

# 4

## Apoptosis and Cell Death

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### Introduction

Cell death plays a central role in multicellular organisms during their early development in sculpting the body parts and in their adult life by controlling cell numbers (homeostasis), and cell death further protects the organism overall by removing all cells damaged by disease, aging, infection, genetic mutation, and exposure to toxic agents. Kerr et al<sup>1</sup> coined the term “apoptosis,” referring to a particular morphology of physiological cell death involving cell shrinkage, nuclear condensation, membrane blebbing, and cellular and nuclear fragmentation into membrane-bound apoptotic bodies (Fig. 4.1). The lipid changes that occur in the membrane eventually lead to phagocytosis of the apoptotic cellular fragments. Often apoptosis is used synonymously with programmed cell death, implying that death results from the regulated activation of a preexisting death program that is encoded in the genome. The first evidence of a genetic program that orchestrates physiological cell death came from the developmental studies of the nematode *Caenorhabditis elegans*.<sup>2</sup> Our knowledge of cell death and the mechanisms of its regulation increased dramatically in the past two decades with the discovery of several death genes in *C. elegans*<sup>3</sup> and their counterparts in mammals. Now it is clear that apoptotic cell death has important biological roles not only in development and homeostasis but also in the pathogenesis of several disease processes. Dysregulation of apoptosis is found in a wide spectrum of human diseases, including cancer, autoimmune diseases, neurodegenerative diseases, ischemic diseases, and viral infections.<sup>4</sup> Interestingly, death programs other than apoptosis with different morphological features were also considered in controlling the cell numbers. These death programs could be directed by the condemned cell itself or by neighboring cells, with or without the help of humoral factors.

### Alternative Forms of Cell Death

Previously, cell death was broadly categorized into only two distinct types: apoptosis and necrosis. However, it has become increasingly evident that such a categorization is an oversimplification. There are 12 different types of cell death that have been described in the literature, which can be grouped into only five major types: apoptosis, necrosis, autophagy, paraptosis, and autschizis. Other forms of death could be classified under one of these headings. For example, anoikis and oncosis are forms of apoptosis (triggered by cell detachment) and necrosis, respectively. Because of overlapping and shared signaling pathways among different death programs, it is difficult to provide exclusive definitions for each one of these cell death programs. However, there is evidence that inhibition of one form of cell death may lead to another. Although the term “programmed cell death” was used specifically to describe apoptosis, other forms of cell death may also fall into this category, if gene activation is required for their execution.

### Necrosis

Necrosis results from a variety of accidental and lethal actions by toxins or physical stimuli or in association with pathological conditions, such as ischemia. Necrosis is characterized by cellular edema, dissolution of nuclear chromatin, disruption of the plasma membrane, and release of intracellular contents into the extracellular space, resulting in inflammation (see Fig. 4.1). In contrast, membrane damage occurs very late in the apoptotic process, where dead cells are engulfed by neighboring cells or phagocytes, leading to little or no inflammation (see Fig. 4.1). Although necrosis has mostly been regarded as an accidental form of cell death, more recent

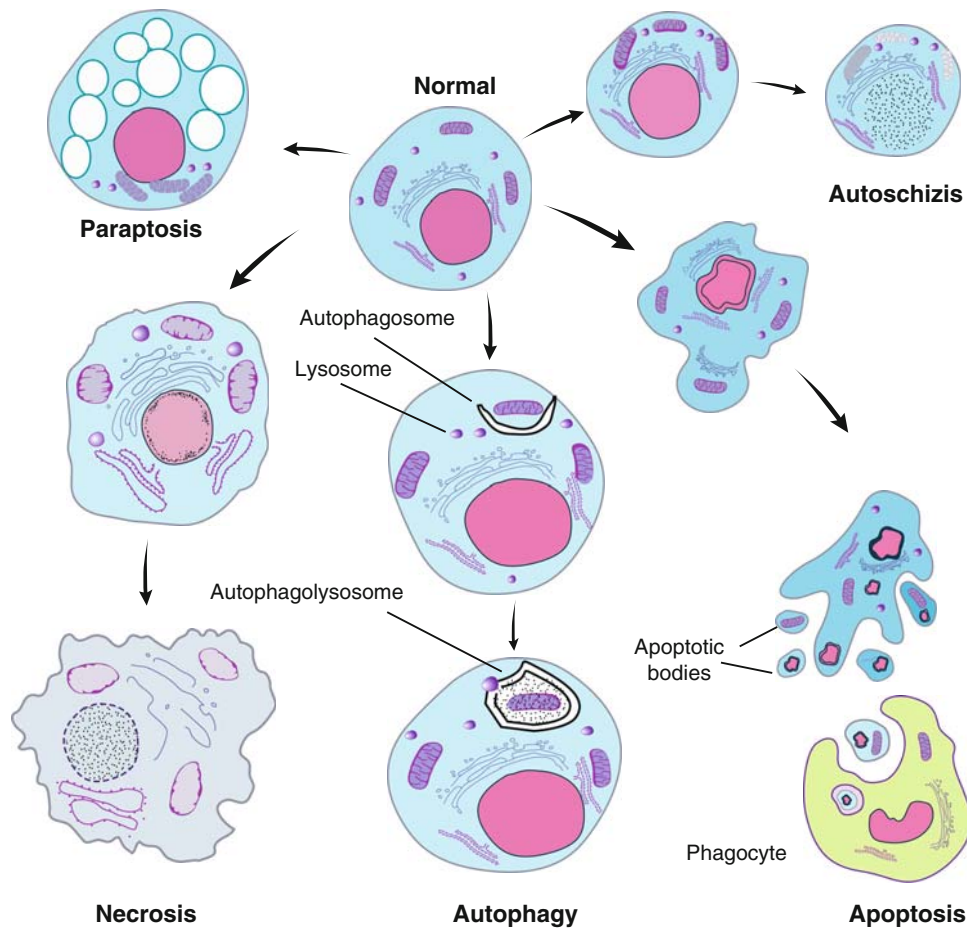


FIG. 4.1. Morphological features of cell death. Necrosis: Cells die by necrosis, and their organelles are characteristically swollen. There is early membrane damage with eventual loss of plasma membrane integrity and leakage of cytosol into extracellular space. Despite early clumping, the nuclear chromatin undergoes lysis (karyolysis). Apoptosis: Cells die by type I programmed cell death (also called apoptosis); they are shrunken and develop blebs containing dense cytoplasm. Membrane integrity is not lost until after cell death. Nuclear chromatin undergoes striking condensation and fragmentation. The cytoplasm becomes divided to form apoptotic bodies containing organelles and/or nuclear debris. Terminally, apoptotic cells and fragments are engulfed by phagocytes or surrounding cells. Autophagy: Cells die by type II programmed cell death, which is characterized by the accumulation of autophagic vesicles (autophagosomes and autophagolysosomes). One fea-

