Apoptosis and gene regulation

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Apoptosis is a highly organized physiologic mechanism of destroying injured and abnormal cells and maintaining homeostatis in multicellular organisms. Apoptotic cells are characterized by cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation and ultimate fragmentation of the cell into membrane enclosed vesicles designated as 'apoptotic bodies'. Apoptosis is particularly important in embryogenesis, in metamorphosis, in various sorts of tissue disorders including tumour regression, nervous diseases and exposure to toxins. Both activation and inhibition of apoptosis are genetically controlled. Several different agents induce apoptosis which require molecules synthesized in the late G1/early S phase of the cell cycle. Many genes such as *p53*, *Fas*, *Ras*, *Myc* are involved in transition from late G1 to early S phase which have also a role to either induce or inhibit the apoptotic process. Recent evidence in the programmed cell death pathway of *C. elegans* has shown that ced-9 (a cell death supressor) and ced-3 (a cell death inducer) encode proteins that share structural and functional similarities with the mammalian proto-oncogene product *Bcl-2* and interleukin-1β converting enzyme respectively. Cysteine protease interlekin-1β converting enzyme (ICE), a family of 10 related cysteine proteases has been identified more recently. These cysteine proteases otherwise called caspases mediate highly specific proteolytic cleavage events in dying cells.

APOPTOSIS (Greek for 'drop-out') is a highly organized physiologic mechanism of destroying injured and abnormal cells. All cells possess a suicide mechanism which plays an indispensable role in the development and maintenance of adult tissue homeostatis. Cell homeostatis is regulated by a balance between proliferation, growth arrest and programmed cell death. Apoptosis is otherwise called 'programmed cell death'. More than 80 years ago the features of cell death were first outlined, but only in the late 1970s, scientists realized the biological significance of negative growth control including growth arrest and programmed cell death[1]. It has been suggested that apoptosis is a form of aberrant mitosis because of rapid chromosome condensation and disassembly of nuclear lamina[2,3]. However it differs mainly in two aspects, first, apoptotic cells do not express the MPM-2 eitope[4], a phosphoepitope that is a universal marker for mitotic cells in both plants and animals; second, the nuclear envelope changes in apoptosis significantly differ from mitosis. The nuclear membrane and nuclear pore complexes remain intact throughout apoptosis[4,5].

Apoptosis is particularly important in the embryonic morphogenesis[6,7], formation of fingers and toes[8,9], maturation of T and B cells in the immune system[10], elimination of excess neurons[11] and those failing to make the right connections during development of the central nervous system[12,13], tissue remodelling in the intestinal lining, cartilage and bone[14–16], and loss of gland epithelial cells in endometrial turnover during secretary, premenstrual and menstrual phases[17,18].

Cell division and cell death are equally important for animal development. These include the lysis of infected cells, foreign germs or incipient neoplasms by the immune system[19–21]. Among these, apoptosis is a special type of cell death in which the cell is destroyed from within. Normally, apoptotic cells appear with highly condensed chromatin and the cells shrink and condense, fragmenting into multiple, membrane bound bodies, called 'apoptotic bodies' which are eventually engulfed by the surrounding cells or sometimes by the same cell without disturbing the surrounding tissue. The phagocytic process has been classified into three mechanisms, according to which the cell disposes the apoptic bodies[22]. Type I: cell death or heterophagy, the lysosomes of neighbouring cells break down the apoptotic body. Type II: cell death or autophagy has been found only in some developing systems. In this mechanism the indigenous lysosomes of the dying cell degrade the apoptotic bodies. Type III: cell death is characterized by macrophage digestion of apoptotic bodies.

Apoptotic bodies are found in the parts of the body where cells are dividing, such as intestinal tissue[23], epithelium of the adrenal cortex[24,25], spermatogonium[26] and in the lymph nodes[27]. Apoptosis is a prominent feature in malignancies[28] where it plays a compensatory role: increase in cell number due to proliferation and cell loss due to death. Both positive and negative signals trigger cells to stop proliferation and undergo apoptosis. Positive signal comprises antigen receptor ligation of immature lymphocytes[29], glucocorticoid-induced thymocytes

