The Bax/Bcl-2 Ratio Determines the Susceptibility of Human Melanoma Cells to CD95/Fas-Mediated Apoptosis

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Defective cytochrome *c* release and the resulting loss of caspase-3 activation was recently shown to be essential for the susceptibility of human melanoma cells to CD95/Fas-induced apoptosis. Cytochrome *c* release from mitochondria is regulated by the relative amounts of apoptosis-promoting and apoptosis-inhibiting Bcl-2 proteins in the outer membrane of these organelles. The assignment of Bax/Bcl-2 ratios by quantitative Western blotting in 11 melanoma cell populations revealed a relation to the susceptibility to CD95-mediated apoptosis. We could show that a low Bax/Bcl-2 ratio was characteristic for resistant cells and a high Bax/Bcl-2 ratio was characteristic for sensitive cells. Low Bax expression was not a consequence of mutations in the p53 coding sequence. The Bax/Bcl-2 ratio was also in clear correlation with sensitivity to another cell death inducer, N-acetylsphingosine. Furthermore, Bcl-2 overexpression abolished apoptosis triggered by both apoptotic stimuli, confirming the critical role of the Bax/Bcl-2 ratio as a rheostat that determines the susceptibility to apoptosis in melanoma cells by regulating mitochondrial function. Interestingly, some chemotherapeutics lead to the activation of death pathways by CD95L upregulation, ceramide generation, direct activation of upstream caspases, or upregulation of proapoptotic genes. Taken together, these signals enter the apoptotic pathway upstream of mitochondria, resulting in activation of this central checkpoint. We therefore assumed that apoptosis deficiency of malignant melanoma can be circumvented by drugs directly influencing mitochondrial functions. For this purpose we used betulinic acid, a cytotoxic agent selective for melanoma, straightly perturbing mitochondrial functions. In fact, betulinic acid induced mitochondrial cytochrome *c* release and DNA fragmentation in both CD95-resistant and CD95-sensitive melanoma cell populations, independent of the Bax/Bcl-2 ratio. ***Key words: Bax/Bcl-2 ratio/CD95/Fas/ceramide/melanoma. J Invest Dermatol 117:333–340, 2001***

The CD95 system has been recognized as a major pathway for the induction of apoptosis in cells and tissues (Nagata and Golstein, 1995). CD95 is a member of the tumor necrosis factor receptor subfamily named death receptors. Human melanoma cells have recently been shown to be heterogenously susceptible to CD95-induced apoptosis. The elucidation of the mechanisms underlying the resistance to CD95-mediated apoptosis in human melanoma cells revealed that the cells resistant to CD95-induced apoptosis failed to translocate cytochrome *c* into the cytosol and to activate the downstream execution process (Raisova *et al*, 2000). Developing resistance within the CD95-signaling pathway may have two potential benefits for the tumor. First, it allows tumor cells to avoid being killed by CD95L-positive cytotoxic T cells and to escape from immune surveillance (Reed, 1999). Second, some anticancer drugs have been shown to involve the CD95 pathway, at least in T cells, so delivering apoptotic signals through an autocrine mechanism (Friesen *et al*, 1999). Therefore, deficiencies in the CD95 apoptotic-signaling pathway might also be a clue to definition of drug resistance in the tumor.

Cytochrome *c* release from mitochondria is a central event in apoptosis. Upon exposure to a variety of proapoptotic stimuli cytochrome *c* enters the cytosol and binds to the cytosolic Apaf-1 (apoptotic protease activating factor 1) and procaspase-9 (Li *et al*, 1997). This results in activation of caspase-9 and processing of the main executioner caspase-3, thus reaching the ''point of no return'' (Enari *et al*, 1996). The release of cytochrome *c* is determined by the relative amounts of apoptosis-promoting and apoptosis-inhibiting Bcl-2 proteins in the outer membrane of the mitochondrion (Adams and Cory, 1998). Inappropriate overexpression of apoptosis-suppressing Bcl-2 protein has been shown to contribute to tumorigenesis in various types of tumors, e.g., breast cancer (Reed, 1996). The apoptosis-preventing effect of Bcl-2 is counteracted by the proapoptotic protein Bax. Imbalance of the Bax/Bcl-2 ratio that tilts the scales toward survival can render tumor cells more resistant to a wide variety of cell death stimuli, including all chemotherapeutic drugs, radiation, hypoxia, or growth factor withdrawal (Reed, 1994). The broad resistance to cell death,

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Abbreviations: CD95L/FasL, CD95/Fas ligand; C_2-ceramide, N-acetylsphingosine; LDH, lactate dehydrogenase; SSCP, single stranded conformational polymorphism.

occurring upon disturbed balance of the Bax/Bcl-2 ratio, can also be relevant for cancer cell behavior, tumor cell invasion, cell adhesion, or metastatic potential.

The *p53* gene product is known to be an upstream regulator of the *bax* gene (Miyashita and Reed, 1995). This tumor suppressor binds to the *bax* gene promoter and directly transactivates the transcription of this proapoptotic gene. The *bax* promoter can be activated by wild-type but not mutant p53, which forms a link between p53 expression, its mutational status, and alterations in Bax expression. Surprisingly, the proapoptotic protein Bax has been shown to be strongly expressed in most tissue samples of malignant melanoma (Tang *et al*, 1998). An explanation for the Bax elevation could be a frequently increased expression of wild-type *p53 and* a low *p53* mutation rate in human melanoma (10%) (Saenz-Santamaria *et al*, 1995). The actions of Bax appear to be neutralized when heterodimerized with Bcl-2 and some other members of the Bcl-2 protein family, such as Bcl-x_L and Mcl-1. Bcl-2 expression is a common finding in melanocytic lesions regardless of their biologic behavior, and the progression of malignant melanoma does not depend on the overexpression of Bcl-2 (Selzer *et al*, 1998). As shown in other tumors, the Bax/Bcl-2 ratio seems to be more important in determining the sensitivity to apoptotic stimuli in melanoma than the expression of each protein individually.

