A novel mechanism of \(\gamma\)-irradiation-induced IL-6 production mediated by P2Y11 receptor in epidermal keratinocytes

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Summary
Skin inflammation is caused by excessive production of cytokines and chemokines in response to an external stimulus, such as radiation, but the mechanisms involved are not completely understood. Here, we report a novel mechanism of γ-irradiation-induced IL-6 production mediated by P2Y11 receptors in epidermal cells. After irradiation of HaCaT cells derived from human epidermal keratinocytes with 5 Gy of γ-rays (137Cs : 0.78 Gy/min), IL-6 production was unchanged at 24 h after γ-irradiation, but was increased at 48 h. IL-6 mRNA was increased at 30 h, and IL-6 production was increased at 33 h after irradiation. The production of IL-6 was sustained at least for 4 days after irradiation. P2Y11 receptor antagonist NF157 inhibited IL-6 production in irradiated cells. Treatment with ATP, a ligand of P2Y11 receptor caused IL-6 production within 24 h. ATP-induced IL-6 production was also suppressed by NF157. Extracellular ATP level was increased after irradiation. The p38 MAPK and NF-κB signaling was involved in the production of IL-6 at the downstream of P2Y11 receptor activation. In addition, the cell cycle was arrested at the G2/M phase, and DNA repair foci were not disappeared at 48 h after γ-irradiation. The protein level of histone methylation enzyme G9a, which inhibits IL-6 production, was decreased after γ-irradiation. In conclusion, we suggest that γ-irradiation induces sustained IL-6 production in HaCaT cells from 33 h after irradiation, which is mediated through P2Y11 receptor-p38 MAPK-NF-κB signaling pathway and G9a degradation. This is a novel mechanism of cytokine production in γ-irradiated cells.

Keywords
γ-irradiation, IL-6, P2Y11 receptor, p38 MAPK, NF-κB, G9a
Introduction

The skin not only protects the body from various external stimuli, but also plays an important role in wound healing and immune response by producing proinflammatory mediators such as cytokines and chemokines in response to various physical or chemical environmental stimuli 1–6). However, potent stimulation causes inflammation due excessive production of cytokines and chemokines 7).

It is known that ionizing radiation, such as γ-irradiation, causes DNA damage 8). This is followed within 1 h by the activation of the protein kinase ataxia-telangiectasia mutated (ATM), formation of phosphorylated histone H2AX (γH2AX), and release of p53 binding protein 1 (53BP1), a tumor suppressor 9,10). Activation of ATM induces phosphorylation of H2AX at the sites of DNA damage and the formation of DNA damage response complexes, such as RAD80, BRCA1 and 53BP1 11).

In recent years, it has been reported that adenosine triphosphate (ATP), an energy donor, is released from epidermal cells into the extracellular space in response to various physical or chemical stimuli 12). Extracellular ATP is involved in epidermal cell proliferation, differentiation, apoptosis, and intercellular communication 13,14) through autocrine and paracrine signaling via P2 receptors expressed on the cell membrane (purinergic signaling) 15,16). The P2 receptors are classified into the ligand-gated ion channel P2X1-7 receptors and metabotropic G protein-coupled P2Y 1, 2, 4, 6, 11-14 receptors 17,18). One of the actions of extracellular ATP is to induce the production of IL-6, one of the inflammatory cytokines, in epidermal cells 19). It has been reported that P2Y11 receptors are involved in the induction of IL-6 production by lipopolysaccharide (LPS) in human monocyte THP1 cells 20,21). Further, we have reported that P2Y11 receptors are involved in the induction of IL-6 production by IFN-γ or silica nanoparticles in human keratinocyte HaCaT cells 22,23). Also, it has been reported that UVA irradiation causes IL-6 production mediated by P2Y11 receptors 20). Though it is also reported that IL-6 production is induced by γ-irradiation in HaCaT cells 24), the mechanism has not yet been established.

