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Apoptosis Protease Activator Protein-1 Expression Is Dispensable for Response of Human Melanoma Cells to Distinct Proapoptotic Agents

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ABSTRACT

Loss of expression of the *apoptosis protease activator protein-1* (*APAF-1*) in human melanoma is thought to promote resistance to programmed cell death by preventing caspase-9 activation. However, the role of the *APAF-1*–dependent pathway in apoptosis activated by cellular stress and/or DNA damage has been recently questioned. We investigated *APAF-1* expression in a large panel of human melanomas and assessed cellular response to several proapoptotic agents in tumors expressing or lacking *APAF-1* protein. In two melanomas with wild-type p53 but with differential expression of *APAF-1*, treatment with camptothecin, celecoxib, or an nitric oxide synthase inhibitor (1400W) significantly modulated expression of 36 of 96 genes in an apoptosis-specific cDNA macroarray, but *APAF-1* mRNA levels were not induced (in *APAF-1*$^-$ cells) nor upregulated (in *APAF-1*$^+$ cells), a finding confirmed at the protein level. Treatment with cisplatin, camptothecin, etoposide, betulinic acid, celecoxib, 1400W, and staurosporine promoted enzymatic activity not only of caspases -2, -8, and -3 but also of caspase-9 in both *APAF-1*$^+$ and *APAF-1*$^-$ tumor cells. Moreover, drug-induced caspase-9 enzymatic activity could be not only partially but significantly reduced by caspase-2, -3, and -8 –specific inhibitors in both *APAF-1*$^+$ and *APAF-1*$^-$ tumor cells. In response to 1 to 100 μmol/L of cisplatin, camptothecin, or celecoxib, *APAF-1*$^+$ melanomas ($n = 12$) did not show significantly increased levels of apoptosis compared with *APAF-1*$^-$ tumors ($n = 7$), with the exception of enhanced apoptosis in response to a very high dose (100 μmol/L) of etoposide. These results suggest that the response of human melanoma cells to different proapoptotic agents may be independent of their APAF-1 phenotype.

INTRODUCTION

When human melanoma progresses to metastatic stage, powerful mechanisms of resistance to chemotherapy, radiation, and biological intervention are established in the neoplastic lesions, thus hampering the efficacy of current medical therapies (1, 2). These mechanisms often depend on the ability of neoplastic cells to evade apoptosis (3). Melanoma resistance to programmed cell death is a complex trait resulting from altered regulation at several steps along the "extrinsic" and the "intrinsic" pathways leading to caspase activation. In the first one, the death receptor-dependent pathway, engagement of CD95 or tumor necrosis factor receptors initiates the apoptotic cascade by activation of apical caspase-8 and -10, which then in turn activate downstream effector caspases such as caspase-3 and -7 (4). In the "intrinsic" pathway, the apoptotic cascade is initiated by cellular stress or DNA damage and is associated with release of cytochrome *c* from mitochondria (4). These two pathways can be affected in human melanoma by several processes. Inactivation of the PTEN tumor suppressor or mutation of N-Ras or B-Raf oncogenes are examples of somatic alterations leading to constitutive activation of antiapoptotic signaling cascades that promote cell survival (2). In addition, upregulation of antiapoptotic factors or down-modulation of proapoptotic genes are frequent in human melanoma. For example, constitutive expression of inhibitors of apoptosis (such as melanoma inhibitor of apoptosis protein and survivin), FLICE-inhibitory proteins, and antiapoptotic members of the Bcl-2 family (such as Mcl-1 and Bcl-X_L) has been described previously (see refs. 2 and 5 for review). In addition, melanoma cells may even express the serine protease inhibitor PI-9/SPI-6, resulting in inactivation of the cytolytic factor Granzyme B (6). Human melanomas may alternatively down-regulate the apoptosis protease activator protein-1 (*APAF-1*). The inactivation of *APAF-1* results from loss of one allele and transcriptional silencing of the other and can promote melanoma chemoresistance to Adriamycin, a chemoterapeutic drug that induces p53-dependent apoptosis (7).

The *APAF-1* molecule, one of the transcriptional targets of p53 in response to DNA damage agents (8, 9), has been initially considered a relevant player in the pathway leading to caspase activation, after triggering the mitochondrial pathway of cell death (10). In fact, on the basis of results obtained by Li *et al.* (11), *APAF-1* was shown to represent one of the essential components of a large protein complex (the apoptosome) that was formed in the presence of dATP by cytochrome *c*, which was released from mitochondria, with *APAF-1* itself, and with procaspase-9 (12). According to this model, procaspase-9 autocatalytically converts itself into an active caspase after recruitment in the apoptosome and then activates the effector caspase-3 (13). Thus, caspase-9 was considered the apical initiator caspase, at least for stress-induced apoptosis. Further support for a relevant, or even essential role of *APAF-1*, in stress-induced programmed cell death, has subsequently come from other studies that used cells from *APAF-1*–deficient mice (14, 15). Thus, ES cells or embryonic fibroblasts from *APAF-1*$^{-/-}$ mice were shown to resist apoptosis induced by anticancer drugs and UV irradiation (14). Similarly, cells from *APAF-1*–deficient mice resisted apoptosis induced by serum deprivation or hypoxia, even after transduction with c-Myc and oncogenic RAS genes (15). However, more recent results have challenged the view that the *APAF-1*–dependent pathway is an initiator of caspase activation, or even an essential player in apoptosis activated by cellular stress and/or DNA damage, in neoplastic cells. In fact, after DNA damage in E1A-transformed fibroblasts and even in some human cancer cell lines, activation of caspase-2 but not of caspase-9 was found to be the apical event required for activation of the proteolytic cascade that is needed for apoptosis before mitochondrial permeabilization (16). Furthermore, in caspase-9$^{-/-}$ and *APAF-1*$^{-/-}$ mice, apoptosis initiated by Bcl-2 was found to be independent of the apoptosome/caspase-9 complex (17). Additional results have indicated that apoptosis promoted by staurosporine can take place in *APAF-1*$^-$ cells, although by a caspase-independent mechanism and with a delayed kinetics (18), further strengthening the notion that programmed cell can take place even in an *APAF-1*–independent fashion. Taken together, these results have suggested that the role of *APAF-1* in programmed cell death might be cell context-dependent

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and that in some cell types the apoptosome might be an amplifier of the caspase cascade rather than an obligate step in induction of apoptosis. In the light of these new findings, we evaluated the cellular responses of human melanoma cells, expressing or lacking *APAF-1* protein, to several anticancer drugs that either induce DNA damage or are known for activating the mitochondrial pathway of apoptosis. The results suggest that in human melanoma, the *APAF-1* phenotype of neoplastic cells may not represent an immediate predictor of response to several proapoptotic agents.

MATERIALS AND METHODS

Normal and Neoplastic Cells. Short-term melanoma cell lines used in this study were established as described previously (19) from surgical specimens of lesions removed from patients admitted to our institute. All melanoma cell lines were isolated from patients not subjected previously to chemotherapy. The melanoma cell line SK-Mel-5 was obtained from American Type Culture Collection (Manassas, VA). Melanoma clones were isolated by cloning in soft agar from a metastatic melanoma (Me665/2), as described previously (20). All cell lines and clones were maintained in RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 10% FCS (BioWhittaker), 2 mmol/L L-glutamine (BioWhittaker), 20 mmol/L HEPES buffer (BioWhittaker), 200 units/ml penicillin (Pharmacia, Milan, Italy), and 40 μg/ml Gentalyn (Italfarmaco, Milan, Italy). Normal human newborn melanocytes (Clonetics Corp., Walkersville, MD) were cultured in melanocyte growth medium as indicated by the provider.