Ceramide is a proapoptotic molecule, recently shown to induce cytochrome *c* release from isolated mitochondria (Ghafourifar *et al*, 1999). It is a signaling molecule involved in cellular responses to a variety of apoptotic stimuli and its significant role in the CD95 apoptotic pathway in human melanoma cells has recently been demonstrated (Raisova *et al*, 2000). Ceramide generation in this pathway has been molecularly ordered downstream of the adaptor protein FADD (Fas-associated death domain) and upstream of mitochondria and effector caspases (Okazaki *et al*, 1998). The tumor-specific toxicity of some anticancer drugs was attributed to a functional CD95 pathway. Betulinic acid is a new cytotoxic agent, with a selective anticancer activity in tumors of neuroectodermal origin, directly targeting mitochondria without triggering the CD95/CD95L system (Pisha *et al*, 1995). Some forms of drug resistance can be circumvented using betulinic acid most probably by straight perturbation of mitochondrial functions (Fulda *et al*, 1998b, c).

In this study, we investigated the molecular mechanisms of resistance to CD95- and ceramide-induced apoptosis in 11 melanoma cell populations. We could show by quantitative Western blotting that the Bax/Bcl-2 protein expression ratios determine the sensitivity to these apoptotic stimuli. The central role of the Bax/Bcl-2 rheostat was confirmed by Bcl-2 overexpression, which blocked CD95/ceramide-induced apoptosis in sensitive melanoma cells. Furthermore, betulinic acid, which directly targets mitochondria and causes cytochrome *c* release, was able to circumvent the apoptosis deficiency of CD95/ceramide-resistant melanoma cells.

MATERIALS AND METHODS

Materials The agonistic anti-CD95 monoclonal antibody CH-11 (Chemicon, Hofheim, Germany) was used at a final concentration of 1 μg per ml. N-acetylsphingosine (C_2-ceramide) was purchased from Alexis (Gruenberg, Germany) and dissolved in ethanol to give a 20 mM stock solution. pIRES1neo was purchased from Clontech (Palo Alto, CA), and betulinic acid was provided from Sigma (Munich, Germany) as pure substance and dissolved in dimethylsulfoxide to give a stock solution of 4 mg per ml betulinic acid. Geneticin (G418) was from Life Technologies (Karlsruhe, Germany).

Cell culture Two melanoma cell populations (M186, M221) were obtained from patients with histologically confirmed metastatic melanoma by surgical intervention. Four out of nine established human melanoma cell lines used in this study originated from primary tumors [A-375 (Giard *et al*, 1973), Bro (Lockshin *et al*, 1985), JPC-298 (Aubert *et al*, 1993); Mel-HO (Holzmann *et al*, 1988)] and five cell lines originated from metastases [Mel-2 A (Bruggen *et al*, 1981); MeWo (Bean *et al*, 1975), SK-Mel-13, SK-MEL-23, SK-Mel-28 (Carey *et al*, 1976)]. All melanoma cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U per ml penicillin, and 100 μg per ml streptomycin.

Lipofection of melanoma cells The construction of pIRES/mbcl-2 has been reported previously and has been shown to be functional in HaCaT keratinocytes (Müller-Wieprecht *et al*, 2000). Transfection of melanoma cells was carried out using the lipofection reagent pFx-2 from Invitrogen (Groningen, The Netherlands) following the supplier's instructions. Briefly, the reagent was diluted 1:200 in OPTI-MEM medium (Life Technologies) without FBS and 1.5 μg pIRES1neo/mbcl-2 or pIRES1neo were added. The solution was added to melanoma cells, grown to 30%–50% confluency in 3.5 cm dishes, and incubated for 2 h at 37°C. Selection with 1.0 mg per ml geniticin started 48 h after transfection. After a selection and subcultivating period of approximately 10 wk individual cell clones were isolated. Bcl-2 overexpression in these cell lines was confirmed by Western blotting (data not shown).

Western blot analysis Cells were lyzed in lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl containing 1 μM leupeptin, 1 μM pepstatin, and 100 μM phenylmethylsulfonyl fluoride) followed by dounce homogenization. The homogenate was centrifuged for 10 min at 400*g* to get rid of cell debris. Protein concentration was assayed using bicinchoninic acid (Pierce, Weiskirchen, Germany). Then, 35 μg of cytosolic protein were separated on a 15% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and blotted to nitrocellulose membrane as described previously (Wieprecht *et al*, 1996). Membranes were blocked for 1 h with 3% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.25% Tween-20 and then incubated with mouse monoclonal anti-Bcl-2 antibodies (100/D5, Novocastra, Dossenheim, Germany) at a 1:100 dilution, with mouse monoclonal anti-Bcl-2 antibodies (C-2, Santa Cruz, Heidelberg, Germany) at a 1:1000 dilution, with mouse monoclonal anti-Bax antibody (YTH-2D2, Trevigen, Gaithersburg) at a 1:10,000 dilution, or with rabbit polyclonal anticaspase-9 antibodies (Pharmingen, Hamburg, Germany) at a 1:1000 dilution. Primary antibodies were labeled with goat antimouse IgG at a 1:10,000 dilution or with goat antirabbit IgG at a 1:4500 dilution conjugated with horseradish peroxidase (Dako, Hamburg, Germany) for 1 h. Immunoreactive bands were visualized by incubation of the membrane with Super Signal West Pico reagent from Pierce.

