INTRODUCTION
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1.1.0 What is apoptotic cell death?

Programmed cell death is a phenomenon where cell death occurs due to the intrinsic activation of a genetic death program. The view that some cells die as normal part of both development and homeostasis was established more than 40 years ago, and that in vertebrates, cell death is observed in all the tissues (Glucksmann, 1950). It is also believed that death is the default pathway, and cell survival is a dynamic superpositioning of the death program and survival signals (Raff, 1992).

The word 'apoptosis' was coined to describe cell death observed in thymocytes and in prostate gland due to castration in rats. The etymological origin of the word ‘apoptosis’ is from the Greek word for leaves falling off the trees in autumn. The first observed description of apoptotic type of cell death, in contrast to the necrotic cell death due to cellular lysis, was detailed by Kerr and Wyllie, in 1972. (One can distinguish ‘pathological type of cell death’, which is not part of the organism’s developmental program, as against ‘programmed cell death’ which is activated in accordance with the developmental program of organisms such as C.elegans. Apoptotic cell death can be defined as pathological cell death since it is not invoked as a part of development, even though the pathways may be similar or converging molecularly). Cell death is also observed in other invertebrates such as Drosophila, Manduca sexta, leeches and Hydra.

The morphological features of apoptotic cell death include membrane blebbing, nuclear disintegration into ‘apoptotic bodies’ and the cleavage of DNA into nucleosomal multimers, which resolves in an agarose gel electrophoresis as ‘DNA ladder’. The DNA ladder is considered as the hallmark of apoptotic cell death.
1.2.0 Invertebrate systems of cell death:

1.2.1 Caenorhabditis elegans:

Genetic evidence for programmed cell death was acquired from studies on an elegant organism, *Caenorhabditis elegans*, a free-living nematode. The organism has a fixed number of cells in its body (1090), and each precursor cell and its descendents can be observed microscopically, as the organism is transparent. It was observed that of the 1090 cells, 131 cells undergo cell death during development, each cell, precisely the same cell and at its own characteristic time. When viewed with Nomarski optics, the programmed cell death shares a characteristic morphology: the dying cells condense, become round, refractile and the corpses are degraded quickly. This process is generally completed within an hour after the birth of the cell (Ellis et al. 1991). Ultrastructural studies (Robertson et al 1982) reveal that the chromatin of the dying cell condenses, the cytoplasm contracts, membranous whorls form and autophagic vacuoles appear. Dying cells in both the embryo (Sulston et al 1983) and the larva (Robertson et al 1982) are engulfed by the neighbouring cells after which the dead cells fragment into membrane bound pieces that are degraded within the engulfing cells. However, engulfment is not the cause of cell death, since mutations that prevent engulfment do not prevent cell death (Hedgecock et al 1983; Ellis et al 1991).

Mutants for cell death, *ced-3, ced-4, ced-9, egl-2* were isolated where there was a defect in the death pathway which allowed the cell to defy its fate and survive (Ellis et al, 1991). The *ced-3* and *ced-4* (cell death defective) recessive mutations prevent these programmed cell deaths and many of the survived cells differentiate into recognizable cell types. Genetic mosaic experiments indicated that cells in which either of these genes is active, undergo programmed cell death while others do not (Yuan et al 1990). The 'undead' cell can adopt a specific cell
fate, differentiate as well as be functional. For example, mutations in egl-1 (egg-laying defective) gene causes programmed cell death of HSN neurons, two cells innervate the vulval muscle and are necessary for egg laying. When these deaths are prevented by ced-3 or ced-4 mutations, the surviving HSN neurons differentiate and control egg laying normally (Trent et al 1983; Ellis et al 1986). Thus, mutations in ced-3 and ced-4 seem to block cell death completely. Further, ced-3 and ced-4 are negatively regulated by ced-9. A dominant mutation in ced-9 prevents all cells from dying (Hengartner et al 1994) and mutations that inactivate ced-9 kill the animal. These observations suggest that ced-9 prevents cells that should survive, from undergoing programmed cell death.

The protein coded by ced-3 was found to be homologous to a mammalian protein interleukin-beta-1-converting enzyme (Miura et al 1993); while the ced-4 gene codes for a protein homologous to APAF1, which is a regulatory protein. These proteins are termed as caspases (cysteine aspartate proteases), and are the downstream effectors of the death program. These proteins are well conserved and fall in two categories: the initiator caspases and the executioner caspases. CED-3 is an executioner caspase (Bergmann et al 1998). Mutational analysis of the ced-3 gene by site directed mutagenesis indicated that the presumptive active-site cysteine of the CED-3 protease and aspartate residues at sites of processing of the CED-3 proprotein are required for programmed cell death in vivo. Of the 30 amino acid residues altered by ced-3 missense mutations, 29 are conserved with at least one other caspase, indicating that these residues define sites important for the function of all caspases. The worm homozygous for the ced-3 allele which has a deletion of the protease domain, incompletely blocked cell death, indicating that programmed cell death can occur independently of the protease domain (Shaham et al 1999). The protein coded by ced-9 is homologous to the mammalian bcl-2 gene and acts as a protector of cell death. From C.elegans system, it is
derived that the CED-9, CED-3, CED-4 proteins form a complex similar to the mammalian APAF1-pro-caspase-9-Bcl-2 complex (Bergmann et al 1998). The EGL-1 protein has a BH3 homologous nine amino acid region and acts as a death activator, upstream of ced-9 and physically interacting with CED-9 (Conrad et al 1998). There are other genes of *C.elegans*, ces-1 and ces-2 (cell death specification) acting upstream of egl-1. These mutations provide an insight into the problem of how the cells are marked for death. Dominant mutations in ces-1 prevent four of the pharyngeal neurons from dying and ces-2 appears to control these deaths by regulating ces-1. The data on the ces genes indicate that these could be cell-type specific, acting in some cells but not in others. Ces-2 encodes for a protein which has a basic helix-loop-helix motif, a leucine zipper and acts as a transcription factor, indicating that programmed cell death is controlled at the level of differential gene expression. It could also be envisaged that mammalian members, the bZIP proteins similar to CES-2 such as the proline and acid rich (PAR) family might have a role in cell-specific programmed cell death (Conradt et al 1998).

