Involvement of CD73 and A2B receptor in radiation-induced DNA damage response and cell migration in human glioblastoma A172 cells

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Summary

Glioblastoma is the most common malignant tumor of the central nervous system and is treated with a combination of surgery, radiation and chemotherapy. However, the tumor often acquires radiation resistance, which is characterized by an increased DNA damage response (DDR). Here, we show that CD73, which generates extracellular adenosine from ATP, and A2B receptor, which is activated by adenosine, are involved in the γ-radiation-induced DDR and the enhanced migration ability of human glioblastoma cell line A172. To investigate DDR, we evaluated ATM activation and focus formation of γH2AX and 53BP1 in the nucleus of A172 cells after γ-irradiation. Antagonists of A2B receptor and CD73, or knockdown with siRNA, suppressed γ-radiation-induced DDR and promoted γ-radiation-induced cell death, as well as suppressing γ-radiation-induced cell migration and actin remodeling. These results suggest that activation of A2B receptor by extracellular adenosine generated via CD73 promotes γ-radiation-induced DDR, leading to recovery from DNA damage, and also enhances cell migration and actin remodeling. The CD73-A2B receptor pathway may be a promising target for overcoming radiation resistance and the acquisition of malignant phenotypes during radiotherapy of glioblastoma.

Keywords

glioblastoma; DNA damage response; cell migration; A2B receptor; CD73; radiation resistance
Introduction

Radiation therapy and chemotherapy are commonly used in addition to surgery to treat glioblastoma, but the tumors frequently acquire resistance to radiotherapy, which significantly reduces the efficacy of the treatment. Indeed, the 5-year survival rate for highly malignant glioblastoma is about 15%, and the median survival time is about 15 months. Therefore, there is an urgent need to elucidate in detail the mechanism of acquisition of radioresistance in order to improve the outcome. Furthermore, local irradiation is known to induce tumor metastasis, and this issue is as important a therapeutic challenge as the acquisition of radioresistance.

The cellular DNA damage response (DDR) machinery is activated by various genotoxic events such as ionizing radiation. DDR involves a complex signaling network that contributes to the regulation of cell cycle checkpoints and DNA repair mechanisms, as well as to cellular functions such as transcription and chromatin remodeling, and the induction of cell death in response to a lethal DNA break or excessive DNA damage.

In many cancers, the DDR mechanism is activated by DNA replication stress, and this might suppress the effects of genotoxic anti-tumor therapy. Furthermore, the radiation-resistant cell population is characterized by the activation of radiation-induced DDR and DNA repair mechanism pathways such as ataxia telangietactasia mutated (ATM) or PI3K-PTEN-Akt-mTOR, which play a role in tumorigenesis in glioma. Therefore, the DDR mechanism could be a therapeutic target to overcome radioresistance in glioblastoma radiotherapy.

Nucleosides (adenosine) and nucleotides (ADP, ATP, UDP and UTP) have recently been recognized as extracellular messengers. Extracellular nucleosides and nucleotides influence multiple cellular responses, including cell death, proliferation, migration, differentiation, growth factor secretion, and inflammation. CD39 is a member of the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family, and is a membrane-bound enzyme that degrades ATP and ADP to AMP. AMP is degraded to adenosine by CD73, which is the only 5'-ectonucleotidase enzyme family expressed on the external surface of the cell membrane. Continuous activation of CD39 and CD73 thus produces extracellular adenosine, which acts specifically on cell membrane P1 receptors. Adenosine is so lipophilic that these receptors would be activated on the cell membrane surface in close proximity to the site of adenosine generation, i.e., in the tumor microenvironment (TME). We recently showed that the adenosine A2B receptor is involved in DDR specifically in cancer cells using three lung cancer cell lines and a normal lung cell line. We also established an important role of the A2B receptor in DDR in mouse melanoma. Thus, A2B receptor appears to be involved in resistance to...
radiation therapy in various types of tumors. However, it is not known whether this
pathway is also involved in γ-radiation-induced DDR in glioblastoma cells.

Therefore, in this study, we investigated the involvement of A2B receptor and CD73 in
γ-radiation-induced DDR in glioblastoma cells. As DDR-related factors, we evaluated
phosphorylated ATM, DNA damage mediator histone H2A isoform γ (γH2AX), and
DNA damage repair factor tumor suppressor p53-binding protein 1 (53BP1). Our
results indicate that activation of the A2B receptor via extracellular adenosine generated
by CD73 plays an important role in γ-radiation-induced DDR in glioblastoma cells. The
A2B receptor and CD73 also appear to be involved in γ-radiation-induced cell migration
and actin remodeling, suggesting that the CD73-mediated pathway of A2B receptor
activation contributes to radioresistance and radiation-induced cell migration in
glioblastoma. Thus, this pathway may be a promising target for improving glioblastoma
radiotherapy.
Materials & Methods

Agonists and antagonists
Adenosine (a P1 receptor agonist) and adenosine 5′-(α,β-methylene) diphosphate (APCP) (an ecto-5′-nucleotidase (CD73) inhibitor) were purchased from Sigma-Aldrich (St Louis, MO, USA). BAY60-6583 (a selective agonist of A2B receptor), PSB36 (a selective antagonist of A1 receptor), SCH442416 (a selective antagonist of A2A receptor), PSB603, MRS1706, MRS1754 (selective antagonists of A2B receptor), MRS3777 (a selective antagonist of A3 receptor) and CGS15943 (a non-selective antagonist of P1 receptors) were from Tocris Bioscience (Ellisville, Mo, USA).

Cell culture
A172 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (FUJIFILM Wako Pure Chemical Corporation, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Gibco, U.S.A), penicillin (100 U/mL) and streptomycin (100 μg/mL). All cultures were performed in an incubator under an atmosphere of 5% CO2 in air, at 37°C. Irradiation with γ rays was carried out with a Gammacell 40 (137Cs source) (Nordin International, Inc., Ontario, Canada; 0.71 Gy/min) at room temperature for a specified time. After irradiation of cells, incubation was continued at 37°C.

