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Resistance of Human Astrocytoma Cells to Apoptosis Induced by Mitochondria-Damaging Agents: Possible Implications for Anticancer Therapy

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ABSTRACT

The success of anticancer chemotherapy is often hampered by resistance to apoptosis, which may depend on defects in intracellular cell death pathways. Characterizing the alterations of these pathways is a prerequisite for developing alternative and effective antitumoral strategies. Here, we investigated the susceptibility of a human astrocytoma cell line, ADF, to apoptotic cell death induced by mitochondria-damaging agents. Neither the anticancer agent betulinic acid nor the "mitochondriotropic" poisons 2-deoxy-D-ribose and potassium cyanide induced apoptosis of these cells, despite induction of highly significant mitochondrial depolarization, eventually resulting in necrotic death. Resistance to apoptosis was not due to presence of the multidrug re-sistance pump or to impaired expression of caspase-8, caspase-9, or "executioner" caspase-3. Cloning of caspase-9 revealed the presence of full-length caspase-9α and a short variant (caspase-9β), which, in other tumors, acts as a dominant negative of the long isoform. All analyzed clones showed a point mutation in the prodomain region that is known to interact with mitochondria-released factors. Thus, in these human astrocytoma cells, mitochondria-damaging agents induce a regulated form of mitochondrial-dependent necrotic cell death (oncosis). Resistance to apoptosis is due to an intrinsic defect of caspase-9, leading to inhibition of enzyme activation and/or impaired interaction with proteins released from depolarized mitochondria. These results may have implications for developing strategies aimed at overcoming tumor resistance to chemotherapy.

In multicellular organisms, many of the mechanisms that control tissue homeostasis are linked to apoptosis. In principle, two main pathways of apoptosis can be identified in mammalian cells, both having proteases called caspases as the main actors in controlling the biochemical steps leading to death (Igney and Krammer, 2002). The so-called "extrinsic pathway" is driven by the activation of extracellular death receptors, leading to the recruitment and activation of initiator caspase-8 and -10. Conversely, death at the mitochondrial level (the "intrinsic pathway") is initiated by a perturbation of the mitochondrial membrane potential, followed by release of cytochrome *c* in the cytosol that in turn contributes to the formation of a protein complex called apoptosome (Igney and Krammer, 2002). This complex eventually allows the activation of effector caspase-9. Both the intrinsic and the extrinsic pathway converge on downstream "executioner" caspases, mainly caspase-3, which are responsible for the cleavage of structural cytoplasmic and nuclear proteins, with consequent cell collapse and death.

Over the past few years, it has become clear that anticancer drugs are able to induce apoptosis and that this process is involved in the mediation of their cytotoxic effects. Furthermore, the induction of apoptosis by different classes of anticancer agents was demonstrated to converge into similar downstream mechanisms that are mainly represented by the two above-described biochemical pathways (Ferreira et al., 2002).

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ABBREVIATIONS: BetA, betulinic acid; dRib, 2-deoxy-D-ribose; PI, propidium iodide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; FL2, fluorescence channel 2; FL1, fluorescence channel 1; FSC, forward light scatter; PS, phosphatidylserine; FITC, fluorescein isothiocyanate; LDH, lactate dehydrogenase; Mdr-1, multidrug resistance protein-1; TBS, Tris-buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; PCR, polymerase chain reaction; Fw, forward; Rw, reverse; ANOVA, analysis of variance; bp, base pair(s); KCN, potassium cyanide.

However, the success of chemotherapy is often hampered by the appearance of broad drug resistance, in several cases due to defective common apoptosis-inducing pathways. It is now clear that these defects are probably at the basis of the initial expansion of the population of neoplastic cells eventually originating the tumor (Igney and Krammer, 2002). Thus, it is very important to identify the biochemical defect(s) borne by a specific tumor to predict the possible outcome of a given drug treatment and to plan more focused and effective therapies aimed at bypassing the observed alterations. This is particularly important in the case of central nervous system tumors (e.g., gliomas, in particular those of astrocytic origin), which account for more than 40% of all brain tumors and are often characterized by a poor prognosis (Kleihues et al., 1995).

We have previously demonstrated that 2-chloro-2′-deoxyadenosine, a widely used chemotherapeutic drug for leukemia and lymphoma (known as cladribine; Bryson and Sorkin, 1993), is able to induce apoptotic cell death of human astrocytoma ADF cells through recruitment of an atypical caspase-2-dependent pathway (Ceruti et al., 2003). No activation of the mitochondria/caspase-9-dependent intrinsic pathway of apoptosis was detected in our experimental model, despite evidence demonstrating a key role played by this pathway in cladribine-induced cell death in other tumor cell lines (Marzo et al., 2001). Given the importance of the intrinsic pathway in the activation of apoptosis by chemotherapeutic agents (see above), we wondered whether insensitivity to this chemotherapeutic agent reflects a peculiar behavior of cladribine in these cells or is the result of a more generalized defect of the intrinsic pathway of apoptosis in astrocytoma cells. In the present study, we show that several mitochondria-damaging agents, including betulinic acid, a new, promising anticancer drug (Fulda and Debatin, 2000; Fulda et al., 2004), do not induce apoptosis in astrocytoma ADF cells, and we raise several hypotheses to explain this insensitivity that might have important implication in resistance to chemotherapy.

Materials and Methods

Cell Culture

Human Astrocytoma ADF Cells. ADF cells (Malorni et al., 1994) were maintained in culture in standard conditions (37°C, 95% humidity, 5% CO_2) in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% nonessential amino acids (all from Euroclone, Celbio, Italy), as described previously (Ceruti et al., 2003). Betulinic acid (BetA, 10–100 μg/ml; Alexis Biochemicals, Vinci-Biochem, Vinci, Italy), 2-deoxy-D-ribose (dRib, 10–50 mM; Sigma-Aldrich, Milan, Italy), and KCN (100–600 μM; Sigma-Aldrich) were added to cultures 24 h after plating cells on six-well plates (600,000 cells/well) and left in the culture medium for 24 to 96 h.

Cytofluorimetrical Studies

Evaluation of Apoptosis. The percentage of apoptotic cells in the total population (adhering + detached cells) was evaluated immediately at the end of the incubation period by means of propidium iodide (PI) staining of DNA followed by flow cytometric analysis, as described previously (Ceruti et al., 2000, 2003).

Analysis of Mitochondrial Membrane Potential. Changes in mitochondrial membrane potential ($\Delta\Psi m$) induced in the total cell population by different pharmacological treatments were analyzed by means of the fluorescent dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1; Molecular Probes, Società Italiana Chimici, Rome, Italy), as described previously (Ceruti et al., 1997, 2003). J-Aggregate fluorescence was recorded by flow cytometry in the fluorescence channel 2 (FL2) and monomer fluorescence in the fluorescence channel 1 (FL1). Necrotic fragments were electronically gated out on the basis of morphological characteristics on the forward light scatter (FSC) versus the side light scatter dot plot.

Analysis of Phosphatidylserine Externalization and Membrane Integrity. Phosphatidylserine (PS) exposure on the outer leaflet of the plasma membrane was evaluated by staining ADF cells with Annexin V-FITC (apoptosis detection kit; Vinci-Biochem), following the manufacturer's instruction. Annexin V binding to PS was evaluated in the FL1. Cells were counterstained with an isotonic PI solution (20 μg/ml in phosphate-buffered saline) to evaluate cell membrane integrity. In fact, under these experimental conditions intact cell membranes exclude PI, which can only permeate cells undergoing necrotic cell death (Scholz et al., 2005). PI positivity was detected in the FL2. On the same experimental samples, cell swelling was evaluated by analyzing cell size distribution as a function of the FSC values.

Detection of Lactate Dehydrogenase Activity in Culture Supernatants. Lactate dehydrogenase (LDH) release into the cell culture supernatant occurs upon damage of the plasma membrane, and it is therefore considered a typical hallmark of necrosis. LDH activity was measured by means of a spectrophotometric assay kit (cytotoxicity detection kit-LDH; Roche, Milan, Italy), following the manufacturer's instructions. At the end of the incubation with the tested agents, culture supernatants from both control and drug-treated ADF cells were collected and centrifuged to discard floating cells. Determination of LDH activity was then carried out in a 96-well plate in a total volume of 200 μl (100 μl of supernatant in each sample and 100 μl of reaction mixture). After a 10- to 20-min incubation at 25°C, LDH enzyme activity was measured in a spectrophotometer at 490 nm. To assess maximal LDH activity, cells were exposed for 18 h to 1% Triton X-100 and processed as described above. To detect spontaneous LDH activity due to the presence of 10% FCS (blank), parallel samples were run with medium alone. Each condition was run in triplicate.

Detection of Caspase Activity

Caspase activity was measured by means of a spectrophotometric assay kit (CaspACE assay system colorimetric; Promega, Milan, Italy), following the manufacturer's instructions with some minor modifications, as described previously (Ceruti et al., 2003). The determination of caspase activity was carried out in a 96-well plate in a total volume of 100 μl, with a total amount of 70 μg of protein in each sample, and in the presence of tetrapeptide substrates conjugated to paranitroaniline (i.e., *N*-acetyl-Asp-Glu-Val-Asp-paranitroaniline, *N*-acetyl-Leu-Glu-His-Asp-paranitroaniline, and *N*-acetyl-Ile-Glu-Thr-Asp-paranitroaniline in caspase-3, -9, and -8, respectively, final concentration 200 μM). Extracts were incubated for 4 h at 37°C and released paranitroaniline was then measured in a spectrophotometer at 405 nm. Each condition was run in triplicate, and for each experimental condition at least three independent experiments were performed.

