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ORIGINAL ARTICLE

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Actinomycin D and staurosporine, potent apoptosis inducers in vitro, are potentially effective chemotherapeutic agents against glioblastoma multiforme

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Abstract *Purpose*: Although chemotherapeutic protocols that include chloroethylnitrosoureas (CENUs), such as 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), have been a mainstay of treatment for glioblastomas, the clinical outcomes have been unsatisfactory. More effective chemotherapeutic protocols for these tumors will require clear delineation of more cytocidal and cytostatic chemotherapeutic drugs. *Methods and Results*: In this study, we measured the cytocidal effects of ACNU, cisplatin, actinomycin D, and staurosporine, administered within their therapeutic dose ranges, in the treatment of glioblastoma cells. As assessed by WST-1 colorimetric assay, the number of viable cells decreased markedly in T98G cultures treated with actinomycin D or staurosporine, to less than 20% of the level in control cultures at 72 h, but did not decrease or even increased after 6 days of treatment with ACNU. After treatment with cisplatin for 5 days, cell viability decreased to 30% of control. As assessed by fluorescence microscopic examination of nuclear staining by Hoechst 33258 and by electron microscopy, the majority of dead cells treated with actinomycin D, staurosporine, or cisplatin had morphologic features of apoptosis. Caspase-3 activity increased more than 20-fold in cells treated with actinomycin D, staurosporine, or cisplatin but increased less than fivefold in ACNU-treated cells. In addition to caspase-3 activation, western blot analysis demonstrated cleavage of caspase-2 during the apoptotic process. These findings indicate that actinomycin D and staurosporine potently induce apoptosis, whereas ACNU exerts mainly a cytostatic rather than a cytocidal effect. *Conclusion*: Actinomycin D and staurosporine and their derivatives are potentially effective chemotherapeutic agents against glioblastoma cells at least in vitro.

Key words Apoptosis · Glioblastoma · Caspase · Actinomycin D · Staurosporine

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Introduction

The prognosis of patients harboring glioblastoma multiforme remains poor, with a 5-year survival rate of only 8% despite multidisciplinary treatment with combinations of surgery, radiotherapy, and chemotherapy [1]. Adjuvant chemotherapy with radiotherapy does not increase the 5-year survival rate much, but it does significantly lengthen the median survival and relapse-free survival times. Therefore, more potent adjuvant chemotherapy might well increase the long-term survival rate. Chloroethylnitrosoureas (CENUs) such as 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) have been widely used to treat glioblastomas [2–4]. Although their antineoplastic properties against glioblastoma cells in vitro and in vivo are well known [5–7], the clinical outcome remains unsatisfactory [2–4]. Other agents including cisplatin [8–10], vincristine [4, 11, 12], etoposide [11, 13, 14], adriamycin [15, 16], and methotrexate [17] have also been used against glioblastomas with rather unsatisfactory results.

To identify the agents that are the most potent against glioblastomas, we need to re-evaluate chemotherapeutic drugs in terms of their cytocidal potential. So far, the

growth-suppressive effects of chemotherapeutic agents have been assessed by colony-formation assays [5–7], which reflect both the cytostatic and the cytocidal properties of a drug. Recent evidence suggests that apoptotic mechanisms mediate drug-induced cell death and that the activation of effector caspases plays a central role in the execution of apoptosis [18, 19]. Therefore, to estimate the cytocidal potential of each chemotherapeutic agent, it is important to quantify its ability to induce apoptosis and activate caspases. In the present study, we investigated the cytocidal potential of several chemotherapeutic drugs by directly assessing cell death and caspase activity in the process of drug-induced apoptosis. Chemotherapeutic drugs surveyed included ACNU and cisplatin, which are currently used to treat glioblastomas, and actinomycin D and staurosporine, whose use against these tumors has not been described.

Material and methods

Cell culture and treatment

Human glioma cell lines T98G [20] (purchased from American Type Culture Collection, Manassas, Va.), U251MG [21], SF126 [21] (gifts from Dr. Mark Rosenblum, University of California, San Francisco, Calif.), and NMCG1 [22] (a gift from Dr. Tatsuya Kondo, International Medical Center of Japan, Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 m*M* glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin in 6-cm dishes in a 95% air/5% CO_2 atmosphere at 37 °C. Medium and serum were purchased from Gibco BRL (Paisley, UK). Staurosporine was a gift from Asahi Chemicals (Tokyo, Japan). Acetyl-asp-glu-val-asp a-4-methyl-coumaryl-7-amide (DEVD-MCA), acetyl-asp-lu-val-asp aldehyde (DEVD-CHO), and acetyl-tyr-val-ala-asp a-4-methyl-coumaryl-7-amide (YVAD-MCA) were from the Peptide Institute (Tokyo, Japan), and benzyloxycarbonyl-val-ala-asp fluoromethylketone (ZVAD-FMK) was from Enzyme Systems (Dublin, Calif.). DEVD-MCA and YVAD-MCA are MCA-labeled caspase-3 and -1 substrates, respectively. DEVD-CHO is an inhibitor of caspase-3-like enzymes, while ZVAD-FMK is a general inhibitor of caspases. These peptides were dissolved in dimethyl sulfoxide. Actinomycin D and cisplatin were purchased from Sigma (St. Louis, Mo.). ACNU was purchased from Sankyo (Tokyo, Japan). Staurosporine and actinomycin D were dissolved in dimethyl sulfoxide, and cisplatin and ACNU were dissolved in distilled water. To induce cell death, 2×10^5 cells were incubated with antitumoral drugs at various concentrations for various lengths of time. In some experiments, cells were preincubated with caspase inhibitors to assess the role of caspases in cell death induced by staurosporine.

