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γ-Irradiation induces $P2X_7$ receptor-dependent ATP release from B16 melanoma cells

Yasuhiro Ohshima [a,1], Mitsutoshi Tsukimoto [a,b,*,1], Takato Takenouchi [c], Hitoshi Harada [d], Akina Suzuki [a], Mitsuru Sato [c], Hiroshi Kitani [c], Shuji Kojima [a]

[a] Department of Radiation Biosciences, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda-shi, Chiba, Japan
[b] Strategic Promotion Program for Basic Nuclear Research, Japan
[c] Transgenic Animal Research Center, National Institute of Agrobiological Sciences, 1-2 Ohwashi, Tsukuba-shi, Ibaraki, Japan
[d] Faculty of Pharmaceutical Sciences, Suzuka University of Medical Science, 3500-3 Minamitamagaki, Suzuka-shi, Mie, Japan

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ABSTRACT

Background: Ionizing irradiation causes not only growth arrest and cell death, but also release of growth factors or signal transmitters, which promote cancer malignancy. Extracellular ATP controls cancer growth through activation of purinoceptors. However, there is no report of radiation-induced ATP release from cancer cells. Here, we examined γ-irradiation-induced ATP release and its mechanism in B16 melanoma.
Methods: Extracellular ATP was measured by luciferin–luciferase assay. To investigate mechanism of radiation-induced ATP release, we pharmacologically inhibited the ATP release and established stable $P2X_7$ receptor-knockdown B16 melanoma cells using two short hairpin RNAs targeting $P2X_7$ receptor.
Results: Cells were exposed to 0.5–8 Gy of γ-rays. Extracellular ATP was increased, peaking at 5 min after 0.5 Gy irradiation. A selective $P2X_7$ receptor channel antagonist, but not anion transporter inhibitors, blocked the release of ATP. Further, radiation-induced ATP release was significantly decreased in $P2X_7$ receptor-knockdown cells. Our results indicate that γ-irradiation evokes ATP release from melanoma cells, and $P2X_7$ receptor channel plays a significant role in mediating the ATP release.
General Significance: We suggest that extracellular ATP could be a novel intercellular signaling molecule released from cancer cells when cells are exposed to ionizing radiation.

1. Introduction

The cytoplasm contains large amounts of ATP, which is released into the extracellular space in response to stress stimuli, such as shear stress, stretch, hypoxia, inflammation, osmotic swelling, and cell death [1]. Extracellular ATP interacts with and activates P2 receptors in an autocrine/paracrine manner [1]. Although the mechanism of ATP release is not yet fully understood, there have been several studies of stress-induced ATP release. The mechanism is dependent on cell type or the nature of the stress stimulus, and is mediated by mechanisms, such as a maxi-anion channel [2], a volume-sensitive outwardly rectifying chloride channel [3] or $P2X_7$ receptor channel [4]. The concentration of ATP in peri-plasmalemmal space after stress-induced ATP release reaches the micromolar range, which is sufficient to activate P2 receptors [1]. P2 receptors are classified into two major subtypes, ionotropic P2X receptors and metabotropic P2Y receptors, and their activation regulates many physiological functions [5]. In addition, the released ATP is rapidly degraded to ADP, AMP and adenosine by ecto-nucleotidases [1]. ADP is a ligand of $P2Y_1$ receptor and adenosine is a ligand of P1 receptors (A_1, A_{2A} A_{2B}, A_3) [1]. Thus, cytosolic ATP release causes activation of various purinergic and adenosinergic signaling pathways in an autocrine/paracrine manner.

The $P2X_7$ receptor is the seventh member of the P2X receptor subfamily, and its expression is increased in human melanoma [6]. It has been revealed that activation of $P2X_7$ receptor channel plays important roles in various physiological functions, such as apoptotic and/or necrotic cell death, production of pro-inflammatory cytokines, shedding of CD62L [7,8]. We have also studied the $P2X_7$ receptor-dependent functions including the mechanisms of cell death in lymphocytes [9–11], regulation of both IL-1β release and autophago-lysosome in microglia [12,13]. Activation of $P2X_7$ receptor channel induces an increase of cationic permeability, followed by plasma membrane depolarization; intense or prolonged activation leads to the opening of large non-selective pores, allowing the passage of

Abbreviations: A438079, 3-[[5-(2,3-dichlorophenyl)-1*H*-tetrazol-1-yl]methyl]pyridine hydrochloride; BzATP, 2′(3′)-O-(4-benzoyl)benzoyladenosine 5′-triphosphate; EtBr, ethidium bromide; FBS, fetal bovine serum; FFA, flufenamic acid; P2X7-KD (1C), stable $P2X_7$ receptor-knockdown B16 melanoma clone which was transfected with $P2X_7$-shRNA (1C) vector; P2X7-KD (3A), stable $P2X_7$ receptor-knockdown B16 melanoma cell which was transfected with $P2X_7$-shRNA (3A) vector; WT, wild type; shRNA, short hairpin RNA

* Corresponding author. Department of Radiation Biology, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki Noda-shi Chiba, 278-8510, Japan. Tel.: +81 4 7124 1501x6443; fax: +81 4 7121 3613.
E-mail address: tsukim@rs.noda.tus.ac.jp (M. Tsukimoto).
[1] These authors contributed equally to this work, and share the first authorship.

