A Key Role for Caspase-2 and Caspase-3 in the Apoptosis Induced by 2-Chloro-2′-deoxy-adenosine (Cladribine) and 2-Chloro-adenosine in Human Astrocytoma Cells

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ABSTRACT

Both the anticancer agent 2-chloro-2′-deoxy-adenosine (Cladribine) and its derivative 2-chloro-adenosine induce apoptosis of human astrocytoma cells (*J Neurosci Res* **60:**388–400, 2000). In this study, we have analyzed the involvement of caspases in these effects. Both compounds produced a gradual and time-dependent activation of "effector" caspase-3, which preceded the appearance of the nuclear signs of apoptosis, suggesting a temporal correlation between these two events. Moreover, the caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone (fmk) suppressed both caspase-3 activation and apoptosis induction. "Initiator" caspase-9 and caspase-8 were only marginally activated at later times in the apoptotic process. Accordingly, at concentrations that selectively inhibit these caspases, neither *N*-benzyloxycarbonyl-Leu-Glu-His-Asp-fmk nor *N*-benzyloxycarbonyl-Ile-Glu-Thr-Asp-fmk could prevent adenosine analog-induced cell death. To definitively rule out a role for the caspase-9/cytochrome *c*-dependent mitochondrial pathway of cell death, neither adenosine analog had any effect on mitochondrial membrane potential, which was instead markedly reduced by other apoptotic stimuli (e.g., deoxyribose, NaCN, and betulinic acid). Consistently, although the latter triggered translocation of mitochondrial cytochrome *c* to the cytoplasm, no cytosolic accumulation of cytochrome *c* was detected with adenosine analogs. Conversely, 1 to 7 h after addition of either adenosine analog (i.e., before the appearance of caspase-3 activation), caspase-2 activity was surprisingly and markedly increased. The selective caspase-2 inhibitor *N*-benzyloxy carbonyl-Val-Asp-Val-Ala-Asp-fmk significantly reduced both adenosine analogs-induced caspase-2 activation and the associated cell death. We conclude that adenosine analogs induce the apoptosis of human astrocytoma cells by activating an atypical apoptotic cascade involving caspase-2 as an initiator caspase, and effector caspase-3. Therefore, these compounds could be effectively used in the pharmacological manipulation of tumors characterized by resistance to cell death via either the mitochondrial or caspase-8/death receptor pathways.

Apoptosis is a cell death process characterized by cell shrinkage, chromatin condensation, membrane blebbing, and fragmentation of the cell into "apoptotic bodies", which are recognized and eliminated by phagocytes (Allen et al., 1997).

Multicellular organisms eliminate abnormal, damaged or unwanted cells through apoptosis. Besides genetic mutations promoting unrestrained cellular proliferation, the transformation of a normal to a neoplastic cell (and then to more malignant phenotypes) is linked to the acquisition of genetic

ABBREVIATIONS: 2-CdA, 2-chloro-2′-deoxy-adenosine; ΔΨm, mitochondrial transmembrane potential; 2-CA, 2-chloro-adenosine; ANOVA, analysis of variance; BetA, betulinic acid; DEVD-pNA, *N*-acetyl-Asp-Glu-Val-Asp-paranitroaniline; dRib, 2-deoxyribose; Eto, etoposide; FBS, fetal bovine serum; IETD-pNA, *N*-acetyl-Ile-Glu-Thr-Asp-paranitroaniline; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol-carbocyanine iodide; LEHD-pNA, *N*-acetyl-Leu-Glu-His-Asp-paranitroaniline; NaCN, sodium cyanide; PI, propidium iodide; TBS, Tris-buffered saline; zDEVD-fmk, *N*-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone; zIETD-fmk, *N*-benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone; zLEHD-fmk, *N*-benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone; zVAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone; zVDVAD-fmk, *N*-benzyloxy carbonyl-Val-Asp-Val-Ala-Asp-fluoromethylketone; zVDVAD-pNA, *N*-benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-paranitroaniline.

resistance to induction of apoptosis (Kleihues et al., 1995). For this reason and because of their high invasiveness, these tumors are often characterized by a poor prognosis and by unsatisfactory response to pharmacological agents.

Several anticancer drugs are therapeutically effective in that they trigger the apoptotic death of malignant cells via activation of different (in many cases yet-to-be identified) mechanisms (Kaufmann and Earnshaw, 2000). Thus, the use of apoptosis-inducing molecules and the evaluation of their mechanism(s) of action in appropriate in vitro models might not only disclose new possible strategies for the pharmacological manipulation of cancer but may also help understanding the intracellular pathways of apoptosis that are still active in cancerous cells and could therefore be exploited in anticancer therapy.

Purine-based molecules inducing apoptosis have been used for years as anticancer agents (Plunkett and Saunders, 1991). Among these, the 2-chloro derivative of deoxy-adenosine (2-CdA; Cladribine) has been employed for the pharmacological control of slow growing leukemias and lymphomas (Bryson and Sorkin, 1993) and has become the drug of choice in hairy cell leukemia (Lauria et al., 1997), even though its mechanism of action remains unclear. It is worth noting that Cladribine has been extensively evaluated in blood tumors but only very limited information is available regarding its possible use in nonhematological tumors. In this respect, a single study in a human carcinoma cell line (HepG2 cells) has been reported (Graziadei et al., 1998).

All apoptotic pathways converge on a family of cysteine-aspartases, named caspases (Earnshaw et al., 1999), whose activity drives the biochemical events leading to cellular disassembly and death. Caspases are present as inactive precursors. "Initiator" caspases are thought to autoactivate themselves proteolytically (see also below), whereas "effector" caspases are activated by initiator caspases and are responsible for execution of cell death (Earnshaw et al., 1999). Two main pathways leading to the activation of initiator caspases have been recognized: the "mitochondrial" and the "death-receptor" pathways of cell death, involving caspase-9 and caspase-8, respectively (Scaffidi et al., 1998; Slee et al., 1999). In several different experimental models, the main effector caspase activated by caspase-9 and caspase-8 is caspase-3 (Earnshaw et al., 1999). The role of other caspases is still a matter of debate. For instance, caspase 2, known as a downstream "effector" caspase (Paroni et al., 2001) has recently been hypothesized to also play an upstream role in some cellular model systems (Robertson et al., 2002; Caballero-Benitez and Moran, 2003; Mueller et al., 2003).

The apoptotic effects of 2-CdA have been correlated to activation of distinct caspase cascades. For instance, in MOLT-4 leukemia cells, 2-CdA activates the death receptor/caspase-8 pathway through activation of the Fas/Fas-L system (Nomura et al., 2000). Conversely, in cell-free extracts, 2-chloro-deoxyATP (the product of the intracellular phosphorylation/activation of 2-CdA) can activate caspase-9 (and subsequently caspase-3) by participating to the formation of the so-called "apoptosome" (Leoni et al., 1998; Genini et al., 2000a). Moreover, in chronic lymphocytic leukemia cells, nucleotide derivatives can damage mitochondria, leading to a drop of mitochondrial membrane potential and release of

cytochrome c and apoptosis inducing factor (Genini et al., 2000b).

We have previously demonstrated that, upon their intracellular phosphorylation/activation by two distinct kinases, 2-CdA and its chemically-related adenosine analog 2-chloro adenosine (2-CA) can trigger apoptosis of human astrocytoma cells (ADF cells; Ceruti et al., 2000). Because of the poor prognosis and unsatisfactorily pharmacological response that often characterize this central nervous system tumor (see above), the identification of new proapoptotic agents and the characterization of their mechanisms of cell death are likely to disclose new pharmacological tools of potential therapeutic importance. Moreover, adenosine analogs can be used as experimental tools to dissect the apoptotic pathways that are still expressed in cancerous astrocytoma cells. On this basis, the present study has been aimed at analyzing the caspase cascade activated by 2-CdA and 2-CA in the same experimental model.

Results show that, in these cells, induction of apoptosis by adenosine analogs occurs through activation of an atypical apoptotic cascade involving caspase-2 as an "initiator" caspase and "effector" caspase-3, with no concomitant involvement of either mitochondrial depolarization, release of cytochrome c, or activation of caspase-9 or caspase-8.