Quantification of the Bax/Bcl-2 ratio Quantitative analysis of the Bax/Bcl-2 protein expression was performed using a scanning densitometer and Multianalyst Software Version 1.0.2 (Biorad, Munich, Germany) as described previously (Prokop *et al*, 2000). Standardization of protein loading was achieved as follows: (i) protein measurements of all samples were performed using the bicinchoninic acid assay and equal amounts of protein (35 μg per lane) were loaded on the gel; (ii) transfer efficiency of the Western blots was routinely checked by staining the membranes with 0.5% Ponceau Red in 1% acetic acid; (iii) for chemiluminescent detection, films were exposed exactly the same length of time, which was optimized for each antibody used in this study; and (iv) detection of Bax and Bcl-2 was performed separately on the same membrane. Thus, this procedure is valid to determine the protein ratios in the same sample (on the same lane of the gel). Values of protein expression are given in arbitrary units. Linearity of protein detection was checked for Bax and Bcl-2 using standard cell extracts from BJAB cells (Prokop *et al*, 2000).

Cytotoxicity assays Cytotoxicity was determined with the cytotoxicity detection kit (LDH) from Roche Diagnostics (Mannheim, Germany) as described previously (Bektas *et al*, 1998). After treatment of cells for different times, cell culture supernatants were removed and clarified by centrifugation at $300 \times g$ for 5 min. 100 μl of the supernatants were transferred to 96-well microtiter plates and lactate dehydrogenase (LDH) activity was determined by addition of substrate solution; formation of the formazan salt was measured at 492 nm. Extinction values of control cells were set as 100% and the rate of LDH release from treated cells was calculated as a percentage of control.

Cell death detection Confluent cells in 24-well plates were treated with the respective agents or control vehicle in DMEM. After distinct times of incubation, cell death was measured in a photometric enzyme immunoassay for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) with the commercially available kit "Cell death detection ELISA^{PLUS}" from Roche Diagnostics, as described previously (Wieder *et al*, 1998). Briefly, cells were centrifuged at $300 \times g$, washed with PBS, pH 7.2, and lyzed with lysis buffer. Then, a clear cytosolic

supernatant was prepared by centrifugation (300 × *g*, 5 min). 20 μl of the respective supernatants were added to a streptavidin-coated 96-well microtiter plate. After addition of 80 μl of a solution containing antihistone-biotin and anti-DNA-peroxidase conjugates in incubation buffer the samples were incubated under moderate shaking for 2 h at room temperature. Subsequently, the wells were washed three times with incubation buffer, 100 μl substrate solution was added, and the samples were incubated for 10 min at room temperature in the dark. Then, the extinction was measured at 405 nm. The values of cells that were treated with control medium were set at 100% and the apoptosis rates of treated cells were calculated as a percentage of control.

Mutational analysis of *p53* DNA from 11 melanoma cell populations was extracted using the QIAamp Tissue Kit (Qiagen, Hilden, Germany). *p53* mutations in the DNA binding region (exons 5–8) were investigated by single stranded conformational polymorphism (SSCP) polymerase chain reaction (PCR) analysis. Oligonucleotide primer sequences, PCR conditions, and the SSCP protocol for *p53* mutational analysis were as described elsewhere, with one modification: PAGE was performed at 22°C (Sturm *et al*, 1999).

PCR products that showed an aberrant SSCP pattern were sequenced with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's recommendation with the same primers used for PCR amplification. Sequencing was performed with an ABI Prism 310 sequencer. Sequence analysis was performed with the software Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI).

Preparation of cytosolic extracts and mitochondria Mitochondrial and cytosolic extracts were prepared according to a method described previously (Liu *et al*, 1996). After incubation with betulinic acid, cells were harvested in PBS, equilibrated in hypotonic buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM ethylenediamine tetraacetic acid), and centrifuged at 300 × *g* for 5 min. The resulting pellets were then dissolved in hypotonic buffer containing phenylmethylsulfonyl fluoride (final concentration 0.1 mM) and incubated on ice for 15 min. Cells were homogenized by passing the cells through a syringe (G 20) approximately 20 times. The membranes were isolated by 2-fold centrifugation at 10,000 × *g* at 4°C for 10 min and the supernatant of the second centrifugation was used as cytosolic extract. The resulting mitochondrial pellets were solubilized in lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl containing 1 μM leupeptin, 1 μM pepstatin, and 100 μM phenylmethylsulfonyl fluoride) and together with the clear supernatants stored at –80°C. Western blot analyses with cytosolic and mitochondrial extracts were performed as described before and conducted with mouse monoclonal anti-cytochrome *c* antibodies (7H8.2C12, Pharmingen) at a 1:1000 dilution. The second horseradish-peroxidase-coupled antimouse antibody (Dako) was used at a 1:10,000 dilution.

RESULTS

Bax and Bcl-2 protein expression in melanoma cell populations A panel of 11 melanoma cell populations was screened for expression of two counteracting proteins of the Bcl-2 family, Bax and Bcl-2. For this, proteins extracted from distinct melanoma cell populations were analyzed by Western blot. Antibodies against both proteins were used subsequently on the same membrane. The protein levels of the two cytoplasmic proteins in the different cell populations showed great variations for both proteins (**Fig 1**). We observed a difference in protein expression between CD95-sensitive and CD95-resistant melanoma cells, however. Whereas CD95-sensitive cell lines revealed a higher expression of Bax in relation to Bcl-2, CD95-resistant cells showed low Bax/Bcl-2 ratios (**Fig 1**).

Quantification of the Bax/Bcl-2 ratio Quantitative analysis of the Bax and Bcl-2 protein expression was performed using a scanning densitometer. The quantitative Bax and Bcl-2 protein expression levels and the Bax/Bcl-2 ratios are listed in **Table I**. Interestingly, we found that a Bax/Bcl-2 ratio < 1.00 was characteristic for resistant cells and a Bax/Bcl-2 ratio > 1.00 for sensitive melanoma cells. Statistical analysis revealed that this finding was significant ($p < 0.0022$, Fisher's exact test; $p < 0.0009$, chi-squared test). Thus, our data clearly support the hypothesis that the Bax/Bcl-2 ratio forms a rheostat that determines the sensitivity to CD95-induced apoptosis in human melanoma cells.