Furthermore, we have shown that γ-irradiation induces ATP release, and activates P2Y receptors in an autocrine/paracrine manner 25–30). However, the involvement of P2 receptors in the IL-6 production in γ-irradiated cells has not yet been revealed. It is also reported P2Y6 receptors induce cyclooxygenase 2 (COX2) expression in HaCaT cells via p38
mitogen-activated protein kinase (MAPK) in response to UVB irradiation. Thus, P2 receptors are involved in inflammatory responses to various stimuli, and we anticipated that they might also play a role in γ-irradiation-induced IL-6 production. The IL-6 production in γ-irradiated cells has been reported in various cancer cells. NF-κB mediates the production of IL-6 in γ-irradiated glioma cells, and also mediates IL-6 production in the ileal muscularis layer of whole body irradiated rats. However, the report of IL-6 production in irradiated normal cells is quite little. The involvement of P2Y11 receptor in IL-6 production in irradiated cells has not yet been reported in both normal cells and cancer cells.

Although radiation treatment is commonly used in the treatment of cancer, it is associated with dose-dependent inflammatory side effects, such as depilation, skin dryness, erythema, pigmentation, bubbles, erosion, skin ulcer and necrosis. Also, chronic inflammation can lead to cancer. However, little is known about the mechanism of inflammation caused by radiation in epidermal cells.

In this study, we investigated the mechanism of γ-ray-induced cytokine production in HaCaT epidermal cells. We observed that IL-6 production in HaCaT cells at 48 h after γ-ray irradiation. Our results indicate that extracellular ATP and P2Y11 receptor would be involved in the production of IL-6. Further, p38 MAPK, NF-κB and G9a are also involved in the pathway of γ-irradiation-induced IL-6 production. Our data suggest a novel mechanism of cytokine production in γ-irradiated cells.

Materials and methods

Reagents and antibodies

Paroxetine was purchased from Wako Pure Chemical Industries (Japan). MG132 was purchased from Cayman Chemical (USA). NF449 was purchased from Abcam (UK). Apyrase, ATP, Brilliant Blue G (BBG), oxidized ATP (oxATP), SB203580 and suramin were purchased from Sigma-Aldrich (USA). AZ10606120, BDBD, clopidogrel, MRS2179, MRS2211, MRS2578 and PPADS were purchased from Tocris Bioscience (UK). Affinity-purified anti-human interleukin-6 mAb and biotin-conjugated anti-human interleukin-6 mAb were purchased from eBioscience (USA). Alexa Fluor 594-conjugated anti-mouse IgG antibody was purchased from Molecular Probes. Mouse anti-ATM (phospho S1981) antibody was purchased from Abcam (UK). Rabbit anti-53BP1 antibody (NB100-305) was purchased from Novus Biological and Pharmaceutical Bulletin Advance Publication
Biologicals (USA). Anti-P2Y11 antibody was purchased from Alomone Labs (Israel). Hoechst 33258 solution and peroxidase-conjugated anti-GAPDH monoclonal antibody were purchased from Wako Pure Chemical Industries (Japan). FITC-conjugated anti-rabbit IgG antibody and propidium iodide (PI) were purchased from Sigma-Aldrich (USA). Rabbit anti-Phospho-histone H2AX (Ser 139) antibody, HRP-conjugated anti-rabbit IgG antibody, rabbit anti-G9a/EHMT2 (C6H3) mAb, HRP-conjugated anti-mouse IgG antibody, rabbit anti-phospho-p38 MAPK (Thr180/Tyr182) (12F8) mAb, mouse anti-p38 MAP kinase (5F11) mAb and mouse anti-IκBα (L35A5) mAb (amino-terminal antigen) were purchased from Cell Signaling Technology, Inc. (USA).

Cell culture and irradiation
Immortalized human-derived epidermal keratinocytes, HaCaT cells, were kindly supplied by Drs. M. Ichihashi and M. Ueda (Kobe University School of Medicine, Kobe, Japan) with the permission of Dr. N. E. Fusening (German Cancer Research Center, Heidelberg, Germany)\(^ {37,38}\). Cells were cultivated in Dulbecco's modified Eagle's medium (low glucose) (Wako, Japan), containing 10% fetal bovine serum (FBS) (Gibco, USA), 100 units/ml penicillin (Sigma-Aldrich, USA), and 100 μg/ml streptomycin (Sigma-Aldrich, USA) in an atmosphere of 5% CO\(_2\) in air at 37 °C. The cells were irradiated with γ-rays from a Gammacell 40 (\(^{137}\)Cs source) (Nordin International, Inc., Japan; 0.78 Gy/min) at room temperature for a suitable time. After irradiation, the cells were incubated in an atmosphere of 5% CO\(_2\) in air at 37 °C.