In the *C.elegans* adult hermaphrodite, there is massive cell death (of over 300 germ cells which have the potential to develop as oocytes and sperm). These cells use the same machinery of ced-3, ced-4 and ced-9, as in all the previously described 131 somatic cell deaths. These germline cell deaths are different from the somatic cell deaths in that they require the activation of ras/MAPK pathway and maintain the germline homeostasis (Gumienny et al 1999).

### 1.2.2 Pathological cell deaths:

Other mechanisms can also kill cells in *C. elegans*. For example: Two cells in the male are murdered by specific engulfing cells. Dominant mutations in the genes *lin-24* and *lin-33* cause
an abnormal form of cell death that might also require engulfment by a killer cell, since they can be reversed by concomitant mutations in *ced-2*, *ced-5* or *ced-10* genes which are involved in the process of engulfment initiation (Ellis et al 1991).

Dominant mutations in genes *deg-1* (degeneration) and *mec-4* (mechano-sensory abnormality) cause specific neurons to swell and form large vacuoles. These dominant mutations do not eliminate the *deg-1* or *mec-4* gene function, but result in abnormal proteins. The DNA sequence comparisons indicate that these proteins could be similar, but novel transmembrane receptors. In *deg-1* mutants, the neurons degenerate late in development, long after acquiring maturity and functionality. This late onset of neuronal degeneration is analogous to those seen in some of human neurodegenerative disorders. The fate of these neurons can be reversed if another gene *mec-6* is inactivated by a mutation, indicating that these deaths are controlled by a common pathway distinct from the *ced-3* and *ced-4* pathway (Chlafie et al 1990; Driscoll et al 1991).

1.2.3 *Drosophila:*

In *Drosophila*, a large number of cells die during embryonic and imaginal disc development as well as during metamorphosis (Truman et al 1992). These cells have characteristic apoptotic morphology resulting from the activation of caspase pathway (McCall et al 1997). Particularly, the *Drosophila* visual system is regulated by adjusting both the rate of cell proliferation as well as the rate of cell death through competitive interactions between developing cells in retina and brain (Steller et al 1987, Selleck et al 1991, Campos et al 1992). Cells which fail to form appropriate neuronal circuits in the *Drosophila* visual system, undergo cell death (Steller et al 1994). Mutation analysis indicated that the deletion Df (3L) H99 at the chromosomal location 75C1,2 was found to be virtually essential for all cell deaths occurring during normal embryogenesis (White et al 1994). It was also found that in the H99 mutant embryos, X-
irradiation could induce cell death, which was morphologically indistinguishable from that of the wild type. This observation indicated that the H99 deletion was upstream to the 'execution' genes and contained the 'activator(s)' of cell death genes. The molecular characterisation of the H99 mutation led to the identification of three genes, rpr (reaper), hid (head-involution defective) and 'grim'. All the three genes encode novel proteins and the deduced amino acid sequences revealed small regions of homology at the N-terminus (Grether et al 1995, Chen et al 1996). The N-terminal region can interact with the baculoviral IAP (Inhibitor of apoptosis) proteins. The RPR protein too exhibits weak homology to the mammalian 'death domain' of type I Tumor Necrosis Factor alpha Receptor (TNFRI) and Fas. The significance of this finding is not clear since the conserved region of the protein is not required for the cell killing activity of RPR. A detailed analysis of the rpr promotor revealed that there are distinct promoter elements, which are required for the regulation of rpr expression in response to different stimuli (Bergmann et al 1998); but in case of hid, the regulation is at the transcriptional as well as translational level. These three proteins seem to be differentially expressed in different cell types and could be cell type specific. Rpr and grim transcripts accumulate prior to apoptosis in a certain subset of neurons called the N4 cluster whereas hid transcripts do not (Robinow et al 1997). During metamorphosis, in response to the steroid hormone ecdysone, expression of hid and rpr are upregulated but the expression of grim is not (Jiang et al 1997). There is further evidence for the synergistic interaction between rpr and hid during apoptosis of midline glial cells (Zhon et al 1997).

Apoptosis of secondary and tertiary pigment cells, after laser ablation of the primary pigment cells in the compound eye, is blocked by simultaneous expression of an activated ras allele ras vl (Miller et al 1998). Also, overexpression of argos, a secreted inhibitor of the Ras signalling pathway, in post-mitotic cells resulted in induction of apoptosis (Sawamoto et al 1998). It has
been posited that the HID inhibiting activity of Ras is largely mediated by the activation of MAPK (MAP kinase), as the MAPK phosphorylation consensus sites in HID protein are critical for the inhibition (Bergmann et al 1998).

1.2.4 Drosophila IAPs:

The IAPs (Inhibitor of Apoptosis Proteins) were originally proteins of baculoviral strains which have the ability to functionally replace the p35 protein in blocking apoptosis (Hay et al 1995; Rothe et al 1995; Liston et al 1996; Duckett et al 1996; Uren et al 1996; Ambrosini et al 1997). Structurally all of these proteins share at least one, usually two or three, baculoviral IAP repeat (BIR) motifs and have a RING finger domain. One of the Drosophila IAP homologs, diap1, whose inactivation due to a loss of function mutation, enhances the expression of grim, hid and rpr and induced cell death. In contrast, overexpression of diap1 suppresses apoptotic cell death (Hay et al 1995). The second homologue diap2 has a similar function to diap1. Drosophila offers a unique model system to study some of the human diseases. Mutations in the rhodopsin or associated proteins can cause retinitis pigmentosa in humans, mice and a very similar pathological condition in flies (Chang et al 1993; Kurada et al 1995; Gregory-Evans et al 1998). In Drosophila, the retinal degeneration due to mutations in the rhodopsin and rhodopsin phosphatase genes was due to apoptosis and could be blocked by caspase inhibitors (Davidson et al 1998), which restored visual function to the otherwise blind flies. It meant that the 'undead' cells can be functional. Inactivation of the Drosophila homolog of tumor suppressor APC (Adenomatous Polyposis Coli) causes neuronal degeneration which results from apoptotic cell death, a condition remarkably similar to that found in the human germline mutations in APC. The degenerating neurons can be rescued by the expression of the caspase inhibitor, p35 (Ahmed et al 1998). Drosophila is a good model
system for glutamine repeat neurological disorders such as Huntington’s disease and Joseph-Machado disease. The polyglutamine expanded alleles of human genes expressed in *Drosophila* compound eye causes massive degeneration of photoreceptor neurons by apoptotic cell death (Jackson et al 1998; Warrick et al 1998).