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hydrophilic molecules of up to 400–900 Da in size, such as ethidium bromide (EtBr) [7]. Although $P2X_7$ receptor channel mediates ATP release, the mechanism of $P2X_7$ receptor-mediated ATP release has not yet been uncovered.

Radiotherapy by ionizing irradiation, including γ-rays, is an effective modality of cancer treatment, because ionizing irradiation causes DNA damage, followed by cell cycle arrest or cell death. Ionizing irradiation also evokes the release of various transmitters, such as reactive oxygen species, nitric oxide and cytokines, and these signaling molecules affect not only irradiated cells, but also adjacent and far-away non-irradiated cells through intercellular communication—the so-called bystander effect [14]. There have been several studies of secondary intercellular signaling between irradiated and non-irradiated cells [15–17], but the mechanisms involved have not yet been fully understood.

Stress-induced ATP release is a well-known physiological response of various cells. However, it is not yet clear whether ionizing irradiation induces ATP release from cells. Here, we set out to examine whether or not ionizing γ-radiation induces ATP release in B16 melanoma cells, and if it does, what mechanisms are involved. We found that extracellular ATP was increased in response to γ-irradiation. The increase was abolished by an antagonist of $P2X_7$ receptor channel. The $P2X_7$ receptor-dependent ATP release in response to γ-irradiation was also confirmed by using $P2X_7$ receptor-knockdown B16 melanoma. Our results indicate that γ-irradiation evokes ATP release from melanoma cells, and $P2X_7$ receptor channel plays significant role in mediating this irradiation-induced ATP release.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium, RPMI1640 medium (phenol red free), penicillin, streptomycin and dithiothreitol (DTT) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Geneticin was purchased from Invitrogen (Carlsbad, CA). ATP, 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate (BzATP), $GdCl_3$, arachidonic acid, EtBr, glibenclamide and flufenamic acid (FFA) were purchased from Sigma-Aldrich (St Louis, MO). Selective inhibitor of $P2X_7$ receptor, 3-[[5-(2,3-dichlorophenyl)-1*H*-tetrazol-1-yl]methyl] pyridine hydrochloride (A438079) was purchased from Tocris Bioscience (Ellisville, MO). Fetal bovine serum (FBS) was purchased from Biowest (Nuaillé, France). $P2X_7$-short hairpin RNA (shRNA) expression vector and scramble-shRNA expression vector were purchased from Takara Bio (Shiga, Japan). The target sequence of $P2X_7$-shRNA is 5′-CAA ACT ATG TCA AGT CAG A-3′ ($P2X_7$-shRNA (1C)) and 5′-GAA CGA GTA TTA CTA CAG A-3′ ($P2X_7$-shRNA (3A)), corresponding to nt 317–335 and 1137–1155 of the $P2X_7$ receptor-encoding mRNA sequence (GenBank Accession Number: AJ489297). All other chemicals used were of the highest purity available.

2.2. Cell culture

B16 murine melanoma was routinely maintained in DMEM containing 10% heat-inactivated FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) at 37 °C in 5% CO_2, 95% air.

2.3. Measurement of extracellular ATP

Extracellular ATP concentration was measured using ENLITEN® rLuciferase/Luciferin Reagent (Promega, Madison, WI). Cells (3.0×10^5 cells/well) were incubated in 500 μl of RPMI1640 medium containing 1% FBS for 16 h in a 12-well culture plate. For investigation of radiation-induced ATP release, an aliquot (40 μl) of the conditioned medium was collected as a control sample for background ATP release. The cells were then irradiated with γ-rays from a Gammacell 40 (^{137}Cs source) (Nordin International, Inc., Ontario, Canada, 0.88 Gy/min) at room temperature for a suitable time. For hypotonic stress-induced ATP release, 250 μl of the conditioned medium was collected as a control sample for background ATP release. Hypotonic stress was then applied by gently adding 250 μl of H_2O to each well. After γ-ray irradiation or hypotonic stress, 40 μl of conditioned medium was collected at the indicated time points. Each sample was centrifuged at $600\times g$ for 5 min and 10 μl of the supernatant was used for ATP determination. The concentration of ATP was determined by measuring chemiluminescense with a TR717™ Microplate Luminometer (Applied Biosystems, Foster City, CA) 1.6 s after adding 100 μl of luciferin–luciferase reagent to 10 μl of sample solution.