Immunohistochemical Analysis of Benign and Malignant Melanocytic Lesions. Immunohistochemical analysis was done on routinely formalin-fixed and paraffin-embedded specimens, as described previously (21). To optimize immunodetection of *APAF-1*, antigen unmasking was done as previously described (21). Staining for *APAF-1* was conducted with a monoclonal rat antibody (MAB3503, Chemicon International, Temecula, CA) directed against the CARD (caspase activation and recruitment) domain common to all human *APAF-1* isoforms (22). Tissue sections subjected to the same treatment but without incubation with primary monoclonal antibody (mAb) were used as negative controls. Levels of expression of *APAF-1* found in normal epithelium, skin adnexa, and background inflammatory cells were used as baseline for evaluation of the intensity of the marker in neoplastic lesions and classified as strong, weak, and negative.

Monoclonal Antibodies and Immunofluorescence Analysis. Expression of *APAF-1* was determined by intra-cytoplasmic immunofluorescence in saponin-permeabilized cells as described previously (23). Permeabilized cells were stained with anti-*APAF-1* mAb (Chemicon International) followed by incubation with a fluorescein-conjugated affinity-purified goat antirat antibody (Jackson ImmunoResearch, West Grove, PA). Cells stained with a mAb to human vimentin (Cymbus Biotechnology Ltd., Chandlers Ford, United Kingdom) followed by incubation with fluorescein-conjugated $F(ab')_2$ fragments of a goat-antimouse antibody (Jackson ImmunoResearch) were used as a positive control for cell permeabilization. After staining and washing, the samples were analyzed for expression by a FACS-Calibur cytofluorimeter (Becton Dickinson, Franklin Lakes, NJ).

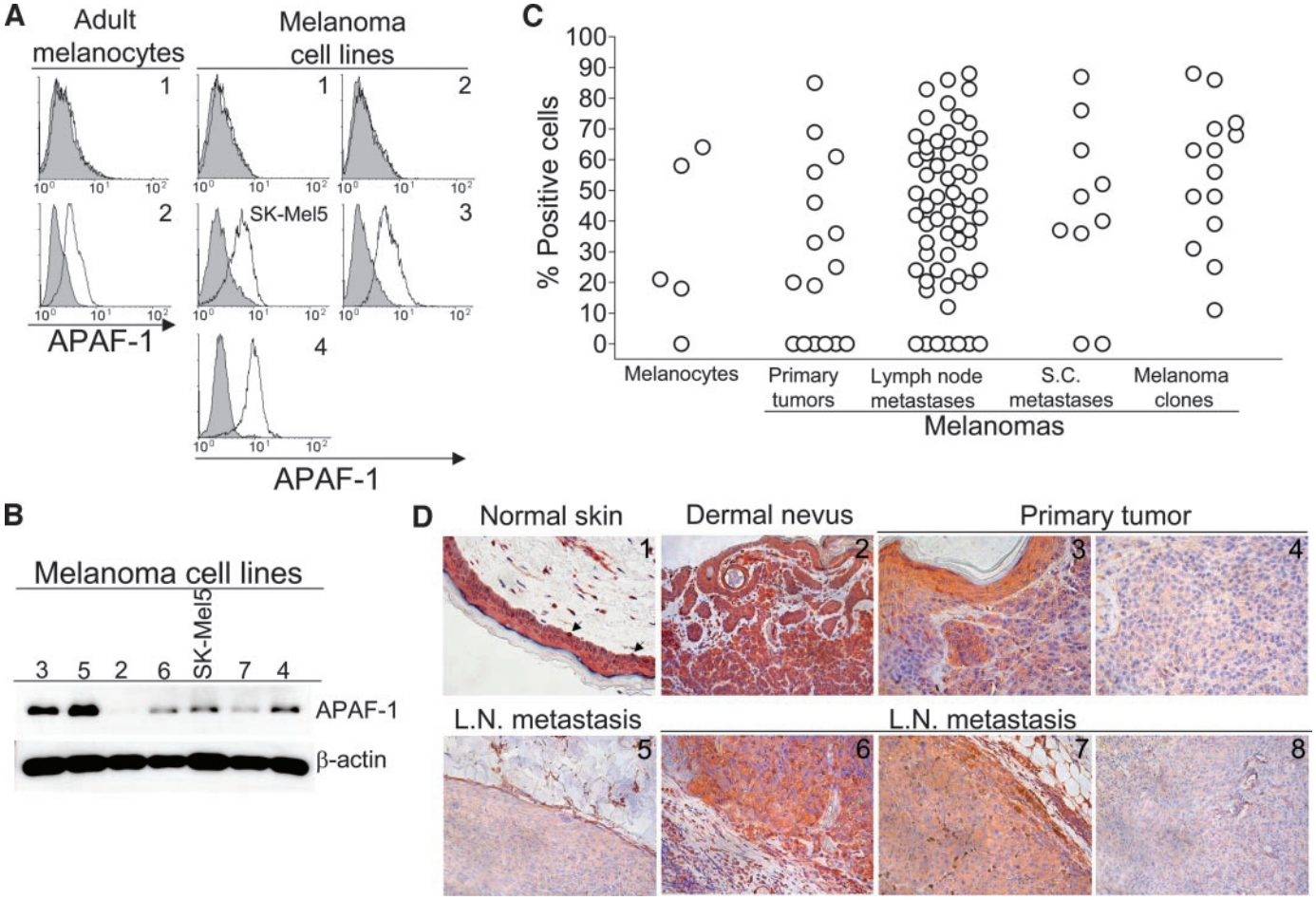

Fig. 1. Expression of *APAF-1* in normal adult melanocytes, melanoma cell lines, and tissue sections. *A*, flow cytometry analysis for *APAF-1* expression in normal adult melanocytes and melanoma cells lines. SK-Mel-5 cell line was obtained from American Type Culture Collection. *Empty histograms*, staining with anti-*APAF-1* mAb; *gray histograms*, cells stained with secondary antibody only. *B*, Western blot analysis for the expression of *APAF-1* in representative melanoma cell lines. *C*, flow cytometry analysis for *APAF-1* expression in five cultures of normal adult melanocytes and in cell lines from 16 primary (vertical growth phase) melanomas, 61 lymph node metastases, 10 subcutaneous metastases, and 14 clones isolated *in vitro* from a single subcutaneous metastasis. Results expressed as a percentage of positive cells. *D*, representative staining patterns for *APAF-1* found in sections of normal skin (1), dermal nevus (2), two distinct areas (3 and 4) of the same primary tumor with positive or negative staining for *APAF-1*, a negative metastatic lesion (L.N.; 5), and three distinct areas (6–8) of a heterogeneous metastasis showing positive, weak, or negative staining for *APAF-1*. *Arrows*, normal melanocytes.

Table 1 *Expression of APAF-1 in benign and malignant melanocytic lesions*

APAF-1 Expression	Benign melanocytic lesions	Primary melanomas	Melanoma metastases to lymph nodes
Strong and homogeneously positive	8*	0	0
Heterogeneous and weak staining	0	5	3
Negative	0	2	3
Total	8	7	6

NOTE. Tissue sections were stained with mAb to *APAF-1*.

* Results expressed as number of lesions showing the indicated staining pattern for *APAF-1* protein. Level of expression of *APAF-1* in normal epithelium, skin adnexa, and background inflammatory cells were used as baseline for evaluation of the intensity of the marker in neoplastic lesions, classified as strong, weak and negative.