Materials and Methods

Cell Cultures

Human ADF astrocytoma cells were kept in culture in standard conditions (37°C, 95% humidity, 5% CO_2) in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 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., 2000). Cells were seeded on Petri dishes according to the experimental protocol (see below) and treated with drugs 24 h after plating to allow them to attach to culture dishes.

Human promonocytic leukemia U937 cells were grown in RPMI 1640 + HEPES (Sigma, Italy) supplemented with 10% FBS, 2 mM glutamine, 100 u/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded on Petri dishes according to the experimental protocol and pharmacological treatments were performed immediately.

Isolation and Activation of Peripheral Blood Lymphocytes. Human peripheral blood lymphocytes (PBL) from healthy donors were isolated from fresh heparinized blood through a Ficoll-Hypaque density gradient centrifugation and grown in standard conditions. In selected experiments, purified T cells were activated for 72 h with phytohemagglutinin (2 μg/ml; Roche, Milan, Italy) and interleukin-2 (60 IU/ml; Invitrogen, Milan, Italy).

Pharmacological Treatments

2-Chloro-2′-deoxy-adenosine (2-CdA; Sigma, Italy), Etoposide (Eto; Sigma, Milan, Italy) betulinic acid (BetA; Vinci-Biochem, Vinci, Italy) and the caspase inhibitors N-benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk; Vinci-Biochem), N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (zDEVD-fmk), N-benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone (zLEHD-fmk), N-benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone (zIETD-fmk), and N-benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethylketone (zVDVAD-fmk) [all from Biomol (Trimital, Italy)] were dissolved in dimethyl sulfoxide (10^{-2}M) and stored at −20°C as stock solutions. 2-Chloro-adenosine (2-CA) and 2-deoxyribose (dRib; both from Sigma) were stored powdered and dissolved immediately before experiments. Solutions of all drugs (10×) were prepared in sterile Hanks' balanced salt solution (Celbio, Milan, Italy). Sodium cyanide (NaCN) stock solution (38 mM) has been purchased from Fluka (Buchs, Switzerland) and

Sigma. In selected experiments, zVAD-fmk was added to cultures 7 h after adenosine analogs (see *Results*); all analyses were performed immediately at the end of the incubation period (1–24 h).

CD95 Triggering. To activate the CD95/Fas pathway, 500 ng/ml of an anti-human Fas IgM monoclonal antibody [anti-CD95/Fas-triggering monoclonal antibody (α-Fas t, clone CH11; Upstate Biotechnology, Lake Placid, NY)] was either added to resting or activated human lymphocytes (see below) or to ADF cells for 48 h.

Western Blotting Analysis

Evaluation of Caspase Activation. Whole-cell lysates were prepared and analyzed by immunoblotting, as described previously (Abbracchio et al., 1997). Sixty micrograms of proteins in each lane were size-fractionated by SDS-polyacrylamide gel electrophoresis in 9 or 15% acrylamide gel for caspase-9 and caspase-3, respectively, followed by electroblotting onto nitrocellulose membrane. Membranes were then overnight-probed with mouse monoclonal anti-caspase-3 antibody (1:750 in 3% nonfat dry milk in TBS; Vinci-Biochem) or with rabbit anti-caspase-9 polyclonal antiserum (1:50 in 3% nonfat dry milk in TBS; Vinci-Biochem), followed by corresponding species-specific IgG antibodies conjugated to horseradish peroxidase (1:2000 for anti-mouse and 1:3500 for anti-rabbit antibodies, respectively; 1 h at room temperature, in 3% nonfat dry milk in TBS; Sigma). Detection of proteins was then performed by enhanced chemiluminescence (Amersham Biosciences, Milan, Italy) and autoradiography.

Evaluation of Cytochrome *c* Release from Mitochondria. At the end of the incubation period with the various pharmacological agents, culture supernatants were collected from petri dishes and adhering cells were detached using phosphate-buffered saline + 0.04% EDTA. The total cell suspension was centrifuged, washed in ice-cold phosphate-buffered saline, and the cytosolic and mitochondrial fractions were separated by means of the cytochrome *c* releasing apoptosis assay kit (BioVision; Vinci-Biochem), following the manufacturer's instructions. Briefly, cells were incubated on ice in cytosol extraction buffer mix (as supplied with the kit) containing 1 mM 1,4-dithio-DL-threitol and protease inhibitor cocktail, homogenized in an ice-cold tissue grinder (40 strokes in ice), and centrifuged at 700g for 10 min at 4°C. Pellets were discarded and supernatants were then centrifuged at 10,000g for 30 min at 4°C to obtain the cytosolic fraction (supernatants). Finally, pellets were resuspended in mitochondrial extraction buffer mix (as supplied with the kit) containing 1 mM 1,4-dithio-DL-threitol and protease inhibitor cocktail, vortexed for 10 s, and saved as mitochondrial fraction.

Protein content in each fraction was determined according to the method of Bradford (1976) and protein samples for SDS-polyacrylamide gel electrophoresis were prepared as described above. Twenty micrograms of proteins in each lane were size-fractionated in 15% acrylamide gel followed by electroblotting onto nitrocellulose membrane. Membranes were then probed overnight with a monoclonal mouse anti-cytochrome *c* antibody (supplied with the kit; 1:200 in 3% nonfat dry milk in TBS), followed by IgG anti-mouse antibody conjugated to horseradish peroxidase (Sigma; 1:2000), enhanced chemiluminescence detection of proteins, and autoradiography. Densitometric analysis of the 12-kDa protein band detected both in the cytosolic and in the mitochondrial fraction and corresponding to cytochrome *c* was performed by means of the NIH Image program (ver. 1.52; http://rsb.info.nih.gov/nih-image/Default.html).

Detection of Caspase Activity

Caspase activity was measured by means of a spectrophotometric assay kit (CaspACE Assay System Colorimetric; Promega, Milan, Italy), following manufacturer's instructions with some minor modifications. Briefly, at the end of the incubation period, cells were collected in Cell Lysis Buffer (as supplied by the manufacturer), exposed to repeated freeze/thawing cycles, and incubated for 15 min on ice. Insoluble fraction was discarded by centrifugation (5 min at

15,000 rpm) and the protein content in the supernatant was determined according to the method of Bradford (1976) and subsequently adjusted to desired concentration in caspase assay buffer (as supplied by the manufacturer). Several different protein concentrations have been tested, and the best results have been obtained with a total amount of 50 to 60 μg in each sample. The determination of caspase activity was carried out in a 96-well plate in the same buffer in a total volume of 100 μl, in the presence of the corresponding tetrapeptide conjugated to paranitroaniline (i.e., DEVD-pNA, LEHD-pNA IETD-pNA, and VDVAD-pNA in the case of caspase-3, -9, -8, and -2, respectively; final concentration, 200 μM). Extracts were incubated for 4 h at 37°C; at the end of the incubation period, released pNA was measured in a spectrophotometer at 405 nm. The specificity of the recorded absorbance was determined by adding the corresponding –CHO tetrapeptide to the reaction mixture (final concentration, 20 μM); a 60 to 80% reduction of the signal was considered significant and specific. Each condition was run in duplicate, and at least four independent experiments have been performed.

Cytofluorimetric Analysis

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 staining of DNA followed by flow cytometric analysis, as described previously (Ceruti et al., 2000).

Evaluation of Cell Death Receptors. Expression of CD95/Fas on the cell surface of activated human lymphocytes and ADF cells was verified by using monoclonal antibodies to human CD95 directly conjugated to (*R*)-phycoerythrin (BD Biosciences, San Jose, CA).

Analysis of Mitochondrial Membrane Potential. Changes in mitochondrial membrane potential ($\Delta\Psi$m) induced in the total cell population by different pharmacological agents were analyzed by means of the fluorescent dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1; stock solution: 10^{-2}M in dimethyl sulfoxide; Molecular Probes, Società Italiana Chimici, Rome, Italy), as described previously (Ceruti et al., 1997). 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 versus side light scatter dot plot.

