APOPTOSIS AND PREDISPOSITION TO ORAL CANCER

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ABSTRACT: The term apoptosis, also known as programmed cell death (PCD), was coined by developmental biologists a number of years ago to describe a form of cell death characterized by several unique morphological and biochemical features. Genetic studies of the round worm *Caeneorhabditis elegans*, a simple multicellular organism, first revealed apoptosis to be an integral part of the developmental program. Subsequently, the importance of apoptosis in higher organisms was demonstrated in several eukaryotic systems. In mammals, apoptosis is widespread during embryogenesis and in adult tissues. It is required for normal tissue homeostasis and for clonal selection in the immune system. In both developing and adult organisms, apoptosis plays a central role in reinforcing appropriate cellular patterns and in regulating cell number by eliminating cells that are harmful or no longer needed. It is becoming increasingly clear that disruption in the apoptosis pathway can contribute to the development of a number of developmental, inflammatory, degenerative, and neoplastic diseases. The effector arm of the apoptotic program includes members of the Bcl-2 gene family that function as either death agonists or death antagonists. These proteins participate in an elaborate genetically controlled biochemical pathway that functions to maintain tissue and organ homeostasis and serve as a critical defense mechanism to guard against malignant transformation. Cancer is the result of a series of genetic lesions that include activation of oncogenes and inactivation or loss of tumor suppressor genes. Several groups of investigators have observed that deregulated expression of oncogenes can subvert apoptotic pathways, resulting in prolonged cell survival. In pathological settings such as cancer, members of the Bcl-2 gene family are able to synergize with oncogenes and tumor suppressor genes to transform cells. In this review, we describe the process of apoptosis in mammalian cells and define the role and biochemical pathways through which the Bcl-2 gene family induce and/or protect cells from apoptosis. Last, we will discuss the evidence which suggests that alterations in this pathway may play a central role in tumorigenesis by allowing genetically damaged cells normally destined for elimination to persist, predisposing them to additional mutations and driving them to malignancy.

Key words. Apoptosis, Bcl-2, carcinogenesis, carcinogens, initiation, oncogenes, oral carcinoma, programmed cell death, promotion, transformation, tumor suppressor genes.

The Apoptotic Program

Apoptosis is a tightly regulated process designed to remove superfluous, damaged, or senescent cells from an organ or tissue (Glucksmann, 1951; Kerr *et al.*, 1972; Wyllie, 1980; Arends and Wyllie, 1991; Collins, 1991; Goldstein *et al.*, 1991; Williams, 1991; Koli and Keski, 1992; Sen, 1993). Induction of this endogenous "suicide" program is triggered by a variety of environmental signals such as DNA or protein damage, toxins, growth or nutrient factor withdrawal, and ligand binding (Ijiri, 1989; Barry *et al.*, 1990; Muschel *et al.*, 1991; Bazar and Deeg, 1992; Hardin *et al.*, 1992; Hickman, 1992; Potten *et al.*, 1992; Raff, 1992; Waters, 1992; Manchester *et al.*, 1993; Nagata, 1994, 1997; Venkatatachalam *et al.*, 1993). This process (Table 1), which occurs over several hours or days, is characterized by cytoskeletal disruption, cell shrinkage, condensation and margination of chromatin, activation of endonucleases, and cellular fragmentation into small membrane-bound structures termed apoptotic bodies, that are phagocytosed by macrophages or neighboring cells (Barinaga, 1994). The DNA degradation that occurs is a two-step process that involves separate pools of endonucleases (Pandey *et al.*, 1994). The first step is initiated by endonuclease activity at interloop sites in chromatin and leads to DNA fragments in the 50- to 300-kilobase range. The second stage of fragmentation is catalyzed by calcium/magnesium-dependent endonucleases. It results in internucleosomal DNA cleavage and production of multimers of 180-200 base pairs that can be visualized as a "ladder" on agarose gels (Walker and Sikorska, 1994; Pandey *et al.*, 1997). All apoptotic cells undergo DNA fragmentation; however, not every cell type undergoing apoptosis exhibits internucleosomal DNA cleavage. The first pool of endonuclease activity is sufficient for cell death (Walker *et al.*, 1994).

DNA fragmentation can be reliably detected at the single-cell and tissue level by means of the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling technique, also known as the TUNEL

TABLE 1

Comparisons between Apoptosis and Necrosis

Necrosis	vs.	Apoptosis
Morphological Changes		
Loss of membrane integrity		Membrane blebbing without loss of membrane integrity
Random fragmentation of chromatin		Aggregation and margination of chromatin
Cellular swelling		Cellular shrinkage
Cell lysis		Formation of apoptotic bodies
Swelling and disintegration of organelles		Persistence of apoptotic bodies
Biochemical Changes		
Loss of ion homeostasis		Genetically controlled activation of enzymes
Passive process (no energy requirement)		ATP-dependent process
Random digestion of DNA (DNA smear on agarose gel)		Generation of non-random oligonucleosomes of DNA (ladder pattern on agarose gel)
Late post-lytic DNA fragmentation		Early pre-lytic DNA fragmentation
Physiological Significance		
Death of large contiguous groups of cells or organ segments		Death of individual or small groups of cells
Evoked by pathological stimuli		Evoked by physiological stimuli
Phagocytosis by macrophages		Phagocytosis by macrophages or neighboring cells
Acute inflammation		No inflammation

