Components of the Cell Death Machine and Drug Sensitivity of the National Cancer Institute Cell Line Panel

Phyllis A. Svingen,1 David Loegering,1 Joe Rodriguez,3 Xue Wei Meng,1 Peter W. Mesner, Jr.,1 Susan Holbeck,5 Anne Monks,4 Stan Krajewski,6 Dominic A. Scudiero,4 Edward A. Sausville,5 John C. Reed,6 Yuri A. Lazebnik,3 and Scott H. Kaufmann1,2

1Division of Oncology Research, Mayo Clinic, and 2Department of Molecular Pharmacology, Mayo Graduate School, Rochester, Minnesota; 3Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; 4SAIC-Frederick, Inc., National Cancer Institute, NIH, Frederick, Maryland; 5Developmental Therapeutics Program, National Cancer Institute, NIH, Bethesda, Maryland; and 6Burnham Institute, LaJolla, California

ABSTRACT

Purpose: According to some studies, susceptibility of cells to anticancer drug-induced apoptosis is markedly inhibited by targeted deletion of genes encoding apoptotic protease activating factor 1 (Apaf-1) or certain caspases. Information about levels of these polypeptides in common cancer cell types and any possible correlation with drug sensitivity in the absence of gene deletion is currently fragmentary.

Experimental Design: Immunoblotting was used to estimate levels of Apaf-1 as well as procaspase-2, -3, -6, -7, -8, and -9 in the 60-cell-line panel used for drug screening by the National Cancer Institute. Sensitivity of the same lines to >80,000 compounds was determined with 48-hour sulforhodamine B binding assays. Additional 6-day assays were performed for selected agents.

Results: Levels of Apaf-1 and procaspases varied widely. Apaf-1 and procaspase-9, which are implicated in caspase activation after treatment of cells with various anticancer drugs, were detectable in all of the cell lines, with levels of Apaf-1 ranging from ~1 × 10^5 to 2 × 10^6 molecules per cell and procaspase-9 from ~5 × 10^4 to ~1.6 × 10^5 molecules per cell. Procaspase-8 levels ranged from 1.7 × 10^5 to 8 × 10^6 molecules per cell. Procaspase-3, a major effector caspase, varied from undetectable to ~1.6 × 10^6 molecules per cell. Correlations between levels of these polypeptides and sensitivity to any of a variety of experimental or conventional antineoplastic agents in either 2-day or 6-day cytotoxicity assays were weak at best.

Conclusions: With the exception of caspase-3, all of the components of the core cell-death machinery are expressed in all of the cell lines examined. Despite variations in expression, levels of any one component are not a major determinant of drug sensitivity in these cells in vitro.

INTRODUCTION

Studies performed over the past decade indicate that chemotherapeutic agents induce apoptosis in vitro and in vivo (1–4). Conversely, failure to activate the apoptotic machinery has been correlated with resistance to multiple chemotherapeutic agents (5–9). These observations raise the possibility that factors regulating the apoptotic process might play an important role in sensitivity to anticancer drugs (2, 4, 10, 11).

Present understanding suggests that a family of aspartate-directed cysteine proteases called caspases play critical roles in the apoptotic process (12–16). Among the 12 human caspasess, procaspase-2, -3, -6, -7, -8, and -10 have accepted or postulated roles during apoptosis (13–16). Caspases-3 and -7 seem to be the major effector caspases responsible for events leading to cellular disassembly; caspases-8, -9, and -10 are involved in the initiation steps that result in caspase activation; and the exact roles of caspases-2 and -7 remain to be established (reviewed in refs. 12–17; see also refs. 18–22).

Because of its importance to subsequent apoptotic events, caspase activation has been extensively studied (12–17). One mechanism of activation involves ligation of cell surface death receptors, recruitment of adapter molecules to the cytoplasmic surfaces of the ligated receptors, and binding of initiator procaspases to the adaptor molecules in a fashion that results in activation (17–19, 23, 24). For example, binding of the cytotoxic cytokine tumor necrosis factor a-related apoptosis-inducing ligand (TRAIL) to its receptors DR4 and DR5 results in recruitment of the adaptor molecule Fas-associating death domain protein (FADD; ref. 5), which, in turn, binds procaspase-8 and facilitates its activation (25). Once activated, procaspase-8 can proteolytically activate procaspase-3 and -7 (26); and the resulting activated caspase-3 can cleave procaspase-6. Alternatively, caspase-8 can cleave the proapoptotic Bcl-2 family member Bid to yield a M_15,000 fragment that binds to mitochondria and facilitates release of cytochrome c to the cytoplasm (14, 27, 28). Once released to the cytoplasm as a consequence of Bid cleavage or other signals (28–30), cytochrome c binds the docking molecule apo-
Caspase Expression and Drug Sensitivity

The preceding preclinical and clinical studies have left a number of important questions unanswered. First, quantitative information about the abundance of procaspases and Apaf-1 is difficult to find. Second, it is unclear from existing studies whether genes encoding procaspases and/or Apaf-1 are frequently silenced in common cancer cell types. Third, the effects of alterations short of gene deletion or complete silencing on drug sensitivity remain to be elucidated. To begin to address these issues, the present study examined expression of procaspases and Apaf-1 in the 60 cell lines used by the National Cancer Institute (NCI) to screen new anticancer drugs. These cell lines, which have been previously described in detail (70, 71), have been tested for their response to 30,000 agents, ~50% of which are nonproprietary. The assay for drug sensitivity yields quantitative information about drug concentrations that induce cell death, which is apoptotic cell death in the vast majority of instances in which it has been investigated. The present study focused on (a) quantitating levels of procaspases and Apaf-1 in widely used cancer cell lines; (b) determining the frequency of alterations in the core apoptotic machinery in a variety of common neoplastic cell types, including colon, breast, lung (non–small-cell), prostate, and ovarian cancers; and (c) identifying any possible relationship between expression of various components of the core death machinery and sensitivity of the cell lines to anticancer agents.