Results

Induction of Apoptosis and Activation of Caspase-3 by 2-CA and 2-CdA in Human Astrocytoma Cells. Figure 1 shows significant and concentration-dependent induction of apoptotic cell death in human astrocytoma cells upon exposure to either 2-CA or 2-CdA at micromolar concentrations, which are pharmacologically relevant, based on the estimated plasma and intracellular concentrations of cladribine nucleotides in patients with hematologic tumors treated with this anticancer agent (Liliemark, 1997).

To investigate the role of caspases in this effect, we have started our analysis from the most important "effector" caspase [i.e., caspase-3 (Earnshaw et al., 1999)].

Because caspase activation is a relatively early event in the apoptotic program, to evaluate in detail the time-dependence of apoptosis induction, human astrocytoma cells were exposed for graded (1–24 h) periods of time to 100 μM 2-CA or 2-CdA. At the end of the incubation period, both caspase-3 activation and induction of apoptosis were evaluated. As shown in Fig. 2, both adenosine analogs induced a statistically significant activation of caspase-3 starting from a 10-h exposure, as evaluated by spectrophotometric analysis of yellow pNA release caused by the cleavage of the synthetic

tetrapeptide substrate DEVD-pNA. Maximal caspase-3 activation was present at 20/22 h, when an 18-fold increase of enzyme activity with respect to basal levels was detected. Activation tended to decrease starting from 24 h. At any time point showing a significant activation of the enzyme (10–24 h), an 80 to 92% reduction of absorbance in the presence of DEVD-CHO with respect to corresponding adenosine analog alone was detected (data not shown), therefore confirming that pNA release was indeed specifically caused by the action of active caspase-3 (see *Materials and Methods* for more detail). Activation of caspase-3 preceded the appearance of nuclear signs of apoptosis, as evaluated by cytofluorometric analysis of PI stained nuclei (Fig. 2, line graphs), therefore

suggesting the existence of a temporal relationship between caspase-3 activation and induction of cell death by adenosine analogs. Activation of caspase-3 was also confirmed by immunoblotting analysis, with an antibody recognizing both the inactive proenzyme (detected as a 32-kDa band) and its active proteolytic fragments (detected as 17- and 12-kDa protein bands; Fig. 3). In agreement with the enzymatic activation data, active caspase-3 fragments were detected starting from 10 h after beginning exposure of cells to either 2-CA or 2-CdA. To support a role of caspase-3 in adenosine analog-induced apoptosis, prevention of the intracellular phosphor-
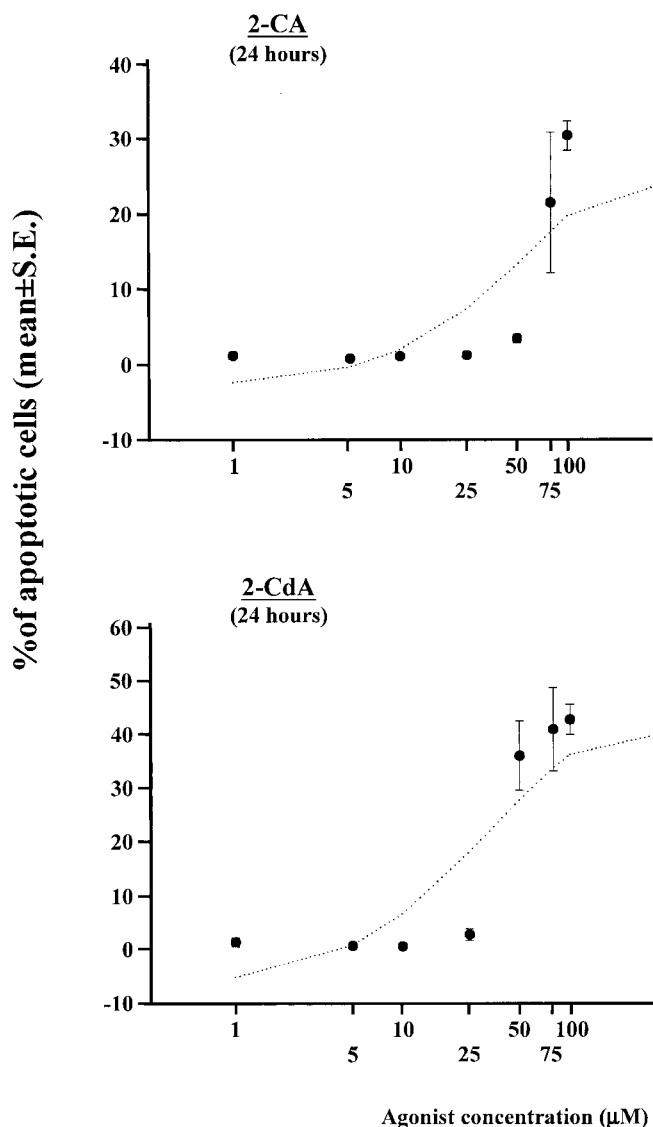

Fig. 1. Concentration-dependent induction of apoptosis by adenosine analogs. Human astrocytoma ADF cells were exposed to graded concentrations (1–100 μM) of either 2-CA (upper) or 2-CdA (lower) for 24 h. At the end of the incubation period, the percentage of apoptotic nuclei was evaluated by flow-cytometric analysis of PI-stained nuclei. Data represent the mean of four independent experiments. EC_{50} values were calculated using a four-parameter logistic model according to Ratkovsky and Reedy (1986) with adjustment by nonlinear regression using the Levenberg-Marquard algorithm in Sigma Plot 7.10 software. Estimated EC_{50} values are 67.6 $\pm$ 0.31 μM and 32.3 $\pm$ 0.3 μM for 2-CA and 2-CdA, respectively.
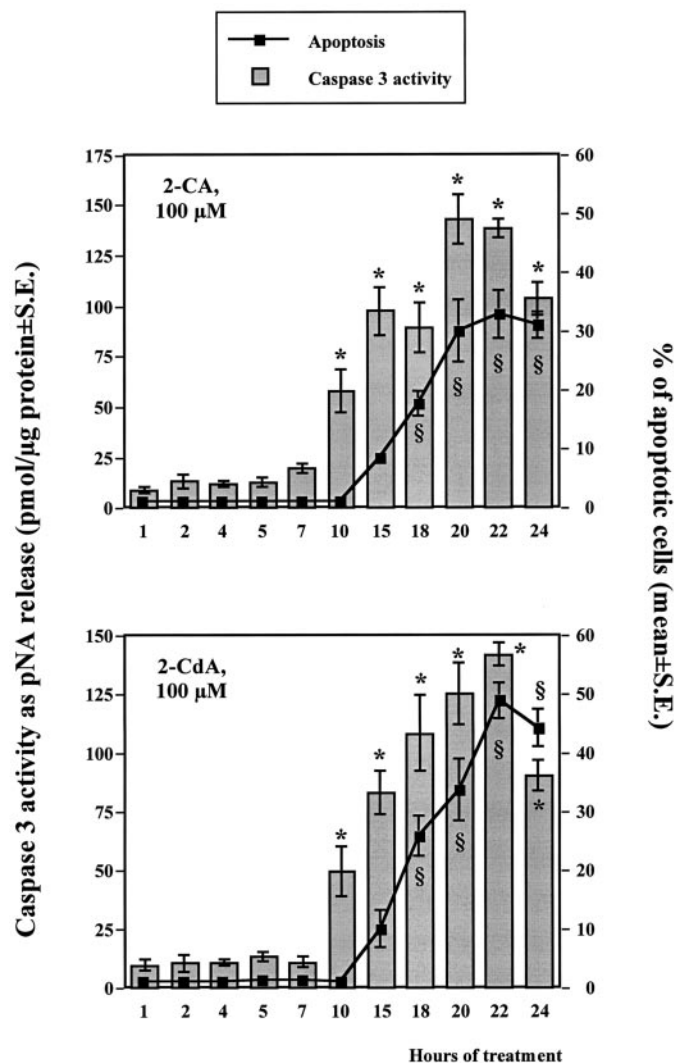

Fig. 2. Time-dependent induction of apoptosis and activation of caspase-3 by 2-CA (top) and 2-CdA (bottom). Human astrocytoma ADF cells were exposed for graded (1–24 h) time periods to either adenosine analog and at the end of the incubation period the percentage of apoptotic nuclei was determined by flow cytometric analysis of PI-stained nuclei (line graphs). The mean value of spontaneous apoptosis in control cultures was 0.7 $\pm$ 0.02%. §, $p < 0.05$ with respect to corresponding control, one-way ANOVA (Scheffé F test). At the same time points, caspase-3 activity was determined in parallel samples by evaluating cleavage of the specific caspase-3 substrate DEVD-pNA and release of pNA at 405 nm (histogram graphs). Mean control value was 7.8 $\pm$ 0.5 pmol of pNA/μg of protein. The specificity of the release was determined by adding the reversible caspase-3 inhibitor DEVD-CHO during the assay in vitro (see *Results*). Data represent the mean $\pm$ S.E. of at least four independent experiments, run in triplicate. *, $p < 0.05$ with respect to corresponding control, one-way ANOVA (Scheffe F test).

ylation/activation of 2-CA and 2-CdA by specific inhibitors (Ceruti et al., 2000) abolished both caspase-3 activation and induction of cell death (Ceruti et al., 2003).

