

Apoptosis in Drug Response

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Abstract: Apoptosis, the cell's intrinsic death program, plays an important role in the regulation of tissue homeostasis. Imbalances between cell death and survival may result in premature death, uncontrolled proliferation or tumor formation. Also, killing of cancer cells by various cytotoxic approaches such as anticancer drugs, -irradiation, suicide genes or immunotherapy, is predominantly mediated through induction of apoptosis in target cells. Thus, defects in apoptosis programs that suppress cell death can also confer drug resistance. Understanding the molecular events that regulate apoptosis and how tumor cells evade apoptotic deletion have provided a paradigm to link cancer genetics and response to cancer therapy. Insights into the signaling cascades that regulate drug-induced apoptosis provide rational targets for therapeutic interventions. Also, genetic variability in apoptosis regulatory genes found in tumors of individual patients may contribute to variability in drug response. Thus, monitoring expression profiles of apoptosis genes in tumors of individual patients or in response to specific pharmacological agents may serve as predictive markers of drug response.

1. INTRODUCTION

Apoptosis or programmed cell death is an intrinsic cell death program that is involved in the regulation of various physiological and pathological processes (Hengartner, 2000). Apoptosis is a key regulator of tissue homeostasis, which critically depends on the balance between proliferation and cell death (Evan and Vousden, 2001). One of the most important recent advances in cancer research is the recognition that apoptosis plays a major role in both tumor formation and treatment response (Johnstone, 2002; Lowe and Lin, 2000; Reed, 1999; Herr and Debatin, 2001; Kaufmann and Gores, 2000). Since apoptosis is a gene-directed program, it can be disrupted by genetic mutations (Johnstone, 2002). Oncogenic mutations often result in an increased growth rate or a block in apoptosis, leading to tumor initiation, progression and resistance to current treatment approaches (El-Deiry, 1997). Defects in apoptosis pathways may create a permissive environment for genetic instability and accumulation of mutations, promote resistance to immune-based deletion and support survival (Igney and Krammer, 2002). Moreover, killing of tumor cells by current anticancer therapies such as cytotoxic drugs, -irradiation, suicide genes or immunotherapy, is mediated by triggering apoptosis in target cells (Herr and Debatin, 2001; Kaufmann and Earnshaw, 2000). To this end, elucidation of the core machinery of apoptosis has provided new insight into cancer biology, revealing novel strategies for cancer therapy. In addition, it is emerging that not only toxicity, but also response to chemotherapy is influenced by pharmacogenetic determinants (Innocenti, 2002; Johnson, 2001). Genetic variability in apoptosis regulatory genes found in tumors of individual patients may contribute to the variability in drug response (Innocenti, 2002; Innocenti, 2000; Johnson, 2001). Thus, monitoring expression profiles

of apoptosis regulating genes in tumors of individual patients or in response to specific pharmacological agents, e.g. by microarray or proteome analysis, may serve as predictive markers of drug response (Innocenti, 2002).

2. APOPTOSIS IN CANCER THERAPY

Most chemotherapeutic agents currently used for cancer treatment were developed by empirical screens designed to identify substances which selectively or non-selectively kill cancer cells. Studies on drug action initially focused on intracellular drug targets, drug-target interaction or resistance mechanisms that prevent drug target interaction. However, it is now well established that most cytotoxic agents primarily act by inducing apoptosis in cancer cells (Herr and Debatin, 2001; Kaufmann and Earnshaw, 2000). This implies that cellular responses occurring after drug-target interaction have a profound impact on drug-induced cytotoxicity. The mechanisms for triggering apoptosis upon cytotoxic therapy may differ for individual stimuli and are only partially understood. However, damage to DNA or to other key signaling molecules appears to be a common inciting event (Herr and Debatin, 2001; Rich, 2000). This initial lesion is then propagated by the cellular stress response and multiple stress-inducible molecules, e.g. JNK, MAPK/ERK, NF B or ceramide may have a profound impact on apoptosis pathways (Leppa and Bohmann, 1999; Davis, 2000). Since the cytotoxic effects of current therapies are mediated by apoptosis, defects in apoptosis signaling pathways can reduce or abrogate treatment response. Since agents with different primary intracellular targets can trigger apoptosis through similar mechanisms, defects in apoptosis programs may result in multi-drug resistance.