2.4. Plasmid transfection and selection of stably transfectants

Cells were transfected with 3 μg of $P2X_7$-shRNA expression vector ($P2X_7$-shRNA (1C) and $P2X_7$-shRNA (3A)) or scramble-shRNA expression vector. Each experiment was performed in six wells containing cells at 50–60% confluence, using the transfection reagent FuGENE6 and FuGENE HD (Roche Diagnostic Systems, Branchburg, NJ). The transfected cells were selected in Geneticin-containing (2 mg/ml) culture medium for 3 weeks. $P2X_7$-shRNA (3A)-transfected polyclonal transfectant (P2X7-KD (3A)) was obtained. On the other hand, a stably $P2X_7$ receptor-knockdowned clone was selected from $P2X_7$-shRNA (1C)-transfected cells. We have pre-confirmed that incubation with 2 mM ATP for 12 h induces cell death in B16 melanoma. To help prescreening of highly $P2X_7$-knockdowned clones, $P2X_7$-shRNA (1C)-transfectants were incubated with 2 mM ATP for 12 h and ATP-high sensitive clones were excluded from prescreening process by flow cytometry. Clones were prescreened for gene-silencing by flow-cytometric assessment of pore formation induced by activation of $P2X_7$ receptor. Representative clones were chosen and used for detailed characterization of the gene-silencing effects of the shRNA expression vector construct. Integration of $P2X_7$-shRNA into the host genome was confirmed by genomic PCR analysis. A $P2X_7$ receptor-knockdowned clone (P2X7-KD (1C)) was established. These two $P2X_7$-shRNA-transfectants (monoclonal P2X7-KD (1C) cells and polyclonal P2X7-KD (3A) cells) were used to reduce a risk of off target effect.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using a Fast Pure RNA kit (Takara Bio). The first-strand cDNA was synthesized from 0.5 μg of total RNA with PrimeScript Reverse Transcriptase (Takara Bio). The sequences of specific primers for $P2X_7$ receptor were 5′-CTT GGC CAC TGT GTG CAT TGA CTT-3′ (sense) and 5′-TCA TGG AGC AGC TGG ATT TCC TCA-3′ (antisense) and those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-TGA TGA CAT CAA GAA GGT GGT GAA G-3′ (sense) and 5′-TCC TTG GAG GCC ATG TGG GCC AT-3′ (antisense). PCR was carried out by incubating each cDNA sample with the primers (0.5 μM each), Blend Taq polymerase (1.25 U: Toyobo, Osaka, Japan), and deoxynucleotide mix (0.2 mM each: Toyobo). Amplification was carried out for 35 cycles (94 °C for 30 s, annealing at 55 °C for 30 s, 72 °C for 1 min). The products were then subjected to 2% agarose gel electrophoresis. Bands were stained with EtBr and photographed.

2.6. Immunoblotting

Cells were dissolved in sample buffer (25% glycerin, 1% SDS, 62.5 mM Tris–Cl, 10 mM DTT) and boiled for 5 min. Aliquots of samples containing 20 μg of protein were analyzed by 10% SDS-PAGE and transferred onto a PVDF membrane. Blots were incubated at 4 °C overnight in TBS-Tween 20 (TBST: 10 mM Tris–HCl, 100 mM NaCl, 0.1% Tween 20, pH 7.5) with 5% skim milk, and then with rabbit anti-

rat $P2X_7$ receptor antibody (P8232) (1:200) (Sigma-Aldrich) [11] or mouse anti-actin antibody (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1.5 h. After having been washed with TBST, blots were incubated with goat HRP-conjugated anti-rabbit IgG antibody (1:20,000) (Cell Signaling Technology, Inc., Beverly, MA) or goat HRP-conjugated anti-mouse IgG antibody (1:20,000) (Santa Cruz Biotechnology) for 1.5 h at room temperature. The blots were further washed with TBST, and specific proteins were visualized by using ECL Western blotting detection reagents (GE Healthcare, Piscataway, NJ).

2.7. Flow cytometry

Uptakes of EtBr were determined by flow cytometry. Cells (1.0×10^6 cells/ml) were incubated with EtBr (25 μM) for 3 min at 37 °C in RPMI1640-based buffer (102 mM NaCl, 5 mM KCl, 0.4 mM $CaCl_2$, 0.4 mM $MgSO_4$, 23.8 mM $NaHCO_3$, 5.6 mM Na_2HPO4, 11.1 mM glucose and 10 mM HEPES-NaOH (pH 7.4)) after addition of $P2X_7$ agonist. After incubation, 10,000 cells for each sample were analyzed using a flow cytometer with laser excitation at 488 nm, and ethidium fluorescence (FL2-A) was measured. Mean fluorescence intensity was analyzed using WINMDI software version 2.9 (Joseph Trotter, The Scripps Institute, La Jolla, CA).

2.8. Statistics

Results are expressed as mean ± SEM. The statistical significance of differences between two groups was calculated by using the unpaired Student's *t*-test. The statistical significance of differences between control and other groups was calculated by using Dunnett's test. The criterion of significance was $P<0.05$ as determined with the Instat version 3.0 statistical package (Graph Pad Software, San Diego, CA).

3. Results

3.1. γ-Ray irradiation-induced ATP release from B16 melanoma and its blockade by $P2X_7$ receptor antagonist

We measured the concentration of ATP in culture medium of B16 melanoma cells after γ-irradiation by means of luciferin–luciferase assay. The concentration of extracellular ATP increased upon γ-irradiation of the cells and reached a peak at 5 min (Fig. 1A), then decreased to the basal level within 30 min. As shown in Fig. 1B, the concentration of extracellular ATP peaked at a γ-irradiation dose of 0.5 Gy; higher radiation doses resulted in a smaller increase of ATP concentration. In the following experiments, we examined the ATP release at 5 min after 0.5 Gy γ-irradiation.