To further confirm the causal relationship between caspase-3 activation and induction of apoptosis, 2-CA- and 2-CdA-treated cultures were exposed to the pan-caspase inhibitor zVAD-fmk, and both caspase-3 activation and the percentage of nuclear apoptosis induced by the two analogs alone or in combination with this synthetic tetrapeptide were evaluated (Fig. 4). As described in Figs. 2 and 3, significant caspase-3 activation can be detected 10 h after addition of either 2-CA or 2-CdA. Hence, in this set of experiments, cells were exposed to either adenosine analog, and the pan-caspase inhibitor (20–40 μM final concentration) was added 7 h later to inhibit the enzyme as soon as it was activated; both the extent of caspase-3 activation and the degree of apoptosis were determined after a total of 24 h in culture. As expected, 2-CA and 2-CdA significantly activated caspase-3 (Fig. 4A) and induced the appearance of an hypodiploid DNA peak, typical of apoptosis (Fig. 4, B and C). zVAD-fmk completely prevented both the activation of caspase-3 (Fig. 4A) and the appearance of the nuclear signs of apoptosis (Fig. 4, B and C) induced by either adenosine analog, therefore confirming the key role played by caspases in our experimental model. A partial, but statistically significant, protection against 2-CA and 2-CdA-induced apoptosis was already detected at a lower zVAD-fmk concentration (10 μM; data not shown). To further confirm the above results, when added to intact cells during the incubation with adenosine analogs, the specific caspase-3 inhibitor zDEVD-fmk was also able to prevent apoptosis (Fig. 9, see also below).

Apoptosis by Adenosine Analogs Is Not Preceded by Changes of $\Delta\Psi$m. In an attempt to characterize the apoptotic pathways activated by adenosine analogs upstream of caspase-3, we focused on the possible role of mitochondria, because this pathway has been involved in 2-CdA-induced apoptosis in other experimental models (Genini et al., 2000b; Marzo et al., 2001). Possible changes in $\Delta\Psi$m have been evaluated by means of cytofluorometric analysis after loading cells with the mitochondrial specific cationic dye JC-1 (Ceruti et al., 1997). In healthy cells with polarized mitochondria, JC-1 accumulates in the transmembrane space and forms the so-called "J-aggregates", emitting orange fluorescence at 590 nm when excited at 490 nm and thereby detectable by flow cytometry in fluorescence channel 2 (FL2; see y-axis in Fig. 5 graphs). Drops in $\Delta\Psi$m result in disappear-

ance of J-aggregates and formation of JC-1 monomers that emit a greenish-yellow fluorescence at 530 nm when excited at 490 nm, corresponding to fluorescence channel 1 (FL1, see x-axis in Fig. 5 graphs). It is therefore possible to monitor changes in $\Delta\Psi$m by looking at the number of cells showing fluorescence emission in the two channels. In control untreated cultures, because of the equilibrium existing between J-aggregates and monomers in healthy cells, the majority of cells (96.25%) shows a high emission of fluorescence in both channels and is therefore found in the upper right quadrant of the plot (Fig. 5A). Only a very small percentage of control untreated cells (3.75%) shows low emission of fluorescence in FL2 and can therefore be found in the lower right (LR) quadrant of the plot (Fig. 5A). In initial experiments, we have evaluated the changes of $\Delta\Psi$m induced by agents known to
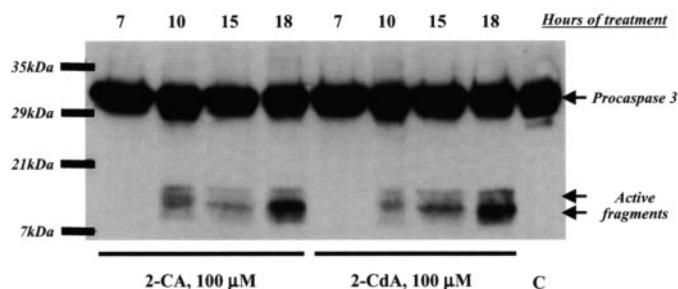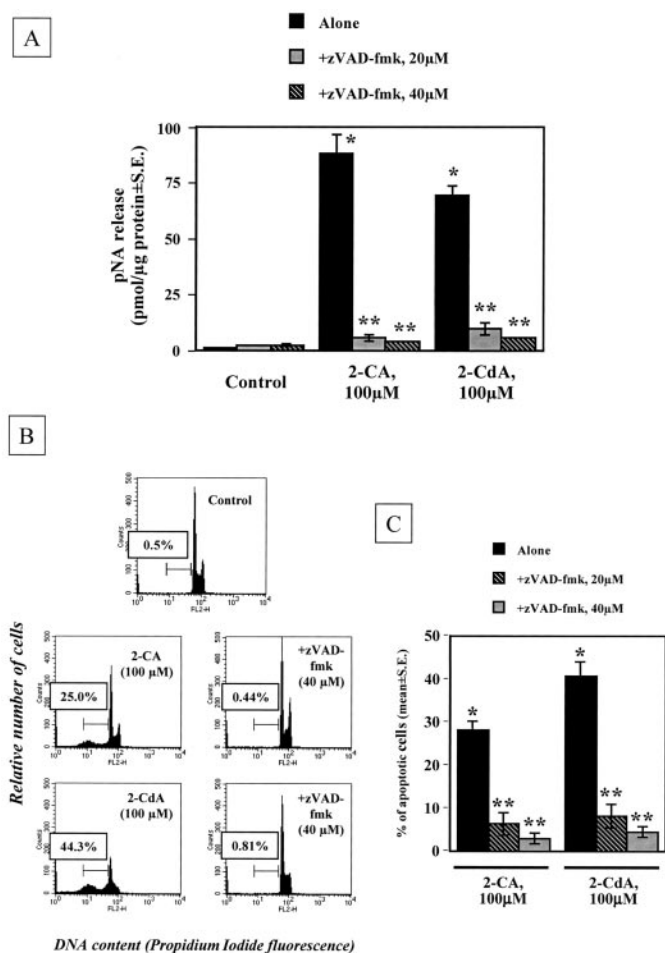
Fig. 3. Time-dependent activation of caspase-3 by 2-CA and 2-CdA: Western blotting analysis. ADF cells were exposed to either adenosine analog for 7 to 18 h, as indicated, and activation of caspase-3 was evaluated by Western blotting analysis. The 17-/12-kDa protein bands that appear starting from 10-h incubation with both 2-CA and 2-CdA represent the active fragments of the enzyme.

