Cyclosporin A and sanglifehrin A enhance chemotherapeutic effect of cisplatin in C6 glioma cells

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Abstract. Glioma is the most common type of brain tumors in adults, and treatment of high-grade gliomas is still palliative. Studies to date have revealed only modest effect in attenuating growth of these tumors with single agent therapy, but combination treatment appears to be more effective. Cyclophilin A (CypA), a target of immunosuppressive drugs cyclosporin A (CsA) and sanglifehrin A (SFA), is an intracellular protein that has peptidyl-prolyl *cis-trans* isomerase (PPIase) enzymatic activity. Previously, we showed that overexpressed CypA induced chemoresistance in cancer cells. Here we provide evidence that combination of cisplatin with either CsA or SFA synergistically enhances apoptotic cell death in C6 glioma cells, compared with single agent treatment. Enhanced apoptotic cell death is a result of an increase in ROS generation and a decrease in intracellular glutathione levels. Consistently, CypA knockdown by siRNA also enhances cisplatin-induced apoptosis. Immunohistochemical analysis showed increased expression of CypA in human glioblastoma multiforme, but not in normal human astrocytes. CypA was also shown to be up-regulated in C6 glioma cells during hypoxia. In conclusion, CsA or SFA in combination with cisplatin synergistically enhances cisplatin-induced apoptosis in C6 glioma cells via inhibition of PPIase activity of CypA, indicating that development of new drugs that selectively inhibit the CypA PPIase activity without immune suppression may facilitate alleviation of chemoresistance in treatment of high-grade glioma.

Introduction

Glioma is the most common type of brain tumors in adults, and accounts for 25% of all brain tumors (1,2). Despite significant advances in cancer therapy, treatment of high-grade gliomas is still palliative. To date, the median survival for patients with high-grade gliomas, including glioblastoma multiforme (GBM; World Health Organization grade IV), is less than 1 year (3,4). Therefore, improved systemic treatment strategies are urgently required.

Cyclophilin A (CypA), the prototypical member of the cyclophilin family, is a highly conserved protein in mammalian cells (5). CypA possesses enzymatic peptidyl-prolyl *cis-trans* isomerase (PPIase) activity, which is essential for protein folding *in vivo*. Although little is known about the function of CypA in cancer cells, it was recently reported that CypA is overexpressed in many cancer cells (6-11). In addition, our studies, as well as others, previously showed that overexpressed CypA protects cancer cells against cellular stresses, including hypoxia and cisplatin treatment, at least in part as a result of its antioxidant function (12-15). These reports show that CypA might be important for tumorigenesis in solid tumors. CypA is also an immunophilin and a cytosolic receptor for the immunosuppressive drugs cyclosporin A (CsA) (5) and sanglifehrin A (SFA) (16). CsA binds to CypA, and this complex inhibits calcineurin, a calcium-dependent phosphatase, that regulates the expression of various cytokine genes

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in activated T cells (17). The effects of CsA and SFA have been characterized in lymphocytes, but recent data suggest that CsA also affects signaling pathways in other cell types, such as adipocytes (18) and myocytes (19,20). Recently, CsA and SFA have been reported to trigger tumor suppressor p53-mediated growth inhibition in some tumor cells (21), indicating their potential usefulness in cancer therapy.

Cisplatin is a DNA-damaging agent, and is among the most active and widely used cytotoxic anticancer drug (22). A major limitation in clinical use of cisplatin is acquisition of resistance by initially responsive tumors (23). Combinational use of cisplatin with other drugs, such as perillyl alcohol, other platinum(II) compounds, or AG1478, accelerates cisplatin-induced apoptosis in several glioma cell lines (24-28). Moreover, recent studies have indicated that CsA enhances docetaxel-induced apoptosis in human gastric carcinoma cells (29). In this study, we demonstrate that either CsA or SFA significantly improves the anticancer effects of cisplatin by enhancing cisplatin-induced apoptosis in C6 glioma cells. The molecular mechanism responsible for this enhanced apoptosis is closely related to inhibition of CypA's protective activity in cancer cells. We also show that CypA is over-expressed in GBM cells relative to normal astrocytes. Thus, the current study will facilitate opening new avenues of therapy in patients with high-grade gliomas.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and 2'-7'-dichlorodihydrofluorescein diacetate (DCF-DA) were purchased from Invitrogen Life Technologies (Grand Island, NY), cisplatin, 3-(4,5-dimethyl-thiasol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-acetyl-L-cysteine (NAC) and Hoechst 33342 were purchased from Sigma Aldrich (St. Louis, MO). Cyclosporin A (CsA) was purchased from Calbiochem (Darmstadt, Germany). Sanglifehrin A (SFA) was obtained from Novartis Pharma AG (Basel, Switzerland). Antibodies against poly (ADP-ribose) polymerase-1 (PARP), cytochrome *c* and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against caspase-3 was purchased from Assay Designs (Ann Arbor, MI). Antibodies against CypA and glial fibrillary acidic protein (GFAP) were from Abcam (Cambridge, MA). GenePORTER was purchased from Genlantis (San Diego, CA).

Cell culture. C6 rat glioma cells were grown in 10-cm diameter dishes containing DMEM supplemented with heat inacti-vated 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were seeded in 6-well culture plates at a density of $1x10^4$ cells/ml for the microscopic assessment of cell death.

MTT assay. Cell viability was evaluated using the MTT conversion assay in a 12-well plate. The culture medium was replaced with 1 ml medium containing 0.5 mg/ml MTT and incubated for 60 min at 37˚C. The blue colored tetrazolium crystals resulting from mitochondrial enzymatic activity on MTT substrate were solubilized with 150 μl DMSO. The optical density was read at 595 nm in a microplate reader

(Bio-Rad, Richmond, CA). Cell survival was expressed as percentage of absorbance relative to that of untreated cells.