1.3.0 *Vertebrate systems of cell death:*

1.3.1 *Cell death in thymocytes:*

Thymocyte cell death occurs characteristically on a large scale during the process of thymic education. Most thymocytes die because they express inappropriate T-cell receptors. In the process of clonal selection, thymocyte recognizing self-MHC bound to antigens are signalled to survive (Teh et al 1988; Sha et al 1988). Thymocytes that bear T-cell receptors capable of reacting strongly to self-antigens associated with MHC glycoproteins are removed by a process called negative selection or clonal deletion, which helps prevent autoimmune diseases (Kappler et al 1987; MacDonald et al 1988). Programmed cell death seems to play a major role in the maintenance of thymic homeostasis. The size of the thymus is normally regulated by hormones secreted by the adrenal gland. Removing the adrenal gland surgically causes the thymus to increase in size, while injection of corticosteroids causes it to regress (Perris et al 1970).

Thymocytes can be made to undergo cell death *in vitro* by co-culturing them with glucocorticoids, which act through a steroid receptor (Wyllie et al 1984). The morphology of this type of death is described as condensation of chromatin around the periphery of the nucleus, fragmentation of nucleoli into coarse osmiophilic particles, dilation of the endoplasmic reticulum under the cell membrane and cytoplasmic contraction. Other organelles such as the mitochondria remain unaffected. These changes in the cell during cell death are characteristic of what was termed as ‘apoptosis’ (Wyllie et al 1980). During such deaths there
is activation of an endonuclease that cleaves the DNA into 180 base pair (the size of one nucleosome) multimers (Wyllie 1980). This endonuclease is calcium dependent, and can be induced by increasing the levels of intracellular calcium (Cohen et al 1984; Wyllie et al 1984).

1.4.0 Genes and proteins involved in cell death pathway:

1.4.1 bcl-2

bcl-2 was discovered as a proto-oncogene in B-cell lymphomas which had arisen due to chromosomal translocation t(14;18). The lymphomas represent quintessential malignancies due to failed cell death rather than rapid cell division (Reed 1994; Zamzami et al 1998a). The bcl-2 gene is evolutionarily conserved and its homolog in C.elegans is ced-9, which implies that the death pathway too is conserved in evolution. This hypothesis was supported by the observation that bcl-2 was capable of rescuing partially, the ced-9 deficient worms (Hengarter et al 1996; Vaux et al 1992; Vaux et al 1996). The BCL-2 protein is a mitochondrial inner membrane protein, and has evolutionarily conserved domains termed as the BH (bcl-2 homology) domains. BCL-2 and members of its family have multiple mechanisms of action. Some of its members are known to be pro-apoptotic in function. Of the anti-apoptotic members, BCL-2 and BCL-xL have the ability to dimerise with other BCL-2 family member proteins, bind to non-homologous proteins, and lead to the formation of ion channels as well as pores (Reed 1997; Schendel et al 1998; Vaux et al 1996). BCL-2 and many of its homologs contain a hydrophobic C-terminal domain that results in the post-translational insertion of the protein into the mitochondrial membrane, nuclear envelope and endoplasmic reticulum (Krajewski et al 1993; Lithgow et al 1994; Yang et al 1995; Zha et al 1996b). It was also found that the BCL-2 protein was capable of dimerization with its pro-apoptotic relative BAX, and the decision of the cell to opt for death or survival depends on the ratios of BCL-2 and BAX proteins (Oltvai et al 1993). BCL-2 and BAX appear to have intrinsic effector
function of survival or death which do not depend on the interaction of these proteins with each other, since it was observed that the mutants of bcl-2 and bax which are incapable of forming heterodimers with one another retain their function of repressing or inducing cell death (Cheng et al 1996). Knockout studies on bcl-2 and bax alternatively indicated that BCL-2 was capable of its anti-apoptotic function without the requirement of BAX and BAX was capable of executing its pro-apoptotic function without BCL-2, indicating that dimerization of these two proteins is distinct and is not necessary for their respective functions (Hanada et al 1995; Ink et al 1997; Juergensmeier et al 1997; Kane et al 1993).

1.5.0 Receptors of cell death:

1.5.1 TNF Family of receptors and their ligands:

Homeostasis in mammalian cells is dependent on the continuous integration of cell survival and death signals from the extracellular environment that activate apoptotic or survival pathways in the intracellular milieu (Raff, 1992). Extracellular signals that activate apoptotic or survival pathways include peptide growth factors, cytokines, interleukins etc. Signals can be triggered by either cell-cell interactions or by soluble factors. One of the most intensive and well-studied family of receptors in apoptotic signalling is the TNFR superfamily (Bazzoni and Beautler, 1996). This family of proteins are multimeric transmembrane receptors that upon activation by their respective ligands can initiate either death or survival fates in the cell. The extracellular domains of TNFR family members are related while their intracellular domains differ extensively both in their amino acid sequence as well as in their propensity to bind to adaptor molecules and second messengers. The sub-family which contains receptors such as Fas, TNFR type I, and DR3, contain an intracellular conserved sequence region called the 'death domains' This term 'Death Domain' was first coined by Tartaglia et al 1993, as a result of deletion mutagenesis studies involving TNFRI (p55) mediated apoptosis. These domains
associate with a number of downstream molecules, which themselves contain death domains, and are used to transduce the signal. For example, the intracellular death domain of activated Fas receptor interacts with that of a linker molecule FADD/MORT-1 (Fas-Associated Death Domain containing protein) (Chinnaiyan et al 1995; Boldin et al 1995). Subsequent transduction of the signal is effected by another domain called the ‘Death Effector Domain’, in FADD, which interacts with additional downstream repertoire of molecules. One of the downstream molecule is caspase-8, which after activation by FADD can initiate a cascade of caspase activation, which leads to intracellular substrate degradation and cell death (Muzio et al 1996). TNFRI similarly induces cell death by interaction with a different but related molecule termed as TRADD (TNFR-Associated Death Domain containing protein) (Hsu et al 1996).

