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Apoptosis and Lung Cancer: A Review

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Abstract It is important to understand the molecular events that contribute to drug-induced apoptosis, and how tumors evade apoptotic death. Defects in apoptosis are implicated in both tumorigenesis and drug resistance, and these defects are cause of chemotherapy failures. These studies should explain the relationship between cancer genetics and treatment sensitivity, and should enable a more rational approach to anticancer drug design and therapy. Lung cancer is a major cause of cancer deaths throughout the world. Small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC) represent the two major categories of lung cancer that differ in their sensitivity to undergo apoptosis. The role of apoptosis regulation in lung cancer with major focus on the differential sensitivities of the major subtypes is reviewed. J. Cell. Biochem. 88: 885–898, 2003. © 2003 Wiley-Liss, Inc.

Key words: chemosensitivity; p53; lung cancer; apoptosis

APOPTOSIS AND CANCER

Apoptosis is an evolutionarily conserved and genetically regulated form of cell suicide which plays an important role in development and in the maintenance of tissue homeostasis in multicellular organisms [Webb et al., 1997; Wyllie, 1997]. In large, long-lived multicellular organisms, such as man, there exists a substantial and continuous need for cellular proliferation for development as well as maintenance and repair [Evan and Littlewood, 1998; Evan and Vousden, 2001]. This life-long need for cellular proliferation has to be balanced against the constant threat of cancer arising as a result of deregulated growth caused by mutations in the same genes which control normal cellular proliferation [Evan and Littlewood, 1998; Evan and Vousden, 2001]. Thus, the organism must find a way to allow cellular proliferation only when needed while effectively suppressing this activity at other times. One of the main mechanism by which the above balance is achieved is by coupling cellular proliferation to apoptosis—the tendency of cells to undergo apoptosis is a normal consequence of engaging the cell's proliferative machinery [Evan and Littlewood, 1998; Evan and Vousden, 2001].

One of the best examples of the above hypothesis is provided by the oncogene *C-MYC*. Deregulated expression of *MYC* genes is frequently seen in many forms of human cancer including lung cancer, and the growth promoting ability of MYC protein is well documented. However, MYC is also a powerful inducer of apoptosis, especially under conditions of stress, genotoxic damage or depleted survival factors, which has led to the hypothesis that the innate apoptotic potential of MYC serves as an in-built foil to its oncogenic capacity [Amati et al., 1998; Evan and Littlewood, 1998; Prendergast, 1999; Evan and Vousden, 2001]. Although the exact mechanism of *MYC*-induced cell death is unclear, it is believed to sensitize cells to a wide range of mechanistically different triggers of apoptosis including DNA damage, nutrient deprivation, interferon, hypoxia, and

Abbreviations used: SCLC, small cell lung carcinoma; NSCLC, non-small cell lung carcinoma.

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death ligands such as TNF, FasL, and TRAIL [Prendergast, 1999]. As many of the above stimuli are also encountered by the incipient tumor cells, these results support the hypothesis that growth-deregulating mutations sensitize cells to a wide variety of apoptotic triggers, which unless neutralized, automatically remove the affected cells [Evan and Littlewood, 1998; Evan and Vousden, 2001].

Apoptosis plays an essential role in the elimination of mutated or transformed cells from the body and as such evasion of apoptosis is considered to. Thus, in order to survive, cancer cells and their precursors must develop highly efficient, and usually multiple, mechanisms to avoid apoptosis. In fact, the avoidance of apoptosis is regarded as one of the hallmarks of cancer cells [Hanahan and Weinberg, 2000]. A frequent, apparently paradoxical, finding in tumors and their precursor lesions is an increased rate of apoptosis (as measured by the apoptotic index) and increased resistance to apoptosis. The increased apoptotic index reflects the enormous pressure of these abnormal cells to undergo death, while the increased resistance reflects defense mechanisms developed by the abnormal cell in an effort to survive. Without the development of apoptotic resistance early during tumorigenesis, the preneoplastic cells would not survive long enough to become invasive cancers. Because apoptosis involves a complex network of interacting checks and balances utilizing over 150 known genes [Aravind et al., 2001], tumor cells must develop resistance to apoptosis at multiple levels.

Apoptotic cell death is orchestrated by the activation of caspases, a family of cysteine proteases with specificity for aspartic acid residues [Stennicke and Salvesen, 2000]. To date, 14 mammalian caspases have been identified, a subset of which is involved in the regulation of apoptosis. Caspases (*CASP*s) are normally expressed in the cells as inactive zymogens and are converted into their active form at the onset of apoptosis by a process involving proteolytic processing followed by assembly of the subunits into active tetramers. Caspases can be broadly divided into initiator (upstream) and effector (downstream) caspases. Initiator caspases, such as *CASP8*, *CASP9*, and *CASP10*, usually possess a long prodomain. They are activated following a proapoptotic stimulus and are responsible for activating the effector caspases. The effector caspases, primarily *CASP3*, *CASP6*, and *CASP7*, carry out the majority of substrate proteolysis during apoptosis. To date, two major pathways for caspase activation have been described, which are headed by *CASP8* and *CASP9*, respectively (Fig. 1). One major pathway involves responses to death inducing ligands including TNF, FasL, or TRAIL [Ashkenazi and Dixit, 1999; Bodmer et al., 2000]. Binding of death ligands to their receptors, including *FAS* (for FasL), *TNFR1* (for TNF), or *DR4* and *DR5* (for TRAIL) trigger apoptosis via the recruitment of adaptor protein FADD, which subsequently helps in the recruitment of procaspase 8. Complexing of ligand receptor, FADD and *CASP8* results in the formation of a Death Inducing Signaling Complex (DISC). Upon its recruitment to the DISC, *CASP8* is activated by cross-proteolysis which leads to activation of downstream effector caspases either directly (Type I cells) or via an amplification loop involving truncated Bid (tBid) mediated release of cytochrome *c* from the mitochondria (Type II cells) [Li et al., 1998; Luo et al., 1998; Scaffidi et al., 1998]. Methylation and silencing of *CASP8* has been described in neuroblastomas, and is associated with *N-MYC* amplification and poor prognosis [Teitz et al., 2000a]. The role of *CASP10*, a closely related molecule whose gene is located in the same complex on chromosome 2q as *CASP8*, was uncertain until recently. We demonstrated that both *CASP8* and *CASP10* can participate in DISC formation and that both caspases may have important roles in apoptosis initiation [Kischkel et al., 2001].

