Betulinic Acid-Induced Apoptosis in Glioma Cells: A Sequential Requirement for New Protein Synthesis, Formation of Reactive Oxygen Species, and Caspase Processing

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ABSTRACT

Betulinic acid (BA), a pentacyclic triterpene, is an experimental antineoplastic agent for malignant melanoma. Here, we show that BA triggers apoptosis in five human glioma cell lines. BA-induced apoptosis requires new protein, but not RNA, synthesis, is independent of p53, and results in p21 protein accumulation in the absence of a cell cycle arrest. BA-induced apoptosis involves the activation of caspases that cleave poly-(ADP-ribose)polymerase. Interactions of death ligand/receptor pairs of the CD95/CD95 ligand family do not mediate BA-induced caspase activation. BA enhances the levels of BAX and BCL-2 proteins but does not alter the levels of BCL-xL or BCL-xL. Ectopic expression of BCL-2 prevents BA-induced caspase activation, DNA fragmentation, and cell death. Furthermore, BA induces the formation of reactive oxygen species that are essential for BA-triggered cell death. The generation of reactive oxygen species is blocked by BCL-2 and requires new protein synthesis but is unaffected by caspase inhibitors, suggesting that BA toxicity sequentially involves new protein synthesis, formation of reactive oxygen species, and activation of crmA-A-insensitive caspases.

Betulinic acid (BA), a pentacyclic triterpene, is an experimental antineoplastic agent that induces apoptosis in melanoma cells in vitro and in vivo (Pisha et al., 1995), as well as in neuroectodermal tumor cell lines in vitro (Fulda et al., 1997). BA is abundant in the plant kingdom and easily prepared in high yields from betulin of white-barked birch trees (Betula spp.). Cultured neoplastic glial cells are rather resistant to most proapoptotic stimuli (Weller, 1996). This corresponds to the poor response to radiotherapy or chemotherapy of human malignant gliomas in vivo. Numerous in vitro studies performed in recent years have shown that most types of drug-induced cytotoxicity in human tumor cell lines are associated with the induction of apoptosis. Drugs reported to induce apoptosis in glioma cells not only include common cancer chemotherapy drugs such as N,N′-bis(2-chloroethyl)-N-nitrosourea and cisplatin but also several drugs that, among other actions, interfere with the activation of protein kinase C, including staurosporine, calphostin C, hypericin, and the hydroxy-methylglutaryl-CoA reductase inhibitor, lovastatin. To characterize a novel potential antglioma agent, the relationship to the CD95 and p53 systems needs to be defined, as well as the relationship to the caspase cascade.

CD95 is a cell surface receptor of the tumor necrosis factor/nerve growth factor receptor superfamily expressed on a variety of normal and neoplastic cells (Nagata, 1997). To transmit the apoptotic signal (Schlottmann and Coggeshall, 1996), CD95 associates via its death domain with the Fas-associating protein with death domain (FADD/MORT1), which binds FADD-like ICE (interleukin 1-converting enzyme) (FLICE/caspase 8/MACH-1). FLICE/MACH-1 contains a caspase (ICE/ced-3-like protease) domain that seems to be activated upon ligation and trimerization of CD95 by a conformational change of FLICE (Medema et al., 1997). FLICE then transmits the activation signal to ICE and CPP32 (caspase 3), which is thought to trigger cell death. In contrast to untransformed astrocytes, malignant glioma cells acquire CD95 expression during malignant progression (Tachibana et al., 1995) and become sensitive to CD95-mediated apoptosis.

The p53 tumor suppressor gene (TP53) is the most frequently altered gene in human cancer and also is found mutated in human gliomas. Transfer of TP53 can induce apoptosis in different cell types, including human glioma cells (Li et al., 1997). Evasion of the pathways leading to apoptosis is critical for the development of tumors. For example, p21 is an important downstream mediator of p53-dependent, cisplatin-induced apoptosis (Kondo et al., 1996).

However, here we characterize a cascade of apoptosis triggered by the novel antineoplastic agent BA in cultured ma-

ABBREVIATIONS: ActD, actinomycin D; BA, betulinic acid; CD95L, CD95 ligand; CHX, cycloheximide; DEVD-CHO, N-acetyl-Asp-Glu-Val-Asp-aldehyde; NAC, N-acetyl-cysteine; PARP, poly(ADP-ribose)polymerase; PBN, N-tert-butyl-α-phenylnitrone; ROS, reactive oxygen species.
lignant glioma cells that is independent of CD95-ligand/receptor interaction and the p53 status of the tumor cells. We find that BA-induced apoptosis of glioma cells is characterized by a sequential requirement for new protein, but not RNA, synthesis, generation of reactive oxygen species, and caspase processing associated with poly(ADP-ribose)polymerase cleavage. BA-induced apoptosis of glioma cells does not involve net changes in the BCL-2/BAX rheostat.