Western Blotting Analysis

Whole-cell lysates were prepared and analyzed by immunoblotting, as described previously (Ceruti et al., 2003). For caspase-3 detection, 50 μg of proteins in each lane was size fractionated by SDS-polyacrylamide gel electrophoresis in 11% acrylamide gel, whereas for gp-170 (Mdr-1), 100 μg of proteins was subjected to electrophoretic separation in 7.5% acrylamide gel. After electroblotting onto nitrocellulose membrane, an overnight incubation with primary antibodies was performed. In particular, mouse monoclonal

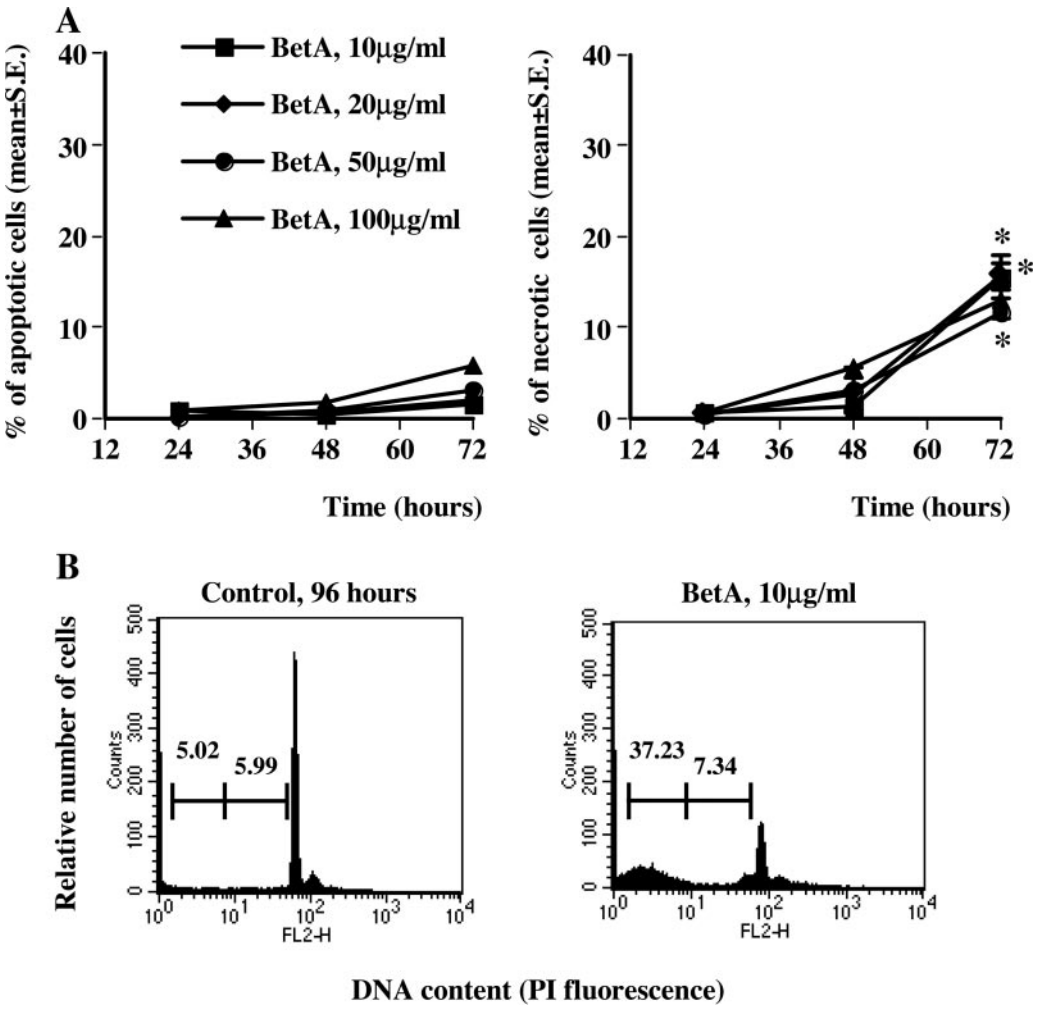

Fig. 1. Human astrocytoma ADF cells are resistant to BetA-induced apoptosis. A, ADF cells were exposed to 10 to 100 μg/ml BetA for 24 to 72 h, and induction of cell death (either apoptotic or necrotic) was evaluated by flow cytometric analysis of PI-stained nuclei. No significant induction of apoptosis was detected at any of the time points tested (also see B). $*$, $p < 0.05$ with respect to corresponding control, one-way ANOVA (Scheffé F-test). B, typical flow cytometry histograms obtained after a 96-h exposure to 10 μg/ml BetA and PI staining of nuclei. From left to right, markers represent necrotic and apoptotic cell death, respectively. Only an increase in the percentage of necrosis was detected, with no significant changes in the percentage of apoptotic cell death.

anti-caspase-3 antibody (1:750 in 3% nonfat dry milk in TBS; Alexis Biochemicals), followed by goat anti-mouse secondary antibody conjugated to horseradish peroxidase (1:2000 in 3% nonfat dry milk in TBS, 1 h at room temperature; Sigma Aldrich), was used to detect caspase-3 activation. A goat polyclonal anti-Mdr-1 antibody (1:100 in 5% nonfat dry milk in TBS; Santa Cruz Biotechnology, Inc., Santa Cruz, CA; kindly provided by Dr. Paola Perego, Istituto Nazionale dei Tumori, Milan, Italy), followed by monkey anti-goat secondary antibody conjugated to horseradish peroxidase (1:4000 in 5% nonfat dry milk in TBS, 1 h at room temperature; Sigma-Aldrich) was used to detect gp-170 expression. In this case, whole-cell lysates from LoVo/DX cells, known to express Mdr-1 (Soranzo et al., 1990) were processed in parallel as positive control. Afterward, to check for correct protein loading in the gel, the same filters were probed using a rabbit anti-actin polyclonal antibody (1:500 in 3% nonfat dry milk in TBS; Sigma-Aldrich) followed by a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:3000 in 3% nonfat dry milk in TBS, 1 h at room temperature; Sigma-Aldrich). Detection of proteins was performed by enhanced chemiluminescence (Amersham Biosciences, Inc., Milan, Italy) and autoradiography.

Total RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction Analysis

Total RNA was extracted as described previously (Fumagalli et al., 2004). For RT-PCR analysis, 1 μg of RNA was treated with RQ1 RNase-free DNase (Promega, Milan, Italy), and RNA was then reverse-transcribed with Superscript II RNA H^- reverse transcriptase (200 U/sample; Invitrogen, Milan, Italy). cDNAs were amplified in each PCR assay with Platinum *Taq*DNA polymerase (1.25 U/sample; Invitrogen) in a 25-μl reaction mixture containing 20 pmol of 5′ and 3′ primers in a standard PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, and 20 mM Tris-HCl, pH 8.4). Amplification were performed in a GeneAmp 9700 thermal cycler (Applied Biosystems, Milan, Italy) for 35 to 40 cycles (typically, 94°C/45 s, 45 s at the annealing temperature of 68°C, 56°C and 67°C for caspase-3, caspase-9, and caspase-8, respectively, followed by 45 s at 72°C) after an initial denaturation at 95°C for 2 min.

The following forward (Fw) and reverse (Rw) oligonucleotide primers were used: caspase-3, Fw 5′-TTCAGAGGGGATCGTTGTAGAAGTC-3′ and Rw 5′-CAAGCTTGTCGGCATACTGTTTCAG-3′; caspase-9, Fw 5′-ATGGACGAAGCGGATCGGCGGCTCC-3′ and Rw 5′-GCACCACTGGGGTAAGGTTTTCTAG-3′; caspase,-8 Fw 5′-CACTAGAAAGGAGGAGATGGAAAG-3′ and Rw 5′-CTATCCTGTTCTCTTGGAGAGTCC-3′; and β-actin, Fw 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ and Rw 5′-CTAGAAGCATTGCGGTGGACGATGGAGGG-3′. Ten microliter aliquots of the PCR products were finally size separated by electrophoresis in 1% agarose gels.