The other major apoptotic initiator pathway involves release of cytochrome *c* from mitochondria in response to several internal and some external stimuli, such as growth factor withdrawal, osmotic stress, and hypoxia. Cytochrome *c* forms a multiprotein complex with the adaptor molecule *Apaf-1* and procaspase 9 in the presence of dATP to form a multiprotein complex, called an apoptosome [Adrain and Martin, 2001]. Procaspase 9 is activated upon recruitment to this complex and in turn activates the effector caspases. However, cytochrome *c*-mediated caspase activation may not be sufficient to lead to cell death. This is due to the presence of a family of proteins, called the IAPs (inhibitor of apoptosis protein), which can bind and inhibit the active caspases in the apoptosome. This inhibition is relieved by the release of another mitochondrial protein, called

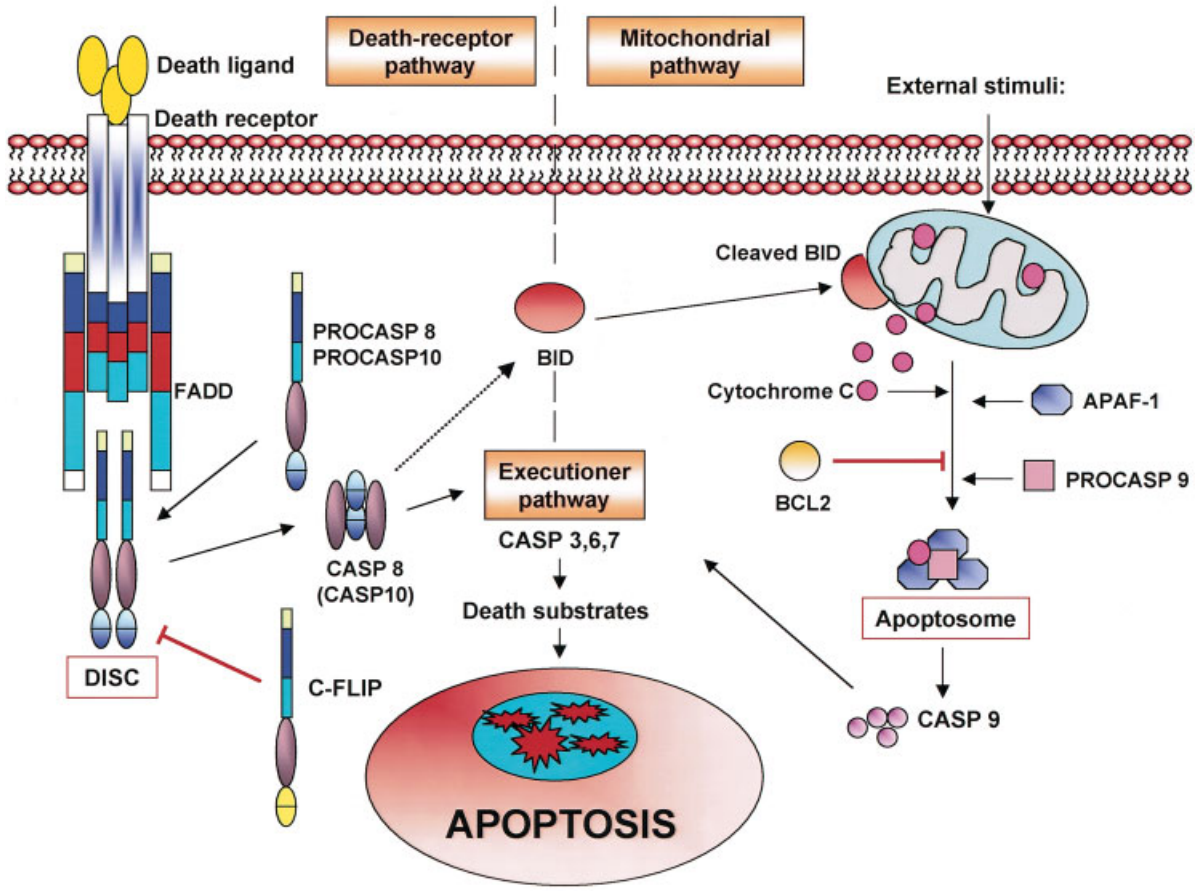

Fig. 1. The major pathways to apoptosis. Two alternative, but overlapping, pathways lead to apoptotic death.; the extrinsic pathway acts via death receptors while the intrinsic pathway acts via release of mitochondrial proteins. Activation of either pathway leads to activation of a common executioner pathway. In both pathways, the extrinsic and intrinsic pathways, initiator caspases are activated, *CASP8* (and probably *10*) for the extrinsic pathway and *CASP9* for the intrinsic pathway. The initiated caspases active executioner caspases which cleave death substrates, leading to cell death. The two major initiator pathways interact via the Bcl-2 family member Bid. See text for further details.