While activation of Fas, TNFR I and DR3 receptors induces apoptosis by associating with cytoplasmic factors such as FADD, TRADD and RIP, other receptors such as CD40, TNFR II, and CD30 promote cell survival by interacting with another distinct downstream adaptor molecules called as TRAFs (TNF Receptor Associated Factors) (Arch et al 1998).

About six mammalian TRAF proteins have been identified. Recruitment of TRAF2 protein to TNF receptors has been reported to promote survival by the activation of JNK (c-Jun N-terminal Kinase) and NF-kB (Nuclear Factor Kappa B) (Yeh et al 1997). A number of recent studies have linked NF-kB activation to cell survival pathway (Liu et al 1996; Beg and Baltimore 1996). Additional regulatory proteins are capable of modifying the signalling cascade downstream to TRAFs. These adaptor molecules include proteins such as A20, cIAPs (cellular Inhibitors of Apoptosis), TRIP (TRAF Interacting Protein) and 1-TRAF or TANK (TRAF family member Associated with NF-kB).
1.5.2 Fas:

Fas/CD95/APO-1 was identified independently by monoclonal antibodies anti-APO-1 or anti-Fas, which induced apoptotic cell death in malignant lymphocyte tumors expressing APO-1, which is also called Fas (Trauth et al 1989; Yonehara et al 1989). Cloning and sequence analysis of the protein indicated that it was a type I trans-membrane receptor belonging to the TNFR superfamily. Subsequently other family members capable of transducing apoptotic cell death were identified as TNFRI, DR3 (APO-3/TRAMP), DR4 (TRAIL-R1), DR5 (TRAIL-RII). These proteins share a common 68 amino acid domain called as the Death Domain (DD) in their cytoplasmic regions (Nagata et al 1995).

The genetic evidence for the existence of such a death receptor molecule came from the studies of homozygous lpr/lpr (lymphoproliferative disease) mice, which showed extensive lymphoproliferation, lymphadenaopathies and autoimmune disorders which were mapped to the Fas receptor (Watanbe-Fukunaga et al 1992); a different mutation with similar phenotype was identified as gld/gld (generalised lymphoproliferative disease). The gld mutation was mapped on chromosome 1, and identified to be the Fas ligand. The mutation was characterised to be a missense point mutation, phe to leu in the C-terminal tail of the FasL protein, which was well conserved in all the TNFR superfamily proteins (Takahashi et al 1994).

Fas Ligand is a type II transmembrane protein of the TNFR superfamily, is trimeric, and is effective either in the membrane bound or in the soluble form. The death signalling is effected when the ligand or an agonist antibody binds to the receptor, which results in oligomerization of the receptor molecules. This leads to the clustering of the C-terminal death domain (Itoh et al 1993) and rapid recruitment of the death domain containing adaptor molecule termed as FADD (Fas-associated death domain containing protein) (Boldin et al 1995; Stanger et al 1995; Chinnaiyan et al 1995). FADD in turn recruits pro-caspase-8 (also termed as FLICE or
MACH, Boldin et al 1996; Muzio et al 1996) through another of its domains termed as the Death Effector Domain (DED) or CARD (Caspase Recruitment Domain). CD95 along with FADD and pro-caspase-8 form what is termed as Death Inducing Signalling Complex (DISC) (Muzio et al 1996). Pro-caspase-8 is a proteolytic proenzyme which can be autoproteolytically cleaved to form the active Caspase-8 (Medema et al 1997), by induced proximity (Martin et al 1998; Muzio et al 1998; Yang et al 1998). The cleavage of pro-caspase-8 gives rise to two active peptide fragments p18 and p10 that are released into the cytosol (Muzio et al 1996), where they activate the pro-caspase-3 and other downstream caspases including caspase-6 and caspase-7 (Salvesen et al 1997).

An alternate pathway, which is mitochondria dependent, can be induced by the DISC. A small amount of pro-caspase-8 recruited to DISC cleaves the pro-apoptotic BH3 domain protein called BID (Scaffidi et al 1998a; Li et al 1998). The cleaved C-terminal product of BID, which is pro-apoptotic, translocates to mitochondria and causes clustering of the mitochondria around the nucleus followed by the release of cytochrome C. Cytochrome C in association with the Apoptotic Protease Activating Factor (APAF-1) initiates the processing of pro-caspase-9, which then activates caspase-3 and the downstream caspase cascade (Susin et al 1998). It was observed that there was a distinction between the cell types in activating either of the CD95 triggered apoptotic pathways. These cell types were denoted as Type I, which utilize the pathway that activates caspase-8 directly through DISC, followed by the activation of the downstream caspases; while the Type II cells were defined as those which activate pro-caspase-3 through mitochondrial damage, and cytochrome C release (Scaffidi et al 1998).