Table 1. Agents that induce cell cycle arrest and apoptosis

Cell line	Phase	Agent	Reference
Murine B cell lymphoma-WEHI-231	S	Anti Igm	128, 129
Human lymphoid cell	G1	Dexamethasone	130
Bursal stem cell	S	γ-radiation	131
L 929 cells	G1	Tumour necrosis factor-a	132, 133, 134
HeLa cells	G1	TNF-a & Cycloheximide	135
HeLa cells	S	Staurosporine, 6-dimethyl-aminopurine, Okadaic acid Caffeine, γ-radiation	53 Meikrantz, and Schlegel (unpublished observations)
Human promyelocytic leukemia (HL-60)	G1	Nitrosomustard	136
HL-60	G2-M	γ-radiation	136
Mouse embryo fibroblasts	G2	X-ray	137
Human liver cells	G1-S	Staurosporine	138
Rat embryo fibroblasts	G1	Ultraviolet	139
Tumour cells (L 1210/0)	G2-M	Cisplatin	140
Burkitt's lymphoma SV2	G2-M	Oracin	141
PaCa-2 cells	G1-M	Eicosapentaenoic acid	142
Breast cancer MCF-7	G0-G1	Tamoxifen	143
	G2-M	Cisplatin	
L 1210 cells	G2	Imidazoacridinones	144
Mouse embryo fibroblast and human cell lines	early M and G1	Taxol	145
Rat thymocytes	G0-G1	Dexamethasone	146
	G2-M	Etoposide	
Chinese hamster ovary cells (CHO)	–	Cleistanthin B	147
CHO cells	–	Fluchloralin	148

Table 2. Apoptosis versus necrosis

Apoptosis	Necrosis
Solitary	Massive
Cell shrinks	Cell swellls
Condensation of cytoplasm, chromatin and organelles occur (apoptotic bodies)	Cytoplasm, chromatin and organelles swell due to an influx of water
Cytoplasm condensed and dehydrated	Swelling of ER and mitochondria due to abnormal accumulation of watery fluid
Increase of intracellular calcium level was sustained	Intracellular calcium level was elevated
DNA were cleaved systematically	No systematic DNA cleaving
Cells undergo membrane blebbing, but retain membrane integrity	Complete loss of membrane integrity
Formation of membrane-bound apoptotic bodies keeps organelles intact	Cells swell until they lyse, leaving critically-damaged organelles and membranes
Autolysis did not occur	Necrosis was thought to reflect an autolytic digestion of the cell (suicide bag-hypothesis)
Possible to rescue	Impossible if its cause is not removed

death[30]. Negative signaling comprises withdrawal of necessary growth factors, e.g. mouse myeloma lines will undergo apoptosis following withdrawal of IL-2 (interleukin) and IL-6 (ref. 31). The signals indicate that apoptotic programmes are related or interrelated with the cell cycle. For example, inhibitors of the replication fork-associated enzymes prevent DNA fragmentation and apoptosis in lymphocytes, suggesting that apoptosis is dependent on cell proliferation[32]. Table 1 shows the different agents that induce apoptosis and arrest the cell cycle at different stages. However, some agents directly activate apoptosis regardless of the cell-cycle stage. The separate morphologic and biochemical changes occurring in apoptosis represent a highly coordinated specific response that differs entirely from necrosis. In 1964, Bassis[33] first pointed out that accidental cell death due

to environmental disruption may fundamentally differ in mechanism from natural cell death. Later, in 1972, Kerr *et al.*[1] classified cell death into two categories – apoptosis and necrosis based on the morphological observations. Necrosis lacks the tight regulation and organization of apoptosis. Table 2 shows the important differences between apoptosis and necrosis.

Cellular consequences of apoptosis

The entire cell is reorganized during apoptosis. The chromatin becomes fragmented and condensed, the cell shrinks and the cell-surface blebs leading to budding off the membrane bound apototic bodies[34].

Cell membrane alterations during apoptosis

The cell membrane undergoes extensive alterations, the *in vitro* cell detaches from substrate or the *in vivo* cell from the matrix. The membrane loses specialized structures such as microvilli. During apoptosis the neighbouring cells act as eliminators of apoptotic bodies to ensure the rapid clearance. Many other cells perform the phagocytes to remove the apoptotic bodies termed as 'semiprofessional' or 'amateur phagocytes'. These phagocytic cells recognize apoptotic cells by a change in the lipid composition of the outer plasma membrane[35].

Cytoplasmic changes

The most important morphological feature is the formation of apoptotic bodies. During this process, the microfilament network is completely rearranged. The microtubule disrupting agents colchicine, vinblastine and nocadazole induce apoptosis, suggesting that disruption of the microtubule network initiates the apoptotic events[36].