3. THE CORE APOPTOTIC MACHINERY

The majority of apoptosis signaling pathways ultimately result in activation of caspases, a family of cysteine proteases that act as common death effector molecules in

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many forms of cell death (Thornberry, 1998; Los, 1999; Earnshaw, 1999). 11 human caspases with different substrate specificity have been identified that cleave next to aspartate residues. Caspases are synthesized as inactive zymogens and activated by proteolytic cleavage. The fact that active caspases can activate each other results in amplification of caspase activity through a protease cascade.

Caspases which are involved in apoptosis signaling are categorized into initiator and effector caspases, respectively (Thornberry, 1998). Initiator caspases transduce various signals into protease activity. Caspase-8 or caspase-10 are directly linked to death inducing signaling complexes (DISCs) by interacting via their death effector domain (DED) with adaptor proteins (FADD) recruited and bound to activated death receptors. Caspase-9 is recruited to the apoptosome via its CARD domain. Effector caspases cleave numerous cytoplasmic or nuclear substrates which mark many of the morphologic features of apoptotic cell death (Hengartner, 2000). For example, polynucleosomal DNA fragmentation is initiated by cleavage of ICAD (inhibitor of caspase-activated DNase), the inhibitor of the endonuclease CAD (caspase-activated DNase) that cleaves DNA into the characteristic oligomeric fragments (Hengartner, 2000). Likewise, loss of overall cell shape is due to proteolysis of cytoskeletal proteins including fodrin, gelsolin, actin, plectrin, cytokeratin, while nuclear shrinking and budding occurs after degradation of lamin (Hengartner, 2000).

Activation of caspases can be initiated through different entry sites, e.g. at the plasma membrane by death receptor mediated signaling (receptor pathway) or at the mitochondria (mitochondrial pathway) (Fulda and Debatin, 2002b; Fig. (1)). Ligation of death receptors of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or TRAIL receptors leads to receptor aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD) and caspase-8 to form the death inducing signaling complex (DISC) (Scaffidi, 1998; Walczak and Krammer, 2000). Caspase-8 becomes activated upon recruitment and propagates apoptosis by direct cleavage of downstream effector caspases. The mitochondrial pathway is initiated by the release of apoptogenic factors such as cytochrome c, apoptosis inducing factor (AIF), Smac/Diablo, Omi/HtrA2, endonuclease G, caspase-2 or caspase-9 from the mitochondrial intermembrane space into the cytosol (Kroemer and Reed, 2000; Constantini, 2000). The release of cytochrome c from mitochondria results in caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex. Smac/Diablo and Omi/HtrA2 promote caspase activation by antagonizing the inhibitory effects to IAPs, while AIF and endonuclease G cause large scale DNA fragmentation and chromatin condensation (Du, 2000; Martins, 2002; Daugas, 2000; Li, 2001).

Also, there are multiple connections between the receptor and the mitochondrial pathway (Roy and Nicholson, 2000). Activation of caspase-8 may lead to cleavage of Bid, a BH3 domain containing protein of the Bcl-2 family, which upon cleavage triggers cytochrome c release from mitochondria thereby initiating a mitochondrial amplification loop (Roy and Nicholson, 2000). Moreover, mitochondria-triggered

caspase-6 cleavage may feed back to the receptor pathway by cleaving caspase-8 (Slee, 1999).

4. SIGNALING PATHWAYS IN CANCER THERAPY

Apoptosis in response to cancer therapy is mediated by activation of the core apoptotic machinery described above, including the receptor and the mitochondrial signaling pathway (Herr and Debatin, 2001; Kaufmann and Earnshaw, 2000). The relative contribution of the death receptor versus the mitochondrial pathway has been a subject of controversial discussion and may depend on the cytotoxic drug, dose and kinetics or on differences between certain cell types similar to the cell type dependent signaling in the CD95 pathway (Herr and Debatin; 2002; Friesen, 1996; Eischen; 1997; Fulda, 2001). Collectively, the data point to a crucial role of the mitochondrial pathway in drug-induced apoptosis. The net outcome of signaling through the core apoptotic machinery is regulated by multiple pro- and anti-apoptotic signaling paths as discussed below.