Experiments conducted to reveal the proteins involved in CD95 triggered apoptotic pathway using yeast-two-hybrid system led to the identification of several other proteins such as Receptor Interacting Protein (RIP) that can associate with CD95 through its death domain (Stanger et al 1995). RIP has been shown to activate caspase-2, through an adaptor molecule
Flow chart showing the non-mitochondrial caspase activation pathway during apoptotic cell death.
Death receptors

Pro-caspase-8

BCL-2

Active

Inactive

Mitochondrial caspase activation pathway during apoptotic cell death.
RAIDD (Duan et al 1997). Other events such as activation of the JNK kinase pathway (Wilson et al 1996) can be initiated by the binding of Daxx to the receptor, an interaction, which results in its binding to the death domain of Fas via its C-terminal end (Yang et al 1997). Overexpression of Daxx enhanced Fas mediated apoptosis. Further analysis of the Daxx protein revealed that this protein interacts with the N-terminal region of ASK1 (a MAP3K). Based on deletion mutagenesis, it has been proposed that ASK1 normally exists in an inactive state due to interactions of the N-terminal and C-terminal ends, which opens up in an active conformation, upon interaction with Daxx (Chang et al 1998). The activated ASK1 triggers a downstream phosphorylation cascade resulting in the phosphorylation of JNK, which in turn phosphorylates c-Jun. The mechanism adopted by c-Jun leading to apoptotic cell death is yet unclear (Baker et al 1998). Studies involving targeted deletions in mice, of FADD (Yeh et al 1998), caspase-8 (Varfolomeev et al 1998), caspase-9 (Hakem et al 1998; Kuida et al 1998) and acid sphingomyelinase (Santana et al 1996), indicate that the CD95 signalling through FADD-caspase-8 is probably the primary, non-redundant pathway, though different pathways could be followed by cells where caspase-8 is a limiting factor. It has been shown that FADD knock-outs are embryonic lethal in mice (Yeh et al 1998; Zhang et al 1998).

1.5.3 Inhibition of Fas induced apoptosis:

CD95 induced apoptosis can be blocked by various viral proteins, some of which have cellular homologs. CrmA (Cytokine response modifier A), a serpin from the cowpox virus (Ray et al 1992) and p35 from baculoviral strains (Xue et al 1995; Bump et al 1995) can prevent apoptotic cell death by acting as non-cleavable substrates of caspases. Another viral gene product termed v-FLIP (viral- FLICE Inhibitory Protein), and its cellular homologue termed as c-FLIP, both have two death effector domains (DEDs) and an N-terminal region which resembles caspase-8. c-FLIP lacks the amino acid residues which are in the catalytic domain of
caspase-8 and are essential for proteolytic activity (Irmler et al 1997). This protein functions by competing and preventing caspase-8 recruitment for DISC. Another protein named 'sentrin', was identified, which itself contains a death domain (DD) and binds to the CD95 death domain, preventing the recruitment of FADD (Scaffidi et al 1998b).

Recently, another protein called TOSO was identified which inhibited T-cell apoptosis, and probably inhibited caspase-8 by upregulating c-FLIP (Hitoshi et al 1998).

Other stimuli such as TGF-beta (Transforming growth factor-beta) are potent and rapid inhibitors of cell proliferation and induce growth arrest and apoptotic cell death (Hoffinan et al 1994, Arch et al 1998; Ashkenazi et al 1998).

1.6.0 Caspases:

Genetic analysis of programmed cell death in the nematode C. elegans led to the identification of two genes ced-3 and ced-4. Cloning of ced-3 and its characterisation indicated that it encodes a protein with homology to interleukin-1-beta converting-enzyme (ICE). This was the first evidence of a cysteine protease involved in the cell death process (Yuan et al 1993). Subsequently, many members of this family of proteases were identified. They form a family termed as CASPASEs (Cysteine Aspartate Proteases) (Alnemri et al 1996). These enzymes are synthesised in the cell as inactive precursors composed of four distinct domains: the N-terminal polypeptide or prodomain, a large subunit, a small subunit, and a linker region between the large and small subunits flanked by aspartate residues (reviewed in Nicolson and Thornberry 1997). Activation is effected when (either by autoproteolytic processing, or by proteolysis by other caspases) there is proteolytic processing between the domains resulting in the removal of the prodomain and linker regions, and the assembly of the two large and small subunits to form an active enzyme complex. Crystal structures of caspase-1 and caspase-3
bound to substrate analogs show that the active enzyme is a tetramer of two heterodimers with two catalytic sites. (Walker et al 1994; Wilson et al 1994; Rotonda et al 1996). Caspases are highly specific even though they are all common in that they require aspartate residues for cleavage. The substrate specificity is determined by a sequence of four amino acids amino terminal to substrate P1 site, and the P4 site which binds to a conserved site in the small subunit (Talanian et al 1994; Thornberry et al 1997).

Mammalian caspases are classified into two categories based on their hierarchy in the proteolytic cascade as initiator or upstream caspases, which have long prodomains and specific interaction modules such as DED or CARD (Boldin et al 1996; Muzio et al 1996) and which can interact with the specific receptors intercepting the death signal; and effector or downstream caspases which have shorter prodomains. Mammalian Caspase-1, -2, -4, -5, -8, -9, -10, -11, -12, -13 and the nematode CED-3 belong to the former category of initiator caspases, while caspase-3, -6, -7, -14 are the effector caspases. Further, initiator caspases have substrate specificities that are similar to caspase recognition sites present in their own sequence (Thornberry et al 1997), implying that the caspases can autocatalyze their own activation. Even though there is a large amount of data available regarding caspases, the specific role of each of these caspases is yet unclear. Targeted disruption studies in mice of some of these caspases provided an insight regarding their exact role. Targeted disruption of caspase-1, caspase-2, or caspase-11 has little or no effect on apoptotic cell death in vivo (Kuida et al 1995; Li et al 1995; Bergeron et al 1998; Wang et al 1998). In contrast, the disruption of caspase-3, caspase-8 or caspase-9 show significant effects (Kuida et al 1996; Kuida et al 1998; Woo et al 1998; Hakem et al 1998; Varfolomeev et al 1998). Mice deficient in caspase-8, which is linked to death receptors, exhibit abnormal heart development and die early during embryonic development (Varfolomeev et al 1998). Mutant mice deficient in caspase-9 or caspase-3 die in utero, and exhibit brain malformation due to decreased
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<th>CASPASE NOMENCLATURE</th>
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<td>Caspase-1</td>
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Table for caspase nomenclature
programmed cell death in the proliferative neuroepithilium (Kuida et al 1996; Kuida et al 1998; Woo et al 1998; Hakem et al 1998). It is known from in vitro studies that caspase-9 activates caspase-3 (Li et al 1997). The knockout data suggest that, in the developing brain, caspase-9 is an upstream activator of caspase-3. Studies on Mouse Embryonic Fibroblasts (MEFs) derived from the caspase-8 deficient mice indicate that this caspase is required for apoptotic cell death induced by TNF receptors, Fas and DR3, but not by treatment such as serum deprivation, ceramide and several chemo-therapeutic agents (Varfolomeev et al 1998). Similar studies in the caspase-9 knock-out background indicate that this caspase is important in mouse embryonic fibroblast apoptosis induced by dexamethasone, chemotherapeutic agents and Gamma-irradiation, but not when apoptosis is induced by death receptor stimulation (Kuida et al 1998; Hakem et al 1998). Caspase-3 and caspase-9 are not indispensable for the apoptotic cell death that occurs in T cells during thymic selection. Therefore, it appears that caspases can be redundant to some extent, but they can be necessarily activated depending on the cell type and the death stimulus.