Western Blot Analysis for *APAF-1* Expression. Melanoma cells were lysed with a buffer containing 500 mmol/L Tris-HCl (pH 7.4), 1.5 mol/L NaCl, 1% SDS, 10% NP40, 10 mmol/L EDTA (N2), 10 mmol/L EGTA, 100 mmol/L sodium fluoride, 100 mmol/L sodium orthovanadate, and 1:25 dilution of Protease Inhibitor Cocktail (Roche, Milan, Italy). Protein samples (5 μg) were subjected to electrophoresis under reducing conditions on 8% polyacrylamide minigels. The proteins were then electroblotted to a polyvinylidene difluoride membrane (Hybond-P; Amersham Bioscience, Buckinghamshire, United Kingdom), and the membrane was blocked with 10 mmol/L Tris-HCl and 150 mmol/L NaCl (TBS) plus 0.1% Tween containing 5% nonfat milk. The membrane was rinsed with 0.1% TBS/Tween and then incubated for 1 hour at room temperature in the presence of antibody specific for *APAF-1* (Chemicon International). After washing, the membrane was incubated for 1 hour at room temperature with horseradish peroxidase-conjugated affinity-purified goat-antirat IgG secondary antibody (Chemicon International). The membrane was also incubated with an antibody specific for β-actin (Sigma-Aldrich, Milan, Italy) and then with horseradish peroxidase-conjugated affinity-purified goat-antirabbit IgG secondary antibody (Amersham Biosciences). Development was done by the chemiluminescence method following the manufacturer's protocol (Pierce Technology Corp., Iselin, NJ).

Gene Expression Levels by Pathway-Specific Array Analysis. Melanoma cells in the log phase of growth were cultured for 24 hours in serum-free medium with 50 μmol/L camptothecin (Aventis Pharma, Milan, Italy), celecoxib (Pfizer, New York, NY) and with the nitric oxide synthase (iNOS) inhibitor *N*-(3-(aminomethyl)benzyl) acetamidine (1400W, Cayman Chemical, Ann Arbor, MI). Levels of expression of 96 genes involved in the apoptotic pathway were then compared in treated and control cultures by the Human-Apoptosis GEArray Q Series (SuperArray, Bethesda, MD). To this end, TriPure Isolation Reagent (Roche Molecular Biochemicals, Mannheim, Germany) was used to prepare total cellular RNA. cDNA was obtained from 5 μg RNA of each sample by reverse transcription with Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies, Inc. CA) in the presence of [α-^{33}P]dATP (Amersham Biosciences). The resulting cDNA probes were hybridized to gene-specific cDNA fragments spotted on the GEArray membranes. The membranes were exposed to X-ray film (Amersham Bioscience) and scanned with a Typhoon 8600-Variable Mode Imager (Amersham Biosciences). The amount of radioactive signal from expression of each specific gene was quantitated with ImageQuant software (Amersham Biosciences) and after background subtraction, it was normalized to the aver-

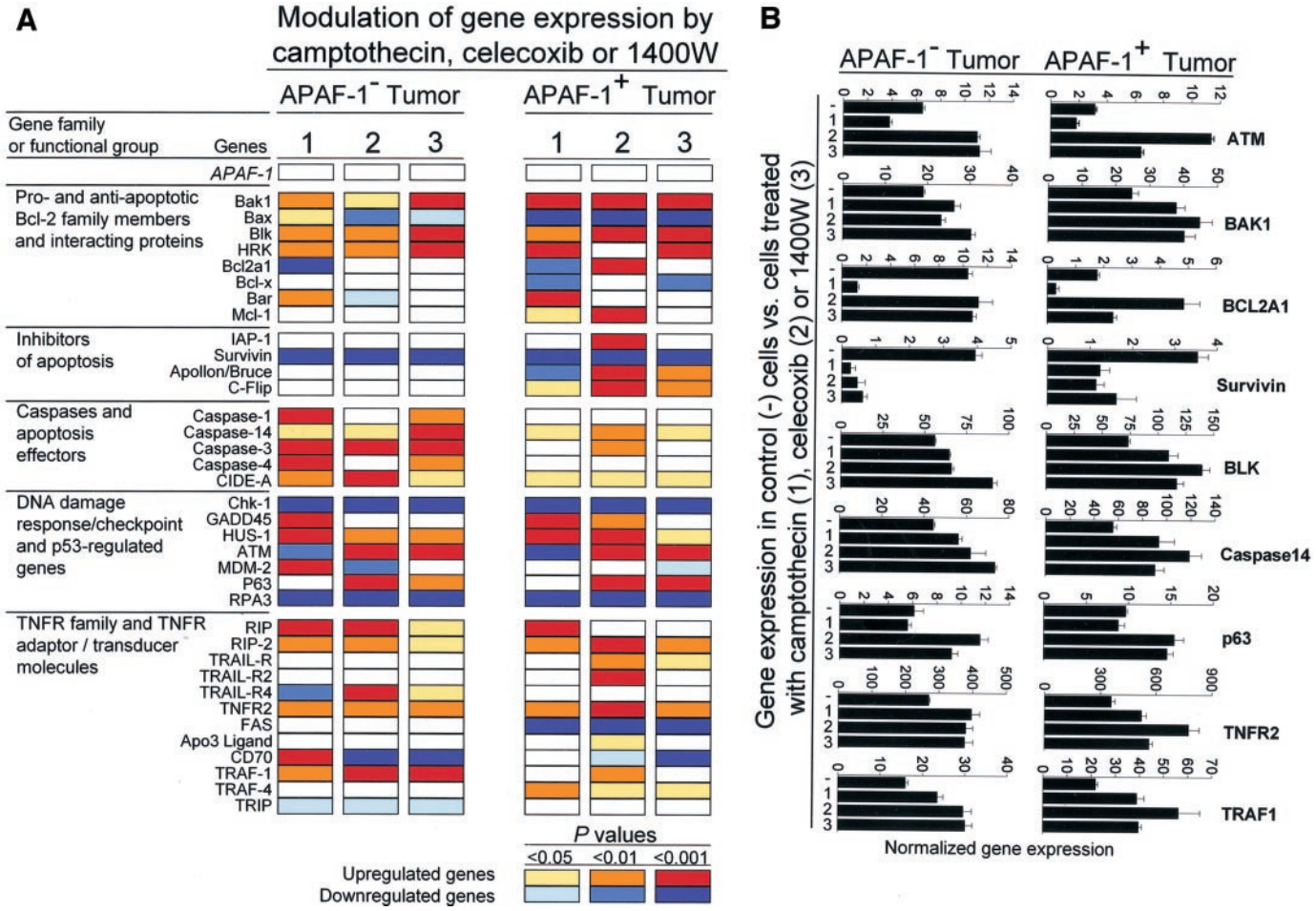

Fig. 2. Modulation of gene expression in melanoma cells after treatment with camptothecin, celecoxib or the iNOS inhibitor 1400W. *A*, melanoma cells from an *APAF-1*$^{-}$ and an *APAF-1*$^{+}$ tumor, both bearing wt p53, were cultured for 24 hours with 50 μmol/L camptothecin (1), celecoxib (2), or with the iNOS inhibitor 1400W (3). mRNA expression levels were then compared in control and treated cells by a pathway-specific expression array (GEArray Human Apoptosis Array). Significance of changes in the mRNA levels induced by each drug compared with control cultures (average of three determinations) was shown by a color code (see legend at the bottom of the figure). Genes with normalized expression not significantly affected by drug treatment, compared with control cultures, were coded in *white*. *B*, normalized expression levels of nine representative genes in control cultures (−) *versus* cultures treated with camptothecin (1), celecoxib (2), or 1400W (3).