Smac/Diablo, which binds to the IAPs and releases active caspases from their inhibitory influence [Du et al., 2000; Verhagen et al., 2000]. Apoptosis-inducing factor (AIF) and endonuclease G are two additional proteins which are released from the mitochondria and are involved in chromatin condensation and DNA fragmentation associated with apoptosis [Susin et al., 1999; Li et al., 2001]. The death receptor and mitochondrial pathways for caspase activation are also referred to as the "extrinsic" and "intrinsic" apoptosis pathways, respectively.

APOPTOSIS AND LUNG CANCER

Lung cancer is the leading cause of cancer deaths in the world with over one million cases diagnosed every year [Parkin et al., 2001], and the vast majority of cases are smoking related. Human lung cancers are classified into two major types, small cell lung cancer (SCLC) and non-small cell lung carcinoma (NSCLC), the latter consisting of several types [Travis et al., 1995]. Adenocarcinoma is the most common of the NSCLC subtypes, and its frequency is rising in the USA and other parts of the world [Travis et al., 1995]. Adenocarcinoma is the most frequent type of lung cancer in women and never smokers. SCLC cancers are neuroendocrine tumors, are very strongly smoking associated, and they differ from NSCLC cancers in biology, response to therapy, and prognosis. Except for a few very recent reports, differences in apoptosis evasion mechanisms between SCLC and NSCLC have not been investigated.

Because SCLC tumors are seldom surgically resected, fresh tumor tissue is difficult to obtain and continuous cell lines are usually utilized for laboratory studies.

Far fewer articles appear each year on apoptosis in lung cancer than of the other major cancers [Fine et al., 2000]. Therefore, our knowledge of this aspect of lung cell physiology and malignancy is relatively rudimentary. However, recent reports are beginning to define the crucial roles for apoptosis in normal lung cell turnover, lung development, and the pathogenesis of diseases such as interstitial pulmonary fibrosis, acute respiratory distress syndrome, and chronic obstructive pulmonary disease [Fine et al., 2000]. However, studies with lung cancer tend to investigate one or a limited number of aspects of this highly complex multigene process, and we are aware of only a single review of this important area [Joseph et al., 2000]. An important relatively early finding was that the mean apoptotic index of SCLC tumors was significantly different from that of NSCLC, and was higher than the mean indices of many common tumor types [Soini et al., 1998] (Fig. 1). A seminal finding by Joseph et al. found that SCLC cell lines had higher spontaneous apoptotic rates than NSCLC lines, had high expression of Bcl-2 and had frequent loss of caspases 1, 4, 8, and 10 [Joseph et al., 1999]. Although only a modest number of cell lines were studied, this report indicated that the apoptotic mechanisms in SCLC and NSCLC were very different.

APOPTOSIS EVASION DEVELOPS DURING PRENEOPLASIA

Almost all epithelial tumors (carcinomas) arise after a series of progressive histological and molecular changes. In the bronchial epithelium, a continuing series of steps have been identified during the formation of squamous cell carcinomas [Wistuba et al., 1999]. For SCLC, these steps are largely unknown [Wistuba et al., 2000]. Peripherally arising adenocarcinomas are believed to arise from lesions known as atypical adenomatous hyperplasias (AAH) [Kitamura et al., 1999].

Preneoplastic lesions for lung cancer are exceedingly difficult to detect, are usually small, and require histological examination and identification. For these reasons, studies on apoptosis have been limited to paraffin embedded materials, usually of bronchial preneoplastic lesions (squamous dysplasia and carcinoma in situ). A high apoptotic index (Fig. 2), and increased expression of p53 and Bcl-2 proteins have been reported in these lesions [Ferron et al., 1997; Zhang et al., 1998; Chen et al., 1999; Tormanen et al., 1999].

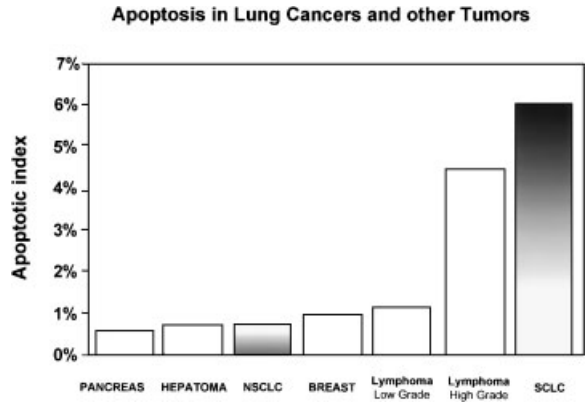

Fig. 2. Apoptotic indices of some common cancers. Note that the index for SCLC is among the highest for any tumor type studied, while that of NSCLC is intermediate. Data extracted from Soini et al. [1998]; #16330].

DEATH RECEPTOR PATHWAY IN LUNG CANCERS

The finding by Joseph et al. [1999] that several caspases including *CASP8* and *10* were not expressed in some SCLC cell lines suggested that major perturbations in the death receptor pathway (and other aspects of apoptosis) characterize this tumor type. While we were initially unaware of this report, we began to study the same pathway because of a different observation. We investigated genes located in homozygous deletions during a genome wide search as the potential location of tumor suppressor genes involved in the pathogenesis of cancers. We noted that a SCLC cell line initiated by us had a homozygous deletion at 2q33 encompassing the chromosomal location of the *CASP8* gene [Shivapurkar et al., 2002b]. We also noted a homozygous deletion in a breast line encompassing the location of the *CASP3* gene. RT-PCR studies indicated that gene expression was lost in most (27 of 34, 79%) of SCLC cell lines, but expression was retained in all 22 non-SCLC (NSCLC) lines tested. Loss of gene expression at the RNA level was associated with absent protein expression by Western blotting and lack of *CASP8* enzymatic activity. All lung cancer

lines and almost all breast lines expressed *CASP10* and *CASP3*.

