0022-3565/99/2893-1306$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 289:1306–1312, 1999

Vol. 289, No. 3
Printed in U.S.A.

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

WOLFGANG WICK, CORNELIA GRIMMEL, BETTINA WAGENKNECHT, JOHANNES DICHGANS, and MICHAEL WELLER

Laboratory of Molecular Neuro-Oncology, Department of Neurology, University of Tübingen, School of Medicine, Tübingen, Germany

Accepted for publication January 28, 1999 This paper is available online at http://www.jpet.org

ABSTRACT

Betulinic acid (BA), a pentacyclic triterpene, is an experimental cytotoxic 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-x_S or BCL-x_L. 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 crm-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, lovostatin. To characterize a novel potential anti-glioma 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-

Received for publication October 23, 1998.

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 [3β-hydroxy-20(19)lupaen28-carbonic 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-tert*-butyl-α-phenylnitrone (PBN), 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 Tribolet (Lausanne, Switzerland). T98G were obtained from the American Type Culture Collection (Manassas, VA). U87 MG cells 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 p53val^{135} proteins or the viral caspase inhibitor, crm-A, 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 crm-A mRNA. Experiments were performed with control transfectants harboring plasmids without insert (neo for BCL-2, hygro for p53val^{135}, and puro for crm-A).

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, dichlorofluorescein 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 Biomol (Hamburg, Germany).

Immunoblot Analysis. Cells were rinsed in PBS, harvested, centrifuged at 1000*g*, 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, poly(ADP-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 (10^6 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 EC_{50} 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 EC_{50} 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

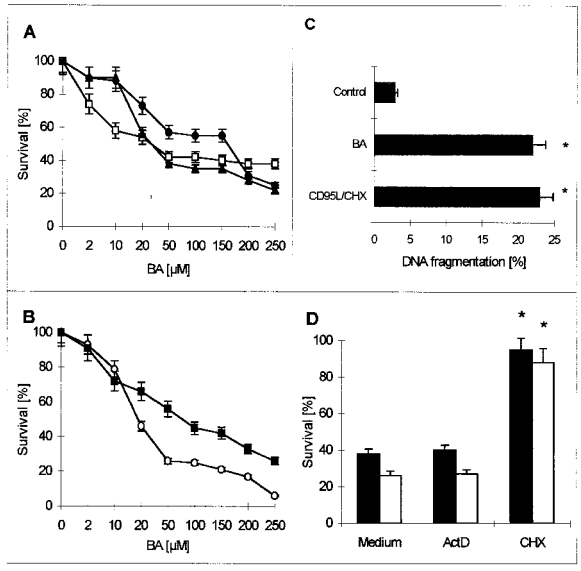

Fig. 1. BA toxicity of human glioma cells: requirement for new protein but not RNA synthesis. A and B, LN-18 (●), U87 (□), or T98G (▲) (A) or LN-229 (○) or LN-308 (■) human glioma cells were seeded in 96-well plates (10^4/well) for 24 h and then exposed to BA for 24 h. Survival was assessed by crystal violet assay. Data are expressed as mean percentages and S.E.M. of survival ($n = 3$). C, LN-229 cells were untreated or exposed to BA (50 μM) for 24 h or to CD95L (32 U/ml) plus CHX (10 μg/ml) for 16 h. DNA fragmentation was measured by DNA fluorometry as described (Weller et al., 1997a). Data are expressed as mean percentages and S.E.M. ($n = 3$). D, T98G (solid columns) or LN-229 (open columns) cells were exposed to BA (50 μM) for 16 h without or with a 2-h preexposure to ActD (0.5 μg/ml) or CHX (10 μg/ml). At these concentrations, RNA and protein synthesis were inhibited by more than 80% (data not shown; Winter and Weller, 1998). Data are expressed as mean percentages of survival and S.E.M. relative to untreated cells or cells exposed to the inhibitors alone (*$P < .05$, *t* test).

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 G_0-G_1 or G_2-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 p53val^{135} 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.