Nuclear changes and DNA fragmentation

The mechanism of chromatin condensation and nuclear envelope breakdown are unknown during apoptosis. It has been suggested that disassembly of lamin by proteolysis occurs during apoptosis[37]. Lamins are intermediate filaments and associate with the inner nuclear envelope. They form the nucleoskeleton and provide a framework for the attachment of chromatin to the nuclear envelope. During mitosis, chromatin condensation and nuclear envelope breakdown occur via the solubilization of nuclear lamin upon phosphorylation by p34 cdc2 kinases[38]. A characteristic DNA cleavage of apoptosis is the result of an endogenous neutral Ca^{2+}- and Mg^{2+}-dependent endonuclease activity capable of inducing double strand breaks at internucleosomal sites[39]. It has been proposed that DNAse I is the main candidate for the endonuclease activity during apoptosis[40]. The DNA is cleaved to generate 180–200 bp length fragments, giving a 'ladder'-like appearance, which is a biochemical hallmark of apoptosis in many cells.

Types of DNA fragmentation

The three types of DNA fragmentation that occur during apoptosis are (a) internucleosomal DNA cleavage; (b) fragmentation into large 50–300 kbp lengths and (c) single strand cleavage events[41].

Internucleosomal DNA cleavage

Most of the authors have reported that internucleosomal DNA cleavage occurs during apoptosis in a wide variety of cells and tissues. This occurs by an activation of an endogenous endonuclease that cleaves the DNA in the linker region between histones on the chromosomes, comprising about 180–200 bp which form the 'apoptotic ladder' pattern. The main candidate of apoptotic nuclease is DNAse I which cleaves the internucleosomal DNA. A second nuclease DNAse II and an endonuclease Nuc 18, are also involved in apoptosis. The occurrence of different types of DNA fragmentation clearly showed the existence of more than one enzyme to be involved in apoptosis. The role of DNAses during apoptosis has been reviewed by Peitsch *et al.*[42].

Large DNA fragmentation

Many researchers have detected large DNA fragments of 50–300 kbp cleaved at the nuclear scaffold region during apoptosis[43–45]. They proposed that these large DNA fragments serve as precursors for the smaller DNA fragments and occur before internucleosomal DNA cleavage. Large DNA fragmentation may also occur during necrosis and may not be observed under all apoptotic conditions. Clearly more studies are needed to confirm the cleavage of large DNA fragmentation.

Single strand DNA cleavage

Recently, it has been suggested that single strand cleavage of DNA also occurs during the apoptotic process[40,46].

Gene regulation during apoptosis

Apoptosis and the G1 restriction point: Activation of CDKs

Cyclins are proteins whose levels rise during the course of the cell cycle and fall in the quiescence state. They

are positive regulating subunits for CDKs (cyclin dependent kinase) which are regulated by activating and inactivating phosphorylations. Activation of *cdk*2 cyclin-A appears to be the final step required for the transition from G1 to S. Cyclin A is unique among the cyclins and is required both for mitosis and for DNA replication[47-49]. Two oncogenes *c-myc* and *adenovirus EIA* are both transcriptional activators of the cyclin A gene which induce apoptosis in the absence of serum or without expression of a co-operating oncogene[50-52]. Many evidences suggest that cdk-cyclin A activity may actually be simulated by apoptotic-inducing agents. For example, in S phase arrested HeLa cells, apoptosis was induced by agents like caffeine, 6-dimethylaminopurine, staurosporine and okadaic acid. These agents activate CDKs with activation of cyclin A dependent kinases occurring within 2 h after exposure[53].

cdk2 becomes activated as a protein kinase at the time of entry into S phase[54]. Inactivation of cdk2 prevents cells from entering S phase by microinjecting neutralizing antibodies in cyclin A[48] or treatment with antisense cyclin A nucleotide[47]. It seems that cdk2-cyclin A affect DNA replication directly. cdk2 is dephosphorylated and activated by CDC25A. The transcription of CDC25A begins in early G1 and peaks in late G1. In addition to cdk2, another gene *cdc*2 is also involved in apoptotic process. *cdc*2 was rapidly activated at the initiation of apoptosis whereas DNA fragmentation and nuclear condensation could be prevented by an excess of cdc2 peptide substrate. Both cdc2-cyclin A and cdk2-cyclin A are activated when the cells (HeLa) undergo apoptosis. It is possible that apoptosis can be inhibited at a greater level due to elimination of all cyclin-A dependent kinase activity. Moreover the predominance of cdc2-cyclin A complexes versus cdk2-cyclin A complexes vary from cell type to cell type in the cell cycle and as a function of growth conditions[55].