Cloning of Human Caspase-9α and Caspase-9β

Amplification of caspase-9α and caspase-9β mRNAs was performed with specific oligonucleotide primers (Fw 5′-CCATGGACGAAGCGGATC-3′ and Rw 5′-GCCCTGGCCTTATGATGTTTT-3′) for 35 cycles (initial denaturation at 95°C for 2 min, 95°C/60 s, 60 s at the annealing temperature of 58.8°C, followed by 70 s at 72°C). The PCR products were cloned into the pcDNA3.1 expression vector using the pcDNA3.1/V5-His TOPO TA expression kit (Invitrogen). Positive colonies were identified with a double enzymatic digestion, the first with HindIII and EcoRV (Roche) and the second with PvuII

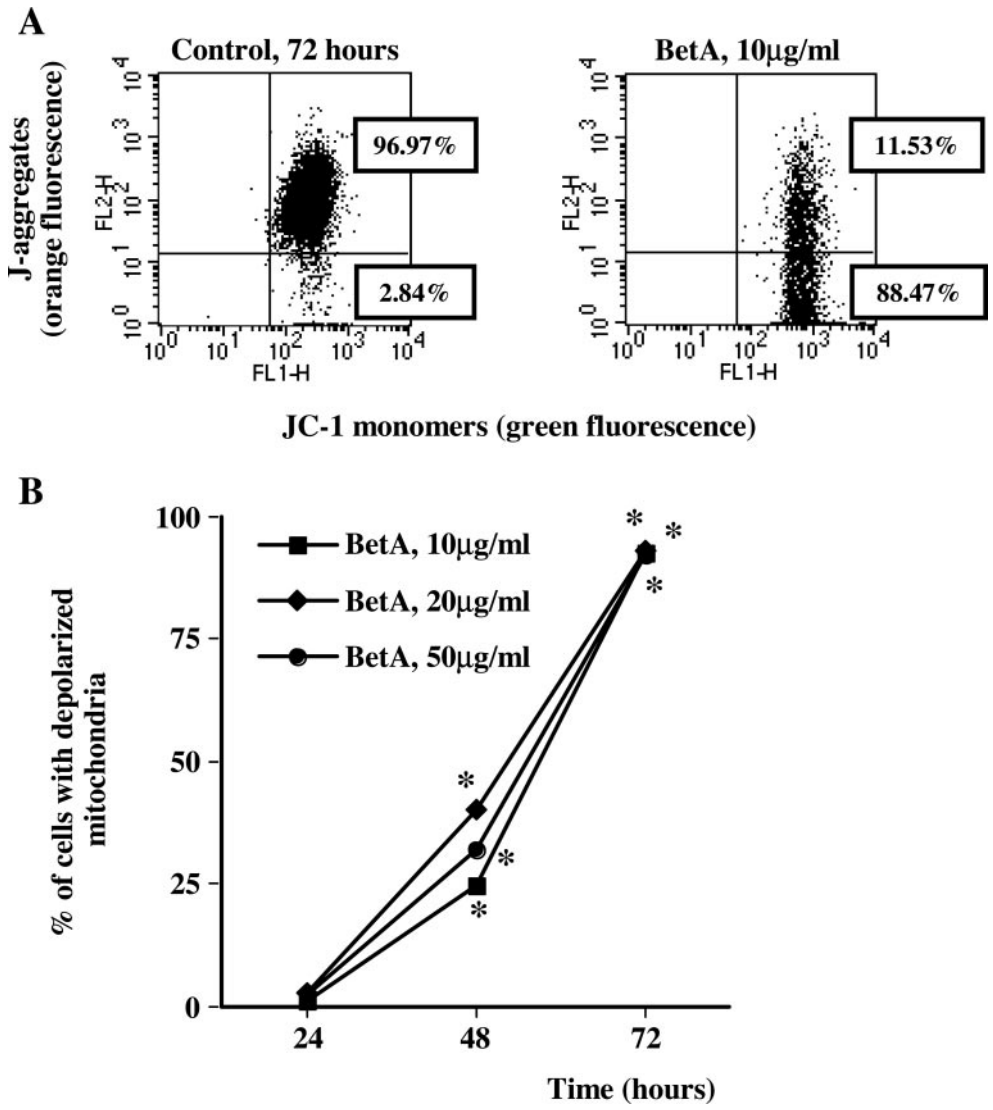

Fig. 2. BetA induces a marked time-dependent depolarization of mitochondria in ADF cells. A, BetA-induced drop in $\Delta\Psi m$, as evaluated by flow cytometric analysis of JC-1-stained cells. Healthy cells are found in the top right quadrant of the plots, corresponding to high emission of fluorescence in both FL1 (green; *x*-axis) and FL2 (orange; *y*-axis) channels. After a 72-h exposure to 10 μg/ml BetA, mitochondrial depolarization becomes evident as a dramatic drop in the FL2 and an increase in the FL1 emission. B, time-dependent depolarization of mitochondria induced by increasing BetA concentrations. Results represent the percentage of depolarized cells found in the bottom right quadrant of plots. $*$, $p < 0.05$ with respect to corresponding control, one-way ANOVA (Scheffé F-test).

(Promega). The construct was verified by sequencing using the Applied Biosystems Terminator cycle sequencing kit.

Statistical Analysis

All results are expressed as mean ± S.E.M. of three independent experiments. Statistical significance between groups was derived from one-way ANOVA followed by the Scheffé's F-test. A p value less than 0.05 was considered significant.

Results

Lack of Effect of Mitochondria-Damaging Agents on the Survival of Human Astrocytoma ADF Cells. BetA has been claimed to be a very promising drug in central nervous system tumors for its ability to activate the mitochondrial apoptotic pathway of cell death (Fulda et al., 1999; Fulda and Debatin, 2000). Hence, we exposed human astrocytoma ADF cells to increasing concentrations of the drug (10–100 μg/ml) for various times (24–72 h; Fig. 1A). No significant induction of apoptosis was detected at any of the times tested, with only a slight, although significant increase in the percentage of necrosis obtained after a 72-h incubation with the drug. Not even prolongation of the incubation time to a total of 96 h (Fig. 1B) was able to activate apoptotic cell death, but it did further increase the necrotic signs of toxicity.

Despite the lack of nuclear signs of apoptosis, BetA was able to induce a highly significant depolarization of mitochondria. This is clearly shown by graphs in Fig. 2A, which were obtained after staining both control and BetA-treated cultures with the mitochondria-specific dye JC-1. A high fluorescence emission of the dye in both the yellow (FL2; *y*-axis) and the green (FL1; *x*-axis) channels of the flow cytometer is representative of healthy and polarized mitochondria. Depolarization induces a significant drop in the yellow emission, accompanied by a contemporary increase of the green emission, due to the prevalence of the monomeric form of the dye with respect to J-aggregates. Depolarization is time-dependent, starting from a 48-h incubation time (Fig. 2B), and it can be achieved with any of the drug concentrations tested (10–50 μg/ml).

We next wondered whether the inability to activate the mitochondrial pathway of apoptosis represented a drug-related effect or whether it was a more general behavior of our cell line, mirroring some defects in the intrinsic pathway of death. Hence, we exposed ADF cells to dRib and KCN, two

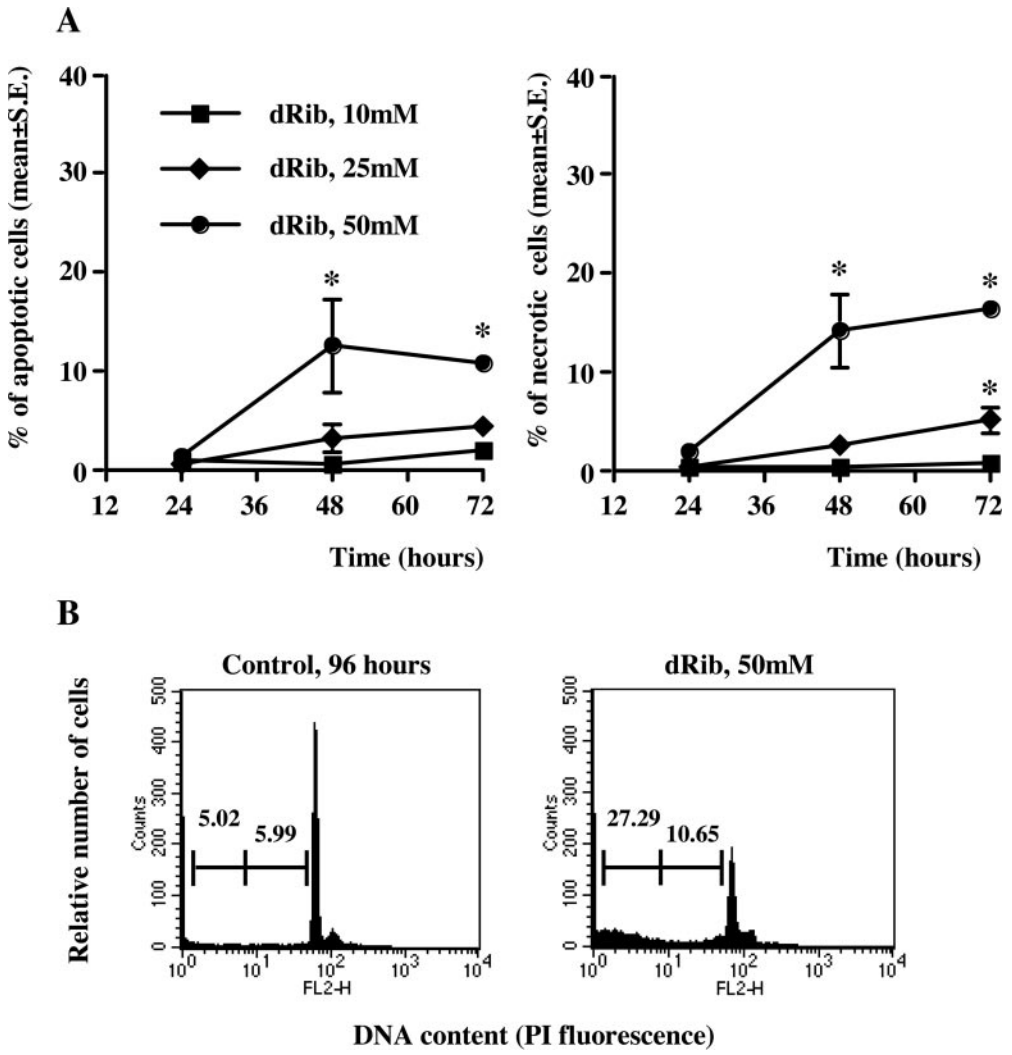

Fig. 3. Human astrocytoma ADF cells are resistant to dRib-induced apoptosis. A, ADF cells were exposed to 10 to 50 mM dRib for 24 to 72 h and induction of cell death (either apoptotic or necrotic) was evaluated by flow cytometric analysis of PI-stained nuclei. A very low, although statistically significant percentage of apoptosis was detected starting from a 48-h incubation with the highest concentration tested (50 mM; B). *, $p < 0.05$ with respect to corresponding control, one-way ANOVA (Scheffé F-test). B, typical flow cytometry histograms obtained after a 96-h exposure to 50 mM dRib and PI staining of nuclei. From left to right, markers represent necrotic and apoptotic cell death, respectively. As previously observed after BetA exposure (see Fig. 1), only an increase in the percentage of necrosis was detected. The percentage of apoptosis is not statistically different from control at this very late time, when control cells also started suffering spontaneously.