Real-time RT-PCR
Real-time RT-PCR was performed as described previously.9) Total RNA was extracted from A172 cells using a ReliaPrep™ RNA Cell Miniprep System (Promega). The first-strand cDNA was synthesized with PrimeScript Reverse Transcriptase (Takara Bio). The cDNA was used as a template for real-time RT-PCR analysis; reactions were performed in a CFX Connect Real-Time System (Bio-Rad). RT2-qPCR® primer assays for human A1 receptor, A2A receptor, A2B receptor, A3 receptor, CD39 and CD73 were purchased from Qiagen. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was determined as a positive control. Each sample was assayed in a 20 μL amplification reaction mixture, containing cDNA, primers and 2×KAPA SYBR ® FAST qPCR Master Mix (Kapa Biosystems). The samples were incubated at 95 °C for 1 min, then amplification was carried out for 40 cycles (each cycle, 95 °C for 3 sec, annealing at 60 °C for 30 sec), followed by incubation at 95 °C for 1 min. Fluorescent products were detected at the last step of each cycle. The obtained values were normalized to GAPDH mRNA expression.
Detection of DNA damage response

We examined the formation of γH2AX and 53BP1 foci at DSB sites as described previously. A172 cells were grown on cover glasses. The cells were reseeded at a density of 1 × 10^5 cells in 35 mm cell culture dishes containing sterile coverslips, pretreated with/without P1 receptor antagonists, agonists or APCP for 0.5 h, and then irradiated. At 0.5-24 h post irradiation, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature, and permeabilized in 0.1% Triton X-100 for 5 min on ice. After incubation in blocking buffer (10% FBS in PBS) for 1 h, the fixed cells were incubated with primary antibody (mouse anti-H2AX phosphorylated (Ser139) antibody (Bio Legend, U.S.A.) 1 : 200 and 53BP1 antibody rabbit polyclonal (Novus, U.S.A.) 1 : 200) for 24 h at 4°C, and then with secondary antibody (Alexa Fluor 594 goat anti-mouse immunoglobulin G (IgG) (Invitrogen, U.S.A.) 1 : 200, or goat anti-rabbit IgG-fluorescein isothiocyanate (Sigma-Aldrich, U.S.A.) 1 : 200) for 1 h. Counterstaining with Hoechst 33342 (1 µg/mL) was used to verify the location and integrity of nuclei. Fluorescence images were obtained with a laser-scanning confocal microscope (FV1000 IX81; Olympus, Japan). We observed and counted co-localized foci of γH2AX and 53BP1 in nuclei as a measure of DDR.

Western blotting

Western blotting was performed as described previously. A172 cells were lysed in cold lysis buffer containing 1% Triton X-100, protease inhibitor cocktail (Sigma-Aldrich) and PhosSTOP at 4°C for 30 min. Lysates were centrifuged at 10000×g for 15 min. To the supernatant was added 2× sample buffer (FUJIFILM Wako Pure Chemical Corporation, Ltd.) supplemented with 10 mM dithiothreitol, and the mixture was boiled for 10 min at 95°C. Protein (20 µg/lane) was analyzed by means of 7.5%, 10% or 12% SDS-PAGE, and bands were transferred onto a PVDF membrane. The blots were blocked overnight in 1% bovine serum albumin at 4°C. For detection, the blots were incubated with primary antibody (mouse anti-H2AX phosphorylated (Ser139) antibody (Bio Legend, U.S.A.) 1 : 200, ATM phosphorylated (S1981) antibody (abcam, U.K.) and rabbit anti-A2B receptor (Merck Millipore, U.S.A.) overnight at 4°C, and further incubated with goat horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Cell Signaling Technology) (1:5000) or (1:20000) for 1.5 h at room temperature. Blots were also incubated with anti β-actin monoclonal antibody, peroxidase conjugated (FUJIFILM Wako Pure Chemical Corporation, Ltd.) (1:50000) for 1 h at room temperature. Specific proteins were visualized by using ImunoStar® LD (FUJIFILM Wako Pure Chemical Corporation, Ltd.) according to the manufacturer’s instructions.
**Flow cytometry**

The cells were reseeded at a density of $1 \times 10^5$ cells in 35 mm cell culture dishes, pretreated with/without P1 receptor antagonists or APCP for 0.5 h, and then irradiated. A172 cells were harvested by trypsinization, washed with PBS, and then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature, and permeabilized in 0.1% Triton X-100 for 5 min on ice. After incubation in blocking buffer (10% FBS in PBS) for 1 h, the fixed cells were incubated with fluorescence-conjugated γH2AX mAb (phospho-histone H2AX (Ser139) rabbit mAb (Alexa Fluor® 488 conjugate) (Cell Signaling Technology) 1 : 200 at room temperature for 1.0 h in the dark. The cells were then washed twice in PBS and immediately subjected to flow cytometry (FACSCalibur Flow Cytometer, Becton, Dickinson and Co., U.S.K.).

**MTT assay**

The cells (1.0×10^5 cells/mL) were incubated for 24 h in 35 mm cell culture dishes, exposed to 2 Gy of γ-rays, incubated for a further 24 h, and then trypsinized and scored. They were reseeded at 1.0×10^3 cells per 96-well plate in DMEM. The cells were incubated at 37°C for 96 h in an atmosphere of 5% CO_2 in air. After 96 h, the MTT solution (5 mg/ml PBS) was added. The plate was incubated under 5% CO_2 at 37°C for 3 h, then the blue formazan crystals generated by metabolically active cells in each well were dissolved in stop solution. The absorbance of each well was read on an ELISA Reader (490 nm).

**Annexin V-PI assay**

We examined cell death as described previously. Briefly, cells (1.0×10^5 cells/mL) were incubated for 24 h in 35 mm cell culture dishes, pretreated with/without P1 receptor antagonists or APCP for 0.5 h, and then irradiated. After 24 hours, the medium was replaced with fresh medium. After incubation for a further 24 hours, A172 cells were harvested by trypsinization, washed with PBS, and then stained with Annexin V-FITC/PI (MEBCYTO® Apoptosis Kit (Annexin V-FITC Kit), Medical & Biological Laboratories Co., Ltd., Japan) for flow-cytometric analysis on a FACSCalibur (Becton, Dickinson and Co., United States). The data were analyzed with FlowJo software (FlowJo, LCC).