MATERIALS AND METHODS

Materials. Recombinant human caspases-3, -6, -7, and -8 were from PharMingen (San Diego, CA). Recombinant human caspase-2 was from Biomol (Plymouth Meeting, PA). Recombinant human caspase-9 and Apaf-1 were purified as described previously (31). During the course of these studies, the following commercially available immunologic reagents were used: monoclonal antibodies to procaspase-2 and -7 from Transduction Labs (Lexington, KY) and BD PharMingen (San Diego, CA), respectively; and polyclonal rabbit sera that recognize procaspase-3 and procaspase-6 from Cell Signaling Technology (Beverly, MA) and Upstate Biotechnology (Lake Placid, NY), respectively. Monoclonal antibodies that recognize procaspase-9 and Apaf-1 were generated as described previously (31). Rabbit antisera that recognize procaspase-3 and -8 were raised by injecting female New Zealand White rabbits with untagged recombinant human caspase-3 large subunit or with recombinant His6-tagged caspase-8 enzyme (72). All of these reagents recognize epitopes that are present in the active caspases as well as the corresponding zymogens. Peroxidase-coupled affinity-purified secondary antibodies were from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

Tissue Culture. 2K562 human leukemia cells from American Type Culture Collection (ATCC; Manassas, VA) were cultured in RPMI 1640 containing 5% heat-inactivated fetal calf serum, 100 units/ml penicillin G, 100 µg/ml streptomycin, and 2 mmol/L glutamine. Cultures were incubated at 37°C in humidified air with 5% (v/v) CO2 and were maintained in exponential growth phase. Cell size was determined on a Coulter counter equipped with a channel analyzer and calibrated with polystyrene beads of defined diameter (Becton-Dickinson, San Jose, CA). To produce an internal standard that would be reproducible from blot to blot, we

The preceding gene disruption has demonstrated that the loss of procaspase-8 or FADD produces murine cells that are resistant to the cytotoxic effects of death receptor ligands but not to agents such as etoposide or doxorubicin (34, 35). Conversely, deletion of Apaf-1 (36), procaspase-9 (37, 38), or cytochrome c (39) results in cells that are relatively resistant to the cytotoxic effects of several agents, including glucocorticoids, etoposide, or staurosporine. These observations have suggested that many small-molecule anticancer agents induce apoptosis through the cytochrome c/Apaf-1/caspase-9 pathway (3, 4). There are, however, exceptions to this generalization. Tillman et al. (40) have reported that p53-mediated induction of Fas ligand plays a critical role in apoptosis induced by 5-fluorouracil (5FU) in some colon cancer cell lines, a finding corroborated by subsequent observations in Fas-deficient thymocytes (41). Additional studies suggest that a number of other agents, including 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (42, 43), fenretidine (44), CI-1040 (45), etoposide (46), and camptothecin (47), also trigger apoptosis in certain cell types through a process that involves caspase-8 activation.

There is also growing evidence that alterations in the core apoptotic machinery might occur in cancer cells. The MCF-7 breast cancer cell line lacks procaspase-3 polypeptide as a consequence of a 47-bp deletion that alters the reading frame of the message and results in a truncated, unstable translation product (48). Transfection of procaspase-3 into MCF-7 cells reportedly enhances apoptosis induced by cisplatin (49), doxorubicin, or etoposide (50), but not paclitaxel (51), which suggests possible drug-specific dependence on certain components of the apoptotic machinery. A number of cancer cell types, including small-cell lung cancer (52, 53), neuroblastoma (54–56), and primitive neuroectodermal tumors (57), lack procaspase-8, most likely as a consequence of gene methylation (53, 54, 57). Resistance to the cytotoxic effects of TRAIL has been demonstrated in these caspase-8-deficient cells (55–57). Finally, it has been reported that Apaf-1 is absent from ~40% of metastatic melanoma cell lines and clinical specimens (58). These observations raise the possibility that alterations in the levels of caspases and/or Apaf-1 might occur commonly in human cancer cell lines and might affect drug sensitivity.

On the basis of these findings, there is growing interest in assessing expression of Apaf-1 and procaspases in the clinical setting. Previous studies have detected variable levels of several procaspases and Apaf-1 in blasts from patients with acute leukemias but failed to establish a consistent relationship with clinical outcome (59–61). Additional reports have suggested that high procaspase-3 levels are associated with a better prognosis in patients with neuroblastoma (62) or squamous cell cancer of the esophagus (ref. 63; for alternative view, see ref. 64), a poorer prognosis in patients with endometrial (65) or non–small-cell lung cancer (66), and are unassociated with prognosis in patients with cervical (67) or bladder (68) cancer. In addition, diminished caspase-8 expression has been associated with a particularly poor prognosis in patients with neuroblastoma (69).
prepared a large aliquot of these K562 cells for electrophoresis as described in the Immunoblotting section and lyophilized them in multiple single-use vials.

The cells of the NCI 60-cell-line panel were cultured in RPMI 1640 containing 5% fetal bovine serum and 2 mmol/L glutamine (73). Sensitivity of cells to various agents was determined by exposing cells to graded concentrations of each drug for 48 hours and assessing viable cell mass with sulforhodamine B, as previously described in detail (70). For each agent, the GI₅₀ (the drug concentration that inhibits proliferation by 50%) and the LC₅₀ (the drug concentration that reduces the sulforhodamine signal to 50% of the input number of cells) were determined (70). In addition, because of concern that many cell lines might require >48 hours to die after treatment with cytotoxic agents, the LC₅₀ was also determined from multiple dose-response curves after 6-day exposure to the >100 agents in the standard agent database.

