Regulation of caspase activation in apoptosis: implications for transformation and drug resistance

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Abstract

Recent developments in the apoptosis field have uncovered a family of cysteine proteases, the Caspases, that act as signalling components as well as effectors of the cell death machinery. Caspases are constitutively present as inactive precursors within most cells and undergo proteolytic processing in response to diverse death-inducing stimuli to initiate the death programme. Active caspases can process other caspases of the same type as well as process caspases further downstream in the pathway that ultimately leads to collapse of the cell. This cellular collapse is thought to occur as a consequence of caspase-mediated cleavage of a diverse array of cellular substrates. Regulation of entry into the death programme is controlled at a number of levels by members of the Bcl-2 family, as well as by other cell death regulatory proteins. Recent data has shed light upon the mechanism of action of these regulatory molecules and suggests that the point of caspase activation is a major checkpoint in the cell death programme. Because many transformed cell populations possess derangements in cell death-regulatory genes, such as *bcl-2*, such cells frequently exhibit elevated resistance to cytotoxic chemotherapy. Thus, a deeper understanding of how apoptosis is normally regulated has therapeutic implications for disease states where the normal controls on the cell death machinery have been subverted.

Introduction

Apoptosis, the dominant mode of cell death that is invoked to eliminate excess, aged or damaged cells, is currently the focus of intense research activity. Accumulating evidence indicates that apoptosis is under complex molecular control and that disruption of these controls may underlie various disease states such as neurodegeneration, autoimmunity and cancer (Martin and Green, 1995a; Thompson, 1995; Nagata and Golstein, 1995). The molecules that act as regulators and effectors of apoptosis are currently being uncovered at a frenetic pace. The picture that is emerging is one in which a newly discovered family of proteases – the Caspases – are the key effectors of the cell death machinery (see Salvesen and Dixit (1997) for a recent review). The activities of the caspases are regulated post-translationally – typically being produced as inactive precursor proteins – and are also regulated

by a variety of caspase-inhibitory proteins that can neutralize the proteolytic activities of active caspases.

Other work in this area suggests that many events that result in cellular transformation affect genes that are involved in the regulation of the cell death machinery (see Martin, 1997 for an overview). Much evidence now indicates that oncogenes cooperate with dysregulated cell death genes to achieve the fully transformed state (Wyllie *et al.*, 1987; Strasser *et al.*, 1990; Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992; Reed, 1997a; Amarante-Mendes and Green, 1997; Bellamy *et al.*, 1997).

Here, we provide an overview of recent developments in the apoptosis field with particular emphasis on the role that the caspases play in the cell death process. The role that dysregulation of the apoptosis machinery plays in transformation and the implications that this has for the phenomenon of drug resistance are also discussed.

Apoptosis: A Mechanism for the Selective Removal of Aged, Infected, Damaged or Excess Cells

The morphological and functional aspects of apoptosis have been reviewed exhaustively (Wyllie *et al.*, 1980; Ellis *et al.*, 1991; Raff, 1992; Steller, 1995; Jacobson *et al.*, 1997) and for this reason we will only briefly touch upon this topic. In essence, apoptosis represents a mode of cell death that enables multicellular organisms to eliminate cells, at will, in a safe and efficient manner. Cells targeted to undergo apoptosis are typically cells that are only required for a limited period of the animal's life-span, cells that have suffered injury, that have become infected, or have simply reached the end of their useful life. While it is readily appreciated that very significant numbers of cells die during development – during the sculpting processes that shape organs or form cavities, for example – it is often less readily appreciated that cells die on an incredible scale in mature organisms.

For example, billions of new erythrocytes are released from the bone marrow into the circulation every day, and it follows that a similar number must be eliminated to make way for these new arrivals. In a similar manner, millions of neutrophils are also produced and eliminated daily and the cells of the skin and gut epithelia also undergo constant renewal. Within the adaptive immune system, activated T and B lymphocytes clonally expand to produce many millions of effector cells upon each encounter with foreign antigen. While a proportion of these cells are retained as 'memory' cells to help fight future infections, clearly most are quickly eliminated (by apoptosis) since T and B cell numbers are maintained at relatively constant levels throughout our lives.