**Cell transfection**

SiRNA transfection was performed as described previously. SiRNA targeting A2B
receptor or CD73 and negative control siRNA (TriFECTa Kit® DsiRNA Duplex) were purchased from Integrated DNA Technology. A172 cells (1.6 × 10^5 cells in 24-well plates) were seeded in culture medium and transfected with negative control siRNA (si-cont), A2B receptor or CD73 siRNA1, siRNA2 and siRNA3 duplex oligonucleotides (10 nM) by using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer’s instructions. Reduction of A2B receptor or CD73 was confirmed by real-time PCR at 24 h after transfection and by western blotting at 48 h after transfection. The cells were used for experiments at 48 h after transfection.

**Cell migration assay**

Cell migration assay was performed as described previously.\(^{13, 14}\) γ-Radiation-induced cell migration was analyzed by using 24-well Transwell® plates (6.5 mm diameter; 8 µm pore size polycarbonate membrane, Corning, Lowell, MA). Cells (1.0×10^4 cells/mL) were incubated for 72 h in 35 mm cell culture dishes, exposed to 2 Gy of γ-rays, incubated for a further 48 h, and then trypsinized and scored. The insert compartment was seeded with A172 cells (6×10^4 cells/mL) in basal culture medium. After 24 hours, the medium was replaced with fresh medium. The insert chamber contained 5% FBS and the bottom chamber contained 10% FBS. After incubation for a further 24 hours, cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, and incubated with 1 µg/ml 4′,6-diamidino-2-phenylindole (DAPI) and 50 µg/ml propidium iodide (PI) for 30 minutes at room temperature. Non-migrated cells on the insert chamber surface of the membrane were removed and cells that had migrated through the membrane to the lower surface were counted using a fluorescence microscope (BZ-9000; KEYENCE).

**Actin remodeling**

Actin remodeling staining and observation were performed as described previously.\(^{13, 14}\) γ-Radiation-induced actin remodeling was analyzed by using glass-bottomed dishes. Cells (2.0×10^4 cells/mL) were incubated for 24 h in 35 mm glass-bottomed dishes, exposed to 2 Gy of γ-rays, and incubated for a further 48 h. For F-actin staining and immunofluorescence staining, cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, and permeabilized with 0.5% Triton X-100 for 5 minutes on ice. For staining of F-actin, fixed cells were incubated with 100 nM Rhodamine–phalloidin for 30 minutes at room temperature. Counterstaining with Hoechst33342 (1 µg/ml) was used to verify the location and integrity of nuclei. Fluorescence images were obtained with a laser-scanning confocal microscope (FV1000 IX81; Olympus, Japan).
Statistics

Results are expressed as the mean ± standard error of the mean (s.e.m.). The statistical significance (p values) of differences between control and other groups was calculated using Student’s t test (two-group test, irradiated and non-irradiated), Bonferroni’s test (time-dependent and concentration-dependent experiments) or Dunnett’s test. Calculations were done with the Instat version 3.0 statistical software package (Graph Pad Software, U.S.A.). A value of p<0.05 was considered statistically significant.
Results

**DDR is induced by γ-irradiation in A172 cells**

γ-Radiation causes DNA damage and induces DDR, which we evaluated in terms of co-staining of γH2AX foci and 53BP1 accumulation (Fig. 1A). We irradiated glioblastoma cell line A172 cells with various doses of γ-rays, and examined γH2AX and 53BP1 focus formation in the nuclei after incubation for various periods. γ-Irradiation induced focus formation in a dose-dependent manner with a peak at 0.5 h after irradiation at the dose of 2 Gy (Fig. 1B, C). These results show that γ-radiation induces DDR in A172 cells immediately after irradiation, and the DDR subsequently declines.

**Involvement of adenosine receptors in DDR**

We examined the expression of adenosine receptors in A172 cells and confirmed that A1 and A2B receptors were highly expressed (Fig. 2A). Next, we investigated the involvement of adenosine receptors in γ-radiation-induced DDR using adenosine receptor antagonists. Specifically, we examined the effects of PSB36 (an A1 receptor-specific antagonist), SCH442416 (an A2A receptor-specific antagonist), PSB603 (an A2B receptor-specific antagonist), MRS3777 (an A3 receptor-specific antagonist), and CGS15943 (a non-specific P1 receptor antagonist) on focus formation of γH2AX and 53BP1 at 0.5 h after 2 Gy of γ-irradiation as a measure of the early phase of DDR. Focus formation of γH2AX within a short time after irradiation (0.5 h) is a useful marker for DDR, because γH2AX is dephosphorylated and defocused after the DNA repair reaction has been performed. Thus, residual foci of γH2AX and 53BP1 at 24 h after irradiation are considered to represent unrepaired DNA damage. In the present study, γ-irradiation at the dose of 2 Gy did not induce a sufficient number of residual foci to evaluate unrepaired DNA damage at 24 h after irradiation. Therefore, in order to investigate unrepaired DNA damage, we evaluated residual foci of γH2AX and 53BP1 at 24 h after the higher dose of 4 Gy. SCH442416, PSB603 and CGS15943 markedly suppressed focus formation at 0.5 h after irradiation, and in particular, PSB603 and CGS15943 increased residual foci at 24 h (Fig. 2B, C), indicating that PSB603 and CGS15943 attenuated DDR. PSB36 and MRS3777 did not affect γ-radiation-induced focus formation at 0.5 h or 24 h after irradiation. Also, SCH442416 did not affect residual foci at 24 h after irradiation.

Furthermore, other A2B receptor antagonists, MRS1706 and MRS1754, similarly reduced the focus formation at 0.5 h after irradiation and increased the residual foci at 24 h after irradiation (Fig. 2D, E). Conversely, adenosine receptor ligand adenosine and...
A2B receptor-specific agonist BAY60-6583 increased the focus formation at 0.5 h after irradiation and decreased the residual foci at 24 h after irradiation (Fig. 2F, G). These results suggested that the highly expressed A2B receptor positively regulates γ-radiation-induced DDR and promotes repair of DNA damage.