Loss of gene expression of tumor suppressor genes may occur by multiple mechanisms including homozygous deletion or by a combination of monaoallelic loss, point mutations, or aberrant promoter methylation. While some genes such as *TP53*, frequently demonstrate point mutations that result in an abnormally functioning protein, it has become increasing clear during the last decade that the major mechanism of gene inactivation is via methylation of normally unmethylated promoter regions of genes [Baylin et al., 2001]. Methylation only occurs at CpG sites that tend to be concentrated in approximately half of all human genes (known as CpG islands). Methylation at certain crucial CpG sites in these islands may result in transcriptional silencing. Methylation, while it may be biallelic, is usually combined with allelic loss of the other allele, resulting in loss of function of both alleles, and satisfying the Knudson's hypothesis [Knudson, 1985]. Of great biological importance, methylation is an epigenetic phenomenon—i.e., it does not change the basic nucleotide sequence of the genome, and it is potentially reversible by application of demethylating agents. Methylation is a mechanism to down regulate proapoptotic genes including *CASP8*, *Apaf-1*, DAP kinase, TMS1, and various components of the p53 pathway [Conway et al., 2000; Teitz et al., 2000b; Jones, 2001; Soengas et al., 2001]. For these reasons, we explored the mechanism of gene silencing of *CASP8* and *CASP10* in lung cancers and its relationship to methylation.

An initial investigation by our collaborators at Genentech, Inc., indicated that there appeared to be discrepancies between *CASP8* and *CASP10* RNA and protein expression, indicating a post-transciptional mechanism of silencing [Kischkel et al., 2001]. Thus, while both of these caspases appeared to be frequently deregulated in lung cancer cell lines, the mechanisms of inactivation appeared to differ between SCLC and NSCLC. *CASP8* is believed to play an obligatory role in apoptosis initiation by death receptors, but the role of its structural relative, *CASP10*, remains controversial. However, our findings indicated that apoptosis signaling by death receptors involves not only *CASP8* but also *CASP10*, and that both caspases may have equally important roles in apoptosis initiation [Teitz et al., 2000b].

Our further studies indicated that *CASP8* RNA expression was absent in a subset of both high-grade (SCLC) and low-grade (carcinoid) neuroendocrine lung tumors and cell lines but not in NSCLC, which usually lack neuroendocrine features [Shivapurkar et al., 2002a,b]. Methylation was the mechanism of gene silencing in some SCLC cell lines, but other mechanisms were also responsible. In contrast, methylation and gene silencing were absent in NSCLC, although protein expression was frequently absent. These observations suggested that *CASP8* may function as a tumor suppressor gene in neuroendocrine lung tumors.

Because *CASP8* (and, in all proabability *CASP10*) are key components of the DISC, we examined expression of the other components of the DISC complex in lung cancer cell lines [Shivapurkar et al., 2002a]. *MYC* family members are frequently amplified (*MYC* + ve) in SCLC, and *MYC* is a potent inducer of apoptosis. In addition, in neuroblastomas, *CASP8* methylation and silencing is related to N-*MYC* ampliciation [Teitz et al., 2000b]. Thus, we examined the relationship between *MYC* amplification and loss of DISC components. *MYC* amplification was present in 45% of SCLC cell lines, which had lost *CASP8* expression, but not in any of the *CASP8* positive lines. The frequency of *CASP8* loss was significantly higher in *MYC* + ve SCLC compared to *MYC* − ve SCLC or in NSCLC. Analyses of other DISC components showed significantly higher rates of loss of expression of *CASP10*, *DR5*, FAS, and FasL in SCLC as compared to NSCLC. The loss of expression of proapoptotic DISC components was significantly higher in *MYC* + ve SCLC cell lines and these lines were completely resistant to TRAIL. Expression of c-FLIP (proteolytically inactive homologue of *CASP8*) was inversely related to expression of *CASP8*. Clearly, the death receptor pathway is differently inactivated at multiple levels in lung cancer cell lines; and *MYC* amplification in SCLC is associated with inactivation of most components of the DISC complex, with resistance to TRAIL and with expression of c-FLIP.

COMPLEX ROLE OF MITOCHONDRIA IN APOPTOSIS

Our knowledge of the complex role of the mitochondrion in the regulation of apoptosis was initiated by the pioneering work performed

in the roundworm *C. elegans* [Horvitz, 1999], for which Robert Horvitz was awarded the Nobel prize in medicine in 2002. While the role of mitochondria in apoptosis was identified nearly a decade ago, its role came into focus when several mitochondrial proteins were identified that can activate apoptosis directly [Wang, 2001]. A variety of apoptotic stimuli release these proteins from their usual locations in the intermembrane space of mitochondria into the cytoplasm or nucleus. Caspase activation is initiated by release of cytochrome *c*, a key component of the mitochondrial electron transfer system. The cytosolic cytochrome *c* binds to *Apaf-1* protein, with formation of the apoptosome (Fig. 1). The caspase recruitment domain (CARD) of *Apaf-1* becomes exposed, resulting in recruitment and activation of several procaspase 9 molecules. In turn, activated *CASP9* activated the executioner pathway. In *C. Elegans*, *Ced-3* and *Ced-4* (homologues of *CASP9* and *Apaf-1*, respectively) are essential for developmental apoptosis, *Apaf-1* knockout mice survive. These findings may reflect the greater complexity of the role of mitochondria in mammals, where release of three further mitochondrial proteins contribute to apoptosis. The *C. elegans* studies indicate that the mitochondrial pathway is evolutionarily more ancient than the death receptor pathway.