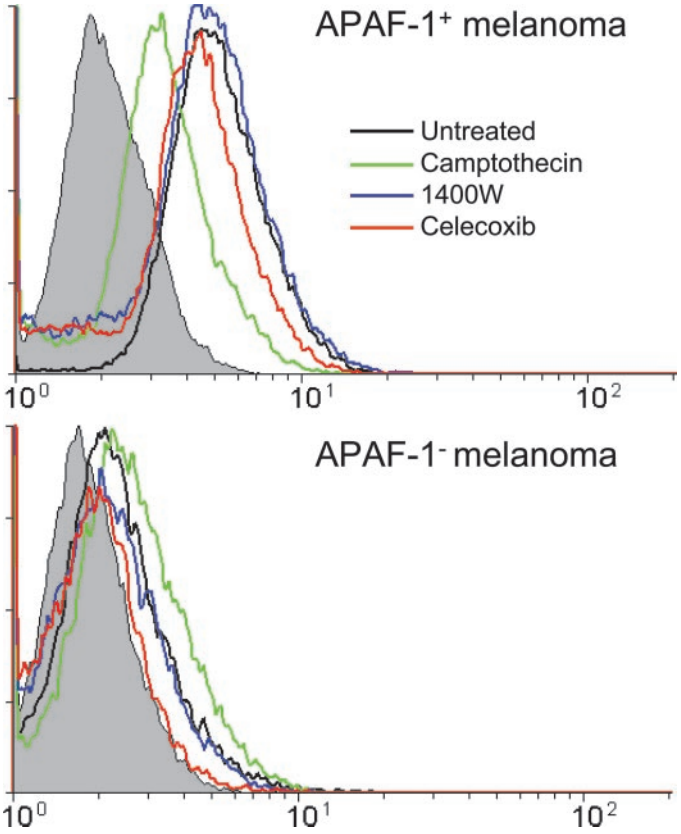

Fig. 3. *APAF-1* protein expression in melanoma cells treated with proapoptotic agents. Two tumors (shown even in Fig. 2), differing for *APAF-1* expression but bearing both wt p53 were treated with 50 μmol/L camptothecin, celecoxib, or 1400W. Expression of *APAF-1* protein in permeabilized cells was monitored after 24 hours by flow cytometry. *Gray histogram*, cells stained with secondary FITC-labeled mAb only.

age signal derived from housekeeping genes present on the membrane (glyceraldehyde-3-phosphate dehydrogenase, cyclophilin A, ribosomal protein L13A, and β-actin).

Detection of Caspase Activity and Apoptosis. BD ApoAlert Caspase Assay kit (BD Biosciences, Clontech, Palo Alto, CA) was used to evaluate detection of enzymatic activity of caspase-2, -3, -8, and -9 in melanoma cells. To this end, 1×10^6 melanoma cells were cultured from 30 minutes to 18 hours without serum in the presence or absence of 50 μmol/L cisplatin (Pharmacia & Upjohn, Milan, Italy), betulinic acid (Sigma Aldrich), 1400W (Cayman Chemical), etoposide (Bristol-Meyers Squibb, Rome, Italy), camptothecin (Aventis Pharma), and celecoxib (Pfizer). In the same experiment, melanoma cells were treated even with 700 nmol/L staurosporine (Sigma Aldrich) for 5 hours, as positive control for caspase induction. Treated and untreated cells were collected, resuspended in cold lysis buffer, and incubated for 10 minutes on ice. After centrifugation (12,500 rpm for 5 minutes at 4°C), cell lysates (50 μL) were incubated for 2 hours at 37°C with different caspase substrates covalently linked to the fluorogenic dye 7-amino-4-methyl coumarin in a reaction buffer containing DTT (1 mol/L). A fluorescence spectrophotometer (TECAN ULTRA 384, Gentronix, Manchester, United Kingdom) with a 380 nm excitation filter and a 480 nm emission filter was used to measure the release of 7-amino-4-methyl coumarin fluorochrome. As control, before reaction with specific caspase substrates, cell lysates from drug-treated cells were incubated with caspase-2, -3, -8, and -9 –specific inhibitors supplied with the BD ApoAlert Caspase Assay kit (BD Biosciences). In some experiments, melanoma cell lines were preincubated for 45 minutes with 20 μmol/L of the following caspase inhibitors: Z-VDVAD-FMK (caspase-2 inhibitor), Z-DEVD-FMK (caspase-3 inhibitor), or Z-IETD-FMK (caspase-8 inhibitor, Kamyia Biomedical Company, Seattle, WA). The cells were subsequently cultured for 5 hours in the presence of 50 μmol/L camptothecin, etoposide or celecoxib and then assessed for caspase enzymatic activity by the BD ApoAlert Caspase Assay kit (BD Biosciences).

For the evaluation of apoptosis, sub-confluent monolayers of melanoma cells were incubated for 48 hours in DMEM (EuroClone, Pero, Italy) without serum and in the presence of 1 to 100 μmol/L of cisplatin (Pharmacia & Upjohn), etoposide (Bristol-Meyers Squibb), camptothecin (Aventis Pharma), or celecoxib (Pfizer). Quantification of apoptotic cells was done by flow cytometry with the Annexin V-FITC kit (BD Biosciences, PharMingen, San Diego, CA) as described previously (21).

Statistical Analysis. Gene expression levels in cDNA array experiments, caspase enzymatic activities, and apoptosis assays were analyzed by ANOVA followed by Student-Newman-Keuls multiple comparison test.

RESULTS

Reduced Expression of *APAF-1* Is Frequent in Human Melanomas, but Total Loss Is Not a Predominant Feature. *APAF-1* expression was evaluated by flow cytometry after cell permeabilization and by staining with a mAb directed against the CARD domain common to all *APAF-1* isoforms (22). Positive and negative tumors were found among short-term melanoma cell lines isolated in our laboratory from patients not subjected previously to chemotherapy (Fig. 1*A*). Interestingly, normal melanocytes were not always *APAF-1* positive (Fig. 1*A*). Differences in expression of *APAF-1* among melanoma cells lines were confirmed by Western blot analysis (Fig. 1*B*). In all positive tumors, a single band of ~142 kDa was identified by Western blot, consistent with the molecular weight of the functional *APAF-1* isoforms (22). Evaluation of *APAF-1* protein expression by flow cytometry in a large panel of normal and neoplastic cells of the melanocyte lineage confirmed *APAF-1* expression, although not in 100% of the cells in most instances (Fig. 1*C*). Complete loss of *APAF-1* was observed in one of five normal melanocytes. Moreover, complete lack of *APAF-1* was observed in 6 of 16 vertical growth-phase primary tumors, in 7 of 61 lymph node metastatic lesions, and in 2 of 10 subcutaneous metastases. In the *APAF-1*$^+$ primary and metastatic cell lines, *APAF-1* was expressed only on a fraction of the cells, suggesting intratumoral heterogeneity, a finding confirmed by the *APAF-1* profile of 14 clones isolated from the same metastatic melanoma (Fig. 1*C*). In addition, *APAF-1* phenotype in the panel of 16 primary and 66 metastatic tumors was not significantly associated with patient survival (data not shown). The heterogeneity and often-reduced expression for *APAF-1* seen *in vitro* was confirmed in tissues sections of primary and metastatic tumors by immunohistochemistry (Fig. 1*D*). Normal melanocytes within the surface epithelium showed equal or greater intensity of stain when compared with epithelial cells (Fig. 1*D*, *arrows*). All benign melanocytic lesions (8 compound or dermal nevi were analyzed) showed a homogeneous strong reaction to the marker (Fig. 1*D* for representative results and Table 1). In contrast, heterogeneity for *APAF-1* and/or weaker staining or even loss of *APAF-1* compared with benign lesions was observed in seven primary tumors and six lymph node metastatic lesions (Fig. 1*D* and Table 1).