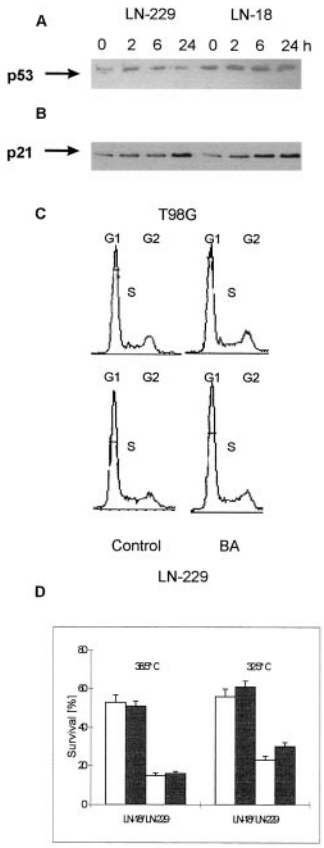

Fig. 2. No role for p53 in BA-induced apoptosis. A and B, LN-18 or LN-229 cells were treated with BA (50 μM) for the indicated time intervals. Immunoblot analysis for p53 (A) or p21 (B) was performed as described in *Materials and Methods*. C, T98G or LN-229 cells were untreated (left) or treated with BA (50 μM) (right) for 24 h. Cell cycle analysis was performed as described in *Materials and Methods*. D, LN-18 (left) or LN-229 (right) hygro (open columns) or p53val^{135}-transfected (shaded columns) cells were exposed to BA (50 μM) for 24 h at 38.5°C (mutant p53, left) or 32.5°C (wild-type p53, right). Survival was assessed by crystal violet assay. Data are expressed as mean percentages and S.E.M. of survival (n = 3).

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, crm-A, 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

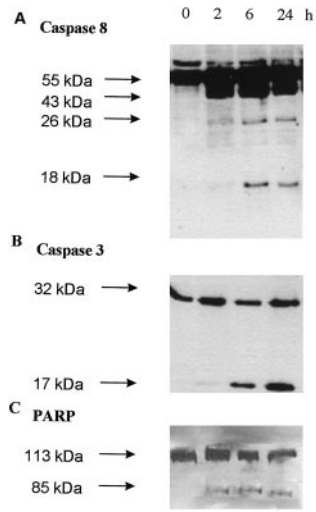

Fig. 3. BA-induced apoptosis of human malignant glioma cells involves activation of caspase 8 and 3 and PARP cleavage. LN-229 cells were untreated or exposed to BA (50 μM) for 2 to 24 h. Immunoblot analysis was performed as described in *Materials and Methods*. A, processing of caspase 8, which migrates as a double band corresponding to two caspase 8 isoforms (caspase 8/a and 8/b) (Scaffidi et al., 1998), results in p43 and p41 cleavage intermediates derived from caspase 8/a and 8/b, respectively, and the p18 active subunit. B, activation of caspase 3 is documented by the time-dependent occurrence of caspase 3/p17. C, PARP cleavage is visualized by the occurrence of a PARP/p85 proteolytic product.