5. APOPTOSIS REGULATORS IN CANCER THERAPY

Caspases

Given the important role of caspases as key effector molecules in numerous forms of cell death including drug-induced apoptosis, the ability of chemotherapeutic agents to trigger caspase activation appears to be a crucial determinant of drug response (Fulda and Debatin, 2002b). As a consequence, defects in caspase activation often results in chemoresistance (Fulda and Debatin, 2002b; Faderl, 2001; Svingen, 2000).

First, expression levels of individual caspases may influence their overall activity, since activation of caspases may simply be impaired by deficient expression levels of caspases (Teitz, 2000; Fulda, 2001; Estrov, 1998; Koomagi, 2000). For example, MCF-7 breast carcinoma cells completely lack caspase-3 expression due to a frameshift mutation within exon 3 of the caspase-3 gene (Janicke, 1998). These cells can be sensitized by transfection of procaspase-3 towards treatment with cytotoxic drugs (Yang, 2001). Because of the key role of caspases for the execution of cell death one might expect a high frequency of caspase mutations in tumors. Interestingly however, screening for mutations in caspases in various human tumors has not revealed a high frequency of genomic aberrations in caspase genes (Mandrizzato, 1997; Teitz, 2000). Instead, caspase expression and function may be impaired by epigenetic changes such as promotor hypermethylation (Teitz, 2000). To this end, caspase-8 expression was reported to be frequently inactivated by hypermethylation of regulatory sequences of the caspase-8 gene in a number of different tumors cells both *in vitro* and also *in vivo* in primary tumor samples (Teitz, 2000; Fulda, 2001). Importantly, restoration of caspase-8 expression by gene transfer or by demethylation also restored sensitivity of resistant tumor cells for apoptosis induction (Fulda, 2001). In addition, enhanced transcription of caspase genes has been reported in response to cytotoxic therapy. Thus, treatment with IFN resulted in increased expression of caspase proteins, which was mediated by direct