ture that distinguishes apoptosis from autophagic cell death is the source of the lysosomal enzymes used for most of the dying cell degradation. Apoptotic cells use phagocytic cell lysosomes for this process, whereas cells with autophagic morphology use the endogenous lysosomal machinery of dying cells. Paraptosis: Cells die by type III programmed cell death, which is characterized by extensive cytoplasmic vacuolization and swelling and clumping of mitochondria, along with absence of nuclear fragmentation, membrane blebbing, or apoptotic body formation. Autoschizis: In this form of cell death, the cell membrane forms cuts or schisms that allow the cytoplasm to leak out. The cell shrinks to about one-third of its original size, and the nucleus and organelles remain surrounded by a tiny ribbon of cytoplasm. After further excisions of cytoplasm, the nuclei exhibit nucleolar segregation, and chromatin decondensation followed by nuclear karyorrhexis and karyolysis.

data have suggested that necrosis can also occur as a programmed form of cell death. There is growing evidence that necrotic and apoptotic forms of cell death may share some similarities.<sup>5</sup> Nevertheless, necrosis has been shown to occur in cells having defective apoptotic machinery or upon inhibition of apoptosis,<sup>6</sup> and this form of cell death is emerging as an important therapeutic tool for cancer treatment.<sup>7</sup> Some forms of cell death may have a mixture of morphological features of both necrosis and apoptosis and are referred to as "aponecrosis."<sup>8</sup>

## Autophagy

Autophagy, which is referred to as "*macroautophagy*" or *type II programmed cell death*,<sup>9</sup> is characterized by sequestration of cytoplasm and organelles by double or multimembrane structures called *autophagic vacuoles*, followed by degradation of the contents of these vacuoles by fusing to the cell's own lysosomes (see Fig. 4.1). Although the precise role of autophagy in cell death is not clear, yet it has long been regarded as a cell survival mechanism whereby starving

cells produce energy-generating basic molecules from complex polymers such as proteins, lipids, polysaccharides, and nucleic acids through sequestration and degradation of some of the cytoplasm and organelles. In this regard, it is argued that autophagy may help cancer cells to survive under nutrient-limiting and low-oxygen conditions and against ionizing radiation.<sup>10,11</sup> However, recent observations that there is decreased autophagy during experimental carcinogenesis and heterologous disruption of *Beclin 1* (Atg6),<sup>12–14</sup> an autophagy gene, in breast and ovarian cancers suggest that breakdown of autophagic machinery may contribute to development of cancer.<sup>15</sup> In addition, Atg6 heterozygous mice develop tumors spontaneously, suggesting tumor suppressor activity of Atg6.<sup>12</sup> Similarly, mice deficient in another autophagy gene, Atg4C, a cysteine protease, are susceptible to chemical carcinogenesis.<sup>16</sup> However, the mechanism of how autophagy suppresses tumorigenesis is still unclear. Other studies have shed some light on the relationship between autophagy and apoptosis. For example, reduction of *Atg7* and *beclin 1* inhibited zVAD-induced death in human U937 cells<sup>17</sup> and knockdown of *Atg5* and *beclin 1* protected Bax/Bak double-knockout cells from staurosporine- or etoposide-induced nonapoptotic death.<sup>18,19</sup> However, these studies were done in cells whose apoptotic pathways had been compromised. Thus, it remains to be seen whether cells with intact apoptotic machinery can also die by autophagy and whether apoptosis-competent cells lacking autophagy genes will be resistant to other death stimuli. In the in vivo setting, the complicated relationship between autophagy and apoptosis needs to be clarified.

Because some autophagic cells would undergo caspase-independent gene-activated cell death but not display any of the characteristic ultrastructural features of apoptosis including DNA laddering, autophagy is still considered as programmed cell death. Similar to apoptosis, cells do require de novo gene expression with an increase in the expression of the ubiquitin-like gene.<sup>20,21</sup> The ubiquitin-like protein conjugation system and formation of protein complexes that direct membrane docking and fusion of the lysosomes and vacuoles are main components of autophagy. Although the molecular details are still being elucidated, this process appears to be regulated by various kinases, phosphatases, and guanosine triphosphatases (GTPases).<sup>22,23</sup>

## Paraptosis

Paraptosis was recently described as a form of cell death characterized by extensive cytoplasmic vacuolation (see Fig. 4.1) involving swelling of mitochondria and endoplasmic reticulum. This form of cell death was not inhibited by the caspase inhibitors but is inhibited by translation and transcription inhibitors, cycloheximide and actinomycin D, suggesting a requirement for new protein synthesis.<sup>24</sup> Paraptosis has been shown to be triggered by the tumor necrosis factor (TNF) receptor family TAJ/TROY and insulin-like

growth factor I receptor. TAJ/TROY-induced paraptotic cell death was enhanced by overexpression of programmed cell death 5 (PDCD5).<sup>25</sup> This form of cell death was shown to be mediated by mitogen-activated protein (MAP) kinases and inhibited by AIP1/ALIX, a protein interacting with the calcium-binding death-related protein ALG-2.<sup>26</sup> Inhibition by Na<sup>+</sup>/H<sup>+</sup> exchanger also led to cell death resembling paraptosis.<sup>27</sup> Recently, glucocorticoids were shown to induce retinal toxicity by mechanisms associated with paraptosis.<sup>28</sup> Although this form of cell death has been shown to be evoked by a variety of reagents, the exact molecular mechanism is far from clear.