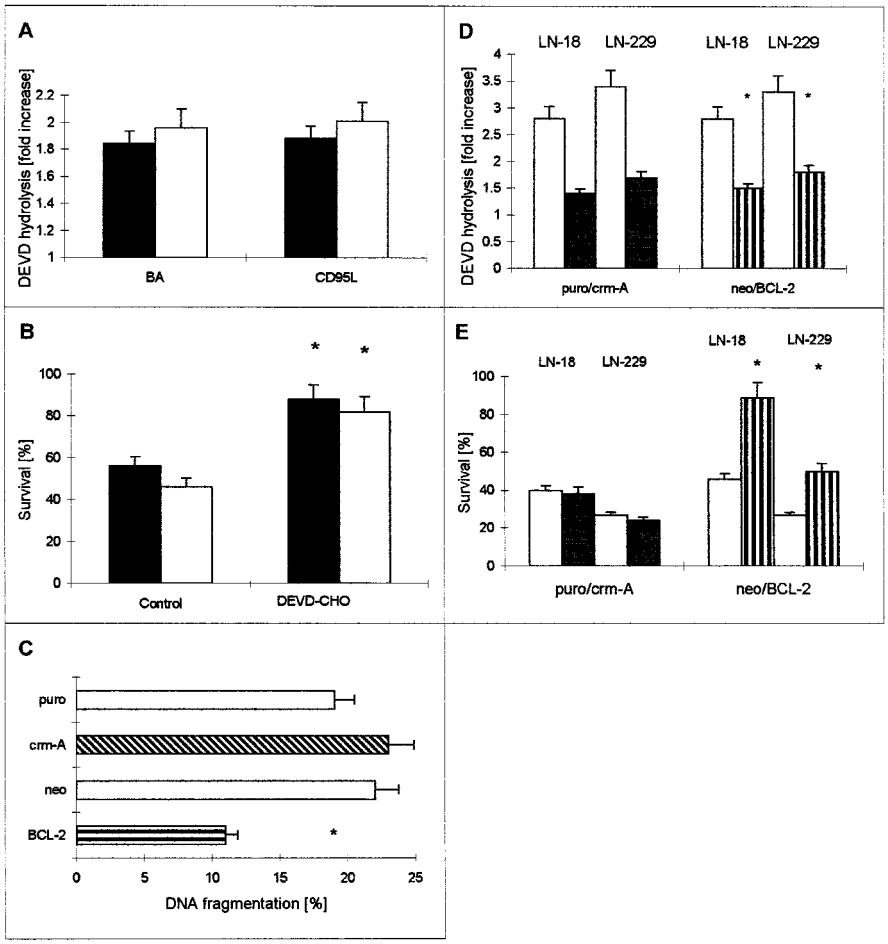

Fig. 4. Inhibition of BA-induced apoptosis and caspase 3 activity by ectopic expression of crm-A or Bcl-2. A, T98G (solid columns) or LN-229 (open columns) glioma cells were plated as in Fig. 1. Caspase 3 activity was measured by the conversion of DEVD-AMC (12.5 μM) at 12 h after exposure to BA (50 μM) or 6 h after exposure to CD95L (32 U/ml) plus CHX (10 μg/ml), which was included as a positive control. Data are expressed as means and S.E.M. from three independent experiments performed in triplicate. B, T98G (solid columns) or LN-229 (open columns) cells were exposed to BA (50 μM) for 72 h in the absence or presence of DEVD-CHO (600 μM). Data are expressed as mean percentages of survival and S.E.M. relative to untreated cells or cells exposed to the inhibitors alone ($^*P < .05$, t test). C, LN-229 puro, crm-A, neo, or BCL-2 cells were exposed to BA (50 μM) for 24 h. DNA fragmentation was measured by DNA fluorometry as described (Weller et al., 1997a). Data are expressed as mean percentages and S.E.M. ($n = 3$, $^*P < .05$, t test). D and E, LN-18 or LN-229 puro (open columns) or crm-A (shaded columns) cells (left) or LN-18 or LN-229 neo (open columns) or BCL-2 (striped columns) cells (right) were exposed to BA (50 μM) for 12 h. D, caspase 3 activity was assessed by DEVD-AMC cleavage. Data are expressed as optical density ratios relative to untreated controls. E, survival was assessed by crystal violet assay in parallel experiments. Data are expressed as mean survival and S.E.M. ($n = 3$, $^*P < .05$, t test).

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_s protein were unaffected (data not shown). Similarly, expression of the antiapoptotic BCL-x_l 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 (Fig. 6A), 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.

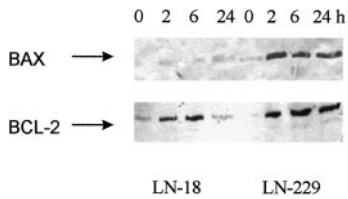

Fig. 5. Bcl-2 family protein expression during BA-induced apoptosis. LN-18 (left) or LN-229 (right) cells were untreated or treated with BA (50 μM) for 2, 6, or 24 h. Immunoblot analysis for BAX (A) or BCL-2 (B) proteins was performed as described (Weller et al., 1997b).