assay (Gavrieli *et al.*, 1992). As an indicator of cell death, it detects 3'-OH DNA ends generated by DNA fragmentation that are known to occur early in cells destined to die by apoptosis. The technique is useful for identifying cells in the early stages of apoptosis by labeling intact apoptotic nuclei and apoptotic bodies with large DNA fragment breaks as well as single-stranded breaks. The technique involves a template-independent addition of deoxyuridine triphosphates to the 3'-OH ends of double- or single-stranded DNA with either blunt, recessed, or overhanging ends that is catalyzed by the enzyme TdT. Depending on the chromogen/substrate used to detect the labeled 3'-hydroxyl ends, one can readily identify strand breaks in nuclei and apoptotic bodies in either cell suspensions or tissue sections. This approach has been shown in most instances to correlate with other morphological and biochemical features of apoptosis. However, a word of caution should be noted. While DNA fragmentation is certainly an indicator of apoptosis, it is also a feature of cell replication. During cell replication, DNA single-strand breaks are required to uncoil the double helix for binding of enzymes necessary for replication (Bohr *et al.*, 1986). Thus, the identification of apoptotic cells by the TUNEL assay in the absence of other cytopathic or biochemical changes indicative of apoptosis should be interpreted with guarded confidence. The potential for erroneous interpretation of the TUNEL assay data was recently demonstrated by Wrone-Smith *et al.* (1997). These investigators reported that despite the presence of TUNEL-positive keratinocytes derived from psoriatic plaques, psoriatic keratinocytes were observed to be resistant to the induction of apoptosis as measured by electrophoretic detection of DNA fragmentation (DNA ladder). This demonstrates that one should not rely on a single assay for assessing apoptosis.

A loss of mitochondrial function is also an important feature of apoptosis, and several lines of evidence suggest that the integrity of mitochondrial membranes and changes in cytochrome c efflux have a profound influence on the subsequent biochemical events that lead to cell death (Adachi *et al.*, 1997; Kluck *et al.*, 1997; Yang *et al.*, 1997). This chronic, genetically regulated, and predictable form of cell death typically involves single cells or small groups of cells and does not provoke an inflammatory response. This is in distinct contrast to cell necrosis, which results from acute toxic injury. This form of cell death usually involves large contiguous groups of cells and major segments of an organ or tissue. Typically, cellular necrosis is associated with rapid cellular swelling due to the inability of cells to maintain ion homeostasis. As a consequence, the membrane integrity of organelles is severely compromised early in the process, with random DNA degradation occurring late in the cell's demise (Kerr *et al.*, 1972). The induction of an acute inflammatory response invariably accompanies this form of cell death.

The process of apoptosis has been described as consisting of three distinct phases: initiation, effector, and degradation (Kroemer, 1997). The initiation phase is mediated by a variety of diverse stimuli that act to trigger the response. These triggers include, among others: radiation-induced DNA and protein damage, reactive oxygen molecules, or proteases; withdrawal of essential nutrients, growth factors, or hormones during critical stages in cell growth and maturation; and binding of ligands such as Fas, tumor necrosis factor-α, interferon gamma, interleukin-1, or transforming growth factor-β to their corresponding cell membrane receptors. The signal transduc-

tion pathways that regulate apoptosis and initiate the downstream effector and degradation phases are common to most apoptotic processes. It is known that the apoptotic regulatory machinery involves several groups of molecules, including the Bcl-2 family of proteins (to be discussed in greater detail below), and a number of enzymes (see Mundel *et al.*, 1996, for review), including: granzymes or serine proteases (Greenberg and Litchfield, 1995; Shi *et al.*, 1992), ceramides (Obeid *et al.*, 1993; Zhang *et al.*, 1996), tissue transglutaminases (Fesus *et al.*, 1987, 1989; Piacentini *et al.*, 1991), lamin proteinases (Lazebnik *et al.*, 1995), phosphatases (Morana and Eastman, 1995), inducible nitric oxide synthase (Ankarcrona *et al.*, 1994; Kitajima *et al.*, 1994; Blanco *et al.*, 1995), the caspases or interleukin-1β-converting enzyme (ICE) family of cysteine proteases (Barinaga, 1994; Duan *et al.*, 1996; Henkart, 1996), poly(ADP-ribose) polymerases (PARP) (Henkart, 1996; Ray *et al.*, 1992; Rice *et al.*, 1992), and nucleases (Arends *et al.*, 1990; Hughes and Cidlowski, 1994). Knowledge of the mechanisms underlying the apoptotic process continues to expand at an exponential rate, and key signal transduction pathways that regulate apoptosis and control the function of these family members are just now being identified. Several excellent reviews have been published in the last several years on the structure, function, and mechanism of action of the apoptotic program (Arends and Wyllie, 1991; Goldstein *et al.*, 1991; Sen, 1993; Korsmeyer, 1995; Manning and Patierno, 1996; Mundel *et al.*, 1996; Nagata, 1997). Thus, details regarding these aspects of apoptosis will not be discussed here. For the remainder of this review, we will focus on a key component of the effector arm of the apoptotic program, the Bcl-2 gene family of proteins, and discuss how they contribute to the pathogenesis of disease, specifically, their role in the development of cancer.

