Celecoxib enhances radiosensitivity of hypoxic glioblastoma cells through endoplasmic reticulum stress

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Background. Refractoriness of glioblastoma multiforme (GBM) largely depends on its radioresistance. We investigated the radiosensitizing effects of celecoxib on GBM cell lines under both normoxic and hypoxic conditions.

Methods. Two human GBM cell lines, U87MG and U251MG, and a mouse GBM cell line, GL261, were treated with celecoxib or γ-irradiation either alone or in combination under normoxic and hypoxic conditions. Radiosensitizing effects were analyzed by clonogenic survival assays and cell growth assays and by assessing apoptosis and autophagy. Expression of apoptosis-, autophagy-, and endoplasmic reticulum (ER) stress–related genes was analyzed by immunoblotting.

Results. Celecoxib significantly enhanced the radiosensitivity of GBM cells under both normoxic and hypoxic conditions. In addition, combined treatment with celecoxib and γ-irradiation induced marked autophagy, particularly in hypoxic cells. The mechanism underlying the radiosensitizing effect of celecoxib was determined to be ER stress loading on GBM cells.

Conclusion. Celecoxib enhances the radiosensitivity of GBM cells by a mechanism that is different from cyclooxygenase-2 inhibition. Our results indicate that celecoxib may be a promising radiosensitizing drug for clinical use in patients with GBM.

Keywords: autophagy, celecoxib, ER stress, glioblastoma, hypoxia, radiosensitivity.

Glioblastoma multiforme (GBM) is the most frequently occurring malignant tumor of the central nervous system. Despite standard care comprising maximum surgical resection, 60 Gy of conventional radiotherapy, and chemotherapy with temozolomide (TMZ), the median survival time among patients with GBM is ~1 year.

The molecular mechanisms underlying the initiation and progression of GBM reported to date fall into 2 categories: (i) primary GBM with increased epidermal growth factor receptor (EGFR) expression and mutation of the phosphatase and tensin homolog and (ii) secondary GBM that is transformed from benign glioma types with stepwise mutations in the p53 and retinoblastoma genes. It is known that these genetic alterations in GBM are significantly associated with clinical prognosis. It has also been reported that methylation of O6-methylguanine-DNA methyltransferase increases the susceptibility of GBM tumor cells to alkylating agents, such as TMZ, and mutation in the isocitrate dehydrogenase 1 gene was recently reported to significantly affect the prognosis in patients with GBM.

On the basis of these findings, therapy that targets these specific molecules could be a promising approach for controlling GBM. One possible target is cyclooxygenase-2 (COX-2) because a positive association has been reported among the levels of COX-2, EGFR/EGFRvIII, and activated signal transducer and activator of transcription 3 (STAT3). COX-2 expression was reportedly increased in human GBM and was negatively correlated with clinical outcomes in patients.

Celecoxib, a nonsteroidal anti-inflammatory drug (NSAID), selectively inhibits COX-2. This drug is used clinically both for patients with osteoarthritis and articular rheumatism for pain relief and for patients with familial adenomatous polyposis as an adjuvant to chemotherapy. We previously confirmed that concomitant administration of celecoxib and irinotecan (CPT-11) inhibited the proliferation of neuroblastoma xenografts. However, COX-2 expression in our original neuroblastoma cell lines was low; therefore, there was no clear evidence that celecoxib inhibited COX-2. Thus, we considered the possible existence of
other pharmacological actions of celecoxib, independent of COX-2 inhibition.\textsuperscript{10}

Kardosh et al.\textsuperscript{11,12} reported that, in addition to its anti-inflammatory and analgesic effects, celecoxib could trigger an endoplasmic reticulum (ER) stress response, as shown by calcium leakage from ER to the cytosol. They also reported that celecoxib exhibited ER stress loading–induced anti-proliferative activity for GBM cell lines.\textsuperscript{11} When cells are exposed to endogenous or exogenous stimuli, unfolded proteins accumulate in ER, which engenders unfavorable stress on cells. Although cells respond to reduce ER stress to maintain homeostasis, which engenders unfavorable stress on cells. Although cells respond to reduce ER stress to maintain homeostasis, which engenders unfavorable stress on cells.