Cytoplasmic vacuolation, as seen in paraptosis, has been shown to occur in a wide range of cell lines either spontaneously or as induced by a variety of stimuli. The extent to which a cell becomes vacuolated depends on the cell type. The process of vacuolation seems to follow a definite pattern, with the vacuole number and size increasing gradually. Cells can recover from vacuolation up to a certain threshold, beyond which they succumb to death.<sup>29</sup> The presence of a definite underlying program that decides whether a cell undergoing cytoplasmic vacuolation should be destined to death is currently unknown. However, there are instances in which a decidedly vacuolated cell undergoes cell death, which suggests the existence of some underlying programs. During the salivary gland development in metamorphosis of blowfly larva (*Calliphora vomitoria*), cells are eliminated by a process of intensive vacuolation in a particular order.<sup>30,31</sup> Furthermore, in *Dictyostelium discoideum*, stalk cells undergo extensive cytoplasmic vacuolation while proceeding toward programmed cell death.<sup>32</sup> In addition, cytoplasmic vacuolation was also observed in aspirates of lobular and ductal breast carcinoma.<sup>33</sup> These studies further emphasize that cytoplasmic vacuolation-induced cell death has a physiological role and warrants further investigation. The most studied cytoplasmic vacuolation-induced cell death is autophagy.

## Autoschizis

A new form of cell death, which has been shown to be triggered by oxidative stress,<sup>34</sup> differs from apoptosis and necrosis and is characterized by reduction of cytoplasm to a narrow rim around the nucleus with chromatin marginating the entire nucleus from inside.<sup>35</sup> Mitochondria and other organelles aggregate around the nucleus as a consequence of cytoskeletal damage and loss of cytoplasm. Interestingly, the rough endoplasmic reticulum is preserved until the late stages of autoschizis, in which cells fragment and the nucleolus becomes condensed and breaks into smaller fragments.<sup>35</sup> The nuclear envelope dissipates eventually with the remaining organelles after cell death. In this type of death, cells lose cytoplasm by self-morsellation or self-excision (see Fig. 4.1). Autoschizis usually affects contiguous groups of cells both in vitro and in vivo but can also occasionally affect

scattered individual cells trapped in subcapsular sinuses of lymph nodes.<sup>36</sup>

## Apoptosis

Genetic studies in the nematode worm *C. elegans* led to the initial characterization of apoptosis. Activation of specific death genes during the development of this worm results in death of exactly 131 cells, leaving 959 cells intact.<sup>3</sup> Further studies revealed that apoptosis can be divided into three successive stages: (1) commitment phase, in which death is initiated by specific extracellular or intracellular signals; (2) execution phase; and (3) cleanup phase, in which dead cells are removed by other cells with eventual degradation of the dead cells in the lysosomes of phagocytic cells.<sup>37</sup> The apoptotic machinery is conserved through evolution from worm to human.<sup>38</sup> In *C. elegans*, execution of apoptosis is mediated by CED-3 and CED-4 proteins. Commitment to a death signal results in the activation of CED-3 by CED-4 binding. The CED-9 protein prevents activation of CED-3 by binding to CED-4.<sup>39</sup>

## Mechanisms of Apoptosis

The mechanisms of apoptosis are very complex and involve a cascade of energy-requiring molecular events. Past research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway, and the intrinsic or mitochondrial pathway. An additional pathway also operates during T-cell-mediated cytotoxicity involving perforin-granzyme-mediated cell killing. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A. All three pathways then converge on the same terminal execution pathway, which is initiated by the cleavage and activation of effector caspase-3 and -7, resulting in the degradation of cytoplasmic, cytoskeletal, and nuclear proteins, fragmentation of nuclear DNA, formation of apoptotic bodies (membrane blebbing), expression of cell-surface ligands for phagocytosis, and finally uptake by phagocytic cells.

## Caspases

The executioners in both intrinsic and extrinsic pathways of cell death are the caspases,<sup>40</sup> which are cysteine proteases with specificity to cleave their substrates after aspartic acid residues. The first member of the caspase family, caspase-1, was initially known as interleukin-1 $\beta$ -converting enzyme (ICE), an enzyme required for the maturation of IL-1 $\beta$ . Later, the *C. elegans* cell death gene *ced-3* was found to be similar to ICE and a developmentally regulated protein Nedd2 (now referred to as caspase-2). Subsequently, many caspase genes have been cloned from mammalian and non-mammalian sources. The central role of caspases in apopto-

sis is underscored by the observation that apoptosis and all classic changes associated with apoptosis can be blocked by inhibition of caspase activity. To date, 12 mammalian caspases (caspase-1 to -10, caspase-14, and mouse caspase-12) have been identified. Caspase-13 was later found to represent a bovine homologue, and caspase-11 appears to be a murine homologue of human caspase-4 and -5, respectively. These caspases have been broadly categorized into initiators (caspase-2, -8, -9, -10), effectors or executioners (caspase-3, -6, -7), and inflammatory caspases (caspase-1, -4, -5). While murine caspase-11 is reported to regulate apoptosis and cytokine maturation during septic shock, caspase-12 appears to mediate endoplasmic reticulum-specific apoptosis and cytotoxicity by amyloid- $\beta$ . Caspase-14 is highly expressed in embryonic tissues but not in adult tissues.<sup>41</sup>

Caspases are normally produced as inactive zymogens containing an N-terminal prodomain followed by a large and a small subunit that constitute the catalytic core of the protease. They have been categorized into two distinct classes: initiator and effector caspases. The upstream initiator caspases contain long N-terminal prodomains and one of the two characteristic protein-protein interaction motifs: the death effector domain (DED; caspase-8 and -10) and the caspase activation and recruitment domain (CARD; caspase-1, -2, -4, -5, -9, and -12). The downstream effector caspases (caspase-3, -6, and -7) are characterized by the presence of a short prodomain. Apart from the structural differences, a prominent difference between initiator and effector caspases is their basal state. Both the zymogen and the activated forms of effector caspases exist as constitutive homodimers, whereas initiator caspase-9 exists predominantly as a monomer both before and after proteolytic processing.<sup>42</sup> Initiator caspase-8 has been reported to exist in equilibrium between monomers and homodimers.<sup>43</sup> Although the initiator caspases are capable of autocatalytic activation, the activation of effector caspases requires formation of oligomeric complexes with their adapter proteins and often intrachain cleavage within the initiator caspase.