Materials and Methods

Chemicals. BA [3α-hydroxy-20(19)]lupane28-carboxylic acid] was purchased from Aldrich (Steinheim, Germany) and dissolved in 50 mM dimethyl sulfoxide. Aliquots were stored at −20°C. Cycloheximide (CHX), actinomycin D (ActD), N-tet-butyl-α-phenylnitritin (PNB), and N-acetylcysteine (NAC) were purchased from Sigma (St. Louis, MO). Soluble CD95 ligand (CD95L) was obtained from CD95L cDNA-transfected N2A neuroblastoma cells (Roth et al., 1997).

Cell Lines and Cell Culture. LN-18, LN-229, LN-308, and U87 MG cells were kindly provided by Dr. N. de Trinquet (Lausanne, Switzerland). T98G were obtained from the American Type Culture Collection (Manassas, VA). U87 MG are wild type for p53. LN-229 cells are heterozygous for p53 and exhibit p53 transcriptional activity, whereas LN-18 and T98G cells are mutant for p53. LN-308 cells do not express p53 protein (Van Meir et al., 1994). The human glioma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. The generation of glioma cell clones expressing murine BCL-2 or p53val135 proteins or the viral caspase inhibitor, crmA, has been described (Weller et al., 1995; Trepel et al., 1996; Wagenknecht et al., 1997). Transgene expression was assessed by immunoblot analysis for murine BCL-2 and p53 protein and by Northern blot analysis for crmA mRNA. Experiments were performed with control transfectants harboring plasmids without insert (neo for BCL-2, hygro for p53val135, and puro for crmA).

Viability and Apoptosis Studies. Cell growth, generation times, and survival were assessed by crystal violet staining (Weller et al., 1997a). Apoptotic cell death was measured by quantitative assessment of DNA fragmentation (Reber et al., 1997).

Biochemical Studies. The generation of reactive oxygen species was monitored using a fluorescent probe, dihydrofluorescein diacetate (Reber et al., 1997). Caspase 3 activity was measured by conversion of the fluorescent substrate, DEVD-AMC (Schulz et al., 1997). The caspase-3 inhibitor, N-acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO), was purchased from Bioworld (Hamburg, Germany).

Immunoblot Analysis. Cells were rinsed in PBS, harvested, centrifuged at 1000g, lysed in 0.1 M Tris-HCl (pH 7.2) containing 0.1% Nonidet P-40, 0.1 mM EDTA, and 5 μg/ml phenylmethylsulfonyl fluoride for 40 min on ice, and centrifuged at 10,000 g for 10 min. Protein concentration was assayed using Bio-Rad reagents (München, Germany) with photometric analysis. Twenty micrograms of protein per lane was separated by 10 to 15% SDS-PAGE and electroblotted onto nitrocellulose (Amersham, Braunschweig, Germany). Equal protein loading was controlled by Ponceau red staining. After blocking for 1 h in PBS supplemented with 2% bovine serum albumin and 0.1% Tween 20, immunodetection of caspase 8 and 3, polyADP-ribose polymerase (PARP), Bax, Bcl-x, Bcl-2, p53, and p21 proteins was done using the following antibodies: mouse anti-caspase 8 monoclonal antibody C15 (1:5 dilution of hybridoma supernatant kindly provided by Dr. P. H. Krammer, Heidelberg, Germany), mouse anti-caspase 3 monoclonal antibody (1:1000; Transduction Laboratories, Lexington, KY), rabbit anti-PARP polyclonal antibody (1:10,000; Boehringer, Mannheim), rabbit anti-BAX and anti-BCL-x polyclonal and mouse anti-BCL-2, and rabbit anti-p21 polyclonal and mouse anti-p53 monoclonal antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). Goat anti-mouse IgG or anti-rabbit IgG (1:5000; Santa Cruz Biotechnology) and enhanced chemiluminescence (Amersham) were used for detection.

