Original Article

Purine receptor P2Y₆ mediates cellular response to γ -ray-induced DNA damage

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(Received October 9, 2013; Accepted October 28, 2013)

ABSTRACT — We previously showed that nucleotide P2 receptor agonists such as ATP and UTP amplify γ -ray-induced focus formation of phosphorylated histone H2A variant H2AX (γ H2AX), which is considered to be an indicator of DNA damage so far, by activating purine P2Y₆ and P2Y₁₂ receptors. Therefore, we hypothesized that these P2 receptors play a role in inducing the repair response to γ -ray-induced DNA damage. In the present study, we tested this idea by using human lung cancer A549 cells. First, reverse-transcription polymerase chain reaction (RT-PCR) showed that P2Y₆ receptor is highly expressed in A549 cells, but P2Y₁₂ receptor is only weakly expressed. Next, colony formation assay revealed that P2Y₆ receptor antagonist MRS2578 markedly reduced the survival rate of γ -ray-exposed A549 cells. The survival rate was also significantly reduced in P2Y₆-knock-down cells, compared with scramble siRNA-transfected cells. Since it has reported that phosphorylation of ERK1/2 after activation of EGFR via P2Y₆ and P2Y₁₂ receptors is involved in the repair response to γ -ray-induced DNA damage, we next examined whether γ -ray-induced phosphorylation of ERK1/2 was also inhibited by MRS2578 in A549 cells. We found that it was. Taken together, these findings indicate that purinergic signaling through P2Y₆ receptor, followed by ERK1/2 activation, promotes the cellular repair response to γ -ray-induced DNA damage.

Key words: DNA damage repair, Radiation, P2Y₆ receptor, ADP, γ H2AX

INTRODUCTION

It is well known that exposure of cells to ionizing radiation causes DNA damage, including double-strand breaks (DSBs), which can lead to cell death or genomic instability. DSBs can be repaired via two major mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ) (Lieber, 2010; Wang and Lees-Miller, 2013). The master controller of the DSB response is ataxia telangiectasia mutated (ATM), a member of the phosphoinositide 3-kinase-related protein kinase (PIKK) family that also includes DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and ataxia telangiectasia and Rad3-related kinase (ATR) (Bekker-Jensen and Mailand, 2010; Ciccica and Elledge, 2010; Rass *et al.*, 2013; Shiloh, 2003; Thompson, 2012). In response to DSBs, ATM is activated to phosphorylate multiple DSB response proteins, including histone H2A variant H2AX, MDC1, BRCA1 and NBS1. Rapid phosphorylation of H2AX on serine 139 of the carboxyl-terminal SQEY motif (affording γ H2AX) proceeds over a

large chromatin domain flanking the DSBs, and initiates a series of downstream reactions including protein recruitment and posttranslational protein modifications. However, many questions remain regarding the mechanisms, and it is also not clear whether autocrine signaling is involved in the response to DNA damage.

Cytoplasmic ATP is released into the extracellular space in response to various stimuli and activates P2 receptors (Yegutkin, 2008). P2 receptors are classified into two major subtypes, ionotropic P2X receptors and metabotropic P2Y receptors, and their activation regulates many physiological functions (Burnstock, 2006, 2009). The released ATP is rapidly degraded to ADP, AMP, and adenosine by ectonucleotidases (Zimmermann and Braum, 1999). ADP and adenosine are ligands of P2Y and P1 receptors, respectively (Yegutkin, 2008). Thus, cytosolic ATP release causes activation of various purinergic and adenosinergic signaling pathways in an autocrine/paracrine manner. Recently, several studies have shown that purinergic signaling via extracellular ATP stimulates DNA repair (Nishimaki *et al.*, 2012;

Pines *et al.*, 2005) and antioxidant activity and protects cells from oxidative damage (Ohshima *et al.*, 2012a; Shinozaki *et al.*, 2005).