p53 and p21 protein in apoptosis

The nematode *C. elegans* is a model system to analyse apoptosis of multicellular animals[56]. Many signaling systems are involved in the apoptotic process. Oncogenes like *c-fos*, *junB*, *junD* and *c-jun* are involved in apoptosis during transition from G1 to S phase[31,57]. In general, it appears that cells must progress to late G1 of the cell cycle for apoptosis to occur. In late G1, passage of normal cells through this point of the cell cycle is atleast partially regulated by p53. There are generally two types of apoptotic cells, p53 dependent and p53 independent. It is evident that many types of apoptosis-inducing agents are p53 dependent.

What is the role of p53 in apoptosis and how it is related to its role as a cell cycle regulator? Generally, G1 cell cycle stage in mammalian cells is referred to as the 'R' (restriction) point[58] where cells become irreversibly committed to DNA replication. Any cell that passes through this point requires activation of specific cyclin and cyclin-dependent kinases (CDKs). The p53 phosphoprotein is a tumour suppressor gene whose inactivation leads to human cancer[59]. Growth arrest in the G1 phase of the cell cycle and induction of apoptosis are two distinct and dissectable functions of p53 (refs 60–63). Ionizing radiations induce increase in p53 protein level, which in turn leads to transcriptional activation of a number of genes including p21, WAF1/CIPI, GADD45 and MDM2. When mammalian cells are exposed to ionizing radiation, cells are transiently arrested at the G1 due to increased intracellular levels of p53 protein and G2 phases of the cell cycle[64] and driven into apoptosis[65]. p53 proteins arrest the cell cycle at the G1 check point and allow the cell to repair the DNA damage or to commit to apoptosis if the damage is too great and thereby halt progression of cells towards carcinogenesis. Hence, p53 is referred to as 'guardian of the genome'[66]. Many findings proved that cell lines derived from p53-deficient animals fail to undergo apoptosis in response to DNA-damaging agents[67-71]. If p53 fails to function, the cells undergo DNA replication even in the presence of DNA damage. So p53 maintains the integrity of the cell/organism.

*p*21 is an another gene associated with the *p*53 gene. *p*21 was discovered as a senescent cell derived inhibitor of DNA replication in human diploid fibroblasts[72]. *p*21 is induced in p53-dependent G1 arrest in normal human diploid fibroblasts following irradiation, resulting in inhibition of cdk2-cyclin E kinase activity, preventing phosphorylation of pRB and thereby blocking entry into S phase[73]. A similar expression was observed in colorectal carcinomal lines RKO and HCT-116 treated with doxorubicin[74]. When tumour cells are exposed to irradiation, cells containing wild type p53 were induced with p21 and underwent apoptosis; while p21 and apoptosis were not induced in p53-deficient cells[74]. Thus induction of p21 and the consequent inhibition of CDKs may be a first step in p53-dependent apoptosis.

Apart from p53-dependent apoptosis, some cell lines showed interesting features of several p53-independent pathways of apoptosis, for example T cell hybridoma[75] and in HeLa cells which are human papillomavirus positive functional p53. These cells can promote apoptosis by p53 independent apoptosis-inducing agents. Another example, okadaic acid (OA)-induced apoptosis in human breast carcinoma cells (HBC) occurs independently of cell cycle arrest and the wild type p53 function is not an absolute prerequisite for cell death. OA-induced apoptosis is associated with up-regulation of endogenous p21 waf1/cip1 and Bax protein levels[76].

Fas gene

Fas (APO-1, CD95) has control of cell death and survival through engaging members of the Fas/TNF recep-

tor 1 (TNFR 1 – tumor necrosis factor receptor) family[77]. The *Fas* gene contains a cytoplasmic region of 60–70 amino-acids termed 'death domains' which is important in inducing apoptosis[78]. The genes encoding three proteins MORT 1/FADD[79,80], TRADD[81] and RIP[82] have been identified recently which contain the death domains[83] and it is likely that these domains associate with the death domains of Fas and/or TNFR1.

Ras gene

Ras is a member of a super family containing more than 50 small GTPases, functioning as GDP/GTP regulated switches. The three Ras proteins H, K and N-Ras have a vital role in signal transduction, linking the receptor and non-receptor tyrosine kinases including the mitogen-activated protein (MAP) kinases. The Ras/Raf/MAP kinase cascade have a role in the inhibition of apoptosis. Ras-related proteins regulate BCl-2 proteins and inhibit apoptosis[84,85].

