Celecoxib Increased Expression of 14-3-3σ and Induced Apoptosis of Glioma Cells

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Abstract. *Background: Celecoxib, a cyclooxygenase-2 inhibitor, has been found to inhibit the proliferation of several kinds of cancer cells; however, the effects of celecoxib on glioma cells are not clear. Materials and Methods: A172 glioma cells were treated with various concentrations of celecoxib for 4, 24 or 48 h. Cytotoxic drug effects were studied by MTT (3-[4,5-dimethylthiazole-2-yl]-2,5- diphenyltetrazolium bromide)-based colorimetric assay, and celecoxib-induced apoptosis of glioma cells was investigated by FACScan. Western blot analysis was used to study celecoxib effects on the expression of mitogen-activated protein kinases (MAPKs), p53, p21, 14-3-4σ, Bcl-2 and Bax. Caspace-3 activity in glioma cells was analyzed by caspase activity assay. Results: Celecoxib exerted cytotoxic effects upon and induced apoptosis of the A172 glioma cells in a concentration and time-dependent manner ($p<0.05$). Celecoxib had no effects on expression of MAPKs, Bax, or p21; however, it increased expression of p53 and 14-3-4σ, and reduced expression of Bcl-2. Celecoxib also increased the activity of caspace-3 in glioma cells. The apoptotic fraction of A172 cells induced by 24-h treatment with 100 μM celecoxib was reduced from 39% to 23% by pretreatment with caspace-3 inhibitor (DEVD-CHO) ($p<0.001$). Conclusion: The results suggest that celecoxib induced cytotoxicity and apoptosis in this line of glioma cells and that such effects might be related to activation of p53 and 14-3-3σ, reduced Bcl-2 and Bcl-2/Bax ratio, and increased caspace-3 activity.*

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Prostaglandins are important mammalian physiological mediators that affect blood vessel tone, platelet aggregation and immune reactions (1). In addition to normal physiological functions, prostaglandins may influence the development of cancers that overexpress prostaglandins relative to their production levels in normal tissues (1). Cyclooxygenase (COX) is the rate-limiting enzyme in the synthesis of prostaglandins from arachidonic acids (2). COX has two isoforms, COX-1 and COX-2 (2). COX-1, constitutively expressed in many tissues, is important for the production of prostaglandins responsible for normal physiological functions (2). In contrast, COX-2 usually is not expressed or is minimally expressed in normal tissues; it is induced after stimulation by proinflammatory stimuli, mitogens, or hormones (2). Increased expression of COX-2 has been found in colon, pancreatic, prostate, gastric, and head and neck cancer (3-8). Furthermore, glioma cells have shown increased expression of COX-2 (4, 9), with such expression correlated with the degree of malignancy: more cells in high-grade gliomas express COX-2 than do these in low-grade gliomas (1, 4, 9). High expression of COX-2 in gliomas suggests that COX-2 inhibitors might be useful for the treatment of gliomas.

As a class, non-steroidal anti-inflammatory drugs (NSAIDs) have been found to inhibit proliferation of tumor cells *in vitro* and *in vivo* (9-11). Because most NSAIDs affect both COX-1 and COX-2, they have toxicity on normal tissues, with the gastrointestinal tract being most vulnerable (9-11). Therefore, selective COX-2 inhibitors are considered better than non-selective COX inhibitors for use in the oncology setting. In the literature, several COX-2 inhibitors have suppressed proliferation and invasion of glioma cells (9, 10). Celecoxib, a COX-2 inhibitor, has also been found to exert preventive or therapeutic effects on cancer such as mesothelioma, neuroblastoma, and lung, oral cavity, gastric, and prostate cancer (12-19). However, few reports have investigated the effects of celecoxib on glioma cells (20). In this study, the

cytotoxic effects of celecoxib on glioma cells was investigated; in addition, we explored celecoxib-induced apoptosis of glioma cells and its effects on the apoptosis-related signaling pathway including mitogen-activated protein kinases (MAPKs), p53, p21, 14-3-3σ, Bcl-2, Bax, and caspace-3.

Materials and Methods

Cell line and cell culture. The cell line used in this study was the rat A172 glioma cell line (American Type Culture Collection, Manassas, VA, USA). All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37˚C in a 5% CO_2 incubator.