Cell viability

The net number of viable cells was assessed by direct counting or by using the WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2.4-disulfophenyl)-2H-tetrazolium, sodium salt; Wako, Tokyo, Japan] colorimetric assay, a modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [23]. Briefly, 5×10^3 cells were seeded in 100 µl of medium in each well of 96-well flat-bottomed plates and incubated overnight at 37 °C. The cultures were then incubated with each antitumoral drug for 1 to 6 days. At selected points, 10 µl of WST-1 reagent was added to each well, and the cells were incubated for 2 h at 37 °C. Then, absorbance at 450 nm against a reference wavelength of 650 nm was measured with a microtiter plate reader (BioRad, Calif.; Model 3550) on day 0 (AB_{cont}) and at each time-point (AB_{each}). The blank value (BL) was the absorbance of the culture medium without cells. Since absorbance is proportional to the number of viable cells, the number of viable cells was calculated from the following equation:

$$\text{Number of viable cells} = 5 \times 10^3 \times (AB_{each} - BL)/(AB_{cont} - BL)$$

Assessment of apoptosis

Tumor cells treated with chemotherapeutic agents were examined for features of apoptosis by electron and fluorescence microscopy. For electron microscopic examination, the cells were fixed in 2.5% glutaraldehyde and embedded in Epon. For fluorescence microscopic examination, the cells were stained with 20 µ*M* Hoechst 33258 for 15 min at room temperature. Apoptotic cells were defined as cells with chromatin condensation and fragmentation on fluorescence microscopy [24]. We examined at least 1000 cells per specimen and identified apoptotic cells.

Caspase-1 and caspase-3 activity

Caspase-1 and caspase-3 activities in cell lysates were measured as described previously [25]. Briefly, treated cells were collected, washed twice with phosphate-buffered saline, suspended in extraction buffer (10 m*M* Hepes-KOH, 2 m*M* EDTA, 0.1% CHAPS, 1 m*M* phenylmethylsulfonyl fluoride, and 5 m*M* dithiothreitol), and sonicated. Lysates were centrifuged at 8000 *g* for 5 min, and the supernatants were collected. The concentration of each cell extract was determined with a protein assay (BioRad). Lysates (80 ng) were incubated at 37 °C for 1 h with 20 µl of 10 m*M* YVAD-MCA or DEVD-MCA for measurement of caspase-1 or caspase-3 activity, respectively, and the reaction was stopped by adding 1 ml cold distilled water. Before and after the reaction, the fluorescence intensity of free 7-amino-4-methylcoumarin (AMC) released from the substrates was measured with a spectrofluorometer set at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The fluorescence intensity of purified control AMC in an amount equal to that of peptide-MCA added to the reaction mixture was used as a reference (fluorescence intensity per nanomole). The amount (nanomoles) of free AMC released in the reaction mixture was calculated by dividing the increase in fluorescence intensity by the reference. The enzyme activity (nanomoles per minute per milligram of protein) of each cell lysate was determined by dividing the amount (nanomoles) of released AMC by the reaction time (60 min) and the amount of protein (milligrams) in each lysates. The relative caspase-3 activity was defined as the ratio of enzyme activity of each cell lysate to that of the control lysate.