Fig. 4. Effect of the pan-caspase inhibitor zVAD-fmk on 2-CA- and 2-CdA-induced activation of caspase-3 (A) and apoptosis (B and C) in human astrocytoma cells. Cultures were exposed to either adenosine analog; 7 h later, the pan-caspase inhibitor zVAD-fmk (final concentration, 20–40 μM) was added to inhibit the enzyme as soon as it is activated (see Figs. 2 and 3). After a total of 24 h in culture, caspase-3 activity was determined by evaluating cleavage of the specific substrate DEVD-pNA and release of pNA at 405 nm (A). *, $p < 0.05$ with respect to corresponding control and **, $p < 0.05$ with respect to corresponding adenosine analog alone, one-way ANOVA (Scheffé F test). On parallel samples, apoptosis was evaluated by PI staining and cytofluorometric analysis. B shows results from a typical experiment, whereas histograms in C represent the mean values ± S.E. of the percentage of apoptotic cells obtained in four independent experiments. *, $p < 0.05$ (with respect to corresponding control); **, $p < 0.05$ (with respect to corresponding adenosine analog alone) by one-way ANOVA (Scheffé F test).

interfere with mitochondrial function, such as NaCN (Rutter and Rizzuto, 2000), dRib (Ceruti et al., 1997), and the anticancer agent BetA (Fulda et al., 1998), which have been used as positive controls. Also in human astrocytoma cells, these agents induced dramatic drops in $\Delta\Psi$m (Fig. 5A); for example, a 4-h exposure to 3.8 mM NaCN resulted in a dramatic decrease of J-aggregate fluorescence (y-axis; FL2), accompanied by a concomitant increase of fluorescence in FL1, so that 80.75% of cells were now found in the lower right quadrant of the plot (Fig. 5A). In a similar way, marked increases of the percentage of cells with depolarized mitochondria were detected with the other two depolarizing agents (Fig. 5A). For all agents, induction of mitochondrial depolarization was followed, a few hours later, by induction of cell death (data not shown). These results demonstrate that: 1) alterations of $\Delta\Psi$m can be induced in ADF cells and 2) with the above method, changes of mitochondrial function induced by toxic stimuli can be detected *before* appearance of cell death. We therefore evaluated possible changes in $\Delta\Psi$m after exposure of cultures to adenosine analogs. To check whether changes in $\Delta\Psi$m preceded or followed caspase-3 activation, we exposed cultures to 2-CA and 2-CdA: 7 h showed no significant activation; 10, 15, and 18 h showed partial activation; 20 and 22 h showed maximal activation, and 24 h showed declining activation (see Fig. 2). As shown in Fig. 5B, which reports the percentage of cells in the upper or lower right quadrants of the $\Delta\Psi$m analysis plot, significant alterations of $\Delta\Psi$m could be detected only starting from an 18-h exposure to either adenosine analog, a quite late time at which cells are already committed to apoptotic cell death. Therefore, we conclude that the mitochondrial depolarization detected at quite late stages of adenosine analog-induced apoptosis probably reflects a consequence, rather than a cause, of cell death.

Apoptosis Induced by Adenosine Analogs Does Not Involve Cytochrome *c* Release from Mitochondria nor Caspase-9 Activation. Activation of the mitochondrial pathway of cell death is coupled, sequentially, to a drop in $\Delta\Psi$m, cytochrome *c* release, formation of the apoptosome, stimulation of the "initiator" caspase-9, and activation of effector caspases (e.g., caspase-3; Li et al., 1997). Lack of early mitochondrial depolarization upon exposure to adenosine analogs (Fig. 5B) would rule out a role for cytochrome *c* release and caspase-9 in our experimental model. However, because a significant release of cytochrome *c* can also be detected in the absence of changes of $\Delta\Psi$m (Grubb et al., 2001), specific experiments were done to investigate the involvement of both cytochrome *c* and caspase-9 in the apoptosis induced by adenosine analogs. The subcellular localization of cytochrome *c* was evaluated by Western blotting analysis in the mitochondrial and cytosolic fractions of control untreated cells and of cells exposed to either 2-CA or 2-CdA for various time periods (7, 10, 15, and 24 h). Cytochrome *c* was easily detected in the mitochondria of both control and treated cells (Fig. 6A); however, in no case were we able to detect any accumulation of cytochrome *c* in the cytoplasm of cells exposed to either 2-CA or 2-CdA. We have applied the same methods to monitor cytochrome *c* release upon exposure to either NaCN, dRib, or BetA, which induced mitochondrial depolarization in these cells (Fig. 5A). Exposure to these agents indeed resulted in marked release of cytochrome *c* from mitochondria to the cytosolic fraction (Fig. 6B), hence confirming the suitability of these methods to follow changes of cytochrome *c* subcellular localization in these cells.

Finally, we assessed the presence and activation of caspase-9 in cells treated with either 2-CA or 2-CdA. Evaluation of caspase-9 activation by the same methods used to detect caspase-3 (i.e., cleavage of the specific caspase-9 substrate LEHD-pNA and release of pNA; Fig. 7A) revealed no significant stimulation of enzyme activity. A small not statistically significant stimulation of caspase-9 was detected after 20 h, a time when the apoptotic process in these cells is already well established. We have applied exactly the same methods to measure caspase-9 activity in another cell line (U937 cells) that has been previously described to undergo caspase-9 activation and cell death upon exposure to 2-CdA
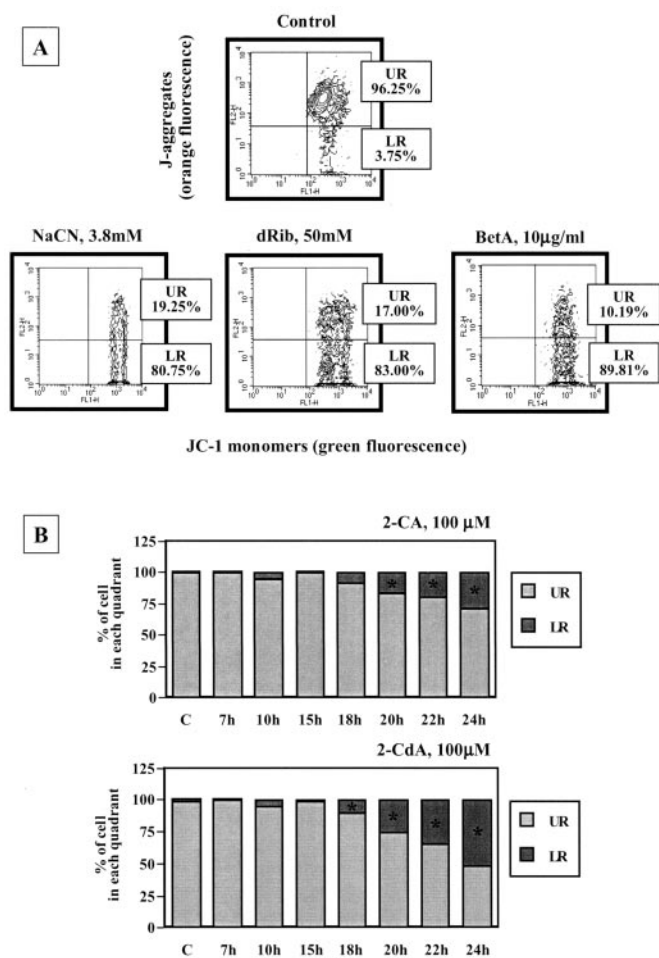

Fig. 5. A, evaluation of $\Delta\Psi$m by JC-1 staining in NaCN-, dRib-, and BetA-treated ADF cells. Healthy cells are found in the upper right quadrant of the plots, corresponding to high emission of fluorescence in both FL1 (green; x-axis) and FL2 (orange; y-axis) channels. After NaCN, dRib, and BetA exposure, mitochondrial depolarization becomes evident as a drop in the FL2 and an increase in the FL1 emission. The majority of cells are now in the lower right quadrant of the plots. A typical experiment is shown and similar data have been obtained in three independent experiments. B, evaluation of $\Delta\Psi$m by JC-1 staining in 2-CA- and 2-CdA–treated ADF cells. Human astrocytoma ADF cells were treated with either adenosine analog for graded time periods (7–24 h, as indicated) and $\Delta\Psi$m evaluated by means of cytofluorimetric analysis of JC-1 stained cells (see above). Histograms show the mean values of the percentage of cells found in three independent experiments either in the upper right (UR; polarized mitochondria) or in the lower right (LR; depolarized mitochondria) quadrant of the $\Delta\Psi$m analysis plots. *, $p < 0.05$ with respect to corresponding control, one-way ANOVA (Scheffé F test).