Cytotoxic effects of celecoxib on glioma cells. The sensitivity of A172 glioma cells to celecoxib (kindly given by Pfizer Limited, New York, NY, USA) was determined *in vitro* by an MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide)-based colorimetric assay. For this purpose, $5x10^3$ cells were seeded in triplicate wells of a flat-bottomed 24-well microtiter plate and cultured overnight prior to celecoxib treatment. The glioma cells were exposed to different concentrations (0, 1, 5, 10, 25, 50 or 100 μM) of celecoxib dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) for various time periods including 4, 24 or 48 h. Subsequent to removal of the drug, cells were incubated for a total of five days following cell seeding. The extent of cell proliferation and cell viability was then determined by MTT assay. The celecoxib concentration at which 50% of the A172 cells were killed was designated as the LC_{50}.

Analysis of celecoxib-induced apoptosis and cell cycle distribution by flow-activated cell sorter flow cytometry. Briefly, after treatment with one of the test concentrations of celecoxib (0, 1, 5, 10, 25, 50 or 100 μM) for 4, 24 or 48 h, 10^6 A172 glioma cells were trypsinized and washed twice with phosphate-buffered saline (PBS). Following this, cells were stored in 1.0 mL 80% ethanol/PBS at –20˚C for subsequent experiments. For flow-activated cell sorter flow cytometry (FACScan) analysis, cells were centrifuged at 6,000 rpm for 5 min and washed twice with PBS. Following this, cells were incubated with 0.5 mL 0.5% Triton X-100/PBS and 5 μg RNase A for 30 min, then stained with 0.5 mL 50 μg/mL propidium iodide/PBS in the dark and analyzed using FACScan flow cytometry (FACSCalibur, Becton Dickinson Immunocytometry System, San Jose, CA, USA). The cell cycle distribution of glioma cells treated with celecoxib (0, 10 or 100 μM) for 24 hours was also studied using FACScan.

Terminal deoxynucleotidyl transferase mediated dUTP nick-end label (TUNEL) staining. A total of 10^6 glioma cells cultured on Lab-Tek chamber slides (Nunc, Inc., Naperville, IL, USA) were either treated or not treated with 100 μM of celecoxib for 24 h, after which adherent cells were stained using MEBSTAIN Apoptosis Kit Direct (Immunotech, Marseille, France). All procedures were conducted according to the manufacturer's instructions. Slides were observed on a Zeiss Axioskopz epifluorescence microscope (Carl Zeiss Jena GmbH, Zeiss Gruppe, Germany). The number of positively TUNEL-stained cells was counted under the microscope, and the ratio of the number of positively TUNEL-stained cells to the total cell number in the observed field was calculated. The apoptotic fraction was determined by averaging the ratio from five independent fields. The difference in apoptotic fraction was compared between celecoxib-treated and control groups.

Whole cell extract preparation and western blot analysis. Expression of COX-2 and MAPKs including JNK1, phospho-JNK1, ERK1/2, phospho-ERK1/2, p38, phospho-p38, p53, p21, 14-3-3σ, Bcl-2 and Bax in glioma cells treated with 100 μM celecoxib for different drug-exposure times (0, 15, 30 min, 1, 4 and 24 h) was analysed by western-blot analysis. Cells were lysed in a buffer containing 20 mM HEPES (pH 7.6), 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM Na_3VO_4, 50 mM NaF, 0.5 μg/ml leupeptin, 1 μg/mL aprotinin, and 100 μg/mL 4-(2-aminoethyl) benzenesulfonyl fluoride. The cell lysate was rotated at 4˚C for 30 min and then centrifuged at 10,000 rpm for 10 min; precipitates were discarded.

The concentration of protein in the supernatant was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin used as a reference standard. For western blot analysis, cellular protein (20-50 μg) was loaded onto 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Protein bands were then transferred electrophoretically to PVDF membranes (Micron Separations Inc., Westborough, MA, USA). Membranes were probed with anti-β-actin, anti-COX-2, anti-JNK1, anti-phospho-JNK1, anti-ERK1/2, anti-phospho-ERK1/2, anti-p38, anti-phospho-p38, anti-p21, anti-14-3-3σ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Bax (Mobio, Nagoya, Japan), anti-Bcl-2 (Pharmingen, San Diego, CA, USA), or anti-p53 (Calbiochem-Novabiochem Corporation, San Diego, CA, USA) antibody, followed by a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Detection of antibody reactions was performed with Western blotting reagent ECL (Santa Cruz Biotechnology), with resultant chemiluminescence detected through exposure of the filter to Kodak Medical X-ray film (Eastman Kodak Company, Rochester, NY, USA). Differences in expression of proteins were analyzed by ABC-Tiger Gel V2.0 (software from Taigen Bioscience Corp., Taipei, Taiwan, ROC).

