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Autocrine regulation of γ-irradiation-induced DNA damage response via extracellular nucleotides-mediated activation of P2Y6 and P2Y12 receptors

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ABSTRACT

A key component of the response to DNA damage caused by ionizing radiation is DNA repair. Release of extracellular nucleotides, such as ATP, from cells plays a role in signaling via P2 receptors. We show here that release of ATP, followed by activation of P2Y receptors, is involved in the response to γ-irradiation-induced DNA damage. Formation of phosphorylated histone variant H2AX (γH2AX) foci, which are induced in nuclei by DNA damage and contribute to accumulation of DNA-repair factors, was increased at 1–3 h after γ-ray irradiation (2.0 Gy) of human lung cancer A549 cells. Focus formation was suppressed by pre-treatment with the ecto-nucleotidase apyrase. Pre-treatment with ecto-nucleotidase inhibitor ARL67156 or post-treatment with ATP or UTP facilitated induction of γH2AX, indicating that extracellular nucleotides play a role in induction of γH2AX foci. Next, we examined the effect of P2 receptor inhibitors on activation of ataxia telangiectasia mutated (ATM; a protein kinase) and accumulation of 53BP1 (a DNA repair factor), both of which are important for DNA repair, at DNA damage sites. P2Y6 receptor antagonist MRS2578, P2Y12 receptor antagonist clopidogrel, and P2X7 receptor antagonists A438079 and oxATP significantly inhibited these processes. Release of ATP was detected within 2.5 min after irradiation, but was blocked by A438079. Activation of ATM and accumulation of 53BP1 were decreased in P2Y6 or P2Y12 receptor-knockdown cells. We conclude that autocrine/paracrine signaling through P2X7-dependent ATP release and activation of P2Y6 and P2Y12 receptors serves to amplify the cellular response to DNA damage caused by γ-irradiation.

1. Introduction

Exposure of cells to ionizing radiation causes DNA damage, followed by activation of ataxia telangiectasia mutated (ATM; a serine/threonine protein kinase), formation of phosphorylated histone variant H2AX (γH2AX), and accumulation of tumor suppressor p53-binding protein 1 (53BP1) [1,2]. The DNA damage response pathways protect genomic integrity and form a barrier against cancer [2]. The overall response involves multiple mechanisms that detect damaged DNA, activate DNA repair machineries, delay cell cycle progression, and cause permanent replicative arrest or activate apoptosis [1]. In general, DNA damage sensors, such as ATM, mediate phosphorylation of other proteins, thereby stimulating repair pathways. Further, ATM promotes repair of heterochromatin following exposure to ionizing radiation. Activated ATM induces phosphorylation of H2AX [1]. The resulting γH2AX then triggers the assembly of a large DNA damage response complex consisting of MDC1, RNF8, BRCA1, DNMT, and 53BP1 [1]. However, involvement of autocrine signaling in the response to DNA damage has not been reported.

Extracellular nucleotides (ATP, ADP, UTP and UDP) are known to serve as intercellular signaling molecules [3,4]. ATP and UTP are released from cells in response to various stimuli through maxi-anion channels, volume-sensitive outwardly rectifying chloride channels and gap junction hemichannels interacting with P2X7 receptor [5–9]. Extracellular nucleotides bind to and activate P2 receptors, which are expressed on the cell membrane. There are two subfamilies of P2 receptors [3,4]. The P2X1–7 receptors are extracellular ATP-gated nonselective cation channels that are permeable to calcium. The P2Y1–14 receptors are metabotropic G protein-coupled receptors that possess seven transmembrane hydrophobic domains with short extra amino and intracellular carboxyl terminals. Activation of P2 receptors is involved in various physiological responses, such as cell proliferation or cell death [3,4]. It is also reported that activation of P2Y receptor induces activation of apurinic/apyrimidinic endonuclease, which works in the repair of DNA damage [10].

Abbreviations: ATM, ataxia telangiectasia mutated; γH2AX, phosphorylated histone variant H2AX; shRNA, short hairpin RNA plasmid; 53BP1, tumor suppressor p53-binding protein 1.

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Recently, we have reported that γ-irradiation induces ATP release from cells and that activation of P2Y6 receptor is involved in radiation-induced EGF receptor activation (translocation into the nucleus), followed by activation of ERK1/2, in human lung cancer A549 cells and human keratinocytes HaCaT cells [11,12]. Induction of anti-oxidative proteins after low-dose irradiation is also mediated by P2Y6 receptor in mouse macrophage RAW264.7 cells [13]. P2X7 receptor is involved in release of ATP through connexin43 after γ-irradiation in mouse B16 melanoma [14,15]. However, it is unknown whether activation of P2 receptors by released nucleotides is involved in the mechanisms of DNA damage response after ionizing irradiation, such as activation of ATM or accumulation of DNA-repair factors at sites of damage.