APAF-1 Is Not Induced/Modulated in Melanoma Cells by Different Proapoptotic Agents. *APAF-1* has been considered an important effector of p53-dependent apoptosis (10), and *APAF-1* mRNA and protein levels can be up-regulated in different cells after treatment with agents that induce p53 accumulation (8–10). To evaluate gene modulation by proapoptotic agents, two melanoma cell lines with wild-type (wt) p53 (24), either expressing *APAF-1* on most cells (~90%) or completely lacking it, were treated with different drugs. To this end we used a DNA topoisomerase I inhibitor (camptothecin), a drug that induces apoptosis by the phosphatidylinositol 3′-kinase/Akt and the apoptosome/caspase-9 pathways (celecoxib), and we also used an inducible iNOS inhibitor (1400W) that promotes cell death by a mitochondrial-dependent pathway (21, 25–27). Gene expression levels were compared in treated and control cells by an apoptosis pathway-specific cDNA array allowing to profile 96 different genes. Cells were analyzed after 24 hours of drug treatment, a time matching

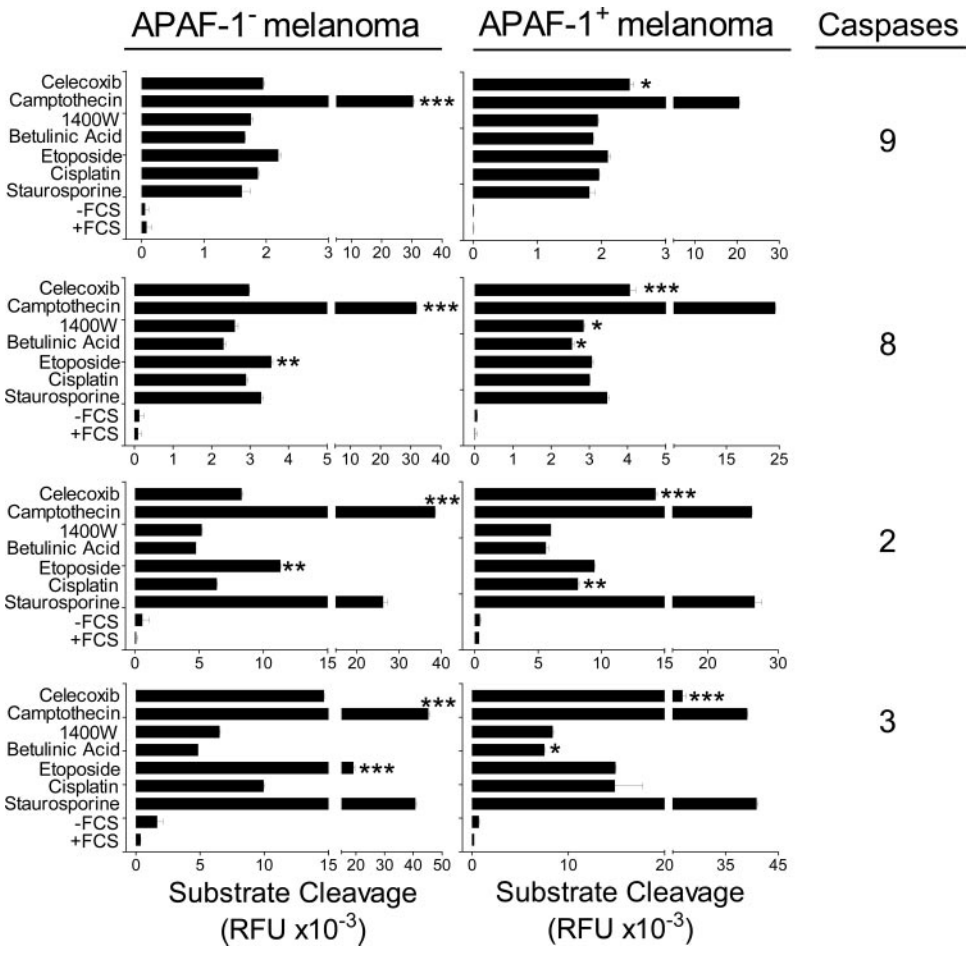

Fig. 4. Caspase activation induced in melanoma cells by proapoptotic drugs. The two melanoma cell lines shown in Fig. 2 and 3 and expressing or lacking *APAF-1* protein were cultured in medium with (+FCS) or without (−FCS) serum or in the presence of cisplatin, etoposide, betulinic acid, 1400W, camptothecin, or celecoxib. Staurosporine was used as positive control for caspase activity. Caspase enzymatic activity, induced by each of the drugs, was assessed at 5 hours by a fluorimetric assay that evaluates the cleavage of substrates specific for each of the four caspases. Data expressed as relative fluorescence units (RFU) of the fluorogenic product resulting from substrate cleavage. The data set for each caspase was subjected to ANOVA followed by Student-Newman-Keuls multiple comparison test. Response to each drug *versus* control cultures (−FCS) was always significant at $P < 0.001$. For each caspase, significantly higher enzymatic activity induced by each drug in one of the cell lines compared with the other is annotated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

the known kinetics of p53-dependent gene and protein modulation including *APAF-1* in different cell types treated with proapoptotic drugs (8–9, 28). Thirty-six genes, belonging to different functional groups and including the Bcl-2 family, inhibitors of apoptosis, caspases and the tumor necrosis factor receptor (TNFR) family, were significantly modulated by drug treatment in one or both cell lines (see Fig. 2*A* for significance of gene expression levels in treated *versus* control cells and Fig. 2*B* for the extent of modulation of nine representative genes). Camptothecin and 1400W up-regulated p53 levels in both cell lines (data not shown). However, *APAF-1* was not induced in the *APAF-1*-negative line nor significantly affected in the positive line, although several p53-dependent genes were modulated by drug treatment in one or both cell lines (such as survivin, caspase-1, gadd45, MDM2, c-FLIP, and TRAILR2). The lack of drug-treatment effect on *APAF-1* expression was confirmed at the protein level. At 24 hours, the *APAF-1*$^-$ melanoma remained negative after treatment with camptothecin, celecoxib, or the iNOS inhibitor (Fig. 3). The same proapoptotic agents did not increase *APAF-1* protein expression in the *APAF-1*-positive tumor, although a partial *APAF-1* reduction after cell exposure to camptothecin was detected (Fig. 3). Similar results were obtained even after 48 hours of drug treatment (data not shown). Taken together, these results indicate that treatment of melanoma cells with distinct proapoptotic agents leads to modulation of several genes involved in the extrinsic and the intrinsic pathways of cell death. However, *APAF-1* gene and protein even when expressed seem resistant to modulation by distinct proapoptotic drugs.

APAF-1 Expression Is Dispensable for Caspase Activation in Response to Anticancer Drugs. Caspase-9 activated by the cytochrome *c*/apoptosome pathway has been initially considered a major initiator caspase for the execution of apoptosis triggered by the intrinsic pathway (13). However, the evidence that caspase-2 activation may precede mitochondrial permeabilization (16) has challenged this model. Moreover, caspase-9 activation in response to TNFR cross-linking may occur in an *APAF-1*-independent fashion through the action of caspase-8 (29). Thus, we hypothesized that *APAF-1* in melanoma cells may not necessarily represent a main determinant of caspase-9 activation in response to different proapoptotic drugs. To evaluate this possibility, activation of enzymatic activity of four caspases by seven different proapoptotic agents including five chemotherapeutic drugs was compared in an *APAF-1*$^-$ and in an *APAF-1*$^+$ melanoma, both bearing wt p53. As shown in Fig. 4, all drugs, but not serum deprivation, determined marked enzymatic activation of the four caspases in both cell lines ($P < 0.001$ for all comparisons of treated *versus* untreated cells). Activated caspases in both the *APAF-1*$^+$ and *APAF-1*$^-$ cell lines included not only the initiator caspases 8 and 2 and the effector caspase-3 but even the "initiator" caspase-9, the enzymatic activity of which should in principle require *APAF-1* expression (13). Matched comparison of the two cell lines for the levels of caspase activity induced by each of the proapoptotic agents revealed that only celecoxib could induce a higher enzymatic activity for all four caspases in the *APAF-1*$^+$ cell line compared with the *APAF-1*$^-$ line. Interestingly, camptothecin and etoposide induced higher caspase activity for most caspases in the *APAF-1*$^-$ line (Fig. 4). Analysis of the kinetics of caspase-2, -3, -8,