Caspase activity assay. Following 24-h 100 μM celecoxib treatment, cells were washed twice with ice-cold PBS and then harvested. The catalytic activity of caspace-3 was measured by use of a caspase activity assay. Briefly, 10 μg total proteins was incubated with 200 μM fluorogenic peptide substrates Ac-DEVD-AFC (Bio-Rad Lab, Richmond, VA, USA) in a 50-μL assay buffer at 37˚C for four hours. The release of 7-amino-4-trifluoromethyl coumarin was measured with a spectrofluorometer (PerSeptive Biosystems, Inc., Framingham, MA, USA) at an excitation wavelength of 390 nm and an emission wavelength of 510 nm, with all procedures being conducted according to the manufacturer's instructions.

Effects of caspace-3 inhibitor on the celecoxib-induced apoptosis of glioma cells. To investigate the effects of a caspace-3 inhibitor upon celecoxib-induced apoptosis, the glioma cells were pretreated with the caspace-3 inhibitor, DEVD-CHO (100 μM, cell permeable; Biosource International, Camarillo, CA, USA), for two hours immediately prior to 24-h 100 μM celecoxib treatment. The apoptotic fraction of cells was analyzed with FACScan flow cytometry as described above.

Statistical analyses. One-way analysis of variance (ANOVA) by Scheffe's multiple comparison was used for statistical analyses of the extent of cytotoxicity and cellular apoptosis of glioma cells

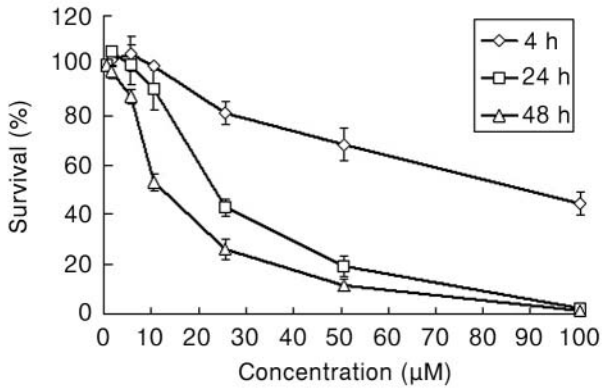

Figure 1. *Cytotoxic effects of celecoxib on glioma cells. A total of 5x10^3 A172 cells were seeded in triplicate wells in flat-bottomed 24-well microtiter plates. Subsequently, cells were exposed to celecoxib (0, 1, 5, 10, 25, 50 or 100 μM) for 4, 24 or 48 h. Following removal of the drug, cells were incubated for a total of five days following cell seeding. Cell proliferation and viability were then determined by an MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide)-based colorimetric assay. Percentage survival was defined as the optical density at a given drug concentration divided by the optical density for controls treated with dimethyl sulfoxide (DMSO) alone, multiplied by 100. Each point is the average of three independent trials (nine determinations for each concentration); data are presented as mean±standard deviation.*

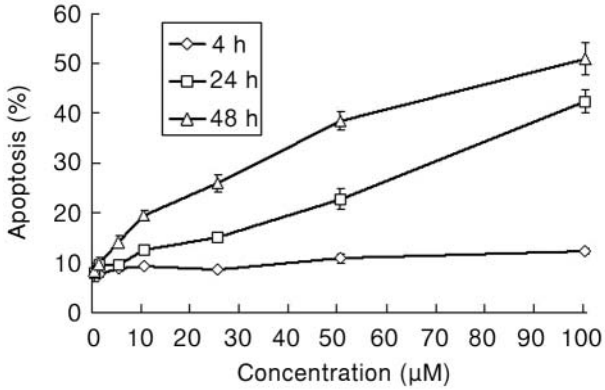

Figure 2. *Apoptosis of glioma cells subsequent to celecoxib treatment. After treatment with celecoxib (0, 1, 5, 10, 25, 50 or 100 μM) for 4, 24 or 48 h, the apoptotic fraction of A172 glioma cells was analyzed with flow-activated cell sorter (FACS) flow cytometry. Each point is the average of three independent trials (nine determinations for each concentration); data are presented as mean±standard deviation.*

induced by various regimens. The Student's *t*-test was used for analysis of the difference in apoptotic fraction revealed by the TUNEL stain between the celecoxib-treated and control groups. Statistical significance was accepted as $p<0.05$.