We previously demonstrated the participation of purinergic P2Y receptors in this DNA injury-repairing mechanism (Nishimaki *et al.*, 2012). Focus formation of γ H2AX was induced in human lung cancer A549 cells by exposure to more than 0.5 Gy γ -rays. The number of foci was decreased by pretreatment with an ecto-nucleotidase apyrase, whereas the enzyme inhibitor ARL67156 increased the focus formation. In addition, the number of γ H2AX foci was increased when P2 receptor agonists, such as ATP and UTP, were added immediately after the irradiation. These results suggested that release of nucleotides from the cells promoted γ H2AX focus formation. Thus, we examined the involvement of P2 receptors in γ H2AX focus formation, which is generally considered to be an index of DNA damage, by using inhibitors of the receptors. We found that P2Y₆ receptor antagonist MRS2578, and P2Y₁₂ receptor antagonist clopidogrel significantly inhibited the focus formation, suggesting involvement of these receptors in the repair of γ -ray-induced DNA injury. On the other hand, ATP and UTP had no effect on γ H2AX focus formation in non-irradiated A549 cells, suggesting that these nucleotides themselves do not induce DNA injury. Furthermore, knock-down of the P2Y₆ and P2Y₁₂ receptors decreased γ H2AX focus formation, supporting the idea that these receptors are involved in repair of γ -ray-induced DNA injury. The aim of the present work was to directly confirm the role of P2 receptor activation in repair of DNA damage by using colony formation assay to evaluate cell survival after irradiation. Our results confirm that release of extracellular nucleotides and activation of P2 receptors, especially P2Y₆, participate in the repair response to γ -ray-induced DNA damage, supporting the idea that autocrine/paracrine signaling mediated by nucleotides plays a key role in the cellular DNA repair response.

MATERIALS AND METHODS

Reagents and antibodies

DMEM was purchased from Wako Pure Chemical (Osaka, Japan). FBS was purchased from Biowest (Nuaillé, France). Phospho-histone H2AX (Ser139) rabbit monoclonal antibody, rabbit anti-rat phospho-ERK1/2 (Thr202/Tyr204) antibody, rabbit anti-rat ERK1/2 antibody and goat anti-rabbit IgG-FITC were purchased from Cell Signaling Technology (Beverly, MA, USA). Purified mouse anti-H2AX phosphorylated (Ser139) antibody was from BioLegend (San Diego, CA, USA). Alexa

Fluor 594 goat anti-mouse IgG was from Molecular Probes (Eugene, OR, USA).

Agonists, antagonists, and inhibitors

ATP diphosphohydrolase (ecto-nucleotidase) apyrase was purchased from Sigma-Aldrich (St Louis, MO, USA). Agonists of P2 receptors, ADP and UDP, were from Sigma-Aldrich. Antagonists of P2 receptors, MRS 2179 (a selective antagonist of P2Y₁), MRS2211 (a selective antagonist of P2Y₁₃), MRS2578 (a selective antagonist of P2Y₆), NF157 (a selective P2Y₁₁ antagonist), and clopidogrel (a selective antagonist of P2Y₁₂), were from Tocris Bioscience (Ellisville, MO, USA). Suramin (a non-selective antagonist of P2Y) was from Sigma-Aldrich. U0126 (a specific inhibitor of MEK1/2), SP600125 (a specific inhibitor of JNK), and SB203580 (a specific inhibitor of p38 kinase) were obtained from Calbiochem (La Jolla, CA, USA).

Cell culture and irradiation

A549 human adenocarcinoma cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μ g/ml) in a humidified atmosphere of 5% CO₂ in air at 37°C. The cells were then irradiated with γ -rays from a Gamma-cell 40 (¹³⁷Cs source) (Nordin International, Inc., Ontario Canada; 0.87 Gy/min) at room temperature for a suitable time. After irradiation, the cells were incubated in a humidified atmosphere of 5% CO₂ in air at 37°C.

Colony formation assay

A549 cells (1.0 $\times$ 10⁵ cells/ml) were seeded in a 40 mm dish and incubated for 24 hr in a 5% CO₂ incubator at 37°C, followed by exposure to γ -rays at a dose of 2 Gy and further incubation for another 24 hr. Then, 1 $\times$ 10³ cells were seeded in a 100 mm dish. After incubation for 1 week, the cells were stained with 0.5% crystal violet. Colonies containing more than 50 cells were counted. The averages of triplicate dishes were obtained for each sample. The results were normalized with respect to corresponding non-irradiated cells, and the survival rates were calculated. Antagonists were added to the medium 30 min before irradiation.