Overexpression of Bcl-2 prevents CD95- and ceramide-induced apoptosis Bcl-2 has been shown to maintain cell

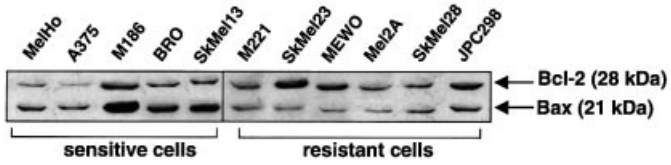

Figure 1. Determination of Bax and Bcl-2 protein expression in human melanoma cells. Protein extracts from 11 melanoma cell populations were prepared and equal amounts of protein (35 μg per lane) were separated by SDS-PAGE. After transfer on nitrocellulose membrane, immunodetection was carried out using anti-Bax antibodies and anti-Bcl-2 antibodies. *Arrows* at the right margin indicate the migration positions of Bax and Bcl-2 proteins.

Table I. Correlation of CD95 sensitivity with the intraexperimental quantification of the Bax/Bcl-2 ratio

Cell populations	Susceptibility to CH-11[a]	Susceptibility to C_2-ceramide	Bax O.D. [arbitrary units][b]	Bcl-2 O.D. [arbitrary units][b]	Bax/Bcl-2 ratio[c]
MelHo	++	++	0.067	0.045	1.49
A375	+++	+++	0.048	0.025	1.93
M186	+	+	0.693	0.186	3.73
BRO	++	++	0.208	0.089	2.34
SkMel13	+	+	0.518	0.062	8.36
M221	–	–	0.047	0.058	0.82
SkMel23 (p53 mut.)[d]	–	–	0.071	0.396	0.18
MEWO (p53 mut.)[d]	–	–	0.030	0.201	0.15
Mel2A	–	–	0.034	0.096	0.36
SkMel28	–	–	0.040	0.098	0.41
JPC298	–	–	0.111	0.285	0.39

[a]+, low sensitivity (apoptotic cells 200% of control); ++, mean sensitivity (apoptotic cells 200%–300% of control); +++, high sensitivity (apoptotic cells 300%–600% of control) as measured by cell death detection ELISA

[b]Values of Bax and Bcl-2 expression in different melanoma cell populations were quantified by scanning densitometry of the Western blot shown in **Fig 1** using the Multianalyst Software Version 1.0.2 (Biorad) and are given in arbitrary units.

[c]A Bax/Bcl-2 ratio < 1.00 characterizes CD95-resistant cells and a Bax/Bcl-2 ratio > 1.00 CD95-sensitive melanoma cells.

[d]p53 mut., p53 mutated.

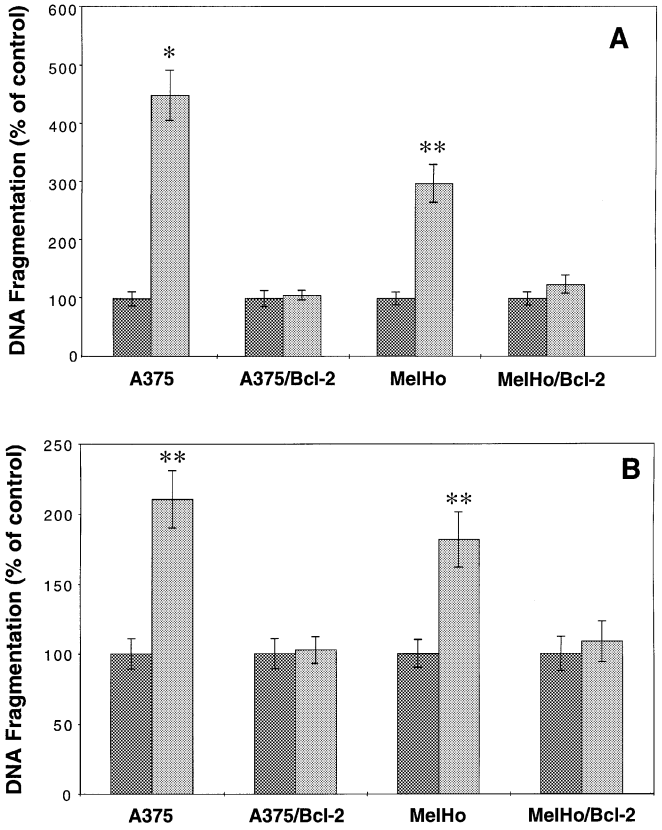

Figure 2. The involvement of Bcl-2 in CD95- and ceramide-induced apoptosis. Nontransfected (A375 and MelHo) and *bcl-2* transfected (A375/Bcl-2 and MelHo/Bcl-2) melanoma cell lines were treated either with 1 μg per ml agonistic anti-CD95 monoclonal antibody CH-11 for 12 h (*A*) or with 30 μM C_2-ceramide for 6 h (*B*). DNA fragmentation was measured photometrically using an enzyme-linked immunoassay for determination of cytoplasmic histone-associated DNA fragments. *Gray columns* represent CH-11- or C_2-ceramide-treated cells and *black columns* untreated control cells. DNA fragmentation of control cells was set as 100% and DNA fragmentation of treated cells was calculated as a percentage of control. Values represent the mean of three experiments ± SD (*$p < 0.0001$; **$p < 0.001$).