Establishing the radiosensitizing effects of celecoxib on GBM cells in vitro under both normoxic and hypoxic conditions. We examined the radiosensitizing effects of celecoxib on GBM cell death even in a hypoxic state. In this study, stress triggers, celecoxib and radiation, might promote radioresistance of tumor cells in hypoxic regions.

With regard to clinical trials with celecoxib, Gilbelet al.\textsuperscript{13,14} recently reported that a combination of TMZ and other drugs, including celecoxib, was feasible and safe in a phase I factorial design study. Kesari et al.\textsuperscript{15} conducted a phase II trial using a combination of TMZ, thalidomide, and celecoxib for patients with GBM. Although they confirmed that these combination protocols were safe, no significant clinical efficacy was found. One reason for this might be that they limited the celecoxib dose to that used for preventing colorectal cancer.\textsuperscript{16}

One of the major obstacles to radiotherapy for GBM is the hypoxic conditions of tumor tissues.\textsuperscript{20} Hypoxia is also as a known source of ER stress for cells.\textsuperscript{21} This suggests that ER stress overloading in hypoxic cells in combination with other ER stress triggers might induce cellular removal through the unfolded protein response pathway.\textsuperscript{22} This may be an approach to overcome the radioresistance of tumor cells in hypoxic regions.

Thus, we hypothesized that a combination of 2 ER stress triggers, celecoxib and radiation, might promote GBM cell death even in a hypoxic state. In this study, we examined the radiosensitizing effects of celecoxib on GBM cells in vitro under both normoxic and hypoxic conditions. Establishing the radiosensitizing effects of celecoxib could contribute to developing a novel therapeutic approach for treating patients with GBM.

**Materials and Methods**

**Cell Lines and Normoxic and Hypoxic Cell Culture Conditions**

Two human GBM cell lines, U87MG\textsuperscript{23} and U251MG,\textsuperscript{24} and a mouse GBM cell line, GL261,\textsuperscript{25} were obtained from the RIKEN Cell Bank (Tsukuba, Ibaraki, Japan). U87MG cells had wild-type p53,\textsuperscript{26,27} whereas U251MG and GL261 cells had mutated p53,\textsuperscript{26,28} These cell lines were selected on the basis of COX-2 expression screening by immunoblotting. The p53 and COX-2 expression levels of each cell line are shown in Supplementary Fig. S1. All cells were grown in minimum essential medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 100 mg/mL streptomycin, 100 U/mL penicillin (Sigma-Aldrich), and 10% fetal bovine serum (Sigma-Aldrich). For cell transfer, the cells were rinsed with Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free phosphate-buffered saline (PBS; Sigma-Aldrich) and dispersed with 0.25% trypsin solution containing 0.5 mM ethylenediaminetetraacetic acid (Sigma-Aldrich).

The cells were maintained at 37°C in a humidified incubator. Normoxic conditions were defined as 5% CO\textsubscript{2} in air. Hypoxic conditions were established using a chamber containing AnaeroPack (Mitsubishi Gas Chemical Company, Tokyo, Japan). According to the manufacturer’s data sheet, oxygen concentration in the chambers was adjusted to <1% (available at http://www.mgc.co.jp/seihin/a/an aeropack/pdf/pamphlet.pdf [last accessed date 7 April 2013]). The cells were carefully protected from other sources of stress, including nutrient deficiency and confluence. All experiments were conducted before cultures had reached a state of 70%–80% confluence.

**Irradiation**

Cells were irradiated with \textsuperscript{137}Cs γ-rays (γ cell 40; Atomic Energy of Canada, Ontario, Canada) at a dose rate of 0.78 Gy/min, which was calculated on the basis of the decay curve of \textsuperscript{137}Cs. The cells were irradiated under normoxic or hypoxic conditions at room temperature (25–26°C). The doses used were as follows: 2, 4, 6, and 8 Gy for clonogenic survival assays and 6 Gy for cell growth assays, cell cycle analysis, detecting apoptosis and autophagy, and immunoblotting analysis. The dose of 6 Gy was selected on the basis of the results of a clonogenic survival assay, as noted in the Results section.