Hoechst 33342 staining. Cells were incubated for 30 min with Hoechst 33342 loading dye and fixed for 20 min in 4% formaldehyde. After washing with cold PBS three times, stained cells were monitored using the confocal laser micro-scope (Meta 510, Zeiss, Thornwood, NY). Apoptotic cells were identified by nuclear condensation and fragmentation.

Analysis of cellular ROS levels. ROS were measured using DCF-DA dye. The cells were grown to 80% confluence in complete medium, treated with or without cisplatin (30 μM), loaded with 10 μM DCF-DA at 37˚C for 30 min and then resuspended in 1 ml of PBS. Fluorescence was measured by flow cytometer (Beckman Coulter Flow Cytometry). The mean DCF fluorescence intensity was measured with excitation at 488 nm and emission at 525 nm. Untreated cells were used as a reference for ROS levels.

Glutathione determination. The cells were washed in PBS and harvested by scraping in 5% MPA for the determination of intracellular glutathione (GSH) levels. After homogeni-zation, the homogenates were incubated for 30 min on ice and centrifuged for 20 min at 18000 g to discard precipitated proteins. GSH content was assessed via HPLC (30). The levels of GSH were determined via comparison with standards and normalized to protein content.

Immunohistochemistry. Formalin-fixed, paraffin-embedded human tissues, including GBM and morphologically normal brain derived from surgical procedures and autopsy, respec-tively, were obtained from the Department of Neurosurgery at the First Hospital of Jilin University in China under approval by the Jilin University Institutional Review Board. Whole tissue sections (5-μm thick) were mounted on conventional charged glass slides. All sections were deparaffinized in three changes of xylene, and rehydrated through a descending series of ethanol. Heat antigen retrieval was done by immersing the section in 0.01 mol/l sodium citrate (pH 6.0) in a pressure cooker (Nordic Ware, Minneapolis, MN) placed in a 1100-W microwave set to 'high' for 15 min. Following endogenous peroxidase blocking with a 0.03% hydrogen peroxide solution for 5 min, 5% normal goat serum was applied for 30 min to block non-specific protein binding sites. The sections were then incubated for 1 h at room temperature with rabbit polyclonal anti-CypA or mouse polyclonal anti-GFAP antibodies (1:1000). A biotin-free immunohisto-chemical staining detection technique was used for antibody visualization (EnVision+ System, HRP Rabbit kit, Dako, Carpinteria, CA). Subsequently, the sections were incubated for 30 min at room temperature with the goat anti-rabbit or horse anti-mouse secondary antibody conjugated with peroxidase-labeled polymers. The sections were visualized with application of 3,3-diaminobenzidine substrate chromagen solution. Pooled non-immune irrelevant rabbit or mouse IgG (Vector Laboratories, Burlingame, CA), applied at the same final concentration, was used as a negative control for all samples. Sections of morphologically normal human hepatic tissue were used as positive tissue controls for CypA expres-
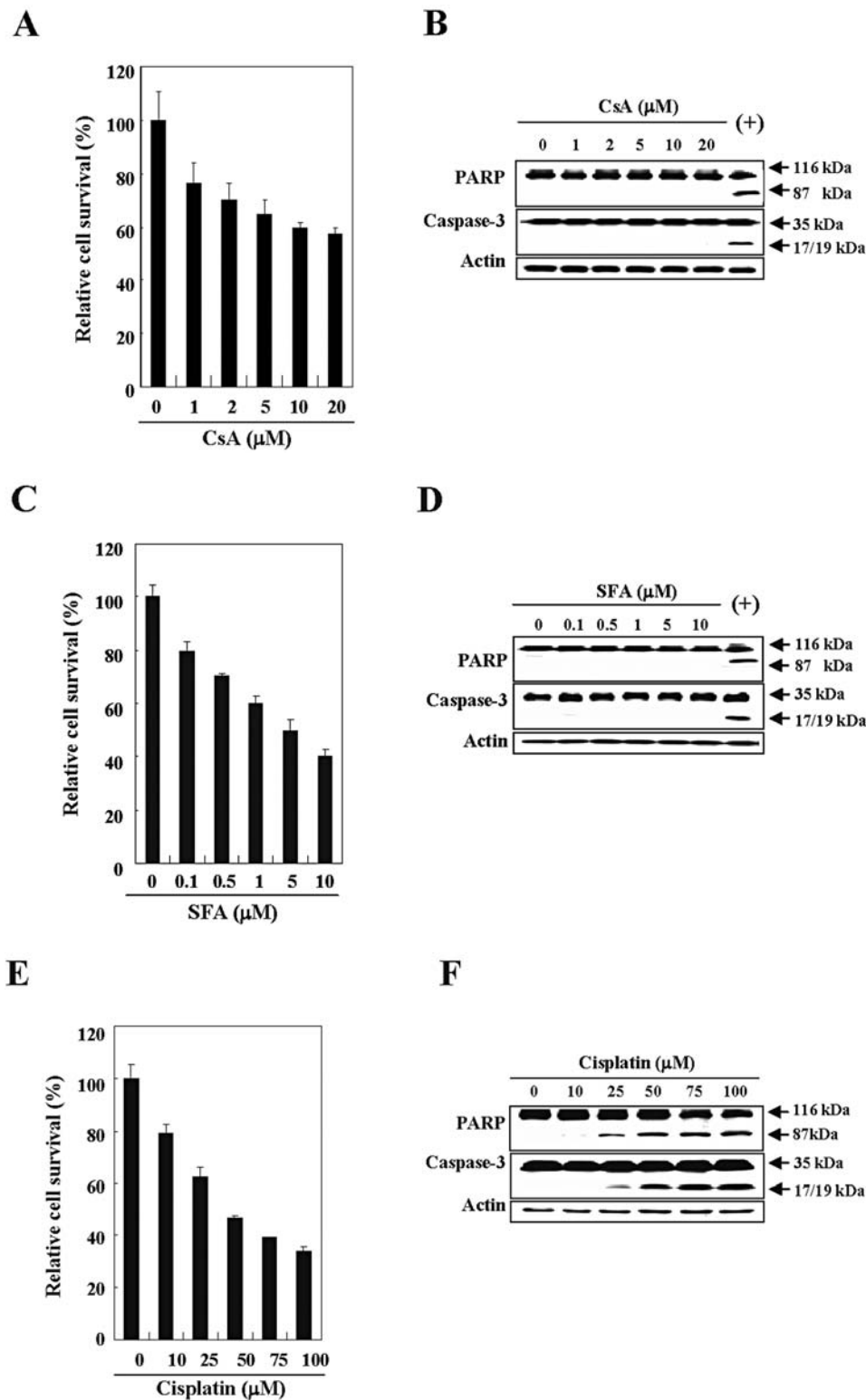
Figure 1. Dose responsiveness of C6 glioma cells. C6 cells were treated with the indicated concentrations of CsA (A), SFA (C) or cisplatin (E), and cell viability was determined via the MTT assay at 60 h. Data represent the means ± SE of at least three independent experiments. C6 cells were treated in the same ways, and then equal amounts of whole-cell extracts were resolved by SDS-PAGE and analyzed by Western blot analysis with antibodies specific to caspase-3 or PARP (B, D and F).