viability by blocking cytochrome *c* release and preventing loss of mitochondrial membrane potential (Yang *et al*, 1997). In order to further elucidate the role of the Bax/Bcl-2 rheostat in our experimental system, we overexpressed the *bcl-2* gene in two melanoma cell lines, A375 and MelHo, which were shown to be highly sensitive to CD95- and ceramide-induced apoptosis. The overexpression shifted the Bax/Bcl-2 ratio from 1.93 and 1.49 in A375 and MelHo, respectively, to approximately 0.04 and 0.03 in the corresponding transfected cell lines as determined using the mouse and human reactive anti-bcl-2 antibodies C-2. We could show that Bcl-2 overexpression in A375 and MelHo cell lines rendered these cells completely resistant to treatment with 1 μg per ml agonistic anti-CD95 monoclonal antibody CH-11 (**Fig 2*A***). Furthermore, the sensitivity to ceramide-induced apoptosis was tested. For this purpose, *bcl-2* transfected A375 and MelHo cells were treated with C_2-ceramide, a cell-permeable short acyl chain ceramide. These experiments revealed that overexpression of the *bcl-2* gene also prevented apoptosis induced by 30 μM C_2-ceramide after 6 h of incubation (**Fig 2*B***). These findings suggest that mitochondrial alterations are the key events, determining the fate of melanoma cells upon treatment with different apoptotic stimuli, such as CD95L and C_2-ceramide.

Low Bax expression is not exclusively caused by *p53* mutations Induction of Bax expression has been attributed to

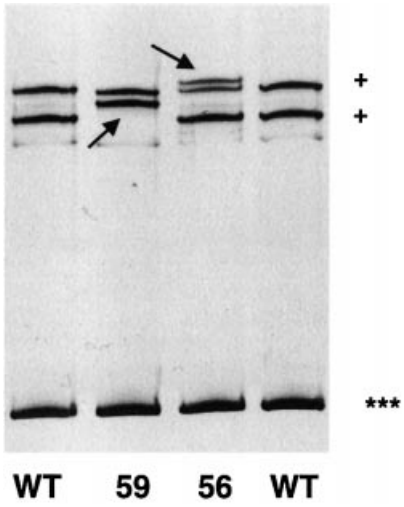

Figure 3. Mutational analysis of *p53* in melanoma cell lines. The mutational analysis of p53 in melanoma cell lines was assessed by SSCP-PCR. Two of the CD95-resistant cell lines, SkMel23 and MEWO, revealed a mutation of *p53* in exon 7. Migration positions of single-stranded DNA (+), double-stranded DNA (+ + +), and aberrant bands (*arrows*) are indicated (WT, wild-type *p53*; 59, SkMel23; 56, MEWO).

the wild-type form of the tumor suppressor p53, which binds to typical recognition elements located in the *bax* gene promoter and directly transactivates this proapoptotic gene. Therefore, we evaluated whether the differential expression of Bax in melanoma cells is influenced by the *p53* mutational status. More than 90% of *p53* mutations occur in the DNA-binding site of *p53*, which is encoded by exons 5–8. *p53* mutations, as assessed by mutational analysis using SSCP-PCR, were found in exon 7 of the *p53* gene in two melanoma cell lines, SkMel 23 and MEWO (**Fig 3**). Sequencing revealed a codon 248 mutation in SkMel23 (CGG → TGG, arginine → tryptophan) and a codon 258 mutation in MEWO (GAA → AAA, glutamic acid → lysine). Both cell lines showed a Bax/Bcl-2 ratio < 1 and were resistant to CD95-induced apoptosis, thereby providing evidence that low Bax levels in these cells might be a direct consequence of the loss of functional p53. p53 mutation, however, cannot serve as an explanation for low Bax expression and resistance to CD95-induced apoptosis in other cell populations, e.g., M221, Mel2A, SkMel28, and JPC 298. Furthermore, there was no statistically significant correlation between low Bax expression and p53 mutational status ($p < 0.5$, Fisher's exact test; $p < 0.2$, chi-squared test).

Betulinic acid bypasses the resistance to CD95-induced apoptosis In the previous experiments we could show that the mitochondrion is a central player in CD95- and ceramide-induced apoptosis in human melanoma cells. We therefore reasoned that the apoptosis deficiency of resistant melanoma cells can be circumvented by drugs directly targeting mitochondria and causing cytochrome *c* release. For this purpose, we tested betulinic acid, a selective cytotoxic agent for melanoma and other cells of neuroectodermal origin (Fulda *et al*, 1999). In a first set of experiments, cytotoxicity of betulinic acid on melanoma cells was determined by measurement of the release of LDH into the cell culture supernatants. In these experiments, 10 μg per ml betulinic acid was shown to be the most effective concentration triggering apoptosis in melanoma cells without affecting plasma membrane integrity (data not shown). Additionally, CD95-sensitive as well as CD95-resistant melanoma cells that had been treated with betulinic acid exhibited typical morphologic features of apoptotic cells as observed under the light microscope (data not shown). Apoptotic cell death was confirmed by measurement of DNA fragmentation in betulinic-acid-treated cells. As shown in **Fig 4**, betulinic acid treatment of A375, M186 (CD95-sensitive cells) and M221, Mel2A

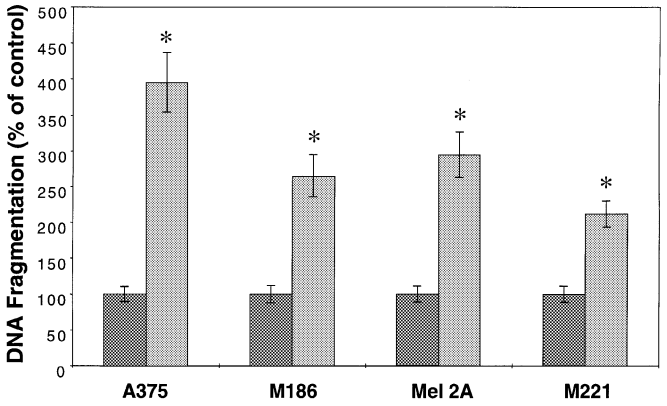

Figure 4. Betulinic acid bypasses resistance to CD95-induced apoptosis. CD95-sensitive (A375 and M186) and CD95-resistant (Mel2A and M221) melanoma cell lines were treated with 10 μg per ml betulinic acid for 24 h. DNA fragmentation was measured photometrically using an enzyme-linked immunoassay for determination of cytoplasmic histone-associated DNA fragments. *Gray columns* represent betulinic-acid-treated cells and *black columns* untreated control cells. Control cells were set as 100% and treated cells were calculated as a percentage of control. Values represent the mean of three experiments ± SD (*$p < 0.0001$).