Results

Cytotoxic effects of celecoxib on A172 glioma cells. To comprehend the inhibitory effects of celecoxib on A172 glioma cells, a cytotoxicity assay was conducted as described above. Figure 1 reveals the survival curve of glioma cells treated with different concentrations of celecoxib for different exposure times. The relative viability of cells subsequent to celecoxib treatment proved to be both concentration- and time-dependent. As celecoxib dose or drug exposure time increased, the proportion of cells that were killed increased significantly ($p=0.004$). Survival curves shifted to the left when longer drug-exposure times were used. LC_{50} was 92.2, 22.7 and 11.8 μM following 4, 24 and 48 hours of celecoxib treatment, respectively. The LC_{50} of celecoxib was in the micromolar range with the drug exposure times selected for this study.

Induction of apoptosis by celecoxib. Apoptosis of glioma cells treated with celecoxib was analyzed by FACS flow cytometry; the apoptotic fraction was found to be directly related to both the selected celecoxib concentration and the drug exposure time (Figure 2). As celecoxib dose increased, the proportion of apoptotic cells also increased ($p=0.013$). Glioma cells treated with dimethyl sulfoxide for 4, 24, or 48 hours (controls) showed apoptotic fractions of 7.9% to 8.3% (mean); by contrast, treatment with 100 μM (the highest dosage we tested in this study) celecoxib for 4, 24 or 48 h induced apoptotic fractions of 12.3%, 42.4% and 51.0% (mean), respectively. Because treatment with 100 μM celecoxib elicited significant apoptosis of glioma cells, such a dose was selected for subsequent experiments. In order to study the degree of apoptosis of glioma cells treated with 100 μM celecoxib for 24 hours, a TUNEL staining method was used. The apoptotic fraction of cells treated with celecoxib was 36.5%±8.7%, significantly higher than the fraction for the control cells (6.6%±3.2%) ($p=0.001$).

Celecoxib induced G2/M accumulation of glioma cells. Following treatment with 0, 10 or 100 μM celecoxib for 24 hours, the cell cycle distribution was analyzed by way of FACS flow cytometry. For control glioma cells (treated with 0 μM celecoxib), the fraction of cells in the G0/G1-, S- and G2/M-phase was 57.5%±3.1%, 11.3%±1.0% and 31.2%±2.2%, respectively. Glioma cells treated with 10 μM celecoxib showed no significant change in cell cycle distribution (G0/G1-phase: 57.0%±2.8%, S-phase: 12.4%±1.5%, G2/M-phase: 30.6%±1.5%) compared with the distribution for controls ($p=0.774$). In contrast, 100 μM celecoxib treatment reduced the S and increased the G2/M fractions (G0/G1-phase: 54.8%±5.1%, S-phase: 1.1%±0.8%, G2/M-phase: 44.2%±4.4%) ($p=0.0001$).

Celecoxib did not change expression of COX-2 or MAPKs in glioma cells. Expression of COX-2 and MAPKs including JNK-1, ERK 1/2, and p38 MAPK, phospho-JNK-1, phospho-ERK 1/2, and phospho-p38 MAPK in glioma cells

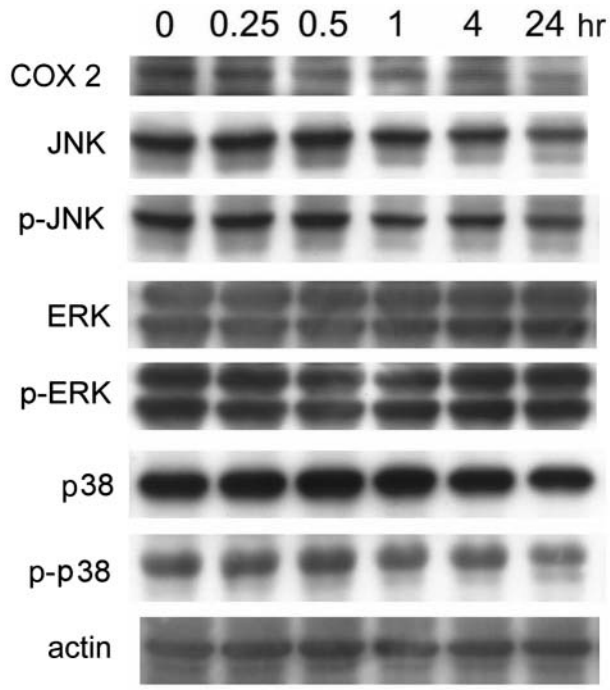

Figure 3. *Effects of celecoxib on expression of COX-2 and MAPKs in glioma cells. Western blot analysis of mitogen-activated protein kinases (MAPKs) in glioma cells treated with 100 μM celecoxib for different drug exposure times showed that celecoxib did not affect expression of COX-2 or MAPKs including JNK-1, ERK 1/2, and p38 MAPK, phospho-JNK-1, phospho-ERK 1/2, and phospho-p38 MAPK. The control was β-actin.*