Effects of A2B receptor antagonist on γ-radiation-induced DNA damage response via ATM activation and on cell death

It is known that radiation-induced DNA damage induces phosphorylated ATM and γH2AX expression as a part of the DDR pathway, so we examined phosphorylated ATM and γH2AX expression by means of western blotting. γ-Radiation induced phosphorylated ATM and γH2AX at 0.5 h after irradiation, and this induction was suppressed by A2B receptor antagonist PSB603 and by P1 receptor antagonist CGS15943 (Fig. 3A-C). We also examined the generation of γH2AX in the cells after irradiation by means of flow cytometry using FITC-labeled γH2AX antibody. Figure 3D shows that γH2AX production increases as the cell distribution shifts to the right. Figure 3D and E show that γ-radiation increased γH2AX generation, and the γH2AX generation in irradiated cells was reduced by PSB603 or CGS15943 (Fig. 3D, E), supporting the results obtained by immunostaining (Fig. 2) and western blotting (Fig. 3A-C). We also examined the effect of an A2B receptor antagonist on γ-radiation-induced cell death using MTT assay (Fig. 3F). Though PSB603 alone reduced cell viability to about 80%, γ-irradiation of PSB603-treated cells caused a significant reduction of cell viability (about 50%). Considering that γ-irradiation (2 Gy) alone only reduced the cell viability to about 90%, PSB603 strongly enhanced the radiation-induced cell death. Similar results were observed in CGS15943-treated cells (Fig. 3F). γ-Radiation-induced cell death was also examined using the Annexin V-PI assay. The results confirmed that γ-irradiation induces cell death, which was enhanced by A2B receptor antagonists (Fig. 4A, B). On the other hand, adenosine and A2B agonist BAY60-6583 almost completely suppressed the radiation-induced decrease of cell viability. These results indicate that A2B receptor activation promotes DDR and suppresses γ-radiation-induced cell death.

Involvement of A2B receptor in γ-radiation-induced DDR and cell death

To investigate the involvement of A2B receptor in γ-radiation-induced DDR mediated by ATM activation and in cell death, we performed knockdown of the receptor in A172 cells with siRNA. We first confirmed the effectiveness of knockdown by the siRNA (Fig.
A2B receptor decreased focus formation of γH2AX and 53BP1 at 0.5 h after irradiation and increased residual foci at 24 h after irradiation (Fig. 5D, E). We also investigated the involvement of A2B receptor in the activation of ATM and γH2AX expression by γ-radiation. Knockdown significantly suppressed the formation of phosphorylated ATM and γH2AX after irradiation (Fig. 5F-H). Since these results indicated involvement of the receptor in DDR, we also examined its role in γ-radiation-induced cell death. Knockdown of the receptor further enhanced γ-radiation-induced cell death (Fig. 5I). These results strongly support the involvement of A2B receptor in radiation-induced DNA repair.

Effect of CD73 inhibitor on γ-radiation-induced DDR and cell death

Adenosine receptors are activated by binding of extracellular adenosine produced by dephosphorylation of extracellular ATP by CD39 and CD73. We previously reported that ATP is released extracellularly after γ-irradiation in lung cancer cells. In A172 cells, this extracellularly released ATP may be metabolized by CD39 and CD73 to adenosine, which would activate the A2B receptor. We examined the involvement of CD73 in γ-radiation-induced DDR by using α,β-methylene adenosine 5′-diphosphate (APCP), a CD73 inhibitor. First, we examined the expression of CD39 and CD73, which are membrane-bound ATP-metabolizing enzymes, in A172 cells. We confirmed that CD73 is highly expressed in A172 cells (Fig. 6A). When we examined the effects of CD73 inhibitor on the γ-radiation-induced DDR, we found that APCP reduced focus formation of γH2AX and 53BP1 at 0.5 h after irradiation and increased residual foci at 24 h after irradiation (Fig. 6B, C). APCP also suppressed γ-radiation-induced phosphorylated ATM and γH2AX expression (Fig. 6D-F). Furthermore, γH2AX generation was suppressed in γ-irradiated cells pretreated with APCP (Fig. 6G, H). γ-Radiation-induced cell death was enhanced by APCP, which is consistent with the suppressing effect of APCP on DDR (Fig. 6I, 4C and D). These results indicate that activation of CD73 promotes γ-radiation-induced DDR and suppresses γ-radiation-induced cell death.

Involvement of CD73 in γ-radiation-induced DDR and cell death

We further investigated the role of CD73 in γ-radiation-induced DDR in A172 cells by knockdown with siRNA in A172 cells. We first confirmed the effectiveness of knockdown of CD73 with the siRNA (Fig. 7A). Knockdown of CD73 reduced focus formation of γH2AX and 53BP1 at 0.5 h after irradiation and increased residual foci at 24 h after irradiation (Fig. 7B, C). Knockdown also reduced γ-radiation-induced cell death.

Involvement of CD73 in γ-radiation-induced DDR and cell death
phosphorylated ATM and γH2AX (Fig 7D-F). Further, knockdown of CD73 enhanced γ-radiation-induced cell death, which is consistent with suppression of γ-radiation-induced DDR by knockdown of CD73 (Fig. 7G). These results suggest that the generation of extracellular adenosine by CD73 is involved in radiation-induced DNA repair.

**Involvement of A2B receptor in γ-radiation-induced cell migration and actin remodeling**

Many studies using a variety of cell types, including glioma and lung cancer, have demonstrated that radiation enhances cell migration *in vivo* and *in vitro*. We also reported that γ-radiation induces migration of lung cancer and melanoma cells. Since migration is an essential step in tumor metastasis, we examined the involvement of A2B receptor in γ-radiation-induced cell migration by using Transwell® plates. We confirmed that γ-radiation induced migration of A172 cells at 96 h after irradiation (Fig. 8A, B). This migration was significantly suppressed by A2B receptor antagonist and by knockdown of A2B receptor (Fig. 8C, D). Actin remodeling is related to the acquisition of cell motility, and is considered a characteristic of invasive and metastatic cells. We found that γ-irradiation induced formation of actin stress fibers at 48 h after irradiation, whereas cells treated with A2B receptor antagonist or subjected to A2B receptor knockdown exhibited only modest polymerization of actin after irradiation (Fig. 8E, F). These results indicate that A2B receptor is involved in γ-radiation-induced cell migration and actin remodeling.