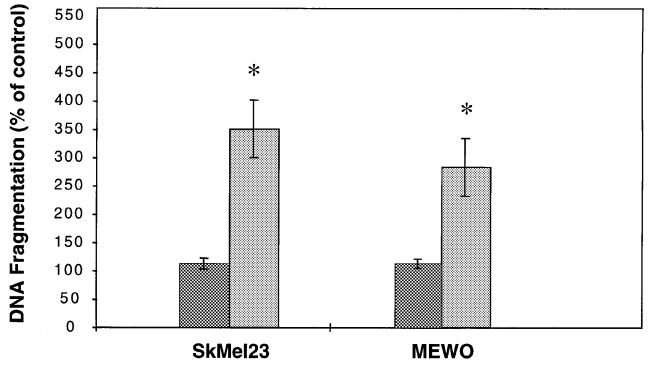

Figure 5. Betulinic acid induces apoptosis in p53 mutated cell lines SkMel23 and MEWO. Melanoma cell lines were treated with 10 μg per ml betulinic acid for 24 h. DNA fragmentation was measured photometrically using an enzyme-linked immunoassay for determination of cytoplasmic histone-associated DNA fragments. *Gray columns* represent betulinic-acid-treated cells and *black columns* untreated control cells. Control cells were set as 100% and treated cells were calculated as a percentage of control. Values represent the mean of three experiments ± SD (*$p < 0.0001$).

(CD95-resistant cells) clearly induced DNA fragmentation. Additionally, apoptosis induction was also observed in SkMel23 and MEWO, two cell lines with mutated p53 (**Fig 5**), indicating a p53-independent mechanism. In some cases DNA fragmentation in betulinic-acid-treated cells reached 400% compared with control cells, indicating that this compound represents an effective inducer of apoptosis, even in cells with a blockade in the upstream apoptotic pathway.

Bcl-2 overexpression partially inhibits betulinic-acid-induced apoptosis in melanoma cells To elucidate the role of Bcl-2 in betulinic-acid-induced cell death, we measured DNA fragmentation in Bcl-2 transfected A375 and MelHo cells. We could show that Bcl-2 overexpression in A375 and MelHo cell lines reduced the apoptosis triggered by betulinic acid in A375/Bcl-2 by 79% and in MelHo/Bcl-2 by 74% (**Fig 6**). In contrast to CD95- and ceramide-induced apoptosis, however, Bcl-2 overexpression did not completely block the effect of betulinic acid.

Betulinic-acid-triggered cytochrome *c* release occurs in CD95-sensitive cells as well as CD95-resistant melanoma cells To gain insight into the molecular effector pathway of betulinic acid, we investigated the effect of betulinic acid on cytochrome *c* release from mitochondria. Cytosolic extracts of melanoma cells were prepared and cytochrome *c* release after stimulation was determined by Western blot analysis. Mitochondrial extracts of the different cell lines were included as positive controls. As shown in **Fig 7**, stimulation of the CD95-sensitive melanoma cells A375 and M186 as well as the CD95-resistant cell lines Mel2A and M221 with betulinic acid provoked the translocation of cytochrome *c* into the cytosol. Thus, these results exclude a general defect in cytochrome *c* release at the level of mitochondrial transition pore formation in CD95-resistant melanoma cells but rather point to the key role of the upstream Bax/Bcl-2 rheostat.

Betulinic acid induces activation of caspase-9 in sensitive and resistant cell lines In order to determine the status of caspase-9 level and activation in our experimental set-up, we performed Western blot analyses using anticaspase-9 antibodies. As shown in **Fig 8**, both sensitive and resistant melanoma cell populations showed the active cleavage forms of caspase-9 upon treatment with betulinic acid. This further confirmed the role of

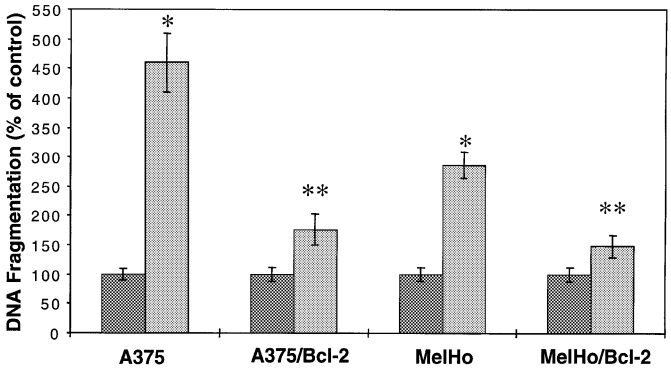

Figure 6. The involvement of Bcl-2 in betulinic-acid-induced apoptosis. Nontransfected (A375 and MelHo) and bcl-2 transfected (A375/Bcl-2 and MelHo/Bcl-2) melanoma cell lines were treated with 10 μg per ml betulinic acid for 24 h. DNA fragmentation was measured photometrically using an enzyme-linked immunoassay for determination of cytoplasmic histone-associated DNA fragments. *Gray columns* represent betulinic-acid-treated cells and *black columns* untreated control cells. DNA fragmentation of control cells was set as 100% and DNA fragmentation of treated cells was calculated as a percentage of control. Values represent the mean of three experiments ± SD (*$p < 0.0001$; **$p < 0.02$).

mitochondrial factors in betulinic-acid-treated melanoma cells. Interestingly, Jurkat cells did not show activation of caspase-9 (**Fig 8**), thereby verifying the specificity of betulinic acid for cells of neuroectodermal origin.