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-

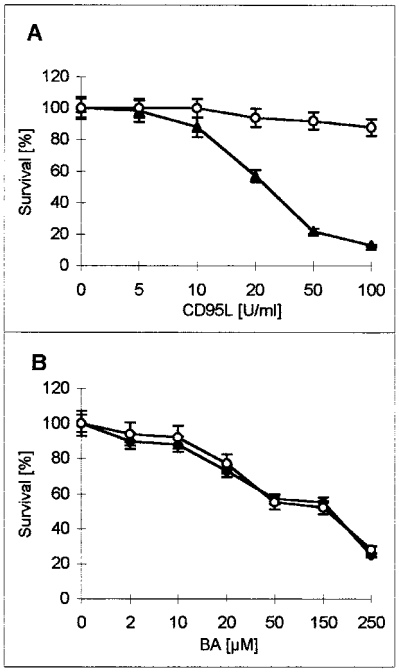

Fig. 6. BA induces apoptosis in LN-18 cells with acquired resistance to cytotoxic cytokines. Parental LN-18 (▲) or LN-18-R (resistant; ○) glioma cells were treated with different concentrations of CD95L (A) or BA (B) for 24 h. Survival was assessed by crystal violet staining. Data are expressed as mean percentages and S.E.M. ($n = 3$).

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

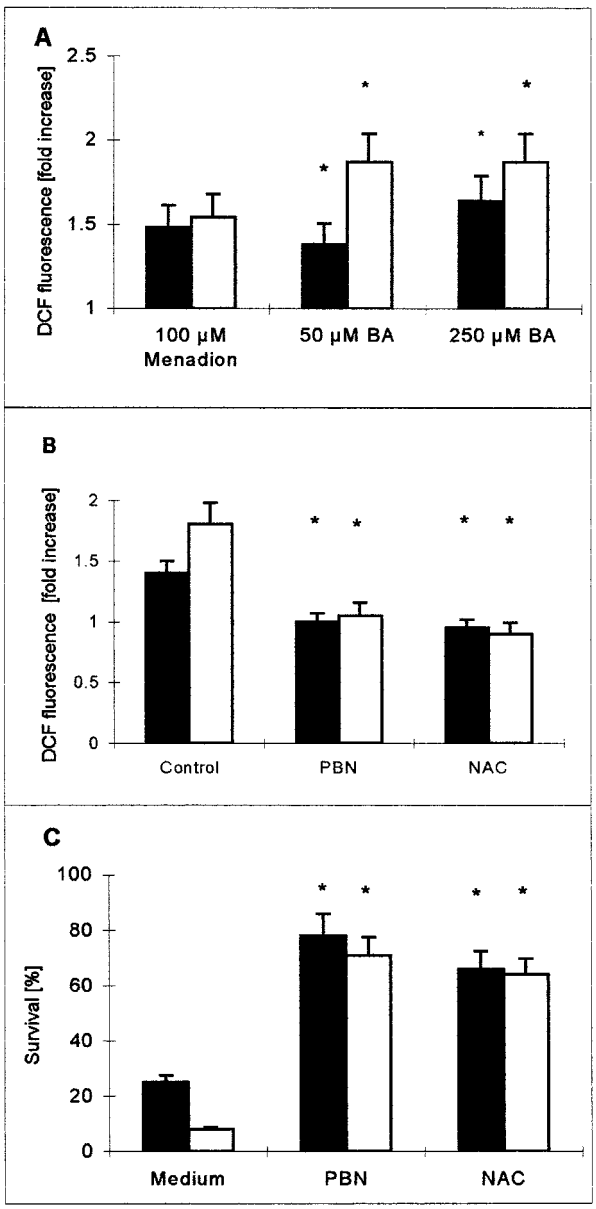

Fig. 7. A role for ROS in BA-induced cytotoxicity of human glioma cells. A, T98G (solid columns) or LN-229 (open columns) cells were treated for 4 h with menadione (100 μM) or 24 h with BA (50 μM or 250 μM). ROS generation was monitored by fluorescence generated by the dichlorofluorescein diacetate fluorescent probe relative to untreated control cultures. The amount of fluorescence generated by untreated cells was normalized to 1. Data are mean percentages and S.E.M. of triplicates of three independent experiments. B and C, T98G (solid columns) or LN-229 (open columns) cells were not pretreated or pretreated with PBN (10 μM) or NAC (1 μM) for 2 h and then exposed to BA (50 μM) in the absence or presence of these agents. B, ROS formation was assessed as in A. C, survival was assessed by crystal violet assay. Data are expressed as mean percentages and S.E.M. ($n = 3$) (*$P < .05$, t test).