The huge scale of this ongoing cell death would present a major problem for multicellular organisms were it not for the existence of a mechanism that prevents the escape of cellular constituents from dying cells and ensures safe removal of the dead cell corpses (Savill *et al.*, 1993). Apoptosis represents such a mechanism and appears to have evolved in primitive multicellular organisms (Ameisen, 1996). There are also indications that a related programmed cell death mechanism is present in many unicellular eucaryotes, although genetic evidence for this has yet to be found (Ameisen, 1996).

Significantly, the phenotypic changes that occur during numerous instances of programmed cell death, from nematodes to man, are strikingly similar. This

suggests that the molecular events that underlie these events have been preserved through evolution. Advances in the field over the past 3–4 yr have confirmed this suspicion and have identified an array of gene products that appear to have evolved for the sole purpose of killing the cells in which they are expressed when called upon to do so.

Switching on the Death Machinery

Apoptosis is under complex molecular control and can be triggered by diverse signals. Many of these signals are physiological and are used to exert tight control over cell numbers in multicellular organisms (Raff, 1992). For example, many cells depend upon growth factors for their continued survival (for historical reasons the term 'growth factor' has been applied to factors that confer survival as well as factors that confer growth, however, it should be noted that a survival factor does not necessarily promote growth). By limiting the supply of such factors, tight control can be exerted over cell numbers since cells that fail to receive sufficient quantities of survival factor(s) will engage their cell death machinery (Raff, 1992, 1996). This sets up a competition between cells that sets the cell number at a steady-state level, unless the supply of growth factors are increased or decreased. An example of such a situation is found in the developing nervous system where there are initially many extra neurons and oligodendrocytes – the cells that myelinate them – produced. As neural networks are established, neurons that fail to synapse with others are progressively lost via apoptosis, as are those that fail to obtain sufficient neurotrophic factors (Raff *et al.*, 1993).

A particularly important physiological trigger of apoptosis in man and other mammals is the Fas ligand (FasL) molecule (Nagata and Golstein, 1995; Nagata, 1997). FasL is constitutively present on the surface of a relatively restricted range of cells, most notably on a subset of cytotoxic T lymphocytes, but can be upregulated on many others. FasL triggers apoptosis by binding to the Fas receptor, a molecule that is present on many cell types within the immune system, as well as in other tissues. Fas is a member of a growing family of related cell surface receptors (the TNF/NGF receptor family) many of which appear to be involved in the regulation of cell death and survival (Nagata and Golstein, 1995; Nagata, 1997). Binding of FasL to Fas results in clustering of this receptor and the propagation of a death signalling cascade into

Figure 1. Schematic representation of the proximal portion of the Fas signalling pathway that culminates in apoptosis. (A) The Fas receptor molecule is a type I transmembrane protein, the cytoplasmic portion of which contains a region (called the Death Domain; DD) that is involved in propagating death signals. (B) Upon clustering of neighbouring Fas receptors via the trimeric Fas ligand molecule (FasL), the FADD (Fas-Associated protein with Death Domain) adaptor protein binds to the death domain of Fas, probably due to a conformational change in the Fas cytoplasmic tail. (C) Recruitment of FADD to the receptor complex promotes binding of caspase-8 (also called FLICE/MACH/Mch5) to FADD due to homology between the Death Effector Domain (DED) of FADD and regions within the prodomain of the caspase-8 molecule. Clustering of caspase-8 molecules results in their proteolytic processing (probably via autocatalysis or adjacent molecules acting *in trans*) and liberation of the active caspase-8 enzyme to initiate the cascade of proteolytic events that produce the apoptotic phenotype.

the cell (Trauth *et al.*, 1989; Yonehara *et al.*, 1989; Suda *et al.*, 1993). Clustering of Fas initiates the death cascade due to recruitment of the inactive pro-form of one of the caspase family (caspase-8) to the plasma membrane via the adaptor protein, FADD (Boldin *et al.*, 1996; Muzio *et al.*, 1996; Kischkel *et al.*, 1995; Medema *et al.*, 1997; Nagata, 1997). Membrane-bound caspase-8 molecules then become activated and set up a cascade of proteolytic events that culminate in death of the cell (Figure 1). The FasL/Fas system is involved in regulating T and B cell lifespans, peripheral deletion, immune privilege, a component of cytotoxic T cell killing, as well as many other instances of programmed cell death (Nagata, 1997).