Regulation of the mechanisms of DNA repair is usually discussed in terms of intracellular signaling. However, based on our recent studies, we hypothesized that autocrine signaling mediated by extracellular nucleotides and P2 receptors also plays a role in the response to γ-irradiation-induced DNA damage. Therefore, in this study, we investigated the involvement of P2 receptors in the formation of DNA repair foci at sites of DNA damage after irradiation. Our results indicate that release of extracellular nucleotides and activation of P2Y6 and P2Y12 receptors do participate in the DNA repair response. We propose that autocrine/paracrine signaling mediated by nucleotides serves to amplify the cellular response to DNA damage caused by γ-irradiation.

2. Materials and methods

2.1. Reagents and antibodies

Dulbecco's modified Eagle's medium was purchased from Wako. Fetal bovine serum was purchased from Biowest. The primary antibodies used were ATM (phosphoS1981) antibody (abcam), 53BP1 antibody rabbit polyclonal (Novus), Phospho-Histone H2AX (Ser139) rabbit monoclonal antibody (Cell Signaling Technology), and purified mouse anti H2AX phosphorylated (Ser139) antibody (Bio Legend). The secondary antibodies used were Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes) and goat anti-rabbit IgG-FITC (Sigma–Aldrich).

2.2. Agonists, antagonists and inhibitors

ATP is a non-selective agonist for P2X and P2Y receptors. UTP is a P2Y2, P2Y4 and P2Y6 receptor-specific agonist. Apyrase (Sigma–Aldrich) is an ATP diphosphohydrolase (ecto-nucleotidase). ARL67156 (Sigma–Aldrich) is ecto-nucleotidase inhibitor. Oxidized ATP (oxATP) (Sigma–Aldrich) is P2X receptor antagonist. A438079 (Tocris Bioscience) is a selective antagonist of P2X7 receptor. MRS2578 (Tocris BioScience) is a selective antagonist of P2Y6 receptor. Clopidogrel (Tocris Bioscience) is a selective antagonist of P2Y12 receptor. MRS2179 (Tocris Bioscience) is a selective antagonist of P2Y1 receptor. MRS2211 (Tocris Bioscience) is a selective antagonist of P2Y13 receptor.

2.3. Cell culture and irradiation

A549 human adenocarcinoma cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO_2 in air at 37 °C. The cells were then irradiated with γ-rays from a Gammacell 40 (^{137}Cs source) (Nordin International, Inc.; 0.87 Gy/min) at room temperature for a suitable time. After irradiation, the cells were incubated at 37 °C.

2.4. 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 h, the fixed cells were incubated with primary antibody (γH2AX 1:200, 53BP1 1:200, ATM 1:1000) for 24 h at 4 °C and with secondary antibody (1:200) for 1 h. 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).

2.5. Determination of extracellular ATP concentration

The release of ATP was quantified by using the luciferin-luciferase-based Enlighten ATP assay system (Promega) according to the manufacturer's instructions. Briefly, A549 cells were grown in a 40-mm dish to 100% confluence. The culture medium was replaced with RPMI1640-based buffer containing 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_2HPO_4, 11.1 mM glucose and 10 mM HEPES–NaOH (pH 7.4), then the cells were irradiated with γ-rays (2.0 Gy). Cell-conditioned buffer was obtained up to ten minutes after stimulation. Luciferin-luciferase reagent (100 μL) was injected into 10 μL of conditioned buffer and chemiluminescence was measured with a WALLAC ARVO SX multilabel counter (PerkinElmer, Inc.). ATP concentration in each sample was determined by comparing the luminescence of samples with those of standards in the concentration range of 10^{-8} to 10^{-10} M. It is important to note that ATP released from cells is diluted in the culture medium and is rapidly metabolized by ecto-nucleotidases on the plasma membrane. Therefore, the detected concentration of ATP must be much lower than the real concentration at the cell surface.

2.6. siRNA Transfection for knockdown of P2Y6 receptor

P2Y6 receptor knockdown was done by transfection with siRNA as described in our previous report, and was confirmed to cause a significant decrease of P2Y6 receptor protein [see ref. [11]]. The expression levels of P2Y6 protein were decreased in P2Y6-knockdown cells to 48% of those in the cells transfected with scrambled shRNA. Briefly, 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. Forty-eight hours after transfection, cells were irradiated with γ-rays.

2.7. Short hairpin RNA plasmid (shRNA) stable transfection

Stable transfection with shRNA was performed using the Sure-SilencingTM shRNA plasmid Kit for human P2Y12 receptor (SA Biosciences). Two different shRNA plasmids encoding GFP targeting P2Y12 or scrambled shRNA plasmid (negative control) were transfected by electroporation with the Amaxa system (Nucleofector solution T and Nucleofector program X-01) (Lonza). The GFP-positive cells were sorted with a flow cytometer (FACSAriaII, BD Biosience). The expression levels of P2Y12 mRNA and protein were decreased in P2Y12-knockdown cells to 62% and 76%, respectively, of those in the cells transfected with scrambled shRNA.

2.8. Statistics

Results are expressed as means ± SE. The statistical significance of differences between control and other groups was calculated using Dunnnett's test. Multiple groups were compared using ANOVA followed by pairwise comparisons with Bonferroni's post