**Chemicals**

Celecoxib, 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-pyrazol-1-yl]benzenesulfonamide (Pfizer Co., Ltd., Groton, CT), was dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries, Osaka, Japan) at 10 mM and diluted immediately before each experiment. Celecoxib toxicity was assessed using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kamasicsikigun, Kumamoto, Japan) according to the manufacturer’s protocol. This method provided for sensitive and colorimetric assays to determine cell viability during cell proliferation and cytotoxicity assays (available at http://www.dojindo.com/store/p/456-Cell-Counting-Kit-8.aspx [last accessed date 7 April 2013]). The amount of formazan dye (yellow) generated by the activities of dehydrogenases on a water-soluble tetrazolium salt, WST-8, was directly proportional to the number of viable cells. Each cell line was exposed to celecoxib at 10–70 μM for 48 h under normoxic or hypoxic conditions. For precise assays, DMSO was added to a control sample at the same concentration as that to the celecoxib samples. These experiments were repeated in triplicate. Results were normalized to the value of control cells set at 1 and are presented as means ± standard deviations (SDs).
Clonogenic Survival Assay

A standard colony survival assay was performed immediately after exposing the cell lines to \( \gamma \)-rays. After irradiation, the cells were trypsinized, suspended in PBS on ice, and plated on a 60-mm dish (Falcon, Becton Dickinson, Franklin Lakes, NJ). Colonies were fixed and stained \( \sim 14 \) days later. Three replicate dishes were used for each dose, and colonies of \( >50 \) cells were scored as survivors. The doses corresponding to 10% survival (D10) and surviving fractions at 2 Gy (SF2) were determined from a linear quadratic equation (LQ) model that was fit using DeltaGraph5.4 software (RedRock Software Company, Salt Lake, UT).

Doses used were 0, 2, 4, 6, and 8 Gy. Three independent experiments were performed for each dose.

Cell Growth Assay

The effects of celecoxib and/or radiation on tumor cell growth were assessed. Celecoxib concentrations and radiation doses were determined from previous toxicity and clonogenic assays. All cell lines were plated at \( 1 \times 10^4 \) cells/well in 24-well plates. When the cells entered the logarithmic growth phase \( 48 \) h later, they were treated with celecoxib (50 \( \mu \)M for U87MG and U251MG cells; 30 \( \mu \)M for GL261 cells) for \( 48 \) h. The cells were then irradiated with \( \gamma \)-rays (6 Gy), after which the medium was immediately changed to fresh control medium. Cell numbers were determined using a Coulter Counter (Beckman Coulter, Fullerton, CA) every 2 days for a period of 10 days after irradiation.

Cell Cycle Analysis

Cells were harvested on days 4, 5, and 10 after celecoxib treatment and used for cell cycle analysis. These time points corresponded to before irradiation (celecoxib alone), 1 day after irradiation, and 5 days after irradiation, respectively. At each time point, the cells were trypsinized and harvested along with the cells floating in the medium, respectively. At each time point, the cells were trypsinized, suspended in PBS on ice, and plated on a 60-mm dish (Falcon, Becton Dickinson, Franklin Lakes, NJ). Colonies were fixed and stained \( \sim 14 \) days later. Three replicate dishes were used for each dose, and colonies of \( >50 \) cells were scored as survivors. \( \sim 10^8 \) The doses corresponding to 10% survival (D10) and surviving fractions at 2 Gy (SF2) were determined from a linear quadratic equation (LQ) model that was fit using DeltaGraph5.4 software (RedRock Software Company, Salt Lake, UT).

Doses used were 0, 2, 4, 6, and 8 Gy. Three independent experiments were performed for each dose.


treated with celecoxib (50 \( \mu \)M for U87MG and U251MG cells; 30 \( \mu \)M for GL261 cells) for 48 h followed by \( \gamma \)-irradiation (6 Gy). Then, the cells were harvested at 6, 48, 72, and 96 h; washed twice in cold PBS; and resuspended in cold binding buffer. Annexin V-FITC (Ann-V) solution (5 \( \mu \)L) and propidium iodide (10 \( \mu \)L) were added to the cell suspension and incubated at room temperature for 10 min in the dark. For each sample, \( >10^4 \) cells were examined by flow cytometry.