sion. Rabbit anti-vimentin antibody (Santa Cruz Biotechno-logy) was used as a positive assay control.

Peptidyl prolyl cis-trans isomerase assay. A modified method was adopted in order to determine PPIase activity in a coupled assay with chymotrypsin, as previously described (31). The

peptide substrate N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide was dissolved in trifluroethanol with 470 mM LiCl to create a 100-mM stock solution, which was then diluted to 4 mM immediately prior to use. GST-CypA was diluted in 50 mM HEPES and 86 mM NaCl, pH 8.0 (PPIase buffer), yielding a 2-μM stock. In one cubic centimeter cuvette, 10 μl of protein

(20 nM final concentrations) was added to 860 μl of PPIase buffer. The reaction was initiated via addition of 30 μl (120 μM) of peptide substrate followed by rapid mixing and the measurement of the absorption at 400 nm with an Amersham Biosciences spectrophotometer.

Preparation of total cellular fraction. Cells were washed three times with PBS and harvested in buffer (pH 7.4 50 mM Tris-HCl, 1% nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 200 mM PMSF, 200 mM NaF, 100 mM Na_3VO_4). Cellular debris was removed via 15 min of centrifugation at 14000 g. Whole cell extracts were snap-frozen in liquid nitrogen and stored at -80˚C until use for immunoblotting.

Small interfering RNA (siRNA). siRNAs specific to either CypA (CypA-siRNA) or control sequence (control-siRNA) were prepared by Eurogene tech (Intron Biotechnology, GyeongGi-do, Korea). siRNA (0.5 μg) was transfected into cells using GenePorter transfection reagent. siRNA target sequences were as follows: CypA-siRNA (sense, 5'-UGACU UCACACGCCAUAAUdTdT-3'; antisense, 5'-AUUAUGGC GUGUGAAGUCAdTdT-3') and control-siRNA (universal negative control). The efficiency of siRNA-based interference of CypA was monitored by immunoblot.

Western blot analysis. Cell extracts were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Transfer of proteins was assessed by Ponceau S red staining. After blocking, the membrane was incubated with the indicated primary antibody followed by incubation with a secondary antibody. Unless specified, actin protein was immunoblotted to standardize the amount of sample proteins for the Western blot analysis.

Statistical analysis. Results were expressed as means ± standard errors (SE) from at least three independent experiments. Statistical analysis was performed by using Student's t-test. Unless otherwise indicated, P<0.05 was deemed significant.

Results

Cyclosporin A, sanglifehrin A and cisplatin suppress C6 glioma cell proliferation. Although CsA and SFA were initially identified for their immunosuppressive activity, they were later shown to have direct antiproliferative effects on some tumor cells (32). Therefore, we wanted to determine if CsA or SFA could enhance cisplatin-induced apoptosis in C6 glioma cells. First, we observed the effects of CsA, SFA, and cisplatin on proliferation of C6 glioma cells using the MTT assay (Fig. 1A, C and E). As expected, CsA, SFA and cisplatin inhibited the proliferation of C6 glioma cells in a dose-dependent manner.

Activation of caspases during apoptosis results in the cleavage of various cellular substrates including caspase-3 and PARP (33,34). To determine if CsA, SFA or cisplatin induced apoptosis in C6 cells, we examined cleavage of caspase-3 and PARP by Western blot analysis. The cleaved form of caspase-3 and PARP proteins were undetectable in cells treated with CsA or SFA alone, even at relatively high concentrations 60 h after treatment (Fig. 1B and D). However, the cleaved forms of caspase-3 and PARP proteins increased in a dose-dependent manner 60 h after cisplatin treatment, suggesting that cisplatin directly induces apoptosis in C6 glioma cells (Fig. 1F).