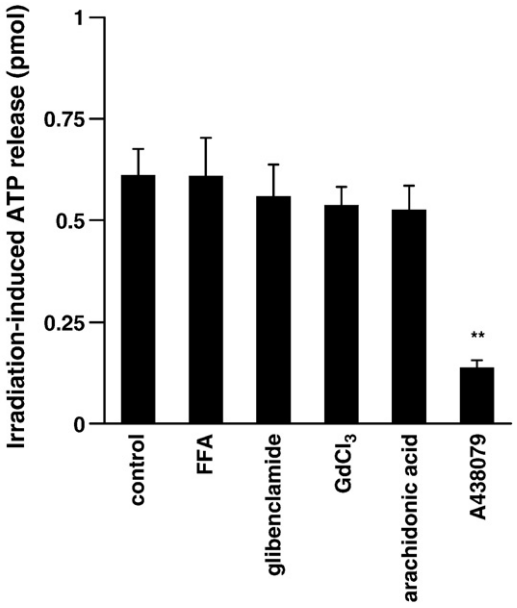

Fig. 2. Effect of pharmacological inhibition on ionizing irradiation-induced ATP release. Cells were pretreated with FFA (50 μM), glibenclamide (100 μM), $GdCl_3$ (50 μM), arachidonic acid (20 μM) and A438079 (100 μM) for 30 min. At 5 min after 0.5 Gy of γ-irradiation, supernatants were collected and ATP contents were measured ($n=11$–25). The *Y*-axis shows the increase of ATP contents induced by γ-irradiation. The data represent the means ± SEM. A statistically significant difference from the control is indicated by ** ($P<0.01$).

To determine the pathway of the ATP release, we examined the effect of anion channel/transporter blockers and an antagonist of $P2X_7$ receptor channel on radiation-induced ATP release. The ATP release was not affected by application of a potent chloride channel inhibitor, glibenclamide (100 μM) [3], an inhibitor of anion transporters, FFA (50 μM) [18], inhibitors of maxi-anion channel, $GdCl_3$ (50 μM) [2,19] and arachidonic acid (20 μM) [20]. However, treatment with 100 μM A438079, a selective $P2X_7$ receptor antagonist [21], significantly inhibited the radiation-induced ATP release (Fig. 2). These results indicate a contribution of $P2X_7$ receptor to the ATP release.

3.2. Establishment of $P2X_7$ receptor-knockdown B16 melanoma cell line

In order to demonstrate involvement of $P2X_7$ receptor in radiation-induced ATP release, B16 melanoma cells were stably transfected with

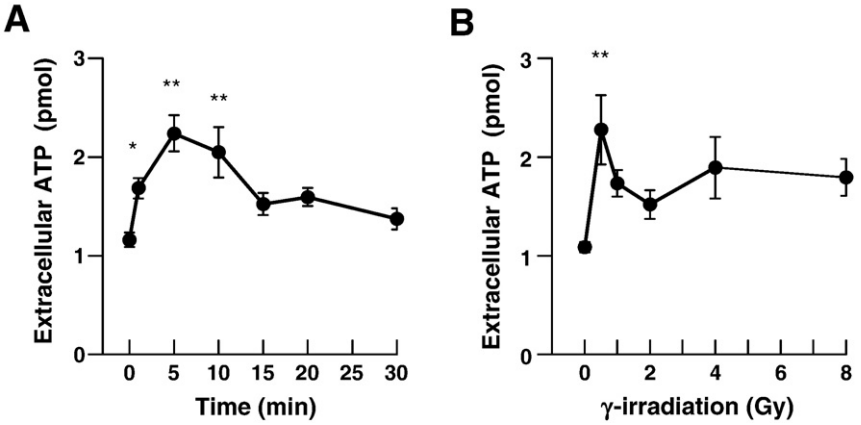

Fig. 1. Ionizing irradiation-induced ATP release from B16 melanoma. Cells were exposed to 0.5 Gy of γ-rays and incubated for indicated times (A), or exposed to various doses of γ-rays and incubated for 5 min (B). After incubation, the contents of ATP in culture medium were measured ($n=6$–8). The data represent the means ± SEM. A statistically significant difference from the control is indicated by * ($P<0.05$) or ** ($P<0.01$).

$P2X_7$-shRNA (1C) vector. Cells were also stably transfected with scramble-shRNA vector as a negative control (mock). The expression levels of $P2X_7$ mRNA and protein were analyzed in a $P2X_7$ receptor-knockdown clone (P2X7-KD (1C)). The mRNA expression of $P2X_7$ receptor in P2X7-KD (1C) cells was decreased to 15% of that in wild-type (WT) cells (Fig. 3A). In contrast, transfection with scramble-shRNA did not affect $P2X_7$ receptor mRNA expression. The protein expression of $P2X_7$ receptor was also decreased in P2X7-KD (1C) cells, as shown in Fig. 3B. Integration of both $P2X_7$-shRNA (1C) and scramble-shRNA into the host genome was confirmed by genomic PCR analysis (data not shown).