Many non-physiological stimuli (such as DNA-damaging drugs and UV-irradiation) also appear to engage the Fas/FasL pathway, typically by stimulating the upregulation of FasL on cells that already

express the Fas molecule (Herr *et al.*, 1997; Sheard *et al.*, 1997). This results in an autocrine death signal where the newly-expressed FasL engages Fas on the cell surface, resulting in death. Because many tumors arise due to DNA damage, this suggests that mutations that disable the Fas pathway are likely to favor tumor growth.

Cell Division and Death: Striking a Balance

The simple fact that we do not continue to grow throughout our lives suggests that at some point during our development into mature adults the cells within our tissues either receive signals to stop dividing, or the rate of cell death within dividing tissues competes on more or less equal terms with the rate of cell division. This seems obvious enough, so it is all the more remarkable therefore that the role of cell death

in the maintenance of cell populations at equilibrium was practically ignored until recent years.

Clearly, the size of a dividing cell population is dictated by the rates of death as well as division of its constituent cells. If the rate of cell death exceeds the rate of cell proliferation, involution will occur, conversely if proliferation outstrips cell death a population expansion will result. The rate of cell death, like division, is dictated by many factors, both extra- as well as intracellular. These factors interact with an array of gene products that either induce or facilitate apoptosis, or conversely, block or delay it. These inducers or repressors of apoptosis are thus perfect targets for mutagenic events that result in a transformed phenotype since disruption of their function will affect the life-span of the cell.

It is well known that net growth rates of tumors generally do not correlate well with the rate of division of the cells within the tumor – as assessed by the abundance of mitotic figures – suggesting that rates of cell death and/or other factors (such as cell migration) play a significant role in dictating the tumor growth rate (see Toft and Arends, 1997 for a review of this topic). Accumulating evidence now suggests that dysregulated cell death regulatory genes are very commonly found in transformed cell populations. Indeed, much evidence also points to the fact that defects in such genes may be essential for tumor development. Since this topic has been reviewed extensively in recent years (see Martin, 1997 and references therein), only one example of a cell-death regulatory gene with co-transforming properties will be presented here. Before doing so, it should be noted that somatic mutations that result in overexpression of proteins that can block or repress apoptosis would not in themselves be expected to be transforming events since a cell possessing this type of mutation would simply live longer. However, extending the lifespan of a cell increases the probability of it acquiring a second mutation. In the case where a second mutation affects a proliferation-related gene, such mutations would be expected to have cotransforming properties, and indeed this has been found to be the case (Wyllie *et al.*, 1987; Strasser *et al.*, 1990; Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992). Similarly, if a mutation in a cell death regulator enhances the normal lifespan of a cell type that is normally rapidly dividing, a marked population expansion would be expected to result.

Bcl-2: A Potent Repressor of Apoptosis

Bcl-2 was originally discovered at the breakpoint between chromosomes 14 and 18 that is characteristic of many B cell lymphomas (Tsujimoto *et al.*, 1985). Wild-type Bcl-2 is typically expressed at constitutively low levels in many tissues. The t(14;18) translocation results in a massive upregulation of Bcl-2 expression in B cells since this translocation places the *bcl-2* gene under the control of the IgH promoter-enhancer at 14q32. Initially, *bcl-2* was thought to be a proliferation-related oncogene, however, it was subsequently established that Bcl-2 was in fact a potent repressor of many forms of apoptosis (Reed, 1997a).

Mice transgenic for a *bcl-2*-Ig minigene which mimics the t(14;18) rearrangement seen in follicular lymphoma initially develop a polyclonal lymphoid hyperplasia as a result of an accumulation of B cells in these animals (McDonnell *et al.*, 1989). However, these mice then go on to develop clonal malignant B cell lymphomas (after ~15 months), which in many cases was found to be due to secondary rearrangements of *c-myc* (McDonnell and Korsmeyer, 1991). Thus, cells with elevated levels of Bcl-2 in these animals exhibited abnormally long lifespans and were therefore available for a second mutagenic event. Additional mutations involving proliferation-associated oncogenes facilitated progression of the hyperplasia to a much more aggressive malignant phenotype. Direct evidence that *bcl-2* and *c-myc* can cooperate in lymphomagenesis was provided by experiments where mice transgenic for *c-myc* were crossed with *bcl-2* transgenic mice (Strasser *et al.*, 1990). Mice carrying both transgenes developed tumors much more rapidly than either of the parental strains alone.