**COMPARE Analysis.** The COMPARE program to analyze data generated by the NCI Drug Screen can be accessed at http://www.dtp.nci.nih.gov. For much of this study, analysis was limited to the standard agents database, which includes cytotoxicity data on >100 prototypic compounds against the NCI cell line panel, the molecular targets database of proteins and genes examined in the 60 cell lines, and the microarray database containing gene expression profiles of the 60 cell lines. The COMPARE software calculated pairwise correlations between the measured protein levels and the patterns in these databases. Analyses were reported as Pearson correlation coefficients. Type II errors described in the present article were not corrected for the effects of multiple comparisons.

**Immunoblotting.** Log phase cells were washed with serum-free RPMI 1640 and were lysed in buffer A [6 mol/L guanidine hydrochloride, 250 mmol/L Tris-HCl (pH 8.5 at 21°C), and 10 mmol/L EDTA] that was supplemented with 1% (w/v) β-mercaptoethanol and 1 mmol/L α-phenylmethylsulfonyl fluoride immediately before use. This method was used because it gives more rapid cell lysis and better antigen preservation in certain cell types than does treatment with buffers containing neutral detergent or SDS (74). After incubation for >24 hours, samples were treated with iodoacetamide to block free sulfhydryl groups and then were dialyzed sequentially into 4 mol/L urea containing 50 mmol/L Tris-HCl (pH 7.4 at 4°C), 4 mol/L urea (four passes) and 0.1% (w/v) SDS (3 passes). After an aliquot was removed for determination of protein by the bicinchoninic acid method (75), multiple aliquots of each sample were lyophilized to dryness and stored at −20°C.

Before electrophoresis, samples were solubilized at a concentration of 5 mg of protein per mL in SDS sample buffer [4 mol/L urea (deionized over AG1-X8 beads), 2% (w/v) SDS, 62.5 mmol/L Tris-HCl (pH 6.8) and 1 mmol/L EDTA] and were heated to 65°C for 20 min. All of the cell lines were run at a minimum of two different protein concentrations. Aliquots containing 5, 10, or 50 μg of protein were applied to adjacent wells of SDS-polyacrylamide gels containing a short hairpin RNA (79) targeting Apaf-1 mRNA (nucleotides 190–208) were synthesized and annealed at 70°C, as follows: 5'-GATCCCCGATAATGTTCCATGGATTTCGAGAAGAATACTGAGAATCTTATCTTTTGAAAAA-3', and 5'-AGCTTCTCCAAAAAGATATTGCTATTATCCTGTTGAA-AATACGATGAGATTTGGG-3'. After digestion with BglII and HindIII, the double-stranded oligonucleotide was cloned into the corresponding site of pSS-Hip vector (kindly provided by Dan D. Billadeau, Mayo Clinic, Rochester, MN). Once integrity of the insert was confirmed by sequencing, the plasmid was transfected into Jurkat T cell leukemia cells by electroporation at 240 V for 10 milliseconds on a BTX 820 square wave electroporator. Beginning 48 hours after transfection, stable transfecants were selected in 400 μg/mL geneticin and cloned by limiting dilution.

**RESULTS**

**Levels of Procaspases and Apaf-1 in K562 Cells.** Before assessing the expression of various components of the core cell-death machinery in the cell lines that make up the NCI human
tumor cell line screen, we endeavored to quantitate levels of various procaspases and Apaf-1 in a widely available human tissue culture cell line that could serve as an internal control in subsequent experiments. K562 cells were chosen for these studies based on previous reverse transcription-PCR experiments that indicated that this cell line expresses all caspases examined (78). To provide an estimate of caspase expression at the protein level, the signals observed on immunoblots containing serial dilutions of ATCC K562 cells and each purified polypeptide were compared. Examples of this analysis are illustrated in Fig. 1. These blots demonstrate that \(3 \times 10^5\) K562 cells yield the same procaspase-7 signal on immunoblots as 12 ng (0.41 pmol) of purified caspase-7 (Fig. 1A and B). This corresponds to \(8 \times 10^5\) procaspase-7 molecules per K562 cell. On the basis of a cell diameter of 15 \(\mu\)m, this translates into a procaspase-7 concentration of \(24 \mu\)g/mL (800 nmol/L) if the zymogen were uniformly distributed in the cytoplasm of K562 cells (49, 51).8 On the basis of the data shown in Figs. 1C–H, similar calculations indicate that ATCC K562 cells contain \(5 \times 10^5\) procaspase-2 molecules, 4 \(\times\) \(10^5\) procaspase-3 molecules, and \(1 \times 10^6\) procaspase-6 molecules per cell. Levels of procaspase-8 and Apaf-1 (6 \(\times\) \(10^5\) molecules of each per K562 cell) are similar to levels of the effector caspases, whereas procaspase-9 is less abundant at \(8 \times 10^4\) molecules per K562 cell.

Expression of Initiator Caspases. In additional experiments, expression of the same polypeptides was examined in the 60 cell lines that make up the NCI in vitro drug screen. Blots were loaded with 5, 10, or 50 \(\mu\)g of protein from each cell line, along with a serial dilution of the same K562 cell extract used in Fig. 1, and were probed with antibodies to procaspase-2, -3, -6, -7, and -9, as well as Apaf-1 and histone H1. The latter protein, which is present in equal amounts in all diploid cells and has a mobility on gels similar to that of the procaspase-3, -6, and -7, not only served as a control to confirm appropriate loading and transfer of the gels, but also provided a method for normalizing caspase content in other cell lines (i.e., caspase levels per cell equivalent). Results of this analysis are illustrated in Figs. 2 and 3 and are summarized in Fig. 4.

In view of the importance of Apaf-1 and procaspase-9 in initiating the apoptotic response to many anticancer agents (3, 15, 37), levels of these polypeptides are shown in Fig. 2 for all 60 cell lines. Both Apaf-1 and procaspase-9 were detectable in each of the lines, including the melanoma cell lines.