Immunofluorescence staining

A549 cells were fixed in 4% paraformaldehyde in 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 hr, the fixed cells were incubated with primary antibody (γ H2AX 1:200, phospho-ERK1:1,000 and ERK 1:1,000) for 24 hr at 4°C

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Table 1. Primers for RT-PCR Used in the present study

	Accession no.	Primer sequences	Expected size (bp)
P2X1	NM_002558	GGCTCGGAATTGGCATCTT CGGGTTGGATAATGAGAGTC	520
P2X2	NM_016318	CAGCATCCACTACCCCAAAT TAGCAAGATCCAGTCGCACA	490
P2X3	NM_002559	GAGGAGAAATACCGCTGTGT TCAGGTTGGGAAGGAGGTTT	256
P2X4	NM_031594	GATCCCTTCTGCCCCATATT TAGGCGTCACTGGTTCATCT	504
P2X5	NM_002561	AAGAACCACTACTGCCCCAT GTTCAACCATCACGTCAAAGC	310
P2X6	AF065385	CTGCCGCTATGAACCACAAT GCAGTAGCAGGTCACAGAAA	398
P2X7	NM_002562	CCATCACTGCCATCCCAAAT ATATGGGAGCGACAGCAGTT	303
P2Y1	NM_002563	GGTCTAGCAAGTCTCAACAG AAGCTAAGTGTGGATGTGGG	359
P2Y2	NM_002564	TTGCCGTCATCCTTGTCTGT CTGCCCAACACATCTCCTAT	433
P2Y4	NM_002565	TGTCCTTTTCTCACCTGCA AGTAAATGGTGCGGGTGATG	440
P2Y6	NM_004154	CACATCACCAAGACAGCCTA TCTTAACTCCATGCCAGCT	340
P2Y11	NM_002566	TCCACCCCTCTACTCTACATG TGTAGAGTAGAGGGTGGACA	443
P2Y12	NM_022788	GTGTCAAGTTACCTCCGTCA ATGCCAGACTAGACCGAACT	274
P2Y13	NM_176894	TGAGGAACTGAAGTGGGTCAGCAA AGGCCAGAAAGGTAGGCAAGTTCT	813
P2Y14	BC034989	TGCTGCAAATGTATGCTTGGACCC TGCAAACAGCTCTCTAGCCCTTCA	640
GAPDH	NM_031144	TGATGACATCAAGAAGGTGGTGAAG TCCTGGAGGCCATGTGGGCCCAT	240

and with secondary antibody (goat anti-rabbit IgG-FITC 1:200) for 1 hr. Counterstaining with Hoechst 33258 (1 mg/ml) was used to verify the location and integrity of nuclei. Fluorescence images were obtained with a confocal laser scanning microscope (DMIRBE; Leica, Deerfield, IL, USA).

RT-PCR

Total RNA was isolated from cells using a FastPure

RNA kit (Takara Bio, Shiga, Japan). The first-strand cDNA was synthesized from 0.5 µg of total RNA with Prime Script Reverse Transcriptase (Takara Bio). The primers used for amplification were designed based on the cDNA sequences of human P2 receptors, as summarized in Table 1. Polymerase chain reactions were performed by incubating each cDNA sample with the appropriate primers (0.5 µM each), dNTP mix (0.2 mM each) and Blend Taq polymerase (Toyobo, Osaka, Japan) (1.25

U each). Amplification was carried out for 35 cycles (94°C for 30 sec, annealing at 60°C for 30 sec, 72°C for 1 min), followed by extension at 72°C for 5 min. The products were then subjected to 2% agarose gel electrophoresis. The bands were stained with ethidium bromide and photographed.

siRNA transfection for knockdown of P2Y₆ receptor

P2Y₆ receptor knockdown was done by transfection with siRNA as described in our previous report (Tamaishi *et al.*, 2011), and was confirmed to cause a significant decrease of P2Y₆ receptor protein. The expression level of P2Y₆ protein in P2Y₆-knockdown cells was decreased to 48% of that in cells transfected with scrambled siRNA. Briefly, siRNA duplex oligonucleotides (10 nM) for reduction of human P2Y₆ receptor were transfected into cells (5 × 10⁴ cells) by using HiperFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were irradiated with γ-rays.