The Bcl-2 Gene Family of Proteins

The centerpiece of the apoptotic program and the major effector arm of the cell death program is the Bcl-2 family of related proteins (Table 2) (Nuñez and Clarke, 1994; Reed, 1994; Korsmeyer, 1995; Kroemer, 1997). The prototype member of this family is Bcl-2, an acronym for B-cell lymphoma/leukemia-2 gene, that was first discovered at the breakpoint of the t(14;18) in a follicular non-Hodgkin's B-cell lymphoma (Tsujimoto *et al.*, 1985). In the translocation, the Bcl-2 gene is moved from its normal chromosomal location at 18q21 into proximity with powerful enhance elements in the immunoglobulin heavy-chain (IgH) locus at 14q32. This results in deregulation of the translocated Bcl-2 gene and overproduction of Bcl-2 mRNAs and their encoded proteins (Bakhshi *et al.*, 1985). The Bcl-2 gene family consists of two opposing groups of proteins: death antagonists (Bcl-2, Bcl-x_L, Bcl-w, Bfl-1, Brag-1, Mcl-1, and A-1) and death agonists (Bax, Bak, Bcl-x_S, Bad, Bid, Bik, and Hrk) (Kroemer, 1997). They differ in their tissue- and activation-dependent patterns as well as in their structure (Hockenbery *et al.*, 1991; LeBrun *et al.*, 1993; Krajewski *et al.*, 1994a,b). The proteins encoded by the Bcl-2 family localize to the outer mitochondrial membrane, the nuclear membrane, and the endoplasmic reticulum (Korsmeyer, 1995; Kroemer, 1997). The protein products of the gene family share two highly conserved domains, BH1 and BH2 (Bcl homologue 1 and 2), that regulate heterodimerization between the death antagonists such as Bcl-2 and Bcl-X_L and death agonists like Bax. Cell death is induced by this gene family through competing dimerization (Oltvai and Korsmeyer, 1994). If, for example, the level of Bcl-2, which protects cells from undergoing apoptosis, is higher than the level of the death inducer Bax, Bcl-2 homodimers prevail and cells are protected. If, on the other hand, Bax is in excess, the opposite effect occurs and cells become susceptible to apoptosis. Thus, it is the ratio (relative level of expression) of proteins that either protect or induce cell death which determines the ultimate fate of a cell (Korsmeyer, 1995). Indeed, there is now substantial evidence that these domains play a critical role in how a cell responds to death-inducing and death-protecting signals (Kroemer, 1997). The BH3 domain of death agonists such as Bax and Bak is required for these proteins to heterodimerize with Bcl-2 and Bcl-x_L to promote apoptosis (Zha *et al.*, 1996; Kroemer, 1997). Overexpression of those proteins containing only the BH3 domain is sufficient for interacting with and inhibiting the function of Bcl-2-like survival proteins. Thus, this class of proteins thus functions as dominant negative inhibitors of cell death.

TABLE 2

Bcl-2 Gene Family

Anti-apoptotic Members
Bcl-2
Bcl-x_L
Bcl-w
Bfl-1
Brag-1
Mcl-1
A1
Pro-apoptotic Members
Bax
Bak
Bcl-x_S
Bad
Bid
Bik
Hrk

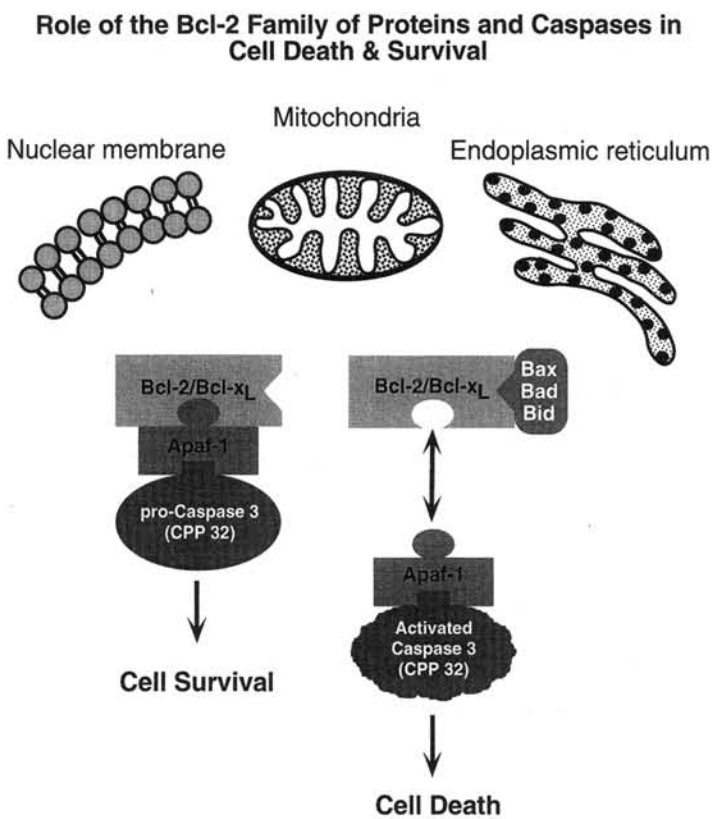

Figure. The illustration depicts the interaction of the Bcl-2 gene family members with key Cytosolic Aspartate-Specific cysteine Proteases (Caspases) (Mignotte and Vayssiere, 1998). Bcl-2 is located at the contact points between the inner and outer mitochondrial membranes, and along the nuclear membrane and endoplasmic reticulum. Bcl-x_L is present in both soluble and membrane-bound forms while Bax is located predominantly in the cytosol. The current speculation is that Bcl-2/Bcl-x_L and perhaps other members of the Bcl-2 family inhibit apoptosis by maintaining the Apaf-1/pro-Caspase 3 (CPP 32) complex bound tightly to membranes. When Bax, Bid, or Bad heterodimerizes with Bcl-2/Bcl-x_L, the complex dissociates, resulting in activation of pro-Caspase 3 and cell death.