Bcl-2 and related genes

Many mammalian genes such as *bcl*-2, *bcl*-x, *bax*, *mcl*-1, *bak*, *bak*-2 and *bak*-3 have been identified that encode proteins with significant amino-acid sequence similarity to *ced*-9 gene of *C. elegans*. The ratio of the apoptosis-suppressing genes *Bcl*-2, *Bcl*-xl, *Mcl*-1 to apoptosis promoting genes *Bax*, *Bcl*-xs, *Bak* and *Bad* may control the susceptibility of mammalian cells to apoptosis. Among these genes, *Bcl*-2 is the most important member of a family of genes that control cell homoeostatic process in the course of development and adult life. The *Bcl*-2 exibits two main biochemical properties: (i) it acts in an antioxidant metabolic pathway aimed at eliminating oxygen-free radicals that induce lesions in DNA, lipids and proteins; (ii) it modulates intracellular Ca^{++} fluxes[86]. Many recent data have demonstrated that *Bcl*-2 gene protects tumour cells from apoptosis induced by a variety of agents. *Bcl*-2 is able to antagonize the induction of apoptosis by p53, but not growth arrest in G1. However, coexpression of *Bcl*-2 and of the oncogene *c-myc* efficiently antagonizes effects of p53 on G1 arrest and apoptosis[63,86]. *Bcl*-2 and ICE probably affect the same apoptosis pathway suggesting either that ICE lies upstream of *Bcl*-2 or that they act at the same stage[87]. Recently another *Bcl*-2 family member, *Mcl*-1, a gene expressed when ML-1 human myeloid leukemic cells are exposed to DNA damaging agents, was found. These findings demonstrate that cytotoxic DNA damage causes an increase in the expression of *Mcl*-1 along with increases in GADD45 and BAX and a decrease in *Bcl*-2. The increase in GADD45 is seen both in cells that undergo growth arrest and in cells that undergo apoptosis in response to DNA damage[88].

Myb gene

Myb expression can induce apoptosis[89]. Inhibition of c-myb expression induces apoptosis in neuroblastoma cells and increased *c-myb* expression induces apoptosis in T' cells; so it is apparent that *myb* expression induces apoptosis which depends upon the specific cell type.

Myc

c-myc proto-oncogene is an important positive regulator of cell growth and proliferation in early G1 phase of the cell cycle. Recently, *c-myc* has also been demonstrated to be a potent inducer of apoptosis when expressed in the absence of serum or growth factors. However, coexpression of *Bcl*-2 with *c-myc* in serum-deprived rat fibroblasts led to a 10-fold increase in the number of live cells and a significant decrease in DNA fragmentation; thus *Bcl*-2 effectively inhibits Myc-induced apoptosis[90].

Caspases

In the last few years, many of the molecules that participate in a conserved biochemical pathway that mediates the highly ordered process of apoptotic cell suicide have been identified. Cysteine proteases otherwise called caspases are at the heart of a conserved cell-death pathway. Caspases are synthesized as inactive proenzymes which are activated by cleavage at specific Asp residues to active enzymes containing both large (p20) and small (p10) subunits. Early studies concentrated on the role of nucleases in apoptosis, more recently a role has been proposed for a number of different proteases, including serine proteases, calpains and proteasomes[91–93]. Most attention has focused on the interleukin-1β (IL-1β)-converting enzyme (ICE)-like proteases which forms the caspase-1 subfamily. Two genes, *ced*-3 and *ced*-4, are vital for cell death in *C. elegans*, while the *ced*-9 gene antagonizes their function and prevents cell death[94]. The ced-9 protein bears sequence similarity to mammalian BclI-2, which acts to prevent cell death in mammals. To resolve the confusion, as a result of different groups isolating the same proteases, a unified nomenclature has recently been suggested. The trivial name proposed for all family members is caspase, the 'c' denoting a cysteine protease and the 'aspase' referring to the ability of these enzymes to cleave after an aspartic acid residue. Phylogenetic analysis of the caspases reveals that there are three subfamilies: an ICE subfamily, comprising caspases –1, –4 and –5, a CED-3/CPP32 (32 kDa cysteine protease) subfamily, comprising caspases –3, –6, –7, –8, –9 and –10, and an ICH-1 (where ICH is Ice and *ced*-3 homologue)/Nedd2 subfamily (Table 3). All caspases contain an active-site pentapeptide of general structure QACXG (where X is R, Q or G). It is evident from studies that all caspases are required for cell death,