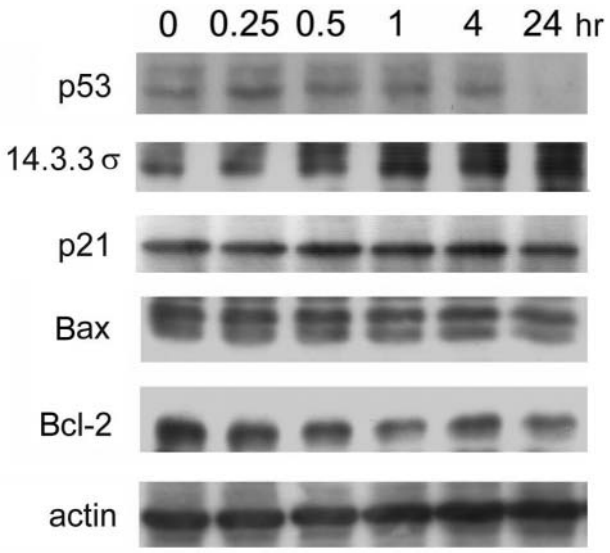

Figure 4. *Western blot analysis of Bax, Bcl-2, p53, p21 and 14-3-3σ proteins in glioma cells treated with celecoxib. Western blot analysis of the Bax, Bcl-2, p53, p21 and 14-3-3σ proteins in A172 glioma cells treated with 100 μM celecoxib for different drug exposure times. The control was β-actin.*

treated with 100 μM celecoxib for various drug exposure times was evaluated by western blot analyses (Figure 3). Celecoxib did not affect the expression of these proteins.

Celecoxib increased expression of p53, 14-3-3σ and Bcl-2, but did not change expression of p21 or Bax. Expression of p53, p21, 14-3-3σ, Bcl-2 and Bax in glioma cells treated with 100 μM celecoxib for different drug exposure times was analysed by western-blot analysis (Figure 4). Expression of p53 increased up to 15 min (2.3-fold), then decreased to baseline from 30 min to 4 h subsequent to initiation of celecoxib treatment. Expression of p53 was further reduced at 24 and 48 hours after the initiation of celecoxib treatment. In contrast, expression of p21 showed no change after celecoxib treatment. Expression of 14-3-3σ slightly increased from 1 h to 24 h subsequent to initiation of celecoxib treatment (up to 2.7-fold the value of the control). The expression of Bax showed no significant change after celecoxib treatment. In contrast, expression of Bcl-2 decreased from 15 min to 48 h subsequent to initiation of celecoxib treatment (0.82- to 0.37-fold), with the lowest level obtained one hour after the initiation of celecoxib treatment.

Celecoxib-treated cells had increased caspace-3 activity and caspace-3 inhibitor suppressed celecoxib-induced apoptosis. To elucidate the role of caspace-3 in celecoxib-induced apoptosis, the relative caspace-3 activity of celecoxib-treated cells was measured by caspase activity assay. Following 24 h of 100 μM celecoxib treatment, caspace-3 activity appeared to increase 3.4-fold compared with the corresponding value for the control. Caspace-3 inhibitor (DEVD-CHO; 100 μM) induced significantly more apoptotic cells than the control (9.5%±1.1% *vs.* 5.9%±0.7%, $p=0.005$) (Figure 5). In contrast, the apoptosis fraction significantly decreased from 38.9%±4.8% with celecoxib treatment to only 22.7%±2.5% with DEVD-CHO pretreatment ($p=0.001$) (Figure 5).

Discussion

In this study, we demonstrated significant concentration- and time-dependent celecoxib-induced cytotoxic effects for A172 glioma cells. The celecoxib LC_{50}s for the A172 glioma cells were 92.2, 22.7 and 11.8 μM after 4, 24 and 48 h of treatment, respectively. The A172 glioma-cell LC_{50} was similar to values reported for other types of cancer (12-19) and glioma cells (20). The results of the current study and previous report (20) indicate that glioma cells have a sensitivity to celecoxib similar to that for other cancer cell types reported in the literature. Celecoxib also induces apoptosis for several types of cancer cell lines (12, 13, 15-17, 19, 21). Similarly, celecoxib was also found to induce apoptosis for glioma cells in this study and a previous report (20). Celecoxib-induced apoptosis in the current study appeared to be both concentration- and time-dependent: the greater the concentration of celecoxib, the more apoptotic the exposed cells became.