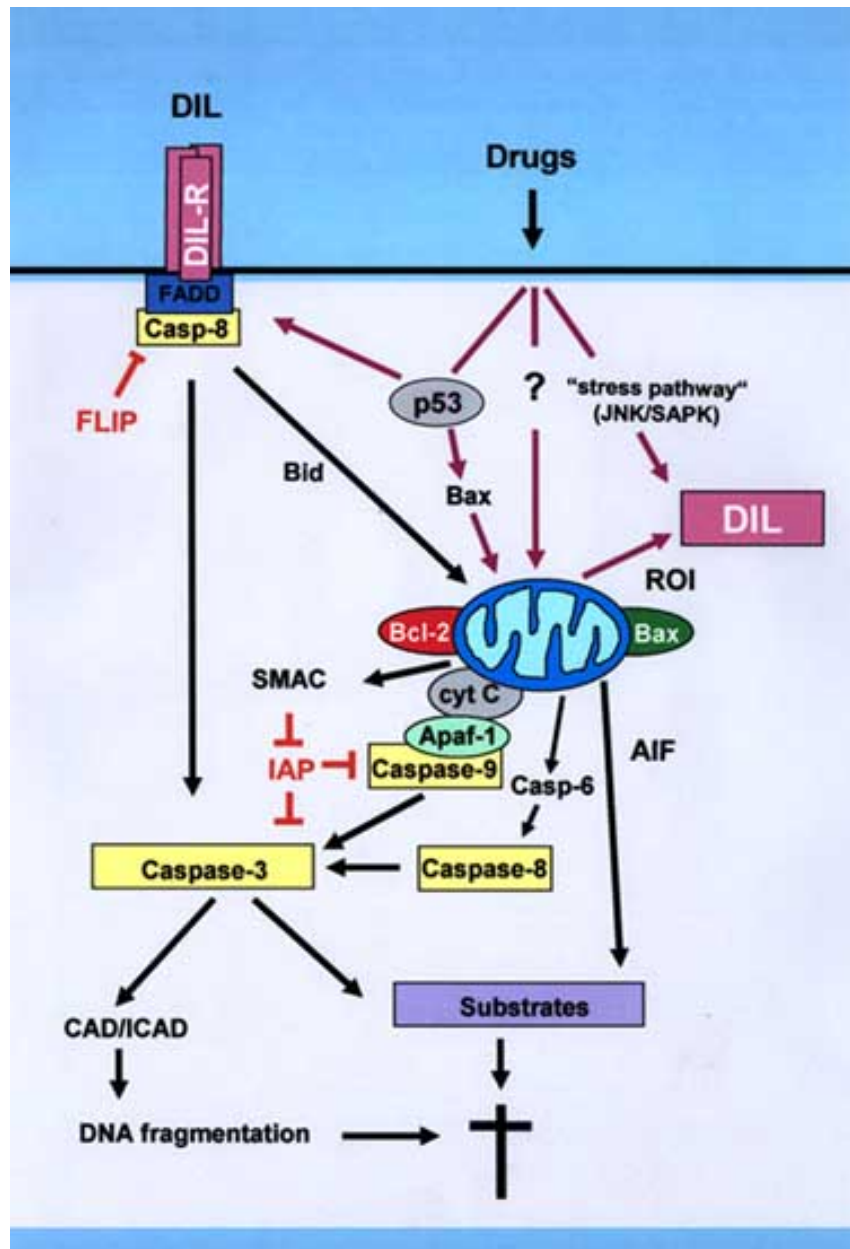


Fig. (1). Activation of apoptosis pathways by anticancer therapy.

Apoptosis pathways can be initiated through different entry sites, e.g. at the plasma membrane by death receptor mediated signaling (receptor pathway) or at the mitochondria (mitochondrial pathway). Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily (DIL-R) such as CD95 (APO-1/Fas) or TRAIL receptors by death-inducing ligands (DIL) results in receptor aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD) and caspase-8. Upon recruitment caspase-8 becomes activated and initiates apoptosis by direct cleavage of downstream effector caspases. The mitochondrial pathway is initiated by the release of apoptogenic factors such as cytochrome c, apoptosis inducing factor (AIF), or Smac from mitochondria into the cytosol. The release of cytochrome c into the cytosol triggers caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex. Smac promotes caspase activation through neutralizing the inhibitory effects to IAPs, while AIF causes DNA condensation. The receptor and the mitochondrial pathway can be interconnected at different levels, e.g. by Bid, a BH3 domain containing protein of the Bcl-2 family which assumes cytochrome-c-releasing activity upon cleavage by caspase-8. Activation of caspases is negatively regulated at the receptor level by FLIP which block caspase-8 activation, at the mitochondria by Bcl-2 family proteins and by inhibitor of apoptosis proteins (IAPs). See text for more details.

activation of STAT-1, a downstream transcription factor involved in IFN signaling (Fulda and Debatin, 2002a). Also, transcriptional upregulation of caspases was found independent of STAT-1 in response to drug treatment (Micheau, 1999).

Inhibitor of Apoptosis Proteins (IAPs)

The family of endogenous caspase inhibitors called "inhibitor of apoptosis proteins" (IAPs) are highly conserved throughout evolution and comprise human analogues such as XIAP, ciap1, cIAP2, survivin, livin (ML-IAP). IAPs have been reported to directly inhibit active caspase-3 and caspase-7 and to block caspase-9 activation (Deveraux and Reed, 1998; Holczik and Korneluk, 2001). In addition to modulation of apoptosis, IAP members such as survivin have been implied in regulation of mitosis (Altieri, 2001). The activity of IAPs are controlled at various levels, e.g. at the transcriptional level by the transcription factor NF B that can induce expression of cIAP1, cIAP and XIAP (Deveraux and Reed, 1998). IAPs are negatively regulated by caspase-mediated cleavage. In addition, Smac/Diablo and Omi, proteins that are released from mitochondria into the cytosol upon apoptosis induction, neutralize IAPs' function through binding to IAPs thereby displacing them from their caspase partners (Du, 2000; Suzuki, 2001; Martins, 2002). Overexpression of Smac or Smac peptides sensitized even resistant tumor cells for apoptosis induction and strongly synergized with TRAIL to eradicate established tumors in an orthotopic mouse model of malignant glioma indicating that Smac agonists represent novel promising cancer therapeutics (Fulda, 2002).

