Springer Semin Immunopathol (1998) 19:279–288

Springer Seminars in Immunopathology

Targeting cancer cell death with a bcl-x_S adenovirus

Jeffrey S. Han[1], Gabriel Núñez[2], Max S. Wicha[1], Michael F. Clarke[1]

[1] Department of Internal Medicine, University of Michigan Medical School, 1500 E. Medical Center Dr., Bldg. Room 4–303, Comprehensive Cancer Center, Ann Arbor, MI 48109, USA
[2] Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109, USA

Introduction

Recent advances on the molecular basis of cancer have indicated that transformation is not solely a matter of increased cellular proliferation [60]. It has become widely accepted that more than one genetic alteration is necessary for a cell to become cancerous [80]. This is due to built-in cellular mechanisms that can detect abnormalities and activate genetic programs to remedy them, making single adverse events less likely to cause significant problems. For example, tumor suppressor genes are best known for their ability to halt cell cycle progression and allow time for repair and maintenance of genomic integrity [83]. Malfunction of this system constitutes a second defect that can lead to malignant transformation [80].

More recently, a second protective mechanism known as apoptosis has been identified as important in deleting potentially dangerous cells from an organism [28]. Programmed cell death (PCD) is a genetic program that initiates a series of events resulting in cell suicide. This process leads to apoptosis, a morphologically distinct form of death with physical characteristics such as nuclear fragmentation, membrane blebbing, and DNA degradation [86]. Apoptosis plays a vital role in the normal development of an organism. Its many functions include regression of the tadpole tail in frogs [44] as well as the removal of self reactive lymphocytes in the formation of mammalian immunity [72].

DNA damage and the improper expression of oncogenes have been shown to induce programmed cell death [28]. This is presumably a safeguard against cancer. Genetic defects that permit a cell to constitutively block apoptosis confer a selective growth advantage to that cell. Thus, it makes sense that this is an essential step in the progression to cancer. The discovery and characterization of several apoptosis modulators have verified that this is indeed often the case. Improper regulation of these genes can increase cell survival and provide tumor resistance to traditional forms of cancer treatment (radiation and chemotherapy) that function by activating PCD [32]. This generally leads to poor clinical prognosis.

Fortunately, these same pathways that block cell death represent a potential target for the rational design of new therapies. In this review, we will introduce the factors

Correspondence to: M. F. Clarke

regulating apoptosis in cancer and discuss possible areas for intervention. We will also provide an example of such intervention, in which an adenovirus is used to deliver a pro-apoptotic gene, *bcl-x$_S$*.

Programmed cell death

PCD is best understood in the nematode *Caenorhabditis elegans*, where the fate of all 1090 cells have been tracked. Of these cells 131 undergo cell death during development, and genetic studies revealed the master controls behind this phenomenon. A gain of a function mutant of the *ced*-9 (*ced* for cell death abnormal) gene caused all 131 cells that are normally eliminated to survive, while loss of function *ced*-9 mutations caused more than the usual 131 cells to die [38]. In contrast to *ced*-9, the *ced*-3 and *ced*-4 genes cause cell death [27]. In *ced*-4;*ced*-9 and *ced*-3;*ced*-9 double mutants all cells live, which suggests that Ced-3 and Ced-4 are cell death effectors, and Ced-9, which functions upstream, is an antagonist of these proteins. It is known that Ced-4 binds to Ced-3 resulting in activation of the caspase [42, 85]. Ced-9 binds to Ced-4, blocking its ability to activate Ced-3 [73, 85]. This apoptosis machinery has been well conserved throughout evolution. We now know that Bcl-2 family members are mammalian homologues of Ced-9 [39] and the caspases (or ICE proteases) are homologues of Ced-3 [90]. No homologue of Ced-4 has yet been identified, but the apparent interchangability of these proteins between nematode and mammalian systems has facilitated the ordering of these genes [14, 15, 84].

Improper growth stimulation leads to apoptosis

The expression of key genes is involved in the signal to proliferate. Due to their mitogenic properties many of these are proto-oncogenes and have been implicated in human carcinogenesis, since uncontrolled growth is one of several steps along the path towards cancer. Recent evidence suggests that inappropriate expression of these genes leads not only to proliferation but to activation of the PCD pathway.

Deregulated expression of the c-*myc* gene occurs in up to 30% of human cancers [61]. In normal cells, expression of the c-*myc* proto-oncogene is rapidly up-regulated when a cell initiates proliferation [2]. Although all of the functions of c-Myc are not completely understood, it is a transcription factor that, when highly expressed, is able to overcome growth arrest and to block differentiation [5, 6, 25, 62]. Interestingly, c-Myc, whose expression is so tightly linked with cell growth, has also been shown to induce cell death. When c-Myc is highly expressed in cells that are deprived of growth factor, they undergo apoptosis [29]. Therefore, the consequences of c-Myc expression depends on the context of other proliferative signals.