Levels of Apaf-1 varied over a >20-fold range among the

---

8 This calculation is based on the observation that procaspase-3 is excluded from nuclei (40) and the assumption that the nucleus occupies \(50\%\) of the volume of K562 cells.
cell lines (Figs. 2 and 4 A), ranging from 17% (CCRF-CEM cells) to 470% (UACC-62 melanoma cells) of the levels observed in the ATCC K562 cells after normalization with histone H1 content. Among the various cell types, leukemia cell lines had lower levels (median, 37% of ATCC K562 cells), and renal carcinoma cells had higher levels (median, 160% of ATCC K562 cells). Interestingly, a polypeptide of lower molecular weight that reacted with the anti-Apaf-1 monoclonal antibody was detected in some of the colon and brain tumor cell lines (Fig. 2, row marked by asterisk), with lower levels in several leukemia, lung, prostate, and breast lines as well. Because this polypeptide is smaller than Apaf-1 in K562 cells, it is unlikely to correspond to the previously described longer splice variant of Apaf-1 (80, 81). Instead, this species might reflect another splice variant or posttranslational modification of Apaf-1. Because the origin and importance of this species was unclear, it was not quantified in the present study.

Levels of procaspase-9 displayed a 30-fold variation among the cell lines (Figs. 2 and 4B), ranging from 6% of the levels observed in ATCC K562 cells (M14 melanoma cells) to 200% of the levels found in K562 cells (SK-MEL-2 melanoma cells). Levels of procaspase-9 were particularly low in breast cancer cell lines (median, 20% of ATCC K562 cells after normalization with histone H1 content). Although a truncated, dominant-negative version of procaspase-9 has been reported in a number of cell types (82–84), this truncated version of procaspase-9 was not evident in these 60 cell lines.

Expression of Effector Caspases. Caspase-3 has been identified as the major effector caspase in most mammalian cells (86, 87). In addition, several recent reports have suggested that caspase-3 plays a critical role in determining the sensitivity of cells to a number of different stimuli, including doxorubicin (50, 88), cisplatin (49), and etoposide (50). The zymogen form of this enzyme varied from undetectable in MCF-7 cells, which had previously been shown to lack procaspase-3 (48, 51), to four times as high as the reference K562 cells in NCI-H226 lung cancer cells (Fig. 4D). Aside from MCF-7 cells, none of the lines had levels below 30% of those found in ATCC K562 cells. Thus, loss of procaspase-3 does not appear to be a prominent feature of breast cancer cell lines or any other tumor type.

Examination of procaspase-6 (Fig. 3 and 4E), the other major effector caspase in cells undergoing drug- or death ligand-induced apoptosis (86, 87), revealed that levels of this zymogen varied over a >30-fold range, from 9% (MAME-3M and M14 melanoma cells) to 380% (A49B renal carcinoma cells) of the levels found in ATCC K562 cells. Levels of procaspase-6 were particularly low in melanoma cell lines, with a median of 28% of the level observed in ATCC K562 cells (Fig. 4E). Once again, this polypeptide was detected in every cell line examined.

Procaspase-2 is activated downstream of caspase-6 in some models of apoptosis (89) but has been reported to be critical for genotoxic stress-induced apoptosis in mouse oocytes (90) and a variety of cancer cell lines (21, 91). Levels of this zymogen also varied 20-fold among the cell lines (Figs. 3 and 4F), ranging...
from 34% (Colo205) to 700% (Molt4 acute lymphocytic leukemia) of the levels found in ATCC K562 cells.

When monoclonal antibodies to procaspase-7 became available, this zymogen was present in much higher amounts in most of the cell lines than in the K562 cells used as an internal standard (Fig. 3). Accordingly, it was not possible to accurately quantitate this zymogen even when 20-fold less protein was loaded on gels for the other cell lines.

**Procaspase Expression Patterns.** Previous studies have suggested that levels of procaspase-1, -2, and -3 are under transcriptional control of the signal transducer and activator of transcription 1 (Stat1) protein (92). In particular, Stat1−/− fibroblasts have diminished levels of procaspase-1, -2, and -3. In addition, coordinate Stat1-independent up-regulation of caspases-2, -3, and -8 has been reported under certain conditions (93). To determine whether levels of Apaf-1 and any of the procaspases were coordinately expressed in the 60 human cancer cell lines, we searched for correlations between levels of these polypeptides and sensitivity to the therapeutic agents, we examined the relationship between levels of these polypeptides and sensitivity to the >40,000 agents in the public database. This approach is similar to one previously used to determine whether drug sensitivity in this model system is affected, for example, by pretreatment expression of P-glycoprotein (95) or Bcl-2 family members (96). For this analysis, we used LC50 values, which represent the drug concentrations required to decrease cell number by 50% relative to the starting number of cells. These LC50 values are a reflection of cell death that occurs during the 48-hour assays. Of the 40,000 nonproprietary compounds analyzed, 19,000 were examined at high enough concentrations to allow assessment of LC50 values. When caspase levels and sensitivity to these 19,000 compounds were examined with the COMPARE algorithm, few significant correlations between levels of these polypeptides and cytotoxic effects of the drugs were observed.7 In particular, there were only weak correlations with levels of Apaf-1 or procaspase-3 or -9. Results obtained with 10 representative chemotherapeutic agents are shown in Table 3. The strongest correlations were observed between paclitaxel sensitivity and relative caspase-3 levels (r = 0.38) or Apaf-1 levels (r = 0.34).

Because of concern that this lack of correlation might reflect poor killing by the agents in some of the cell lines during the relatively short 48-hour standard assays, further analysis was performed with cytotoxicity results (LC50 data) from an alternative set of assays in which cell lines were exposed to various concentrations of more than 100 standard cytotoxic antineoplastic agents for 6 days. For purposes of illustration, the correlation coefficients between LC50 values and levels of procaspases or Apaf-1 are presented in Table 4 for the same 10 commonly used antineoplastic agents. Results of this analysis indicated that even the weak correlations observed in the 48-hour assays generally disappeared when cells were given a longer time to die.