Bcl-2 is an Antagonist of Cell Death

Bcl-2 functions to confer a survival advantage to a variety of cell types by inhibiting apoptosis. The death-inhibitory effect of Bcl-2 was first reported in studies of an IL-3-dependent B-lymphocyte line (Vaux *et al.*, 1988). Growth and survival of these cells are strictly dependent on the presence of IL-3 in the culture media. When Bcl-2 was overexpressed in these cells, they were able to survive in the absence of IL-3. Also, B-lymphocytes immortalized with the Epstein-Barr virus constitutively overexpress Bcl-2 and exhibit prolonged cell survival in culture (Henderson *et al.*, 1991; Oren, 1992). The c-myc oncogene, when introduced into rat fibroblasts, induces cell proliferation in serum-supplemented culture media. With serum deprivation, apoptosis ensues but can be blocked by Bcl-2 (Bissonnette *et al.*, 1992; Evan *et al.*, 1992; Wagner *et al.*, 1993). Overexpression of Bcl-2 has been shown to inhibit apoptosis induced by a number of agents, including calcium ionophores, ethanol, irradiation, corticosteroids, cyclic AMP, and heat-shock (Barry *et al.*, 1990; Baxter and Lavin, 1992; Ray *et al.*, 1992). Interestingly, the introduction of Bcl-2 into a variety of epithelial and mesenchymal cells inhibits cell death but does not enhance cell proliferation (Oren, 1992; Manning and Patierno, 1996). Further insights into the function of Bcl-2 during apoptosis and the consequences of its inappropriate expression have been revealed in both gain-of-function and loss-of-function experiments (McDonnell *et al.*, 1989; Veis *et al.*, 1993). Transgenic mice bearing a Bcl-2-immunoglobulin minigene initially showed a polyclonal follicular expansion of B-cells in G0/G1 of the cell cycle. Cells accumulated because they lived longer rather than due to increased cell proliferation. Eventually, the expanded B-cells progressed to high-grade lymphomas due to the occurrence of a translocation involving the c-myc oncogene in a large number of these tumors (McDonnell *et al.*, 1989; McDonnell and Korsmeyer, 1991; Korsmeyer, 1995). Thus, the proliferative advantage afforded by c-myc overexpression was complemented by and synergized with the increased survival caused by overexpression of Bcl-2. In the mouse embryo, Bcl-2 is expressed much more widely than in adult tissues. This raised the question by Korsmeyer and colleagues whether this key repressor of cell death would be required for the successful development of multiple lineages (Korsmeyer, 1995). Mice with a targeted disruption in Bcl-2 exhibited normal development until the first week after birth, suggesting the function of redundant genes that control apoptosis during this phase and that can overcome the lack of Bcl-2. Hematopoiesis appeared normal, indicating that Bcl-2 was not required for the development of these lineages. Most organs in these mice develop normally. However, one week after birth, homozygous mutants exhibited growth retardation, with small ears and immature facial features. The mice died at an early age and exhibited diffuse apoptosis of their kidneys which progressed to severe polycystic kidney disease (Veis *et al.*, 1993).

Mechanism of Action of the Bcl-2 Protein

The mechanism by which Bcl-2 inhibits apoptosis is still uncertain and thus remains the subject of intensive investigation. Bcl-2 has been localized to areas of contact between the outer and the inner mitochondrial membranes, suggesting that Bcl-2 may protect cells from apoptosis by altering mitochondrial function. There is evidence that Bcl-2 might act to prevent calcium influx into mitochondria induced by growth factor withdrawal and prevent cytochrome c outflow from the intermem-

brane space (Adachi *et al.*, 1997). The observation that suppression of DNA fragmentation by Bcl-2 overexpression is associated with decreased levels of cytosolic calcium and increased levels of mitochondrial calcium suggests that Bcl-2 may be involved in the regulation of intracellular calcium distribution. This is further supported by the observation that calcium-ionophore-induced apoptosis is substantially reduced in cells overexpressing Bcl-2. The localization of Bcl-2 to the nuclear membrane and in mitotic nuclei suggests that it may protect DNA from damage by endonucleases.

Recently, a homologue of Bcl-2, called Bcl-x, has been cloned and characterized. As a result of alternative splicing, two mRNA species, designated Bcl-x_L and Bcl-x_S, were identified in humans (Boise *et al.*, 1993). The former, like Bcl-2, inhibits apoptosis. The latter is a dominant negative repressor of Bcl-2 and enhances apoptotic signals in cells which express Bcl-2. The structure of Bcl-x_L is strikingly similar to the pore-forming domain of diphtheria toxin. This suggests that this Bcl-2-related protein and perhaps Bcl-2 itself may function as ion channels (Minn *et al.*, 1997; Schendel *et al.*, 1997). The mitochondrial permeability transition (depolarization of the transmembrane potential) can cause the outflow of mitochondrial proteins (*e.g.*, apoptosis-inducing factor, cytochrome c) into the cytosol and activate specific apoptogenic proteases such as caspase-3 (Kroemer, 1997). The activation of this caspase is dependent on the presence of cytochrome c in the cytosol and results in cleavage of PARP and subsequent DNA fragmentation (Yang *et al.*, 1997). Therefore, both Bcl-2 and Bcl-x_L may prevent apoptosis by inhibiting cytochrome c translocation and thus prevent caspase activation and the subsequent downstream events in the apoptotic process (Kluck *et al.*, 1997).