Western blotting to detect caspase-2 and poly(ADP-ribose) polymerase

Because specific caspase-2 substrate was not available, it was difficult to assess pure caspase-2 activity directly. Therefore, we assessed it indirectly, by western blot analysis of caspase-2 cleavage. Cells treated with antitumoral drugs were pelleted at 200 *g*, washed twice in ice-cold phosphate-buffered saline, resuspended in extraction buffer (25 m*M* Tris-HCl, pH 7.6, 50 m*M* NaCl, 2% NP-40, 0.5% deoxycholic acid, 0.2% sodium dodecyl sulfate (SDS), 1 m*M* phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, and 20 µg/ml aprotinin), and sonicated. Lysates were centrifuged at 8000 *g* for 5 min, and supernatants were collected. Each protein sample (15 µg) was separated with 15% polyacrylamide/SDS gels to detect caspase-2 and with 7.5% polyacrylamide/SDS gels to detect poly(ADP-ribose) polymerase (PARP). Proteins were electrically transferred to nitrocellulose membranes (Protran ba 85; Schleicher & Schuell, Germany). After incubation with 5% skimmed milk and 0.05% Tween 20 in Tris-buffered saline to block nonspecific protein-binding sites, the membranes were incubated with anti-caspase-2 or anti-PARP polyclonal antibodies (Santa

Cruz Biotechnology, Santa Cruz, Calif.). The membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody and subjected to chemiluminescence detection (ECL, Amersham-Pharmacia Biotech, N.J.).

Results

Chemosensitivity of glioma cells

Under normal culture conditions, the number of viable glioma cells increased exponentially during the first 4 days after passage (normal growth) (Fig. 1A). As shown by the WST-1 colorimetric assay, cell viability decreased markedly to less than 40%, after 4 days of treatment with staurosporine (Fig. 1C) or actinomycin D (Fig. 1D) or after 5 days of treatment with cisplatin (Fig. 1E). T98G, U251, SF126, and NMCG1 cells were all sensitive to these drugs. Caspase-3 activity was 10- to 30-fold higher on days 2 and 3 in treated cultures than in untreated (day-0) cultures (Fig. 2B–D). However, in cell lines treated with ACNU, the number of viable cells did not decrease, or even increased after 6 days of treatment (Fig. 1B). Comparison of the time courses of the ACNU-treated cells (Fig. 1B) and untreated cells in normal growth (Fig. 1A) showed that ACNU had a cytostatic rather than a cytocidal effect. In cultures treated with ACNU, caspase-3 activity increased less than tenfold (Fig. 2A). Thus, actinomycin D, staurosporine, and cisplatin exerted a cytocidal effect on various glioma cell lines by activating caspase-3, whereas ACNU had only a cytostatic effect and exhibited little caspase-3 activity.