**Effect of Apaf-1 RNA Silencing.** Because the results of this analysis appeared to be at odds with data showing procaspase-6 mRNA and protein. Similarly, correlation coefficients of 0.61–0.63 were observed for levels of procaspase-2, -3, and -9 and their corresponding messages. For procaspase-8, however, the correlation between mRNA and protein levels was not as strong (r = 0.49). A similar dissociation between polypeptide and mRNA levels was recently observed when procaspase-10 was examined in multiple cell lines (18). These observations raise the possibility that additional factors, such as posttranslational modifications or binding to other polypeptides, might differentially affect stability of some of the procaspases in some of the cell lines.

In further analysis, levels of Apaf-1 and the procaspases were compared with other polypeptides that have been quantitated in these cell lines. These included drug targets (e.g., topoisomerases), DNA repair proteins (e.g., DNA-PK), pro- and antiapoptotic Bcl-2 family members (e.g., Bcl-2, Bcl-xL, and Bax), and other regulators of apoptosis (e.g., XIAP, cIAP1, cIAP2, survivin). There was no significant correlation between levels of any of these polypeptides and the procaspases or Apaf-1 (data not shown).

**Relationship between Procaspase Levels and Drug Sensitivity.** To determine whether procaspase levels were a prominent factor in determining sensitivity of the cell lines to chemotherapeutic agents, we examined the relationship between levels of these polypeptides and sensitivity to the >40,000 agents in the public database. This approach is similar to one previously used to determine whether drug sensitivity in this model system is affected, for example, by pretreatment expression of P-glycoprotein (95) or Bcl-2 family members (96). For this analysis, we used LC50 values, which represent the drug concentrations required to decrease cell number by 50% relative to the starting number of cells. These LC50 values are a reflection of cell death that occurs during the 48-hour assays. Of the 40,000 nonproprietary compounds analyzed, 19,000 were examined at high enough concentrations to allow assessment of LC50 values. When caspase levels and sensitivity to these 19,000 compounds were examined with the COMPARE algorithm, few significant correlations between levels of these polypeptides and cytotoxic effects of the drugs were observed.7 In particular, there were only weak correlations with levels of Apaf-1 or procaspase-3 or -9. Results obtained with 10 representative chemotherapeutic agents are shown in Table 3. The strongest correlations were observed between paclitaxel sensitivity and relative caspase-3 levels (r = 0.38) or Apaf-1 levels (r = 0.34).

Because of concern that this lack of correlation might reflect poor killing by the agents in some of the cell lines during the relatively short 48-hour standard assays, further analysis was performed with cytotoxicity results (LC50 data) from an alternative set of assays in which cell lines were exposed to various concentrations of more than 100 standard cytotoxic antineoplastic agents for 6 days. For purposes of illustration, the correlation coefficients between LC50 values and levels of procaspases or Apaf-1 are presented in Table 4 for the same 10 commonly used antineoplastic agents. Results of this analysis indicated that even the weak correlations observed in the 48-hour assays generally disappeared when cells were given a longer time to die.

**Effect of Apaf-1 RNA Silencing.** Because the results of this analysis appeared to be at odds with data showing...
diminished anticancer drug-induced cell death in cells lacking Apaf-1 (36, 58), caspase-9 (38), or caspase-3 (88), a final series of experiments was performed by creating stable clones of the Jurkat T cell leukemia line that contained various levels of Apaf-1 as a consequence of RNA interference. Immunoblotting demonstrated down-regulation of Apaf-1 protein in each of the clones (Fig. 5A). Despite Apaf-1 down-regulation, these clones remained as sensitive as parental cells to the induction of apoptosis by etoposide (Fig. 5B) and paclitaxel (Fig. 5C), two agents that require the Apaf-1/caspase-9 pathway for completion of apoptosis (97–99). Although the cells with diminished Apaf-1 exhibited diminished sensitivity to the topoisomerase I poison camptothecin at 48 hours (Fig. 5D), this difference disappeared with longer drug exposure (Fig. 5E and F). Thus, results in this isogenic system complement the analysis of the 60-cell-line panel by showing that quantitative alterations in Apaf-1 levels might slow the rate of apoptosis induced by some
Caspase Expression and Drug Sensitivity

present on the arrays of Brown and Botstein. Apaf-1 was not included in this analysis because sequences corresponding to this transcript were not available at http://www.dtp.nci.nih.gov. mRNA levels were determined by Brown and Botstein (94) and are one–four. The attempt to correlate drug sensitivity with levels of various components of the cell death machinery was prompted by previous studies indicating that drug-induced apoptosis is markedly diminished when certain key components of the core cell-death machinery, particularly Apaf-1, procaspase-9, or procaspase-3, are genetically or functionally deleted (36–38, 58, 88, 99). Conversely, Apaf-1 overexpression was previously reported to enhance paclitaxel and etoposide sensitivity in HL-60 leukemia cells (97). In addition, it was recently demonstrated that certain agents can impinge directly on the cell death machinery. In the case of fludarabine, for example, phosphorylated metabolites were shown to directly activate the cytochrome c/Apaf-1/caspase-9 complex (100). All of these observations raised the possibility that variations in levels of Apaf-1 or certain caspases might be important determinants of drug sensitivity.