Caspases have also been divided into three categories based on substrate specificity. Group I members (caspase-1, -4, and -5) have a substrate specificity for the WEHD sequence with high promiscuity; group II members (caspase-2, -3, and -7 and CED-3) prefer the DEXD sequence and have an absolute requirement for aspartate (D) at P4; and members of group III (caspase-6, -8, and -9 and the "aspase" granzyme B) have a preference for the (I/L/V)EXD sequences. Several reports have suggested a role for group I members in inflammation and that of group II and III members in apoptotic signaling events.

The roles of various caspases in apoptotic pathways and their relative importance for animal development have been examined in genetic studies involving knockout of different caspase genes (Table 4.1). A caspase-1 (interleukin-1 $\beta$ -converting enzyme, ICE) knockout study suggested that ICE plays an important role in inflammation



TABLE 4.1. Summary of effects of loss of function of caspases in vivo.

Genotype	Phenotype
Caspase-1 <sup>-/-</sup>	Viable mice. No defects in cell death. Defects in pro-IL-1b and pro-IL-18 processing and resistant to lipopolysaccharide (LPS)-induced septic shock
Caspase-2 <sup>-/-</sup>	Viable and fertile mice. No defects in cell death except during development. Excess germ cells in the female mutant ovaries. Oocytes and neurons show resistance to apoptosis in vitro. Mutant mouse embryonic fibroblasts (MEFs) show some resistance to killing by heat shock and some drugs
Caspase-3 <sup>-/-</sup>	Postnatal death in mixed background mutants (129/SvJ and C57BL/6). Mice are viable in C57BL/6 background. Decreased apoptosis in brain
Caspase-7 <sup>-/-</sup>	Viable mice. Mild resistance to apoptosis in mutant MEFs
Caspase-3 <sup>-/-</sup> and caspase-7 <sup>-/-</sup>	Double-knockout (DKO) mice die perinatally. Exencephaly in about 10% of DKO embryos. Resistant to apoptosis. Defects in cardiac development. MEFs show resistance to apoptosis
Caspase-8 <sup>-/-</sup>	Embryonic lethal. Defects in cardiac and T-cell development. Impaired heart muscle development and congested accumulation of erythrocytes. Degeneration of yolk sac vasculature and circulatory failure. Defective hematopoietic progenitor function and macrophage differentiation. Mutant MEFs are resistant to death receptor-mediated apoptosis
Caspase-9 <sup>-/-</sup>	Perinatal lethal. Defective brain development associated with decreased apoptosis. Mutant MEFs are resistant to apoptosis
Caspase-11 <sup>-/-</sup>	Viable mice. Defects in IL-1 production. Defects in LPS-induced apoptosis
Caspase-12 <sup>-/-</sup>	Viable mice. Reduced endoplasmic reticulum (ER) stress-induced apoptosis. Improved bacterial clearance and resistance to sepsis

by activating cytokines such as IL-1 $\beta$  and IL-18. However, caspase-1 was not required to mediate apoptosis under normal circumstances and did not have a major role during development.<sup>44</sup> Surprisingly, ischemic brain injury was significantly reduced in caspase-1 knockout mice compared with wild-type mice,<sup>45</sup> suggesting that inflammation may contribute to ischemic injury. Caspase-3 deficiency leads to impaired brain development and premature death. Also, functional caspase-3 is required for some typical hallmarks of apoptosis such as formation of apoptotic bodies, chromatin condensation, and DNA fragmentation in many cell types.<sup>46</sup> Lack of caspase-8 results in the death of embryos at day 11 with abnormal formation of the heart,<sup>47</sup> suggesting that caspase-8 is required for cell death during mammalian development. In support of this finding, knockout of FADD, which is required for caspase-8 activation, resulted in fetal death with signs of abdominal hemorrhage and cardiac failure.<sup>48</sup> Moreover, caspase-8-deficient cells did not die in response to signals from members of the TNF receptor family.<sup>47</sup> However, cells lacking either FADD or caspase-8, which are resistant to TNF- $\alpha$ -mediated or CD95-mediated death, are susceptible to chemotherapeutic drugs, serum deprivation, ceramide,  $\gamma$ -irradiation, and dexamethasone-induced killing.<sup>48</sup> In contrast, caspase-9 has a key role in apoptosis induced by intracellular activators, particularly those that cause DNA damage. Deletion of caspase-9 resulted in perinatal lethality, apoptotic failure in developing neurons, enlarged brains, and craniofacial abnormalities.<sup>49</sup> In caspase-9-deficient cells, caspase-3 was not activated, suggesting that caspase-9 is upstream of caspase-3 in the apoptotic cascade. As a consequence, caspase-9-deficient cells are resistant to dexamethasone or irradiation, whereas they retain their sensitivity to TNF- $\alpha$ -induced or CD95-induced death<sup>49</sup> because of the presence of caspase-8, the initiator caspase involved in death recep-

tor signaling that can also activate caspase-3. Overall, these observations support the idea that different death signaling pathways converge on downstream effector caspases (Fig. 4.2). Indeed, caspase-3 is regarded as one of the key executioner molecules activated by apoptotic stimuli originating either at receptors for exogenous molecules or within cells through the action of drugs, toxins, or radiation.

A cascade of caspases plays the central executioner role by cleaving various mammalian cytosolic and nuclear proteins that play roles in cell division, maintenance of cytoskeletal structure, DNA replication and repair, RNA splicing, and other cellular processes. This proteolytic carnage produces the characteristic morphological changes of apoptosis. Once the caspase cascade is initiated, the process of cell death has crossed the point of no return.