Table 3. Members of the caspase family

Caspase	Other names	Active site	Cleavage site(s) between large and small subunits
Caspase-1	ICE	QACRG	WFKD↓ S; FEDD↓ A
Caspase-2	Nedd2, ICH-1	QACRG	DQQD↓ G; EESD↓ A
Caspase-3	CPP32, Yama, Apopain	QACRG	IETD↓ S
Caspase-4	ICE II, TX, ICH-2	QACRG	WRVD↓ S; LEED↓ A
Caspase-5	ICE III, TY	QACRG	WRVD↓ S; LEAD↓ S
Caspase-6	Mch2	QACRG	DVVD↓ N; TEVD↓ A
Caspase-7	Mch3, ICE-LAP3, CMH-1	QACRG	IQAD↓ S
Caspase-8	MACH, FLICE, Mch5	QACQG	VETD↓ S; LEMD↓ L
Caspase-9	ICE-LAP6, Mch6	QACGC	DQLD↓ A
Caspase-10	Mch4	QACQG	SQTD↓ V; IEAD↓ A

and that some are more important than others. Some caspases contain only a short prodomain (Caspases –3, –6 and –7), whereas others contain long prodomains (Caspases –1, –2, –4, –5, –8, –9 and –10). The importance of the FADD-like prodomains of caspase-8 and possibly caspase-10 in directly linking CD95- and TNFR-1-mediated apoptosis has been emphasized[95,96]. The significance of other prodomains is not known, but they may be important in the regulation of the activation of caspases[97].

Caspase-1 (ICE)

Interleukin-1β converting enzyme (ICE) was first discovered in the cytosol of monocytes and monocyte-like cell lines. The amino-acid sequence of ICE shows similarity to ced-3 of *C. elegans*[98]. The gene encoding ICE has been mapped to chromosome 11 band q22.2–q22.3 which is frequently involved in rearrangement in human cancer, including a number of leukemias and lymphomas[99,100]. ICE is a cysteine protease that converts pro-interleukin1 to its active form by cleaving it at aspartate residues. Over-expression of the murine ICE gene induces programmed cell death in different cell lines[87,94]. IL-1 is released exclusively in the inactive precursor form, when cells are injured by scraping[101]. Many experimental results suggest that not only cysteine proteases but also serine proteases play important roles in controlling the apoptotic process in vertebrates. Another ICE gene family called TX showing similar ICE sequence[102] plays an active role in apoptosis. The apoptosis-inducing properties of ICE-like proteins have been demonstrated only in cultured cells by their overexpression; so their physiological levels of apoptosis have yet to be determined. Moreover, ICE cannot be the only initiator of mammalian apoptosis since cells from ICE deficient mice also undergo apoptosis[103,104].

Caspase-1 is a 45 kDa protein and comprises two subunits of 20 kDa and 10 kDa, both of which are required for catalytic activity and are derived from a single proenzyme following removal of an 11 kDa N-terminal peptide and a 2 kDa linker peptide. Caspase-1 is found predominantly in the cytoplasm of cells as the p45 pro-form[105]. Some of it is also localized to the external cell surface membrane, where it activates pro-IL-1β to its mature form during secretion[106]. Caspase-1 is a novel type of cysteine protease containing an active-site cysteine residue in the p20 subunit, the mutation of which results in loss of activity. The active enzyme is a tetramer of two p20 subunits surrounding two adjacent p10 subunits, with most of the area of contact between the dimers occurring between the p10 subunits[107,108]. Based on the crystal structure, two models were proposed for maturation of the proenzyme. In the first, two precursor p45 proteins associate and are then processed, with the p10 subunit from one caspase-1 molecule complexing with the p20 subunit from another caspase-1 molecule, hence creating the active site. The alternative, but less favoured, model suggested processing followed by association of the subunits[108]. Caspase-1 and CED-3 share 28% sequence identity, and the active-site pentapeptide, QACRG, is completely conserved[109]. Overexpression of the murine ICE gene induces apoptosis in Rat-1 fibroblasts, which is abrogated by point mutation in the cysteine or glycine residues of the active site pentapeptide and either *bcl*-2 or *crm*A[87]. Mice deficient in caspase-1 develop normally, appear healthy and are fertile, with no apparent abnormalities, suggesting that there are no gross defects in normal physiological processes involving apoptosis[103,104].