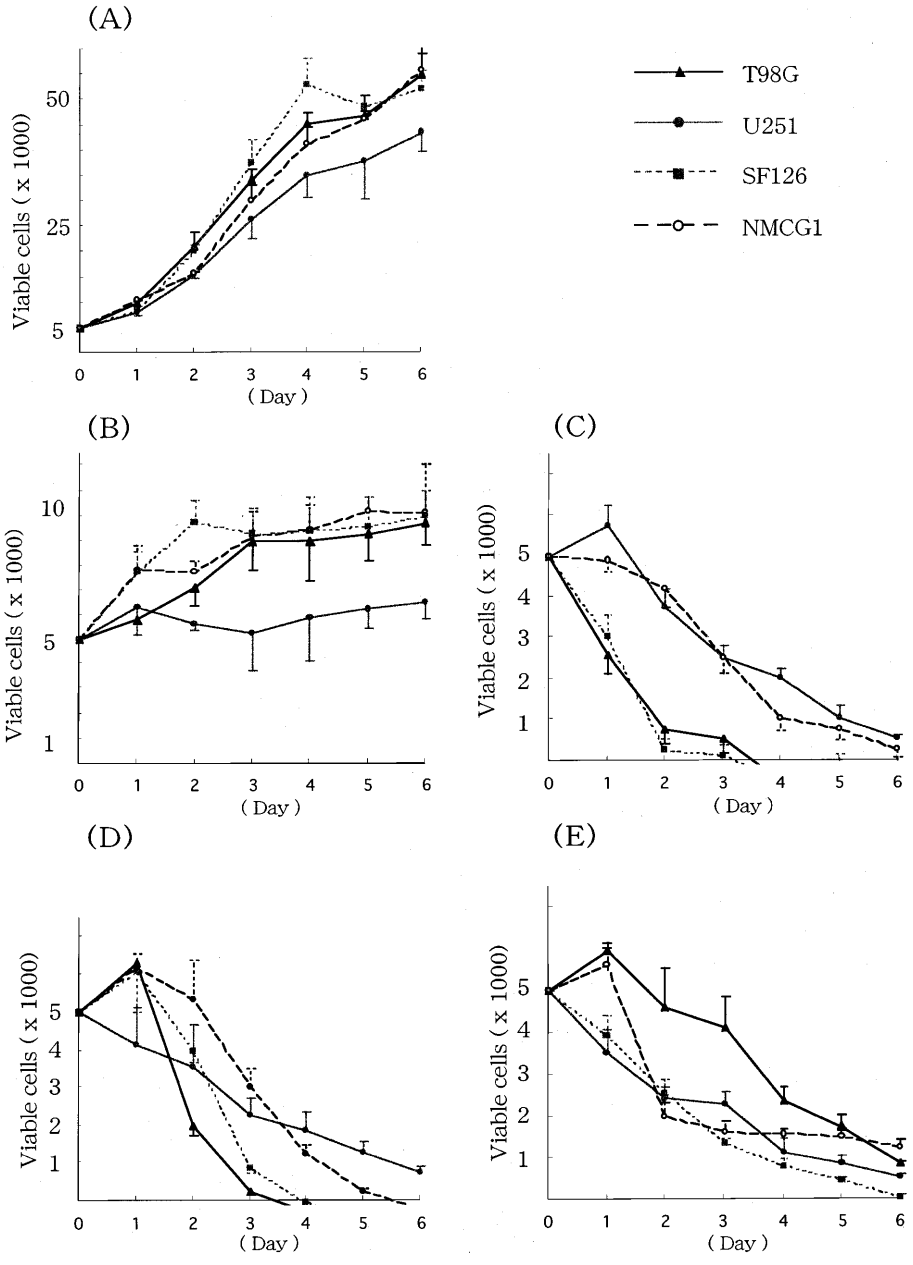

Fig. 1A–E Estimated number of viable cells after treatment with anticancer drugs. The number of viable glioma cells under normal culture conditions increased exponentially during the first 4 days after passage (**A**). The number of viable cells decreased to less than 40% within 4 days in cultures treated with 100 n*M* staurosporine (**C**) or 50 ng/ml actinomycin D (**D**). In cultures treated with 10 μg/ml cisplatin, the number of viable cells decreased to less than 40% within 5 days (**E**). In cultures treated with 100 n*M* ACNU, the number of viable cells did not decrease even after 6 days (**B**). The number of viable cells was calculated as described in Materials and methods

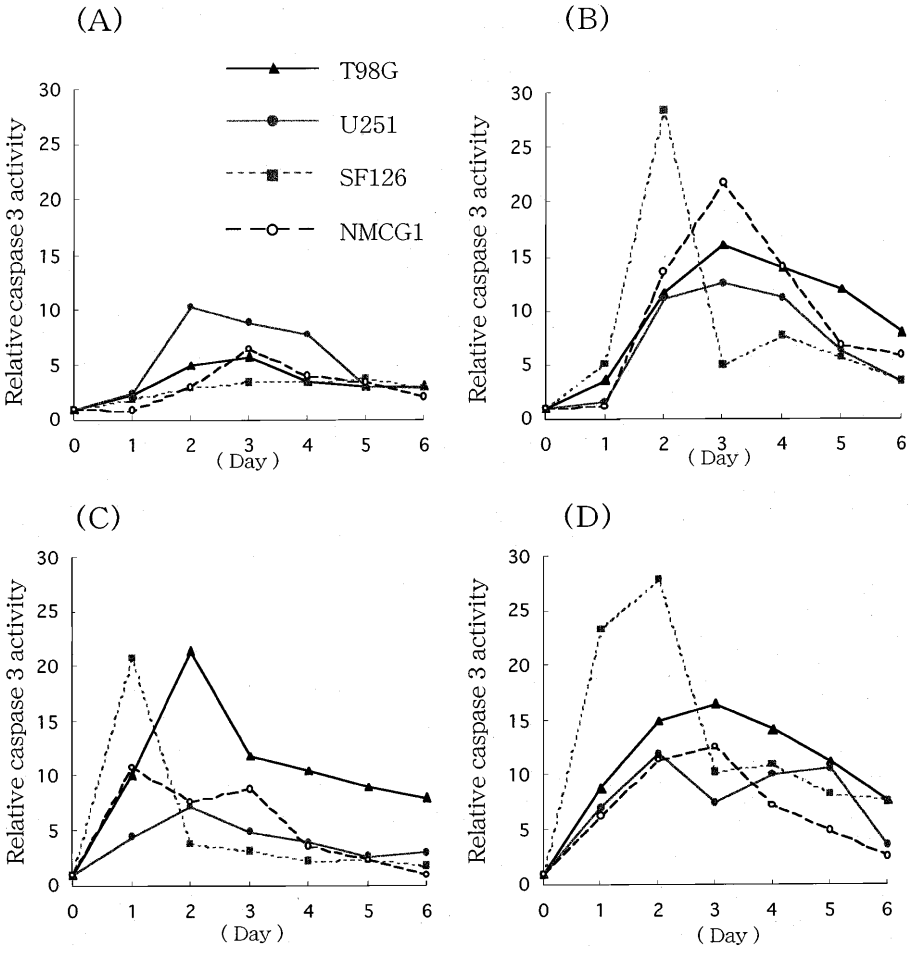

Fig. 2A–D Caspase-3 activity after treatment with anticancer drugs. Relative caspase-3 activity increased 10- to 30-fold in cells treated with staurosporine (**B**), actinomycin D (**C**) and cisplatin (**D**), but increased less than tenfold in all cells treated with ACNU (**A**). Caspase-3 activity was measured using MCA-labeled tetrapeptide substrates, which release fluorogenic AMC when digested. Relative caspase-3 activity was calculated as the caspase-3 activity of treated cells divided by that of control (untreated) cells. All experiments were performed in triplicate. Error bars designate the standard error