Blockade of apoptotic cell death by IAPs in response to chemotherapy has been suggested by several experimental studies. XIAP, cIAP1 or cIAP2 inhibited apoptosis *in vitro* following treatment with cisplatin, cytarabine, TRAIL, staurosporine or after γ -irradiation (Datta, 2000; Altieri, 2001). Also, enhanced IAPs expression in primary myeloid leukemia cells correlated with poor treatment response (Tamm, 2000). Interestingly, survivin represents the fourth most common transcriptome of the human genome, since it is expressed at high levels in the majority of human cancers, indicating that survivin may contribute to the malignant phenotype of cancer cells (Altieri, 2001; Velculescu, 1999). High survivin expression has been associated with poor prognosis in a variety of human neoplasms including neuroblastoma, colon carcinoma, gastric carcinoma or leukemia (Adida, 1998; Adida, 2000; Altieri, 2001). The mechanisms by which survivin regulates apoptosis and cell division remain a point of controversial discussion and may differ from other members of the IAP family, since survivin has also been reported to fail to inhibit caspase-3 *in vitro* (Altieri, 2001).

Bcl-2 Proteins

Bcl-2 family proteins play an important role in the regulation of the mitochondrial pathway, since these proteins localize to intracellular membranes such as the mitochondrial membrane (Antonsson, 2000). They comprise both anti-

apoptotic members, e.g. Bcl-2 or Bcl-X_L, as well as pro-apoptotic molecules such as Bax and BH3 domain only molecules, e.g. Bid (Antonsson, 2000; Zhang, 2000). Upon apoptosis induction proapoptotic Bcl-2 proteins with multidomains such as Bax translocate from the cytoplasm to the outer mitochondrial membrane to promote cytochrome c release. This translocation to mitochondria can be triggered by Bcl-2 proteins which have a BH3 domain only. Bcl-2 or Bcl-X_L block apoptosis by sequestering BH3 domain only proteins in stable mitochondrial complexes and/or by preventing cytochrome c release from mitochondria (Cheng, 2001). Imbalances in the ratio of anti- and pro-apoptotic Bcl-2 proteins may favor tumor cell survival instead of cell death (Antonsson, 2000). Altered expression of Bcl-2 family proteins have been reported in various human cancers (Reed, 1999). In most cases aberrant expression of Bcl-2 proteins are regulated at the transcriptional or posttranscriptional level. Also, some of these alterations involve structural gene alterations, e.g. single nucleotide substitution or frameshift mutations that inactivate the Bax gene in certain types of colon cancer and hematopoietic malignancies (Reed, 1999). Mutations or altered expression of pro- or anti-apoptotic Bcl-2 family proteins can drastically alter drug response in experimental systems *in vitro*. In addition, several clinical correlative studies have provided support that high level expression of antiapoptotic Bcl-2 proteins confers a chemoresistant phenotype in a variety of tumors, including AML, ALL, CLL, multiple myeloma, prostate carcinoma, malignant brain tumors and neuroblastoma (Campos, 1993; Prokop, 2000). Likewise, reduced Bax levels have been associated with poor responses to chemotherapy and shorter overall survival in breast or colorectal carcinoma (Sturm, 2001; Bargou, 1995). Conversely, enhanced Bax levels correlated in several cell types with response to chemotherapy *in vivo* (Sturm, 2001). Bcl-2 inhibitors are considered as attractive therapeutics, since Bcl-2 is overexpressed in various malignancies (Reed, 1999). To this end, encouraging results without severe toxicities have been observed in phase I clinical studies using Bcl-2 antisense oligonucleotides in lymphoma patients (Waters, 2000).