A similar story holds true for the c-*fos*/*jun* proto-oncogenes. Their protein products associate to form a transcription factor called activator protein-1 (AP-1) [17]. Like c-Myc, AP-1 is induced upon mitogenic stimulation and appears to be involved in mediating cell cycle progression [1]. c-Fos and Jun expression have also been correlated with PCD in response to unfavorable growth conditions or cell injury [10, 23]. Further implicating c-Fos in apoptosis were experiments demonstrating that ectopic expression of c-Fos led to cell death under conditions in which cells were normally quiescent [63]. The converse experiment (inhibition of c-Fos expression) increased cell survival under

conditions which normally led to mass apoptosis [23]. This supports the hypothesis that c-Fos somehow regulates apoptosis.

At first these two opposing functions of both c-Myc and AP-1 may appear to be contradictory. However, closer scrutiny reveals that induction of apoptosis by growth effectors may be an important safety means by which proliferation can be halted if such factors are expressed at inappropriate times. This can selectively eliminate cells with potentially carcinogenic alterations, which often result in proliferation regardless of the external signals provided by their environment. The mechanism by which this takes place is as yet unknown. It is possible that the proto-oncogenes that induce proliferation also inherently produce a continuous death signal that can only be stopped under favorable growth conditions. For example, certain growth factors apparently function to inhibit the cell death pathway [12, 21, 34]. On the other hand, apoptosis may be the result of conflicting growth and quiescence signals within the same cell.

In addition, some tumor suppressor genes also function by regulation of PCD. One of the most common abnormalities in human malignancy is a mutation of the *p53* tumor suppressor gene [79]. Up to 50% of human cancers harbor such mutations. p53 exerts its tumor-suppressing effects in two ways: cell cycle regulation and apoptosis. p53 controls the cell cycle through transcriptional activity. Putative p53 DNA binding sites have been identified and can direct mRNA synthesis upon activation of p53 [43, 64]. Several genes have thus far been shown to be under the direct transcriptional control of p53. One of these, *p21* (also called *waf1* or *cip*-1) is activated by p53 in response to cellular damage [26]. Elevated levels of p21 can cause growth arrest by binding to cyclin-Cdk complexes and inhibiting kinase activity [51]. In addition, p53 has been implicated in the repair of radiation-induced DNA damage and transcriptional repression [49, 70].

Perhaps more importantly, p53 has been shown to induce apoptosis under several conditions. Sometimes the simple restoration of p53 in a transformed cell is enough to cause cell death [89]. In another example, mouse thymocytes lacking p53 are resistant to apoptosis caused by radiation and various forms of chemotherapy, while p53-positive cells die when treated in the same way [18, 52]. This suggests that p53 is a major downstream effector of current methods of cancer treatment.

The ability of p53 to induce apoptosis usually does not require transcription, and some p53 mutants that cannot bind DNA and stimulate RNA synthesis are still able to activate the cell death pathway [36, 82]. Therefore, in some systems the apoptotic function of p53 can be separated from the cell cycle regulation, which does require transcription. However, it should be noted that p53-mediated apoptosis is not always transcription independent. Some studies indicate that p53 promotion of cell death can be transcription dependent [67]. This is further supported by the finding that the Bcl-2 family member (see below) Bax, which is pro-apoptotic, has a promoter containing p53 binding sites and is transcriptionally activated by p53 [55].

Members of the bcl-2 family regulate apoptosis

The *bcl*-2 gene was discovered at the breakpoint of a t(14;18) translocation that commonly occurs in B cell lymphomas [20, 76]. Bcl-2 was shown in culture to suppress apoptosis normally induced by a variety of factors including growth factor withdrawal, γ-irradiation, and chemotherapeutic drugs [58, 69]. The protective effects of Bcl-2 are not universal, as T cell deletion still occurs in the presence of Bcl-2 overexpression

[69]. When constitutively expressed in transgenic mice, Bcl-2 led to an accumulation of B cells [53]. The expansion of the B cell population was not due to enhanced cell proliferation but to decreased cell death. The enhanced B cell life presumably allowed secondary genetic abnormalities to accumulate, and eventually ended in lymphoma. Further investigation demonstrated that Bcl-2 knockout mice were subject to increased apoptosis and loss of mature lymphocytes [78]. Nevertheless, these mice were able to survive through development, implying a functional redundancy with respect to Bcl-2.