Results presented here provide the first broad quantitative analysis of Apaf-1 and various procaspases in human tissue culture cells. Previous results have demonstrated the presence of mRNA encoding procaspases and Apaf-1 in a variety of tissue culture cell lines and human tissues. Immunoblotting has also demonstrated the presence of procaspases in a variety of tissue culture lines and clinical tumor samples. Aside from a single report indicating that procaspase-3 levels in 293 human embryonic kidney cells are 100 nmol/L (26), it has been unclear whether the apoptotic procaspases and Apaf-1 are rare or abundant proteins and whether they are present in relatively similar amounts. Our analysis indicates that procaspase-3, -6, and -7 are relatively abundant polypeptides, present in up to several million copies per cell. Procaspase-8 is present in similar amounts. Even procaspase-9 and Apaf-1, which are expressed at somewhat lower levels in most cell lines, are usually present in tens or hundreds of thousands of copies per cell. It is important to emphasize that the methods used to generate these results readily reproduced previously published results in individual cell lines, including the absence of procaspase-3 in MCF-7 breast cancer cells (48) and the paucity of Apaf-1 in CEM human leukemia cells (101). Moreover, in contrast to previous

Table 1  Correlations between polypeptide levels

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procaspase-2</td>
<td>0.63 (P = 7.8 x 10^-3)</td>
</tr>
<tr>
<td>Procaspase-3</td>
<td>0.64 (P = 3.7 x 10^-3)</td>
</tr>
<tr>
<td>Procaspase-6</td>
<td>0.77 (P = 5.4 x 10^-3)</td>
</tr>
<tr>
<td>Procaspase-8</td>
<td>0.49 (P = 7.4 x 10^-3)</td>
</tr>
<tr>
<td>Procaspase-9</td>
<td>0.61 (P = 1.9 x 10^-3)</td>
</tr>
</tbody>
</table>

NOTE. Relative expression of procaspases-2, -3, -6, -8, and -9, as well as Apaf-1, was determined for each of the cell lines as illustrated in Figs. 1–3. Correlation coefficients between levels of various peptides were calculated for the 60 cell lines. None of the correlations reaches statistical significance after correction for multiple comparisons.

drugs but do not appear to play a major role in determining the fraction of cells that ultimately undergo apoptosis.

DISCUSSION

The present study examined levels of various components of the cell death machinery and drug sensitivity of 60 human cancer cell lines to anticancer drugs. With the exception of procaspase-3, which was undetectable in MCF-7 cells, caspases-2, -3, -6, -7, -8, and -9, as well as Apaf-1, were detectable in all 60 cell lines. Although these components of the cell death machinery varied widely in abundance, strong correlations between levels of Apaf-1 or procaspase-2, -3, -6, -8, or -9 and sensitivity to any class of antineoplastic agent were not observed. These results, although negative, have several important implications.

Table 2  Correlation between polypeptide and mRNA levels

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procaspase-2</td>
<td>0.02 (P = 0.09)</td>
</tr>
<tr>
<td>Procaspase-3</td>
<td>0.03 (P = 0.07)</td>
</tr>
<tr>
<td>Procaspase-6</td>
<td>0.04 (P = 0.03)</td>
</tr>
<tr>
<td>Procaspase-8</td>
<td>0.06 (P = 0.01)</td>
</tr>
<tr>
<td>Procaspase-9</td>
<td>0.08 (P = 0.00)</td>
</tr>
</tbody>
</table>

NOTE. Relative expression of procaspases-2, -3, -6, -8, and -9, as well as Apaf-1, was determined for each of the cell lines as illustrated in Figs. 1–3. Correlation coefficients between levels of various peptides were calculated for the 60 cell lines. None of the correlations reaches statistical significance after correction for multiple comparisons.

Table 3  Correlation between polypeptide levels and LC50 measured in 2-day assays

<table>
<thead>
<tr>
<th>Drug</th>
<th>Casp-2: H1</th>
<th>Casp-3: H1</th>
<th>Casp-6: H1</th>
<th>Casp-8: H1</th>
<th>Casp-9: H1</th>
<th>Apaf-1: H1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etoposide</td>
<td>0.03</td>
<td>0.18</td>
<td>-0.13</td>
<td>0.03</td>
<td>0.21</td>
<td>0.13</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.003</td>
<td>0.26</td>
<td>-0.08</td>
<td>0.13</td>
<td>0.32</td>
<td>0.26</td>
</tr>
<tr>
<td>Topotecan</td>
<td>-0.04</td>
<td>0.33</td>
<td>-0.07</td>
<td>0.26</td>
<td>0.07</td>
<td>0.36</td>
</tr>
<tr>
<td>Vincristine</td>
<td>-0.19</td>
<td>-0.06</td>
<td>-0.02</td>
<td>-0.11</td>
<td>-0.004</td>
<td>-0.07</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.01</td>
<td>0.38</td>
<td>-0.08</td>
<td>0.29</td>
<td>0.18</td>
<td>0.34</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>-0.07</td>
<td>-0.02</td>
<td>-0.03</td>
<td>-0.03</td>
<td>-0.12</td>
<td>-0.05</td>
</tr>
<tr>
<td>Melphalan</td>
<td>0.02</td>
<td>0.20</td>
<td>-0.06</td>
<td>0.09</td>
<td>0.19</td>
<td>0.31</td>
</tr>
<tr>
<td>Fludarabine phosphate</td>
<td>0.03</td>
<td>-0.06</td>
<td>-0.04</td>
<td>-0.08</td>
<td>0.04</td>
<td>-0.09</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>0.03</td>
<td>-0.09</td>
<td>-0.02</td>
<td>-0.05</td>
<td>-0.10</td>
<td>-0.13</td>
</tr>
<tr>
<td>5FU</td>
<td>-0.03</td>
<td>0.15</td>
<td>-0.04</td>
<td>0.02</td>
<td>-0.07</td>
<td>0.02</td>
</tr>
</tbody>
</table>

NOTE. Values represent the Pearson correlation coefficients (R) between relative polypeptide levels (ratio of caspase to histone H1) determined as described in Materials and Methods and the mean LC50 for the agent observed during a 2-day incubation. A positive correlation indicates that increasing amounts of target correlate with increasing sensitivity. A negative correlation indicates that increasing amounts of target correlate with decreasing sensitivity. After correction for multiple correlations (see Materials and Methods), none of the correlations reach statistical significance. Abbreviation: Casp, caspase.
studies that have demonstrated a marked disparity between results of proteomic and transcriptional profiling (102), highly significant correlations were observed between polypeptide levels of several of the procaspases and the corresponding messages measured in independent experiments (Table 2). These results argue that the present methods were sufficiently robust to detect the absence of various components across cell lines as well as recurring patterns of caspase expression within various cell types. These quantitative results can serve as a basis for future study of caspase cascades with mathematical modeling techniques or cell-free reconstituted systems.