(Marzo et al., 2001). In these cells, here used as a positive control for caspase-9 activation, both 2-CdA and the pro-apoptotic agent Eto (Plo et al., 2002) induced marked mitochondrial depolarization (Fig. 7B) and significantly increased caspase-9 activity with respect to control untreated cells (Fig. 7C). To further rule out a role for caspase-9 in adenosine analog-induced apoptosis of human astrocytoma cells, Western blotting experiments with an antibody recognizing both the inactive form of pro-caspase-9 and its active proteolytic fragment revealed no activation of this caspase upon exposure to either 2-CA or 2-CdA (data not shown). Globally, these results confirm that the lack of caspase-9 activity in human astrocytoma cells upon exposure to adenosine analogs is actually caused by the inability of these agents to activate this pathway of cell death.

No Role for Caspase-8 in Adenosine Analog-Induced Apoptosis in Human Astrocytoma Cells. In a similar way to caspase-9, a detailed (7–24 h) kinetic analysis of the activity of caspase-8, that is a part of the "death receptor" pathway

and has been demonstrated to be at the basis of 2-CdA-induced cell death in leukemia cells (Nomura et al., 2000), was performed. To this purpose, cell extracts have been incubated with a specific caspase-8 substrate, IETD-pNA, and enzyme activity has been evaluated as described under *Materials and Methods*. A specific activation of the enzyme (corresponding to a 1.8- to 2-fold increase over basal activity) has been detected only starting from a rather late time period of incubation (18 h; Fig. 8), suggesting that its activation is more a consequence than a cause of commitment to cell death, hence ruling out a role for this caspase in triggering the apoptotic cascade activated by adenosine analogs in astrocytoma cells. To rule out a role for the "death" receptor
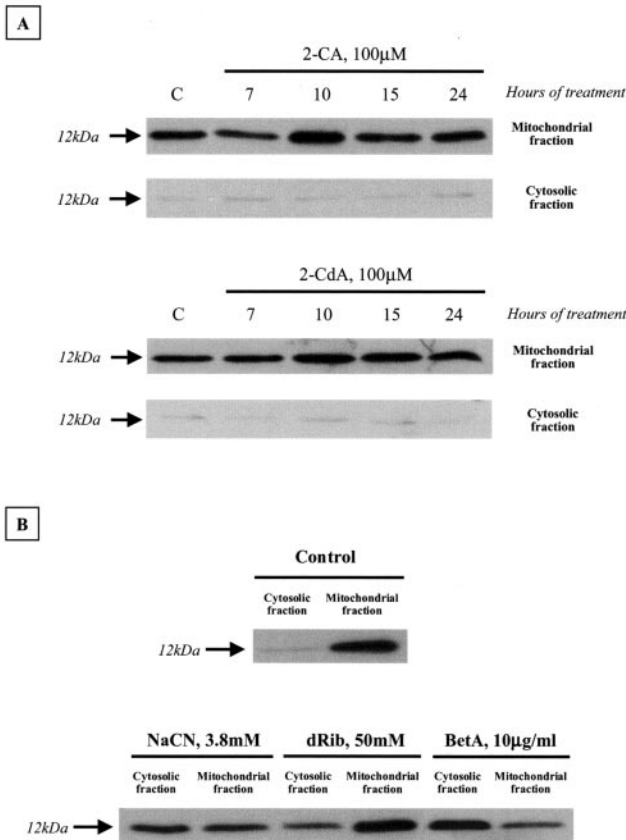
Fig. 6. Evaluation of cytochrome *c* release from mitochondria: Western blotting analysis. Cells were exposed to either 100 μM 2-CA or 2-CdA (7–24 h; A), 3.8 mM NaCN (4 h), 50 mM dRib (24 h), or 10 μg/ml BetA (72 h; B). At the end of each incubation period, cells were collected and the cytosolic and mitochondrial fractions obtained as described under *Materials and Methods*. Cytochrome *c* was detected as a 12-kDa protein band with a specific antibody. For each individual sample, the 12-kDa protein band has been analyzed densitometrically by means of the NIH Image software (ver. 1.52), hence determining the percentage of cytochrome *c* in the cytosolic fraction over the total. This value never exceeded 10% in either control or adenosine analogs-treated cells (A), whereas it was increased to 50.08, 26.11, and 70.50% of total in NaCN-, dRib-, or BetA-treated cells, respectively (B), indicating significant release of cytochrome *c* from mitochondria to the cytosol upon exposure to these agents. No statistically significant differences in total cytochrome *c* among treatments were detected. A typical experiment of three is shown.
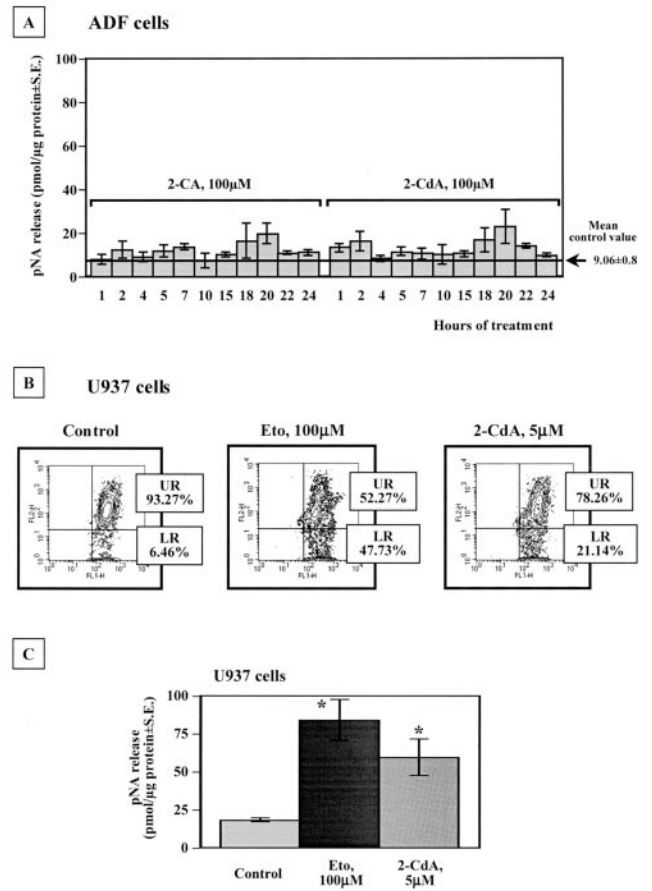
Fig. 7. A, evaluation of caspase-9 activation by 2-CA and 2-CdA in human astrocytoma ADF cells. Cells were exposed for 1 to 24 h to either adenosine analog, and caspase-9 activity was determined by cleavage of the specific caspase-9 substrate LEHD-pNA and release of pNA at 405 nm. For each time point, statistical analysis has been performed with respect to corresponding untreated samples. No statistically significant differences were detected. To allow comparisons, the mean value (picomoles of pNA per microgram of protein) detected in control samples is also reported (see line on histograms). Data represent the mean ± S.E. of at least four independent experiments, run in triplicate. B, evaluation of ΔΨm by JC-1 staining in Eto- and 2-CdA-treated human promonocytic U937 cells. Cells were grown in the absence (control) or presence of either 100 μM Eto or 5 μM 2-CdA for 18 h. At the end of the incubation period, cells were collected and mitochondrial depolarization evaluated as described in the legend to Fig. 5. C, evaluation of caspase-9 activation in Eto- and 2-CdA-treated human promonocytic U937 cells. Cells were grown in the absence (control) or presence of either 100 μM Eto or 5 μM 2-CdA for 18 h. At the end of the incubation period, caspase-9 activation was evaluated by spectrophotometric analysis as described above. *, $p < 0.05$ with respect to untreated control cells, one-way ANOVA (Scheffé F test).

pathway in initiating apoptosis in human astrocytoma cells, activation of the Fas/Fas ligand pathway by exposure to anti-FAS antibodies (a typical trigger of the caspase-8-dependent pathway; Parlato et al., 2000) did not result in any
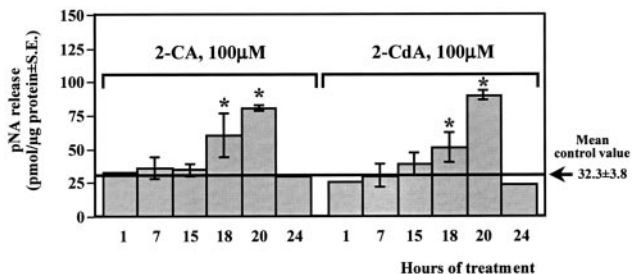