In man, a similar derangement of Bcl-2 expression contributes to the development of follicular (non-Hodgkin's) B cell lymphomas. This type of lymphoma is characterized by elevated levels of Bcl-2 protein, resulting in failure of B lymphocyte death, with the result that these cells accumulate. In this type of malignancy there appears to be no change in proliferation rates (Reed, 1997a). Fortunately, this type of lymphoma usually responds well to chemotherapy. Elevated levels of Bcl-2 expression have also been found to correlate with the progression of prostatic cancer from a treatable androgen-dependent state to a much more aggressive androgen-independent one (McDonnell *et al.*, 1992). Bcl-2 protein levels are also elevated in many other malignancies, possibly due to loss of p53 function in these cases (p53 is a transcrip-

tional repressor of the *bcl-2* gene). Rough estimates indicate that 50% of human cancers may possess abnormally high levels of Bcl-2, implying an important role for this protein in either the development and/or the maintenance of malignancy.

Unmasking the Molecular Face of Death

The fact that apoptosis is under molecular control suggests that it may be possible to modulate the rate or extent of apoptosis in disease states where apoptosis is the dominant mode of cell death. Similarly, in disease situations where a failure to engage the cell death machinery is contributory (as appears to be the case in many tumours), it may be possible to restore sensitivity to agents that would normally trigger death in these cells. Thus, intensive scrutiny of the cell death process has begun and has yielded many of the key players in this process in a remarkably short time.

The current evidence suggests that the core of the cell death machinery is comprised of a family of proteases called caspases (previously called the ICE/CED-3 family proteases), that cleave their substrates after aspartate residues (Martin and Green, 1995b; Alnemri *et al.*, 1997; Salvesen and Dixit, 1997; Nicholson and Thornberry, 1997; Villa *et al.*, 1997). Caspases were originally discovered due to their homology with a gene – *ced-3* – that was known to be required for all of the developmental-related programmed cell deaths in the nematode *C. elegans* (Yuan *et al.*, 1993). To date, eleven human caspases (cysteine **as**partate-specific proteases) have been identified and these proteases appear to comprise a complex proteolytic system, rather like the clotting or complement systems (Martin and Green, 1995b). Stimuli that trigger apoptosis all appear to do so by activating the caspases. Activated caspases then promote activation of other members of the caspase family and proteolysis of a number of cellular proteins, all of which contributes to the controlled collapse of the cell (Figure 2).

Due to the catastrophic consequences for the cell, caspase activation appears to be a tightly-regulated process. Much effort is currently directed towards understanding how caspases become activated at the onset of apoptosis and how spontaneous activation of these proteases is normally prevented.

Caspases: Agents of Cellular Destruction

Caspases are normally present in healthy cells in inactive configurations (zymogens) and undergo proteolytic processing during activation (Darmon *et al.*, 1995; Schlegel *et al.*, 1996; Duan *et al.*, 1996; Martin *et al.*, 1996; Slee *et al.*, 1996; MacFarlane *et al.*, 1997; Harvey *et al.*, 1997). The eleven caspases that have been identified to date all exhibit the same basic structure – an N-terminal prodomain that is discarded during activation, followed by a large and a small subunit, both of which are required for proteolytic activity of the mature enzyme (Figure 3). Two large and two small subunits associate to form the active tetrameric form of the enzyme which possesses two active sites (Rotonda *et al.*, 1996). Residues within both the large and small subunits combine to form the substrate-binding pocket, explaining the requirement for both subunits for enzymatic activity. The sequence QACXG is conserved within the large subunit of all caspases. The cysteine within this sequence which corresponds to Cys 285 in caspase-1 (also known as interleukin-1 β converting enzyme) is involved in catalysis along with His 237 and Gly 238, which are also absolutely conserved in all family members.