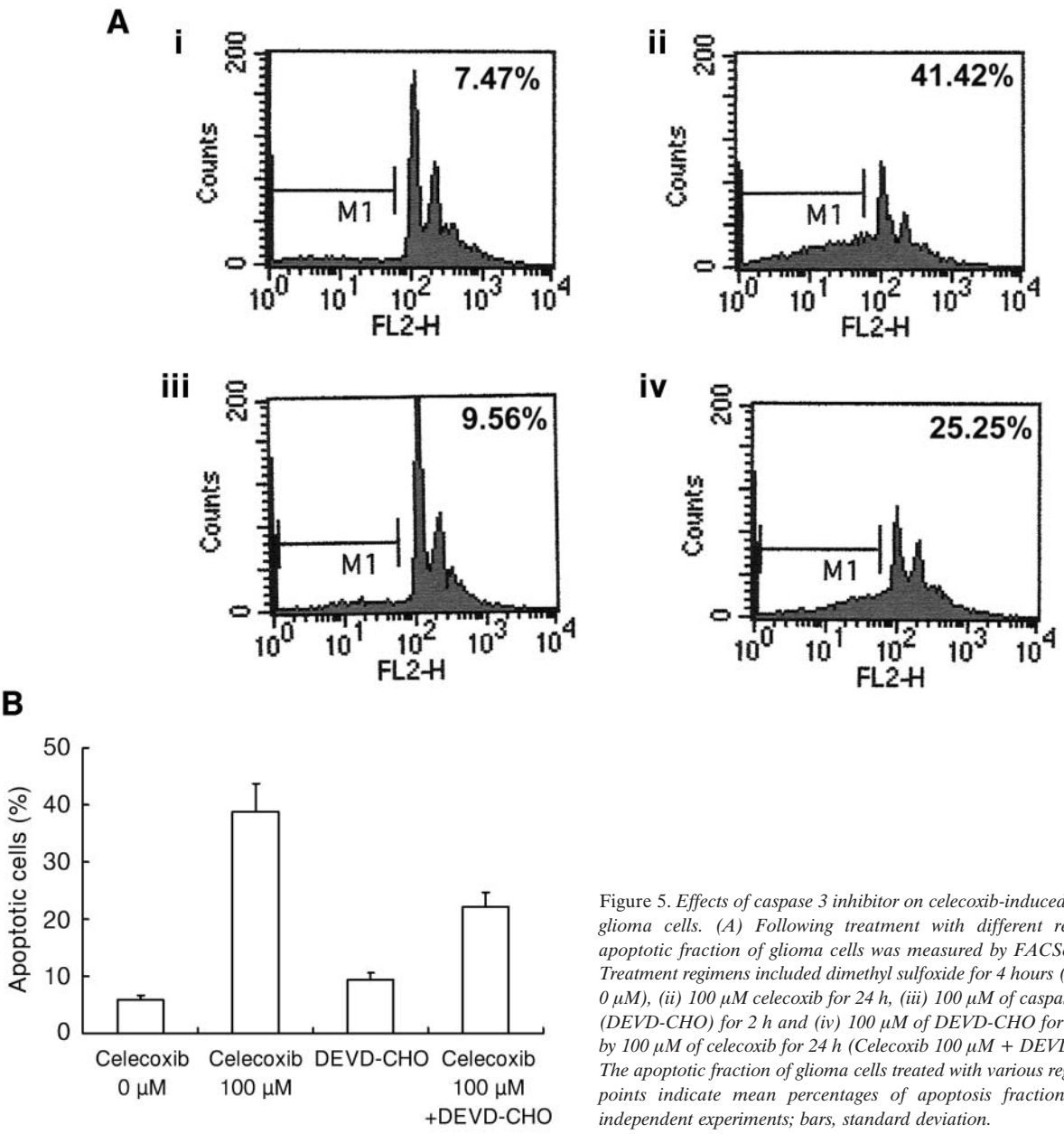

Figure 5. *Effects of caspase 3 inhibitor on celecoxib-induced apoptosis of glioma cells. (A) Following treatment with different regimens, the apoptotic fraction of glioma cells was measured by FACScan analysis. Treatment regimens included dimethyl sulfoxide for 4 hours (i) (Celecoxib 0 μM), (ii) 100 μM celecoxib for 24 h, (iii) 100 μM of caspase 3 inhibitor (DEVD-CHO) for 2 h and (iv) 100 μM of DEVD-CHO for 2 h followed by 100 μM of celecoxib for 24 h (Celecoxib 100 μM + DEVD-CHO). (B) The apoptotic fraction of glioma cells treated with various regimens. Data points indicate mean percentages of apoptosis fraction from three independent experiments; bars, standard deviation.*