The combination of cisplatin and either cyclosporin A or sanglifehrin A induces the synergistic effects on growth inhibition and apoptosis of C6 glioma cells. We next investigated the effects of cisplatin in combination with either CsA or SFA on growth inhibition of C6 glioma cells. To examine the effect of single-agents in combination we used doses of 5 μM CsA (Fig. 1A), 0.1 μM SFA (Fig. 1C) and 10 μM cisplatin (Fig. 1E), all of which produced only partial responses on their own.

Combining 10 μM cisplatin with either 5 μM CsA or 0.1 μM SFA produced a synergistic effect on growth inhibition of C6 glioma cells as measured by the MTT assay (Fig. 2A). Since oxidative stress is responsible for the induction of apoptosis by cisplatin (35), the effect of the antioxidant NAC on cisplatin-induced growth inhibition was studied. Addition of 5 mM NAC markedly rescued the combination-induced cell growth inhibition (Fig. 2A).

Apoptotic changes were analyzed via observation of cellular and nuclear morphological changes, as well as detection of PARP and caspase-3 cleavage. Cells treated with 10 μM cisplatin and either 5 μM CsA or 0.1 μM SFA in combination were more rounded up, and exhibited more blebbing than those treated with cisplatin, CsA, or SFA alone (Fig. 2B). Nuclear staining with Hoechst 33342 demonstrated the typical features of apoptosis, including chromosomal condensation and nuclear fragmentation (Fig. 2C). Clearly, combining cisplatin with either CsA or SFA induced massive apoptosis, which was attenuated by pretreatment with NAC. As demonstrated above, CsA or SFA alone caused no cleavage of caspase-3 or PARP. However, combination of cisplatin with either CsA or SFA led to increased cleavage of caspase-3 and PARP, which is attenuated by NAC pretreatment (Fig. 2D), indicating that cisplatin-induced oxidative stress is required for combination-induced apoptosis.

Combined treatment of cisplatin with cyclosporin A or sanglifehrin A causes an increase in ROS generation and a decrease in intracellular glutathione levels. Using the fluorescent probe DCF-DA, we studied the effect of combining cisplatin with either CsA or SFA on ROS generation. Compared with drugs alone, the combination treatments caused a clear increase in intracellular ROS levels (Fig. 3A). This increased ROS was reversed by 5 mM NAC pretreatment. Consistent with increased ROS, combinational treatment also reduced intracellular GSH levels (Fig. 3B) compared with cisplatin, CsA, or SFA alone, further supporting oxidative stress as the trigger of apoptotic cell death following combination treatment.

Cyclosporin A and sanglifehrin A sensitize cells to cisplatin-induced apoptosis by suppressing PPIase activity of CypA. CsA (36) and SFA (37) have been shown to inhibit PPIase activity of cyclophilins. They also inhibit calcineurin activity
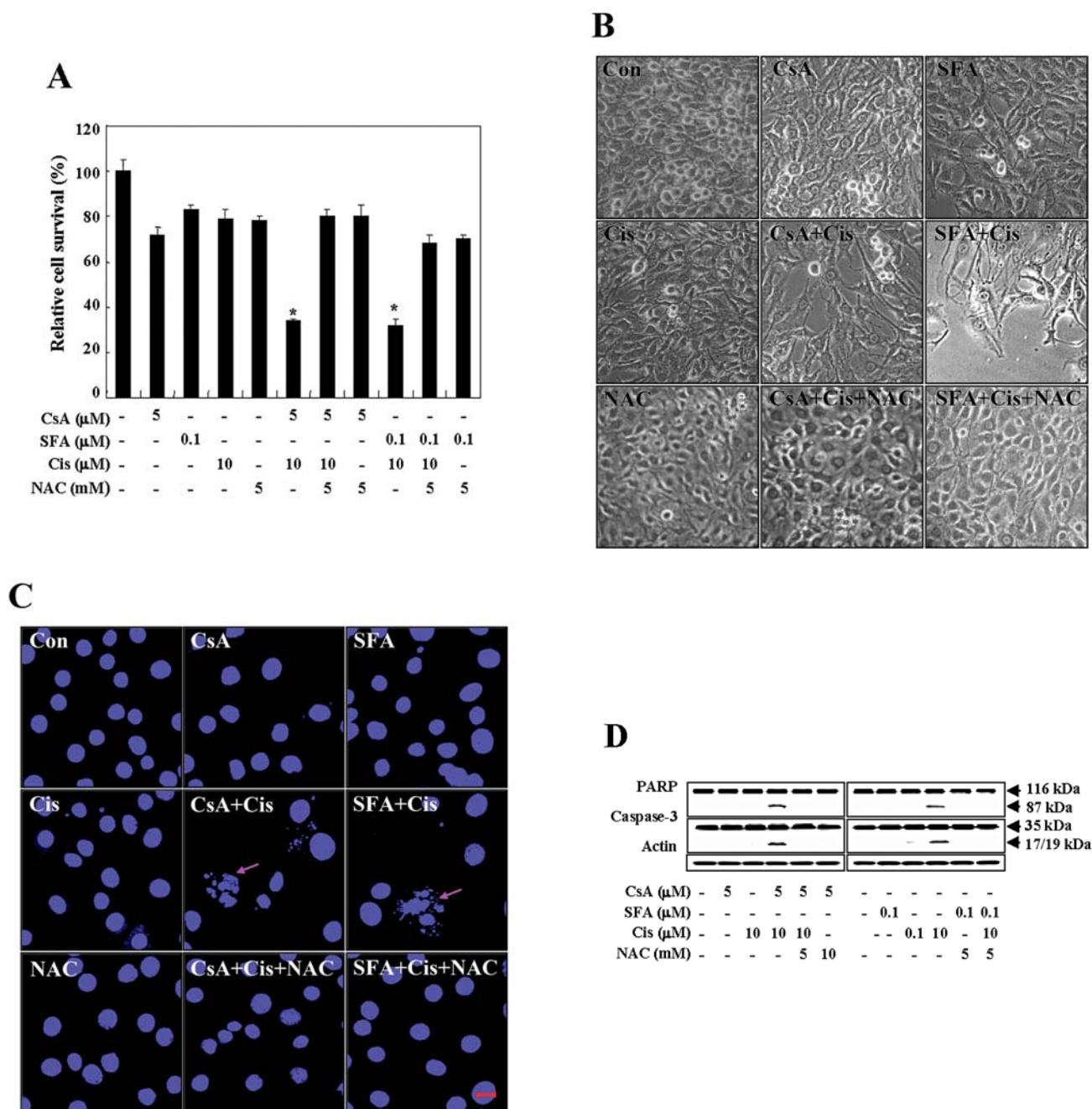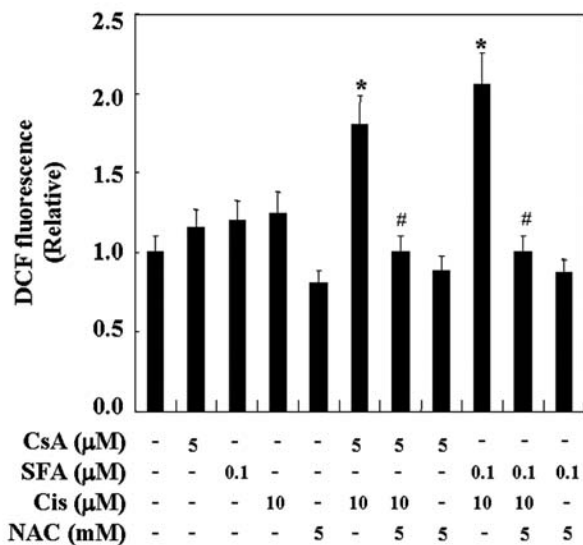
Figure 2. The combination of cisplatin with either cyclosporin A or sanglifehrin A induces the synergistic effects in C6 glioma cells. C6 cells were pre-incubated in the presence or absence of 5 mM NAC for 1 h and then treated with 5 μM CsA, 0.1 μM SFA or 10 μM cisplatin for additional 60 h. (A) Cell viability was assayed by MTT analysis. Data represent the means ± SE of at least three independent experiments. *P<0.05, versus cells treated with drugs alone. (B) Cell number was assessed by phase contrast microscopy. (C) After cells were stained with 10 μM Hoechst 33342 for 15 min, nuclear staining images were captured by confocal microscopy. Arrow, apoptotic cells. Scale bars, 20 μm. (D) Western blot analysis with antibodies specific to caspase-3 or PARP.