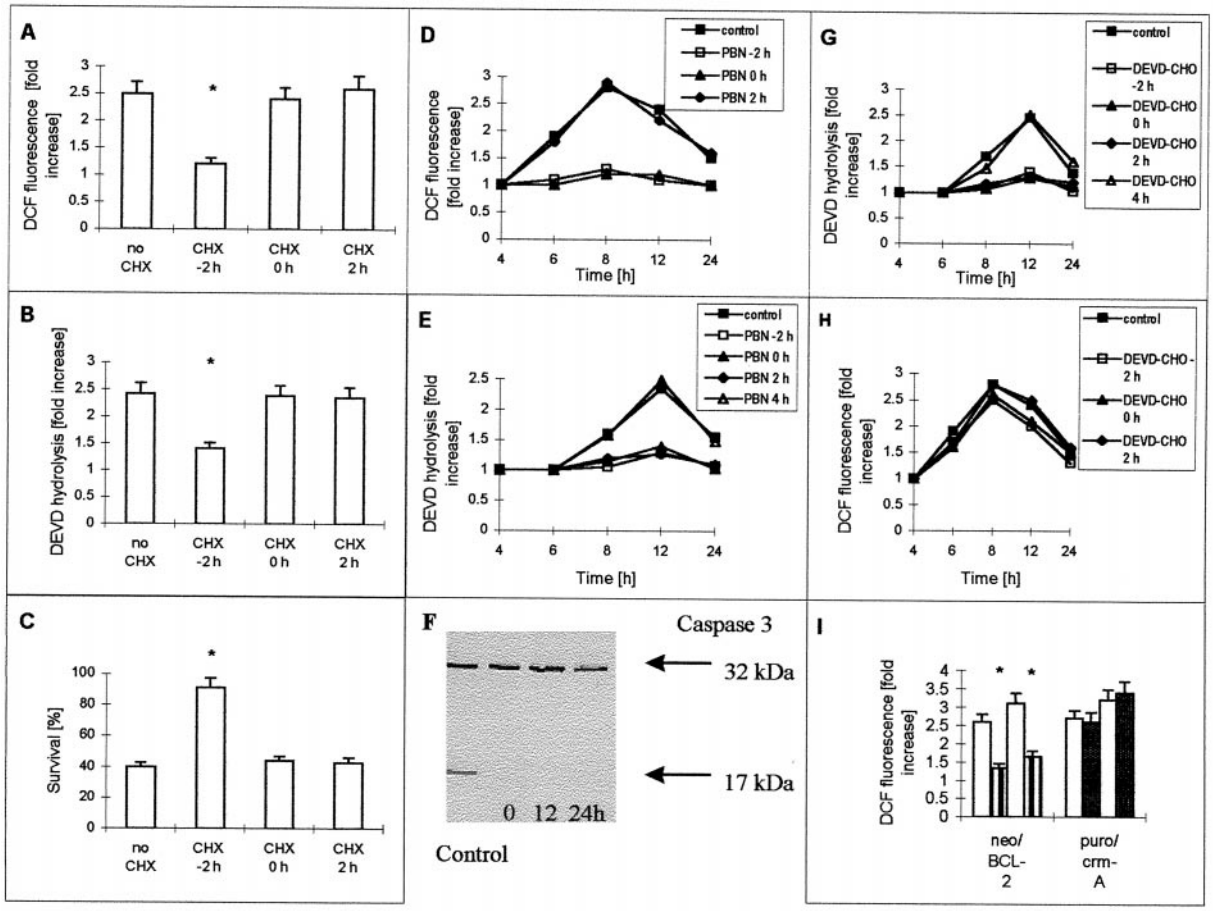

Fig. 8. BA-induced apoptosis requires sequential activation of protein synthesis, ROS synthesis, and caspase activation. A–C, T98G cells were treated with BA (50 μM) for 16 h, without or with the presence of CHX added 2 h before, at the same time and 2 h after addition of BA. A, ROS formation was determined as in Fig. 7, A and B. B, caspase 3 activation was measured as in Fig. 4A. C, survival was assayed as in Fig. 1. Data are expressed as means and S.E.M. (n = 3). D and E, T98G cells were treated with BA (50 μM) for the indicated times, and PBN was added between 2 h before and 4 h after the exposure to BA in 2-h intervals. D, ROS formation was determined as in Fig. 7, A and B. E, caspase 3 activity was measured as in Fig. 4A. Data are expressed as mean values (n = 3, S.E.M. < 10%). F, LN-229 cells were exposed to BA (50 μM) alone for 12 h (lane 1) or preexposed to PBN (10 μM) for 2 h and then treated with BA for 0, 12, or 24 h (lanes 2–4). Immunoblot analysis was performed as described in *Materials and Methods*. Activation of caspase 3 is documented by occurrence of caspase 3/p17 (lane 1). G, T98G cells were treated with BA (50 μM) for the indicated times, and DEVD-CHO was added between 2 h before and 4 h after BA in 2-h intervals. DEVD-AMC cleavage was measured as in Fig. 4A. Data are expressed as mean increase over control (n = 3, S.E.M. < 10%). H, T98G cells were treated with BA (50 μM) for the indicated times, and DEVD-CHO was added between 2 h before and 2 h after the exposure to BA in 2-h intervals. ROS formation was determined as in Fig. 7, A and B. I, LN-18 or LN-229 neo (open columns) or BCL-2 (striped columns) cells (left) or LN-18 or LN-229 puro (open columns) or crm-A (shaded columns) cells (right) were exposed to BA (50 μM) for 12 h. The generation of reactive oxygen species was monitored as in Fig. 7. Data are expressed as increase of fluorescence over control. All experiments were done three times in triplicate.

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, too, indicating that all these changes are p53 independent. p53-independent death mechanisms after administration of chemotherapeutic drugs or γ-irradiation have been described (Liebermann 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 G_0-G_1 and G_2-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