Next, we confirmed the functional knockdown of the $P2X_7$ receptor in P2X7-KD (1C) cells. For this purpose, we examined the effect of knockdown of the $P2X_7$ receptor on pore formation (EtBr uptake) by flow cytometry. Significant uptake of EtBr was observed in WT and mock cells at 3 min after treatment with 1 mM ATP or 300 μM BzATP, an agonist of $P2X_7$ receptor (Fig. 3C and D). As expected, the uptake of EtBr was significantly suppressed in A438079-treated WT cells and P2X7-KD (1C) cells (Fig. 3C and D). This result indicated that early events downstream of activation of the $P2X_7$ receptor were decreased in P2X7-KD (1C) cells. Thus, considering the decrease of both expression and function of the $P2X_7$ receptor in P2X7-KD (1C) cells, we concluded that this melanoma clone exhibits stable knockdown of the $P2X_7$ receptor.

3.3. Radiation-induced ATP release was diminished by knockdown of $P2X_7$ receptor

Before we examined the effect of knockdown of $P2X_7$ receptor on radiation-induced ATP release, we confirmed the utility of P2X7-KD (1C) cells for analyzing $P2X_7$ receptor-dependent ATP release. Since it has been reported that the early phase of ATP release induced by hypotonic stress is mediated through anion channels/transporters and $P2X_7$ receptor channel [4], we examined whether hypotonicity-induced ATP release is suppressed in P2X7-KD (1C) cells. A large amount of ATP was released within 1 min after hypotonic stress in WT