Because procaspase-9 and Apaf-1 play critical roles in initiating or propagating proteolytic signaling in response to many anticancer agents (36–38, 99), it has been suspected that one or the other of these polypeptides might be deleted in a variety of cancer cell types. Consistent with this hypothesis, Soengas et al. (58) reported that ~40% of metastatic melanoma tissue culture cell lines lack Apaf-1. Reinforcement of Apaf-1 markedly enhanced the sensitivity of these cell lines to doxorubicin-induced apoptosis. Interestingly, in the present study, Apaf-1 and caspase-9 were readily detectable in every cell line in the NCI human tumor cell line panel, including the eight melanoma cell lines (Figs. 2 and 4). These results agree with the results of Belmokhtar et al. (103) and Strasser and coworkers,9 who also have observed Apaf-1 expression in a wide variety of melanoma cell lines. The differences between our results and those of Soengas et al. undoubtedly reflect the use of different cell lines and different cell culture conditions. Examination of Apaf-1 protein expression in situ will ultimately be required to determine which set of cell lines more accurately reflects the biology of melanoma. Nonetheless, our observations indicate that deletion and/or silencing of Apaf-1 or procaspase-9 are unlikely to be common features in human cancer cell lines.

The present study demonstrated a correlation between sensitivity to some drugs and levels of Apaf-1 or procaspase-9 across the cell lines (Tables 3 and 4). It is important to emphasize, however, that the largest correlation coefficients (e.g., $r = 0.36$ between relative Apaf-1 level and topotecan LC$_{50}$ in 2-day assays or $r = 0.30$ between relative Apaf-1 levels and doxorubicin LC$_{50}$ in 6-day assays) were sufficiently small that variations in expression of Apaf-1 and procaspase-9 cannot be implicated as the major determinants of drug sensitivity across these cell lines. Moreover, most of the correlations observed in the 2-day assays disappeared when cells were given a longer period of time to die (Table 4). These observations raise the possibility that levels of Apaf-1 or procaspase-9 might play a role in determining how quickly some cells die rather than determining the number of cells that are ultimately killed by chemotherapy. Studies with stable Jurkat clones expressing diminished amounts of Apaf-1 as a consequence of RNA inhibition (Fig. 5) are also consistent with this hypothesis.

The death receptor pathway, which is initiated by procaspase-8 and is amplified in many cell lines by Bid-induced activation of the mitochondrial pathway (14, 104), has been reported to play an important role in 5FU-induced cell death. Although levels of procaspase-8 varied among the cell lines (Fig. 4C), there was no significant correlation between procaspase-8 expression and 5FU sensitivity (Tables 3 and 4). Levels of procaspase-8 also failed to correlate strongly with sensitivity to a variety of additional agents (Tables 3 and 4).7 Although procaspase-8 was readily detectable in each of the cell lines examined, it is important to note that small-cell lung cancer and neuroblastoma cell lines, which previously were reported to lack procaspase-8, are not part of the NCI cell line panel.

Caspase-3 is currently thought to be the major effector caspase in most cells undergoing apoptosis. Interestingly, low tumor cell expression of procaspase-3 was recently reported to correlate with a higher incidence of lymph node metastases and a shorter survival in patients with non–small-cell lung cancer (105) as well as a poorer prognosis in acute lymphocytic leukemia (60). Previous studies have also suggested that cells lacking procaspase-3 are partially resistant to the induction of apoptosis by a variety of agents, including cisplatin (49), doxorubicin (50, 88), and etoposide (50). However, caspase-3 deficiency (e.g., as seen in MCF-7 cells) does not convey resistance to all anticancer agents, as indicated by the ability of staurosporine (106), paclitaxel (51, 107), tumor necrosis factor α (106), and TRAIL (108) to readily induce apoptosis in this cell

### Table 4

<table>
<thead>
<tr>
<th>Drug</th>
<th>Casp-2:H1</th>
<th>Casp-3:H1</th>
<th>Casp-6:H1</th>
<th>Casp-8:H1</th>
<th>Casp-9:H1</th>
<th>Apaf-1:H1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etoposide</td>
<td>−0.15</td>
<td>0.03</td>
<td>−0.14</td>
<td>−0.02</td>
<td>0.24</td>
<td>0.14</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>−0.17</td>
<td>0.37</td>
<td>−0.11</td>
<td>0.06</td>
<td>0.22</td>
<td>0.30</td>
</tr>
<tr>
<td>Topotecan</td>
<td>−0.20</td>
<td>−0.05</td>
<td>−0.07</td>
<td>−0.18</td>
<td>0.05</td>
<td>0.08</td>
</tr>
<tr>
<td>Vincristine</td>
<td>−0.05</td>
<td>−0.09</td>
<td>−0.05</td>
<td>−0.12</td>
<td>0.10</td>
<td>−0.03</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>−0.01</td>
<td>−0.02</td>
<td>−0.08</td>
<td>0.04</td>
<td>−0.01</td>
<td>0.21</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>−0.22</td>
<td>0.00</td>
<td>−0.10</td>
<td>−0.07</td>
<td>−0.17</td>
<td>0.15</td>
</tr>
<tr>
<td>Melphalan</td>
<td>0.06</td>
<td>0.06</td>
<td>0.01</td>
<td>0.11</td>
<td>−0.02</td>
<td>0.17</td>
</tr>
<tr>
<td>Fludarabine phosphate</td>
<td>−0.13</td>
<td>0.11</td>
<td>0.04</td>
<td>0.17</td>
<td>0.12</td>
<td>0.25</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>−0.03</td>
<td>−0.15</td>
<td>−0.05</td>
<td>−0.08</td>
<td>−0.04</td>
<td>0.12</td>
</tr>
<tr>
<td>5FU</td>
<td>−0.22</td>
<td>0.19</td>
<td>0.00</td>
<td>0.18</td>
<td>0.04</td>
<td>0.30</td>
</tr>
</tbody>
</table>