### Extrinsic Death Pathway

The extrinsic pathway involves binding of death ligands such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), CD95 ligand (Fas ligand), and TNF-related apoptosis-inducing ligand (TRAIL) to their cognate cell-surface receptors TNFR1, CD95/Fas, TRAIL-R1, TRAIL-R2, and the DR series of receptors,<sup>50</sup> resulting in the activation of initiator caspase-8 (also known as FADD-homologous ICE/CED-3-like protease, or FLICE) and subsequent activation of effector caspase-3 (see Fig. 4.2).<sup>51</sup> The cytoplasmic domains of death receptors contain the “death domain,” which plays a crucial role in transmitting the signal from the cell’s surface to intracellular signaling molecules. Binding of the ligands to their cognate receptors results in receptor trimerization and recruitment of adapter proteins to the cell membrane, which involves homophilic interactions between death domains of the receptors and the adapter proteins. The adapter protein for the receptors TNFR1 and DR3 is TNFR-associated death domain protein

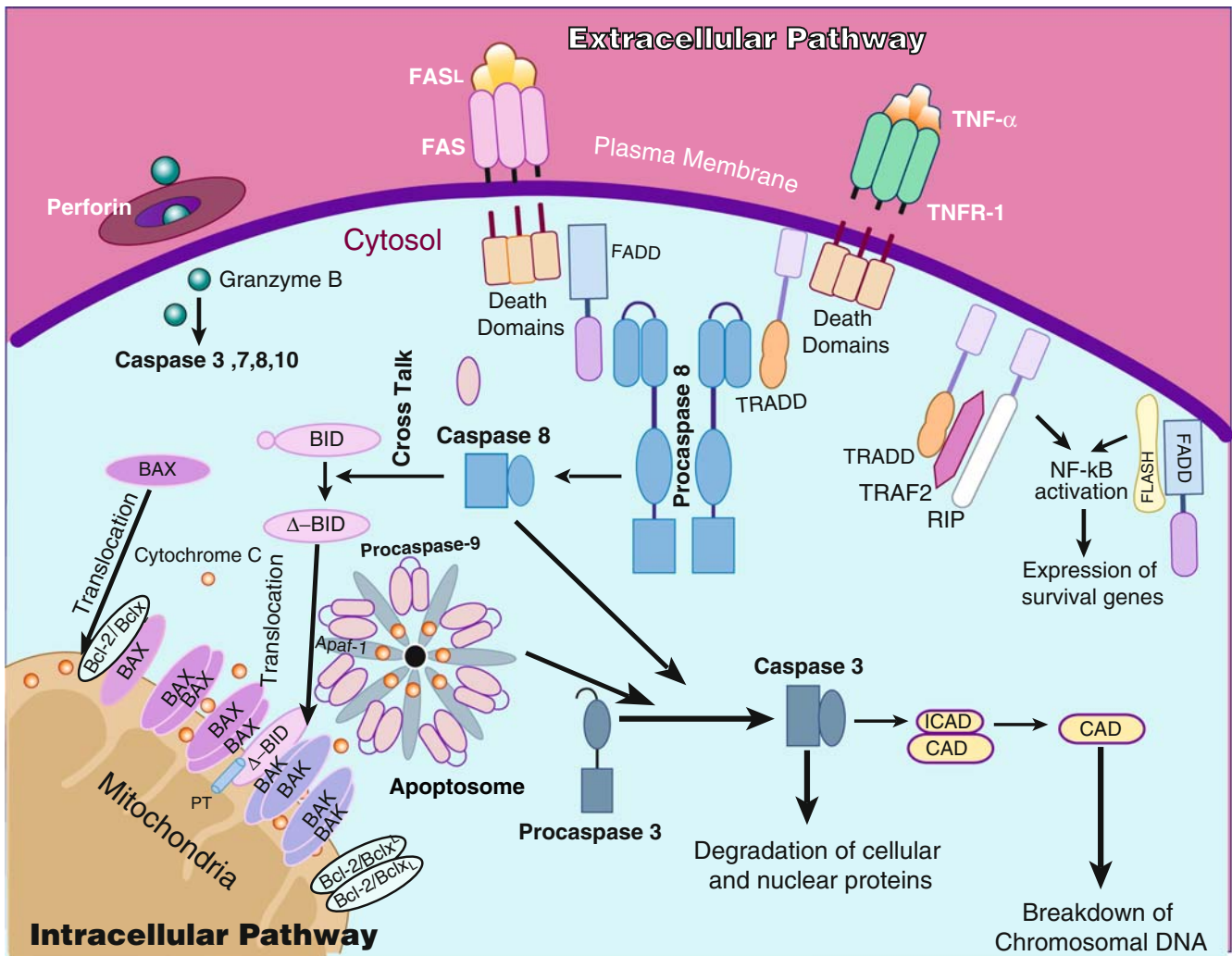


FIG. 4.2. Schematic representation of apoptotic signaling pathways. **Extracellular Pathway:** Following the binding of peptides such as tumor necrosis factor (TNF)- $\alpha$  or Fas ligand (FASL), the receptors oligomerize and recruit adapter proteins [Fas-associated death domain (FADD), tumor necrosis factor receptor (TNFR)-associated death domain (TRADD)] to form death-inducing signaling complexes, causing the activation of the initiator caspase-8, which sequentially activates effector caspases (e.g., caspase-3). Other adapter proteins (FLASH), inhibitory proteins (FLIP), or proteins involved in survival pathways as well as death mechanisms (receptor interaction protein, RIP) may participate in complex mechanisms that determine life or death. The TNF- $\alpha$ -TNFR1 complex can also elicit an antiapoptotic response by recruiting TRAF2, which results in NF- $\kappa$ B-mediated upregulation of antiapoptotic genes. In cytotoxic T-lymphocyte-induced death, granzyme B, which enters the cell through membrane channels formed by the protein perforin, activates caspases by cleaving them directly or indirectly. **Intracellular Pathways:** Lack of survival stimuli (withdrawal of growth factor, hypoxia, genotoxic substances, etc.) is thought to generate apoptotic signals through poorly defined mechanisms, which lead

to translocation of proapoptotic proteins such as Bax to the outer mitochondrial membrane. In some cases, transcription mediated by p53 may be required to induce proteins such as Bax. Translocated Bax undergoes conformational changes in the outer membrane to form oligomeric structures (pores) that leak cytochrome *c* from mitochondria into the cytosol. Formation of a ternary complex of cytochrome *c*, the adapter protein Apaf-1, and the initiator caspase-9 called the "apoptosome" results in the activation of caspase-9, followed by sequential activation of effector caspase(s) such as caspase-3 and others. The actions of caspases, endonucleases, and possibly other enzymes lead to cellular disintegration. For example, the endonuclease CAD (caspase-activated DNase) becomes activated when it is released from its inhibitor ICAD upon cleavage of ICAD by an effector caspase. Antiapoptotic proteins such as Bcl-2 and Bcl-xL inhibit the membrane-permeabilizing effects of Bax and other proapoptotic proteins. Cross-talk between extra- and intracellular pathways occurs through caspase-8-mediated Bid cleavage, which yields a 15-kDa protein that migrates to mitochondria and releases cytochrome *c*, thereby setting in motion events that lead to apoptosis via caspase-9.