Morphologic features

The type of drug-induced cell death in T98G cultures was assessed by nuclear staining with Hoechst 33258 followed by fluorescence microscopy and by electron microscopy. Staurosporine (100 n*M*) and actinomycin D (50 ng/ml) induced more than 90% cell death within 2 and 3 days (Fig. 1C,D), respectively, and cisplatin (10 μg/ml) induced more than 70% cell death within 5 days (Fig. 1D). Staurosporine and actinomycin D were therefore the most potent drugs, both inducing cell death within a short period. Hoechst staining showed condensation and fragmentation of chromatin, which are typical features of apoptosis. We calculated the apoptotic and nonapoptotic cell data from at least 1000 counted cells per specimens in fluorescence microscopy. Swelling and loss of chromatin and nuclei were observed in less than 10% to 40% of the dead cells, which were therefore classified morphologically as nonapoptotic. Apoptosis occurred in about 70% of cells treated with 50 n*M* staurosporine (Fig. 3B), 90% of cells treated with 50 ng/ml actinomycin D (Fig. 3D), and 60% of cells treated with 10 μg/ml cisplatin (Fig. 3F). Electron microscopic examination of cells treated with staurosporine, actinomycin D, or cisplatin also revealed condensation of chromatin (Fig. 3C,E,G). Thus, most of the dead T98G cells in cultures treated with these drugs had morphologic features consistent with apoptosis.

Activation of caspase-2 and -3 in staurosporine-induced cell death

To assess the role of caspases in cell death, we examined the activities of caspase-1, -2, and -3 in T98G cultures treated with staurosporine. In cultures treated with 100 n*M* staurosporine, the number of viable cells began to decrease after 12 h of treatment, reaching less than ≈20%, as shown by the WST-1 assay, within 48 h (Fig. 1C). Caspase-3 activity started to increase after 6 h of treatment and rapidly reached levels 20-fold higher than those in control cells (time 0) after 15 h (Fig. 4E). Caspase-1 (ICE) activity did not increase at all during cell death (data not shown). Caspase-2 (Ich-1) started to be cleaved after 6 h of treatment. Immunoblot analysis showed a decrease in the 45-kDa inactive proenzyme of caspase-2, indicating activation of the enzyme (Fig. 4A). Proteolytic cleavage of the 116-kDa PARP, a common substrate of caspase-2 and -3, into an 89-kDa species was observed after 6 h of treatment, consistent with the activation of caspase-3 and the cleavage and activation of caspase-2 (Fig. 4C).