One of these proteins is Smac/Diablo, which, in its mature post-mitochondrial binding form, is capable of binding to the baculovirus IAP repeat (BIR) domain of inhibitors of apoptosis (IAP, see below). Activated Bid releases Smac/Diablo, providing a link between the death receptor and mitochondrial pathways. Another important mitochondrial protein is AIF. On induction of apoptosis, AIF translocates to the nucleus where it induces caspase-independent chromatin condensation and DNA fragmentation. Another mitochondrial protein released by activated Bid is endonuclease G (EndoG), which also results in DNA fragmentation. The release of proapoptotic mitochondrial proteins is regulated by the Bcl-2 protein family (see below).

The role of most mitochondrial proteins in cancer has not been studied extensively, but indirectly or directly it is involved at multiple levels (Fig. 3). Inactivation of *Apaf-1* or *CASP9* substituted for p53 loss in promoting the oncogenic transformation of Myc-expressing cells [Soengas et al., 1999], suggesting a role for these proteins in controlling tumor development. In metastatic melanomas, *Apaf-1* is frequently lost by a combination of allelic loss and gene promoter methylation [Soengas et al., 1999]. Our preliminary (unpublished) data suggests that *Apaf-1* and *CASP9* are expressed at the RNA level in lung cancer cell lines. Of interest, these proteins are expressed and functionally active in human neuroblastoma tumor cell lines [Teitz et al., 2002]. However, upstream regulators of the mitochondrial pathway (including p53 and DAP kinase) are frequently deregulated in lung cancers, as are the Bcl-2 family proteins (see below).

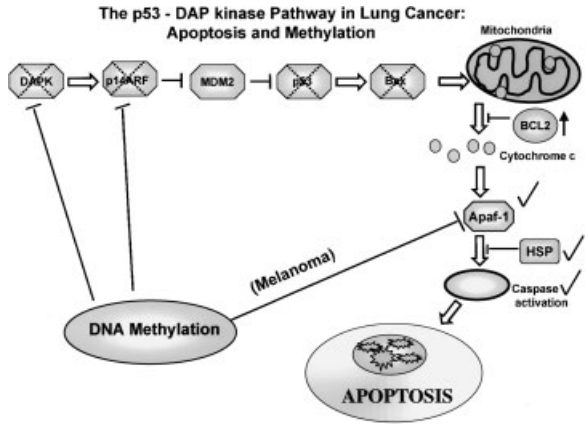

Fig. 3. Disruption of the intrinsic (mitochondrial) pathway in lung cancers. Disruption occurs at several levels including down regulation of DAP kinase, p14/ARF, p53, and Bax, while Bcl-2 is up regulated. *Apaf-1,* heat shock proteins, and *CASP9* are intact. See text for details. Modified from a concept published in Joseph et al. [2000].

DAP KINASE

The DAP (Death Associated Proteins) kinase family is a novel subfamily of pro-apoptotic serine/threonine kinases, consisting of at least five family members which are ubiquitously expressed in various tissues and are capable of inducing apoptosis [Kimchi, 1998; Kogel et al., 1998]. While the sequence homology of these kinases is largely restricted to the N-terminal kinase domain, there is diversity of the adjacent C-terminal regions that link individual family members to specific signal transduction pathways. DAP kinases are involved in both death receptor and mitochondrial pathways of apoptosis. In addition, inactivation of DAPK decreases the induction of p19ARF/p53 resulting in inactivation of the p53 dependent apoptotic pathway [Raveh and Kimchi, 2001]. The

original family member is DAP kinase (DAPK), which is the largest of the family members and is a very large multidomain protein. It contains a death domain at its C-terminal region and was initially isolated as a positive mediator of apoptosis induced by interferon-gamma [Inbal et al., 1997] and is believed to play a role in tumor pathogenesis and with metastasis [Inbal et al., 1997]. Each of the family members has a specific cellular localization, and DAP kinase is associated with the actin filaments of the cytoskeleton. Although the in vivo substrates of the DAP kinase family members have not been identified, DAP kinase is an upstream regulator of p53 activity in response to *c-myc* over expression [Kogel et al., 2001].

Loss of DAP kinase expression has been documented in many cancer types [Esteller et al., 2001]. Functional loss of tumor suppressor genes may occur via point mutations, allelic deletions, homozygous deletions, or by aberrant methylation of the promoter region. Inactivation of DAPK usually occurs by aberrant methylation, although other mechanisms have been described. In lung cancer, methylation of DAPK has been reported at frequencies ranging from 19–44% [Esteller et al., 1999; Tang et al., 2000; Esteller et al., 2001; Kim et al., 2001; Zochbauer-Muller et al., 2001]. Methylation of NSCLC has been reported to be associated with poor prognosis [Tang et al., 2000], advanced pathologic stage increased tumor size and lymph node involvement [Dong et al., 2001], but not with tobacco or asbestos exposure, or with K-ras or p53 mutations [Kim et al., 2001]. Methylation may occasionally be detected in the bronchial epithelium of smokers [Belinsky et al., 2002]. DAPK is located on chromosome 9q34.1 [Feinstein et al., 1995], a region of frequent allelic loss in both NSCLC and SCLC (50–64%) [Girard et al., 2000]. Our recent unpublished work (Toyooka et al., unpublished observation) indicates that DAPK is inactivated in a larger fraction of lung cancers and their cell lines than previously suspected, and that inactivation at the protein level occurs via methylation of the gene as well as at a post-transcriptional level by an as yet unknown mechanism.