after binding to CypA (17). NIM811, a CsA analogue, is also known to block the PPIase activity of CypA but not to be involved in the calcineurin pathway (14). To confirm the effect of CsA, SFA or NIM811 on inhibition of PPIase activity of CypA, we performed PPIase activity assays. As shown in Fig. 4, 20 nM GST-CypA fusion protein showed an increase in the rate of substrate isomerization, which was strongly inhibited by addition of 100 nM CsA, 100 pM SFA or 100 nM NIM811, suggesting that each compound is capable of inhibiting the PPIase activity of CypA at quite low concentrations.

Next, to determine if CsA and SFA enhance cisplatin-induced apoptosis by suppressing PPIase activity of CypA, we performed CypA knockdown experiments using CypA siRNA. The expression of CypA was almost 90% suppressed by specific siRNA interference (Fig. 5A). Knockdown of CypA and treatment with NIM811 showed results similar to those of CsA and SFA treatment in the MTT assay (Fig. 5B), suggesting that inhibition of PPIase activity of CypA is critical to the chemotherapeutic effects of CsA and SFA in combination with cisplatin. Hoechst 33342 staining (Fig. 5C)
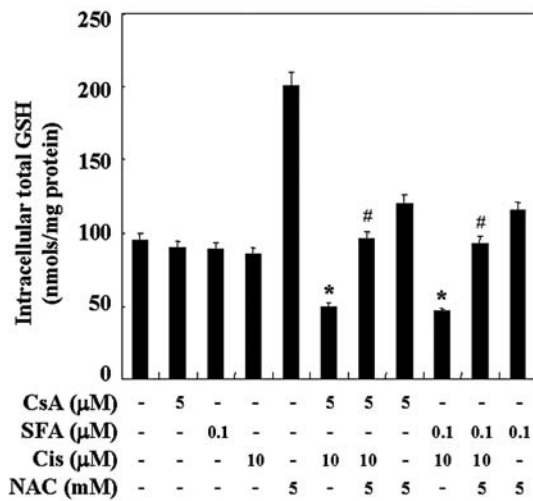
A

B

Figure 3. Combined treatment with cisplatin and either cyclosporin A or sanglifehrin A causes an increase in ROS generation and a decrease in intracellular glutathione levels. C6 cells were pre-incubated in the presence or absence of 5 mM NAC for 1 h and then treated with CsA, SFA or cisplatin for additional 60 h. Thereafter, the intracellular ROS levels (A) and total GSH levels (B) were assessed, as described in Materials and methods. Data represent the means ± SE of at least three independent experiments. *P<0.05, versus cells treated with drugs alone. $^#$P<0.05, versus cells treated with cisplatin and either CsA or SFA in combination.

Figure 4. Inhibition of PPIase activity of cyclophilin A. Recombinant CypA at 20 nmol was preincubated at 10˚C for 5 min in 3.5 ml of PPIase assay buffer with the concentrations of CsA, SFA or NIM811 as described in Materials and methods. The assay was started by the addition of peptide followed by rapid mixing and the measurement of the absorption at 400 nm with an Amersham Biosciences spectrophotometer.

and Western blot analyses of PARP and caspase-3 (Fig. 5D) in CypA siRNA transfected cells further confirmed this observation.