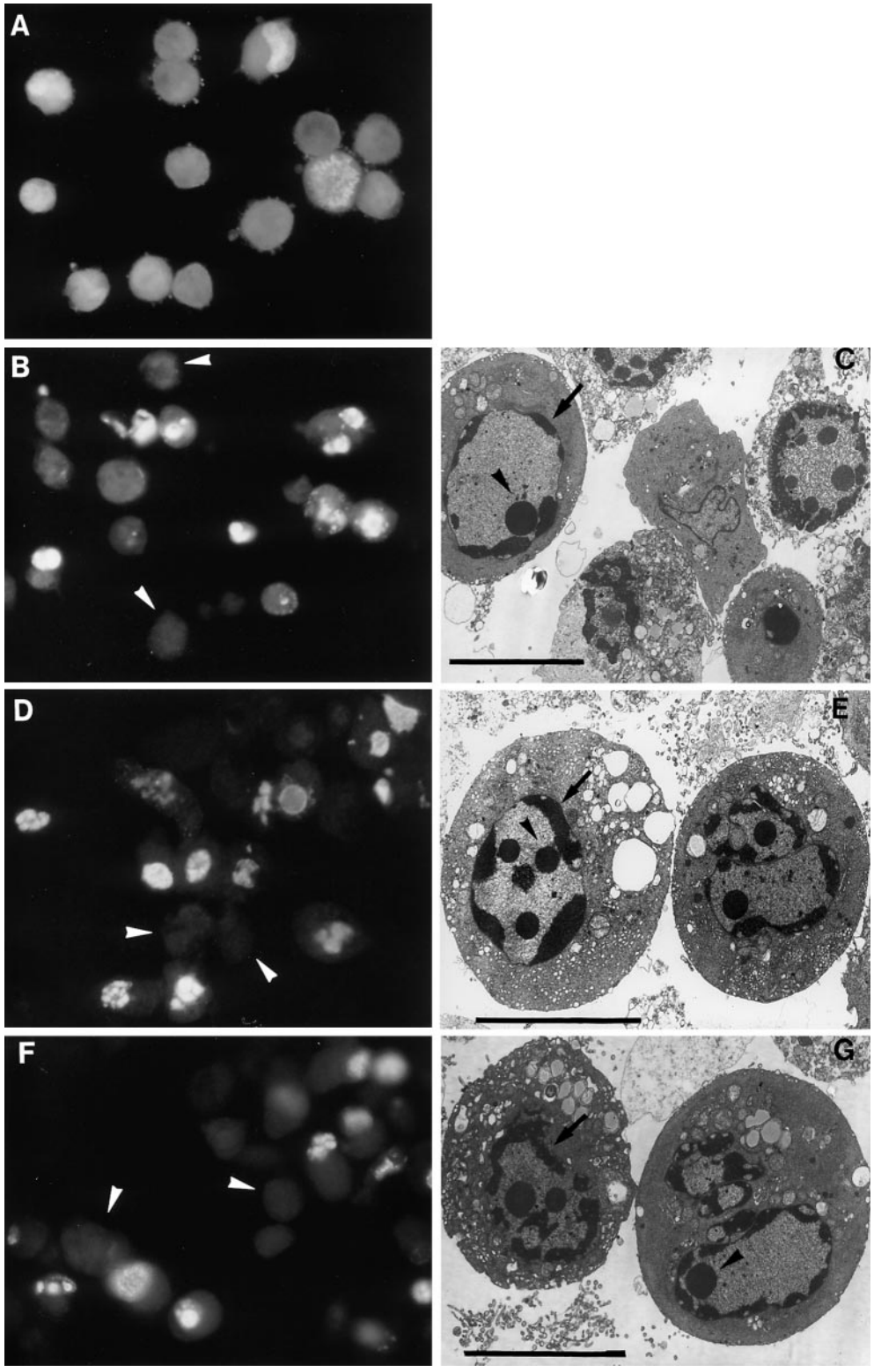

Fig. 3A–G Morphological changes of T98G cells undergoing apoptosis after treatment with anticancer drugs. Fluorescence photomicrographs show apoptotic T98G cells after treatment with 100 n*M* staurosporine (**B**), 50 ng/ml actinomycin D (**D**), or 10 μg/ml cisplatin (**F**) and cells from untreated control cultures (**A**). Condensation and fragmentation of chromatin typical of apoptosis were observed. We determined the apoptotic and nonapoptotic cells by examining at least 1000 counted cells per specimens under the fluorescence microscope. Swelling and loss of chromatin and nuclei were observed in less than 10% to 40% of the dead cells (*arrowhead*). Approximately 70% of cells treated with 50 n*M* staurosporine (**B**), 90% of cells treated with 50 ng/ml actinomycin D (**D**), and 60% of cells treated with 10 μg/ml cisplatin (**F**) were apoptotic (original magnifications, ×600). Cells were stained with 20 μ*M* Hoechst 33258. Electron microscopic examination revealed condensation of chromatin (*arrows*) and nucleoli (*arrowheads*) in T98G cells treated with staurosporine (**C**), actinomycin D (**E**), or cisplatin (**G**). Bars indicate 10 mm. The majority of dead cells have the morphologic features of apoptosis

Inhibitory effects of DEVD-CHO and ZVAD-FMK

To evaluate the effects of the caspase-3 inhibitors DEVD-CHO and ZVAD-FMK [26] on staurosporine-induced apoptosis, cells were incubated with 20 μ*M* of the inhibitors 6 h before the addition of 100 n*M* staurosporine. Caspase-3 and also caspase-1 (data not shown) activities did not change at all in staurosporine-treated cells in the presence of these inhibitors (Fig. 4E). In staurosporine-treated cultures not exposed to inhibitors, the number of viable cells decreased to less than 20% of control at time 0 (Fig. 4F). In cultures pre-