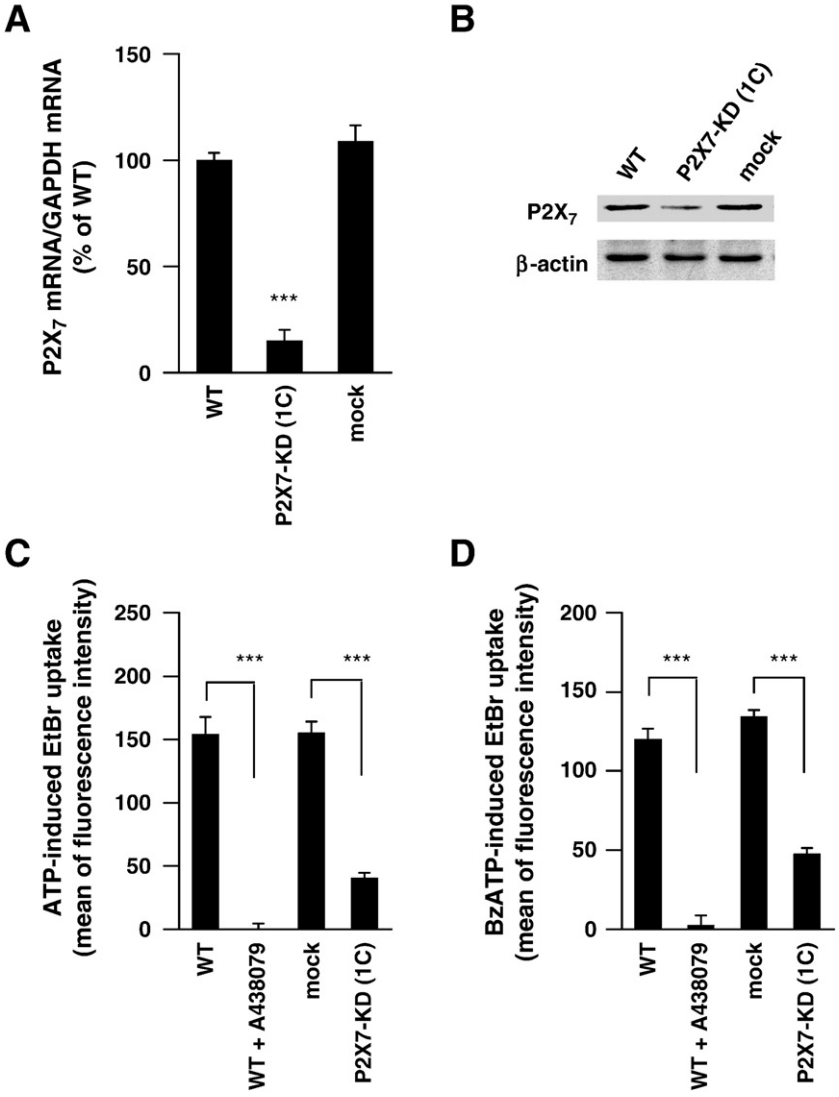

Fig. 3. Expression and activity of $P2X_7$ receptor in $P2X_7$ receptor-knockdown melanoma clone. (A) Expression levels of $P2X_7$ mRNA in WT, mock and P2X7-KD (1C) cells were determined by RT-PCR. Optical density of $P2X_7$ mRNA was normalized to the optical density of GAPDH mRNA. The data represent means ± SEM of four independent experiments. A statistically significant difference between mock cells and P2X7-KD (1C) cells is indicated by *** ($P<0.001$). There was no significant difference of $P2X_7$ receptor expression between WT cells and mock cells. (B) Protein expression of $P2X_7$ receptor was determined by immunoblotting. As a control for equivalent protein loading, beta-actin was simultaneously detected. Representative images from three independent experiments are shown. (C, D) WT, mock and P2X7-KD (1C) cells were incubated with ATP (1 mM) (C), BzATP (300 μM) (D) and EtBr (25 μM) in RPMI1640-based buffer for 3 min. Cells were pre-incubated with A438079 (100 μM) for 15 min, and the change in fluorescence intensity of EtBr was analyzed by flow cytometry. The data represent means ± SEM ($n=4–12$). A statistically significant difference is indicated by *** ($P<0.001$). There was no significant difference of $P2X_7$ agonist-induced EtBr uptake between WT cells and mock cells.

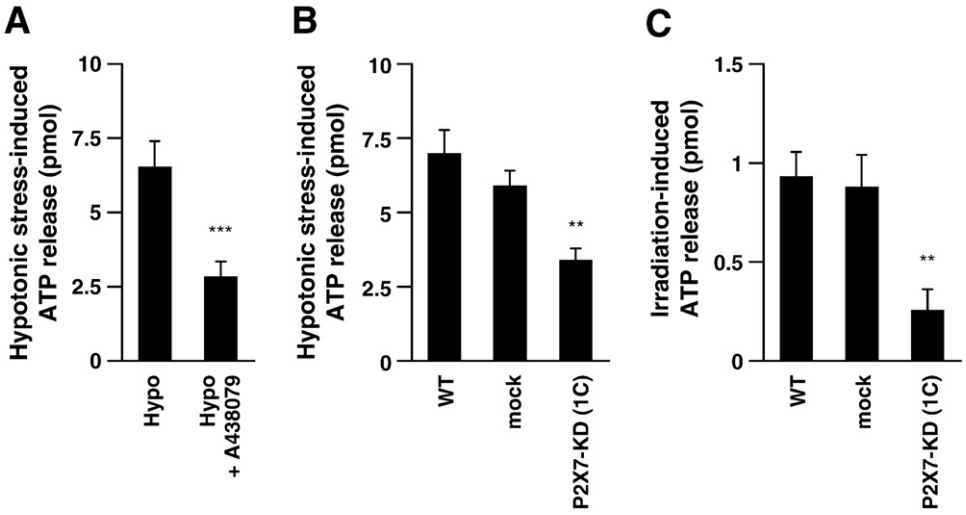

Fig. 4. Knockdown of $P2X_7$ receptor attenuates radiation-induced ATP release. (A, B) WT cells were pre-treated with vehicle or A438079 (100 μM) for 30 min (A, $n=7$–11). WT, mock and P2X7-KD (1C) cells were incubated in RPMI1640 medium containing 1% FBS (B, $n=8$). At 1 min after application of hypotonic stress, supernatants were collected and the contents of ATP were measured. The *Y*-axis shows the increase of ATP contents induced by hypotonic stress. (C) WT, mock and P2X7-KD (1C) cells were incubated in RPMI1640 medium containing 1% FBS. At 5 min after 0.5 Gy γ-irradiation, supernatants were collected and ATP contents were measured (C, $n=14$–16). The *Y*-axis shows the increase of ATP contents induced by γ-irradiation. The data represent the means ± SEM. A statistically significant difference is indicated by ** ($P<0.01$) or *** ($P<0.001$).

cells, and the release was significantly inhibited by treatment with A438079, indicating the involvement of $P2X_7$ receptor (Fig. 4A). Further, the hypotonicity-induced ATP release was significantly suppressed in P2X7-KD (1C) cells (Fig. 4B), indicating that P2X7-KD (1C) cells would indeed be useful for evaluation of $P2X_7$ receptor-dependent ATP release. Next, we evaluated the involvement of $P2X_7$ receptor in radiation-induced ATP release using P2X7-KD (1C) cells. As shown in Fig. 4C, γ-irradiation-induced ATP release was significantly lower in P2X7-KD (1C) cells than in WT or mock cells.

Further, to reduce a risk of potential off-target effect of the $P2X_7$-shRNA (1C), we also transfected with another shRNA targeting to other sequences of $P2X_7$ receptor mRNA ($P2X_7$-shRNA (3A)). As shown in Fig. 5A, the expression of $P2X_7$ receptor in P2X7-KD (3A) cells was decreased to 40% of that in mock cells. The uptake of EtBr in response to ATP was also lower in P2X7-KD (3A) cells than mock cells (Fig. 5B), indicating that the expression and function of $P2X_7$ receptor was decreased in P2X7-KD (3A) cells. We also confirmed that hypotonic stress-induced ATP release in P2X7-KD (3A) cells was also lower than that in mock cells (data not shown), indicating that P2X7-KD (3A) cells are also useful for evaluation of $P2X_7$ receptor-dependent ATP release. Finally, the ATP release induced by γ-irradiation was evaluated in P2X7-KD (3A) cells, and we found that the ATP release was suppressed in P2X7-KD (3A) cells (Fig. 5C). These results strongly support the involvement of $P2X_7$ receptor channel in radiation-induced ATP release from B16 melanoma cells.