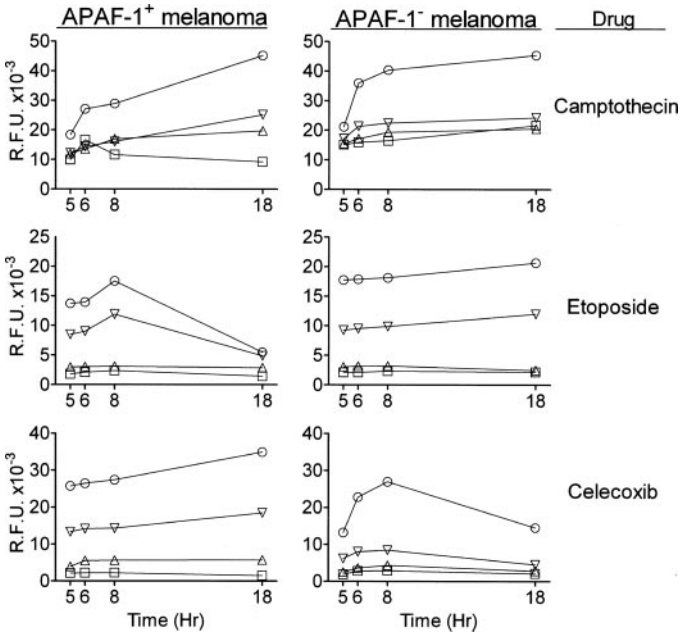

Fig. 5. Kinetics of caspase activation in *APAF-1*$^+$ and *APAF-1*$^-$ melanoma cells by different drugs. Two melanoma cell lines expressing or lacking *APAF-1* but bearing a wt p53 were treated for 5 to 18 hours with 50 μmol/L of the indicated drugs. Enzymatic activity of caspase-2 (▽), -3 (○), -8 (△), and -9 (□) induced by each of the drugs was assessed as described in the legend to Fig. 4. Data expressed as relative fluorescence units (RFU) after subtracting values from control cultures without drugs. Caspase activities in drug-treated cultures were always significantly higher ($P < 0.001$, at all time points by ANOVA followed by Student-Newman-Keuls test) than in control cultures (−FCS, and without drugs, as in Fig. 4).

and -9 activation by different drugs indicated no or very low activity at 30 minutes to 3 hours with the exception of the response to camptothecin (data not shown), whereas marked enzymatic activities (compared with control cultures without drugs) were observed between 5 and 18 hours for all four caspases (Fig. 5). Interestingly, in both *APAF-1*$^+$ and *APAF-1*$^-$ cell lines, the effector caspase-3 was activated earlier and/or at much higher levels than caspase-9 by all drugs and at all time points ($P < 0.001$). Even the initiator caspase-2 was activated at higher levels than caspase-9 in most instances (Fig. 5). To evaluate the possible role of other capases in caspase-9 response, we then evaluated the impact of different inhibitors on caspase-9 enzymatic activity. As shown in Fig. 6, caspase-2, -3, and-8 –specific inhibitors were highly effective in inhibiting the corresponding caspase although they induced even a partial but marked reduction in caspase-9 activity, promoted by celecoxib or etoposide in both *APAF-1*$^+$ and *APAF-1*$^-$ melanoma cell lines. Similar results in the presence of caspase inhibitors were obtained even in the response of *APAF-1*$^+$ and *APAF-1*$^-$ melanoma cells to camptothecin (data not shown).

Taken together, these results suggest that in melanoma cells, expression of *APAF-1* may be dispensable for activation of several caspases, including caspase-9, in response to distinct chemotherapeutic drugs. Moreover, caspase-2, -3, and -8 may contribute to caspase-9 activation, independently from the *APAF-1* phenotype of the tumor cells.

Comparison of *APAF-1*$^+$ and *APAF-1*$^-$ Tumors for Susceptibility to Drug-Induced Apoptosis. A panel of *APAF-1*$^+$ and *APAF-1*$^-$ melanomas was assessed for susceptibility to apoptosis (by the annexin-V/PI assay) following culture with increasing doses (1–100 μmol/L) of four cytotoxic drugs (cisplatin, etoposide, camptothecin, and celecoxib), the apoptosis induction mechanism of which is thought to involve the mitochondrial pathway (15, 25–27, 30). In this panel, all *APAF-1*$^-$ tumors expressed a wt p53 gene as well as 9 of 12 *APAF-1*$^+$ tumors (24). The three tumors with mutant p53 harbored S127F, G187S, and Y236H mutations, respectively (24). No significant differences in the level of apoptosis were found between *APAF-1*$^+$ and *APAF-1*$^-$ tumors in response to all doses of these four drugs (see Figs. 7 and 8 for representative dot plots showing extent of early and late apoptosis by all drugs in two tumors) with the exception of an enhanced susceptibility to apoptosis in *APAF-1*$^+$ tumors, compared with *APAF-1*$^-$ melanomas, at the highest dose of etoposide (Figs. 7 and 8). In addition, no significant differences were observed between tumors harboring wt or mutant p53 (Fig. 7). Moreover, the extent of apoptosis induced by each of the four drugs in the 12

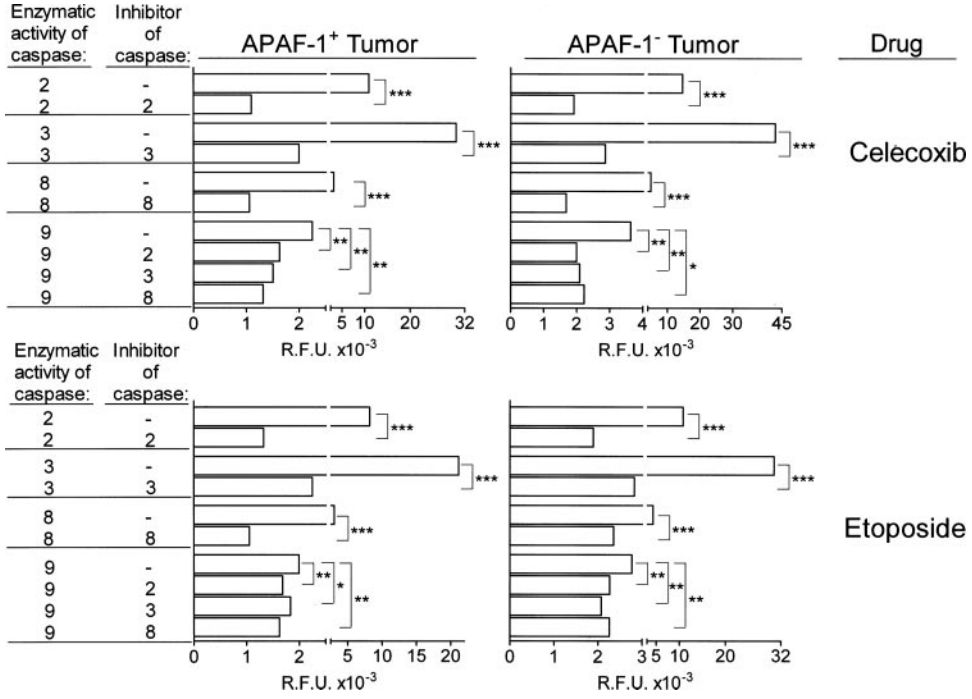

Fig. 6. Inhibition of drug-induced caspase-9 activity by inhibitors of caspase-2, -3, and -8. Two melanoma cell lines expressing or lacking *APAF-1* but bearing a wt p53 were preincubated with caspase-2, -3, or -8 inhibitors and then treated for 5 hours with 50 μmol/L of the indicated drugs. Caspase enzymatic activity induced by each of the drugs was assessed as described in the legend to Fig. 4. Data expressed as relative fluorescence units (RFU), after subtracting values from control cultures without drugs. Significance of inhibition of caspase activity (by ANOVA followed by Student-Newman-Keuls test) in the presence of inhibitors compared with cultures without inhibitors was annotated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