DISCUSSION

Melanoma cell populations exhibit differential sensitivity to CD95-induced apoptosis. The evaluation of the mechanisms underlying the apoptosis deficiency of CD95-resistant melanoma cells revealed a failure to translocate cytochrome *c* into the cytosol and pointed to the central role of mitochondria in this pathway (Raisova *et al*, 2000). The analysis of proteins, regulating mitochondrial functions, showed a correlation between the ratio of two members of the Bcl-2 protein family, Bax and Bcl-2, and sensitivity to CD95-mediated apoptosis. In our experimental system, a Bax/Bcl-2 ratio < 1.00 was characteristic for CD95-resistant cells whereas a Bax/Bcl-2 ratio > 1.00 was characteristic for sensitive melanoma cells. The Bax/Bcl-2 ratio was also in correlation with the sensitivity of melanoma cells to another proapoptotic molecule, C_2-ceramide.

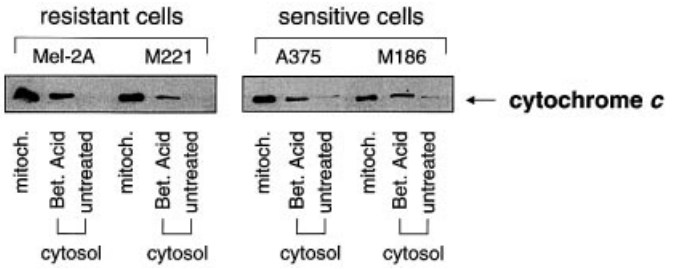

Figure 7. Cytochrome *c* is released upon stimulation with betulinic acid in CD95-sensitive (A375 and M186) as well as in CD95-resistant (Mel2A and M221) melanoma cells. Cells were harvested after 12 h stimulation with 10 μg per ml betulinic acid. For the detection of cytochrome *c* release, cytosolic and mitochondrial extracts were prepared as described in *Materials and Methods*. Western blot was performed with antihuman cytochrome *c* antibodies (mitoch., mitochondrial extracts; Bet. Acid, betulinic acid).

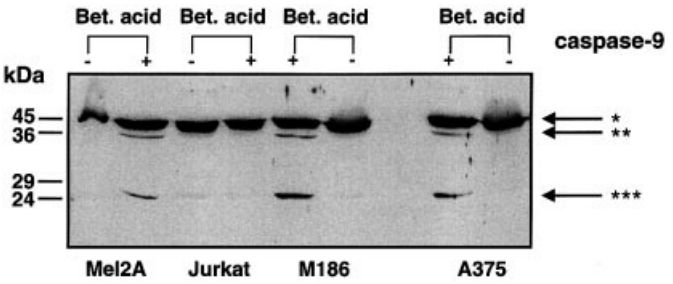

Figure 8. Activation of caspase-9 after treatment with betulinic acid in sensitive and resistant melanoma cell lines A375, M186, and Mel2A. Mel2A, M186, and A375 cells were harvested after 14 h treatment with 10 μg per ml betulinic acid (+) or control medium (–). Additionally the CD95-sensitive lymphoma cell line Jurkat was treated in the same way and used as control. For the detection of caspase-9 activation, Western blot analysis was performed with antihuman caspase-9 antibodies (Bet. Acid, betulinic acid; *procaspase-9, **36 kDa processed form, ***25 kDa processed form).

Ectopic Bcl-2 overexpression in sensitive cell lines prevented apoptosis triggered by both apoptotic stimuli, thereby supporting the role of the Bax/Bcl-2 rheostat as a key checkpoint that determines the susceptibility to CD95- and ceramide-induced apoptosis in melanoma cells. The broad resistance to cell death, occurring upon disturbed balance in the Bax/Bcl-2 ratio, has potential relevance for cancer cell behavior, tumor cell invasion, cell adhesion, or metastatic potential (Reed, 1999). For example, overexpression of Bcl-2 in the murine melanoma cell line B16 strongly enhanced melanoma pulmonary metastasis in allogenic BALB/c nude mice (Takaoka *et al*, 1997). This increased metastatic potential supports the idea of a correlation of melanoma metastasis promotion and the ability of Bcl-2 to protect tumor cells from apoptosis. Disturbances in the Bax/Bcl-2 balance, tilting the scales towards survival, can enhance the resistance of tumor cells to the cytotoxic effects of essential chemotherapeutic drugs. In this context, cytotoxic anticancer drugs have been shown to involve apoptotic pathways entering at different sites: either by a CD95L/CD95-driven amplifier system (doxorubicin, cisplatin) (Fulda *et al*, 1998a), ceramide generation (daunorubicin, adriamycin) (Jaffrezou *et al*, 1996; Lucci *et al*, 1999), or direct activation of upstream caspases (mitomycin C, etoposide) (Wesselborg *et al*, 1999). Thus, most of the interventions by cancer therapy enter the apoptotic pathway upstream of the mitochondrial checkpoint, where they converge into the common effector phase of apoptosis. Modification of the Bax/Bcl-2 protein level as a novel modality has been investigated by *bcl-2* antisense oligonucleotides therapy in melanoma (Jansen *et al*, 1998). Experimental settings using this technique revealed an inverse correlation between chemosensitivity and Bcl-2 levels. This finding indicates that the endogenous Bcl-2 protein levels contribute to melanoma drug resistance. The Bax/Bcl-2 ratio can be altered by the tumor suppressor p53. Wild-type p53 is known to upregulate the expression of Bax, thereby creating a link between Bax expression and the mutational status of *p53* (Miyashita *et al*, 1994). The mutational analysis of *p53* in melanoma cell lines revealed mutations in the coding region of the *p53* gene in two cell lines with a Bax/Bcl-2 ratio < 1. Loss of functional p53 might therefore be responsible for lower Bax expression in these two cell populations but cannot serve as a sufficient explanation for low Bax/Bcl-2 ratios in the remaining resistant cell lines.