During the past few years several homologues of Bcl-2 have been identified. Members of this family of proteins contain one or more of four Bcl-2 homology (BH) regions termed BH1, BH2, BH3, and BH4. Bax, Bad, Bak and Bik negatively regulate apoptosis, apparently by antagonizing Bcl-2 [8, 16, 31, 46, 59, 87]. Another Bcl-2 family member, Bcl-x, can be present in one of two forms depending on how the primary RNA transcript is spliced [7]. The larger of the two Bcl-x proteins, Bcl-x_L, contains all four BH regions and exhibits the highest homology to Bcl-2. In culture Bcl-x_L displays remarkable similarity to Bcl-2 in ability to block apoptotic response to a range of external signals. The smaller protein, Bcl-x_S, contains BH3 and BH4 regions and can actually accelerate apoptosis in certain situations, such as cytokine withdrawal from interleukin-3-dependent cell lines. In addition, Bcl-x_S abrogates the protective functions of both Bcl-2 and Bcl-x_L. The relative levels of Bcl-x_L and Bcl-x_S appears to be an important factor in cell survival.

The actual mechanism by which Bcl-2 family members carry out their actions is an area of intense investigation. The carboxy terminus of Bcl-2 contains a hydrophobic transmembrane domain that localizes Bcl-2 primarily to the outer mitochondrial membrane [48]. Removal of this targeting domain from Bcl-2 and related family members either abolishes or diminishes protective activity, which implies that membrane localization is important for Bcl-2 function [57, 75]. It is likely that Bcl-2 acts by inactivating cell death effectors such as a mammalian version of Ced-4 [15, 84]. In other theories Bcl-2 has been postulated to act by controlling the cytoplasmic level of intercellular species such as p53, Cdks, cytochrome *c*, Ca^{2+}, or reactive oxygen species [3, 41, 47, 50, 54, 66, 88]. How all of these processes relate is still poorly understood.

Apoptosis regulators – who is really in control?

So far we have discussed the c-Myc, AP-1, p53, and Bcl-2 proteins in separate contexts. However, it is obvious that proteins playing such critical roles in the cell must have some degree of interdependence. It is unlikely that there would be so many autonomous apoptosis pathways. A more likely scenario is the existence of multiple ways in which apoptosis can be triggered, all of which converge upon a group of central regulators.

p53 has been shown to activate an apoptosis program not only in response to damage caused by external agents, but also in response to internal cellular dysfunction. This raises the question of whether improper expression of genes such as c-*myc* and *fos*/*jun* induce apoptosis in a p53-dependent fashion. Evidence exists that this is the case. c-Myc-induced apoptosis is not apparent in several cell lines devoid of functional p53, but is restored upon the introduction of wild-type p53 [40, 82]. Similar experiments have shown that this p53 dependence also holds true with c-Fos [63]. Therefore, it appears that p53 acts downstream of Myc and Fos and at least in some cases is an intermediate through which the Myc and Fos cell death signals act.

Bcl-2 and Bcl-x_L can inhibit both c-Myc- and p53-induced apoptosis [4, 30, 33, 68, 77, 81]. This, in addition to the multitude of other death signals that can be antagonized by Bcl-2, suggests that the Bcl-2 family acts downstream of most apoptosis effectors and is one of the final resorts in stopping PCD. Because alteration of Bcl-2 family regulation can block most forms of apoptosis, this represents an efficient manner in which cells could become transformed. Thus, it is logical that Bcl-2 family members might play a role in many cancers. It is currently thought that in up to 60% of all cancers, apoptosis is inhibited through overexpression of a Bcl-2 family member [11, 13, 22, 24, 71].

Therapeutic targeting of apoptosis pathways in transformed cells

Our increased understanding of the hierarchical ordering of apoptosis regulators may be useful in targeting treatment of transformed cells. Since it appears that the majority of cell death pathways converge and are under the control of Bcl-2/Bcl-x_L, negative regulators of Bcl-2 and Bcl-x_L would probably allow a cell to act upon apoptotic signals. The delivery of such a gene to cancerous cells would relieve the protection provided by elevated expression of an apoptosis inhibitor. High expression alone might kill the cells, and lower expression levels could increase cellular sensitivity to radiation or chemotherapy.