Cytokine production (ELISA)
HaCaT cells (1.0×10\(^5\) cells/mL) were seeded in a 35 mm dish and cultured for 24 h. Cells were irradiated and cultured for indicated period. The culture supernatant was collected and centrifuged at 100 g for 3 min at 4 °C. IL-6 was measured by enzyme-linked immunosorbent assay (ELISA). A 96-well plate was coated with purified anti-human IL-6 mAb (1:500), incubated overnight at 4°C, and then washed with phosphate-buffered saline (PBS) (Wako, Japan) containing 0.05% Tween-20 (Bio-Red Laboratories, Germany) 3 times. Nonspecific binding was blocked with PBS containing 1% bovine serum albumin (BSA) for 1 h at room temperature. The plate was washed 3 times, and samples were added. After incubation for 2 h at room temperature, the plate was washed 3 times again, and biotin-conjugated anti-human IL-6 mAb (1:1,000) was added. Incubation was continued for 1 h at room temperature, then the plate was washed 5 times, and peroxidase-conjugated streptavidin solution (Wako, Japan) was added. The plate was incubated for 10 min at room temperature, then washed 10 times, and 3,
3’, 5, 5’-tetramethylbenzidine solution (Wako, Japan) was added. The reaction was stopped by adding 5 N H₂SO₄ (Wako, Japan), and the absorbance at 450 nm was measured with a microplate reader. A standard curve was established with recombinant human IL-6 (R&D Systems, USA), and IL-6 concentrations were estimated by interpolation.

**Intracellular cytokine production (ELISA)**
HaCaT cells (2.0×10⁵ cells) were seeded in a 35 mm dish and cultured for 24 h. Cells were irradiated and cultured for indicated period. The cells were lysed 150 μL/dish lysis buffer (10 mM HEPES-NaOH, 1% Triton/PBS, 5 mM ethylenediaminetetracetic acid (EDTA), pH 8.0, 1% protease inhibitor, PHOSSTOP® (Sigma-Aldrich, USA)) for 30 min at 4°C, and the supernatant was collected by centrifugation (10,000 g, 15 min, 4°C). The samples were diluted two-fold with PBS. ELISA was carried out as described above. The samples were incubated in anti-IL-6 antibody-coated plate overnight at 4°C.

**Reverse transcription polymerase chain reaction (RT-PCR)**
HaCaT cells (1.0 × 10⁵ cells/mL) were seeded in a 35 mm dish and cultured for 48 h. Total RNA was extracted from HaCaT cells using the ReliaPrep™ RNA Cell Miniprep System (Promega, USA). The first-strand cDNA was synthesized from total RNA with PrimeScript Reverse Transcriptase (Takara Bio, Japan). Specific primers were purchased from Sigma Genosys (USA). The sequences of specific primers used in this study were as reported 22,23). GAPDH mRNA was determined as a positive control. PCR was conducted by incubating cDNA with appropriate primers (0.5 μM each), Prime STAR HS DNA polymerase (1.25 U) (Takara Bio, Japan) and deoxynucleotide mixture (0.2 mM each) (Takara Bio, Japan). The samples were incubated at 95 °C for 2 min, then amplification was carried out for 35 cycles (each cycle: 95 °C for 30 s, annealing at 65 °C for 1 min), followed by incubation at 72 °C for 10 min. The products were subjected to 2% agarose gel electrophoresis. Bands were stained with ethidium bromide and photographed.

**Real-time RT-PCR**
HaCaT cells (1.0 × 10⁵ cells/mL) were seeded in a 35 mm dish and cultured for 24 h. Cells were irradiated and incubated for 24-30 h. Total RNA was extracted from cells, and the first-strand cDNA was synthesized as described above. Real-time PCR was performed in a CFX Connect Real-Time System (Bio-Rad Laboratories, Germany). RT2-qPCR® primers for human IL-6 were purchased from Qiagen (Germany). GAPDH mRNA was determined as a
positive control. Each sample was assayed in a 20 μL amplification reaction mixture, containing cDNA, primers and 2x KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, USA). 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 within the linear range of the standard curve and were normalized to GAPDH mRNA expression.