(TRADD)<sup>52</sup> and that for Fas, TRAIL-R1, TRAIL-R2, and DR4 is Fas-associated death domain protein (FADD).<sup>53</sup>

The receptor–ligand and FADD complex in turn recruit caspase-8 to the activated receptor, resulting in the formation of death-inducing signaling complex (DISC) and subsequent activation of caspase-8 through oligomerization and self-cleavage. Depending on the cell type and/or apoptotic stimulus, caspase-8 can also be activated by caspase-6.<sup>54</sup> Activated caspase-8 then activates effector caspase-3. In some cell types, cleavage of caspase-3 by caspase-8 also requires a mitochondrial amplification loop involving cleavage of proapoptotic protein Bid by caspase-8 and its translocation to the mitochondrial membrane, triggering the release of apoptogenic proteins from mitochondria into cytosol (see Fig. 4.2). In these cell types, overexpression of *Bcl-2* and *Bcl-xL* can block CD95-induced apoptosis.<sup>55</sup>

Tumor necrosis factor- $\alpha$  is produced by T cells and activated macrophages in response to infection. Although TNF- $\alpha$ -mediated signaling can be propagated through either TNFR1 or TNFR2 receptors, the majority of biological functions are initiated by TNFR1.<sup>56</sup> Binding of TNF- $\alpha$  to TNFR1 causes release of inhibitory protein silencer of death domain protein (SODD) from TNFR1, which enables recruitment of adapter protein TRADD. Signaling induced by activation of TNFR1 or DR3 diverges at the level of TRADD. In one pathway, nuclear translocation of the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) and activation of c-Jun N-terminal kinase (JNK) are initiated, which results in the induction of a number of proinflammatory and immunomodulatory genes.<sup>57</sup> In another pathway, TNF- $\alpha$  signaling is coupled to Fas signaling events through interaction of TRADD with FADD.<sup>58</sup> The TNFR1–TRADD complex can alternatively engage TRAF2 protein, resulting in activation of transcription factor c-Jun, which is involved in survival signaling. Furthermore, binding of receptor interaction protein (RIP) to TNFR1 through TRADD results in the activation of transcription factor NF- $\kappa$ B, which suppresses apoptosis through transcriptional upregulation of antiapoptotic molecules such as TRAF1, TRAF2, cIAP1, cIAP2, and FLIP. The FLICE-associated huge protein (FLASH) was initially identified to be a CED-4 homologue interacting with the DED of caspase-8 and was shown to modulate Fas-mediated activation of caspase-8.<sup>59</sup> FLASH is also required for the antiapoptotic effects of TNF that occur in some cells, where it participates in the activation of the NF- $\kappa$ B transcription factor.<sup>60</sup> Another class of protein, FLIP (FLICE inhibitory protein), was shown to block Fas-induced and TNF- $\alpha$ -induced DISC formation and subsequent activation of caspase-8.<sup>61</sup>

Cytotoxic T cells play a major role in vertebrate defense against viral infection.<sup>62</sup> They induce cell death in infected cells to prevent viral multiplication and spread of infection.<sup>63</sup> Cytotoxic T cells can kill their targets either by activating the Fas ligand/Fas pathway or by injecting granzyme B, a serine protease, into target cells. Cytotoxic T cells carry Fas ligand on their surface but also carry granules containing the channel-forming protein perforin and granzyme B.

Upon recognizing the infected cells, the lymphocytes bind and secrete granules onto the surface of infected cells. Perforin then assembles into transmembrane channels to allow the entry of granzyme B into the target cell. Upon entry, granzyme B, which cleaves after aspartate residues in proteins (“aspase”), activates one or more of the apoptotic proteases (caspase-2, -3, -7, -8, and -10) to trigger the proteolytic death cascade (see Fig. 4.2). Fas ligand/Fas and perforin/granzyme B systems are the main apoptotic machinery that regulates homeostasis in immune cell populations.

## Intrinsic Death Pathway

Cells can respond to various stressful stimuli and metabolic disturbances by triggering apoptosis. Drugs, toxins, heat, radiation, hypoxia, and viral infections are some of the stimuli known to activate death pathways. Cell death, however, is not necessarily inevitable after exposure to these agents, and the mechanisms determining the outcome of the injury are a topic of active interest. The current consensus appears to be that it is the intensity and the duration of the stimulus that determine the outcome. The stimulus must go beyond a threshold to commit cells to apoptosis. Although the exact mechanism used by each stimulus may be unique and different, a few broad patterns can be identified. For example, agents that damage DNA, such as ionizing radiation and certain xenobiotics, lead to activation of p53-mediated mechanisms that commit cells to apoptosis, at least in part through transcriptional upregulation of proapoptotic proteins.<sup>64</sup> Other stresses induce increased activity of stress-activated protein kinases, which results ultimately in apoptotic commitment.<sup>65</sup> These different mechanisms converge in the activation of caspases.

All stimuli described above change the outer mitochondrial membrane permeability and release two main groups of normally sequestered proapoptotic proteins from the intermembrane space into the cytosol.<sup>4</sup> The first group consists of cytochrome *c*, Smac/DIABLO, and the serine protease HtrA2/Omi.<sup>66–68</sup> Cytochrome *c* binds the adapter protein, Apaf-1, and procaspase-9 to form an “apoptosome”<sup>69</sup> complex and activates caspases. The clustering of procaspase-9 in the apoptosome complex leads to caspase-9 activation. IAPs (inhibitors of apoptosis proteins) in the cell block activate caspase-9 and cause the activation of downstream effector caspases. Smac/DIABLO and HtrA2/Omi are reported to promote apoptosis by inhibiting the activity of IAPs.<sup>70</sup> The second group of proapoptotic proteins, AIF, endonuclease G, and CAD (caspase-activated DNase), are released from the mitochondria during apoptosis, but this is a late event that occurs after the cell dies. The control and regulation of these mitochondrial events during apoptosis occurs through members of the Bcl-2 family of proteins,<sup>71</sup> as discussed next.