To test this hypothesis, our laboratory constructed a recombinant, replication-incompetent adenovirus vector expressing the *bcl-x_S* gene, a functional inhibitor of Bcl-2 and Bcl-x_L [19]. This vector was able to efficiently introduce the gene into a wide variety of cell lines and deliver high levels of expression. As expected, virtually all epithelial-derived transformed cell lines that we have tested to date are killed by the *bcl-x_S* virus via apoptosis. Cancer cells derived from patients with neuroblastoma, kaposi's sarcoma, and breast, colon, ovarian, and head and neck cancers all undergo apoptosis when cells express high levels of Bcl-x_S protein [19]. This is true both for primary cancer cells and established, transformed cell lines. Low level expression of Bcl-x_S sensitizes cells to both chemotherapy [74] and radiation therapy. On the other hand, normal fibroblasts and hematopoietic stem cells are relatively resistant to *bcl-x_S* adenovirus-induced apoptosis.

The use of an adenovirus to deliver *bcl-x_S* has potential clinical utility. Results from our laboratories indicate that resistance of hematopoietic stem cells to the virus is at least in part due to the inability to infect such cells. High-dose chemotherapy and autologous hematopoietic stem cell transplantation is increasingly being used to treat both breast cancer and childhood neuroblastoma [35, 45]. Unfortunately, the autologous stem cells used to rescue the patient from lethal doses of chemotherapy are frequently contaminated with cancer cells [9, 37, 65]. The selective killing of the cancer cells by cytotoxic adenovirus vectors makes such agents ideal for eliminating cancer cells contaminating the stem cells collected for re-infusion. Studies from our laboratories have demonstrated the feasibility of this approach. The *bcl-x_S* adenovirus was able to eliminate 1.5×10^4 cancer cells contaminating 10^6 normal bone marrow cells, whereas the normal human hematopoietic stem cells exposed to the virus were still capable of engrafting the bone marrow of SCID mice [19].

This virus may also be useful for the treatment of cancers in other settings. Because non-replicating viruses will only diffuse for limited distances in solid tissues, they can best be delivered to cells in a cavity. Two diseases in which this virus may be useful

are bladder and ovarian cancer. Early bladder cancer arises in the bladder and ovarian cancer initially spreads in the peritoneal cavity. Furthermore, early bladder cancers are superficial tumors which arise focally or diffusely initially penetrating only a few cell layers of the bladder luminal epithelium. Similarly, early in the course of ovarian cancer, or after initial chemotherapy, there are often microscopic foci of tumor cells remaining. In both of these cases, it is quite possible that all of the cells can be infected and killed by an adenovirus vector.

One area of concern is whether normal, non-transformed cells will be adversely affected by the introduction of Bcl-x_S overexpression. Circumstantial evidence from our laboratory indicates that the virus will preferentially kill transformed cells. This suggests that Bcl-x_S itself does not cause apoptosis. Rather, Bcl-x_S may function by allowing other stimuli to initiate an apoptotic pathway without the interference of Bcl-2 family members. Since transformed cells, by virtue of their genetic lesions, are more likely to deliver these signals than their normal counterparts this may explain the selectivity of Bcl-x_S-mediated killing. This mechanism will only become clearer when we better understand the mode of Bcl-x_S action and the role of the Bcl-2 family in survival of normal and transformed cells.

Summary

Transformation is a complex cellular process that requires several genetic abnormalities. In many cases, one of these abnormalities is an inhibition of PCD, which provides a selective advantage for tumor cells. This has been recently shown in an in vivo model, where overexpression of Bcl-x_L is a crucial step in the progression from hyperplasia to neoplasia and is accompanied by a significant decrease in tumor apoptosis [56].

Frequently, overexpression of a member of the Bcl-2 family results in a block in cell death and appears to nullify many built-in cellular defense mechanisms against cancer. Such a block presents a problem because radiation and chemotherapy, standard cancer treatments, ultimately exert their effect by induction of apoptosis and would also be made less effective. Therefore, to better treat cancer it may be necessary to develop novel methods to overcome the effects of the Bcl-2 family. One way to approach this problem is to target the cause – the molecular machinery that allows a cancer cell to survive. Advances in our understanding of apoptosis has identified the Bcl-2 family as a mediator of most apoptosis pathways, including those initiated by oncogenes, tumor suppressor genes, growth factor withdrawal, and external damaging signals. Therefore, functional inhibition of Bcl-2 family members is lethal to many cancer cells. Using gene transfer technology, we can now deliver genes that accomplish this goal. Further investigation will reveal whether this translates to improved therapy in the future.

Acknowledgements. This work was supported by grants 5 R55 CA67140-02, CA70057, and 5R01 CA61777-04 from the National Institutes of Health; by Department of Defense grant 941407, and by a grant from the U.S. Army Medical Research Command DAMD17-96–6019.

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