Cell Cycle Analysis. For cell cycle analysis, the glioma cells were treated as indicated, washed with PBS, incubated with trypsin for 3 min at 37°C, harvested, washed, and fixed with 70% ice-cold ethanol. Cells (10⁶ per experiment) were stained with propidium iodide (50 μg/ml in PBS containing 100 U/ml RNase A) for 30 min. All samples were analyzed on a FacsCalibur flow cytometer using Cell Quest acquisition and analysis software (Becton Dickinson, Heidelberg, Germany).

Results

BA Induces Apoptosis in Human Glioma Cell Lines. The glioma cells were exposed to increasing concentrations of BA for different time intervals. All five human glioma cell lines were susceptible to BA-induced growth inhibition in a concentration-dependent manner (Fig. 1, A and B). The EC₅₀ values were in the range of 20 μM for LN-229, 25 μM for U87 MG and T98G, 70 μM for LN-18, and 100 μM for LN-308. When the cells were seeded at low density, exposed to BA for 24 h, and then allowed to recover for 5 to 10 generation times (doubling times: T98G, 22 h; LN-229, 24 h; LN-18, 33 h; U87 MG, 39 h; LN-308, 59 h), there was no significant decrease in EC₅₀ values (data not shown), suggesting that the acute cytotoxic effects shown in Fig. 1, A and B, were responsible for the inhibition of clonogenic survival in the pulse/recovery colony-formation assays. Light microscopic monitoring suggested that BA-induced growth inhibition of glioma cells was associated with the induction of apoptosis. Consistent with

Fig. 1. BA toxicity of human glioma cells: requirement for new protein but not RNA synthesis. A and B, LN-18 (●), U87 (○), or T98G (▲) (A) or LN-229 (○) or LN-308 (■) human glioma cells were seeded in 96-well plates (10⁴/well) for 24 h and then exposed to BA for 24 h. Survival was assessed by crystal violet staining. Data are expressed as mean percent survival ± S.E.M (A) or mean survival ± S.E.M. (B) for LN-18 (●), LN-229 (○), U87 MG (▲), T98G (▲), and LN-308 (■) cells after 24 h exposure to BA (50 μM) at different concentrations. The glioma cells were exposed to BA for different time intervals. All five human glioma cell lines were susceptible to BA-induced growth inhibition in a concentration-dependent manner (Fig. 1, A and B). The EC₅₀ values were in the range of 20 μM for LN-229, 25 μM for U87 MG and T98G, 70 μM for LN-18, and 100 μM for LN-308. When the cells were seeded at low density, exposed to BA for 24 h, and then allowed to recover for 5 to 10 generation times (doubling times: T98G, 22 h; LN-229, 24 h; LN-18, 33 h; U87 MG, 39 h; LN-308, 59 h), there was no significant decrease in EC₅₀ values (data not shown), suggesting that the acute cytotoxic effects shown in Fig. 1, A and B, were responsible for the inhibition of clonogenic survival in the pulse/recovery colony-formation assays. Light microscopic monitoring suggested that BA-induced growth inhibition of glioma cells was associated with the induction of apoptosis. Consistent with
that hypothesis, exposure to BA induced DNA fragmentation of up to 25%, a degree of DNA fragmentation commonly induced by other proapoptotic stimuli in these cells, including CD95L (Fig. 1C). Next, we asked whether BA-induced apoptosis was an active type of cell death, that is, requires new mRNA and protein synthesis. We observed that BA-induced cell death was attenuated by a protein synthesis inhibitor, CHX, but unaffected by the RNA synthesis inhibitor, ActD (Fig. 1D), suggesting that translation of pre-existing mRNA mediates BA-induced apoptosis.

**BA Induces p21, but Not p53, Accumulation, in the Absence of a Cell Cycle Arrest.** Wild-type p53 activity is dispensable for BA-induced apoptosis because the p53 mutant cell lines, T98G and LN-18, as well as the p53 null cell line, LN-308, were not resistant to BA-induced apoptosis. However, the cell lines that retain p53 transcriptional activity in CAT assays, LN-229 and U87 MG (Van Meir et al., 1994), were rather sensitive to BA. Figure 2A shows that BA exposure did not result in p53 accumulation, which would be suggestive of wild-type function, in LN-229 cells and does not affect p53 levels in LN-18 cells. However, there was a time-dependent accumulation of p21 in both cell lines after exposure to BA, indicating that p21 expression is induced in a p53-independent manner. Because p21 accumulation is associated with cell cycle arrest, we next examined possible effects of BA on cell cycle progression. Cell cycle analysis of T98G and LN-229 cells treated with BA (50 μM) for 4 and 24 h showed no G0/G1 or G2-M arrest compared with control cells (Fig. 2C). To further determine the role of p53 in BA toxicity, we took advantage of LN-18 and LN-229 transfectants expressing the temperature-sensitive p53val135 mutant that behaves as a mutant p53 protein at 38.5°C but adopts wild-type conformation and activity at 32.5°C (Michalowitz et al., 1990). At 38.5°C and 32.5°C, the p53-transfected cells were as sensitive to BA as hygro control cells (Fig. 2D), confirming the independence of the BA-triggered apoptotic pathway from p53.