well known "mitochondriotropic" molecules, acting on different molecular targets inside the organelle (Kletsas et al., 1998; Shou et al., 2000). Also with dRib, a very low percentage of apoptotic and necrotic death was detected, despite the use of elevated concentrations of the drug (10–50 mM) and the long incubation periods (24–72 h; Fig. 3A). A 96-h incubation with the highest concentration tested was able to induce necrosis in only the 27% of the total cell population (Fig. 3B), despite concentration- and time-dependent depolarization of mitochondria (Fig. 4, A and B).

Similar results were also obtained by exposing cultures to the mitochondrial poison KCN (Fig. 5). Concentrations up to 400 μM were completely ineffective in inducing death of ADF cells (Fig. 5, A and B). A higher concentration (600 μM) was instead able to kill almost all of the cells, mainly by inducing necrosis, through massive mitochondria depolarization (Fig. 5C), accompanied by widespread cytotoxicity.

To consolidate evidence for induction of necrosis by BetA, dRib, and KCN on ADF cells, we have performed additional experiments by looking at other parameters typical of necrotic cell death. In all the following experiments, ADF cells were exposed to 600 μM KCN, 25 mM dRib, or 10 μg/ml BetA for either 48 or 72 h, as indicated in the legend to Fig. 6. A drastic increase in LDH release into cell supernatants, indicating membrane damage and necrotic cell death, was found upon exposure of ADF cells to KCN and upon incubation with either dRib or BetA (Fig. 6A, top). A trend to increase the release of LDH was also detected at earlier times (i.e., 24 and 48 h for KCN and dRib or BetA, respectively, data not shown). Release of LDH induced by an 18-h exposure to 1% Triton X-100 from the same cells was used here as a positive control (Fig. 6A, top). Moreover, as depicted in Fig. 6A (bottom), a flow-cytometric analysis of cell distribution according to their size (see FCS on the *x*-axis) showed that exposure of cells to any of the agents tested induced cell swelling, as shown by a significant shift toward higher cellular size values in comparison with control, vehicle-treated cells (black curve). Finally, induction of necrosis was also confirmed by flow-cytometric analysis of cells stained with an isotonic PI solution. Under these experimental conditions, PI is excluded by cells with intact membranes, and PI positivity can thus be taken as an index of necrosis. Increases of PI staining were found in cells exposed to KCN, dRib, or BetA (Fig. 6B). Interestingly, despite lack of significant nuclear signs of apoptosis and in parallel with the appearance of the above-mentioned signs of necrosis, we found that exposure of ADF cells to the tested agents resulted in phosphatidyl serine externalization as evaluated by Annexin V-FITC positivity by flow cytometry (Fig. 6C). Together, the above-mentioned data clearly demonstrate that, in the human astrocytoma

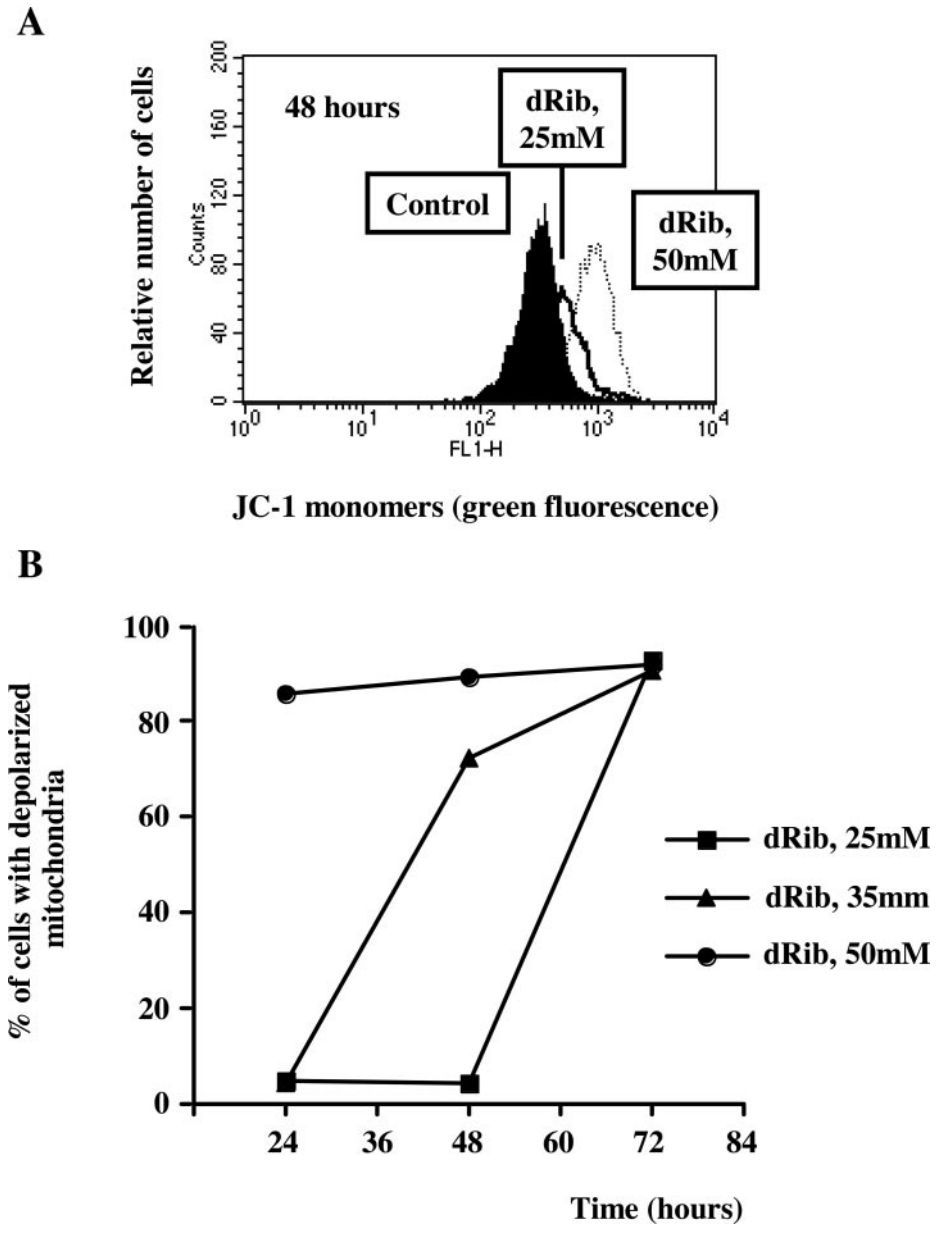

Fig. 4. dRib induces a concentration- and time-dependent reduction of mitochondrial membrane potential. A, after a 48-h incubation with 50 mM dRib, a significant increase of JC-1 monomers emission in FL1 was detected (dotted line). A nonsignificant trend to increase was also detected with a lower dRib concentration (25 mM; open histogram). B, time- and concentration-dependent increase of the percentage of cells with depolarized mitochondria (i.e., cells found in the bottom right quadrant of FL1/FL2 dot plot; see Fig. 3) after exposure to dRib. A typical experiment is shown.

ADF cells, mitochondriotropic agents primarily induce necrotic cell death.

No Activation of Caspases by BetA and dRib in Human Astrocytoma ADF Cells. Resistance to apoptotic stimuli may also arise from defects in the expression of caspases (Philchenkov et al., 2004; Reed, 2004). Thus, we decided to verify whether, in our experimental model, lack of apoptosis by BetA and dRib may arise from a defective expression and/or activation of the most important "initiator" caspases (i.e., caspase-8 and caspase-9 for the intrinsic and extrinsic pathways, respectively) and of "executioner" caspase-3 (Igney and Krammer, 2002). As a first step, we looked at the presence of caspase-8 in the human astrocytoma ADF cells. RT-PCR analysis with specific oligonucleotide primers for this caspase revealed the presence of an amplification product at the expected molecular weight of 411 bp (Fig. 7A). However, despite the presence of caspase-8 mRNA, neither BetA nor dRib used at the same concentrations that induced cell death (see above) were able to activate this caspase (Fig. 7B). To check for the liability of the colorimetric assay, a positive control was used in parallel (see legend to Fig. 7).