*NOTE:* Values represent the Pearson correlation coefficients ($r$) between relative polypeptide levels (ratio of caspase to histone H1) determined as described in Materials and Methods and the mean LC$_{50}$ for the agent observed during a 6-day incubation. A positive correlation indicates that increasing amounts of target correlate with increasing sensitivity. A negative correlation indicates that increasing amounts of target correlate with decreasing sensitivity. After correction for multiple correlations (see Materials and Methods), none of the correlations reach statistical significance.

Abbreviation: Casp, caspase.
Although these observations suggest the possibility of drug-specific dependence on caspase-3, only weak correlations were observed between procaspase-3 levels and drug sensitivity across the 60 cell lines with either 48-hour assays for 40,000 nonproprietary compounds or 6-day assays for >100 standard agents (e.g., Table 4). It is important to note that this weak correlation was observed even when LC\textsubscript{50} values, i.e., drug concentrations that reduced cellular mass to 50% of starting levels, were used for these calculations in preference to the more commonly cited GI\textsubscript{50} values.

There are several potential explanations for the poor correlation between caspase levels and response to various anticancer agents. First, each of the cytotoxic agents examined in this study has primary effects on intracellular targets in addition to any effects on the apoptotic machinery. Camptothecins, for example, stabilize topoisomerase I-DNA cleavage complexes,
leading to replication-associated DNA double-strand breaks and subsequent activation of DNA damage-induced signaling (ref. 109 and references therein). To the extent that those effects are toxic, cells would be expected to die even if the apoptotic machinery could not be activated. Consistent with this notion, Sane and Bertrand (110) have reported that broad spectrum caspase inhibitors alter the features of cell death but do not promote long-term survival of camptothecin-treated cells.

Second, it is important to realize that many chemotherapeutic agents trigger apoptosis through the mitochondrial pathway. Because this pathway involves release of cytochrome c and other mitochondrial polypeptides as an early step (29, 111), it is possible that cells are destined to die when this pathway is triggered, regardless of the amount of caspases that can be activated. A number of caspase-independent mitochondrial death factors have been identified, including the oxidereductase apoptosis inducing factor-1, endonuclease G, and the serine protease HirA2 (29, 111). Activities of these polypeptides and/or the lack of oxidative phosphorylation after cytochrome c release might be responsible for cell death even in cells that are limited in their ability to activate caspases. Consistent with this possibility, Xiang et al. and Cheng et al. (112, 113) have reported that Bax-mediated mitochondrial dysfunction is accompanied by loss of viability even when caspase activation does not occur. Likewise, while the present work was being revised, Scott et al. (114) reported that Apaf-1-deficient B cells die more slowly than wild-type cells after treatment with dexamethasone, etoposide, or γ-irradiation but nonetheless exhibit indistinguishable decreases in long-term viability as measured in colony-forming assays.

Finally, it is possible that caspase levels, although important, might be dwarfed in importance by other factors. The concept that caspases might determine sensitivity to drug-induced apoptosis arose from an examination of cells that lack functional caspase-3, caspase-9, or Apaf-1. Although complete depletion of these polypeptides might inhibit apoptosis after certain agents, low but detectable amounts of these polypeptides might generate enough enzymatic activity to support apoptosis. Consistent with this possibility, preliminary calculations based on reported caspase kcat/Km values indicate that 10-fold variations in caspase levels within the ranges reported above have relatively little effect on the predicted rates of caspase substrate cleavage. In other words, under the conditions encountered in cells, factors that regulate activation of the apoptotic machinery might play a more important role than variations in the apoptotic machinery itself. Consistent with this view, a correlation between p53 status and sensitivity to DNA-damaging agents has been observed (71). Variations in levels of Bcl-2 family members have also been reported to correlate with drug sensitivity in tissue culture (96) and in clinical samples (2). Combined with the present observations, these previous results suggest that additional studies of the apoptotic machinery as a determinant of drug sensitivity should continue to focus more on regulatory molecules such as Bcl-2 family members and inhibitors of apoptosis protein, rather than on components of the core apoptotic apparatus itself.

ACKNOWLEDGMENTS

We gratefully acknowledge Drs. Wm. Earnshaw, Dan Billadeau, and Greg Gores for advice and discussions; Guy Salvesen for the caspase-8 used to raise the antiserum used in this study (Burnham Institute, La Jolla, CA); and Deb Strauss for secretarial assistance.

REFERENCES


87. Faleiro L, Kobayashi R, Fearnhead H, Lazebnik Y. Multiple species of CPP32 and Mch2 are the major active caspases present in apoptotic cells. EMBO J 1997;16:2271–81.


Clinical Cancer Research

Components of the Cell Death Machine and Drug Sensitivity of the National Cancer Institute Cell Line Panel

Phyllis A. Svingen, David Loegering, Joe Rodriquez, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/10/20/6807

Cited articles
This article cites 112 articles, 66 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/10/20/6807.full.html#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
/content/10/20/6807.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.