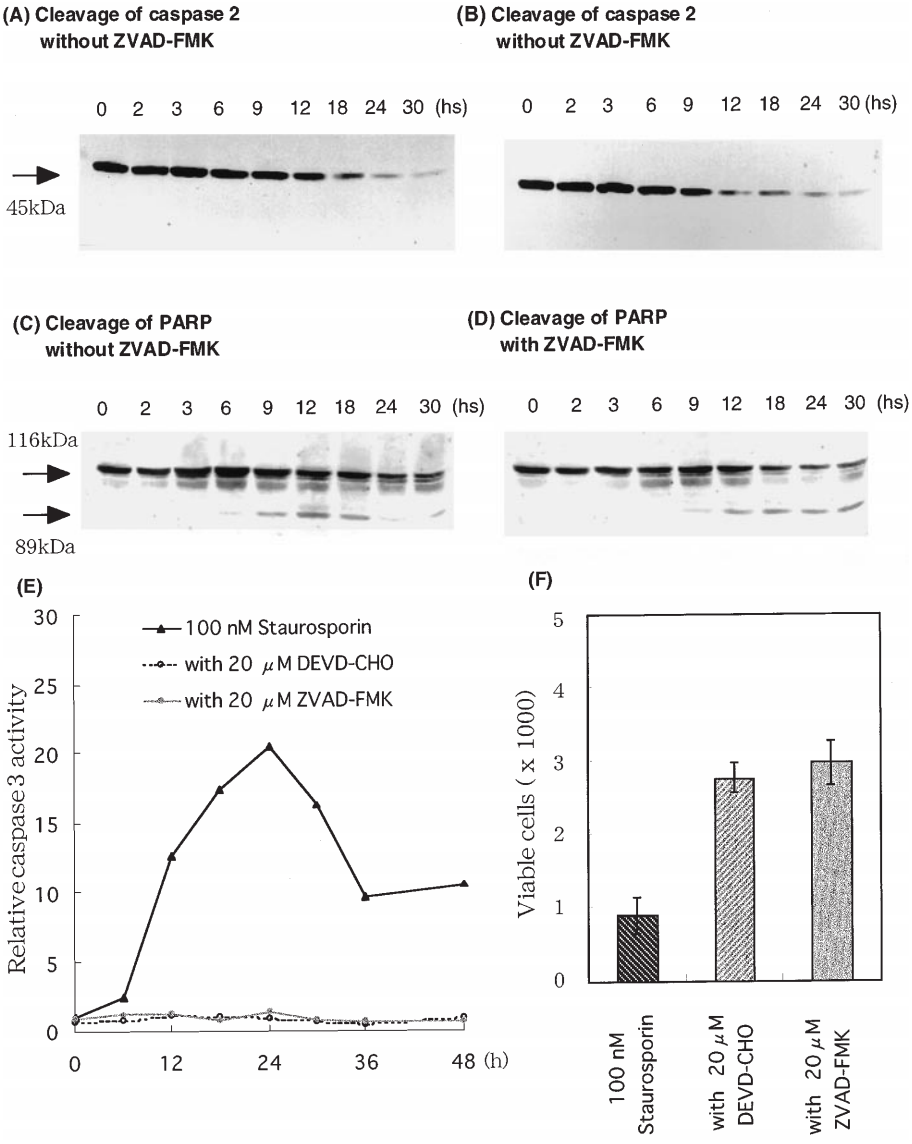

Fig. 4A–F Activation of caspase-2 and -3 during staurosporine-induced apoptosis. Western blots show cleavage of caspase-2 and PARP during the course of apoptosis induced in T98G cells by 100 n*M* staurosporine (**A**,**C**). The 45-kDa inactive proenzyme of caspase-2 begins to undergo cleavage and decrease after 6 h of treatment (**A**). Proteolytic cleavage of 116-kDa PARP into an 89-kDa fragment starts after 6 h of treatment (**C**), in accordance with activation of caspase-3 (**E**) and caspase-2 (**A**). Preincubation with 20 μ*M* ZVAD-FMK for 6 h suppressed the activation of caspase-3 (**E**) but not of caspase-2 (**B**). ZVAD-FMK did not inhibit cleavage of PARP (**D**). Caspase-1 was not activated at all with or without ZVAD-FMK (data not shown). In cultures pretreated with ZVAD-FMK and DEVD-CHO and then treated with 100 n*M* staurosporine for 2 days, the number of viable cells remained higher than in cultures not pretreated with caspase inhibitors (**F**)

treated with DEVD-CHO or ZVAD-FMK, the number of viable cells decreased only to about 70% or 60% of the number in control cultures, respectively, after 48 h of treatment (Fig. 4F), although caspase-3 activity was completely suppressed (Fig. 4E). Despite the presence of these inhibitors, caspase-2 was cleaved (activated) after 6 h of treatment (Fig. 4B), and proteolytic cleavage of PARP was observed after 9 h of treatment (Fig. 3D), indicating that caspase-2 plays an alternate role in the execution of apoptosis in staurosporine-induced apoptosis in glioma cells.

Discussion

This study demonstrated that actinomycin D and staurosporine had stronger apoptosis-inducing potential than ACNU in glioma cells and that the apoptosis was mediated by the activation of caspase-2 and -3. This study also demonstrated that that ACNU exerted a cytostatic rather than a cytocidal effect and did not activate caspase-3 significantly, which was essential to cell death. Electron and fluorescence microscopy revealed that the majority of dead cells treated with actinomycin D, staurosporine, or cisplatin had morphologic features consistent with apoptosis. Staurosporine and actinomycin D were the drugs that most potently activated caspase-2 and -3.