According to our results, it should be possible to circumvent the apoptosis deficiency of melanoma cells by drugs directly influencing the mitochondrial function (as shown in **Fig 9**). For this purpose,

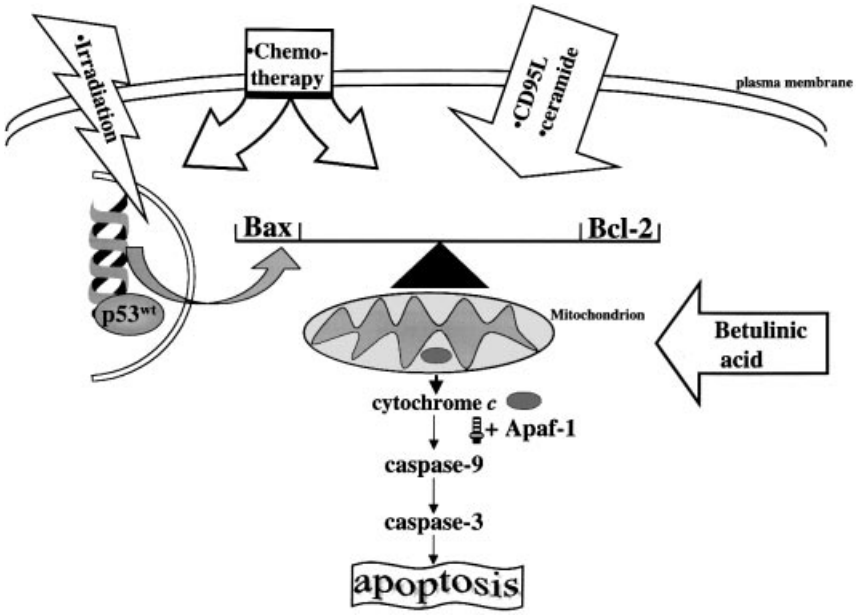

Figure 9. The apoptotic rheostat of the melanoma cell. Imbalances in the ratios of antiapoptotic and proapoptotic Bcl-2 family members that tilt the scales toward survival can render melanoma cells more resistant to different apoptotic stimuli. In contrast, a new class of cytotoxic drugs, exemplified by betulinic acid, may directly trigger mitochondrial activation. On the other hand, p53 is known to upregulate the expression of the *bax* gene, thereby altering the Bax/Bcl-2 ratio. During apoptosis, cytochrome *c* is released from mitochondria and binds to cytosolic Apaf-1 (apoptotic protease activating factor 1) and procaspase-9. This in turn results in activation of caspase-9 and processing of caspase-3.

we tested betulinic acid, a cytotoxic agent selective for melanoma, directly targeting mitochondria (Fulda *et al*, 1999; Selzer *et al*, 2000). Betulinic acid has been shown to act independently of the CD95 pathway and p53 activation by straight perturbance of mitochondrial functions (Fulda *et al*, 1997). A controversy was raised, however, by a report that betulinic acid exerts its inhibitory effect by increasing p53 (Rieber *et al*, 1998). In our system, betulinic-acid-induced apoptosis was in the same range in the p53 mutated cells as in the p53 wild-type cells and thus should be considered independent of the p53 system. Furthermore, betulinic acid was able to bypass the mitochondrial blockade in CD95-resistant melanoma cells and induced cytochrome *c* release and DNA fragmentation in both CD95-resistant and sensitive cells, independent of the Bax/Bcl-2 ratio.

Several experimental and clinical studies of other tumors provided support for higher expression levels of antiapoptotic Bcl-2 proteins as a negative prognostic marker. Furthermore, an apparent upregulation of Bcl-2 in patients with various tumors has been observed at the time of relapse of the disease. On the other hand, high Bax expression could be associated with a better response to chemotherapy in ovarian cancer and decreased Bax levels have been found in correlation with shorter survival in patients with breast cancer and colorectal cancer (Bargou *et al*, 1996; Sturm *et al*, 1999). Disbalances in the Bax/Bcl-2 ratio have been shown to be a determining prognostic factor for breast cancer and a study with 111 patients provided evidence that decrease of Bax was associated with relapse of childhood acute lymphoblastic leukemia (Prokop *et al*, 2000). In addition to transcriptional regulation of Bcl-2 family members, Bcl-2 family molecules are also subject to post-transcriptional regulation. These mechanisms include modifications of proapoptotic proteins, such as Bad, or antiapoptotic proteins, such as Bcl-2 and Bcl-x_L (Gajewski and Thompson, 1996; Wang *et al*, 1999). Phosphorylation by Akt/protein kinase B and other kinases inactivates these proteins and may conceivably be linked to differential responses to cytostatic therapy (Downward, 1998).

In conclusion, mitochondria with the main orchestrators Bax and Bcl-2 play a central role in the regulation of CD95- and ceramide-induced apoptosis in human melanoma cells. Our findings will therefore help in understanding the mechanisms by which apoptosis pathways are regulated in melanoma and provide useful links for developing strategies to improve the efficacy of anticancer therapy.

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