**Involvement of CD73 in γ-radiation-induced cell migration and actin remodeling**

We also examined the involvement of CD73 in γ-radiation-induced cell migration and actin remodeling. CD73 inhibitor and knockdown of CD73 significantly suppressed γ-radiation-induced cell migration (Fig. 9A, B). γ-Radiation induced formation of actin stress fibers at 48 h after irradiation, and their formation was suppressed by CD73 inhibitor APCP and by CD73 knockdown (Fig. 9C, D). These results suggest that CD73-mediated generation of adenosine is also involved in γ-radiation-induced cell migration and actin remodeling.
Discussion

Our results suggest that CD73-mediated adenosine A2B receptor activation plays an important role in DDR in irradiated A172 glioblastoma cells, in accordance with our previous findings in human lung cancer cells and mouse melanoma.\textsuperscript{9,10} We also showed that the A2B receptor is highly expressed in glioblastoma (Fig. 2A), which is consistent with our report showing that A2B receptor is highly expressed in lung cancer cells as compared with normal lung cells.\textsuperscript{9} Although high expression of both A1 and A2B receptors was observed in A172 cells (Fig. 2A), the A1 receptor has a high affinity for adenosine and serves to maintain normal physiological functions in the nervous system, whereas the A2B receptor has a low affinity and functions under pathological conditions, such as in tumors.\textsuperscript{22} Indeed, A2B receptor is upregulated in oral, prostate, colon, lung and hepatocellular cancer compared to normal tissue, and the expression of A2B receptor in astrocytes of mice transplanted with glioblastoma is significantly increased compared to control mice.\textsuperscript{23,24} In short, A2B receptors have been shown to play an important role in the pathogenesis of glioblastoma, and our results are consistent with this. On the other hand, CD73 generates extracellular adenosine, and is induced by HIF-1α under hypoxic conditions in tumors. As a result, the concentration of extracellular adenosine in the tumor microenvironment is at least twice that in healthy tissue. CD73 is involved in tumor growth in prostate cancer and is highly expressed in cancer cells of triple-negative breast cancer patients, contributing to chemoresistance via the A2B receptor.\textsuperscript{8} In accordance with these findings, we previously showed that CD73 is highly expressed in lung cancer cells.\textsuperscript{9} Our present findings show that A172 cells also express CD73 (Fig. 6A), and we found that an inhibitor of CD73 and knockdown of CD73 enhanced γ-radiation-induced cell death in this cell line (Fig. 6I, 7G, 4C, D). Previous studies have found that the expression of CD73 in A172 cells is similar to that in the glioblastoma cell line T98G, while expression of CD73 in T98G cells is moderate compared to that in U-87MG and U-251 cells, which highly express CD73.\textsuperscript{25,26}

We have reported that γ-radiation induces the intracellular translocation of EGFR from the cell membrane via A2B receptor in lung cancer cells.\textsuperscript{9} Furthermore, EGFR overexpression is associated with increased CD73 expression in hepatocellular carcinoma,\textsuperscript{27} and suppression of EGFR activity has a radiosensitizing effect in glioblastoma.\textsuperscript{6} Thus, CD73-A2B receptor signaling may influence EGFR activity and contribute to radioresistance. Taken together, all these findings suggest that extracellular
adenosine generated by CD73 and A2B receptor signaling in a cancer-cell-specific manner promotes physiological functions such as proliferation and survival, and stress responses such as EGFR internalization and radiation-induced DDR.

Purine nucleotides such as ATP are released extracellularly in response to various stimuli and activate P2X receptors with built-in ion channels and P2Y receptors coupled to G protein.\(^{28,29}\) We previously reported that γ-radiation induces the release of ATP in lung cancer cells, and the extracellularly released nucleotides activate P2X7, P2Y6 and P2Y12 and are involved in γ-radiation-induced DDR.\(^{16,30}\) On the other hand, ATP released extracellularly by γ-radiation should be metabolized to adenosine by the membrane-bound enzymes CD39 and CD73 to activate P1 receptors, especially in the tumor microenvironment where CD73 is highly expressed.\(^{8}\) It is difficult to measure extracellular adenosine concentration immediately after γ-irradiation. However, since γ-radiation-induced DDR is suppressed by inhibition of CD73 and A2B receptor (Fig. 5 and 7), it seems likely that the increase of extracellular adenosine by CD73 and the activation of A2B receptor after γ-irradiation are involved. If the CD73-A2B receptor pathway is inhibited before irradiation, the extracellular production of adenosine after γ-irradiation and the binding of adenosine to the A2B receptor would be inhibited. As a result, downstream signaling of the A2B receptor would be suppressed, as would γ-radiation-induced DDR. CD39 expression is small in A172 cells compared to CD73 expression (Fig. 6A), but it was reported that CD39 is highly expressed in immune cells infiltrating glioblastoma tumors.\(^{26,31}\) Therefore, considering the tumor microenvironment, CD73 and A2B receptor may have a greater effect in vivo than in vitro on DDR after ionizing irradiation.