The anti-neoplastic effects of celecoxib have been considered to be cell type-specific and concentration-dependent (20, 21). Celecoxib inhibits COX-2, causes cell cycle arrest and induces apoptosis at different effective concentrations in different types of cancer cells (21). Generally, the effects of celecoxib on cell cycle progression and apoptosis in cancer cell systems *in vitro* require concentrations that are several orders of magnitude greater than that required to inhibit COX-2 activity (21). Furthermore, celecoxib exerts its anti-neoplastic effects through both COX-2-dependent and -independent mechanisms (13, 14, 16, 20-22). In this study, expression of COX-2 in glioma cells was not suppressed by the different concentrations of celecoxib used. Thus, the inhibitory effect of celecoxib on glioma cells might be more closely related to the COX-2-independent mechanisms than COX-2-dependent ones. Possible COX-2-independent mechanisms of celecoxib activity include an increase in intracellular calcium arising from inhibition of endoplasmic reticulum Ca^{2+}-ATPase (23), induction of the mitochondrial apoptosome complex without involvement of the death receptor pathway (24), inhibition of phosphoinositide-dependent kinase 1 (PKB1) and PKB1/Akt pathways (22, 25), an increase in intracellular ceramide concentration (16), and loss of activity of cyclin-dependent

kinases due to the transcriptional down-regulation of cyclin A and cyclin B expression (20). However, the precise underlying mechanisms of the anti-proliferative effects of celecoxib on glioma cells have not been established (19).

Induction of cellular apoptosis may occur through various pathways including the mitogen-activated protein kinase signaling pathway (26-28). MAPKs, belonging to the serine/threonine kinase family, are important signaling mediators from the cell surface to the nucleus (26-28). MAPKs have been demonstrated to be upstream to COX-2 production, and COX-2 activation is dependent upon components of the MAPK signaling cascade, especially the p38 and SAP kinase (SAPK) subfamilies (29, 30). However, the reported effects of celecoxib on expression of MAPKs are inconsistent (15, 19, 21). Celecoxib can reduce the levels of phosphorylated SAPK (MEKK1 and JNK1) in lymphoma cells (19) and cause dephosphorylation of ERK2 in prostate cancer cells (15). In addition, down-regulation of ERK 1/2 has been considered one of the mechanisms involved in celecoxib-induced apoptosis (31, 32). In contrast, celecoxib does not affect the level of phospho-ERK in human umbilical vein endothelial cells (21). Therefore, we specifically studied celecoxib effects on expression of MAPKs; we found celecoxib did not affect expression of various MAPKs including JNK1, p38 and ERK 1/2. The data revealed that MAPKs are not related to celecoxib-induced glioma cell apoptosis.

The major tumor suppressor gene *p53*, which is involved in control of cell cycle progression, DNA integrity and cell survival, has been found to be involved in regulation of COX-2 expression (19, 29). *P53* gene activity may or may not inhibit expression of COX-2 (1, 18); prostaglandins produced by COX-2 can sequester and inactivate p53 in the cytoplasm (18). In addition, celecoxib treatment can sensitize cancer cells to p53-responsive stresses by promoting p53 localization in the nucleus (18). In contrast, one study found that p53 expression did not affect expression of COX-2 (1). In our study, celecoxib did not affect the expression of COX-2, and the expression of p53 in glioma cells was elevated 15 min after initiation of celecoxib treatment. The data suggest that expression of COX-2 is unrelated to expression of p53 in glioma cells, a finding that is consistent with results from a previous study (1). On the other hand, we found that expression of p21 in glioma cells was not changed by celecoxib. Research with a variety of cancer cell types has found that celecoxib increases p21 expression, subsequently inhibiting activity of cyclin-dependent kinases and causes cell cycle arrest (14, 16, 17, 33, 34). In contrast, one study reported p21 was not increased in celecoxib-treated glioma cells (20), which is consistent with our data. Thus we concluded that p21 does not play a significant role in the celecoxib-induced apoptosis of glioma cells.

In the literature, overexpression of Akt has been shown in a variety of cancers including gliomas; furthermore, pharmacological inhibition of Akt has an anti-proliferative effect on gliomas (35, 36). In addition, celecoxib can cause down-regulation of Akt activation in several kinds of cancer cell (12, 19, 21, 25). In this study, we found celecoxib increased expression of 14-3-3σ, a conserved phosphoserine-binding protein, in glioma cells starting from one hour after celecoxib treatment. Expression of 14-3-3σ is down-regulated in various tumors and induced by p53 in response to DNA damage (37). In addition, 14-3-3σ is an important regulator of the Akt function (37); it can bind to Akt and inhibit Akt-mediated cell growth, transformation and tumorigenesis (35, 37). Although we did not study expression and activity of Akt in this study, we speculate that celecoxib activates expression of p53 and 14-3-3σ in glioma cells, subsequently inhibiting Akt and inducing apoptosis.