Immunoblotting

Cells were washed twice with ice-cold PBS and lysed in PBS containing 10 mM Hepes-NaOH (pH 7.4), 1% Triton X-100, 5 mM EDTA, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1.04 mM 4-(2-aminoethyl)- benzenesulfonyl fluoride, 0.8 μM aprotinin, 21 μM leupeptin, 36 μM bestatin, 15 μM pepstatin A and 14 μM E-64 for 30 min on ice. Samples were centrifuged (10,000 g, 10 min) at 4°C and the supernatants were collected. The samples were mixed with 2 × sample buffer (50% glycerin, 2% SDS, 125 mM Tris, 10 mM DTT) and incubated at 95°C for 10 min. Concentration of protein in samples was determined with Bio-Rad Protein assay reagent (BioRad, Hercules, CA, USA). Aliquots of samples containing proteins were analyzed by 10% SDS-PAGE and bands were transferred onto PVDF membranes. The blots were incubated with 1% BSA in TBST (0.1% Tween-20, 10 mM Tris-HCl, 0.1 M NaCl) overnight, then with primary antibody (ERK1/2 1:1,000, p-ERK1/2 1: 1,000) at 4°C overnight. They were then washed with TBST and incubated with an appropriate secondary antibody (1:20,000) for 1.5 hr at room temperature. The levels of specific proteins were visualized by using ECL Western detection reagents (GE Healthcare, Piscataway, NJ, USA).

Flow cytometric analysis of cell cycle

Cells were harvested by trypsinization, washed by PBS, and then fixed in cold 70% ethanol on ice for 1 hr.

Cell suspensions were washed twice with PBS and treated with ribonuclease A (0.34 mg/ml), followed by PI staining (50 μg/ml) for 30 min. Flow cytometric analysis of DNA content and cell cycle distribution was then performed.

Statistics

Results are expressed as means ± S.E. The statistical significance of differences between control and other groups was calculated using Dunnett's test. Multiple groups were compared using ANOVA followed by pairwise comparisons with Bonferroni's *post hoc* analysis. Calculations were done with the Instat version 3.0 statistical software package (Graph Pad Software, San Diego, CA, USA). The criterion of significance was $P < 0.05$.

RESULTS

Identification of P2 receptor subtypes expressed on cell membrane and effect of P2Y₆ receptor antagonists on colony formation after γ-irradiation in A549 cells

First we examined the expression of P2 receptor subtypes in A549 cells by means of RT-PCR. As shown in Fig. 1A, A549 cells strongly expressed P2X_{4,5} and P2Y_{1,6,11} receptor subtypes, but only weakly expressed P2X_{3,6} and P2Y_{4,12,13,14} subtypes. Thus, we focused on P2Y₆ receptor, and examined the effect of P2Y₆ receptor antagonists on colony formation induced by γ-irradiation. As shown in Fig. 1B, apyrase significantly inhibited the survival/colony formation rate. A selective antagonist of P2Y₆ (MRS2578) also markedly inhibited the survival rate, while a selective antagonist of P2Y₁₂ (clopidogrel) and other antagonists, including suramin (a broad P2Y antagonist), A438079 (a selective P2X₇ antagonist), NF157 (a selective P2Y₁₁ antagonist) and MRS2211 (a selective P2Y₁₃ antagonist), had no effect. It is noted that the effective concentrations of antagonists were used in this experiment according to our previous experiments (Nishimaki *et al.*, 2012; Tamaishi *et al.*, 2011).

Effect of P2Y₆ receptor agonists and P2Y₆ receptor knock-down on colony formation after γ-irradiation in A549 cells

The effect of P2Y₆ receptor agonists on colony formation of A549 cells after 2 Gy γ-irradiation was examined. The cells were treated with agonist at 1 min after 2 Gy γ-irradiation and survival was examined by colony formation assay after 1 week. As shown in Fig. 2A, the survival rate was increased in ADP-treated cells, though not significantly. In contrast, UDP significantly increased the survival rate, in comparison with control cells. To

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Fig. 1. Identification of P2 receptor subtypes expressed on A549 cell membrane and effect of P2Y receptor antagonists on survival/colony formation rate after γ -irradiation. (A) Total RNA was isolated from A549 cells and the expression of P2X and P2Y receptor subtypes was analyzed by RT-PCR using specific primers for each P2 receptor subtype, as indicated in Table 1. (B) A549 cells (1.0×10^5 cells/ml) were seeded in a 40 mm dish and incubated for 24 hr in a humidified atmosphere of 5% CO_2 in air at 37°C , then exposed to γ -rays at a dose of 2 Gy, and further incubated for another 24 hr. Then, 1×10^3 cells were seeded in a 100 mm dish. After incubation for 1 week, the cells were stained with 0.5% crystal violet. Colonies that included more than 50 cells were counted. Apyrase (20 U/ml), suramin (100 μM), MRS2179 (10 μM), MRS2578 (10 μM), clopidogrel (30 μM), or MRS2211 (50 μM), was added to the medium 30 min before irradiation. APY, apyrase; SURA, suramin; 2179, MRS2179; 2578, MRS2578; 157, NF157; CLOP, Clopidogrel; 2211, MRS2211. Each value represents the mean $\pm$ SE ($n = 3$). ** $P < 0.01$, *** $P < 0.001$ vs γ -Ray-alone group.