The downstream pathway of A2B receptor activation after irradiation is unknown. We have previously reported that ERK1/2 phosphorylation is involved in γ-radiation-induced DDR in lung cancer cells.\(^{30}\) Radiation-induced DNA damage is known to induce PI3K activity, and in addition, A172 cells have mutations in PTEN that modulate the PI3K/Akt/mTOR signaling pathway.\(^{6}\) These processes might take place downstream of the CD73-A2B receptor after γ-irradiation. Indeed, it has been reported that the PI3K/Akt/mTOR pathway is activated downstream of the A2B receptor in cancer cells.\(^{32}\)

CD73 is associated with migration of breast cancer cells, and promotes lung metastasis of spontaneous breast cancer, and furthermore, in MB-MDA-231 human breast cancer
cells, knockdown of CD73 inhibits adhesion to extracellular matrix and cell migration, though the mechanism was not addressed.\textsuperscript{33} In 4T1.2 tumor cells, blocking of the A2B receptor suppresses cell chemotaxis, which suggests a pathway by which CD73-generated adenosine activates A2B receptor to promote cell migration.\textsuperscript{34} A2B receptor is involved in the promotion of tumor cell motility by increasing intracellular cAMP and cAMP-dependent PKA activity, and phosphorylation of α4 integrin by PKA is required for direct cell migration.\textsuperscript{33} Our results also show that inhibitors of A2B receptor and CD73, as well as knockdown of these molecules, suppress the migration of non-irradiated A172 cells (Fig. 8C, D, 9A, B). The molecular mechanism of radiation-induced cell migration in epithelial cancer cells involves a change in cell phenotype called epithelial mesenchymal transition (EMT), which transforms polar, immobile cells into non-polar, highly motile cells.\textsuperscript{35} This phenomenon is characterized by decreased expression of epithelial cell markers such as E-cadherin and increased expression of mesenchymal cell markers such as vimentin and N-cadherin, associated with actin remodeling.\textsuperscript{36, 37} However, the molecular mechanisms involved are poorly understood. We have reported that TRPV4 channel inhibitors suppress γ-radiation-induced cell migration and actin remodeling in lung cancer cells.\textsuperscript{13} We also reported that the TRPV4 channel is involved in γ-radiation-induced extracellular release of ATP in human epidermal keratinocyte HaCaT cells.\textsuperscript{38} Based on those findings, γ-radiation-induced cell migration and actin remodeling may involve extracellular release of ATP via the TRPV4 channel and subsequent activation of purinergic signaling. In fact, we have reported the involvement of ATP exocytosis and P2 receptor activation in TGF-β1-induced cell migration in lung cancer cells.\textsuperscript{13} Since our present study suggests the involvement of A2B receptor and CD73 in γ-radiation-induced cell migration and actin remodeling in glioblastoma cells, it seems likely that γ-radiation induces extracellular release of ATP, which is metabolized by CD73 to adenosine, which in turn activates A2B receptor to promote cell migration and actin remodeling.

In addition to radiation therapy, the alkylating agent temozolomide (TMZ) is frequently used in the chemotherapy of glioblastoma. The toxicity of TMZ is mainly due to the formation of O6-methylguanine (O6-meG) in DNA, and unrepaired O6-meG triggers an abortive mismatch repair (MMR) cycle, which eventually leads to DSB formation that induces the activation of DDR signaling through both ATM and ATR.\textsuperscript{39} A detailed knowledge of the pathways activated by TMZ and DDR-mediated repair of DNA damage after TMZ treatment might make it possible to increase the therapeutic efficiency. It seems plausible that the CD73-A2B receptor pathway may be activated
after TMZ treatment, and thus the CD73-A2B receptor pathway could be an important molecular target to improve the therapeutic efficacy of both radiation and chemotherapy.

In conclusion, our findings indicate that activation of CD73 and A2B receptor after irradiation has a role in γ-radiation-induced DDR and DNA damage repair in A172 cells, as well as in γ-radiation-induced cell migration and actin remodeling. This is the first evidence that CD73 and A2B receptor are involved in γ-radiation-induced DDR and contribute to radioresistance in glioblastoma, and also that they are involved in the γ-radiation-induced acquisition of motility and enhanced cell migration. The CD73-A2B receptor pathway may be a promising target for overcoming radioresistance and radiation-induced acquisition of malignant phenotypes in the treatment of glioblastoma.

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Conflict of Interest
The authors declare no conflict of interest.
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Figure 1. DDR induced by γ-irradiation in A172 cells

γH2AX/53BP1 focus formation was detected by immunostaining (n = 34-61). Panel A: Immunostaining of non-irradiated A172 cells and A172 cells 0.5 hours after γ-irradiation (2 Gy). Left panel, without irradiation; right panel, after irradiation; red,
γH2AX; green, 53BP1. Scale bars, 50 μm. Panel B: A172 cells were irradiated with various doses (1.0-8.0 Gy) of γ-rays and incubated for 0.5 h, then γH2AX/53BP1 foci were counted. Panel C: A172 cells were irradiated with 2 Gy of γ-rays followed by incubation for various times (0.5-6.0 h), and then γH2AX/53BP1 foci were counted. Each value represents the mean ± s.e.m. In panels B and C, a significant difference from the control group (non-irradiated) is indicated by *** (P < 0.001). (Color figure can be accessed in the online version.)
Figure 2. Involvement of adenosine receptors in DDR

Panels A: mRNA expression levels of P1 receptors were determined by real-time RT-PCR in A172 cells. Each value represents the mean ± s.e.m. Panels B and C: γH2AX/53BP1 focus formation was detected by immunostaining (n = 39-75) and
γH2AX/53BP1 foci were counted. A172 cells were treated with PSB36 (5 μM), SCH442416 (10 μM), PSB603 (10 μM), MRS3777 (10 μM) or CGS15943 (10 μM) for 0.5 h prior to irradiation with 2 Gy (B) or 4 Gy (C) of γ-rays, and then incubated for 0.5 h (B) or 24 h (C). Panels D and E: A172 cells were treated with MRS1706 (5 μM) or MRS1754 (0.1 μM) for 0.5 h prior to irradiation with 2 Gy (D) or 4 Gy (E) of γ-rays, and then incubated for 0.5 h (D) or 24 h (E). Panels F and G: A172 cells were treated with adenosine (10 μM) or BAY60-6583 (1 μM) for 0.5 h prior to irradiation with 2 Gy (F) or 4 Gy (G) of γ-rays, and then incubated for 0.5 h (F) or 24 h (G). Each value represents the mean ± s.e.m. Significant differences are indicated as follows. Panels B-G: *** (P < 0.001 versus non-irradiation control group); ### (P < 0.001 versus irradiation control group (2 Gy (B, D and F) or 4 Gy (C, E and G) of γ-rays)). ns: Not significant.
Figure 3. Effect of A2B receptor antagonist on γ-radiation-induced DNA damage response through ATM activation and cell death