Brady HJ, Salomons GS, Bobeldijk RC and Berns AJ (1996) T cells from bax-α transgenic mice show accelerated apoptosis in response to stimuli but do not show restored DNA damage-induced cell death in the absence of p53. *EMBO J* **15:**1221–1230.

Fulda S, Friesen C, Los M, Scaffidi C, Mier W, Benedict M, Nunez G, Krammer PH, Peter ME and Debatin K-M (1997) Betulinic acid triggers CD95 (APO-1/Fas)- and p53-independent apoptosis via activation of caspases in neuroectodermal tumors. *Cancer Res* **57:**4956–4964.

Kondo S, Bama B, Kondo Y, Tanaka Y, Casey G, Liu J, Morimura T, Kaakaji R, Peterson J, Werbel B and Barnett G (1996) WAF1/CIP1 increases the susceptibility of p53 non-functional malignant glioma cells to cisplatin-induced apoptosis. *Oncogene* **13:**1279–1285.

Li H, Lochmüller H, Yong V, Karpati G and Nalbantoglu J (1997) Adenovirus-mediated wild-type p53 gene transfer and overexpression induces apoptosis of human glioma cells independent of endogenous p53 status. *Exp Neurol* **56:**872–878.

Liebermann DA, Hoffman B and Steinman RA (1995) Molecular controls of growth arrest and apoptosis: p53-dependent and independent pathways. *Oncogene* **11:** 199–210.

Medema J, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH and Peter ME (1997) FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *EMBO J* **16:**2794–2804.

Michalowitz D, Halevy O and Oren M (1990) Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell* **62:**674–680.

Miyashita T and Reed JC (1995) Tumor suppressor p53 is a direct transcriptional activator of the human BAX gene. *Cell* **80:**293–299.

Nagata S (1997) Apoptosis by death factor. *Cell* **88:**355–365.

Pisha E, Chai H, Lee IS, Chagwedera TE, Farnsworth NR, Cordell GA, Beecher CW, Fong HH, Kinghorn AD, Brown DM, Wani MC, Wall ME, Hieken TJ, Das Gupta TK and Pezzuto JM (1995) Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. *Nat Med* **1:**1046–1051.

Polyak K, Xia Y, Zweier J, Kinzler K and Vogelstein B (1997) A model for p53-induced apoptosis. *Nature (Lond)* **389:**300–305.