Caspase-2 (ICH-1/Nedd2)

*Nedd*2 was originally identified as a developmentally down-regulated gene in mouse brain[110]. Using a murine Nedd2 cDNA, a human foetal brain cDNA library was screened at low stringency, and Ich-1, the human homologue of Nedd2 was identified[111]. Both *Nedd*2 and *Ich-1* encode proteins similar to caspase-1, and a sequence alignment shows conservation of many important residues, including the active-site pentapeptide QACRG[110,111]. Overexpression of *Ich*-1 in some, but not

all cell types results in apoptosis whereas overexpression of ICH-1 suppresses apoptosis induced by serum withdrawal, suggesting that Ich-1 may play a role in both the positive and negative regulation of programmed cell death[111]. During embroynic development, Nedd2 is expressed at relatively high levels in various tissues, including the central nervous system, liver, kidneys and lungs. Nedd2 is also expressed to varying extents in several adult tissues, including post-mitotic neurons[110].

Caspase-3 (CPP32/YAMA/APOPAIN)

Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins. Using the DNA sequence encoding the active site of caspase-1 and Ced-3 to search an expressed sequence tag database, a human sequence was identified, cloned and shown to encode a 32 kDa cysteine protease called CPP32 (ref. 112). Independently, two other groups identified caspase-3, one naming it Yama (the Hindu god of death) and other apopain[113,114]. Caspase-3, a member of the Ced-3 subfamily of caspases is widely distributed, with high expression in cell lines of lymphocytic origin, suggesting that it may be an important mediator of apoptosis in the immune system[112]. The active enzyme was shown to be composed of two subunits of 17 kDa and 12 kDa, derived from the precursor protein by cleavage of Asp-28–Ser-29 and Asp-175–Ser-176 (ref. 114). During the execution phase of apoptosis, caspase-3 is responsible either wholly or in part for the proteolysis of a large number of substrates, each of which contains a common Asp–Xaa–Xaa–Asp (DXXD) motif[115]. Caspase-3 has no linker peptide and the prodomain is much shorter in comparison with caspase-1. Caspase-3 prefers a DXXD-like substrate, whereas caspase-1 prefers a YVAD-like substrate.

Caspase-4 (ICE II/TX/ICH-2)

ICE II/TX/ICH-2 were cloned by three groups independently[102,116]. Caspase-4 expression, while lower than that of caspase-1, generally shows a similar tissue distribution, being found in most tissues examined with the exception of the brain. Appreciable levels are found in both the lung and liver, and also in the ovary and placenta, where caspase-1 mRNA is barely detectable[116,117]. Caspase-4 may be involved in the maturation of caspase-1 (ref. 102). At high concentrations, caspase-4 cleaves PARP, but the biological relevance of this is unclear[118].

Caspase-5 (ICE III/TY)

Two groups cloned ICE III/TY (ref. 116). Both caspase-4 and caspase-5 are members of the caspase-1 subfamily, and are more closely related to each other. Caspase-5 is expressed at a much lower level than caspase-4. Caspase-4 and -5 have different substrate specificities from that of caspase-1, being much poorer at cleaving pro-IL-1β[116]. Limited information is available concerning the precise sites at which caspase-4 and caspase-5 are processed.

Caspase-6 (Mch2)

Alnemri and co-workers cloned Mch2 by using PCR with degenerate primers encoding two highly-conserved pentapeptides, QACRG and GSWFI from a human Jurkat T lymphocyte cDNA library. Two transcripts were detected, Mch2α (1.7 kb) encoding the full-length and Mch2β (1.4 kb) encoding the shorter isoform. Mch2α encodes a 293-amino-acid protein with a predicted molecular mass of ~34 kDa and Mch 2β encodes a 204-amino acid protein with a predicted molecular mass of ~23 kDa (ref. 119). Caspase-6 is a member of the CED-3 subfamily, showing high identity with caspase-3.

Caspase-7 (Mch3/ICE-LAP3/CMH-1)

Caspase-7 was cloned independently in three different laboratories and named Mch3/ICE-LAP3/CMH-1 (refs 120–122). It is a member of the CED-3 subfamily, and is a 303-amino acid protein with high similarity to caspase-3. An alternatively spliced isoform of caspase-7, which may act as a negative regulator of apoptosis, has been described. Caspase-7 is constitutively expressed in many foetal and adult tissues, with lowest expression observed in the brain. Caspase-3 and caspase-7 are functionally similar and have similar substrate, specificities[120], cleavage of PARP during apoptosis may be due to a combination of the action of both these caspases. Active caspase-7 is made up of two subunits, similar to other caspases. Caspase-7 is activated to its catalytically active large subunit in intact cells undergoing apoptosis[123].