Panels A-C: Expression of p-ATM (B) and γH2AX (C) induced by irradiation with 2 Gy of γ-rays followed by incubation for 0.5 h was detected by western blotting in A172...
cells (n = 3). A172 cells were treated with PSB603 (10 μM) or CGS15943 (10 μM) for 0.5 h prior to irradiation with 2 Gy of γ-rays, and then incubated for 0.5 h. In panels B and C, each value represents the mean ± s.e.m. (n = 3). Panels D and E: A172 cells were treated with PSB603 (10 μM) or CGS15943 (10 μM) for 0.5 h prior to irradiation with 2 Gy of γ-rays, and then incubated for 0.5 h. A172 cells were stained with fluorescence-conjugated γH2AX mAb and analyzed by flow cytometry. In panel E, each value represents the mean ± s.e.m. (n = 3). Panel F: A172 cells survival rate measured by MTT assay. A172 cells were treated with PSB603 (10 μM), CGS15943 (10 μM), adenosine (10 μM) or BAY60-6583 (1 μM) for 0.5 h prior to irradiation with 2 Gy of γ-rays, and then incubated for 24 h (n = 4). The cells were re-seeded into 96-well plate and cultured for 96 h. Each value represents the mean ± s.e.m. Significant differences are indicated as follows. Panels B, C, E and F: ** (P < 0.01), *** (P < 0.001 versus non-irradiation control group); ## (P < 0.01), ### (P < 0.001 versus irradiation control group (2 Gy of γ-rays)). (Color figure can be accessed in the online version.)
Figure 4. Effects of A2B antagonists or CD73 inhibitor on γ-radiation-induced cell death

The percentage of dead cells was measured by flow cytometry. Panels A-D: A172 cells were treated with PSB603 (10 μM) or CGS15943 (10 μM) (A and B) or APCP (10 μM)
(C and D) for 0.5 h prior to irradiation with 4 Gy of γ-rays, followed by incubation for 48 h. The cells were stained with Annexin V-FITC and PI. Panels B and D: The percentages of Annexin V+ PI− and Annexin V+ PI+ cells were added together, and the quantitative results are presented as bar graphs. Each value is the mean ± s.e.m (n = 3).

Significant differences are indicated as follows. In panels B and D, ** (P < 0.01), *** (P < 0.001 versus non-irradiation control group); ## (P < 0.01), ### (P < 0.001 versus irradiation control group (4 Gy of γ-rays)). (Color figure can be accessed in the online version.)
Figure 5. Involvement of A2B receptor in γ-radiation-induced DDR and cell death

Panels A, B and C: A172 cells were transfected with siRNA for A2B receptor (si-A2BR 1, 2, 3) or negative control siRNA (si-cont) and incubated for 24 h (A) or 48 h (B and C). Decrease of A2B receptor mRNA in A172 cells was confirmed by real-time RT-PCR.
A. Decrease of A2B receptor in A172 cells was confirmed by western blotting (n = 3).

(C). In panels B and C, each value represents the mean ± s.e.m. (n = 3). Panels D and E: γ-Irradiation-induced γH2AX/53BP1 focus formation induced by 2 Gy (D) or 4 Gy (E) of γ-rays followed by incubation for 0.5 h (D) or 24 h (E) was detected by immunostaining (n = 41-69) and γH2AX/53BP1 foci were counted. Panels F-H: Expression of p-ATM (G) and γH2AX (H) induced by irradiation with 2 Gy of γ-rays followed by incubation for 0.5 h was detected by western blotting in A172 cells (n = 3).

Panel I: A172 cell survival rate measured by MTT assay (n = 4). A172 cells were transfected with siRNA for A2B receptor (si-A2BR 1, 2, 3) or negative control siRNA (si-cont), then incubated for 48 h, irradiated with 2 Gy of γ-rays, and further incubated for 24 h. The cells were re-seeded into a 96-well plate and cultured for 96 h. Each value represents the mean ± s.e.m. Significant differences are indicated as follows. Panel C: *** (P < 0.001 versus si-cont). Panels D, E, G, H and I: * (P < 0.05), *** (P < 0.001 versus non-irradiation negative control); ### (P < 0.001 versus irradiation negative control group (2 Gy (D, G, H and I) or 4 Gy (E) of γ-rays)).
Figure 6. Effect of CD73 inhibitor on γ-radiation-induced DDR and cell death

Panel A: mRNA expression levels of CD39 and CD73 were detected by real-time RT-PCR in A172 cells. In panel A, each value represents the mean ± s.e.m. Panels B and C: γH2AX/53BP1 focus formation was detected by immunostaining (n = 44-71) and
γH2AX/53BP1 foci were counted. A172 cells were treated with APCP (10 μM) for 0.5 h prior to irradiation with 2 Gy (B) or 4 Gy (C) of γ-rays, and then incubated for 0.5 h (B) or 24 h (C). Panels D-F: Expression of p-ATM (E) and γH2AX (F) induced by irradiation with 2 Gy of γ-rays followed by incubation for 0.5 h was detected by western blotting in A172 cells (n = 3). A172 cells were treated with APCP (10 μM) for 0.5 h prior to irradiation with 2 Gy of γ-rays, and then incubated for 0.5 h. Panels G and H: A172 cells treated with APCP (10 μM) for 0.5 h prior to irradiation with 2 Gy of γ-rays, and then incubated for 0.5 h. A172 cells were stained with fluorescence-conjugated γH2AX mAb and analyzed by flow cytometry (n = 3). Panel I: A172 cell survival rate measured by MTT assay. A172 cells were treated with APCP (10 μM) for 0.5 h prior to irradiation with 2 Gy of γ-rays, and then incubated for 24 h (n = 4). The cells were re-seeded into 96-well plate and cultured for 96 h. Each value represents the mean ± s.e.m. Significant differences are indicated as follows. Panels B, C, E, F, H and I: * (P < 0.05), *** (P < 0.001 versus non-irradiation control group); ### (P < 0.001 versus irradiation control group (2 Gy (B, E, F, H and I) or 4 Gy (C) of γ-rays)). (Color figure can be accessed in the online version.)
Figure 7. Involvement of CD73 in γ-radiation-induced DDR and cell death