CypA expression in human glioblastoma multiforme and C6 glioma cells, but not in normal human astrocytes and up-regulation of CypA expression during hypoxia in C6 glioma cells. Human samples including pathologically confirmed GBM and normal specimens were used to compare the expression of CypA by immunohistochemisty staining. To identify astrocytes, tissues were stained for GFAP, a sp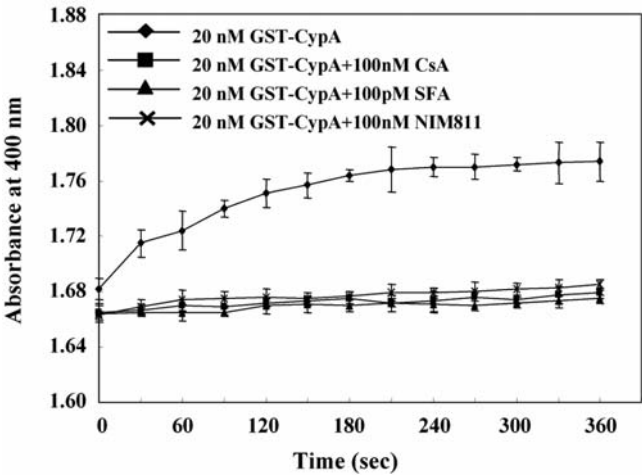ecific marker for astrocytes. Analysis of normal human brain sections revealed complete lack of CypA immunostaining within astrocyte cells. In contrast, strong immunoreactivity of CypA was observed in human GBM samples (Fig. 6A). Since one of the important pathological characteristics of GBM is increased hypoxia-induced necrosis (38), we next examined CypA protein expression in C6 glioma cells under normoxic or hypoxic conditions by Western blot analysis. There was a clear increase in CypA protein after cells were grown for 12 h under hypoxic conditions compared with normoxic conditions (Fig. 6B). Furthermore, we monitored the effect of CsA and cisplatin on the viability of C6 glioma cells under hypoxic conditions for 48 h. As shown in Fig. 6C, the viability of C6 glioma cells was slightly decreased after 48 h of hypoxia exposure. Interestingly, pre-treatment with 5 μM CsA before hypoxia exposure decreased cell survival significantly, while 10 μM cisplatin did not show significant changes. Finally, combinational pre-treatment of cisplatin with CsA before hypoxia synergistically inhibited C6 glioma cell survival under hypoxic conditions. In contrast, all these treatments were less effective after cells were grown under hypoxia for 48 h (data not shown), confirming that up-regulating CypA during hypoxia induces chemoresistance, as previously shown (15).

Discussion

Recent studies have proposed strategies incorporating the use of cisplatin in combination with other drugs such as perillyl alcohol, other platinum(II) compounds, and AG1478 to accelerate cisplatin-induced apoptosis in several glioma cell lines (24-28). Furthermore, combination of CsA with other anticancer drugs has been shown to enhance chemothera-peutic efficacy in leukemia and gastric carcinoma cells (29,39-41). In this study, we found that combining CsA or SFA with cisplatin synergistically enhances cisplatin-induced apoptosis in C6 glioma cells, suggesting that these combi-
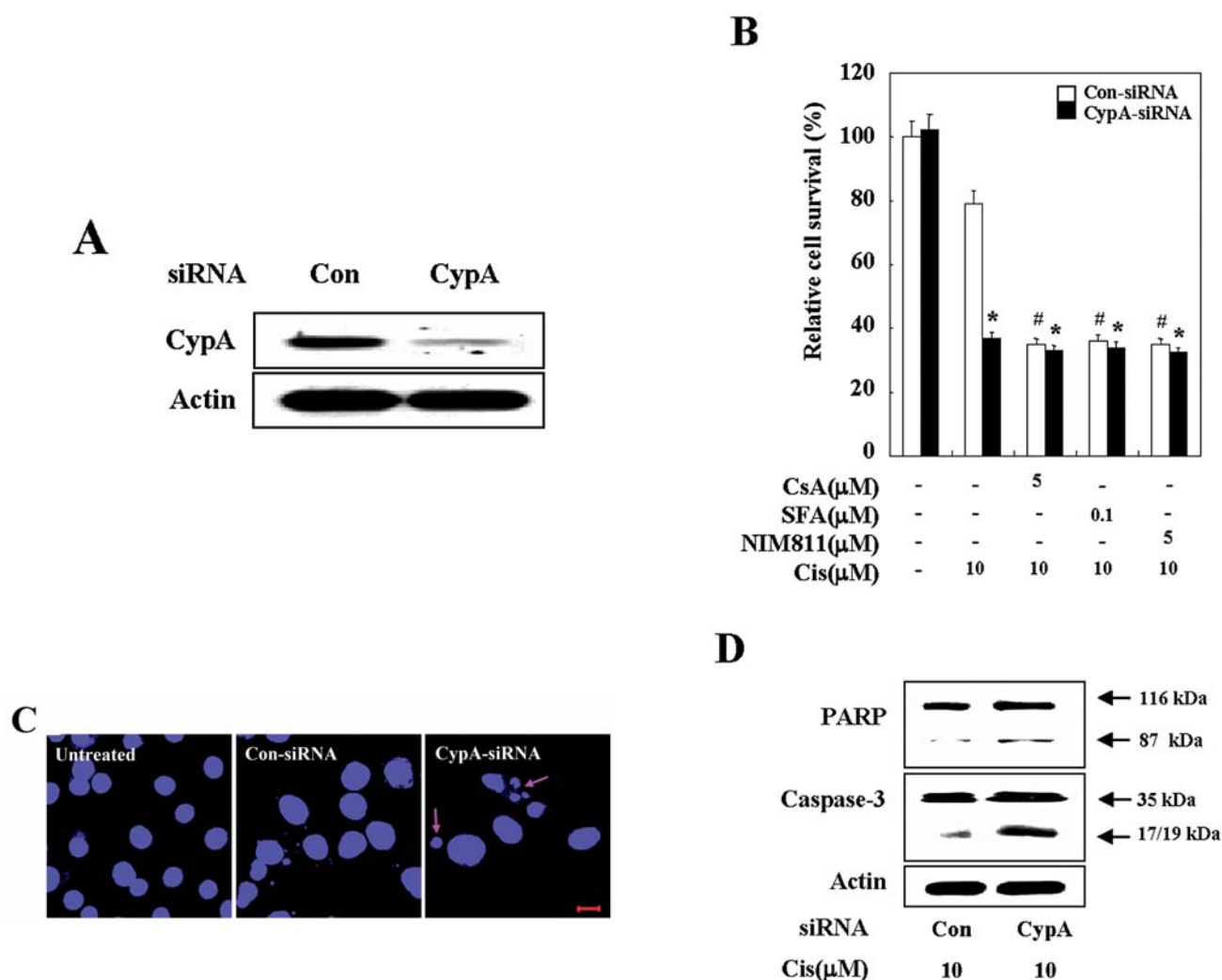
Figure 5. Effect of knockdown of cyclophilin A on cisplatin-induced apoptosis. (A) CypA expression level was monitored by immunoblotting in C6 glioma cells transfected with CypA-siRNA. (B) C6 cells transfected with siRNA specific to CypA were treated with 10 μM cisplatin and either 5 μM CsA or 0.1 μM SFA in combination or alone for 60 h. Cell viability was assayed by MTT. Data represent the means ± SE of at least three independent experiments. #P<0.05, versus con-siRNA transfectants. *P<0.05, versus CypA-siRNA transfectants. (C) After cells were stained with 10 μM Hoechst 33342 for 15 min, nuclear staining images were captured by confocal microscopy. Arrows indicate apoptotic cells. Scale bars, 20 μm. (D) Western blot analysis with antibodies specific to caspase-3 or PARP.