**BA-Induced Apoptosis Involves Activation of Caspases.** To further characterize the apoptotic cascade triggered by BA, we asked whether caspase activation was a critical event in BA-induced cell death. Immunoblot analysis revealed proteolytic cleavage of caspase 8 at 2 h after BA exposure of LN-229 cells (Fig. 3A). Caspase 3 also was proteolytically processed, with a similar time course (Fig. 3B), and PARP, one of the putative substrates of caspase 3, was cleaved to yield an 85-kDa fragment at 2 h as well (Fig. 3C). Consistent with the immunoblot data, BA caused a gradual increase in caspase 3-like enzyme activity, which peaked at 12 h after addition of BA (Fig. 4A). To confirm a critical role for caspase 3 in BA toxicity, we show that the preferential caspase 3-like pseudosubstrate peptide inhibitor, DEVD-CHO, significantly reduced BA toxicity (Fig. 4B). However, ectopic expression of the viral caspase inhibitor, crmA, failed to attenuate BA-induced DNA fragmentation (Fig. 4C), caspase 3 activity (Fig. 4D), and cell death (Fig. 4E) in both cell lines.

Because caspase activity is thought to be under the control of...
of BCL-2 family proteins, we next asked whether BA modulates the expression of endogenous BCL-2-related proteins. The levels of the proapoptotic BAX protein increased after exposure to BA in both cell lines (Fig. 5A), whereas the levels of proapoptotic BCL-x<sub>S</sub> protein were unaffected (data not shown). Similarly, expression of the antiapoptotic BCL-x<sub>L</sub> was unaffected by exposure to BA (data not shown), whereas BCL-2 levels increased after BA exposure (Fig. 5B). Thus, no net shift in the ratio of pro- versus antiapoptotic BCL-2 family proteins after BA exposure was detected. However, consistent with an essential role of caspase activation during BA-induced apoptosis, ectopic expression of BCL-2 in LN-18 and LN-229 cells reduced BA-evoked caspase 3 activity (Fig. 4D), inhibited BA-evoked DNA fragmentation (Fig. 4C), and protected from BA-induced cell death (Fig. 4E).

**BA-Induced Apoptosis Does Not Require CD95/CD95L Interactions.** Because activation of caspases, specifically caspase 8, is thought to mediate apoptosis triggered by cytotoxic cytokines, we asked whether BA-induced caspase activation might depend on interactions of endogenous death ligands and receptors. To this purpose, we used sublines from the CD95L-sensitive LN-18 cell line that exhibit acquired resistance to CD95L (APO2L, and TNF) (data not shown). These cells retained sensitivity to BA (Fig. 6B), suggesting that BA bypasses such ligand/receptor interactions and activates caspases via another pathway.

**BA-Induced Apoptosis Leads to Free Radical Formation.** We have characterized previously a cascade of apoptosis in neurons that involved the sequential requirement for new protein synthesis, caspase activation, and generation of reactive oxygen species (ROS) (Schulz et al., 1996). Here, we report that BA induces the formation of ROS in glioma cells (Fig. 7A) and that the antioxidants PBN and NAC prevent BA-induced ROS formation (Fig. 7B) as well as cell death (Fig. 7C). Next, we elucidated the sequence of events during BA-induced apoptosis of glioma cells. We find that treatment with CHX when given 2 h before exposure to BA abolished ROS formation (Fig. 8A), diminished caspase 3 activity (Fig. 8B), and significantly reduced cell death (Fig. 8C). There was no such effect when CHX was given at the time of or after BA exposure. The free radical spin trap, PBN, had to be given before or at the same time as BA, but not after addition of BA, to prevent the rise in ROS fluorescence (Fig. 8D). PBN significantly attenuated DEVD-AMC cleavage even when added 2 h after BA exposure (Fig. 8E), suggesting that ROS forma-
tion is required for caspase activation. Similarly, no caspase 3 activation became apparent on immunoblot analysis when BA exposure was started after 2-h pre-exposure to PBN (Fig. 8F). DEVD-CHO supplementation up to 2 h after incubation of the cells with BA prevented the rise in caspase 3 activity (Fig. 8G) but had no effect on ROS formation (Fig. 8H).