Autophagy Assay

Autophagy was assessed using a Premo Autophagy Sensors kit (LC3B-FP; Life Technologies, Tokyo, Japan). Cells were placed in a chamber slide under low stress conditions by avoiding nutrient deprivation and confluence. Then, the LC3B reagent was added to the cells and gently mixed according to the manufacturer’s instructions. The cells with reagent were incubated overnight (\( \geq 16 \) h) for LC3B expression. Then, cell nuclei were stained with Hoechst 33342 (Dojindo, Kumamoto, Japan) at a final concentration of 8 \( \mu \)g/mL.

The cells were examined using a fluorescence microscope (Biozero BZ-8000 KEYENCE; Tokyo, Japan) and incorporated software to obtain Z-stacking images. Because we found that the Premo reagent could not be used to detect autophagosomes in GL261 cells, we assessed GL261 cells with use of a Cyto-ID autophagy detection kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer’s protocol. Other methods were the same as described above.

For quantitative analysis, acidic vesicular organelles (AVOs) inside cells were visualized and quantified as a marker of autophagy with use of the method described by Kanzawa et al. In brief, after trypsinization, harvested cells were washed once with PBS, resuspended in PBS containing 1 mg/mL acridine orange (Wako Pure Chemical Industries, Osaka, Japan), and incubated for 10 min at room temperature in the dark. The cells were analyzed by flow cytometry; the acid compartment in AVOs was stained red, and the background was stained green with this fluorescent dye. Green (510–530 nm) and red (650 nm) fluorescence emissions from 10,000 cells after excitation with blue light (488 nm) were determined using FACSCalibur (Becton Dickinson).

Immunoblotting

Cells harvested after celecoxib treatment and irradiation were lysed at room temperature in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Waltham, MA) with a protease inhibitor cocktail (Halt Protease Inhibitor Cocktail; Thermo Fisher Scientific). The buffer included 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate. Protein concentrations were determined using a 660-nm protein assay kit (Thermo Fisher Scientific) containing Ionic Detergent Compatibilization (Thermo Fisher Scientific). Samples that included the same amounts of protein were analyzed by SDS-PAGE and transferred to nitrocellulose membranes (Invitrogen). Membranes were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween 20 (TBS-T) and incubated overnight at 4°C with primary antibodies. Membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies. Proteins were detected using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).
were separated by 12.5%–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by electroblotting on polyvinylidene fluoride membranes (Millipore, Bedford, MA). These blots were incubated with the following antibodies under conditions recommended by the manufacturers. The primary antibodies used were anti-COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p21Waf1 (Santa Cruz Biotechnology), anti-GRP78/BiP (Santa Cruz Biotechnology), anti-GADD153/CHOP (Santa Cruz Biotechnology), anti-microtubule–associated protein 1 light chain 3 (Cosmo Bio, Tokyo, Japan), anti-p53 clone DO-1 (Calbiochem, Darmstadt, Germany), anti-cleaved caspase-3 clone 5A1, and anti-β-actin (Cell Signaling Technology). Horseradish peroxidase–conjugated anti-mouse (Amersham Biosciences, Buckinghamshire, UK) or anti-rabbit antibody (Enzo Life Sciences) was used as the secondary antibody. Signals were detected using an enhanced chemiluminescence system (Millipore, Billerica, MA). Preliminary experiments showed that GRP/BiP, CHOP, and caspase-3 expression was highest at 6 h after each treatment (data not shown); thus, we used data obtained at 6 h in subsequent experiments.

Statistical Analysis

Results are expressed as means ± SDs determined from multiple experiments. Statistical comparisons were made using Student’s t tests. P < .05 was considered to be statistically significant.

Results

Cell Line Characteristics

p53 function was preserved in U87MG cells, whereas it was absent in U251MG and GL261 cells. COX-2 expression was highest in U87MG cells, followed by GL261 and U251MG cells (Supplementary Fig. S1).