p53

p53 was the first tumor suppressor gene linked to apoptosis (Vogelstein, 2000; Vousden, 2000). Mutations of p53 occur in the majority of human cancers and are often associated with advanced disease and poor prognosis (Wallace-Brodeur and Lowe, 1999). p53 functions as a checkpoint protein involved in cell cycle arrest, DNA repair and apoptosis (Vogelstein, 2000; Vousden, 2000). As a sensor of cellular stress, the p53 is activated by a variety of stimuli such as anticancer drugs or irradiation, leading to cell cycle arrest and /or apoptosis (Vogelstein, 2000; Vousden, 2001). The transcriptional activity of p53 is important for mediating its biological function and involves cell cycle regulatory genes such as p21 or GADD45 and apoptosis genes, e.g. Bax (Vogelstein, 2000). In addition, transcription-independent regulation of cell growth and apoptosis by p53 has also been described, e.g. by promoting the translocation of CD95 from intracellular compartment such as the Golgi stores to the plasma membrane or by a direct effect of p53 on mitochondria (Vogelstein, 2000).

However, the relationship of p53, apoptosis and drug response has been controversially discussed. On one hand, loss of p53 function has been reported to attenuate drug-induced apoptosis *in vitro* (Lowe, 1995). In addition, several clinical correlative studies and studies in mice showed an association between wildtype p53 and drug response indicating that the p53 status may predict clinical response to chemotherapy (Wallace-Brodeur and Lowe, 1999). On the other hand, it has been proposed that p53 play little or no role in the sensitivity of cancer cell to chemotherapy or radiation (Brown, 1999). Although wildtype p53 was found to predispose cells to die more rapidly by apoptosis as assessed by short-term assays, the p53 status had no effect on clonogenic survival indicating that the p53 status may determine the threshold and kinetics of cell death rather than the overall survival (Brown, 1999). Moreover, cells harboring wild-type p53 may fail to respond to cytotoxic treatment and those lacking functional p53 may even respond better (Brown, 1999). Also, the contribution of p53 to apoptosis in response to cytotoxic therapies may depend on drug doses or tumor cell type.

NF B

The transcription factor NF B has been connected with multiple aspects of oncogenesis including cell proliferation, inhibition of apoptosis, cell cycle and migration (Mayo and Baldwin, 2000). Multiple human tumors, e.g. Hodgkin lymphoma or pancreatic carcinoma, have evolved mechanisms for disrupting the NF B pathway suggesting that NF B is involved in tumor formation (Karin, 2002). Also, the ability of NF B to suppress apoptosis has been implied to confer drug resistance (Mayo and Baldwin, 2000). To this end, NF B can already be constitutively active in certain tumor types such as pancreatic carcinoma (Mayo and Baldwin, 2000). In addition, NF B activity is induced in response to diverse stimuli, e.g. in response to cellular stress and chemotherapy (Karin and Ben-Neriah, 2000). In most cell types, NF B is sequestered in the cytoplasm by its interaction with I B proteins and therefore remains inactive (Karin and Ben-Neriah, 2000). Once NF B is released from I B following phosphorylation of I B, NF B can translocate into the nucleus to initiate transcription of target genes (Karin and Ben-Neriah, 2000). NFkB target genes include several anti-apoptotic proteins, e.g. cIAP1, cIAP2, or Bcl-X_L (Pahl, 1999; Barkett and Gilmore, 1999). Interestingly, promoter activation of certain pro-apoptotic factors such as CD95L is also controlled by NF B consistent with findings that NF B can enhance apoptosis under certain conditions (Pahl, 1999). Since some anticancer agents can increase NF B transcriptional activity, inhibition of NF B together with chemotherapy enhanced the cytotoxic effect of chemotherapy (Mayo and Baldwin, 2000). Thus, NF B may play a prominent role in inducible chemoresistance and blockade of NF B activity may be a potential new adjuvant approach to chemotherapy (Mayo and Baldwin, 2000).