Reber U, Wüllner U, Trepel M, Baumgart J, Seyfried J, Klockgether T, Dichgans J and Weller M (1997) Potentiation of treosulfan toxicity by the glutathione-depleting agent buthione sulfoximine in human malignant glioma cells: The role of BCL-2. *Biochem Pharmacol* **54:**349–359.

Roth W, Fontana A, Trepel M, Dichgans J, Reed JC and Weller M (1998) Immunochemotherapy of malignant glioma: Synergistic activity of CD95 ligand and chemotherapeutics. *Cancer Immunol Immunother* **44:**55–63.

Schlottmann K and Coggeshall K (1996) CD95/Fas/Apo-1-mediated signal transduction. *Cell Physiol Biochem* **6:**345–360.

Schulz JB, Bremen D, Reed JC, Lommatzsch J, Takayama S, Wüllner U, Löschmann P-A, Klockgether T and Weller M (1997) Cooperative interception of neuronal apoptosis by BCL-2 and BAG-1 expression: Prevention of caspase activation and reduced production of reactive oxygen species. *J Neurochem* **69:**2075–2086.

Schulz JB, Weller M and Klockgether T (1996) A sequential requirement for new mRNA and protein synthesis, ICE-like protease activity and free radicals in potassium deprivation-induced apoptosis of cerebellar granule cells. *J Neurosci* **16:**4696–4706.

Tachibana O, Nakazawa H, Lampe J, Watanabe K, Kleihues P and Ohgaki H (1995) Expression of Fas/Apo-1 during the progression of astrocytomas. *Cancer Res* **55:** 5528–5530.

Trepel M, Dichgans J, Malipiero U, Fontana A and Weller M (1996) p53 gene transfer for malignant glioma: Influence on sensitivity to anticancer drugs (Abstract). *J Neurooncol* **30:**95.

Van Meir EG, Kikuchi T, Tada M, Li H, Diserens AC, Wojcik BE, Huang HJS, Freimann T, De Tribolet N and Cavenee WK (1994) Analysis of the p53 gene and its expression in human glioblastoma cells. *Cancer Res* **54:**649–652.

Wagenknecht B, Trepel M, von Deimling A, Grimmel C, Rollbrocker B, Hayashi Y, Lang F, Dichgans J, Gulbins E and Weller M (1997) p53 accumulation promotes dephosphorylation and proteolytic cleavage of retinoblastoma protein in human malignant glioma cells. *Cell Physiol Biochem* **7:**304–311.

Weller M (1996) Genetic regulation and therapeutic modulation of apoptosis in human malignant glioma. *Cell Physiol Biochem* **6:**376–380.

Weller M, Malipiero U, Aguzzi A, Reed JC and Fontana A (1995) Protooncogene bcl-2 gene transfer abrogates Fas/APO-1 antibody-mediated apoptosis of human malignant glioma cells and confers resistance to chemotherapeutic drugs and therapeutic irradiation. *J Clin Invest* **95:**2633–2643.

Weller M, Trepel M, Grimmel C, Schabet M, Bremen D, Krajewski S and Reed JC (1997a) Hypericin-induced apoptosis of human malignant glioma cells is light-dependent, independent of bcl-2 expression and does not require wild-type p53. *Neurol Res* **19:**459–470.

Weller M, Winter S, Schmidt C, Esser P, Fontana A, Dichgans J and Groscurth P (1997b) Topoisomerase I inhibitors for human malignant glioma. Differential modulation of p53, p21, bax and bcl-2 expression and of CD95-mediated apoptosis by camphotecin and beta-lapachone *Int J Cancer* **73:**1–8.

Winter S and Weller M (1998) Potentiation of CD95L-induced apoptosis of human malignant glioma cells by topotecan involves inhibition of RNA synthesis but not changes in CD95 or CD95L expression. *J Pharmacol Exp Ther* **286:**1374–1382.

Send reprint requests to: Michael Weller, M.D., Department of Neurology, University of Tübingen, School of Medicine, Hoppe-Seyler-Strasse 3, 72076 Tübingen, Germany. E-mail: michael.weller@uni.tuebingen.de