Celecoxib Cytotoxicity and Celecoxib-Induced ER Stress

Celecoxib cytotoxicity results under normoxic and hypoxic conditions as assessed by a CCK-8 assay are shown in Fig. 1A, C, and E. Results for GRP78/BiP expression under normoxic conditions are shown in Fig. 1B, D, and F. These results showed that celecoxib cytotoxicity increased in a dose-dependent manner; however, differences in cell viability between normoxic and hypoxic conditions were insignificant for each cell line investigated. In particular, at celecoxib concentrations of 30 and 50 μM, viability of GL261 cells under normoxic conditions were significantly lower than those of the other cell lines.

Immunoblotting results showed that levels of the ER stress marker GRP78/BiP under normoxic conditions increased as celecoxib concentration increased for each cell line over the concentration range examined (Fig. 1B, D, and F). Cell cytotoxicity and GRP78/BiP expression levels were positively correlated.

On the basis of these results, celecoxib concentration corresponding to a 50% lethal dose for U87MG and U251MG cells was 50 μM, whereas it was 30 μM for GL261 cells under normoxic conditions. Because GRP78/BiP expression significantly increased at either of these concentrations, these concentrations were used in subsequent experiments.

Clonogenic Survival

The results of clonogenic survival assays are shown in Fig. 2. For each cell line, radiosensitivity was significantly reduced under hypoxic, compared with normoxic, conditions. Celecoxib (50 μM for U87MG and GL261 cells; 30 μM for GL261 cells) exhibited significant sensitizing effects under both normoxic and hypoxic conditions, except for U87MG cells.

In addition to evaluations at each dose used, we analyzed the difference between treatment with radiation alone and the combination treatment using SF2 and D10 (Table 1). In this table, SF2 results are presented as means ± SDs and were compared using t tests, whereas D10 results are presented as means ± 95% confidence intervals to determine dose-enhancement ratios. Although the SF2 differences for U87MG cells were insignificant under both normoxic and hypoxic conditions, it was significantly different for the other cell lines under both normoxic and hypoxic conditions. In addition, dose-enhancement ratios calculated at D10 ranged from 1.10 to 1.49 and were higher under normoxic than under hypoxic conditions for each cell line.

Cell Growth

Figure 3 shows the changes in cell number after no treatment, after treatment with celecoxib (50 μM for U87MG and U251MG cells; 30 μM for GL261 cells) or radiation (6 Gy) alone, and after the combination treatment under normoxic and hypoxic conditions. A significant delay in cell growth was observed with the combination treatment under both normoxic and hypoxic conditions. In addition, the combination treatment significantly inhibited cell growth, compared with the treatment with radiation alone under normoxic conditions (Fig. 3B, D, and F). In particular, the effect of combination treatment on GL261 cells was significantly greater under normoxic conditions, and proliferation of these cells was completely arrested after day 12.

Cell Cycle Analysis

DNA histograms of p53 wild-type U87MG cells demonstrated a slight G1 arrest after treatment with radiation alone or the celecoxib plus radiation combination. In contrast, U251MG and GL261 cells, which had mutated p53, did not exhibit G1 arrest; instead, they showed G2/M arrest. In particular, GL261 cells showed strong G2/M arrest after treatment with radiation alone or after the
In addition, G2/M arrest in GL261 cells continued until day 10 after treatment.

Cellular Apoptosis

Figure 5 shows the results for cellular apoptosis after treatment with celecoxib or radiation alone and after treatment with the celecoxib plus radiation combination under normoxic and hypoxic conditions. The proportions of early apoptotic U87MG, U251MG, and GL261 cells under normoxic and hypoxic conditions are shown in Fig. 5A–C. Cellular apoptosis was significantly higher under normoxic than under hypoxic conditions. Treatment with the celecoxib plus radiation combination did not induce any significant increase in apoptosis, compared with treatment with radiation alone, for each cell line.