**Immunoblotting**

HaCaT cells (1.0 × 10⁵ cells/mL) were seeded in a 35 mm dish and incubated for 24 h. And then, cells were irradiated. Cells were pretreated with inhibitors or antagonists 1 h before irradiation. After incubation for indicated period, cells were incubated in lysis buffer containing 1% Triton X-100 and protease inhibitor cocktail (Sigma-Aldrich) at 4 °C for 30 min. The samples were centrifuged at 10,000 g for 15 min, and the supernatant was used as cell lysate. Cell lysate was mixed with 4x Laemmli sample buffer (Bio-Rad Laboratories, Germany) and 10 mM dithiothreitol, and incubated at 95°C for 10 min. Samples containing 2 μg protein were subjected to 7.5% or 10% SDS-PAGE, and protein was transferred to PVDF membranes. The membranes were incubated overnight at 4°C in TBST (0.1% Tween-20, 10 mM Tris–HCl, 0.1 M NaCl) containing 1% BSA or 5% skim milk (Wako, Japan), then further incubated overnight at 4°C with each antibody (1:1,000). Membranes were washed with TBST for 30 min, incubated with HRP-conjugated anti-rabbit IgG antibody (1:20,000) or HRP-conjugated anti-mouse IgG antibody (1:20,000) for 1.5 h at room temperature, and washed again with TBST for 30 min. Membranes were also incubated for 1 h at room temperature with peroxidase-conjugated anti-GAPDH monoclonal antibody (1:50,000), to confirm equal loading, and washed again with TBST for 30 min. Specific proteins were visualized by using ImmunoStar®LD (Wako, Japan). Western blotting detection reagents were from LI-COR (UK), and bands were analyzed with Image Studio 4.0 for C-DiGit Scanner (LI-COR, UK).

**Measurement of extracellular ATP**

HaCaT cells (1.5 × 10⁵ cells/mL) were seeded in a 24 well plate and incubated for 24 h. One hour before irradiation, the culture medium was changed to phenol red free medium containing 0.5% FBS. Eighteen hours or 24 h after irradiation, the culture medium was harvested. The medium was centrifuged at 600 g for 1 min at 4°C. The supernatant (10 μL)
was added into a white 96-well plate, and then 100 µL of rLuciferase/Luciferin Reagent (Promega) was injected into the wells. The chemiluminescence was measured with a WALLAC ARVO SX multilabel counter (PerkinElmer, Inc.).

**Senescence-associated β-galactosidase (SA-β-gal) staining**

HaCaT cells (1.0 × 10^5 cells/mL) were seeded in a 35 mm dish and cultured for 24 h, and then cells were irradiated. After incubation for 24 h or 48 h, SA-β-gal activity was determined using an SA-β-gal staining kit (Cell Signaling Technology, USA) according to the manufacturer’s instructions. SA-β-gal activated cells were identified as blue-stained cells under a fluorescence microscope BZ9000 (BIOREVO) (Keyence, Japan).

**Immunofluorescence staining**

HaCaT cells (1.0 × 10^5 cells/mL) were seeded in a 35 mm dish containing a glass slide and cultured for indicated period. The cells were fixed in 4% paraformaldehyde (Wako, Japan) for 10 min at room temperature and permeabilized in 0.1% Triton X-100 for 5 min on ice. The samples were incubated in blocking buffer (10% FBS in PBS) for 1 h, then incubated with primary antibody (ATM 1:1000, γH2AX 1:200, 53BP1 1:200, G9a 1:50) for 24 h at 4°C and with secondary antibody (Alexa Fluor 594 or FITC 1:200) for 1 h at room temperature. Counterstaining with Hoechst 33258 (1 mg/ml) was used to verify the location and integrity of nuclei. Fluorescence images of the samples were collected with a laser scanning confocal microscope (FV1000 IX81; Olympus, Japan).

**Flow cytometry**

HaCaT cells (1.0 × 10^5 cells/mL) were seeded in a 35 mm dish and cultured for 24 h, and then cells were irradiated. After incubation for 24 h or 48 h, HaCaT cells were dissociated using 0.1% trypsin-EDTA, collected by centrifugation (100 g, 3 min, 4°C), and rinsed with PBS. They were placed in 100% ethanol, incubated for 1 h on ice, and collected by centrifugation (700 g, 8 min, 4°C). They were suspended in PBS, and incubated with RNaseA (0.01 mg/mL) for 30 min at 37°C. Next, the cells were stained with PI solution (50 µg/mL) and incubated for 30 min at 37°C. The resulting single cell suspensions were analyzed by flow cytometry (FACS Caliber cytometer, Becton, Dickinson and Co., USA). Cell cycle profiles were evaluated based on the peaks of the control sample. The data were analyzed by FlowJo software (FlowJo, LLC).
Statistics