PI3K/Akt

The PI3K/Akt pathway is a potent mediator of cell survival (Blume-Jensen and Hunter, 2001). Survival signals

may be delivered by growth factors or by interactions with neighboring cells or with the extracellular matrix (Blume-Jensen and Hunter, 2001; Datta, 1999). The PI3K/Akt pathway is often altered at a variety of steps in tumor cells, e.g. as a result of production of autocrine growth factors, elevated levels of growth factors receptors or constitutively active, mutated receptors. To this end, enhanced signaling from receptor tyrosine kinases often occurs in breast carcinoma as a result of HER2/neu overexpression (Blume-Jensen and Hunter, 2001). Likewise, absence of the tumor suppressor gene PTEN, which antagonizes the prosurvival function of Akt through dephosphorylation of phosphatidylinositol triphosphate, frequently occurs in several tumors including malignant glioma (Simpson and Parsons, 2001). Also, mutated constitutively active Ras isoforms, which are found in 30% of cancers, in particular in pancreatic carcinoma, or the fusion protein bcr/abl, the transforming kinase of CML, can directly activate PI3K (Downward, 1998). Akt regulates multiple signaling pathways involved in cell proliferation, apoptosis, glucose metabolism or angiogenesis through transcriptional and posttranscriptional modifications of key molecules involved in cellular signaling (Datta, 1999). The pro-survival function of Akt is mediated by phosphorylation of apoptosis signaling molecules such as Bad, caspases-9, or by inhibiting cytochrome c release from mitochondria (Datta, 1999). A role of the PI3K/Akt pathway in treatment resistance has been suggested, since deregulated activation of Akt conferred resistance to apoptosis upon death receptor ligation or cytotoxic drug treatment (Datta, 1999). Thus, targeting the PI3K/Akt pathway, e.g. by small molecule inhibitors, may be useful to augment treatment response of tumors (Stein, 2000).

6. CASPASE-INDEPENDENT AND NONAPOPTOTIC MODES OF CELL DEATH

Although numerous studies indicate an essential role of caspase-dependent apoptosis in mediating drug response, this concept has also been challenged (Finkel, 1999). So far, a consistent link between the cells' ability to undergo apoptosis and drug response could not be observed (Finkel, 1999). Thus, nonapoptotic modes of cell death, e.g. necrosis or some forms of cell death that cannot be easily classified at present, may also mediate response to cytotoxic therapy (Leist and Jaattela, 2001; Sperandio, 2001; Borner and Monney, 1999; Johnson, 2000). However, the signaling pathways and molecules involved in these alternative forms of cell death have not yet exactly been defined (Johnson, 2000). The relative contribution of these different modes of cell death for chemoresponses *in vitro* and *in vivo* remains to be defined.

CONCLUSION

Numerous studies over the last years have shown that cell death by apoptosis plays a crucial role in the surveillance of tumor formation and in anticancer therapies which primarily act by triggering apoptosis in tumor cells (Herr and Debatin, 2001; Kaufmann and Earnshaw, 2000; Johnstone, 2002). However, several points remain to be addressed in future studies. Most of the apoptosis signaling components have

not yet been studied in clinical samples. Moreover, the biology that determines the individual responses of different tumors to anticancer therapies warrants further investigations to provide the basis for more specific therapeutic interventions. Also, further insights into the genetic contributions of apoptosis regulating genes to variability in drug efficacy will add to our understanding of the genetic basis for drug response (Innocenti, 2002; Innocenti, 2000; Johnson, 2001). To this end, genetic variability in apoptosis regulatory genes found in tumors of individual patients may contribute to variability in treatment response (Innocenti, 2000). Thus, monitoring the expression profile of apoptosis regulating genes in individual patients or in response to specific pharmacologic agents may serve as predictive markers of drug response. Future studies on the role of apoptosis regulating genes in individual tumors both *in vitro* and *in vivo* in tumor cells of patients under chemotherapy, e.g. by DNA microarrays or proteomic studies, may provide the basis for "tailored" tumor therapy and may identify new targets for therapeutic interventions.

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