ER Stress

Figure 5D shows protein expression 6 h after the combination treatment. For each cell line, GRP78/BiP expression levels increased after treatment with celecoxib alone or the celecoxib plus radiation combination. In addition, GRP78/BiP expression levels under hypoxic conditions were higher than those under normoxic conditions, particularly for GL261 cells. GADD153/CHOP expression also increased concomitant with increased GRP78/BiP expression in U87MG and U251MG cells. In addition, GADD153/CHOP expression under hypoxic conditions was significantly increased, compared with that under normoxic conditions, in each cell line. For GL261 cells, GADD153/CHOP expression was increased after combination treatment, compared with treatment with celecoxib alone under hypoxic conditions. Although GADD153/CHOP expression...
was enhanced in these cell lines, particularly under hypoxic conditions, cleaved caspase-3 expression was not significantly increased.

**Autophagy**

Using fluorescence microscopy, we found that the number of autophagosomes in each cell line increased after treatment with celecoxib or radiation alone or after combination treatment. In addition, combination treatment induced a higher number of autophagosomes than treatment with celecoxib or radiation alone. Figure 6A shows representative images of autophagosomes.

The results of flow cytometry analysis to quantify AVOs under normoxic and hypoxic conditions are shown in Fig. 6B–D. In U87MG and U251MG cells, the ratios of AVOs under hypoxic conditions were...
higher than those under normoxic conditions. The number of AVOs was significantly higher with combination treatment than with treatment alone or celecoxib or radiation alone, and more AVOs were observed under hypoxic than under normoxic conditions for each cell line.

The results for autophagy-related protein LC3 expression are shown in Fig. 6E. A shift from LC3-I to LC3-II was observed with U87MG cells. These results suggest that LC3-II expression was enhanced by celecoxib or radiation alone and combination treatment, compared with the normoxia control. In addition, GRP78/BiP, GADD153/CHOP, and LC3-II expression levels were interrelated in U87MG and U251MG cells after treatment with celecoxib with or without radiation, particularly under hypoxic conditions.

**Discussion**

GBM is one of the most refractory of tumors and has high radioresistance. Because the effects of ionizing radiation strongly depend on oxygen concentration,20 radioresistance of GBM may be in part attributable to the presence of hypoxic regions in tumor tissues.31 This hypothesis is supported, because hypoxia-inducible factor (HIF)–1a is highly expressed in GBM tissues.32 In addition, a hypoxic environment is known to be a niche for cancer stem-like cells in GBM.33 Therefore, increasing the radiosensitivity of hypoxic tumor cells is one of the crucial issues to improve the effects of radiotherapy for GBM. In this regard, our results suggest that celecoxib has a radiosensitizing effect not only under normoxic but also under hypoxic conditions.

The original pharmacological action of celecoxib was found to be COX-2 inhibition.7 COX-2 is a rate-limiting enzyme for cytokines derived from arachidonic acid, and it affects increased production of various cytokines, such as prostaglandin E2, or cancer growth factors, such as TGF-β.34–36 It has also been reported that COX-2 expression was increased in human GBM and that its expression level was associated with poor clinical prognosis.37 One reason for this is that prostaglandin E2, a major product of COX-2, accelerates cell motility, growth, invasion, and angiogenesis, whereas it inhibits apoptosis and immune surveillance.37,38

Celecoxib reportedly had anti-proliferative activity against colorectal cancer by inhibiting neo-vascularization.39 In addition, celecoxib induced apoptosis in colon and prostate cancer cells40,41 when used at concentrations of ~50–100 μM. Kardosh et al11 and Du et al42 reported that this anti-tumor effect was independent of COX-2 inhibition, and Tsutumi et al43 noted that celecoxib induced tumor cells apoptosis through ER stress overloading.

Stress on the ER, a central cellular organelle with crucial biosynthetic sensing and signaling functions,44 includes hypoxia,22 Ca2+ depletion,45 and reactive oxygen species generation.46 In addition, recent results showed that both radiation and celecoxib could induce ER stress11,43,46–48 Although the pharmacological actions of celecoxib that are responsible for inducing ER stress are considered to be involved in suppressing sarcoplasmatic/ER calcium ATPase activity,46–50 which is independent of a COX-2 inhibitory effect,51 further analysis is required to establish this point.