Caspases typically recognize tetrapeptide (P4-P3-P2-P1) motifs in their substrates (e.g. DEVD, YVAD, DEAD), the scissile bond occurring between the P1 amino acid residue and the adjacent C-terminal amino acid in the peptide chain (Villa *et al.*, 1997; Nicholson and Thornberry, 1997). Caspases have an absolute requirement for aspartate at the P1 position, however, it is the residue at the P4 position that is the most critical in determining the substrate specificity of the individual caspases (Thornberry *et al.*, 1997).

The caspases can be broadly classified into two groups on the basis of the lengths of their prodomain regions. Several caspases have long prodomains while the remainder have short prodomains. The prodomain region seems to regulate the targeting and activation of the caspases by facilitating the association of these caspases with other proteins. During conversion of the caspases from their inactive to their active forms the prodomain is removed along with a linker region between the large and small subunits (Figure 3). In all cases to date, these internal cuts occur adjacent to an aspartate residue. This suggests that activation of caspases during apoptosis occurs via a protease cascade – with those caspases possessing long prodomains forming the apex of the cascade. Upon activation of the upstream proteases by recruitment to death-inducing

Figure 2. Schematic representation of the triggering, signalling, effector, and clearance phases of apoptosis. The proximal events in the scheme are likely to be disparate since numerous stimuli can culminate in apoptosis. However, events distal to activation of the caspases are likely to be highly similar given the striking phenotypic similarities between apoptotic cells, irrespective of the nature of the stimulus that initiated the death programme.

Figure 3. Scheme depicting conversion of a caspase pro-form to its active heterodimeric form. At least two proteolytic cleavage events are required to convert inactive caspase zymogens to their active forms. These cleavage events typically occur at caspase consensus-cleavage sites, strongly suggesting that caspases are processed by other caspases (or other non-cysteine aspartases such as granzyme B) or via autocatalysis. The QACXG motif depicted is completely conserved between all of the known caspases, from nematodes to man.

signaling complexes, these caspases proceed to activate – via proteolytic processing – the ‘effector’ caspases, which in turn act upon cellular substrates to effect apoptosis (see Figure 2).

Caspase Substrate Proteins

The list of identified substrates of the caspase family is growing rapidly (Martin and Green, 1995b; Nicholson and Thornberry, 1997; Slee *et al.*, 1998). These substrates represent an eclectic group of proteins although most can be put into one of three categories; those that have a structural role, those that function as regulatory or signaling enzymes, and those that are involved in DNA repair or gene expression. The consequences of these cleavage events are still the subject of speculation at present but it is likely that several of these directly result in some of the gross phenotypic as well as biochemical changes (such as plasma membrane blebbing, nuclear envelope collapse, nuclear fragmentation) that take place during apoptosis. For example, a recently characterised dimeric protein, DNA fragmentation factor (DFF; Liu *et al.*, 1997), is a substrate of caspase-3 that when cleaved acts to initiate the internucleosomal degradation of DNA – a classical hallmark of apoptosis. The murine homolog of this protein – called caspase-activated deoxyribonu-

clease (CAD) – has also recently been cloned (Enari *et al.*, 1998). Studies with CAD have conclusively demonstrated that this enzyme becomes activated during apoptosis as a consequence of caspase-mediated inactivation of its associated inhibitor (ICAD).

The actin-associated protein gelsolin is also targeted by caspases at the onset of apoptosis, the consequences of which appears to be the extensive plasma membrane blebbing that is commonly seen during this process (Kothakota *et al.*, 1997). It is thought that cleaved gelsolin acts to sever actin filaments independently of Ca^{2+} , provoking destabilization of the actin cytoskeleton.

Thus evidence is accumulating to link specific caspase-mediated proteolytic events with the stereotypical destructive changes that have long been known to take place in the cell during apoptosis. The overall picture is one where the cell is coordinately dismantled in preparation for recognition and removal by phagocytes.

Foiling the Death Signal: Modulating Caspase Activation

Evidence to date suggests that the point of no return in the death programme is the stage at which the caspases become explosively activated. The vast majority of

agents that block apoptosis appear to do so prior to the point of caspase activation. Broad spectrum caspase inhibitors that act downstream of caspase activation failed, in some instances, to block apoptosis (Xiang *et al.*, 1996; McCarthy *et al.*, 1997). These inhibitors did however succeed in blocking several features of apoptosis, and death in this context appeared to be necrotic rather than apoptotic in nature. While this suggests that the commitment point in the death pathway is upstream of caspase activation, it is very likely that the caspase inhibitors used in these studies did not efficiently neutralize the activities of all of the caspases activated during apoptosis or that the sheer concentration of caspases activated within the dying cells rendered the inhibitors ineffective.