Eventually, we asked whether ectopic expression of crm-A or BCL-2 modulated ROS formation after BA exposure. Consistent with the data summarized above, BCL-2 inhibited the formation of ROS whereas crm-A had no such effect (Fig. 8I), confirming an important role for ROS in BA toxicity and excluding a role for crm-A-sensitive caspases in that process.

**Discussion**

BA is a novel antineoplastic agent with cytotoxicity against melanoma and neuroectodermal tumor cells. Here, we report that BA triggers an apoptotic cascade in human malignant glioma cells as well. BA-induced apoptosis of glioma cells involves sequentially new protein synthesis, formation of reactive oxygen species, and caspase processing. In detail, we show that BA toxicity depends on new protein but not mRNA synthesis, suggesting that translation of preexisting mRNA mediates BA toxicity. Caspase 8 and 3 activation are critical steps in the killing cascade triggered by BA because cell death ensues whenever caspase activation is detected. Furthermore, pharmacological (PBN, NAC, DEVD-CHO) or genetic (BCL-2) manipulations that inhibit apoptosis do so at the level of, or upstream of, caspase activation. Cytotoxic, cytokine-resistant glioma cells do not exhibit cross-resistance to BA, suggesting that caspase activation is independent of interactions of endogenous CD95/CD95L or related pairs of death ligands and receptors.

Interestingly, we were able to demonstrate that BA-induced ROS formation is necessary for caspase activation and cell death and that new protein synthesis is required for ROS formation. Although BA does not change the BAX/BCL-2 rheostat as assessed by protein levels on immunoblots, ectopic expression of BCL-2 suppressed BA-induced cell death upstream or at the level of ROS generation, confirming that some antiapoptotic properties of BCL-2 are related to antioxidative defense mechanisms.

p53 that mediates growth arrest or apoptosis in response to...
DNA damage in many tumor cells is a direct transcriptional activator of the human bax gene (Miyashita and Reed, 1995) and may mediate apoptosis via induction of oxidative stress (Polyak et al., 1997). However, no accumulation of p53 was seen in p53 wild-type cells after treatment with BA, and enhanced levels of BAX and p21 proteins as well as ROS formation were observed after BA exposure in p53 mutant cell lines, indicating that all these changes are p53 independent. p53-independent death mechanisms after administration of chemotherapeutic drugs or γ-irradiation have been described (Liebmann et al., 1995), and BAX-accelerated apoptosis in response to dexamethasone has been observed in the absence of p53 (Brady et al., 1996). These data suggest that in addition to p53-restricted pathways, alternative pathways are involved in the up-regulation of BAX expression and drug-induced apoptosis. In regard to p21, this protein inhibits cyclin-dependent kinase (cdk) activity and mediates p53-dependent and independent cell cycle arrest in G₀-G₁ and G₂-M. However, cell cycle analysis after exposure to BA revealed no specific pattern of cell cycle redistribution in LN-18 or LN-229 cells despite the induction of p21 expression. This may be a consequence of the retinoblastoma gene product checkpoint disruption in both cell lines, which we have been able to link to a homozygous deletion of p16 in both cell lines (Wagenknecht et al., 1997).

These findings may provide implications for overcoming drug resistance of glioma cells. The CD95-resistant LN-18 glioma cells showed the same response to BA as the CD95-sensitive cells, suggesting that resistance is bypassed by activating death-signaling pathways downstream of CD95L-receptor interactions. BA therefore might be an interesting adjunct to present glioma chemotherapy. BA is especially attractive because of a lack of toxicity reported in the literature (Pisha et al., 1995) and no death-inducing action in concentrations up to 250 μM in nontransformed cultured...
human astrocytes in our lab (unpublished observation). Our data are consistent with the work of Fulda et al. (1997) on neuroblastoma cells in that BA triggers apoptosis in these cells independently of CD95/CD95L interactions and p53 status, but depends on activation of caspases 8 and 3 and involves ROS formation. Furthermore, we define here a critical role for new protein synthesis in BA toxicity and report that new protein synthesis, ROS formation, and activation of caspases are sequential and necessary steps in BA-induced apoptosis.

References

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