We previously reported that a combination of the anticancer drug CPT-11 and celecoxib significantly inhibited the growth of neuroblastomas implanted in mice.9 We considered that the anti-proliferative activity of this combination treatment was probably caused by inducing strong ER stress10 because an increase in cellular apoptosis was closely associated with GADD153/CHOP upregulation. The present study results support this hypothesis that was proposed in our previous report.10

At present, the signaling pathway for radiation-induced ER stress is considered to be binary, with one pathway involving PERK/eIF2α/CHOP and the other involving IRE1/XBP1/JNK32 (Supplementary Fig. 2). This binary pathway can result in both cellular apoptosis and autophagy. In addition, ER stress induced by hypoxia and celecoxib is connected to apoptosis or autophagy through this binary pathway.44,52 Thus, it is conceivable that the combined ER stress induced by radiation,
hypoxia, and celecoxib could be significantly cytotoxic for tumor cells.

With regard to ER stress response markers, GRP78/BiP is a molecular chaperone that is upregulated when ER stress is induced in cells. Moreover, when there is a stress overload, the ER stress response for recovery is overwhelmed and a cell death response is initiated by GADD153/CHOP upregulation. In our study, GADD153/CHOP expression in U87MG cells under normoxic conditions was not increased. Although we cannot fully account for this result, the JNK pathway (Supplementary Fig. 2) may be dominant in this cell line under normoxic conditions. Because the other 2 cell lines that we used and U87MG cells under hypoxic conditions exhibited GADD153/CHOP upregulation concomitant with increased autophagy, we consider

Fig. 3. Cell growth assay. Growth curves of U87MG (A: normoxia, B: hypoxia), U251 (C: normoxia, D: hypoxia), and GL261 (E: normoxia, F: hypoxia) with radiation or celecoxib treatment alone or with a combination of both are shown. The dose of γ-irradiation is 6 Gy, and concentrations of celecoxib are 50 μM for U87MG and U251 and 30 μM for GL261. The bottom arrows indicate the timings for celecoxib (blank arrows) and radiation treatments (black arrows). Each point represents the mean value, and error bars indicate standard deviation calculated from 3 independent experiments. *P < .05 calculated from comparisons between combined treatment and radiation alone.
that GADD153/CHOP plays a crucial role in autophagy induction in GBM cells.

In our results, although we observed increased GADD153/CHOP expression induced by celecoxib/radiation, as noted above, we did not observe caspase-3 upregulation or a significant increase in apoptotic cells (Fig. 5). It is known that ER stress induces not only apoptosis but also autophagy.\textsuperscript{54–56} In addition, recent evidence suggests that GADD153/CHOP has a pro-autophagic function and a pro-apoptotic function in the ER stress response,\textsuperscript{57} and hypoxia accelerates autophagy by inducing HIF-1 expression.\textsuperscript{53} Kang et al\textsuperscript{58} recently reported that celecoxib induced autophagy rather than apoptosis, which is compatible with our results.

Clonogenic survival curves indicated that celecoxib significantly enhanced radiosensitivity in each cell line even under hypoxic conditions, except for U87MG cells (Fig. 2). Similarly, the SF2 values were significantly different between treatment with radiation alone and the combination treatment, except for U87MG cells, under both normoxic and hypoxic conditions (Table 1). Furthermore, the growth curves for U87MG cells after treatment with radiation alone and the combination treatment were very close until day 10 (Fig. 3B). These observations indicate that U87MG cells are more resistant to celecoxib than are U251MG and GL261 cells and suggest that the protective ER stress response against celecoxib is greater in U87MG cells than in other cell lines. Although this phenomenon was probably attributable to wild-type p53 preservation in U87MG cells (Supplemental Fig. 1), selective inhibition of p53 in this cell line will be required to establish this hypothesis.

The dose-enhancement ratio at D10 was higher under normoxic than under hypoxic conditions for each cell line (Table 1). Although a CCK-8 assay showed that the cytotoxic effects of celecoxib were not significantly different between normoxic and hypoxic conditions (Fig. 1A, C, and E), the results of clonogenic survival assays suggest that the radiosensitizing effect of celecoxib was less in the hypoxic state than in the normoxic state. Although this difference may be attributable to the strong radiosensitizing effect of oxygen, a dose-enhancement effect of \textasciitilde10% was observed in each cell line, even in the hypoxic state. We consider that this result has a clinical implication in the treatment of highly refractory GBM.