national therapies may enhance the therapeutic efficacy of cisplatin in GBM patients.

The molecular mechanisms of CsA chemotherapeutic effects have been delineated. Some groups report that CsA enhances the effect of anticancer drugs in leukemia cells by blocking the multidrug resistance pump (MDR1) (39-41). However, other groups suggest that CsA enhances taxotere-induced apoptosis mainly through an MDR1-independent pathway in GCTM-1 cells (29). Consistent with the latter group, we demonstrate here that CsA and SFA enhance cisplatin-induced apoptosis via inhibition of PPIase activity of CypA. Our conclusion is based on the following analyses. First, siRNA-based CypA knockdown aggravated cisplatin-induced apoptosis. Second, combination of cisplatin with the CsA analogue NIM811, a selective inhibitor of CypA's PPIase activity without affecting calcineurin activity, also increased cisplatin-induced apoptosis. This finding indicates that the enhanced apoptosis is due to inhibition of the PPIase activity of CypA, but not due to inhibition of the calcineurin pathway, which is known to be immunosuppressive (42). Therefore,

although we could not completely rule out the possibility that CsA suppresses cisplatin efflux in MDR-expressing cells, we concluded that the combination-induced synergistic effect is due to inhibition of the CypA PPIase activity. Since CypA possesses antioxidant activity (14), and the antioxidant NAC significantly abrogated the combination-induced apoptosis, it is likely that an increase in ROS levels and a decrease in GSH levels (Figs. 2-4) is a result of induced oxidative stress following inhibition of PPIase activity of CypA.