In *C. elegans*, biochemical and genetic studies have indicated a role for CED-4 upstream of CED-3.<sup>72</sup> Upon receiving death commitment signals, CED-4 binds to pro-CED-3 and releases active CED-3.<sup>72</sup> However, when overexpressed,



CED-9 can inhibit the activation of pro-CED-3 by binding to CED-4 and sequestering it away from pro-CED-3. Therefore, CED-3 and CED-4 are involved in activation of apoptosis, and CED-9 inhibits apoptosis. After the discovery of caspases as CED-3 homologues, a search for activators and inhibitors analogous to CED-4 and CED-9 led to the discovery of diverse mammalian regulators of apoptosis. The plethora of these molecules and their functional diversity allowed them to be classified into four broad categories: (1) adapter proteins, (2) the Bcl-2 family of regulators, (3) inhibitors of apoptosis (IAPs), and (4) other regulators.

## Adapter Proteins

As stated earlier, two major pathways of apoptosis, involving either the initiator caspase-8 or the initiator caspase-9 (see Fig. 4.2), have been recognized. Signaling by death receptors (CD95, TNFRI) occurs through a well-defined process of recruitment of caspase-8 to the death receptor by adapter proteins such as FADD. Recruitment occurs through interactions between the death domains that are present on both receptor and adapter proteins. Receptor-bound FADD then recruits caspase-8 through interactions between DEDs common to both caspase-8 and FADD, forming a DISC. In the DISC, caspase-8 activation occurs through oligomerization and autocatalysis. Activated caspase-8 then activates downstream caspase-3, culminating in apoptosis. The inhibitory protein FLIP was shown to block Fas-induced and TNF- $\alpha$ -induced DISC formation and subsequent activation of caspase 8.<sup>61</sup> Of particular interest is cellular FLIP, which stimulates caspase-8 activation at physiologically relevant levels and inhibited apoptosis upon high ectopic expression.<sup>73</sup> Cellular FLIP contains two DEDs that can compete with caspase-8 for recruitment to the DISC; this limits the degree of association of caspase-8 with FADD and thus limits activation of the caspase cascade. It also forms a heterodimer with caspase-8 and caspase-10 through interactions between both the DEDs and the caspase-like domains of the proteins, thus activating both caspase-8 and caspase-10.<sup>74</sup> Apoptotic protease activating factor-1 (Apaf-1), a CED-4 homologue in mammalian cells, affects the activation of initiator caspase-9.<sup>75</sup> This factor binds to procaspase-9 in the presence of cytochrome *c* and 2'-deoxyadenosine 5'-triphosphate (dATP) or adenosine triphosphate (ATP) and activates this protease, which in turn activates a downstream cascade of proteases (see Fig. 4.2).<sup>69</sup> By and large, Apaf-1 deficiency is embryonically lethal, and the embryos exhibit brain abnormalities similar to those seen in caspase-9 knockout mice.<sup>76</sup> These genetic findings support the idea that Apaf-1 is coupled to caspase-9 in the death pathway. Unlike CED-4 in nematodes, Apaf-1 requires the binding of ATP and cytochrome *c* to activate procaspase-9. The multiple WD40 repeats in the C-terminal end of Apaf-1 have a regulatory role in the activation of caspase-9.<sup>77</sup>

## The Bcl-2 Family of Proteins

The CED-9 homologue in mammals is the Bcl-2 protein. Bcl-2 was first discovered in B-cell lymphoma as a proto-oncogene. Overexpression of Bcl-2 was shown to offer protection against a variety of death stimuli.<sup>78</sup> The Bcl-2 protein family includes both proapoptotic (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Nr13, and A1/Bfl1) and antiapoptotic proteins (Bax, Bak, Bok, Diva, Bcl-Xs, Bik, Bim, Hrk, Nip3, Nix, Bad, and Bid).<sup>71,79</sup> These proteins are characterized by the presence of Bcl-2 homology (BH) domains: BH1, BH2, BH3, and BH4 (Fig. 4.3). The proapoptotic members have two subfamilies: a multidomain and a BH3-only group (see Fig. 4.3). The relative ratio of pro- and antiapoptotic proteins determines the sensitivity of cells to various apoptotic stimuli. The best studied proapoptotic members are Bax and Bid. Exposure to various apoptotic stimuli leads to translocation of cytosolic Bax from the cytosol to the mitochondrial membrane.<sup>80</sup> Bax oligomerizes on the mitochondrial membrane along with another proapoptotic protein, Bak, leading to the release of cytochrome *c* from the mitochondrial membrane into the cytosol.<sup>81</sup>

Other proapoptotic proteins, mainly the BH3-only proteins, are thought to aid in Bax-Bak oligomerization on the mitochondrial membrane. The antiapoptotic Bcl-2 family members are known to block Bax-Bak oligomerization on the mitochondrial membrane and subsequent release of cytochrome *c* into the cytosol.<sup>81,82</sup> After release from the mitochondria, cytochrome *c* is known to interact with the WD40 repeats of the adapter protein Apaf-1, resulting in the formation of the apoptosome complex. Seven molecules of Apaf-1, interacting through their N-terminal caspase activation and recruitment domain, form the central hub region of the symmetrical wheel-like structure, the apoptosome. Binding of ATP/dATP to Apaf-1 triggers the formation of the apoptosome, which subsequently recruits procaspase-9 into the apoptosome complex, resulting in its activation.<sup>83</sup> Activated caspase-9 then activates executioner caspases, such as caspase-3 and caspase-7, eventually leading to programmed cell death. However, for the caspase-9 to be active, it must reside on the apoptosome complex.<sup>84</sup>

## Inhibitors of Apoptosis Proteins

The IAPs, first discovered in baculoviruses and then in insects and *Drosophila*, inhibit activated caspases by directly binding to the active enzymes.<sup>85</sup> These proteins contain one or more baculovirus inhibitors of apoptosis repeat domains, which are responsible for the caspase inhibitory activity.<sup>86</sup> To date, eight mammalian IAPs have been identified: they include X-linked IAP (XIAP), c-IAP1, c-IAP2, Melanoma IAP (ML-IAP)/Livin, IAP-like protein-2 (ILP-2), neuronal apoptosis-inhibitory protein (NAIP), Bruce/Apollon, and Survivin. In mammals, caspase-3, -7, and -9 are inhibited by IAPs.<sup>83</sup> There are reports suggesting aberrant expression of IAPs in many cancer tissues. For example, cIAP1



