of the fibrous cap and rendering plaque rupture more probable. Macrophages also

undergo apoptosis in response to the oxidized lipids that are present in atheromatous plaques. Death of the lipid- filled macrophages (foam cells) produces extracellular depots of oxi- dized lipid in the plaque core, a key step in plaque progression.

3.6 Apoptosis in health and disease 279 Although necrosis is the pattern of the cell death that immedi- ately follows episodes of infarction, there is now substantial evidence that apoptosis occurs in the surrounding tissue over several hours thereafter, probably in response to relative ischaemia and the local generation of reactive oxygen species. In animal models of stroke, this apoptosis can be down-regulated by a variety of manoeuvres, including caspase inhibition, with objective evidence of improved cerebral function. These observations have generated enthusiasm for the development of antiapoptotic drugs for use following stroke and myocardial infarction. Another approach, potentially applicable to ischaemic myocardium, is to promote angiogenesis, perhaps by the use of angiogenic stem cells. Experimental models suggest that this improves the remodelling of the peri-infarct tissue, including decreased apoptosis of myocytes and improved cardiac function. Degeneration of the central nervous system Despite the importance of the subject, there is still much doubt over the role of apoptosis in the chronic degenerative disorders such as Alzheimer’s and Parkinson’s diseases. Much of the problem stems from the relative inaccessibility of the brain for sequential studies following injury. In both conditions there is clear evidence of a loss of neurons, and those that remain accumulate abnormal cytoplasmic material, such as presenilins 1 and 2, and amyloid protein Aβ in Alzheimer’s dis- ease. Cell culture and animal models suggest that the presence of these proteins may induce oxidative stress, which can lower the threshold for apoptosis. The protective effect of BCL-2 and caspase inhibition has also been recorded. The difficulties are compounded by the fact that neurons that undergo severe overstimulation (e.g. by local high con- centrations of the neurotransmitter glutamate) can also be induced to die (a phenomenon called excitotoxicity), but it is not clear whether the pathways involved overlap with or are identical to those of apoptosis. Tumour biology Apoptosis is of significance in cancer biology for several reasons. First, carcinogenesis is almost invariably associated with escape from mechanisms that normally activate apoptosis. Second, a large component of tumour regression following therapy is attributable to apoptosis. Third, and perhaps most surprisingly, apoptosis harbours sinister, protumour properties. Carcinogenesis Carcinogenesis involves inappropriate cell proliferation, driven by release from tumour suppressor gene inhibition or by hyperactive oncogene expression. Under normal circumstances, however, the accelerated movement around the cell cycle renders the cells vulner- able to DNA damage, which activates p53 (the ‘DNA damage check- point’) and ensures either cessation of replication or apoptosis of the affected cells. For the inappropriately driven population to progress to tumour growth, the affected cells must silence this p53 response. This affords one reason for the frequent appearance of deletions and loss-of-function mutations of p53 in tumours, and the observation that the cells of many tumours and some premalignant (but pro- gressing) lesions appear to be in a perpetual state of uncompleted DNA repair. A more subtle mechanism couples inappropriate prolif- eration to activation of p14ARF. Uncoupling of this ‘oncogene check- point’ also permits the continuing replication of cells that would otherwise have been arrested in cell cycle or committed to apoptosis, as ARF has the effect of increasing the half-life of p53 thus activating the p53 pathway. Suppression of these pathways in the early genesis of tumours has the effect of permitting repeated escape from the DNA damage or oncogene checkpoints and so giving cancer cells the op- portunity to explore the consequences of further genomic rearrange- ments or mutations that are denied normal cells. Some of these prove incompatible with continuing life but

others lead to selective, pro- gressive growth advantage towards malignancy (Fig. 3.6.8). Tumour regression These considerations have an important bearing on therapeutically induced tumour regression. Many therapeutic agents are effective because they create DNA lesions that activate a DNA damage check- point. However, as discussed earlier, most if not all tumours will already be derived from clones of cells that have lost critical damage- or oncogene-activated checkpoints. If these happen to be the same as is targeted by the therapeutic agent, there is a high likelihood that the tumour will be resistant to the agent. Further, animal experiments have tested the effect on tumour behaviour of selective restoration of p53 function. Significantly, although regression was initiated almost immediately, tumour regrowth often occurred, accompanied by loss of function in the tumour cells of either ARF or the restored p53. The immediately effected regression of these tumours demonstrates that the downstream effectors of the p53 pathway are still intact in these tumours, and still capable of response to p53 when it is provided. However, the swift tumour recurrence also shows that single-agent therapy has a high chance of failure: the tumour's genomic instability leads to rapid selection of alternative resistant clones. Genotoxic damage Injured but surviving NORMAL APOPTOSIS TUMOUR CLONE DSB p53+ p53− Fig. 3.6.8 Failure to activate apoptosis following DNA damage by genotoxic carcinogens, because of the absence of functional p53, leads to the inappropriate survival of clones of cells bearing double-strand breaks (DSB) and illegitimate recombination events. Although some of these clones may fail to proliferate further (purple bars), others survive to become the founder clones of tumours. Constitutionally, these survivors have unstable genomes, as on further exposure to similar genotoxic stimuli they may again undergo genomic rearrangements or other forms of mutation yet fail to enact apoptosis. Although the example given is for cells lacking normal p53, and hence unable to respond appropriately to DNA DSBs, similar mechanisms apply to cells that fail to identify nucleotide mismatches through defective DNA mismatch repair (mutated/inactivated MSH2 or MLH1), or fail to repair DNA interstrand cross-links due to inactivation of the Fanconi DNA repair pathway or other DNA repair pathways (e.g. inactivated BRCA1 and BRCA2). Such cells can tolerate and survive extensive DNA damage and have very high mutation rates.

280 SECTION 3 Cell biology Protumour properties Finally—and on the face of it counterintuitively—apoptosis has protumour properties. True to their description as 'wounds that fail to heal', malignant tumours hijack innate host responses to cell death such as compensatory proliferation and angiogenesis to ensure sus- tained net growth. It is notable that high-grade tumours display high apoptotic indices alongside their high mitotic indices. The main cause of such high constitutive apoptosis is likely to be the out-pacing of oxygen and nutrient supplies through rapid proliferation leading to microenvironmental stress. However, the apoptotic portion of the tu- mour cell population helps to perpetuate and progress the malignant disease through multiple mechanisms. Perhaps the most important of these is the regulation of tumour-associated macrophages, cells of the tumour stroma which tend to be associated with poor prog- nosis. At least in some tumours, apoptosis drives the accumulation of tumour-associated macrophages displaying protumour activities, including stimulation of angiogenesis and inhibition of antitumour immunity. These along with possibly additional 'regenerative' prop- erties of apoptotic tumour cells are likely to be important underlying causes of relapse following apoptosis-inducing cancer therapies. FURTHER READING Adams J, Cory S (2007). The Bcl-2 apoptotic switch in cancer develop- ment and therapy. Oncogene, 26, 1324–37. Anwar S, Whyte MK (2007). Neutrophil apoptosis and infectious dis- ease. Exp Lung Res, 33, 519–28. Czabotar PE, et al. (2014). Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nat Rev Mol Cell Biol, 15, 49–63. Feig C, Peter ME (2007). How apoptosis got the immune system in shape. Eur J

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