Fig. 2. Effect of P2Y₆ receptor agonists and P2Y₆ receptor knock-down on survival/colony formation rate after γ -irradiation in A549 cells. (A) Effect of P2Y₆ receptor agonist on the survival rate. The cells were treated with 100 μM UDP at 1 min after 2 Gy γ -irradiation and survival was examined by colony formation assay after 1 week. *** $P < 0.001$ vs γ -Ray-alone group. (B) Effect of P2Y₆ receptor knock-down on the survival rate. siRNA duplex oligonucleotides (10 nM) for reduction of human P2Y₆ receptor were transfected into cells (5×10^4 cells) by using HiperFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. The expression level of P2Y₆ protein was decreased in P2Y₆-knockdown cells to 48% of that in cells transfected with scrambled siRNA. Forty-eight hr after transfection, cells were irradiated with γ -rays, and subjected to colony formation assay after 1 week. ** $P < 0.01$ vs the scramble siRNA-transfected group.

confirm the involvement of P2Y₆ receptor, we examined the survival rate in P2Y₆ knock-down (P2Y₆-KD) cells. As shown in Fig. 2B, the survival rate was significantly decreased in P2Y₆-KD cells, in comparison with that in scramble siRNA-transfected cells. These results strongly support the idea that amplification of γH2AX focus formation via P2Y₆ receptor is involved in the repair response to γ -ray-induced DNA damage.

P2Y₆ receptor participates in γ -irradiation-induced ERK1/2 activation in A549 cells

We examined whether P2Y₆ receptor participates in ERK1/2 activation in response to γ -irradiation. First, in order to investigate the involvement of the MAPK cascade in radiation-induced γH2AX focus formation, we examined the effects of various inhibitors of MAPK on the induction of γH2AX foci after irradiation of cells with

Fig. 3. Effects of P2Y receptor antagonists on γ -irradiation-induced activation of ERK1/2. (A) Cells were pre-incubated for 30 min with MRS2578 (10 μ M), 0126 (20 μ M), SP600125 (20 μ M), or SB203580 (20 μ M), then irradiated with 2.0 Gy of γ -rays and incubated for 3 hr. The number of γ H2AX foci in nuclei was counted. Each value represents the mean $\pm$ S.E. (n = 158-180). Significantly different: ####P < 0.001 vs the Non γ -ray-irradiated group. **P < 0.001 vs γ -Ray-alone group. (B) Cells were irradiated with 2.0 Gy of γ -rays and incubated for the indicated times. After irradiation, phosphorylation of ERK1/2 was detected by immunoblotting. (C) Cells were pre-incubated for 30 min with apyrase (20 U/ml), MRS2578 (10 μ M), or clopidogrel (30 μ M), then irradiated with 2.0 Gy of γ -rays and incubated for 30 min.

2.0 Gy of γ -rays. As shown in Fig. 3A, the induction of γ H2AX foci was significantly inhibited by pretreatment with MEK1/2 inhibitor U0126, but not with JNK inhibitor SP600125 or p38 kinase inhibitor SB203580. In addition, the induction was significantly inhibited by pretreatment with P2Y₆ antagonist MRS2578, as already reported (Nishimaki *et al.*, 2012). Thus, we focused on the involvement of the ERK1/2 cascade in the DNA repair response.

The effects of apyrase, P2Y₆ and P2Y₁₂ receptor antagonists on activation of ERK1/2 were examined by western blotting. As shown in Fig. 3B, irradiation with 2.0 Gy of γ -rays resulted in phosphorylation of ERK1/2 within 30 min. The activation of ERK1/2 was suppressed by pretreatment with apyrase, MRS2578 or clopidogrel as shown in Fig. 3C. These results support the involvement of ERK1/2 activation through P2Y₆ and P2Y₁₂ receptors in the DNA repair response to γ -ray irradiation.