Bcl-2 may also function as an anti-oxidant. Bcl-2 has been reported to block apoptosis in cells exposed to γ-irradiation which cause damage due to the generation of reactive oxygen species (Hockenbery *et al.*, 1993; Polyak *et al.*, 1997). Moreover, H_2O_2 and dexamethasone-induced apoptosis can be overcome by Bcl-2. Hockenbery *et al.* (1993) have noted that the death-suppressive effects of anti-oxidants such as N-acetyl-L-cysteine and glutathione peroxidase were mediated through inhibition of lipid peroxidation. It was proposed that Bcl-2 could act as an anti-oxidant by a similar mechanism. However, since Bcl-2 expression can also inhibit apoptosis under aerobic conditions, it may be that production of reactive oxygen molecules is a consequence rather than a cause of apoptosis.

Bcl-2 *and the Development of Cancer*

There is now compelling evidence that aberrant expression of one or more members of the Bcl-2 gene family can contribute to the development of a variety of diseases (see review by Thompson, 1995). Table 3 is a list of developmental, degenerative, inflammatory/immunological, and neoplastic diseases associated with either prolonged cell survival or premature or excessive cell death. The evidence implicating Bcl-2 family members in the development of cancer comes from several lines of study. Tumor cells from a wide variety of human cancers have been shown to exhibit increased survival and resistance to apoptosis (Collins, 1991; Williams, 1991; Oren, 1992; Manning and Patierno, 1996). The ability of tumors to exhibit autonomous growth has long been regarded as a hallmark of a neoplasm. Whether in a primary tumor or a metastasis, tumor cells must be able to survive in an environment that is often toxic or severely depleted of essential nutrients or growth factors (Nowell, 1976; Farber and Cameron, 1980). Resistance to cytotoxicity and a reduced requirement for exogenous growth factors

TABLE 3

Apoptosis-associated Diseases*

Disorders Associated with Prolonged Cell Survival
Cancer
Breast
Central nervous system
Head and neck
Follicular lymphoma
Gastro-intestinal tract
Genito-urinary tract
Lung
Oral cavity
Skin
Autoimmune Disorders
Systemic lupus erythematosis
Viral infections
Herpesviruses
Poxviruses
Adenoviruses
Diseases Associated with Increased Apoptosis
AIDS
Neurodegenerative disorders
Alzheimer's disease
Parkinson's disease
Amytrophic lateral sclerosis
Retinitis pigmentosa
Cerebellar degeneration
Myelodysplastic syndromes
Aplastic anemia
Ischemic injury
Myocardial infarction
Stroke
Reperfusion injury
Toxin (Alcohol)-induced liver disease

* Adapted from Thompson (1995).

have been shown to be associated with an increase in the expression of genes that promote the acquisition of a growth-factor-independent phenotype as well as resistance to apoptosis. Several lines of evidence have implicated Bcl-2 and several of its survival homologues in the conversion of normal cells to malignancy (Collins, 1991; Williams, 1991; Oren, 1992; Manning and Patierno, 1996). As described earlier, the Bcl-2 gene was first identified at the site of a translocation between chromosome 14 and 18 in a human non-Hodgkin's B-cell lymphoma. Unlike most other oncogenes which promote cell cycle progression, overexpression of the Bcl-2 oncogene was found to protect cells from undergoing apoptosis in response to a number of stimuli (Miyashita and Reed, 1992, 1993). The ability to confer the malignant phenotype in cells has been demonstrated following the introduction of Bcl-2 into several immortalized cell types (Reed, 1994; Korsmeyer, 1995). Also, the overexpression of the death agonist Bax or related genes in cancer cells is able to counteract the survival effect of Bcl-2 and induce apoptosis by eliminating the effects of survival proteins (Han *et al.*, 1996; Sabbatini *et al.*, 1997). Transgenic mice overexpressing Bcl-2 develop polyclonal expansion of B-cells that eventually progress to high-grade lymphomas (McDonnell and Korsmeyer, 1991). Thus, Bcl-2 appears to function as a checkpoint against unwarranted cell turnover by preventing cells from undergoing apoptosis (McDonnell *et al.*, 1989). In addition to cells of the B-cell lineage and other hematopoietic stem cells, Bcl-2 protein expression has been observed in several epithelial stem cells but not in more differentiated cells, suggesting that Bcl-2 may also have a protective function in stem cells (Hockenbery *et al.*, 1991). Bcl-x has also been encountered to a limited extent in both normal and diseased skin keratinocytes, but it is more highly and widely expressed in malignant keratinocytes of the skin (Krajewski *et al.*, 1994a; Wrone-Smith *et al.*, 1995; Mitra *et al.*, 1997; Pena *et al.*, 1997) and oral mucosa (Polverini, Nickoloff, and Betz, unpublished observations).

In human cancers, overexpression of Bcl-2 protein has been observed in several tumor types, including cancer of the lung (Pezzella *et al.*, 1993), ovary (Henriksen *et al.*, 1995), colon (Sinicrope *et al.*, 1995a,b), prostate (Colombel *et al.*, 1993), esophagus (Sarbia *et al.*, 1996), and breast (Silvestrini *et al.*, 1994). In non-small-cell lung cancer and breast cancer, Bcl-2 protein expression in tumor cells correlates with a poorer prognosis as compared with Bcl-2-negative tumors. Increased expression of Bcl-2 has also been observed in precancerous lesions, and both Bcl-2 and Bcl-$x_{L/S}$ have been detected in early dysplastic lesions of the oral mucosa in humans and in carcinogen-induced papillomas in a mouse model of multistep tumorigenesis (Birchall *et al.*, 1996; Jordan *et al.*, 1996; Stern *et al.*, 1997). Overexpression of Bcl-2 is also associated with resistance of several human tumors to apoptosis-inducing chemotherapeutic agents such as methotrexate, 1-β-D-arabvinofuranosyl-cytosine, vincristin, etoposide, and cisplatin (Barry *et al.*, 1990). Increased Bcl-2 expression has been observed in the progression of prostate cancer from androgen-dependent to androgen-independent growth. McDonnell *et al.* (1992) have reported that the majority of androgen-dependent tumors exhibit low or barely detectable levels of Bcl-2, while androgen-independent tumors express high levels of this gene. Thus, it was hypothesized that Bcl-2 protects tumors from apoptosis upon withdrawal of androgens.