Knock-out analysis of APAF-1, a mammalian homolog of CED-4, indicated that there were multiple developmental defects including brain hyperplasia, craniofacial alterations, eye abnormalities, persistence in inter-digital webs, some of which are similar to the caspase-9 knock-out abnormalities, and are consistent with the caspase-9-APAF-1 death pathway. However, the severity of abnormalities of APAF-1 knockout mice and the persistence of inter-digital webs were not observed in the caspase-9 knockout phenotype, implying that APAF-1 may be regulating other caspases as well (Ceconi et al 1998; Yoshida et al 1998).

The role of caspase-3 in classical apoptotic changes such as chromatin condensation, DNA cleavage, loss of mitochondrial potential, and formation of apoptotic bodies, was clarified in the caspase-3 knockout experiments, in which some cells fail to display chromatin condensation or DNA degradation while undergoing cell death in vitro (Woo et al 1998). Caspases, therefore,
seem to play a major role in the orchestrated dismantling of the cellular machinery. Caspase substrates, can therefore, be divided into two categories: either the regulators of apoptosis or the survival proteins, which are activated or inactivated by cleavage. These include downstream effector caspases, kinases such as MEK kinase1 Mst1, p21-activated kinase2/hPak65 which are all cleaved and activated by caspases resulting in positive feedback through JNK pathway (Cardone et al 1997; Widmann 1998a; Graves et al 1998; Lee et al 1997; Rudel et al 1997). Further, caspase-8 cleavage of BID, a pro-apoptotic protein triggers cytochrome C release from mitochondria, linking the death receptor and caspase-9-APAF1 pathways (Luo et al 1998; Li et al 1998). Similar cross-talk between two apoptotic pathways may occur due to the cleavage of effector caspase-3 by initiator caspase-8, which can trigger the proteolytic activation of caspase-9 (Stennicke et al 1998; Srinivasula et al 1998). Alternately, caspases may cleave proteins which are in the cell survival pathways such as phosphotidylinositol-3-kinase or AKT, RAF-1, Focal Adhesion kinase (Widman et al 1998b; Wen et al 1997; Graves et al 1998; Levkau et al 1998), as well as anti-apoptotic proteins such as CED-9, BCL-2, BCL-XL (Xue et al 1997; Cheng et al 1997; Clem et al 1998). Interestingly, the proteolysis of BCL-2 and BCL-XL produces a pro-apoptotic fragment, which can aid in the positive feedback to enhance the apoptotic signal.

Secondly, housekeeping or structural proteins whose breakdown can collapse the cell in an ordered manner, can be potential caspase substrates; for example, nuclear lamins, which form the major skeletal component of the nuclear envelope, are cleaved by caspase-6 during apoptotic cell death (Lazebnik et al 1995; Orth et al 1996; Rao et al 1996). Cytoskeletal proteins such as actin, alpha-fodrin, alpha-II- and beta-II-spectrins, as well as gelsolin act as caspase substrates (Mashima et al 1997; Cryns et al 1996; Nath et al 1996; Wang et al 1998). Proteins involved in DNA synthesis and repair such as the nuclear replication factor MCM3 and the human RAD5 have been identified as caspase substrates in cells undergoing apoptosis.
(Rheumae et al 1997; Ubeda et al 1997; Schwab et al 1998; Flygare et al 1998). Also, the nuclease DFF40/CAD, which is required for internucleosomal cleavage of chromatin, is activated by effector caspases by the proteolytic chewing of its inhibitor DFF45/ICAD (Liu et al 1997; Sakahira et al 1998). Mutant DFF45/ICAD resistant to proteolysis can protect the cell from nuclear condensation and internucleosomal fragmentation, but not from other morphological features of apoptotic cell death (Sakahire et al 1998; Liu et al 1998)

1.7.0 Oncogenes:

1.7.1 c-myc:

c-myc is a highly conserved protein with at least 90% homology, at the amino acid level between mouse and humans. The gene is controlled by two upstream promoters P1 and P2, which are 160 bases apart. The predicted molecular weight is 49kDa, but it has a higher anomalous resolution on an SDS-polyacrylamide gel, which is characteristic of phosphoproteins with high proline content. There is a long 5’ UTR (Un-Translated Region) of 400-500 base pairs without any initiation codons but has the termination codons. This region is conserved between mouse and humans with 70% homology, and with no significant homology between chicken and humans. However, there is 90% conservation between the coding exons. The first exon, which is also present in other myc-related genes is important for its regulation. There are quantitative differences rather than qualitative (in terms of mutations), between normal and tumor cells, and in their transcriptional as well as post-transcriptional regulation. Further, several lines of evidence suggest that cytoplasmic mRNA stability is the point at which regulation occurs. The levels of myc protein regulates the rate of entry of cells into S-phase, implying that the presence of the protein decreases the cycling time of the cells. Modulation of mRNA stability plays an important role in the regulation of c-myc RNA levels (Cole 1986).
c-myc is a transcription factor with basic, helix-loop-helix (HLH) motifs as well as leucine zipper (Z) domains (Blackwell et al 1990, Murre et al 1989; Prendergast and Ziff 1989) and has sequence-specific DNA binding property when bound with Max. Max is its heterodimeric partner, another basic, HLH, leucine zipper protein whose interaction is necessary for the physiological effects of myc such as cell proliferation, transformation and apoptosis (Amati et al., 1992; Kretzner et al 1992; Makela et al 1992; Mukherjee et al 1992; Prendergast et al 1992; Amati et al 1993; Gu et al 1993). However, alterations in Max do not appear to occur in malignant cells, even though abnormalities in the 14q23 region of the chromosome, where Max is located, are seen in some cancers (Wagner et al 1992). Further, c-myc belongs to the family of non-redundant myc proteins that also include other members such as N-myc, L-myc and B-myc. Myc genes seem to have appeared relatively late in the evolutionary time scale, since functional homologs have not been detected in organisms more primitive than Drosophila (Gallant et al, 1996; Schreiber-Agus et al, 1997). The gene is tripartite in its organization and has a C-Terminal domain (CTD), which includes the basic/ HLH/ Z with specific DNA sequence binding specificity to the CACGTG, the E box motif present in the target genes. CTD can interact with proteins other than MAX, such as YY-1, AP-2 BRCA-1, TFII-I and MIZ-I, which are all capable of influencing the binding of the protein with DNA (Luscher and Larsson 1999). The N-Terminal Domain (NTD) of myc contains the transcriptional activation domain. Two domains of 20 amino acids at the N-terminal region are conserved in all myc proteins and are denoted as myc box 1 and 2 (MB1 and MB2) and are crucial for its biological functions. The evidence that NTD (N-terminal domain) is required for transcriptional activation came from the experiments where, fusing heterologous DNA to the NTD, activated transcription (Kato et al 1990). Activation of transcription was thought to be important for the biological functions of myc proteins, but recent studies using RAT1 fibroblasts nullizygous for myc, (which are viable and replicatively competent though slow
The literature provides counter examples where under growth limiting conditions, c-myc can promote cell survival of B-lymphocytes (Wu et al. 1996a; Sonenshein et al. 1997) or differentiation of normal human keratinocytes (Gandarillas et al. 1997) illustrating the complex cell fate regulating aspects of c-myc. However, a clearer picture emerges from a large body of evidence indicating the role of myc proteins to promote both mitogenesis as well as apoptosis under growth limiting conditions.

c-myc is deregulated in at least one third of the cancers (Cole 1986; Kelly and Siebenlist, 1986; Spencer and Gourdine, 1991). Further, overexpression of c-myc is common in many malignant cancers including hormone independent adenocarcinomas of the breast and prostrate, wherein the prognosis is found to be generally poor (Berns et al. 1992; Borg et al. 1992; Hehir et al. 1993; Kreipe et al. 1993; Shiu et al. 1993; Jenkins et al. 1997). c-myc has a main role to play in cell proliferation and growth. Induction of myc is sufficient to drive quiescent cells into cell cycle (Eilers et al. 1989), and in some cases it is shown that inhibition of myc can block mitogenic signals and facilitate cell differentiation (Heikkila et al. 1987; Holt et al. 1988; Sklar et al. 1991; Sawyers et al. 1992; Hanson et al. 1994). The involvement of c-myc in apoptotic pathways was studied more recently wherein its expression was enforced and uncoupled from growth factor withdrawal. Thus, cells that contain normal c-myc levels, upon growth factor withdrawal, downregulate the c-myc expression and exit the cell cycle while the cells expressing c-myc undergo apoptotic cell death (Evan et al., 1992).

The first exon is present in the other myc family genes and is important for regulation. Further, there is evidence to indicate that the quantitative differences in the levels of c-myc expression exist between tumor and normal cells, and no mutation is required for tumorigenesis. There is a complex pattern of regulation at the transcriptional level as well as at the post-transcriptional
level. Another set of evidence suggests that regulation occurs at the level of mRNA stability (Cole 1986).

### 1.8.0 Signal transduction in apoptotic death pathways:

#### 1.8.1 Protein Phosphorylation:

Receptors on the cell surface can cause apoptotic cell death on being crosslinked by their ligands. As illustrated earlier, Fas is the most widely expressed and to some extent ubiquitous death receptor, which upon binding with its natural ligand or with an IgM monoclonal antibody, is capable of inducing apoptosis in the cell. Other types of death receptors are known, but are limited in their potential as death inducers depending on the cell types. These receptors belong to the TNFα superfamily. TNFα itself whose receptors (typeI and typeII) are related to Fas, can induce apoptosis in myelogenous leukaemia and other cancer cells (Schmid et al 1986) but acts as growth inducer in certain cell types such as fibroblasts (Vilcek et al 1986). Other members of the family such as the TRAIL receptor and APO-2, (Pitt et al 1996) are less well known and can have an implication in cancer therapy (Pan et al 1997; Sheridan et al 1997).

Other cell surface receptors such as the T-cell receptors and B-cell receptors, present respectively on T-cells and B-cells can cause apoptotic cell death, through a second receptor, in the absence of any co-stimulatory signal (Ivanov et al 1997; Valentine et al 1992; Musci et al 1997).

Tissue culture model systems and certain knockout systems began to identify the downstream signalling pathways, which were necessary and sufficient to commit the cell to apoptotic death. Certain kinases, when expressed in the cells were able to cause apoptosis.

Ser/Thr kinases are known to be important mediators of apoptotic cell death. Specific interactions of TNFR family proteins activate ser/thr kinase such as RIP (Receptor Interacting...
Protein), which in acts as an adapter molecule mediating diverse signalling pathways including NF-kB and cell death.