4. Discussion

ATP is released into extracellular space from various cells in response to stress stimuli, such as hypoxia, low glucose, and hypotonicity,

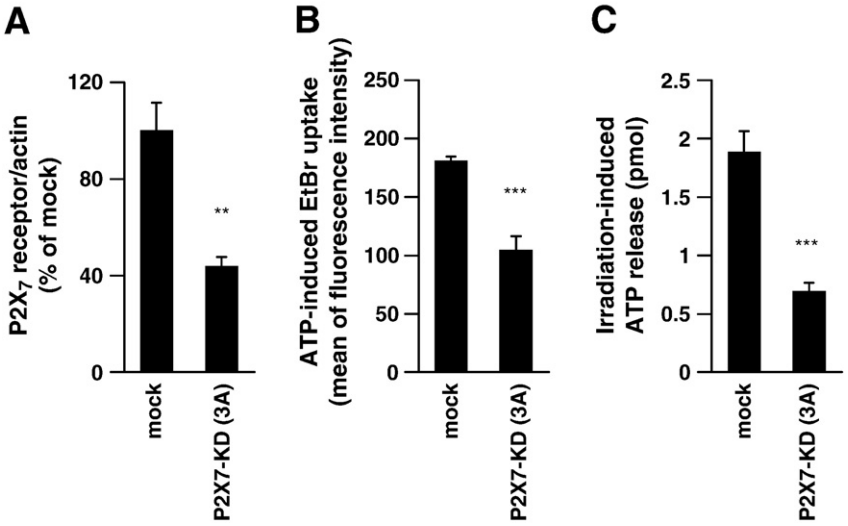

Fig. 5. Attenuation of radiation-induced ATP release by another shRNA knockdown of $P2X_7$ receptor. (A) Protein expression of $P2X_7$ receptor was determined by immunoblotting. As a control for equivalent protein loading, beta-actin was simultaneously detected. Optical density of $P2X_7$ receptor was normalized to the optical density of beta-actin. The data represent means ± SEM ($n=4$). (B) Mock and P2X7-KD (3A) cells were incubated with ATP (1 mM) and EtBr (25 μM) in RPMI1640-based buffer for 3 min and the change in fluorescence intensity of EtBr was analyzed by flow cytometry. The data represent means ± SEM ($n=8$). (C) Mock and P2X7-KD (3A) cells were incubated in RPMI1640 medium containing 1% FBS. At 5 min after 0.5 Gy γ-irradiation, supernatants were collected and ATP contents were measured. The *Y*-axis shows the increase of ATP contents induced by γ-irradiation. The data represent the means ± SEM ($n=6$–11). A statistically significant difference is indicated by ** ($P<0.01$) or *** ($P<0.001$).

and acts as an intercellular signaling molecule, evoking activation of P2 receptors on the cell surface in an autocrine/paracrine manner [1]. Although it is widely known that various stresses induce ATP release from cells, there has been no report on ionizing irradiation-induced ATP release and activation of P2 receptors in irradiated and adjacent non-irradiated cells. In this study, we investigated whether γ-irradiation induces ATP release and further examined the mechanism of the release in B16 melanoma cells.

First, we found that the concentration of ATP in culture medium of irradiated B16 melanoma cells was elevated after γ-irradiation, reaching a peak at 5 min, and then declining to the basal level within 30 min. These results are consistent with γ-irradiation-induced ATP release from the cells, followed by degradation of released ATP by ecto-nucleotidases on the cell surface. A dose of 0.5 Gy of irradiation induced the greatest release of ATP. We speculate that oxidative damage to membrane proteins by higher doses of γ-irradiation might lead to impairment of ATP-release mechanisms, such as $P2X_7$ receptor. Endogenous ATP is released through various pathways, such as the maxi-anion channel, volume-sensitive outwardly rectifying chloride channel and $P2X_7$ receptor channel [2–4]. Treatment with glibenclamide, FFA, $GdCl_3$ and arachidonic acid, which are inhibitors of anion channels or transporters [2,3,18–20], did not abrogate radiation-induced ATP release. However, we observed a significant blockade of radiation-induced ATP release by a highly selective antagonist of $P2X_7$ receptor, A438079 [21], suggesting that $P2X_7$ receptor would be involved in the release.