Astrocytes have been reported not to express CypA (43-45). Consistent with this report, we did not detect CypA expression in human normal astrocytes. However, for the first time we have demonstrated here that CypA is expressed in GBM and C6 glioma cells, which indicates that CypA expression is turned on during the process of transformation. Another factor in the increased expression of CypA in GBM may be hypoxia. We show here that CypA expression is up-regulated by hypoxia in C6 glioma cells, and have previously shown that CypA up-regulation is mediated by the HIF-1α transcription factor in several cancer cells under hypoxic conditions
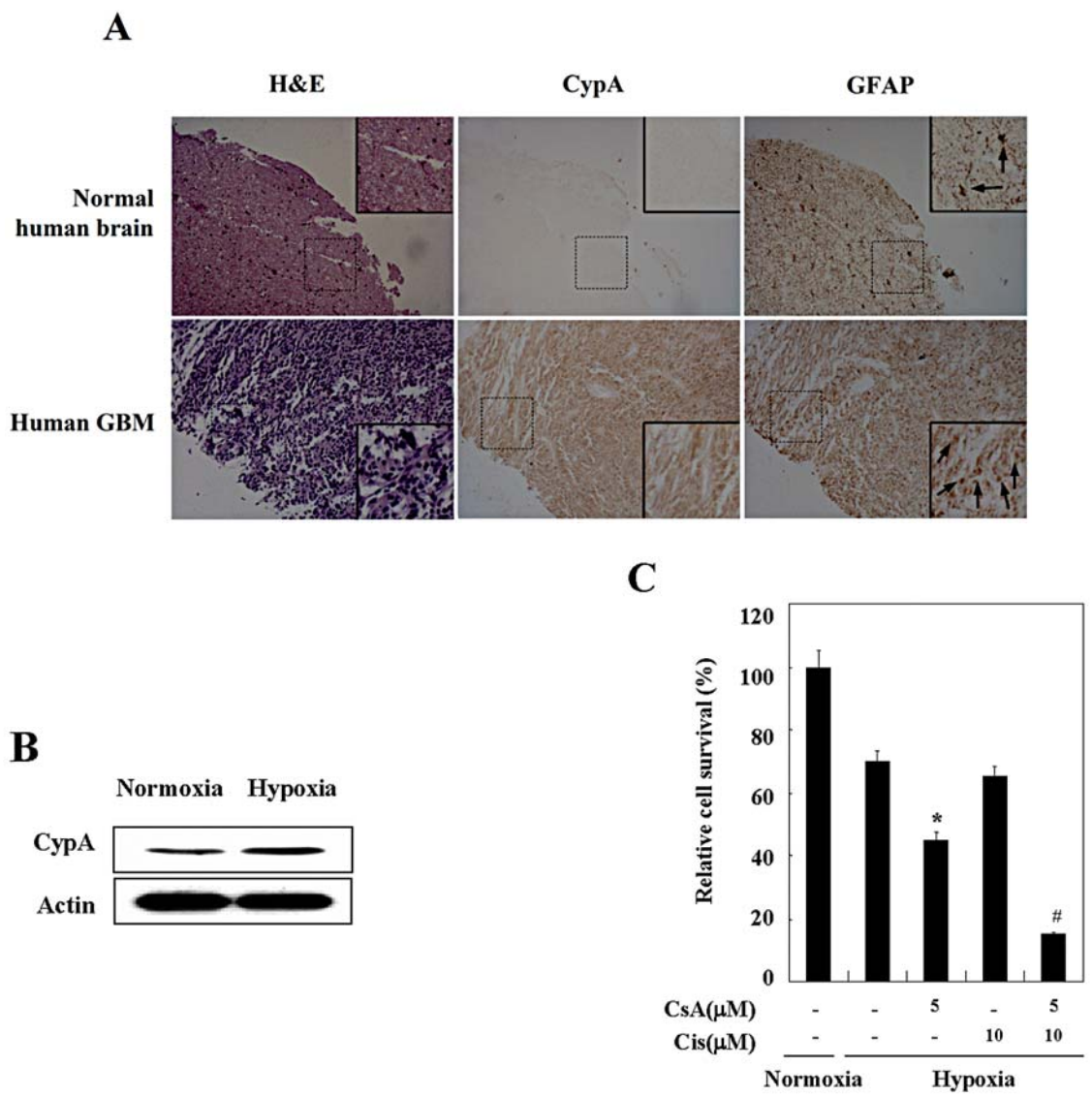
Figure 6. Cyclophilin A is detected in GBM tissues by immunohistochemistry and up-regulated by hypoxia. (A) GBM and normal human brain tissues were stained with either H&E or antibody specific to CypA or GFAP (arrow). The normal glial tissues of the brain show no expression of CypA protein. In contrast, high levels of CypA protein expression are observed in GBM. Original magnifications, x100. Insets, magnifications, x400. (B) Total cell lysates (10 μg) extracted after exposure to hypoxia for 12 h were subjected to Western blot analysis. (C) C6 glioma cells treated with CsA or cisplatin were exposed to hypoxia for 48 h. Data represent means ± SE of results from three independent experiments. *P<0.05, versus cells exposed in hypoxia alone. $^\#$P<0.05, versus cells treated with CsA or cisplatin alone under hypoxic condition.

(15). In addition, one of the remarkable characteristics of GBM is the occurrence of hypoxia in the central region of tumors, which induces necrosis (38). These results led us to suggest that CypA may be epigenetically activated during transformation and further up-regulated by hypoxic conditions in GBM cells.

CsA shows a limited permeability through the intact blood-brain barrier, but a breakdown of this barrier has been observed in brain tumors. High-grade astrocytomas secrete the vascular endothelial growth factor, which increases angiogenesis, opening of tight junctions in highgrade astrocytoma microvessels, and endothelial cell permeability (46). The leaky blood-brain barrier in brain tumors may result in similar CsA concentrations in the brain as in blood. Therapeutic efficacy of CsA for experimental gliomas was apparent at clinically relevant concentrations. A daily CsA dose of 3-7

mg/kg was used for many months without significant side effects in diverse patients including transplantation patients (47,48) and patients with severe psoriasis (49). Therefore, the strategy of combining cisplatin with either CsA or SFA at lower concentrations could be used for cancer patients in the clinic. One intriguing point to consider is that CsA and SFA may rather lessen survival rates of GBM patients due to their immunosuppressive effects. Therefore, we suggest new drugs that inhibit only PPIase activity of CypA without impairing immune activity need to be developed to stimulate cisplatin-induced apoptosis. Although NIM811, a non-immunosuppressive CsA analogue, is currently in phase II clinical trials for the treatment of hepatitis C, its effects on cancer cells still need to be largely investigated.

In conclusion, our data show that CsA or SFA synergistically enhances the chemotherapeutic effect of cisplatin in

C6 glioma cells. Therefore, exploring the full potential of CypA's PPIase activity as a possible target for combination treatment with anticancer drugs could facilitate the alleviation of chemoresistance in high-grade glioma treatment. We are currently studying the synergistic effect of cisplatin combination therapy with CsA or SFA in a murine high-grade glioma model, and attempting to develop inhibitors of CypA's PPIase activity without immune intervention. Further understanding of this combination chemotherapy and development of more selective CypA's PPIase inhibitors will aid in better treatment outcomes for glioma patients.

Acknowledgements

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