Fig. 8. Evaluation of caspase-8 activation by 2-CA and 2-CdA: spectrophotometric analysis. Human astrocytoma ADF cells were exposed for 1 to 24 h to either adenosine analogs, and caspase-8 activity was determined by cleavage of the specific caspase-8 substrate IETD-pNA and release of pNA at 405 nm. For each time point, statistical analysis has been performed with respect to corresponding untreated samples. To allow comparisons, the mean value (picomoles of pNA per microgram of protein) detected in control samples is also reported (see line on histograms). A significant activation of the enzyme was detected only starting from an 18-h exposure (i.e., after the maximal activation of caspase 3) (see Fig. 2). Specificity of the release was determined by concomitant exposure of cellular extracts to the reversible caspase-8 inhibitor IETD-CHO, obtaining 70 and 75% of reduction of pNA release at 18 and 20 h of treatment, respectively (data not shown). Data represent the mean ± S.E. of at least four independent experiments, run in duplicate. *, $p < 0.05$ with respect to corresponding control, one-way ANOVA (Scheffé F test).
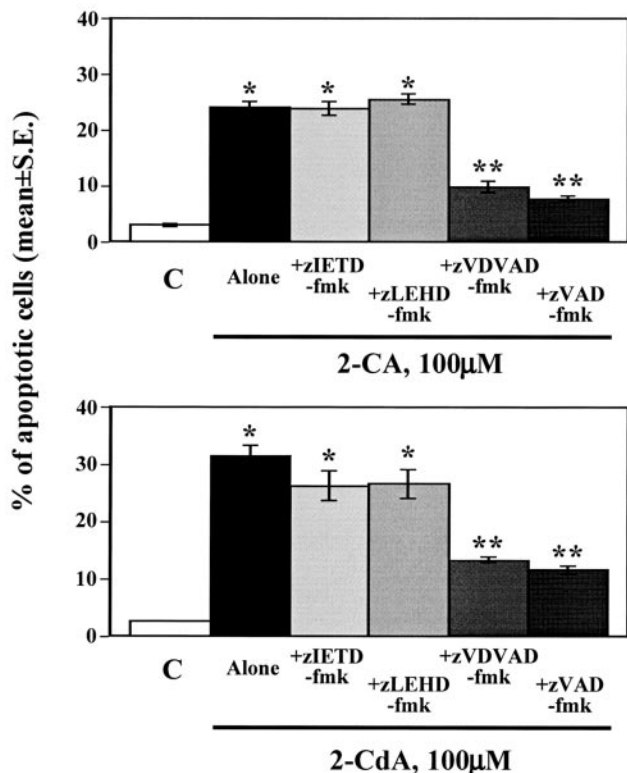

Fig. 9. Effects of various caspase inhibitors on 2-CA- and 2-CdA–induced apoptosis. Cells were exposed for 24 h to either 2-CA or 2-CdA, alone or in combination with various caspase inhibitors (3 μM), as indicated. At the end of the incubation, the percentage of apoptotic nuclei was determined by flow cytometric analysis of PI-stained nuclei. Data represent the mean ± S.E. of three independent experiments. *, $p < 0.05$ (with respect to control); **, $p < 0.05$ (with respect to control and to corresponding adenosine analog alone), one-way ANOVA (Scheffé F test).

increase of the percentage of apoptotic cells (7.1 ± 1.8%) with respect to control untreated cells (6.6 ± 2.2). The same concentration of anti-FAS antibodies induced highly significant apoptosis in human phytohemagglutinin/interleukin-2–activated T lymphocytes, a typical Fas-sensitive cell type (36 ± 3% after 48 h compared with 6.6 ± 1.8% detected in resting cells, mean value of four different experiments, $p < 0.001$, Student's t test), whereas it was ineffective in resting cells. The lack of effect of anti-Fas antibodies on the human astrocytoma cells was not caused by the absence of surface receptors, because both these cells and human T lymphocytes were CD95/Fas positive when analyzed cytofluorimetrically with a specific antibody (data not shown).

Caspase-2 Is the Initiator Caspase Involved in Induction of Apoptosis by Adenosine Analogs in Human As-
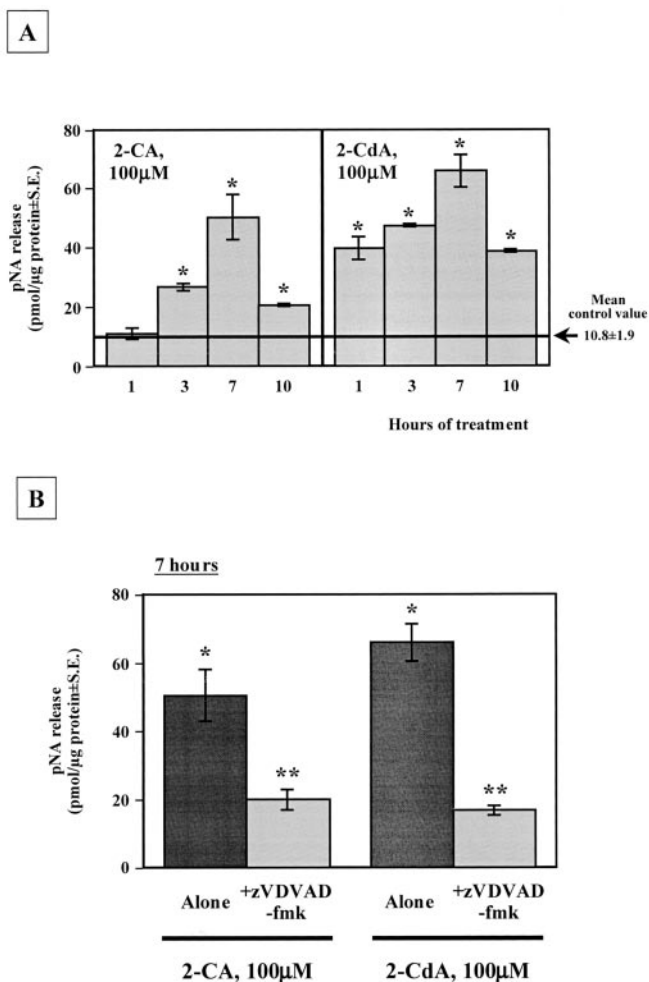

Fig. 10. Role of caspase-2 in adenosine analogs-induced apoptosis of human astrocytoma cells. A, cells were exposed to either 2-CA or 2-CdA for the indicated time periods (1–10 h) and caspase-2 activity was determined spectrophotometrically by cleavage of the specific caspase-2 substrate zVDVAD-pNA and release of pNA at 405 nm. To allow comparisons, the mean value (picomoles of pNA per microgram of protein) detected in control samples is also reported (see line on histograms). Data represent the mean ± S.E. of three independent experiments run in triplicate. *, $p < 0.05$ with respect to corresponding control, one-way ANOVA (Scheffé F test). B, cells were exposed to either 2-CA or 2-CdA in the absence ("alone") or presence of the selective caspase-2 inhibitor zVDVAD-fmk. After 7 h, caspase-2 activation was determined spectrophotometrically as described above. *, $p < 0.05$ (with respect to control); **, $p < 0.05$ (with respect to control and corresponding adenosine analog alone), one-way ANOVA (Scheffé F test).

trocytoma Cells. Although caspase-2 has never been reported to participate to induction of apoptosis by adenosine analogs, we also determined the role of this caspase in our experimental model by assessing whether zVDVAD-fmk, using a concentration (3 μM) that selectively inhibits this caspase (Schotte et al., 1999), could prevent induction of apoptosis by 2-CA or 2-CdA. In parallel, the effects induced by the selective caspase-8 inhibitor zIETD-fmk, the caspase-9 inhibitor zLEHD-fmk, the caspase-3 inhibitor zDEVD-fmk, and the pan-caspase inhibitor zVAD-fmk (all used at 3 μM) have been assessed.