The results of cell growth assays indicated that celecoxib-induced ER stress also had an inhibitory effect on cell cycle progression. Grosch et al\textsuperscript{59} reported that celecoxib modulated cell cycle progression through P21\textsuperscript{Waf1} and P27\textsuperscript{Kip1} upregulation by a mechanism that was independent of COX-2 inhibition. Although we found a significant delay in GBM cell growth after treatment with the radiation plus celecoxib combination, our preliminary results indicated that celecoxib used at the concentrations here did not inhibit COX-2 expression (Supplementary Fig. 3). Therefore, we consider that the proliferative suppression of the GBM cells observed here was most likely caused by ER stress. However, COX-2 inhibition effect might still play a role in tumor cell growth inhibition. This point might be confirmed by clarifying expressions of prostaglandin E2 and/or TGF-\textbeta by immunoblotting after exposure to 30 or 50 \textmu M of celecoxib.

In addition, Chen et al\textsuperscript{60} reported that prolonged ER stress increased the p27 expression level. This was compatible with our results, because celecoxib alone induced slight G1 arrest in p53 wild-type U87MG cells, whereas it caused G2/M arrest in p53-mutated
Because it is known that cells in the G1 or G2/M phase are highly sensitive to radiation, cellular synchronization effects mediated through ER stress could be another mechanism that might explain the radiosensitization effects of celecoxib. Further detailed investigations are required to elucidate the effects of celecoxib on cell cycle regulation, including selective overexpression or inhibition of p53 in the cell lines used in this study.

Because celecoxib is already being used for patients as an NSAID, the hurdle before it can be applied in combination with radiotherapy to patients with GBM is not very high.\(^8\) A limitation of this study was that the concentrations used here (30–50 \(\mu M\)) were significantly higher than the clinically available blood concentrations of <10 \(\mu M\).\(^6\) However, we still consider that 30 \(\mu M\) is feasible on the basis of the celecoxib data sheet provided by Pfizer, which noted that there was no serious toxicity after administering 2400 mg/day for 10 days (available at http://labeling.pfizer.com/ShowLabeling.aspx?id=793 [last accessed date 7 April 2013]). In addition, in a previous study, we confirmed a dominant antitumor effect by combining celecoxib with an anti-cancer drug in vivo.\(^9\) Kardosh et al.\(^{11}\) also noted that ER stress could be induced at a lower concentration in vivo when used in combination with other drugs. Thus, another
A promising approach is combination with other chemotherapeutic drugs. To test the efficacy of these doses or combinations, an orthotopic mouse brain tumor model using GL261 cells would be suitable.

Another interesting strategy might be attributable to the effects on glioma stem-like cells, because Chen et al. reported that celecoxib enhanced the radiosensitivity of cancer stem-like cells. Because hypoxia is
known to provide a niche for CD133-positive GBM stem-like cells, a combination of celecoxib and radiation may be effective for tumor stem cells, which are the main culprits in local recurrence after radiotherapy.

Conclusions

The study results showed that celecoxib inhibited the growth of GBM cells and enhanced the radiosensitivity of these cells under not only normoxic but also hypoxic conditions. In addition, treatment with the celecoxib plus radiation combination caused cell cycle arrest and prominent autophagy in GBM cells under hypoxic conditions by ER stress loading. Our results suggest that celecoxib might contribute to overcoming the radioresistance of GBM cells under hypoxic conditions. These findings should be useful to further the clinical applications of celecoxib for improving outcomes in patients with GBM.

Supplementary Material

Supplementary material is available online at Neuro-Oncology (http://neuro-oncology.oxfordjournals.org/).

Acknowledgments

We thank Ms. Yoko Mori for her competent technical assistance.

Funding

This work was supported by a Grant-in-Aid “Challenging Exploratory Research: No. 24659644” from the Ministry of Education, Culture, Sports, Science & Technology of Japan.

Conflict of interest statement. None declared.

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