BCL-2 FAMILY

Bcl-2 is the mammalian homologue of the *C. elegans* protein Ced-9. The *Bcl-2* gene was discovered when it was found to be linked to an immunoglobulin locus by translocation in follicular lymphomas. The finding that Bcl-2 inactivation prevented apoptosis instead of promoting cell proliferation was a crucial discovery, eventually leading to the concept that inhibition of apoptosis was one of the hallmarks of cancer [Hanahan and Weinberg, 2000]. Bcl-2 has four conserved Bcl-2 homology (BH) domains. In mammals, the Bcl-2 family consists of at least 20 members, all of which share at least one BH domain [Cory and Adams, 2002]. Bcl-2 and at least four other family members are antiapoptotic, and share BH1, 2, and 3 regions. The Bax subfamily contains BH1, 2, and 3 domains, but are proapoptotic in function. Most proapoptotic family members share only a short BH3 domain (BH3 only subfamily). Normally they are sequestered on the cytoskeleton, but proapoptotic signals cause them to insert their BH3 domain into a groove in prosurvival family members. The Bax proteins, Bax and Bak are widely distributed, act further downstream, probably on the mitochondrial membrane. Inactivation of *both* genes is needed for impaired apoptosis. How the Bcl-2 subfamily prevents apoptosis is not fully understood, but stabilizing the mitochondrial membrane (and preventing release of its proapoptotic proteins) as well as regulation of caspases are possibilities [Cory and Adams, 2002].

Bcl-2 and related proteins are among the best studied of the apoptotic molecules in lung cancer, with over 50 references. Bcl-2 is expressed relatively early during bronchial preneoplasia [Ferron et al., 1997; Kalomenidis et al., 2001] and is related to smoke exposure [Hanaoka et al., 2001]. Bcl-2 expression in SCLC is greater than in NSCLC, and the levels in squamous cell carcinomas are higher than in adenocarcinomas [Joseph et al., 2000] (Fig. 4). Since Bax is a proapoptotic member of the Bcl-2 family, the Bcl-2: Bax ratio has been proposed as a measure of tumor resistance to apoptosis. In neuroendocrine lung tumors, there was an inverse correlation between the ratios in low-grade (typical and atypical carcinoids) and high-grade tumors with a predominant Bax expression in the first group and predominant Bcl-2 expression in the second [Brambilla et al., 1996]. The highest levels of Bcl-2 expression and Bcl-2: Bax ratios were associated with a p53 mutant immunophenotype. These and other reports [Ishida et al., 1997; Hwang et al., 2001;

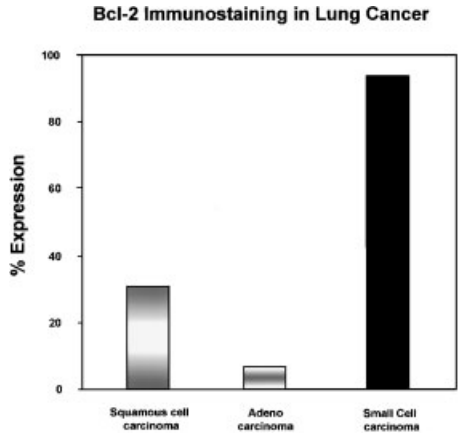

Fig. 4. Upregulation of Bcl-2 in different types of lung cancers. Data extracted from Joseph et al. [2000].

Sartorius and Krammer, 2002] suggest that aggressiveness, response to therapy, and prognosis in lung tumors could be linked to Bcl-2 family proteins.

INHIBITORS OF APOPTOSIS (IAPS)

The IAP class of proteins was originally identified in baculovirus and later in metazoans [LaCasse et al., 1998]. While the antiapoptotic members of the Bcl-2 family inhibit the mitochondrial pathway, IAP molecules are found in the genomes of all metazoans, and are characterized by the presence of 1–3 copies of a ~70 amino acid zinc-finger fold, designated the BIR [LaCasse et al., 1998; Altieri, 2001]. Most IAPs contain other structural motifs including a caspase-recruitment domain (CARD), and a RING finger. IAPs inhibit cell death via a physical association with initiator and effector caspases, thus preventing the proteolytic maturation and enzyme activity of the caspases, especially 3 and 7. IAPs also inhibit apoptosis by modulation of the transcription factor NF-κB. Because their action is downstream of the antiapoptotic Bcl-2 proteins (which acts at the mitochondrial pathway), the IAPs, by inhibiting the executioner pathway, inhibit all of the apoptotic pathways. The mitochondrial protein Smac antagonizes the IAPs by releasing the IAP-bound caspases.

Relatively little has been published on the role of IAPs in lung cancer [Ferreira et al., 2001a,b]. Survivin is unique among the IAPs. Survivin has a single BIR and its expression is cell cycle related [Altieri, 2001]. It is localized to components of the mitotic spindle. It appears to be an oncofetal protein, with strong expression in embryonic and fetal tissues but is absent in most differentiated adult tissues. Considerable over expression has been documented in several tumor types [Altieri, 2001] including lung. Although survivin is expressed in the vast majority of NSCLC tumors, its absence may be associated with improved prognosis [Monzo et al., 1999].