Radiation-induced cell cycle arrest is not involved in purinergic signaling in A549 cells

To investigate the involvement of P2Y₆ and P2Y₁₂ receptors in cell cycle arrest in response to γ -irradiation, we employed flow cytometry to examine whether antagonists of both receptors induce cell cycle arrest. As shown in Fig. 4A, exposure of A549 cells to 2 Gy of γ -rays resulted in G2/M arrest within 6 hr. However, the arrest was not suppressed by pretreatment with MRS2578 or clopidogrel, as shown in Fig. 4B, suggesting that activation of P2Y₆ and P2Y₁₂ receptors is not involved in γ -ray-induced cell cycle arrest in A549 cells.

DISCUSSION

We previously examined the involvement of P2 receptors in the repair response to DNA damage induced by γ -irradiation (Nishimaki *et al.*, 2012). γ H2AX focus formation, considered to be a measure of DNA damage, was induced by irradiation of human lung cancer A549 cells with more than 0.5 Gy of γ -rays. The number of foci was decreased by pretreatment with ecto-nucleotidase apyrase, whereas the enzyme inhibitor ARL67156 increased focus formation. In addition, the number of γ H2AX foci was increased when a P2 receptor agonist such as ATP or UTP was added immediately after irradiation. These results suggested that nucleotide release from the cells amplified γ H2AX focus formation. Thus, we further examined the involvement of P2 receptors in γ H2AX focus formation by using receptor antagonists. We found that antagonists of P2Y₆ and P2Y₁₂ receptors significantly inhibited the focus formation after irradiation, while the agonists ATP and UTP themselves had no effect on γ H2AX focus formation in non-irradiated 594 cells. These findings indicate that these nucleotides promote the repair response to DNA damage via P2Y₆ and P2Y₁₂ receptors. In this study, in order to establish clearly that amplification of γ H2AX focus formation through the P2 receptor leads to amplification of the repair response to γ -ray-induced DNA damage, we made use of colony formation assay.

First, we confirmed that P2Y₆ receptor is strongly

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Fig. 4. Effect of P2Y receptor antagonists on γ -irradiation-induced cell cycle arrest. (A) A549 cells were irradiated with 2.0 Gy of γ -rays and incubated for the indicated times, followed by flow cytometric analysis for DNA content and cell cycle distribution. (B) Cells were pre-incubated for 30 min with MRS2578 (10 μ M) or clopidogrel (30 μ M), then irradiated with 2.0 Gy of γ -rays and incubated for 6 hr.

expressed on the membrane of A549 cells, whereas P2Y₁₂ is only weakly expressed. Then, we examined the effect of non-specific/specific P2 receptor antagonists on colony formation after γ -irradiation. Ecto-nucleotidase apyrase and a selective antagonist of P2Y₆ (MRS2578) significantly reduced the survival/colony formation rate, indicating involvement of P2Y₆ receptor in the formation induced by γ -irradiation in A549 cells.

Next, we examined the survival rate of P2Y₆-KD cells, in order to clarify the participation of P2Y receptors in the survival/colony formation; P2Y₆ was chosen for knock-down based on the finding that it is the most highly expressed P2Y₆ receptor in the cells. The survival rate was significantly decreased in P2Y₆-KD cells, compared with scramble siRNA-transfected cells. This result confirmed that colony formation via P2Y₆ receptor is involved in the

repair response to γ -ray-induced DNA damage. We previously reported that P2Y₆ receptor is involved in radiation-induced activation of EGFR, followed by ERK1/2 activation (Tamaishi *et al.*, 2011). Activation of EGFR is known to contribute to DNA repair (NHEJ) via activation of ERK1/2 (Kriegs *et al.*, 2010). Thus, activation of ERK1/2 through P2Y₆ and P2Y₁₂ receptors may also be involved in the DNA repair response after γ -ray-irradiation. Therefore, we considered that irradiation-induced activation of P2Y₆ receptor might mediate formation of γ H2AX foci via trans-activation of EGFR followed by activation of ERK1/2. Unfortunately, it is unknown that a selective antagonist of P2Y₁₂ (clopidogrel) had no effect on the survival/colony formation rate (Fig. 1B), though the activation of ERK1/2 was suppressed by pretreatment with clopidogrel (Fig. 3C). It may be reason why a significant

Fig. 5. Proposed scheme of purinergic signaling through P2Y₆ receptor for repair of γ-ray-induced DNA damage.

difference may not be provided by the colony assay that was low sensitivity as an evaluation system, due to the very low expression of P2Y₁₂ receptor in the cell used here compared with P2Y₆.