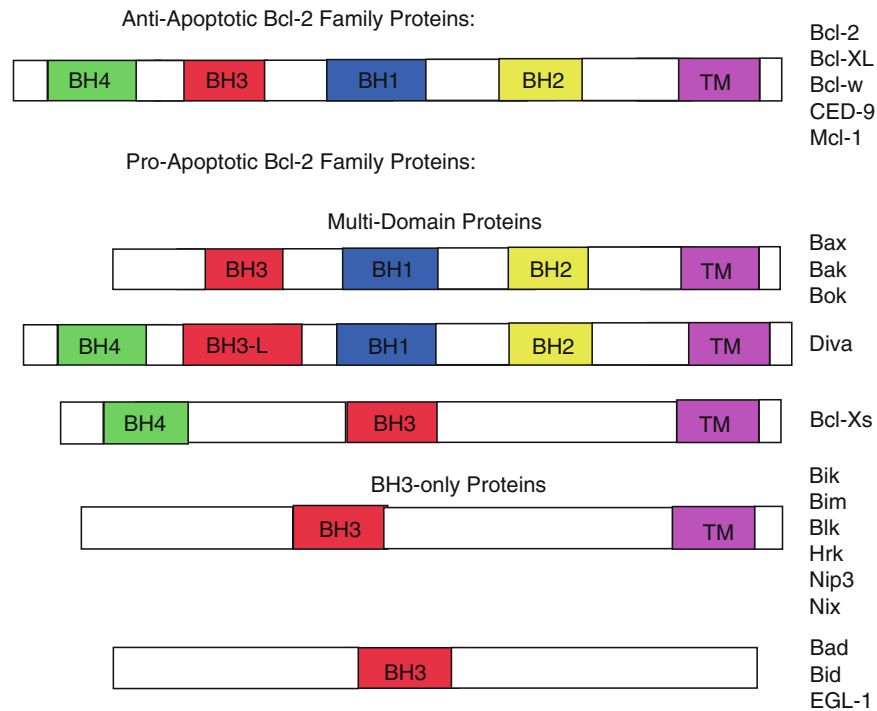


FIG. 4.3. Structural homologies in anti- and proapoptotic proteins of the Bcl-2 family. Anti- and proapoptotic proteins of the Bcl-2 family are depicted, indicating Bcl-2 homologous (BH) regions BH1, BH2, BH3, and BH4 and transmembrane (TM) domains.

is overexpressed in esophageal squamous cell sarcoma<sup>87</sup>; the cIAP2 locus is translocated in mucosa-associated lymphoid lymphoma<sup>88</sup>; and Survivin has been shown to be upregulated in many cancer cells.<sup>89</sup>

### Other Regulators

The caspase inhibitory activity of IAPs is inhibited by proteins containing an IAP-binding tetrapeptide motif.<sup>83</sup> The founding member of this family is Smac/DIABLO, which is released from the mitochondrial intermembrane space into the cytosol during apoptosis. In the cytosol, it interacts with several IAPs and inhibits their function. The other mitochondrial protein, Omi/HtrA2, is also known to antagonize XIAP-mediated inhibition of caspase-9 at high concentrations.<sup>90</sup> A serine protease, Omi/HtrA2, can proteolytically cleave and inactivate IAP proteins and thus is considered to be a more potent suppressor of IAPs than Smac.<sup>91</sup>

It has been reported that the heat shock proteins Hsp90, Hsp70, and Hsp27 can inhibit caspase activation by cytochrome *c* by interacting with either Apaf-1 or other players in the pathway.<sup>92–94</sup> A high-throughput screen identified a compound called PETCM [ $\alpha$ -(trichloromethyl)-4-pyridineethanol] as a caspase-3 activator. Further work with PETCM revealed its involvement in apoptosome regulation.<sup>95</sup> This pathway also includes oncoprotein prothymosin- $\alpha$  and tumor suppressor putative HLA-DR-associated proteins. These proteins were shown to promote caspase-9 activation after

apoptosome formation, whereas prothymosin- $\alpha$  inhibited caspase-9 activation by inhibiting apoptosome formation.

### Protein Targets of Caspases

In an apoptotic cell, the regulatory, structural, and house-keeping proteins are the main targets of the caspases. The regulatory proteins mitogen-activated protein/extracellular signal-regulated kinase kinase-1, p21-activated kinase-2, and Mst-1 are activated upon cleavage by caspases.<sup>96</sup> Caspase-mediated protein hydrolysis inactivates other proteins, including focal adhesion kinase, phosphatidylinositol-3 kinase, Akt, Raf-1, IAPs, and inhibitors of caspase-activated DNase (ICAD). Caspases also convert the antiapoptotic protein Bcl-2 into a proapoptotic protein such as Bax upon cleavage. There are many structural protein targets of caspases, which include nuclear lamins, actin, and regulatory proteins such as spectrin, gelsolin, and fodrin.<sup>47,97</sup>

Degradation of nuclear DNA into internucleosomal chromatin fragments is one of the hallmarks of apoptotic cell death that occurs in response to various apoptotic stimuli in a wide variety of cells. A specific caspase-activated DNase (CAD), that cleaves chromosomal DNA in a caspase-dependent manner, is synthesized with the help of ICAD. In proliferating cells, CAD is always found to be associated with ICAD in the cytosol. When cells are undergoing apoptosis, caspases (particularly caspase-3) cleave ICAD to release CAD and allow its translocation to the nucleus to cleave

chromosomal DNA. Thus, cells that are ICAD deficient or which express caspase-resistant ICAD mutant do not exhibit DNA fragmentation during apoptosis.

## Conclusion

Cell death has become an area of intense interest and investigation in science and medicine because of the recognition that cell death, in general, and apoptosis, in particular, are important features of many biological processes. Apoptosis is a carefully regulated energy-dependent process in which caspase activation plays a central role. Although many of the key apoptotic proteins have been identified, the molecular mechanisms of their action are not yet fully understood and thus continue to be a research focus. Because apoptosis is a vital component of both health and disease that is initiated by various physiological and pathological stimuli, as well as having widespread involvement in the pathophysiology of disease, it lends itself to therapeutic intervention.

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