CENTRAL ROLE OF P53

The p53 protein plays a central role in apoptosis, and its inactivation may be the most frequent method of circumventing apoptosis in tumors. It has two other family members, p63 and p73 [Melino et al., 2002]. The latter two proteins appear to be involved in development, while p53 may have evolved as a mechanism to prevent cancer formation [Vousden and Lu, 2002]. Its activation by various extrinsic or intrinsic factors may result in many responses including senescence, cell cycle arrest, DNA repair, differentiation or apoptosis. It functions as a classic tumor suppressor gene, and it is inactivated by loss of both alleles in about 50% of human cancers, perhaps the most common genetic abnormality described to date [Soussi, 1996]. Inactivation of one allele is usually by allelic loss, while the other allele acquires point mutations. These mutations cause an increased stability of the resultant protein resulting in a dominant-negative form of gene inactivation. Most mutations occur relatively late during the preneoplastic process, indicating that other antiapoptotic events must precede it in order to permit the abnormal preneoplastic clones to survive and eventually become malignant and invasive. It has many downstream targets, some of which may negatively regulate *TP53* transcriptional activity. The best known of these proteins in MDM2. The role of p53 in apoptosis is complex and includes activation of the death receptor and mitochondrial pathways [Vousden and Lu, 2002]. One of its principal mechanisms is the release of cytochrome *c* and Smac from the mitochondrial membranes. p53 activates proapoptotic members of the Bcl-2 family and represses antiapoptotic members. It may also transactivate other genes including *Apaf-1*, *PTEN*, and receptors for death inducing ligands. Thus, p53 may function not only as the

"guardian of the genome" but also as the "master regulator" of apoptosis.

Because of its importance in lung tumorigenesis, there are more than 100 studies related to *TP53* alterations in lung cancers. Mutations of *TP53* tumor suppressor gene occur in about 50% of NSCLC and more than 70% of SCLC [Zochbauer-Muller et al., 2002]. *TP53* mutations are the most extensively studied mutations in lung cancer. Previous reports demonstrated that *TP53* mutational spectra of lung cancer showed specific hotspots that are rarely observed in other types of human tumors, suggesting different carcinogen specific mutations [Hussain et al., 2000]. The mutational spectrum in lung cancers arising in never smokers is different from that present in smoke related tumors [Vahakangas et al., 2001]. While exposure to tobacco smoke is the best known and studied lung cancer mutagen, there are other carcinogens for lung cancers derived from occupational and environmental factors.

TMS1 GENE

Recently, a novel gene called ASC (apoptosis-associated speck-like protein containing a CARD) also called *TMS1* (Target of methylation-induced silencing) that promotes apoptosis and encodes a 22 kDa CARD protein was identified [Masumoto et al., 1999; Conway et al., 2000]. This gene is localized at chromosome 16p11.2-12.1 and was shown to be silenced by aberrant methylation in primary breast tumors [Conway et al., 2000]. The exact role or site of action of TMS1 in apoptosis is not currently understood. While there are no reports about TMS1 inactivation in lung cancers, our recent unpublished data indicate that the gene is silenced by aberrant methylation in an important subset of lung cancers and cell lines (Virmani et al., unpublished communication).

APOPTOSIS AND CHEMOTHERAPY

Although systemic chemotherapy is the only proven way of treating disseminated human malignancies, development of drug resistance prevents satisfactory outcome in a number of cases. A curious and perplexing aspect of response to chemotherapy is that nearly 90% of all drug cures occur in only 10% of cancer types [Chu and DeVita, 2000]. The general failure to overcome drug resistance in the clinic with approaches that attack the classical biochemical or molecular mechanisms of drug resistance has focussed attention on alternate mechanisms of drug resistance which are not specific to a particular agent or group but are more global in nature. For more than 30 years, the classical view of anticancer drug action involved the specific interaction of a drug with its target which resulted in tumor cell death via a direct, injurious effect on the proliferating cells [Chu and DeVita, 2000]. However, emerging evidence, based on an enhanced understanding of the molecular mechanisms underlying the control of cell cycle and process of apoptosis, suggests that rather than being intrinsically toxic, many anticancer drugs merely stimulate tumor cells to self-destruct via apoptosis [Chu and DeVita, 2000].

The realization that anticancer drugs kill tumor cells via the same mechanisms which are utilized during physiological cell death have major implications for our understanding of the development of cancer drug resistance and for the design of more effective chemotherapy regimens. As discussed above, cellular proliferation and apoptosis are coupled and oncogenic mutations are believed to sensitize tumor cells to a wide range of physiological triggers of apoptosis that are encountered in the tumor microenvironment, such as nutrient deprivation, interferon, hypoxia, and death ligands such as TNF, FasL, and TRAIL. In case chemotherapeutic drugs kill tumor cells by utilizing the same apoptotic machinery as used by the above physiological agents, it leads to the conclusion that oncogenic mutations will also sensitize cells to the apoptosis induced by chemotherapeutic agents. In fact, oncogenes-induced sensitization to apoptosis remains the most significant Achilles' heel of the cancer cells and explains the remarkable sensitivity of most primary cancer cells to cancer chemotherapy drugs as compared to their normal counterparts, a fact exploited by most anticancer drugs [Evan et al., 1992; Evan and Littlewood, 1998]. The remarkable chemosensitivity of *MYC*-over expressing primary small cell lung cancers is a good example in support of the above hypothesis.

As discussed above, evasion of apoptosis is a hallmark of cancer and cancer cells ultimately develop resistance to the physiological triggers of apoptosis found in the tumor microenvironment by selecting clones which have lost the expression and/or function of genes critical to cell's apoptotic response. This process is further

hastened by the underlying genomic instability inherent to cancer cells. An unfortunate consequence of sharing of apoptotic machinery by physiological triggers of apoptosis and chemotherapeutic agents is that such cancer cells will also become resistant to apoptosis induced by anticancer drugs. The above model might explain the phenomenon of intrinsic or de novo drug resistance demonstrated by a number of cancers, particularly advance-stage cancers. Furthermore, since apoptosis might represent the final common step in the cytotoxicity exerted by a number of anticancer drugs with different mechanisms of action, the above model might provide one possible mechanism for the phenomenon of multidrug resistance demonstrated by late-stage cancers.