Panel A: A172 cells were transfected with siRNA for CD73 (si-CD73 1, 2, 3) or siRNA for negative control (si-cont) and incubated for 24 h (A). Decrease of CD73 mRNA in...
A172 cells was confirmed by real-time RT-PCR (A). In panel A, each value represents the mean ± s.e.m. Panels B and C: γ-Irradiation-induced γH2AX/53BP1 focus formation induced by 2 Gy (B) or 4 Gy (C) of γ-rays followed by incubation for 0.5 h (B) or 24 h (C) was detected by immunostaining (n=37-67) and γH2AX/53BP1 foci were counted. In panel B and C, each value represents the mean ± s.e.m. Panels D-F: Expression of p-ATM (E) and γH2AX (F) induced by irradiation with 2 Gy of γ-rays followed by incubation for 0.5 h was detected by western blotting in A172 cells (n = 3). Panel G: A172 cell survival rate measured by MTT assay (n = 4). A172 cells were transfected with siRNA for A2B receptor (si-CD73 1, 2, 3) or negative control siRNA (si-cont), then incubated for 48 h, irradiated with 2 Gy of γ-rays, and further incubated for 24 h. The cells were re-seeded into a 96-well plate and cultured for 96 h. Each value represents the mean ± s.e.m. Significant differences are indicated as follows. Panels B, C, E, F and G: * (P < 0.05), *** (P < 0.001 versus non-irradiation si-cont); ## (P < 0.01), ### (P < 0.001 versus irradiation si-cont (2 Gy (B, E, F and G) or 4 Gy (C) of γ-rays)).
Figure 8. Involvement of A2B receptor in γ-radiation-induced cell migration and actin remodeling

Panels A-D: Cell migration was examined by means of Transwell® assay as described in
Materials and Methods. A172 cells were irradiated with 2 Gy of γ-rays followed by incubation for 96 h and the lower membrane surfaces were photographed through a microscope at 20× magnification. Migrated cells in each field were counted. A172 cells were irradiated with various doses (0.5-8.0 Gy) of γ-rays followed by incubation for 96 h (A). A172 cells were irradiated with 2 Gy of γ-rays followed by incubation for 72 or 96 h (B). A172 cells were treated with PSB603 (10 μM) or CGS15943 (10 μM) for 0.5 h prior to irradiation with 2 Gy of γ-rays followed by incubation for 96 h (C). A172 cells were transfected with siRNA for A2B receptor (si-A2BR1, 2, 3) or negative control siRNA (si-cont) for 48 h prior to irradiation with 2 Gy of γ-rays followed by incubation for 96 h (D). In panels A-D, each value represents the mean ± s.e.m. (n=5). Significant differences are indicated as follows. Panels A and B: a significant difference from the control group (non-irradiated) is indicated by ** (P < 0.01), *** (P < 0.001). Panels C and D: *** (P < 0.001 versus non-irradiation control or si-cont); ### (P < 0.001 versus irradiation control or si-cont (2 Gy of γ-rays)). Panels E and F: Actin remodeling was observed by means of Rhodamine phalloidin staining as described in Materials and Methods. F-Actin was stained using Rhodamine–phalloidin (red), and stained cells were analyzed using a confocal laser scanning microscope. To verify the location of nuclei, cells were stained with Hoechst33342 (blue). Without-irradiation, left panel; after irradiation, right panel. A172 were treated with PSB603 (10 μM) or CGS15943 (10 μM) for 0.5 h prior to irradiation with 2 Gy of γ-rays followed by incubation for 48 h (E). A172 cells were transfected with siRNA for A2B receptor (si-A2BR1, 2, 3) or negative control si RNA (si-cont) for 48 h prior to irradiation with 2 Gy of γ-rays followed by incubation for 48 h (F). Scale bars, 50 μm. (Color figure can be accessed in the online version.)
Figure 9. Involvement of CD73 in γ-radiation-induced cell migration and actin remodeling

Panels A and B: Cell migration was examined by means of Transwell® assay as
described in Materials and Methods. A172 cells were irradiated with 2 Gy of γ-rays followed by incubation for 96 h and the lower membrane surfaces were photographed through a microscope at 20× magnification. Migrated cells in each field were counted.

A172 cells were treated with APCP (10 μM) for 0.5 h prior to irradiation with 2 Gy of γ-rays followed by incubation for 96 h (A). A172 cells were transfected with siRNA for CD73 (si-CD73 1, 2, 3) or negative control siRNA (si-cont) for 48 h prior to irradiation with 2 Gy of γ-rays followed by incubation for 96 h (B). In panels A and B, each value represents the mean ± s.e.m. (n=5). Significant differences are indicated as follows.

Panels A and B: *** (P < 0.001 versus non-irradiation negative control); ### (P < 0.001 versus irradiation negative control group (2 Gy of γ-rays)). Panels C and D: Actin remodeling was observed by means of Rhodamine-phalloidin staining as described in Materials and Methods. F-Actin was stained using Rhodamine-phalloidin (red), and stained cells were analyzed using a confocal laser scanning microscope. To verify the location of nuclei, cells were stained with Hoechst33342 (blue). Without irradiation, left panel; after irradiation, right panel. A172 were treated with APCP (10 μM) for 0.5 h prior to irradiation with 2 Gy of γ-rays followed by incubation for 48 h (C). A172 cells were transfected with siRNA for CD73 (si-CD73 1, 2, 3) or negative control siRNA (si-cont) for 48 h prior to irradiation with 2 Gy of γ-rays followed by incubation for 48 h (D). Scale bars, 50 μm. (Color figure can be accessed in the online version.)