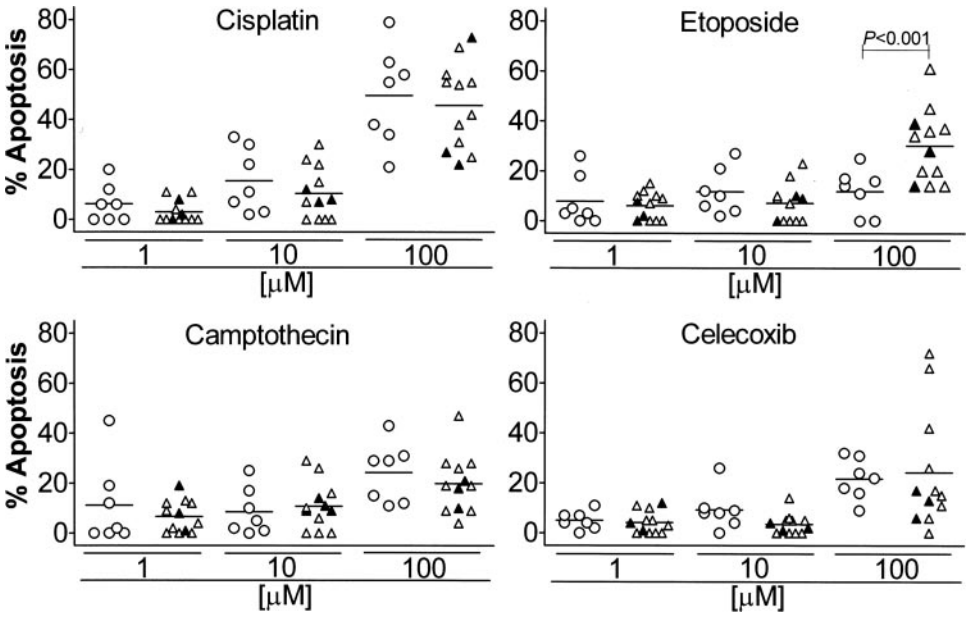

Fig. 7. Drug-induced apoptosis in *APAF-1*$^+$ or *APAF-1*$^-$ melanomas. Melanoma cells expressing *APAF-1* protein (n = 12, △) or lacking it (n = 7, ○) were treated with 1 to 100 μmol/L of the indicated drugs for 48 hours. Apoptosis was then evaluated by flow cytometry after staining with annexin-V and PI. Results expressed as a percentage of cells showing early (annexin-V$^+$/PI$^-$) or late (annexin-V$^+$/PI$^+$) apoptosis. —, average of apoptosis values observed in each of the treatment groups. Three of the *APAF-1*$^+$ tumors harbored a mutant p53 (▲) whereas all of the remaining tumors expressed a wt p53.

APAF-1$^+$ tumors showed no significant correlation with the level of *APAF-1* expression (percentage of positive cells) in such tumors (data not shown). Taken together, these results suggest that the *APAF-1* phenotype of melanoma cells may not represent an immediate predictor of the apoptotic response to several anticancer drugs. On the other hand, in some instances such as with etoposide, *APAF-1* expression may be associated with increased apoptosis but only at very high doses of the drug.

DISCUSSION

The results of this study indicated that *APAF-1* expression is subjected to inter-and intratumor heterogeneity in human melanoma cell cultures and tissue sections. In neoplastic lesions, *APAF-1* expression was weaker than in normal skin melanocytes, but complete loss was not a predominant feature in either primary or metastatic tumors. In agreement with these findings, recent results from Baldi *et al.* (31), obtained by staining tissue sections with a polyclonal antibody to residues 871-1001 of *APAF-1*, indicated that *APAF-1* was retained in 60% of the tumors, although an increased loss of expression was found in association with tumor progression. At the functional level, in response to several proapoptotic agents, we found that *APAF-1* gene and protein levels were not up-regulated, even when *APAF-1* was expressed. Moreover, caspase-activation, including caspase-9 did not require expression of *APAF-1*, and the susceptibility to apoptosis by distinct drugs was not significantly different in *APAF-1*$^-$ and *APAF-1*$^+$ tumors, with the exception of results obtained in response to a very high dose of etoposide. Taken together, these results suggest that the *APAF-1* phenotype of human melanoma cells may not represent an immediate predictor of susceptibility or resistance to apoptosis elicited by several chemotherapeutic drugs.

The identification of *APAF-1*–positive and –negative tumors enabled us to evaluate the impact of several proapoptotic agents on distinct cellular responses, including gene expression, caspase-activation, and apoptosis. At the mRNA level in two p53 wt tumors, different proapoptotic agents promoted marked changes in expression levels of genes acting along the apoptotic pathway. In addition to Bcl-2 family members and to inhibitors of apoptosis, several TNFR family and adaptor/transducer molecules were modulated in one or both cell lines by at least one of the three proapoptotic agents. Although expression of other relevant proapoptotic transcriptional targets of p53, such as PUMA and NOXA (5), was not investigated in drug-treated melanoma cells, nevertheless several other p53-regulated genes, such as survivin and c-Flip (32, 33) were affected by drug treatment, indicating that the p53 pathway was not defective in these cell lines. However, *APAF-1* was neither induced (in a negative line) nor up-regulated (in a positive line). The lack of *APAF-1* gene modulation, after drug treatment, in the *APAF-1*-negative melanoma is consistent with the mechanism of gene inactivation described in this tumor (7). On the other hand, the lack of *APAF-1* gene up-regulation, in the *APAF-1*$^+$ tumor, is in contrast with results obtained in other cell types and suggests that a cell context-dependent effect may dictate whether *APAF-1* gene expression may be responsive or not to proapoptotic agents. In fact, *APAF-1*, a known transcriptional target of p53 and E2F1 (8–9, 28, 34), has been shown to be modulated in lymphoblastoid cell lines, in response to DNA damage induced by ionizing radiation or doxorubicin (8), in neurons in response to camptothecin (9), and in a murine myeloid leukemic cell line expressing a temperature sensitive mutant p53 (34). At the protein level, we observed that *APAF-1* could be down-modulated (in the *APAF-1*$^+$ tumor) after exposure to camptothecin. Such effect is consistent with results showing that *APAF-1* protein can be degraded by caspases-3 and -7 in different cell types including melanoma cells during apoptosis promoted by staurosporine or cisplatin (30, 35).