In line with the data reported above, which rule out a role for caspase-8 and caspase-9 in our experimental model, neither zIETD-fmk nor zLEHD-fmk could prevent the apoptosis induced by either 2-CA or 2-CdA (Fig. 9), whereas a highly significant reduction of adenosine analog-induced apoptosis was observed with both the selective caspase-2 inhibitor zVDVAD-fmk, the selective caspase-3 inhibitor zDEVD-fmk, and, as expected, with the pan-caspase inhibitor zVAD-fmk (Fig. 9). A full protection against adenosine analogs-induced apoptosis was obtained at inhibitor concentrations higher than 3 μM (data not shown); however, these are not selective for any specific caspase (Schotte et al., 1999).

To confirm the involvement of caspase-2, we evaluated the ability of 2-CdA and 2-CA to activate this caspase. By using a colorimetric assay similar to those used for the other caspases, we demonstrated statistically significant increases of caspase-2 activity at times (1–7 h after addition of either adenosine analog to cells; Fig. 10A) that precede the activation of effector caspase-3 (Fig. 2). Stimulation of caspase-2 by 2-CdA seemed to occur slightly earlier (already statistically significant at 1 h) and more robustly with respect to 2-CA (Fig. 10A). For both 2-CA and 2-CdA, caspase-2 activation started to decline at 10 h, a time at which effector caspase-3 is already highly activated in these cells (Fig. 2). Activation of caspase-2 by 2-CA or 2-CdA was specific, because it could be significantly reduced by treating cells with either adenosine analog in the presence of the selective caspase-2 inhibitor zVDVAD-fmk (Fig. 10B).

Discussion

The present data confirm the pro-apoptotic effects induced by adenosine analogs on human astrocytoma cells (Ceruti et al., 2000), and, for the first time, shed light on the role of caspases in such effects.

Main findings of the present study are the following:

1. Induction of apoptosis by adenosine analogs is characterized by a marked activation of caspase-3 starting from 10 h, as shown by both increased caspase enzymatic activity and by proteolytic cleavage of the proenzyme and formation of caspase-3 active fragments. The demonstration that A) activation of caspase-3 temporally precedes the appearance of the nuclear signs of apoptosis and B) the pan-caspase inhibitor zVAD-fmk and the selective capsase-3 inhibitor zDEVD-fmk could both abolish caspase-3 activity and prevent induction of apoptosis is highly suggestive of an important role for this caspase in our experimental model.

2. At variance from previous studies in other experimental models (see below), activation of "initiator" caspase-9 or caspase-8 is not a primary event in adenosine analog-induced apoptosis of human astrocytoma cells. A detailed kinetic analysis of caspase-9 and caspase-8 activity at various times (1–24 h) after addition of adenosine analogs revealed that neither 2-CA nor 2-CdA can activate these caspases. A marginal increase of either caspase-9 and caspase-8 activity was only detected at much later times (18–20 h) with respect to the earlier (10 h) and highly significant activation of caspase-3, indicating that activation of these enzymes is a consequence rather than a cause of caspase-3 activation. Despite limitations in interpretation of studies using caspase inhibitors (Schotte et al., 1999), neither zLEHD-fmk nor zIETD-fmk could prevent the induction of cell death by either 2-CA or 2-CdA when used at low concentrations that should selectively inhibit caspase-9 and caspase-8, respectively. This finding is in line with the other data reported here that rule out a role for caspase-9 and caspase-8 in our experimental model.

3. In line with results reported in *2*, no alteration of mitochondrial potential was induced by adenosine analogs at times consistent with caspase-3 activation. Importantly, this was not caused by a loss of the mitochondrial pathway of cell death in these cells, because this pathway could indeed be recruited by other stimuli (e.g., depolarizing agents such as NaCN, dRib, or BetA). Consistent with these data, although the mitochondrial depolarization induced by these agents was accompanied by release of cytochrome *c* into the cytoplasm, no release of cytochrome *c* from mitochondria was detected in cells exposed to either 2-CA or 2-CdA.

4. Both adenosine analogs induced significant and time-dependent increases of the activity of caspase-2 at times (1–7 h) that preceded the activation of effector caspase-3. To confirm the importance of this caspase in adenosine analog-induced cell death, the selective caspase-2 inhibitor zVDVAD-fmk significantly reduced both the activation of this caspase and the associated cell death. Hence, caspase-2 represents the caspase upstream of caspase-3 in the apoptotic cascade activated by adenosine analogs in human astrocytoma cells.

On this basis, we conclude that adenosine analogs can trigger the apoptosis of astrocytoma cells by a cellular pathway that does not involve mitochondrial depolarization, release of cytochrome *c*, or activation of either caspase-9 or caspase-8 but requires activation of caspase-2 and effector caspase-3. Our data are at variance with results obtained in other experimental systems [i.e., Jurkat cells (Robertson et al., 2002) or transformed human fibroblasts (Lassus et al., 2002)], where caspase-3 activation by caspase-2 has been reported to involve cytochrome *c* release and caspase-9 activation. The mechanistic basis for caspase-3 activation by caspase-2 in our experimental model remains to be elucidated. This atypical pathway of cell death may represent a novel biological target for the pharmacological manipulation of tumor growth. Our results also underline the possibility that, under specific cellular paradigms, caspase-2 may act as an "initiator" caspase. Determining an emergent function for this protease has been difficult, with results assigning this caspase a downstream or feedback role (reviewed in Robertson et al., 2002). Very recently, however, in caspase-9–

blocked testicular cancer cells, initiation of apoptosis by cis-platin has been demonstrated to occur through activation of caspase-2 and caspase-3 (Mueller et al., 2003). Moreover, in cerebellar granule cells, staurosporine-induced apoptosis has been shown to require induction of caspase-2 before caspase-3 (Caballero-Benitez and Moran, 2003). The present data obtained with adenosine analogs in human astrocytoma cells suggest that this apoptotic cascade may be recruited more often than originally suspected.

As underlined above, our results are at variance from results obtained in other experimental models. In MOLT-4 leukemic cells, 2-CdA–induced apoptosis has been shown to crucially involve the Fas/Fas ligand/caspase-8 pathway (Nomura et al., 2000). In other human leukemia cell lines, 2-CdA–induced cell death has been shown to be always preceded by a loss of mitochondrial membrane potential (Genini et al., 2000a,b; Marzo et al., 2001). These differences may be attributable to the differential activation of specific apoptotic programs in cells belonging to different lineages (immune cells in the case of the above studies with respect to astrocytoma cells in the present study) and/or to differential regulation of caspase activity. For instance, in human leukemia cells, caspase-9 activation by 2-CdA can be prevented by Bcl$_2$ (Genini et al., 2000b). No immunoreactivity to this protein can be detected in human astrocytoma ADF cells, which instead express Bcl-X$_L$ at relatively high level (Abbracchio et al., 1997). Interestingly, Bcl-X$_L$ expression is up-regulated by treatment of cells with adenosine analogs (S. Ceruti, R. Brambilla, and M. P. Abbracchio, unpublished data). It may well be that, in our experimental model, this antiapoptotic protein prevents adenosine analogs from promoting the formation of the apoptosome (and thus from activating pro-caspase-9), hence shifting the apoptotic program to other intracellular pathways. The lack of pro- and antiapoptotic proteins in a simplified cell-free system as in that used by Leoni and coworkers (1998) may explain the different outcome of their study (i.e., activation of caspase-9 by 2-chlorodeoxyATP upstream of caspase-3) with respect to the present results.

The present data may have important implications for the pharmacological manipulations of drug-resistant central nervous system tumors. Interestingly, untransformed astroglial cells and neurons are relatively resistant to 2-CdA- and 2-CA–induced apoptosis (Ceruti et al., 1997; 2000), which is quite important in view of a possible exploitation of adenosine-analog induced apoptosis in human astrocytoma/glioblastoma tumors. Moreover, the demonstration that, in human astrocytoma cells, adenosine analogs can induce apoptosis by activating an alternative pathway of cell death has intriguing implications in understanding which apoptosis pathways are still expressed by other neoplastic cells. In this respect, Chandra et al. (2002) have recently selected three 2-CdA–resistant leukemic cell lines. Acquisition of resistance to the apoptosis induced by 2-CdA was accompanied by decreased susceptibility of mitochondria to undergo permeability transition and lack of cytochrome *c* release. Our demonstration that adenosine analogs can also induce apoptosis by bypassing the mitochondrion would predict that these compounds could also act as effective anticancer agents in other kinds of human tumors.

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