CENUs such as ACNU and BCNU have been the mainstays of treatment for glioblastomas [2–4] because they can cross the blood brain barrier (BBB) and be taken up by tumors in vivo [6, 7]. The cell lines we used in this study were all negative for O^6-methylguanine-DNA methyltransferase (MGMT) expression, which is responsible for CENU resistance, and they were sensitive to CENUs in terms of colony-forming ability as previously described [21]. However, they were rather resistant to CENUs in terms of committing apoptosis as

was shown in the present study. The discrepancy between these findings should be elucidated. In the field of radiation and chemotherapeutic oncology, cell death has been classified into two categories: interphase death and reproductive or mitotic death. Interphase death most likely corresponds to acute necrosis and apoptosis (cytocide). Reproductive death is defined as termination of cell proliferation irrespective of cell viability and thus includes dying senescent cells (cytocide) and viable G_0 phase (resting) cells that retain the potential to reproduce when circumstances improve (cytostasis).

Because colony-formation assays cannot discriminate between the two types of cell death, they provide a single estimate that includes both cytostatic and cytocidal effects. As a result, the strictly cytocidal effects of irradiation and chemotherapeutic agents cannot be accurately assessed with this technique. The conflicting data obtained by colony-formation assay and apoptosis assay may imply that CENUs are likely to have strong cytostatic effects and weak cytocidal effects against glioma cells. Actinomycin D and staurosporine had a potent cytocidal effect on glioma cells in vitro, but before applying the drugs to brain tumors in vivo, drug delivery and BBB permeability also have to be considered. To our knowledge, it has not been previously been reported that actinomycin D and staurosporine cannot penetrate the BBB. Even if these drugs cannot penetrate the BBB, they can be used together with BBB-opening drugs [27, 28]. Moreover, the BBB is often damaged in gliomas [29]. Thus, it is possible that these drugs can be taken up by gliomas in vivo. Obviously, in vivo studies will be required to evaluate the efficacy of these drugs on gliomas.

For the estimation of the cytocidal potential of chemotherapeutic agents, evaluating caspase activities as well as directly measuring glioma cell viability was very helpful. Both caspase-2 and caspase-3 were activated in actinomycin D- and staurosporine-induced cell death in glioma cells. Their activation led to proteolytic cleavage of PARP and resulted in apoptosis. Many studies have shown that caspase-3 plays the most critical role in apoptosis induced in glioma cells by chemotherapeutic agents [30–33]. It has also been reported that DEVD-CHO and ZVAD-FMK inhibit apoptosis completely by blocking the processing of caspase-3 [34, 35]. Caspase-3 is composed of two subunits of 17 and 12 kDa. When cells are exposed to apoptotic stimuli, the inactive 32-kDa proenzyme of caspase-3 is cleaved into these two subunits and is then activated. Our studies showed that caspase-3 is activated during apoptosis induced by antitumoral drugs in glioma cells in vitro and is followed by proteolytic cleavage of PARP. However, even when the caspase-3 activity was completely suppressed by two specific inhibitors of caspase-3, DEVD-CHO and ZVAD-FMK, cell death was not fully inhibited, suggesting that other caspases might be activated in the apoptotic process.

Caspase-2 has been reported to be activated in various types of apoptosis [36–40]. When cells are exposed to apoptotic stimuli, caspase-2 (45 kDa) is cleaved into three fragments of 15, 18, and 12 kDa. The last two fragments are active subunits [38]. The polyclonal antibody for the carboxyl terminus of caspase-2 used in this study recognizes the 45-kDa caspase-2 proenzyme but not the active subunits. Our results show that the cleavage of caspase-2 progressed even in the presence of the two inhibitors of caspase-3. Although ZVAD-FMK is a general inhibitor of caspases, it has the minimal inhibitory effect on caspase-2 among ten caspases [41]. Cleavage of caspase-2 was not inhibited in the presence of 20–100 μM ZVAD-FMK. Our finding that PARP is cleaved despite caspase-3 suppression also suggests an alternate means of cleavage by caspase-2, related caspases or other molecules in staurosporine- and actinomycin D-induced apoptosis in glioma cells. Recent studies have revealed that caspase-2 and -3 work independently in apoptosis induced by trophic-factor deprivation in PC12 cells and sympathetic neurons [40]. The mechanism of cleavage and activation of caspase-2 remains unknown. Further investigation is needed to elucidate these issues.

In summary, in glioma cell cultures treated with actinomycin D and staurosporine, the majority of dead cells had morphologic features of apoptosis. Caspase-2 and caspase-3 were both involved in the apoptosis of these cells. We conclude that actinomycin D and staurosporine and their derivatives are most likely to be cytocidal and are potentially effective chemotherapeutic agents against glioma cells.

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