As mentioned previously, Bax, another member of the Bcl-2 family, functions to antagonize the survival effect of Bcl-2. Mice with a targeted disruption in Bax display hyperplasia of thymocytes and B-cells. Recently, it has been reported that loss of Bax expression may be a prognostic indicator of diminished responsiveness to chemotherapy and shorter survival in women with metastatic breast cancer (Krajewski *et al.*, 1995). Thus, loss of a key inducer of apoptosis can also contribute to tumor cell survival and progression. Lack of responsiveness to negative growth controls by tumor cells is another mechanism whereby tumors are able to circumvent apoptosis. It has been shown that resistance to transforming growth factor-β-induced growth arrest and apoptosis correlates with tumor progression (Bursch *et al.*, 1993; Selvakumaran *et al.*, 1994). Thus, these data strongly implicate several members of the Bcl-2 gene family in tumor progression by increasing the resistance of mutated cells against the protective mechanisms of apoptosis. This can be achieved either by overexpression of Bcl-2 homologues that promote cell survival or downregulation of genes that induce cell death.

Relation of Bcl-2 with Cellular Resistance to Apoptosis and Malignant Transformation

Given the inherent role that apoptosis plays in the orderly removal of cells during development and in adult tissues, apoptosis most likely functions to guard against neoplastic development by eliminating genotoxically damaged cells that accumulate in organs and tissues (Manning and Patierno, 1996). This notion is supported by the fact that the majority of cells exposed to carcinogens and chemotherapeutic agents undergo apoptosis. In keeping with the concept that initiation by a carcinogen is a rare event, the process of apoptosis is generally an efficient mechanism for removing genotoxically damaged cells that escape the DNA repair process. This possibility is further supported by *in vivo* studies of liver and skin carcinogenesis. Hepatocellular carcinoma can be readily induced in rats following exposure to an initiating dose of a chemical carcinogen along with a hepatotoxic

agent such as carbon tetrachloride (Farber and Cameron, 1980). In this instance, liver damage is followed by compensatory proliferation of residual hepatocytes. If, on the other hand, the same carcinogen is administered at a similar dose along with lead nitrate, an agent which potently induces cell proliferation as well as apoptosis, cancer development is prevented (Ledda-Columbano and Columbano, 1991). However, when the removal of damaged cells by apoptosis is compromised, or is less efficient, the potential for tumor development increases. This appears to be the case in the large intestine. This organ tends to be the focus of carcinoma development more frequently than the small intestine. This is despite the fact that the small intestine exhibits a higher rate of turnover. In keeping with the concept that apoptosis protects against carcinogenesis by destroying genotoxically damaged cells, the failure to undergo apoptosis may partly explain the protective effects conferred on carcinogen-initiated cells by tumor promoters.

The tumor-promoting agent phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) has been shown to inhibit apoptosis in cultures of C3H-10T1/2 cells following exposure to ionizing radiation, low-energy β-radiation, or acute serum deprivation (Tomei *et al.*, 1988). Also, treatment of chronic leukemia cells with TPA markedly inhibits apoptosis induced by agents such as cholchacine, etoposide, or methylprednisolone (Forbes *et al.*, 1992). The protective action of TPA is likely mediated through modulation of protein kinase C, since its activity can be antagonized by inhibitors of this enzyme. Okadaic acid, a tumor promoter that inhibits protein phosphatases 1 and 2A, has been reported to protect human lymphoid cells from induction of apoptosis by diverse agents (Song *et al.*, 1992). Exposure of liver cells to okadaic acid inhibits apoptosis-associated dephosphorylation of several proteins, including a 40-kDa protein that is common in this tissue (Baxter and Lavin, 1992). These observations suggest that alteration in the phosphorylation state of multiple specific proteins might be an important regulatory mechanism involved not only in cell proliferation but also in apoptosis. *In vivo* studies also support the notion that suppression of apoptosis is a feature of tumor promotion. In the rat hepatocyte model of carcinogenesis, the formation of neoplastic foci can be significantly enhanced if animals are treated with an initiating dose of a carcinogen along with a tumor promoter such as phenobarbital (Garcea *et al.*, 1989; Schulte-Hermann *et al.*, 1990; Bursch *et al.*, 1993). In this model, phenobarbital increases the rate of tumor formation and the number of neoplastic foci. This effect is associated with a decrease in the level of apoptosis within the liver foci, since continuous exposure to phenobarbital does not produce a persistent increase in cell proliferation. Interestingly, cessation of phenobarbital administration results in a decrease in the number of neoplastic foci due to induction of apoptosis. Thus, several *in vitro* and *in vivo* studies suggest that tumor promoters may enhance carcinogenesis by protecting mutagenized cells from apoptotic death and allowing them to proliferate and progress to preneoplasia and frank malignancy. However, other studies (Stern *et al.*, 1997) suggest that this may not be the case.