Since in our experimental model BetA, dRib, and KCN all induced significant mitochondrial depolarization (which, however, was not followed by induction of apoptosis; see above), we also investigated whether ADF cells have defects in caspase-9 or caspase-3. It is known that one of the rate-limiting steps of the mitochondrial intrinsic pathway of apoptosis is release of cytochrome *c* from depolarized mitochondria, which in turn activates initiator caspase-9 and, as a consequence, executioner caspase-3 (Earnshaw et al., 1999; Debatin, 2004). We have already shown a significant release of cytochrome *c* from the mitochondrial to the cytosolic cellular compartment after exposing ADF cells to BetA, dRib, and KCN (Ceruti et al., 2003), suggesting that no alterations in the mechanism(s) of protein release from depolarized mitochondria are detectable in our experimental model.

We thus checked the expression of caspase-9 and caspase-3

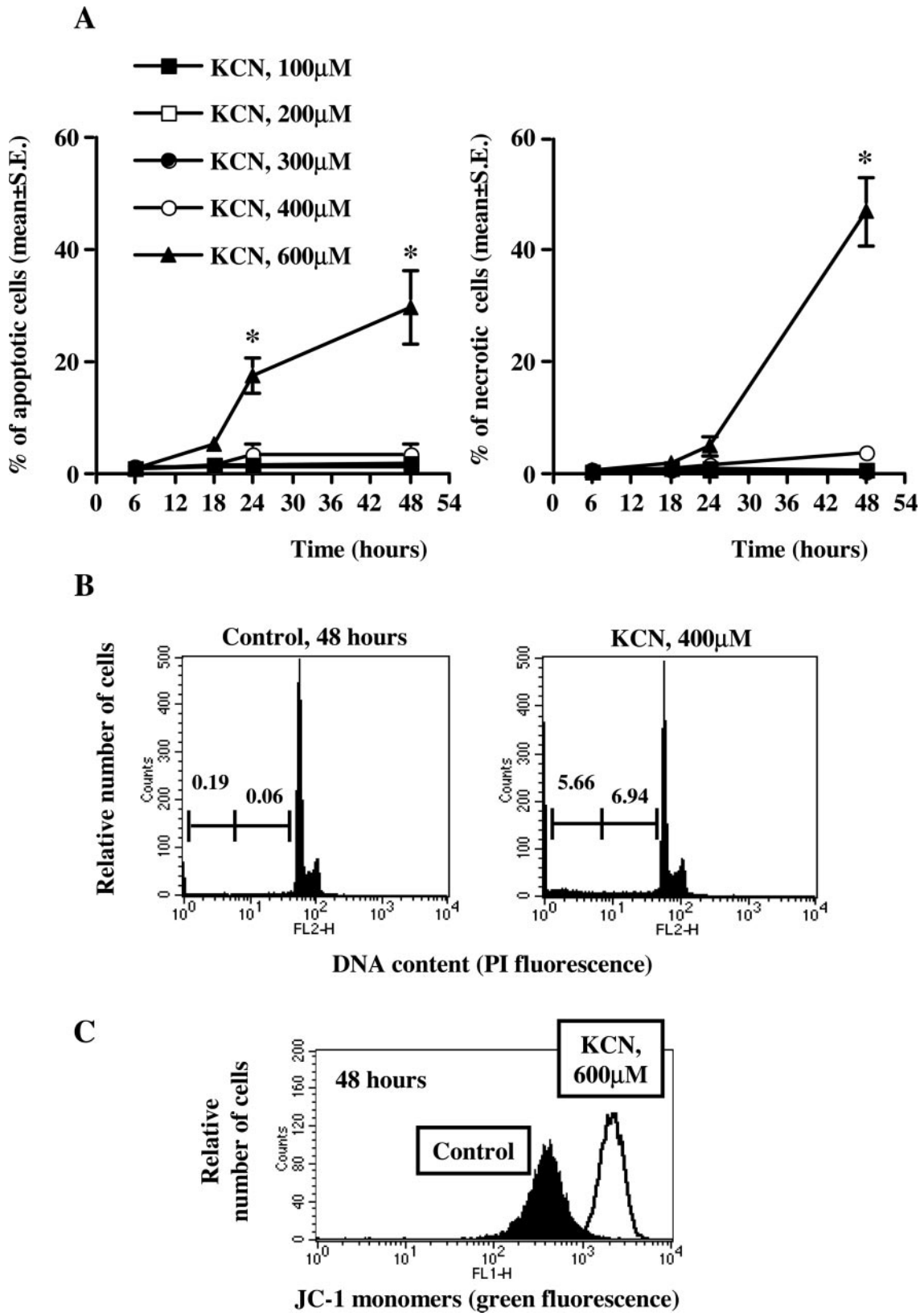

Fig. 5. Human astrocytoma ADF cells are relatively resistant to KCN-induced cell death. A, ADF cells were exposed to 100 to 600 μM KCN for 6 to 48 h, and induction of cell death (either apoptotic or necrotic) was evaluated by flow cytometric analysis of PI-stained nuclei. A significant induction of apoptosis and necrosis was detected only with the highest concentration tested, starting from a 24-h incubation. $*$, $p < 0.05$ with respect to corresponding control, one-way ANOVA (Scheffé F-test). A typical histogram showing DNA content by PI staining of nuclei is shown in B. As shown in A, no significant induction of either necrosis (left marker) or apoptosis (right marker) was detected after a 48-h incubation with 400 μM KCN. C, KCN induced a marked reduction of mitochondrial membrane potential. After a 48-h incubation with 600 μM KCN, a significant increase of JC-1 monomers emission in FL1 was detected (open histogram). A nonsignificant trend to increase was also detected with a lower concentration (400 μM; not shown).