Other kinases related to RIP, such as RIP2 (which is a 61kDa protein kinase) have also been cloned. RIP2 contains an N-terminal domain, which has homology to ser/thr kinases, and a C-terminal caspase activation and recruitment domain (CARD), a homophilic interaction motif that mediates the recruitment of caspase death proteases. Overexpression of RIP2 is capable of NF-kB activation as well as cell death. The pro-apoptotic function of RIP2 was restricted to its C-terminal CARD domain, while the intact molecule was required for the activation of NF-kB (McCarthy et al 1998). Another related protein is RIP3 with extensive homology to RIP and RIP2; but unlike RIP which has a C-terminal death domain and RIP2 which has a C-terminal CARD, RIP3 has an unique C-terminus by which it can interact with RIP and get recruited to the TNFR I signalling complex. RIP mediated TNF-induced activation of the anti-apoptotic NF-kB pathway; but the binding of RIP3 to RIP attenuates the activation of NF-kB pathway. Overexpression of RIP3 is a potent apoptosis inducer (Sun et al 1999). Further, the proteolytic cleavage of RIP, by caspase-8 abrogates the stimulatory role of NF-kB activation. The mutant RIPD324A was less apoptotic, but its ability to activate NF-kB was greater than that of the wild type protein. Ectopic expression of the C-terminal fragment of RIP inhibited the activity of I-kB kinase beta (IKKBeta) which phosphorylates the inhibitor of NF-kB, called I-kB, and triggers its ubiquitin-mediated degradation, thereby acting on NF-kB. The C-terminal fragment also enhanced the association of the TNFR I and death domain proteins resulting in caspase-8 activation and a positive feedback loop (Kim et al 2000).

The kinase related to CD95/Fas/APO-1 receptor death signalling mechanism are RICK, that is composed of a N-terminal serine/threonine catalytic domain and a C-terminal caspase recruitment domain. RICK physically interacts with CLARP, a Caspase-like protein known to be associated with FADD protein and caspase-8, and promoted the activation of caspase-8,
potentiating cell death through FasL, FADD, CLARP, and caspase-8 pathway. Deletion mutational analysis indicated that both the kinase as well as the caspase recruitment domains are essential for apoptotic function. When lysine at position 38 of the ATP binding region of the protein, was replaced by methionine, RICK functioned as an inhibitor of CD95/Fas induced apoptosis (Inohara et al 1998).

Another kinase known as the ZIP kinase belongs to the ser/thr family of kinases; in addition to the N-terminus kinase domain, it has a leucine zipper at its C-terminus. ZIP kinase is shown to physically bind to ATF4, a member of the activating transcription factor/ cAMP response element binding protein (ATF/CREB) family, through the interaction of their leucine zippers. ZIP kinase is also capable of homodimerisation through its leucine zipper, a property necessary for its kinase activity. Further, this protein has been shown to localise in the nucleus. Expression of the catalytically inactive protein does not cause apoptosis. Interestingly, the kinase domain of ZIP kinase shows homology to the kinase domain of death associated protein (DAP kinase) and could represent a novel family of kinases mediating the death signal (Kawai et al 1998).

It has also, been shown that Fas engagement with its ligand activates all major signalling pathways that belong to the family of mitogen-activated protein kinase (MAPK) pathways, by either caspase-dependent or independent mechanisms. Phosphorylation based signals serve as potent modifiers of Fas receptor induced responses, especially extracellular signal-regulated kinase (ERK) and the phosphoinositide-3-kinase pathways are known to be important regulators. Signalling through phosphorylation also regulates the expression of Fas receptor and FasL as well as various proteins that affect the outcome of the receptor stimulation. Although phosphorylation is considered important in the Fas/FasL signalling pathway, the targets, molecular mechanisms and the biological significance of this aspect requires further elucidation (Holmstrom et al 2000).
AK-5 tumor as the model system:

AK-5 is a rat histiocytoma with the property of spontaneous rejection and regression when injected subcutaneously in syngeneic Wistar rats (Khar, 1986), and was characterized by histological, biochemical, and physiological methods (Khar et al 1990). The AK-5 is shown to have markers such as Fc receptors, C3d receptors, MHC class II antigen, leucocyte common antigen (CD45) and MO1 antigen. Enzymes such as lysozyme, non-specific esterase, acid phosphatase and peroxidase were shown to be present. Electron microscopic analysis revealed the presence of a large eccentric kidney-shaped nucleus in the tumor cell, and along with the above cited properties indicated that AK-5 belongs to the macrophage type of cells. Further tests revealed that AK-5 cells demonstrated phagocytosis which vindicate the classification of this tumor in the macrophage class. The tumor is passaged as ascites in Wistar rats and is highly immunogenic. As stated earlier, it has the capacity of spontaneous regression, by day 14 post-transplantation of the tumor subcutaneously, and the process is completed by day 25. Such animals, which have rejected the tumor are rendered immune, and do accept further challenges. This property of the tumor makes it an interesting system to study the molecular as well as immunological aspects of tumor rejection. So far, it is known that AK-5 tumor regression is a consequence of mechanisms such as ADCC (Antibody Dependent Cellular Cytotoxicity) (Khar, 1993), necrosis and NK dependent apoptosis (Khar et al, 1997).

This study was undertaken to investigate the role of apoptosis and some of its pathways in AK-5 regression. The serum collected from animals, which have completely regressed the tumor, and which have been challenged again with the tumor intra-peritoneally was referred to as anti AK-5 antiserum. Anti AK-5 antiserum was capable of inducing apoptotic cell death in vitro in AK-5 cells. This is a unique system, where there is spontaneous regression of a syngeneic tumor, and one of its mechanisms is apoptotic cell death. This is also the first study, where it is shown that antiserum against the tumor causes apoptotic cell death of the tumor.
The hypothesis, thus was that there were factor(s) in anti AK-5 antiserum, which were responsible for the induction of cell death in AK-5 cells. The project focused on the purification and characterization of such factor(s), their downstream signalling pathways. It was found that the apoptotic factor(s) present in anti AK-5 antiserum were the antibodies, generated against the tumor by the host immune system, which act as direct inducers of apoptotic cell death. It was also observed that the death receptor and its initial signalling pathways are different from the ones known in literature, which converge at the caspases. This study, therefore, could prove useful in understanding the various mechanisms of apoptotic cell death and its relevance in anti cancer therapy.