On the other hand, P2Y₁₂ receptor is a G_i protein-coupled receptor, which induces a decrease of cAMP (Foster *et al.*, 2001). Although P2Y₁₂ is only weakly expressed in A549 cells, and the mechanism of γH2AX focus formation mediated by P2Y₁₂ receptor is unknown, our results provide the first evidence of involvement of P2Y₁₂ receptor in a radiation-induced biological effect.

There were no significant changes in the cell cycle distribution when cells were pretreated with either P2Y₆ or P2Y₁₂ receptor antagonist. This indicates that the changes of γH2AX foci are due to altered overall DSB repair, and do not simply reflect differences in cell cycle distribution.

We previously reported that P2X₇ receptor is involved in ATP release after irradiation of A549 cells (Nishimaki *et al.*, 2012). P2X₇ receptor is unique among P2 receptors. Other P2 receptors contribute to intracellular signaling via Ca²⁺ influx or activation of G protein (Burnstock, 2006, 2009), but P2X₇ receptor is a ligand-gated cation channel and its activation contributes to intracellular signaling in various ways (Tsukimoto *et al.*, 2007). This receptor also participates in the mechanism of ATP release through its interaction with hemichannels (Baroja-Mazo *et al.*, 2013).

Recently, we reported that γ-irradiation-induced ATP release from B16 melanoma cells is mediated by P2X₇ receptor and connexin43 hemichannels (Ohshima *et al.*, 2010, 2012b). Considering our previous findings and the present data, P2X₇ receptor appears to participate in the response to DNA damage via induction of ATP release, rather than via a contribution to intracellular signaling.

P2Y₆ receptor is linked to the activation of phospholipase C, leading to generation of inositol 1,4,5-triphosphate and diacylglycerol, which in turn induce elevation of cytoplasmic free Ca²⁺ and activation of protein kinase C isoforms (Communi *et al.*, 1996). Though ATP is a ligand of P2X₇ receptor, the ligands of P2Y₆ and P2Y₁₂ receptors are UDP and ADP, respectively (Burnstock, 2006, 2009). In our previous study, ATP release from A549 cells after irradiation was detected (Nishimaki *et al.*, 2012). However, released ATP and UTP would be readily metabolized by ecto-nucleotidases on the cellular membrane to ADP and UDP, respectively. Therefore, these nucleotides may be involved in autocrine/paracrine signaling to modulate the repair response to DNA damage induced by γ-irradiation.

NHEJ repair is the major mechanism for repair of ionizing radiation-induced DNA double-strand breaks in mammalian cells (Lieber, 2010; Wang and Lees-Miller, 2013). The DNA end-binding heterodimer Ku70/Ku80

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is involved in NHEJ. Since ATM mediates radiation-induced translocation of Ku70 into the nucleus (Morio and Kim, 2008), activation of P2Y₆ and P2Y₁₂ receptors may participate in radiation-induced NHEJ repair.

We have found that the activation of P2Y₆ and P2Y₁₂ receptors in γ -irradiated A549 cells mediates, at least in part, activation of ATM and induction of γ H2AX at sites of DNA damage, and also contributes to accumulation of 53BP1 through activation of ERK1/2. P2X₇ receptor is also involved in DNA repair via mediation of ATP release after irradiation (Ohshima *et al.*, 2010, 2012b; Nishimaki *et al.*, 2012). Since pretreatment with apyrase almost completely suppressed focus formation, we suggest that autocrine/paracrine signaling mediated by nucleotides and P2 receptors contributes to the regulation of DNA repair after γ -irradiation, in addition to intracellular signaling already well established (Fig. 5). Involvement of purinergic signaling through P2Y receptors in the NHEJ repair is now under way.

In conclusion, we propose that release of ATP and autocrine/paracrine positive feedback through P2Y receptors serve to amplify the cellular repair response to radiation-induced DNA damage.

ACKNOWLEDGMENT

There is no conflict of interest with authors in this manuscript. We thank Mr. Mori N. for his technical assistance.

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