In a recent study from the Conti laboratory (Stern *et al.*, 1997), the relationship between apoptosis and tumor progression was examined in mouse skin tumors by means of a two-stage chemical carcinogenesis protocol. Papillomas were generated by exposing the skin of mice to 7,12 dimethylbenz[a]anthracine. The association between tumor progression and apoptosis was analyzed in tumors derived from two strains of mice differing in their susceptibility to tumor development: the SINN and SENCAR B/Pt mice. The TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) assay was used to identify apoptotic cells at various times during tumor promotion. These workers found that the number of apoptotic cells was greater in papillomas that were in the process of undergoing progression to squamous cell carcinoma when compared with benign papillomas. In addition, they found that papillomas from SENCAR P/Bt mice, a tumor-progression-susceptible strain, had more apoptotic cells and exhibited a substantial increase in p53 expression than papillomas from tumor-progression-resistant SSIN mice. These works suggested that, in this model system of skin cancer, the epithelial cells populating tumors still maintained the capacity to undergo apoptosis and that the presence of apoptotic cells in advanced papillomas was an indicator of heightened endogenous mutagenic activity and high genomic instability.

Bcl-2 Synergizes with Oncogenes and Tumor Suppressor Genes to Drive Cells Toward Malignancy

Bcl-2 appears to cooperate in a synergistic fashion with oncogenes and tumor suppressor genes to enhance the survival of and the opportunity for additional mutations to occur in cells. The translocation of the Bcl-2 gene in human follicular B-cell lymphoma, although not sufficient to cause malignant conversion of B-lymphocytes, appears to increase the chance of additional mutations in these abnormally long-lived cells. Studies of Bcl-2 transgenic mice provide support for the observation that activation of an oncogene in cells overexpressing Bcl-2 increases the chances for a second mutational event to occur (McDonnell *et al.*, 1989; McDonnell and Korsmeyer, 1991). The indolent lymphoma that initially develops in these mice after a long latency period progresses to a

high-grade lymphoma. Many of these high-grade lymphomas were found to contain a constitutively activated c-myc oncogene. This apparent synergistic effect was confirmed in studies in which strains of mice overexpressing c-myc and Bcl-2 were crossed with each other. The result of these matings was the rapid development of a disseminated lymphoma in the double-expressing mice (Strasser *et al.*, 1990). The c-myc oncogene is a potent stimulator of cell proliferation but also induces apoptosis when overexpressed in the absence of supporting growth factors. In this case, overexpression of Bcl-2 appears to prevent cell death induced by c-myc, by driving cells toward proliferation and by blocking apoptosis (Vaux *et al.*, 1988). In nonlymphoid mesenchymal cells such as primary rat embryo fibroblasts, Bcl-2 appears to cooperate with the Ha-ras oncogene to produce neoplastic transformation, and does so without directly increasing cell proliferation (Reed *et al.*, 1990). Ras-mediated signal transduction pathways become activated upon stimulation, and are subsequently inactivated to maintain homeostasis. Mutated ras protein, however, has lost the ability to become inactivated, resulting in enhanced cell proliferation. Although the ras signaling pathways have not yet been completely defined, there is evidence that they may be linked to the cell death pathway. The ras oncogenes have been shown to increase resistance of NIH 3T3 cells to gamma-radiation-induced apoptosis (Sklar, 1988). Bcl-2 interacts with ras-related protein R-ras p23. Although the structure of R-ras is similar to other ras proteins, its biological function differs, since R-ras does not share the oncogenic properties of other members of this family of proteins. The potential of ras involvement in the apoptotic pathway is suggested by interaction of ras with Raf-1 kinase (Fernandez-Sarabia and Bischoff, 1993). Bcl-2 together with Raf-1 synergistically suppress apoptosis induced by growth factor withdrawal in 32D.3 hematopoietic cells (Wang *et al.*, 1994).

Inactivation of the p53 tumor suppressor gene has been linked to the etiology of more than 50% of human cancers and is one of the most frequently mutated genes in tumors of the upper aerodigestive tract, including the oral cavity (Hollstein *et al.*, 1991; Levine *et al.*, 1991; Field *et al.*, 1992). Recent studies have provided compelling evidence that alteration in p53 expression may play an important role in selecting for cells resistant to apoptosis during tumor development. Wild-type (WT) p53 regulates the expression of cell cycle genes in normal cells at the G1/S boundary. It functions as a guardian, maintaining the fidelity of genomic information (Hollstein *et al.*, 1991; Levine *et al.*, 1991). Thus, when transduced into tumor cells, WT p53 effectively blocks cell cycle progression. WTp53 also appears to regulate Bcl-2 expression. When introduced into growth-factor-independent tumor cells, these cells become sensitive to growth factor withdrawal and undergo apoptosis. However, the restoration of WT p53 did not cause growth arrest, but rather cells in the G1 phase of the cell cycle displayed enhanced susceptibility to apoptosis (Yonish-Rouach *et al.*, 1991). Expression of WTp53 in the EB tumor line, which lacks endogenous p53 expression, induced apoptosis and prevented tumor growth *in vivo* (Shaw *et al.*, 1992). Inactivation of p53 may therefore be a mechanism by which tumor cells escape negative growth controls that could lead to apoptosis. Mutated p53 genes that encode proteins that act as negative dominant inhibitors of p53 can also inhibit apoptosis (Gottlieb *et al.*, 1994; Zhu *et al.*, 1994). Expression of mutant p53 genes in IL-3-dependent leukemia cells was found to protect cells from apoptosis following growth factor withdrawal (Zhu *et al.*, 1994).