Viruses appear to have evolved much more effective caspase inhibitors that can completely block apoptosis and some of these have been very useful in dissecting the cell death pathway. For example, the cowpox virus encodes a serpin (serine protease inhibitor), CrmA, that can act as a pseudosubstrate for several of the caspases (Zhou *et al.*, 1997). Unlike regular caspase substrates, CrmA fails to be displaced from the caspase active site after undergoing proteolysis, with the result that the activity of the bound caspase is neutralized (Zhou *et al.*, 1997). CrmA can effectively block apoptosis initiated by a multitude of agents, suggesting that caspase activity is indeed essential for death to proceed (Gagliardini *et al.*, 1994; Tewari and Dixit *et al.*, 1995). A functionally similar pseudosubstrate, p35, is encoded by the insect baculovirus *Autographa californica* (Clem *et al.*, 1991). This protein can also bind to and neutralize several of the caspases and has been reported to block death in a number of contexts (Clem *et al.*, 1991; Xue and Horvitz, 1995; Bump *et al.*, 1995). Caspase inhibitory proteins (the vFLIPs) have also recently been found in herpesviruses (Thome *et al.*, 1997). The presence of such proteins in a number of viruses suggests that the ability to repress the host cell death machinery is of significant benefit to viruses, probably because the length of the viral replication cycle can be increased since the host cell cannot undergo apoptosis.

Many cellular proteins that can repress the cell death apparatus are also known. The most well known of these is Bcl-2, the gene product that was discussed earlier in the context of cellular transformation. Bcl-2 is the archetype of a whole family of Bcl-2-related proteins that share regions of sequence similarity (Reed, 1997a,b). Interestingly, while several of the Bcl-2 family behave in a similar manner to Bcl-2 in that they

exhibit potent cell death inhibitory properties (e.g. Bcl-XL, Bcl-w, Mcl-1, A1, BRAG, Ced-9), almost as many again (Bcl-Xs, Bax, Bad, Bik, Bak, Bid) can promote apoptosis, possibly by interfering with the activities of the death inhibitory members of the family.

Recent data suggests that Bcl-2 and other proteins within this family may operate at several points in the death pathway (see Reed, 1997b for a recent review). First, Bcl-2 may repress death by binding to and neutralizing the death-promoting members of the family such as Bax. Second, there is a body of evidence to suggest that Bcl-2 and Bcl-XL may regulate mitochondrial permeability to one or more factors (such as cytochrome c) that have potent death promoting activity (Yang *et al.*, 1997; Kluck *et al.*, 1997). Third, downstream of cytochrome c release, Bcl-2 and/or Bcl-XL may bind to and neutralize the activities of a recently discovered caspase-activating molecule, APAF-1, that requires cytochrome c as a co-factor (Li *et al.*, 1997). Finally, it has also been reported that Bcl-2, Bcl-XL, and the nematode Bcl-2 homologue, Ced-9, can act as caspase substrates (Xue and Horvitz, 1997; Cheng *et al.*, 1997).

This 'Swiss Army Knife' model of Bcl-2 function may seem unlikely at first sight given the relatively small size (26 kDa) of the Bcl-2 protein. However, strong support for each of these separate functions is now accumulating and the idea that the Bcl-2 family of proteins may be able to interrupt the death signal at several points in the death cascade is an appealing one, especially when one considers the finality of the outcome for the cell if the death signal is successfully propagated.

Other cellular regulators of apoptosis have also been found. These include, cFLIP, an inactive caspase-like molecule that acts as antagonist of caspase-8, preventing recruitment of this caspase to the FADD molecule during Fas-initiated cell death (Irmeler *et al.*, 1997). In addition, X-linked Inhibitor of Apoptosis Protein (X-IAP), a potent apoptosis repressor protein has recently been found to act as a direct inhibitor of a number of caspases (Devereaux *et al.*, 1997).

The pattern that is emerging is strikingly consistent. The majority of cell death regulatory molecules either directly target the caspases (by acting as pseudosubstrates) or act just upstream of this point by antagonizing caspase activation.