From the above discussion, one may conclude that as the cancer cells accumulate genetic alterations that protect them against physiological inducers of apoptosis found in the tumor microenvironment, they simultaneously acquire resistance to anticancer drug-induced apoptosis. However, as discussed previously, there are at least two major pathways of apoptosis which are headed by *CASP8* and *9*, respectively. As such, the full impact of any perturbation in the apoptotic machinery on drug-induced apoptosis can not be gauged without considering the pathway(s) of apoptosis induction utilized by the chemotherapeutic drugs.

Chemotherapeutic drugs are, in general, believed to induce apoptosis via the intrinsic (mitochondrial) pathway, which is initiated by the activation of *CASP9*. On the other hand, *CASP8* is believed not be a major player in this process. The lack of involvement of *CASP8* in drug-induced apoptosis has been supported by studies using mouse embryonic fibroblasts derived from *CASP8*-null animals, which were found to be resistant to apoptosis mediated by the death receptors but were as sensitive as the wild-type cells to drug- or irradiation-induced apoptosis [Varfolomeev et al., 1998]. However, one recent study suggested that *CASP8* is not only activated during drug-induced apoptosis in NSLC cell lines but also plays an essential role in this process while *CASP9* is not involved in this process [Ferreira et al., 2000b]. Activation of *CASP8* in this study was found to be independent of signaling via the death receptors as a dominant negative inhibitor of FADD, which could effectively block Fas-induced cell death, had no effect on proCASP 8 cleavage or apoptosis induced by chemotherapeutic drugs. In contrast to NSCLC cell lines, *CASP8* was found to be dispensable for drug-induced apoptosis in Jurkat (human T cell lymphoma) cells, which underwent *CASP9*-dependent apoptosis in response to anticancer drugs [Ferreira et al., 2000b]. These results have led to the suggestion that unlike leukemia cells, *CASP8* might be the apical caspase in anticancer drugs-induced apoptosis in NSCLC cells and this distinction might explain the difference in the chemosensitivity between the two types of malignancies [Ferreira et al., 2000a]. However, activation of *CASP8* during drug-induced apoptosis is not limited to NSCLC cells and has been recently reported in several additional solid tumors [Engels et al., 2000; Goncalves et al., 2000; Seki et al., 2000; Milner et al., 2002]. Although the exact mechanism of FADD- and death receptor-independent activation of *CASP8* during drug-induced apoptosis is not clear at present, *CASP8* might be activated downstream of mitochondria by activated *CASP3* and/or *6* and serve to amplify the caspase cascade by cleaving Bid and promoting further release of cytochrome *c* from the mitochondria. Notwithstanding the exact mechanism of *CASP8* activation, the above studies suggest that inhibition of *CASP8* activity represent one potential mechanism by which solid tumor cells may develop resistant to drug-induced apoptosis. Thus, it is conceivable that down regulation of *CASP8* expression, for example in *MYC*-overexpressing small cell lung cancers and neuroblastomas, might not only protect cancer cells against death receptors-induced apoptosis but also contribute to their resistance against cancer chemotherapy.

An alternative mechanism of blocking *CASP8* activity is through overexpression of its proteolytically inactive homologue cFLIP or MRIT. Based on the studies suggesting the involvement of *CASP8* in drug-induced apoptosis in solid tumors, we have recently investigated the role of MRIT/cFLIP in this process [Matta et al., 2002]. We have discovered that over-expression of cFLIPL/MRITα1 isoform in NCI-H460 (NSCLC) and HeLa (cervical epithelium) cell lines not only protected them against TRAIL-induced cytotoxicity but also against apoptosis induced by a number of commonly used anticancer drugs, such as doxorubicin, etoposide, cytosine arabinoside, daunorubicin, chlorambucil, and cisplatin [Matta et al., 2002]. The

protective effect of cFLIPL/MRITα1 was associated with delayed cleavage of *CASP8* and *3* and was independent of signaling via the death receptors [Matta et al., 2002]. However, MRITα1/cFLIP$_L$ failed to protect against apoptosis induced by paclitaxel and vincristine, two microtubule-damaging agents, suggesting that these agents might have utility in the treatment of cancers which are drug resistant due to MRITα1/cFLIP$_L$ overexpression [Matta et al., 2002].

The above results have important implications for the potential clinical use of death ligands, such as TRAIL/Apo2L, for the treatment of drug resistant cancers as they would suggest that a single genetic alteration, i.e., down regulation of *CASP8* expression or over expression of MRITα1/cFLIP$_L$, may protect cancers against both chemotherapy- and death receptors-induced apoptosis. In other words, tumors that are drug-resistant due to down regulation of *CASP8* expression or overexpression of MRITα1/cFLIP$_L$ may be resistant to TRAIL as well. Furthermore, since TRAIL has been recently shown to play an important role in tumor immune surveillance and prevention of metastasis [Cretney et al., 2002], the above studies provide further examples of genetic alterations which not only promote tumor progression but also provide resistance to cancer chemotherapy.

CONCLUDING REMARKS

Although not as well studied as some other solid human tumors, the complexity of apoptosis resistance in lung cancer is rapidly becoming apparent. The numerous genes involved in apoptosis (one estimate is greater than 300!) indicate not only redundancy of many pathways but also a highly complex interwoven network of checks and balances. In many lung cancers, there is not only loss of proapoptotic proteins by various mechanisms, but also activation or over expression of antiapoptotic molecules. While these multiple changes result in resistance to routine cytotoxic therapies, they offer opportunities to harness them for novel therapeutic approaches. Our ability to effectively develop such approaches will require both a profound knowledge of the apoptotic process and its derailment during tumorigenesis, but also considerable ingenuity.

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