Evaluation of caspase activation in *APAF-1*$^+$ and *APAF-1*$^-$ tumors indicated that seven different drugs and proapoptotic agents could activate caspase-9 independently from the presence of *APAF-1* protein in the neoplastic cells. Moreover, all drugs investigated promoted activation not only of the effector caspase-3, but even of caspase-2 and -8, and experiments with caspase inhibitors indicated a contribution of caspase-2, -3 and -8 to caspase-9 activation. These data suggest that the apoptotic pathways acting upstream of APAF-1, including the mithocondrial-dependent release of mitochondrial proteins (such as Smac/DIABLO and OMI/HtrA2) that are required to release caspase-3- and -9 from IAP inhibition (36), may not be defective in these melanoma cells. In addition, these findings are consistent with the models indicating the role of caspase-2 as an apical caspase in drug-induced apoptosis (16) and with the results suggesting the involvement of the death-receptor pathway, through caspase-8 in the

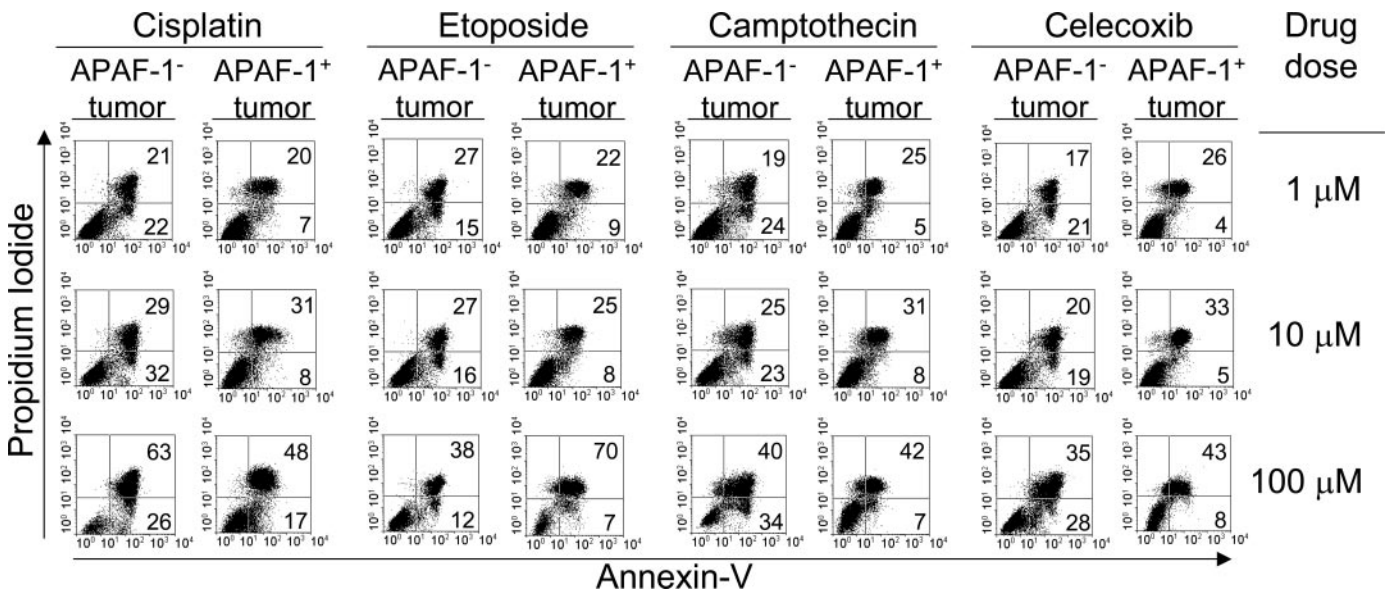

Fig. 8. Drug-induced apoptosis in *APAF-1*$^+$ or *APAF-1*$^-$ melanomas. Extent of early (annexin-V$^+$/PI$^-$, *lower right panel*) or late (annexin-V$^+$/PI$^+$, *upper right panel*) apoptosis induced by the indicated drugs in two representative melanoma cell lines, from the same panel shown in Fig. 7. Both tumors bear a wt p53 but express *APAF-1* or lack it. Cells were treated as indicated in the legend to Fig. 7. Apoptosis was then evaluated by flow cytometry after staining with annexin-V and PI. *Numbers in the dot plots* indicate the percentage of cells showing early (annexin-V$^+$/PI$^-$) or late (annexin-V$^+$/PI$^+$) apoptosis. In control cultures, in the absence of drugs, the percentage of cells in early or late apoptosis was 5 to 10% in both cell lines.

apoptosis promoted by different anticancer drugs (37, 38). The latter mechanism is even in agreement with the finding that several genes of the TNFR family and TNFR adaptor/transducer molecules were upregulated in melanoma cells after drug treatment. In addition, significantly higher levels of caspase enzymatic activity in the *APAF-1*$^+$ tumor compared with the *APAF-1*$^-$ melanoma were observed only after treatment with celecoxib. Interestingly, apoptosis induced by celecoxib is mediated by inhibition of PDK1, which in turn prevents activation of the Akt pathway (27, 39) and has been shown to require *APAF-1* and caspase-9 activity (26). Thus, the enhanced caspase-9 activation that is found in the *APAF-1*$^+$ melanoma after celecoxib treatment is consistent with the role of *APAF-1* expression as an amplifier of celecoxib activity. At the same time, the results of caspase activation with all other drugs argue against a necessary role of *APAF-1* expression for caspase-9 activation in human melanoma cells in response to distinct proapoptotic drugs, and such results are in agreement with those of apoptosome-independent pathways in which apoptosis resulted in response to cellular stress or cytotoxic drugs. For example, in murine leukemia cells lacking *APAF-1*, apoptosis in response to staurosporine could take place in a caspase-9-independent fashion (18). In myoblasts but not in fibroblasts from *APAF-1*$^{-/-}$ embryos, treatment with cytotoxic drugs induced caspase-9–dependent but *APAF-1*–independent apoptosis (40). Similarly, in apoptosis triggered by Sendai virus infection, caspase-9 activation takes place independently from cytochrome *c* release from mitochondria and from presence of *APAF-1* (41). On the other hand, in ovarian cancer cells, the inability to undergo cisplatin-induced caspase-9 activation has been correlated to *APAF-1* deficiency (42). In light of these results in different cell types, it seems that the relevance of *APAF-1* expression for caspase-9 activation may be cell context-dependent. Thus, our results are consistent with the hypothesis that in human melanoma cells, *APAF-1* expression is dispensable for caspase activation and that, in particular, caspase-9 activation can be uncoupled from *APAF-1* expression in such neoplastic cells.

Finally, evaluation of apoptosis by four different cytotoxic drugs indicated that *APAF-1* expression in human melanoma cells, irrespective of p53 status, was not associated with response with the exception of a significantly enhanced susceptibility of *APAF-1*$^+$ tumors to the highest dose of etoposide. The known mechanism of action of etoposide involves the mitochondrial/*APAF-1* pathway, but the relevance of this pathway is cell context-dependent, as exemplified by the model proposed by Fulda *et al.* (37). In this model, depending on the cell type, cytotoxicity induced by anticancer drugs may be triggered by both the death receptors (through CD95 and caspase-8) or by the mitochondrial pathway (through *APAF-1*/caspase-9) rather than being regulated mostly by the mitochondrial pathway alone. For example, in glioma cells, overexpression of *APAF-1* has been found to increase etoposide-induced apoptosis, and cotransduction of *APAF-1* and caspase-9 could further augment susceptibility to this drug (43). On the other hand, in lymphoma cells, a role of caspase-8, the apical caspase in death-receptor-induced apoptosis, has been described in response to etoposide (44), although as an amplifying executioner caspase (45). Moreover, the role of the mitochondrial/*APAF-1* pathway, as an amplifier rather than an initiator of drug-induced apoptosis, has been reinforced by the recent results on the existence of a Bcl-2–dependent pathway of apoptosis that is *APAF-1* and caspase-9 independent (17). Taken together, our results question the role of the *APAF-1* pathway as an obligate initiator of apoptosis induced by several different anticancer agents and suggest that *APAF-1* expression may play an amplifier role in the apoptotic response of human melanoma cells to some cytotoxic drugs.

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