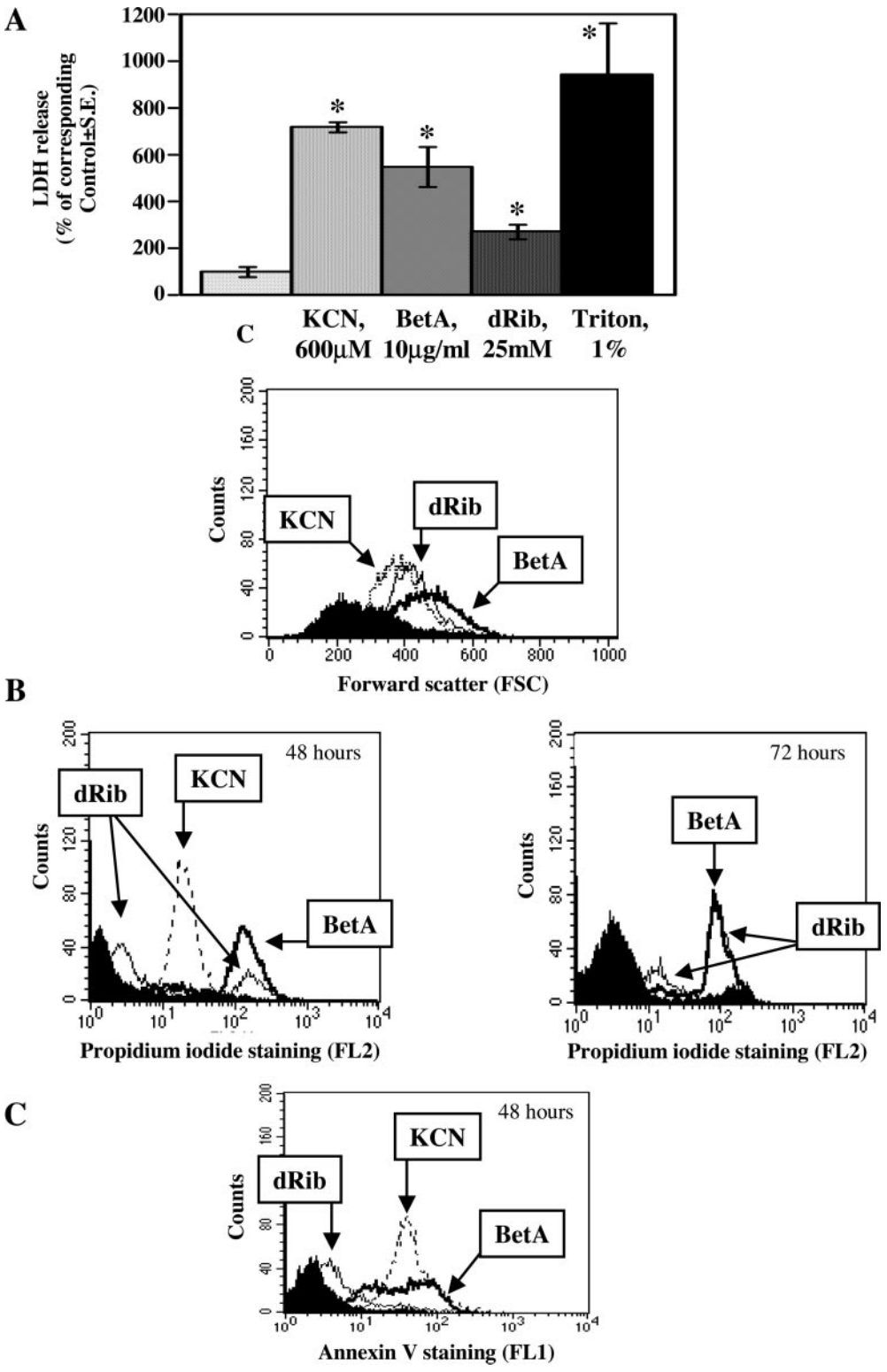

Fig. 6. Typical signs of necrosis are detected after exposure of ADF cells to KCN, BetA, or dRib. A, top, LDH release in culture supernatants after incubation of ADF cells with KCN (48 h) or either BetA or dRib (72 h) in comparison with vehicle-treated cells (C). An 18-h exposure to 1% Triton X-100 was used to check for maximal LDH release (positive control). Results are expressed as percentage of corresponding control ± S.E. *, $p < 0.05$ with respect to corresponding control, one-way ANOVA (Scheffé F-test). Bottom, flow cytometric evaluation of cell swelling. Histograms represent the cell size distribution (FSC) of control cells (black curve) and drug-treated cultures (see labels). A significant shift toward higher size values was observed with any of the tested drug, indicating cell swelling. B, plasma membrane integrity was evaluated by staining cells with an isotonic PI solution followed by flow cytometric analysis (see FL2 on the *x*-axis). The black histogram represents control cells. A significant staining (i.e., a shift toward higher fluorescence values), demonstrating rupture of plasma membrane due to induction of necrosis, was detected after exposure to any of the tested agents for 48 or 72 h. C, PS exposure at the outer leaflet of cell membrane was evaluated by Annexin V-FITC staining of cells followed by flow cytometric analysis (FL1) of control (black curve) or drug-treated cultures (see labels). A significant right shift toward higher fluorescence values indicating increased positivity to the staining was detected after exposure to both 10 μg/ml BetA and 600 μM KCN. A trend to increased fluorescence values was also detected upon incubation with 25 mM dRib.

by RT-PCR in our experimental model. Amplification of cDNA with specific oligonucleotide primers for these caspases revealed two products (Fig. 8A) at the expected molecular weights of 264 and 330 bp for caspase-3 and caspase-9, respectively. Actin was used as an internal control for the amplification reaction. Translation of RNAs to proteins was checked by Western blotting analysis (see lower part of Fig. 8C for caspase-3; data not shown for caspase-9).

Nevertheless, no activation of either caspase-9 (Fig. 8B) or caspase-3 (Fig. 8C) was detectable in ADF cells after exposure to both BetA and dRib at any of the times tested, in agreement with the lack of apoptotic cell death (Figs. 1 and 3). Also for these caspases, positive controls were run in parallel, leading to a statistically significant caspase-9 and -3 activation (see *Discussion*). For effector caspase-3, Western blotting analysis was also performed. Low molecular weight

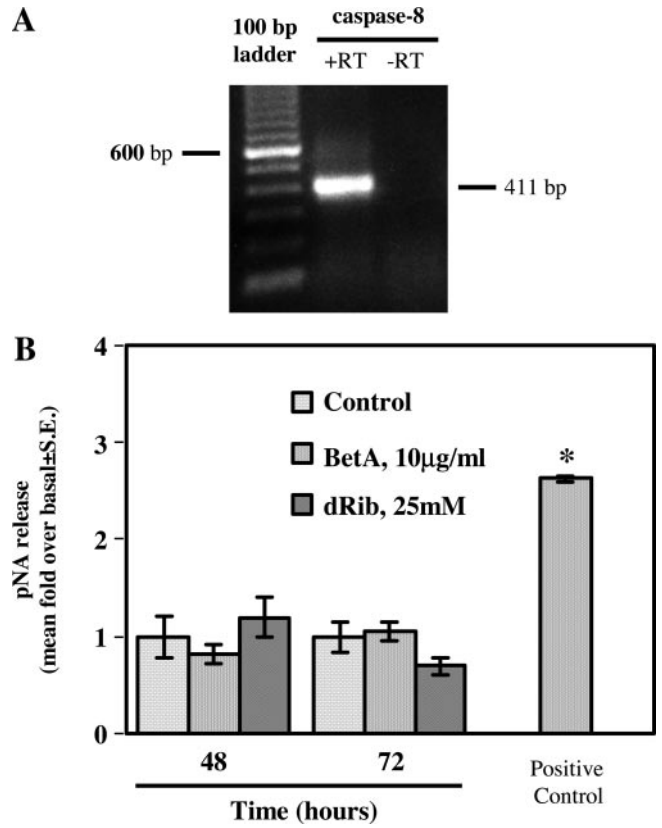

Fig. 7. No activation of caspase-8 is detected in ADF cells after exposure to various pharmacological agents. A, RT-PCR analysis with primers internal to the coding sequence of caspase-8 (see *Materials and Methods*). The negative control for the reaction is indicated as −RT. An amplification product of the expected molecular weight of 411 bp corresponding to caspase-8 was detected. Actin was amplified as internal control (not shown). B, caspase-8 activity was evaluated by a colorimetric assay after 48 to 72 h of exposure to either 10 μg/ml BetA or 25 mM dRib. No significant activation was recorded at any of the experimental condition tested. The reliability of the technique was evaluated by means of a positive control (i.e., ADF cells exposed to 100 μM etoposide in the presence of a proteasome inhibitor; S. Ceruti, A. Mazzola, and M. P. Abbracchio, manuscript in preparation). *, $p < 0.05$ with respect to corresponding control, one-way ANOVA (Scheffé F-test).

active fragments, which are known to be produced upon enzyme activation, were not detected after either dRib or BetA exposure, but only in the lane corresponding to the positive control (Fig. 8C). Lack of caspase activation was also observed in the presence of 100 to 600 μM KCN (data not shown).

Expression of a Caspase-9 Short Isoform in Human ADF Cells. Two splicing variants of caspase-9 have been described previously (Seol and Billiar, 1999; Srinivasula et al., 1999; Angelastro et al., 2001; Waltereit and Weller, 2002): one long isoform corresponding to the full-length protein (called caspase-9α) and one short variant (identified as caspase-9β) missing most of the large subunit (indicated with "B" in Fig. 9A) containing the catalytic site of the enzyme (see Fig. 9A for a schematic representation of the two variants). To check for the presence of these two splice variants, we designed oligonucleotide primers external to the caspase coding sequence (see *Materials and Methods*). Two RT-PCR products were identified in ADF cells (Fig. 9B) with the expected molecular weight of 1250 and 800 base pairs, corresponding to caspase-9α and -9β, respectively.

Furthermore, since mutations in caspase sequence may also account for the inability to activate the apoptotic program of death, we cloned and sequenced the two RT-PCR products. The nucleotide sequences of caspase-9α and -9β obtained from ADF cells are 99% identical to that reported for the human caspase-9 (GenBank accession nos. NM_001229 and NM_032996 for caspase-9α and -9β, respectively), except for a single C-to-T nucleotide substitution in position 83 of the coding sequence, which was found in each of the 11 clones analyzed. Figure 9C shows the deduced amino acid sequence for ADF caspase-9α and its alignment with the previously reported human caspase-9α isoform. The portion of the protein outside the boxes, containing the part that is missing in the short isoform, represents caspase-9β. The reported polymorphism leads to a single amino acid substitution at position 28 (A→V), which belongs to the prodomain portion of the protein (Allan et al., 2003). Therefore, we conclude that not only do ADF cells express the short isoform of caspase-9 but also they have a point mutation in the gene sequence encoding for the enzyme that may contribute to their resistance to various apoptotic stimuli (see *Discussion*).

Finally, to rule out the possibility that resistance to induction of apoptosis may be due to expression, in ADF cells, of the glycoprotein p-170 (gp-170 or Mdr-1) known to act as a pump extruding drugs out of cells (thus reducing their intracellular concentrations and efficacy as chemotherapeutic agents; Moran et al., 1997), we also looked at the presence of this protein in the human astrocytoma cells used here. Western blot analysis with an antibody that specifically recognized the gp-170 protein did not reveal any immunoreactive band in lysates from ADF cells, whereas a specific band with the expected molecular weight of 170 was detected in lysates from LoVo/DX cells (Fig. 10), which have been reported to express the multidrug resistance protein to significant levels (Soranzo et al., 1990).

Discussion

Activation of the mitochondria/caspase-9-dependent pathway of apoptosis by anticancer drugs often represents the key step to a successful therapy. Unfortunately, in cancer cells this is also one of the pathways showing the highest degree of alterations (Igney and Krammer, 2002), which can vary greatly depending upon the histotype, and the degree of malignity. Thus, important needs in the pharmacological approach to cancer are represented by 1) understanding whether apoptotic pathways can be successfully recruited by chemotherapeutic agents in specific tumors, 2) determining whether alterations of the "classical" cell death pathways are present, and 3) assessing the possibility to activate alternative and effective ways of killing cancer cells.