Results were expressed as mean ±SE. 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, USA). The criterion of significance was $P < 0.05$. 
Results

γ-Irradiation-induced IL-6 production in HaCaT cells

HaCaT cells were irradiated with γ-rays from 1 to 20 Gy, and IL-6 production was measured. There was no change in IL-6 production at 24 h after γ-irradiation at any dose, but at 48 h the IL-6 production was increased dose-dependently, with a peak at 10 Gy γ-irradiation (Fig. 1 A). However, the production of IL-6 was lower in 20 Gy-irradiated cells. Subsequent experiments were conducted at an irradiation dose of 5 Gy. We found that IL-6 production remained significantly elevated even at day 5 after γ-irradiation (Fig. 1 B). When the time course of IL-6 production up to 48 h after irradiation was examined in detail, we found that showed little change up to around 33 h after γ-irradiation, but increased thereafter (Fig 1 C). IL-6 production at the mRNA level was increased at 30 h after γ-irradiation (Fig 1 D). The amount of intracellular IL-6 was significantly increased at 33 h after γ-irradiation (Fig. 1 E).

Involvement of P2Y11 receptors in IL-6 production in response to γ-irradiation

It has been reported that ATP is released after γ-irradiation of cells 39), and therefore we focused here on the P2 receptors. Expression of P2X3, 4, 5, 6, P2Y1, 4, 6, 11, 12, 14 was confirmed in HaCaT cells (Fig. 2 A). Thus, we examined the effects of the following agents on γ-irradiation-induced IL-6 production: apyrase (ecto-nucleotidase), PPADS (broad-spectrum P2 receptor antagonist), NF449 (P2X1 receptor antagonist), paroxetine (P2X4 receptor antagonist), BDBD (P2X4 receptor antagonist), oxATP (purinergic receptor antagonist), AZ10606120 (P2X7 receptor antagonist), BBG (P2X7 receptor antagonist), suramin (broad-spectrum P2Y receptor antagonist), MRS2179 (selective P2Y1 receptor antagonist), MRS 2578 (selective P2Y6 receptor antagonist), NF157 (selective P2Y11 receptor antagonist), clopidogrel (selective P2Y12 receptor antagonist) and MRS2211 (competitive P2Y13 receptor antagonist). Apyrase, PPADS, oxATP, suramin, and NF157 suppressed the γ-irradiation-induced IL-6 production increase (Fig. 2 B). We also confirmed the expression of P2Y11 receptors at the protein level, and found no change in P2Y11 receptor expression upon γ-irradiation (Fig. 2 C). We treated of HaCaT cells with a ligand of P2 receptors ATP (1 μM, 10 μM, 100 μM or 1 mM), and incubated for 24 h. Treatment with 100 μM or 1 mM of ATP induced IL-6 production in HaCaT cells (Fig. 2 D). The increase of IL-6 caused by ATP (100 μM or 1 mM) at 24 h was also suppressed by P2Y11 receptor antagonist NF 157 (Fig. 2 D). We measured the change in extracellular ATP concentration in irradiated cells. γ-irradiation caused an increase of ATP concentration in culture medium of irradiated cells at 18 h and at 24 h (Fig. 2 E). Therefore we focused on the P2Y11 receptor. LY294002, an inhibitor of PI3

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kinase regulated by G protein-coupled receptors such as the P2Y11 receptors, suppressed the γ-irradiation-induced increase in IL-6 production (Fig. 2 F). To determine when P2Y11 receptor is activated after irradiation, we post-treated with NF157 at 9, 12, 15 or 18 h after irradiation. Post-treatment with NF157 up to 15 h after γ-irradiation suppressed IL-6 production (Fig. 2 G), but was no longer effective at 18 h, suggesting that the P2Y11 receptors involved in IL-6 production are activated about 15-18 h after γ-irradiation. On the other hand, we also observed the increase of IL-6 production by treatment with P2X4 receptor antagonist BDBD in non-irradiated cells, suggesting that P2X4 receptor would involve in the suppression of IL-6 production in normal state, but irradiation-induced IL-6 production was not affected by BDBD, indicating that P2X4 receptor would not contribute to the production of IL-6 by irradiation.