Implications for Transformation and Drug Resistance

It is now well established that the majority of existing chemo- and radiotherapeutic agents eliminate cells by triggering apoptosis (Martin and Cotter 1990, Lennon *et al.*, 1991; Miyashita and Reed, 1993; Lowe *et al.*, 1993; Toft and Arends, 1997; Reed, 1997a). When apoptosis is not observed it suggests that the cells within the tumor carry a mutation in the apoptosis pathway. In these cases, necrosis is often observed. For this reason, the occurrence of necrosis instead of apoptosis within a tumor is usually a bad prognostic indicator as the tumor is likely to be more difficult to treat (i.e. requiring higher doses of therapeutic agents or a more prolonged period of therapy which the patient may not be able to tolerate). Pathologists and oncologists have known this for some time, but the underlying reason for this was unclear until the importance of apoptosis in the whole equation was finally appreciated.

Because gene products that regulate apoptosis (e.g. Bcl-2, p53, Bcr-Abl, Ras) are commonly involved in cellular transformation, new information concerning how these proteins regulate entry into the cell death programme should have a significant impact on cancer therapy. Prior to the realisation that defective cell death regulatory genes had potent cotransforming properties, cancer was largely thought of as a disease of cellular proliferation. Consequently, the majority of drugs used to treat malignancies targeted aspects of the cell division machinery (nucleotide precursor synthesis, DNA unwinding, DNA repair, chromosome segregation). While these drugs have proved useful, it is likely that therapies that are targeted at death regulatory proteins as well as regulators of cell division will prove much more successful.

Strategies aimed at short-circuiting the components of the death machinery that negatively regulate apoptosis include designing small molecules that can disrupt the function of Bcl-2 and related proteins that repress apoptosis. Because Bcl-2 exerts its effects on the cell death programme in a number of ways, several different approaches can be envisioned. Molecules that disrupt Bcl-2's ability to abrogate the death promoting activities of Bax (and related death-promoting proteins) by heterodimerization could be designed (these would bind to the BH1 and BH2 domains of Bcl-2), as could drugs that bind to the BH4 domain which is likely to be involved in distinct survival-promoting functions of this protein. Another

approach, albeit possibly a more difficult one, would be to use antisense oligonucleotides or ribozymes targeted to Bcl-2 mRNA to drive down Bcl-2 protein levels (even transiently) in an effort to open a window of susceptibility to established cytotoxic drugs. Other potential ways in which Bcl-2 levels could be manipulated within a tumor would be to use biological response-modifiers such as retinoids that have been previously shown to influence Bcl-2 expression levels. The use of such drugs, in combination with existing therapeutics, may very significantly improve the prognosis for patients with tumors that are currently refractory to chemotherapy.

The simple fact that gene products involved in restricting entry into the cell death programme are over-expressed in tumors makes these molecules doubly attractive as potential targets in cancer therapy. Drugs that drive down expression levels or interfere with the function of cell death repressor proteins would not only increase the susceptibility of the tumor to chemotherapeutics, they may even result in spontaneous death of cells within solid tumors due to the harsh environment that typically exists within this type of tumor. Restoration of normal levels of cell death regulatory proteins may overcome the block in the cell death pathway that facilitated growth of the tumor in the first instance.

Tumors that have suffered inactivating mutations in genes that encode apoptosis-inducing proteins (such as p53) are a somewhat more difficult proposition and may require restoration of p53 function using gene therapy, for example. An indication that this approach could be feasible has been provided by reports that liposome or retroviral-mediated delivery of p53 to tumor cells resulted in the restoration of sensitivity to chemotherapeutic agents (Fujiwara *et al.*, 1993; Lowe *et al.*, 1993; Fujiwara *et al.*, 1994; Lowe *et al.*, 1994).

Conclusion

The pace of discoveries in the apoptosis field has been truly breathtaking in recent years. As our understanding of the cell death machinery deepens it is anticipated that new ways of tackling diseases where defects in apoptosis regulation play a role will emerge. Pharmacological agents that can manipulate apoptosis-sensitivity within malignant cells are expected to provide a potent new weapon in the armory of medical oncologists and should significantly expand the growing list of treatable malignancies.

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