Our data clearly show that human ADF astrocytoma cells are resistant to agents known to interfere with cell survival by altering mitochondrial functions. Among the agents tested here, BetA is a natural product derived from the bark of the white birch tree (Eiznhamer and Xu, 2004) and represents a promising new drug for the treatment of various neoplasms. BetA has been demonstrated to potently inhibit the growth of neuroectodermal tumors, such as neuroblastoma, medulloblastoma, and Ewing sarcoma cell lines (Fulda and Debatin, 2000) as well as of several human carcinoma (Zuco et al.,

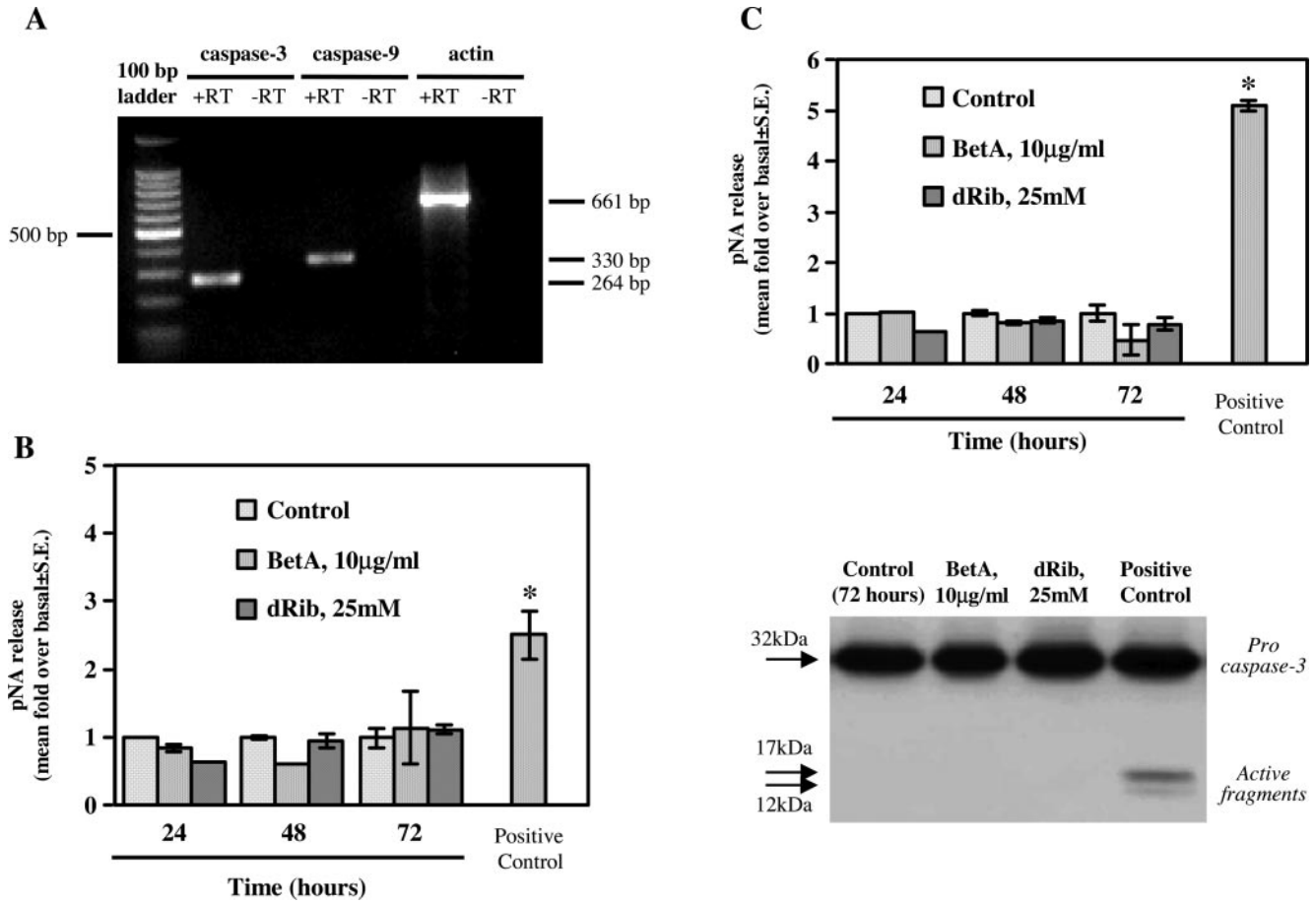

Fig. 8. No activation of either caspase-9 or caspase-3 is detected in ADF cells after exposure to various pharmacological agents. A, RT-PCR analysis with primers internal to the coding sequences of caspase-3 and caspase-9 (see *Materials and Methods*). Actin was amplified as internal control, and −RT lanes represent negative controls for each reaction. Two RT-PCR products were detected at the expected molecular weight of 264 and 330 bp for caspase-3 and caspase-9, respectively. Caspase-9 (B) and caspase-3 (C, top) activity was evaluated by a colorimetric assay after 24 to 72 h of exposure to either 10 μg/ml BetA or 25 mM dRib. No significant activation was recorded at any of the experimental condition tested; the reliability of the technique has been evaluated by means of a positive control (i.e., ADF cells exposed to 100 μM etoposide + proteasome inhibitor; S. Ceruti , A. Mazzola, and M. P. Abbracchio, manuscript in preparation). *, $p < 0.05$ with respect to corresponding control, one-way ANOVA (Scheffé F-test). For caspase-3, the lack of activation was also confirmed by Western blotting analysis (C, bottom). The appearance of the active fragments of the enzyme can only be detected in the positive control lane.

2002). Although its intracellular molecular target has never been described, it is generally claimed that, in neuroectodermal tumors, BetA directly affects mitochondria (Fulda and Debatin, 2000), causing a drop in mitochondrial membrane potential, cytochrome *c* release, and caspase-9 activation (Fulda et al., 1998; Costantini et al., 2000), leading to the typical signs of apoptotic cell death. For glial tumors, conflicting results have been published. In fact, although this drug was originally claimed to also induce apoptosis in glioblastoma cell lines (Fulda and Debatin, 2000), very high concentrations of BetA (as high as 50 μg/ml) were needed to detect signs of apoptosis in quite a few malignant glioma cell lines (Wick et al., 1999), and only the concomitant exposure to BetA and TRAIL was effective in inducing death (Fulda et al., 2004). The present results represent the first in-depth evaluation of BetA-induced effects on a human astrocytoma cell line and might have important clinical implications.

dRib has never been used as an anticancer agent, but it is an extensively used in vitro tool to induce mitochondria-dependent cell death in experimental systems, through glutathione depletion and consequent induction of oxidative stress (Ceruti et al., 1997; Kletsas et al., 1998; Ikeda et al., 2002). On the other hand, KCN represents a well known mitochondrial poison that uncouples the respiratory chain from ATP production, leading to a dramatic energetic defect and cell death, mainly necrotic (Li et al., 2002; Prabhakaran et al., 2004; Fig. 5). Despite very different types of mitochondrial interference, these three agents induced very similar effects on human astrocytoma cells.

In our experimental model, mitochondria-damaging agents are still able to activate the very first steps of the intrinsic pathway of apoptosis (i.e., disruption of mitochondrial membrane potential and release of cytochrome *c* in the cytoplasm; see Figs. 2, 4, and 5 and Ceruti et al., 2003). Nevertheless, despite the appearance of these dramatic biochemical events, caspase-9 activation is defective (Fig. 8). Thus, the prolonged energetic imbalance induced by these agents leads, in turn, to necrotic death (although of modest entity and after very long exposure periods; Figs. 1, 3, and 5). The nature of cell death upon exposure to these agents has been also confirmed by other parameters of necrosis, including LDH release, cellular swelling, and flow cytometry positivity to isotonic PI (Fig. 6, A and B). Interestingly, we also found that, despite lack of significant apoptosis, drug-treated cells show exter-