Involvement of p38 MAPK and NF-κB in γ-irradiation-induced IL-6 production
p38 MAPK and NF-κB are involved in IL-6 production 40,41), so we examined whether they are also involved in IL-6 production induced by γ-irradiation. First, we found that SB203580, a p38 MAPK inhibitor, inhibited the IL-6 production (Fig. 3 A). Phospho-p38 MAPK was increased by γ-irradiation (Fig. 3 B), and IκB was decomposed (Fig. 3 C). The γ-irradiation-induced increase of phospho-p38 MAPK was suppressed by NF157 (Fig. 3 E), and the decomposition of IκB was blocked by SB203580 and NF157 (Fig 3 D and F). These results suggest that p38 MAPK and NF-κB are involved in IL-6 production in response to γ-irradiation.

Cell cycle arrest and DNA damage by γ-irradiation
We stained irradiated cells for SA-β-gal (senescence-associated β-galactosidase), which is a general indicator of cellular senescence. SA-β-gal-positive cells were not observed in irradiated or non-irradiated groups (Fig. 4 A). The cell density was lower in irradiated cells at 48 h. Foci of DNA damage and repair factors γH2AX, 53BP1 and ATM were observed at 30 min after γ-irradiation and then decreased but not disappear even at 48 h after γ-irradiation (Fig. 4 B and C). Next, we examined whether NF-κB is directly activated by γ-irradiation-induced DNA damage, but there was no change in IκB or phospho-NF-κB p65 between 30 min to 6 h after irradiation (data not shown). Micronucleus formation was observed at 27 h after γ-irradiation, together with γH2AX foci (Fig. 4 D). The G2/M phase ratio was significantly increased by γ-irradiation (Fig. 5). Therefore, it appears that cellular senescence was not induced by γ-irradiation, but irradiation-induced cell cycle arrest was indicated. The DNA
damage caused by $\gamma$-irradiation appeared to be persistent.

*Involvement of G9a in IL-6 production by $\gamma$-irradiation*

G9a is involved in IL-6 production associated with DNA damage \(^{42}\), so we next examined changes of G9a. G9a protein was decreased at 24 and 48 h after $\gamma$-irradiation (Fig. 6 A). MG132, a proteasome inhibitor, blocked the $\gamma$-irradiation-induced G9a reduction at 24 h after $\gamma$-irradiation (Fig. 6 B). MG132 treatment suppressed IL-6 production (Fig. 6 C). Since MG132 also suppresses IkB degradation, the effect of MG132 on IL-6 production is caused by inhibition of both G9a and NF-kB signaling. Though we cannot conclude the involvement of G9a in IL-6 production, it is suggested that G9a would play a role in IL-6 production in response to $\gamma$-irradiation.
Discussion

The production of IL-6 was dose-dependently caused by irradiation in HaCaT cells up to 10 Gy. The reason why IL-6 production was decreased by 20 Gy-irradiation was not determined in this study, but it is speculated that 20 Gy of irradiation might cause the cytotoxicity in irradiated cells. Although IL-6 production in response to ATP or UVA stimulation appears within 24 h (Fig. 2 D, 20,21), there was no change in IL-6 production at 24 h after γ-irradiation. However, mRNA expression of IL-6 was increased at 30 h after irradiation, and then intracellular IL-6 protein level and release of IL-6 was increased after 33 h. Although it was previously reported that IL-6 production induced by γ-irradiation is delayed in HaCaT cells 24), the mechanism was not established. This production of IL-6 was sustained up to 5 days, at least. Thus, we focused on the production of IL-6 in irradiated cells.

It has been reported that ATP is released by γ-irradiation 39). On the other hand, extracellular ATP induces IL-6 production in epidermal cells 43). Therefore we examined the role of P2 receptors involved in purinergic signaling of extracellular ATP in the present case. Studies with inhibitors of various P2 receptors suggested that the P2Y11 receptors were involved. This is consistent with the fact that IL-6 production induced by IFN-γ, silica nanoparticles or UVA stimulation is mediated by P2Y11 receptors in HaCaT cells 20,23).