Celecoxib has been found to cause either G0/G1 or G2/M arrest; and the anticancer effects of celecoxib are also thought to be related to celecoxib-induced cell cycle re-distribution (14, 16, 17, 20, 33, 34). The effect of celecoxib on the cell cycle of glioma cells has been studied in one report, which revealed that celecoxib increased the G0/G1 fraction and reduced the S and G2/M fractions (20). In contrast, in this study, we found celecoxib decreased the S and increased the G2/M fraction. In eukaryocytes, the cell cycle is regulated tightly at G1/S and G2/M checkpoints by several protein kinases composed of a cdk subunit and corresponding regulatory cyclin subunit, and cdk inhibitors (14, 38). One of the regulatory molecules in the G2/M check point is 14-3-3σ, which forms a cytoplasmic complex with cdc2 and sequesters cdc2/cyclin B1 in the cytoplasm (39, 40). Thus the celecoxib-induced increase in expression of 14-3-3σ in glioma cells was thought to contribute to G2/M accumulation. Certainly, the effects of the celecoxib-induced cell cycle re-distribution for different cell lines might also be related to drug dose and exposure time.

Generally, protection of cells against apoptosis requires down-regulation of pro-apoptotic activities or up-regulation of anti-apoptotic mechanisms (41, 42). The Bcl-2 family consists of at least 14 kinds of pro-apoptotic or anti-apoptotic proteins, which are important regulators of cellular apoptosis (43). Bcl-2 can regulate the commitment phase of apoptosis, delay cytochrome release *via* influence on functions of mitochondrial membrane channels, and thereby inhibit the apoptosis (44, 45). By contrast, Bax can promote cellular apoptosis. The effects of celecoxib on expression of the Bcl-2 family are inconsistent (12, 15, 19, 25). In malignant mesothelioma cells, Bcl-2 protein level is reduced by celecoxib exposure, accompanied by increased expression of the pro-apoptotic protein Bax (12). Celecoxib treatment of gliosarcoma cells implanted in rats also produced reduced Bcl-2 expression (46). In contrast, celecoxib has been found to induce apoptosis in cancer cells independent of Bcl-2 (19, 25). In B-cell lymphoma cells, celecoxib did not change expression of Bcl-2, Bcl-xL or Bax, although it did induce apoptosis of the lymphoma cells (19).

In this study, we found celecoxib suppressed expression of Bcl-2 in glioma cells; however, expression of Bax was not changed. These data are dissimilar to others in the literature. As a matter of fact, the dynamic changes between the pro-apoptotic and anti-apoptotic mechanisms (the dueling dimers' theory) are important for cellular apoptosis (41, 42). The function of Bax is regulated by Bcl-2 because Bcl-2 can form a Bcl-2/Bax heterodimer with Bax, thus suppressing the pro-apoptotic effect of Bax (47). Therefore, we thought celecoxib altered expression of the same Bcl-2 family protein members, resulting in a shift in their ratios that favored apoptosis (12).

Caspases are cysteine proteases and are responsible for the initiation and execution phases of cellular apoptosis (48, 49). There have been at least 13 caspases identified from mammalian cells and they function in a variety of different fashions (50). Among the 13 caspases (50), caspace-3 plays a decisive role in the execution phase of cellular apoptosis and is a general terminal mediator of apoptosis (2). We studied the role of caspace-3 in celecoxib-induced apoptosis of glioma cells and found that celecoxib stimulated caspace-3 activity by a factor of 3.4 in comparison with the control, which is consistent with results obtained with hepatocarcinoma, malignant mesothelioma and prostate cancer cells (12, 13, 17). Further, we also demonstrated that a caspace-3 inhibitor significantly alleviated the extent of apoptosis.

Conclusion

This study revealed that celecoxib caused concentration- and time-dependent cytotoxicity and apoptosis in A172 glioma cells. Celecoxib caused G2/M accumulation, increased expression of p53 and 14-3-3σ, reduced Bcl-2 and increased caspace-3 activity, without any change in expression of COX-2, MAPKs, or Bax. We hypothesize that celecoxib-induced cell death might be related to activation of p53 and 14-3-3σ, reduced Bcl-2 and Bcl-2/Bax ratio, and increased caspace-3 activity.

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