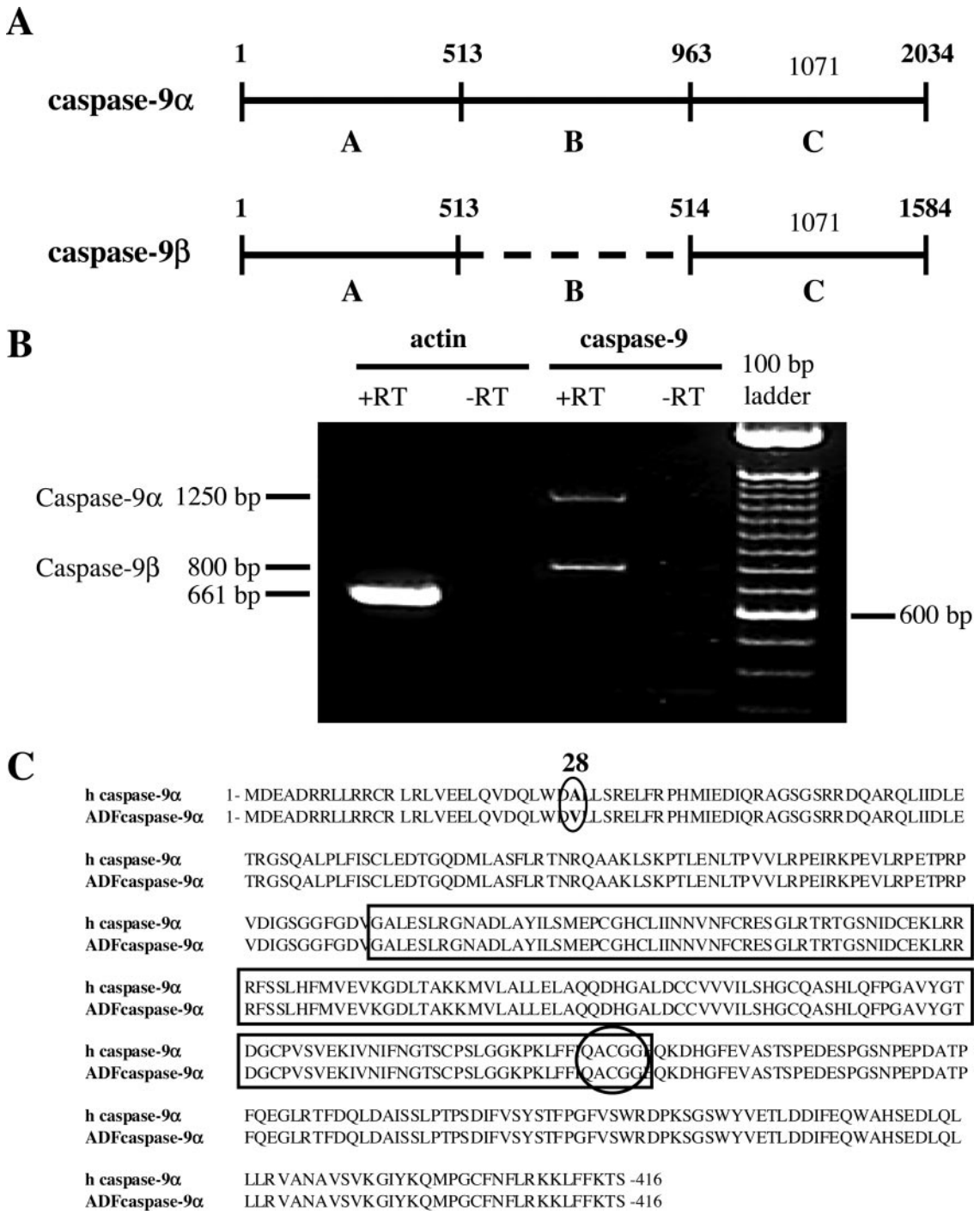

Fig. 9. Human astrocytoma ADF cells express two isoforms of caspase-9. A, schematic representation of the coding sequences of the two caspase-9 isoforms that can be generated by alternative splicing of the caspase-9 gene (Seol and Billiar, 1999; Srinivasula et al., 1999; Angelastro et al., 2001; Waltereit and Weller, 2002). Caspase-9β is missing a 450-bp sequence (fragment B) encoding for part of the large subunit and containing the active QACGG catalytic site (see circle in C). B, primers external to the coding sequence of caspase-9α revealed the presence of two RT-PCR products at the expected molecular weight of 1250 and 800 bp and corresponding to caspase-9α and β, respectively. C, predicted amino acid sequence obtained from cloning of the two caspase-9 isoforms from human astrocytoma ADF cells, and alignment with the corresponding human sequences (GenBank accession nos. NM_001229 and NM_032996 for caspase-9α and -9β, respectively). Boxes indicate the portion of the protein that is missing in the caspase-9β short isoform. A point mutation in the 28 position (A$\rightarrow$V) was detected in every caspase-9α- and -9β-tested clone.

nalization of PS, which is generally taken as a marker of apoptotic death. This confirms recent data from other authors showing that early exposure of PS at the outer face of plasma membrane is not unequivocally associated to induction of apoptosis but also occurs during oncosis (early primary necrosis; Krysko et al., 2004), probably depending upon the loss of aminophospholipid translocase activity, due to the rapid decrease in ATP levels. Moreover, the pathway of death we observed in our experimental model is remarkably similar to what recently reported for arsenic trioxide in several tumoral cell lines. Also in this case, cells underwent potent mitochondrial depolarization, and a regulated form of mito-

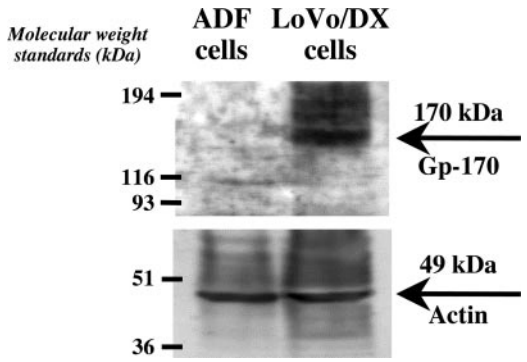

Fig. 10. ADF cells do not express the multidrug resistance glycoprotein gp-170 (Mdr-1). The presence of gp-170 was evaluated by Western blotting analysis with a specific antibody (see *Materials and Methods*). No immunoreactivity was detected in the lane corresponding to ADF cells, whereas a specific band at the expected 170-kDa molecular mass was detected in LoVo/DX cells, used here as positive control (Soranzo et al., 1990). Actin immunoreactivity was performed on the lower part of the nitrocellulose filter to check for correct loading of proteins in the gel.

chondrial-dependent necrotic cell death, with no involvement of caspases (Scholz et al., 2005).

Insensitivity of ADF cells to the agents tested here is not due to a defect in the expression of caspase-9, since both the long and the short forms of this caspase were detected. Even though we cannot rule out that other genetic and/or biochemical alterations may account for ADF cells resistance to chemotherapy (e.g., loss of the apoptotic protease activating factor-1 protein, subcellular distribution of caspases, or overexpression of antiapoptotic proteins), expression of the short caspase-9 isoform (caspase-9β) in these cells might contribute to their insensitivity to chemotherapy. In fact, it has been demonstrated that this form of caspase-9 can behave as a dominant negative of the caspase-9 long isoform (caspase-9α) and inhibit its activation in response to various proapoptotic stimuli (Srinivasula et al., 1999). Both neuronal and non-neuronal cells transfected with caspase-9β were shown to be resistant to death evoked by trophic factor deprivation or DNA damage (Angelastro et al., 2001). Overexpression of caspase-9β inhibited tumor necrosis factor-α- and Bax-mediated apoptosis in MCF-7 carcinoma cells (Seol and Billiar, 1999). Moreover, it has been already demonstrated that other astrocytoma cell lines express very high level of both α and β caspase-9 isoforms with respect to normal astrocytes (Waltereit and Weller, 2002). In these cells, overexpression of both isoforms by exogenous transfection did not modulate their sensitivity to the apoptosis induced by either CD95L plus cycloheximide or teniposide. Nevertheless, it should be emphasized that these apoptotic stimuli do not directly target mitochondria; thus, despite the overexpression of the pro- and antiapoptotic caspase-9 isoforms, the final degree of apoptosis could likely be maintained unchanged by recruitment of other pathways (e.g., the caspase-8- and/or the caspase-2-dependent pathways). This is not the case of the agents used in the present study, which directly act on mitochondria and whose apoptotic activity can be more heavily influenced by alterations of the intrinsic death pathway. Thus, our results seem to confirm the low sensitivity of tumor of glial origin to BetA, suggesting that expression of caspase-9β may represent one of the reasons for this resistance, and may also be used to predict the success of a given chemotherapy. Indeed, a very interesting study have been recently published showing that 13 of 21 glioblastoma cell lines established directly from patients were sensitive to BetA-induced cell death (Jeremias et al., 2004). Although this represents a very high percentage of success (around 62%), the reason for the remaining cell lines to be insensitive to this pharmacological treatment is currently unknown.

We also detected an A-to-V mutation at position 28 of the caspase-9 protein expressed by ADF cells, which may as well influence caspase-9 activity. This polymorphism has already been reported (GenBank single nucleotide polymorphism ID rs1052571), but no information regarding the possible biological significance of this mutation is currently available. The mutated amino acid is localized in the region of the protein corresponding to the enzyme prodomain (Allan et al., 2003), which is responsible for the interactions with apoptotic protease activating factor-1 in the apoptosome complex (Shiozaki et al., 2002). It is tempting to speculate that the introduction of a more lipophilic and ramified amino acid, such as valin, in this region of the protein may somehow modify the ternary structure of the protein, leading to a defective apoptosome formation.

In previous studies, we have been able to activate the apoptotic program, and therefore induce cell death, in ADF astrocytoma cells only by using agents able to bypass the caspase-9-dependent pathway of death. In fact, we have demonstrated that 2CdA is a very effective apoptotic stimulus for this cell line only due to the fact that it is able to recruit a caspase-2-dependent/mitochondria-independent pathway of death (Ceruti et al., 2003). Indeed, ADF cells show resistance to another typical chemotherapeutic agent, etoposide, and this resistance can be overcome by the concomitant exposure to pharmacological agents that can promote the activation of the caspase-2-dependent pathway of apoptosis (S. Ceruti, A. Mazzola, and M. P. Abbracchio, manuscript in preparation). In both pharmacological paradigms, caspase-9 activation can be achieved at very late time points (see positive control in Fig. 8; Ceruti et al., 2003), only as a consequence of the direct intervention of the other caspases that can proteolytically cleave and activate the enzyme, as a positive feedback loop aimed at amplifying the death process.

Thus, since induction of apoptosis represents a common event for different classes of anticancer agents converging into common downstream mechanisms, these observations confirm that disruption and/or alteration of only one of such mechanisms can led to broad drug resistance. For this reason, the setting of therapeutic strategies aimed at overcoming resistance and at recruiting the pathways that are still active in a given tumor represents one of the most important goals in cancer chemotherapy.

Acknowledgments

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