To examine the involvement of $P2X_7$ receptor in radiation-induced ATP release in more detail, we established a stable $P2X_7$ receptor-knockdown B16 melanoma cell line (P2X7-KD (1C)). Integration of shRNA-vector into the host genome was first confirmed by genomic PCR, and we found that expression of $P2X_7$ receptor was significantly reduced in P2X7-KD (1C) at both the mRNA and protein levels. We also evaluated $P2X_7$ receptor activity in P2X7-KD (1C). Activation of $P2X_7$ receptor leads to an increase of cationic permeability, followed by plasma membrane depolarization; intense or prolonged activation leads to the opening of large non-selective pores, allowing the passage of hydrophilic molecules [7]. Treatment with BzATP, a $P2X_7$ receptor agonist, led to EtBr uptake, and this EtBr uptake was blocked by A438079, indicating that the pore formation is mediated by activation of $P2X_7$ receptor in B16 melanoma cells. Little EtBr uptake in response to $P2X_7$ agonists was observed in P2X7-KD (1C) cells, suggesting that $P2X_7$ receptor activity was diminished by knockdown of $P2X_7$ receptor, as expected. These properties indicate that P2X7-KD (1C) is a stable $P2X_7$ receptor-knockdown melanoma clone.

We investigated whether P2X7-KD (1C) cells were suitable for investigation of ATP release mediated by $P2X_7$ receptor. Since the involvement of $P2X_7$ receptor in hypotonic stress-induced ATP release has been reported [4], we examined the ATP release induced by hypotonic stress in P2X7-KD (1C) cells. The rapid phase of ATP release by hypotonic stress was blocked by pretreatment with A438079, and was decreased in P2X7-KD (1C) cells, indicating that P2X7-KD (1C) cells are available to determine the involvement of the $P2X_7$ receptor in irradiation-induced ATP release. The γ-irradiation-induced ATP release was significantly reduced in P2X7-KD (1C) compared with WT or mock. Moreover, the blockade of the ATP release by irradiation was also confirmed in another $P2X_7$-shRNA-transfected polyclonal cell (P2X7-KD (3A)). These results strongly support the involvement of $P2X_7$ receptor in ionizing irradiation-induced ATP release from melanoma cells.

Released ATP would be rapidly (millisecond order) metabolized by ecto-nucleotidase expressed on the cell membrane [22], and diluted in the culture supernatant. Therefore, the concentration of ATP on cell surface actually released from cells in response to γ-irradiation could be substantially higher than that detected in this study. The release of ATP in response to irradiation would serve to activate P2 receptors.

Ionizing irradiation, including γ-rays, not only have an anti-cancer effect, but also may promote cancer malignancy [23,24]. Recent studies have revealed that irradiation enhances malignant behavior of cancer cells by promoting release of growth factors, proteases or inflammatory cytokines from cancer cells [25–27]. Several reports on P2 receptors in cancer cells indicate that release of ATP from cancer cells might enhance cancer growth and contribute to malignancy. For example, it has been reported that activation of P2Y receptors induced proliferation of cancer cells [28] and promoted angiogenesis by activation of vascular endothelial cells [29]. It has also been reported the activation of $P2X_7$ receptor-mediated release of cytokines, such as monocyte chemoattractant protein-1, interleukin-6, interleukin-8 and vascular endothelial growth factor [30,31]. ATP is rapidly degraded to ADP, AMP and adenosine by ecto-nucleotidases after release to extracellular space, and these metabolites also modulate the cancer microenvironment by activation of purinergic or adenosinergic signaling. Human breast carcinoma cells secrete nucleotide diphosphate kinase, which converts ATP to ADP (a ligand of $P2Y_1$ receptor), leading to activation of $P2Y_1$ receptor-mediated angiogenesis [32]. In addition, extracellular adenosine has an immunosuppressive effect via activation of A_{2A} receptor on T lymphocytes, and enhances cancer progression [33]. As well as ATP, other nucleotides such as UTP and UDP activate P2 receptor. UTP activates $P2Y_2$ and $P2Y_4$ receptors, and their activation induces cell proliferation [34,35]. UTP and UDP are also released from cells in response to stress stimuli [36,37]. Recently, it has been reported that extracellular ATP has a radioprotective effect on human blood cells via $P2Y_{11}$ receptor [38]. As mentioned elsewhere, increased expression of $P2X_7$ receptor has been reported in thyroid papillary cancer cells and melanoma [6,30]. Since $P2X_7$ receptor appears to be involved in the mechanisms of radiation-induced ATP release, irradiation might induce greater ATP release from highly $P2X_7$ receptor-expressing cancer cells during radiotherapy. Thus, nucleotides and their metabolites generated following radiotherapy might induce enhancement of cancer progression or radioresistance through purinergic and adenosinergic signaling pathways.

In conclusion, we have established that ionizing irradiation causes cytosolic ATP release from B16 melanoma cells. Our results indicate the involvement of $P2X_7$ receptor channel in the release mechanism. This is the first study to demonstrate that ionizing irradiation induces ATP release from cancer cells and $P2X_7$ receptor plays a significant role in the irradiation-induced ATP release. Because the mechanism of $P2X_7$ receptor-dependent ATP release induced by several stimulations has not yet been understood, we should reveal the mechanism of $P2X_7$ receptor-dependent ATP release induced by various stimuli, including ionizing irradiation, in future. We here suggest that extracellular ATP acts as a novel intercellular signaling molecule between cancer cells and adjacent cells when cells are exposed to ionizing radiation. Further investigation of radiation-induced purinergic and/or adenosinergic signaling is needed.

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