A mechanistic link between apoptosis and the function of the p53 tumor suppressor has recently been described. Prior to the induction of apoptosis, WTp53 rapidly translocates into the nucleus, where it can function as a transcription factor regulating gene expression (Levine *et al.*, 1991). Two proteins regulated by p53 are Bcl-2 and Bax (Miyashita *et al.*, 1994; Miyashita and Reed, 1991). Expression of WT p53 in M1 leukemia cells which lack endogenous p53 resulted in down-regulation of Bcl-2 together with up-regulation of Bax expression prior to the occurrence of apoptosis (Miyashita *et al.*, 1994; Selvakumaran *et al.*, 1994). Mice deficient in p53 have been found to have elevated levels of Bcl-2 protein and reduced levels of Bax in several tissues (Myashita *et al.*, 1994). In human cells exposed to ionizing radiation, induction of Bax was associated with normal p53 function and apoptosis (Zhan *et al.*, 1994). These data suggest that p53 may regulate apoptosis by altering the cellular ratio of Bcl-2/Bax and that it is this perturbation that determines the fate of cells exposed to apoptotic stimuli. In a recent publication from the Vogelstein group (Polyak *et al.*, 1997), it was reported that among 7000 transcripts examined in p53-expressing cells, only 0.19% were found to be markedly increased. Many of these genes were predicted to encode proteins that could generate or respond to oxidative stress. It was concluded that p53 results in apoptosis through a three-step process: (1) the transcriptional induction of redox-related genes; (2) the formation of reactive oxygen species; and (3) the oxidative degradation of mitochondrial components, culminating in cell death. In addition to preventing the generation of reactive oxygen species and protecting mitochondrial components from degradation, loss or inactivation of p53 may also predispose preneoplastic cells to accumulate additional mutations through blocking of the normal apoptotic response to genotoxic damage. Normal p53 has been demonstrated to be critical to the induction of apoptosis in human and murine cells following genotox-

ic damage (Lowe *et al.*, 1993; Clarke *et al.*, 1994; Fujiwara *et al.*, 1994; McCarthy *et al.*, 1994; Merritt *et al.*, 1994). Thus, a p53 deficiency may permit a population of genetically damaged cells to escape the normal process of apoptotic deletion. Recently, Livingstone and colleagues (1992) have suggested that loss of p53 function may predispose cells to further genomic instability and lead to gene amplification. These investigators found that gene amplification occurred at a higher rate in p53-deficient cells, whereas normal fibroblasts with one copy of the normal p53 allele did not demonstrate similar gene amplification.

Activation of Bcl-2 through inactivation or loss of p53 might also enhance the growth of metastatic tumor cells. Metastatic foci often translate to an environment with limited nutrients. In normal cells, nutrient deprivation is a potent stimulus for apoptosis. However, tumor cells that have a decreased dependency on exogenous growth factor or that overexpress survival genes such as Bcl-2 would no longer be subjected to the selection pressure normally associated with nutrient or growth factor deprivation. Indeed, the expression levels of Bcl-2 and Bcl-x_L (a Bcl-2 homologue) are often markedly elevated in invading and metastatic oral squamous carcinomas (Jordan *et al.*, 1996; Polverini, Nickoloff, and Betz, unpublished observations). Many oncogenic viral proteins (such as the simian virus 40 large T-antigen, adenovirus type 5 E1B, and human papillomavirus 16/18 E6) can effectively disrupt the function of p53 by post-translational modification of WTp53 protein (Mormand *et al.*, 1992; Perry *et al.*, 1993). The oncogenic potential of these agents thus appears to work through the p53/Bcl-2 axis, enhancing the survival of infected cells and perhaps predisposing them to additional mutations.

Summary and Conclusions

The earliest step in the carcinogenic process involves the emergence of "initiated" cell populations that are genetically altered. These are biologically indolent cells that do not express any of the conventional markers of premalignancy or malignancy but nevertheless have acquired a selective growth advantage that renders them at risk for further genetic damage. The enhanced expression of Bcl-2 and/or Bcl-x_L in human premalignant and malignant oral keratinocytes suggests that perhaps an early key event in the development of oral squamous carcinoma is the preferential expression of members of the Bcl-2 gene family that protect cells from apoptosis and render them more susceptible to mutation and tumor progression. In normal circumstances, the process of apoptosis effectively eliminates genetically damaged cells from tissues to guard against their continued growth and progression toward malignancy. If, on the other hand, these controls are compromised, then an important mechanism for the removal of damaged cells is lost. Surviving cells that contain sublethal DNA damage would continue to persist in a protected environment and eventually may become hyperresponsive to proliferative signals. Since drug resistance appears to be a feature of cells overexpressing survival genes, this would provide another explanation for the resistance to cytotoxicity that characterizes initiated cell populations. The continued persistence of apoptosis-resistant cells would further increase the probability of acquiring additional mutations. Thus, for neoplastic growth to occur, the selection pressure conferred on cells by the apoptotic program must be overcome. This would in turn create an imbalance between those forces that drive cells toward proliferation and physiological independence and those that keep cells tightly in check through apoptosis. Once this imbalance in favor of cell proliferation and/or survival is achieved, cell growth would predominate. Genetic mutations or epigenetic factors that diminish the tendency to undergo apoptosis may then facilitate their growth and drive them toward malignancy.

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