Caspase-8 (MACH/FLICE/Mch 50)

Two groups independently identified a novel caspase, named MACH/FLICE, which contains both an active subunit with identity with the caspases and an N-terminal prodomain containing two domains with marked identity with the N-terminal DED of FADD/MORT1[95,96]. Caspase-8 transcripts are detectable in the testis, skeletal muscle and brain, with a relatively higher level of expression in peripheral blood leukocytes, consistent with a role for CD95-induced apoptosis in lymphocyte homoeostatis. Caspase-8 contains two N-terminal stretches of approximately 70 amino acids that

are apparently homologous to the DED of FADD. Granzyme B activates caspase-8 to an active protease which cleaves PARP to its characteristic signature fragment. Overexpression of caspase-8 results in apoptosis, and mutation of its catalytic cysteine residue abolishes its apoptotic potential. Mch5 was cloned independently from Jurkat T cells, and its predicted sequence is almost identical with that of caspase-8 (refs 96, 124). A small difference in the prodomain is revealed following sequence comparison of Mch5 and MACH/FLICE.

Caspase-9 (ICE-LAP6/Mch6)

Two groups recently cloned a new member of the caspase family, ICE-LAP6/Mch6 independently[125,126]. Caspase-9 is a member of the CED-3 subfamily, bearing high similarity to caspase-3. The major difference between caspase-9 and other family members is the active-site pentapeptide QACGG, in which Gly is found instead of the usual Arg. Procaspase-9 contains a long N-terminal putative prodomain with high similarity to the prodomains of CED-3 and caspase-2. Granzyme B cleaved procaspase-9 at both sites, with marked preference for Asp-315 over Asp-330, generating an active enzyme capable of cleaving PARP to its signature fragment of ~85 kDa (refs 125, 126).

Caspase-10 (Mch4)

Mch4 was cloned from Jurkat T cells of a novel cDNA encoding a 479-amino acid protein with a molecular mass of ~55 kDa (ref. 124). Caspase-10, a member of the Ced-3 subfamily, is more closely related to caspase-8 than to any other caspase. Like caspase-8, caspase-10 has an active-site QACOG pentapeptide and also contains two FADD-like DEDs in its N-terminal domain, suggesting a possible role in CD95- or TNF-induced apoptosis. Caspase-10 mRNA is present in most tissues, with lowest expression being observed in the brain, kidney, prostate, testis and colon and higher levels in the heart, liver and spleen.

Conclusion

Apoptosis is a highly organized physiologic mechanism of destroying injured and abnormal cells. Early studies concentrated on the role of nucleases in apoptosis, more recently most attention has focused on the interleukin -1β (IL-1β) converting enzyme like proteases, as a result further nine related ICE-like proteases have been identified. Two genes *ced*-3 and *ced*-4, are vital for cell death in *C. elegans*, while the Ced-9 proteins bear sequence similarity to mammalian *Bcl*-2 which prevents cell death in mammals. All caspases are cleaved at specific Asp residues, raising the possibility that some caspases sequentially activate others, thus establishing a hierarchy of caspases. A model has been proposed in which caspase-8 has been termed an 'initiator' protease, which activates an 'amplifier' protease such as caspase-1, which in turn activates a 'machinery' protease such as caspase-3 or caspase-7 (ref. 127). Most of the evidence for this model and the concept of a hierarchy of caspases is based on *in vitro* data with recombinant enzymes. It is important to determine where possible cascade of caspases occurs in cells undergoing apoptosis.

Apoptosis functions effectively in cancer prevention by interfering with the evolution of malignant cells. Most cytotoxic anticancer agents such as etopside and pacilitaxel cause cell death by inducing apoptosis in certain cell lines. The apoptotic elimination of drug-resistant cells amplifies the chemotherapeutic effects in responsive cells. Apoptosis is instrumental in destroying developing neoplasms and facilitating tumour regression. Several genes have been implicated in the apoptotic process, so it is possible to alter the expression or the function of these genes, either positively or negatively, both through pharmacologic and genetic intervention. In addition, the ICE family of proteases appears to be a principle component of the apoptotic trigger. Pharmacologic manipulation to activate these enzymes and/or, as yet undescribed, other regulatory enzymes may aid apoptosis induction.

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