Though we could not precisely determine when P2Y11 receptor is activated, IL-6 production was suppressed by post-treatment with NF157 at 15 h after irradiation, but not 18 h, suggesting the activation of P2Y11 receptor would be occurred between 15-18 h after irradiation. We here showed the increase of extracellular ATP concentration in irradiated cells at 18-24 h after irradiation. We could not determine whether activation of P2Y11 is transient or continuous. Activation of P2Y11 receptor induced by ATP caused IL-6 production at 24 h, and irradiation did not cause IL-6 production at 24 h. Considering these results, P2Y11 receptor might not be activated immediately after irradiation. It is not clear why the activation of P2Y11 receptors is delayed, but a possible explanation is that the ATP concentration does not immediately rise above the threshold for receptor activation, and this in turn leads to a delay of IL-6 production. However, further research is needed to confirm this. The pannexin hemichannel, connexin hemichannel, maxi anion channel, vesicular nucleotide transporter (VNUT) and other anion channel are usually involved in the ATP release pathway, but involvement of an anion channel or a hemichannel has been reported in radiation-induced ATP release 39), and maxi-anion channel is involved in the case of HaCaT cells 25). Therefore,
ATP release from HaCaT cells may be involved in γ-irradiation-induced IL-6 production. On the other hand, since it is reported that activation of ATPase is decreased after γ-irradiation, it is conceivable that a decrease of ecto-nucleotidase activity due to irradiation causes an increase of extracellular ATP concentration. The extracellular ATP concentration was increased after γ-irradiation by either or both release of ATP or/and decrease of ecto-nucleotidase activity, resulting in the activation of P2Y11 receptor.

p38 MAPK or NF-κB are generally involved in IL-6 production, and this was also the case in the present system. Our study has revealed for the first time that P2Y11 receptor, p38 MAPK and NF-κB are involved. Though we also examined the effect of 10 µM SP600125 (JNK pathway inhibitor) and 10 µM U0126 (ERK1/2 pathway inhibitor), treatment with these inhibitors enhanced IL-6 production (Supplemental Figure 1), suggesting JNK and ERK1/2 pathway would not induce IL-6 production in irradiated cells.

Though treatment with ATP induced IL-6 production within 24 h, the IL-6 production in irradiated cells was induced over 24 h. The difference in IL-6 production between ATP treatment and γ-irradiation may be due to the DNA damage caused by γ-irradiation and the occurrence of cell cycle arrest due to its repair. We considered the possibility that cellular senescence is involved in this phenomenon, but γ-irradiated HaCaT cells did not show activation of SA-β-gal or cell cycle arrest at the G0 phase, ruling out this possibility. Since it has been reported that HaCaT cells do not have a SA-β-gal activity, we cannot determine whether cellular senescence was occurred in irradiated HaCaT cells by the result of SA-β-gal. On the other hand, the cell density of irradiated group was lower than control group. Considering the cell cycle arrest in irradiated cells, the cell proliferation could be decreased in irradiated cells. Cellular DNA damages are usually repaired in a few hours, but in this study DNA damage response and repair factors were still not disappeared at 48 h after γ-irradiation. Previously, a phenomenon called senescence-like growth arrest (SLGA) was reported as a growth arrest reaction induced by radiation, in which ATM and γH2AX were continuously activated. The phenomenon in the present study might be similar to SLGA. In addition, micronuclei observed after γ-irradiation arise because fragments of chromosome cut by γ-irradiation lose kinetochores and are left behind in the mitotic process, resulting in accumulation of γH2AX.
Although it was reported that G9a, decreases by DNA damage, is not decreased after UV-irradiation\textsuperscript{42}, we found that G9a was decreased after $\gamma$-irradiation. The reason for this difference may be related to the fact that UV irradiation causes DNA single-strand breaks (SSB), whereas $\gamma$-irradiation causes DNA double-strand breaks (DSB)\textsuperscript{51,52}. There is a report that G9a is involved in repair of DNA damage\textsuperscript{53}. In this study, the decrease of G9a after $\gamma$-irradiation may be involved in the sustained activation of DNA damage markers and repair factors. Although both UV and $\gamma$-rays induce the production of IL-6 via p38 MAPK and NF-$\kappa$B\textsuperscript{54}, their different effects in causing SSBs and DSBs, respectively, may be related to the difference in the change of G9a and in the production time of IL-6. In other words, it is thought that $\gamma$-irradiation-induced DSB induces reduction of G9a expression, which also causes DNA damage, and the difference in the production time of IL-6.

In summary, our results indicate the involvement of P2Y11 receptor, p38 MAPK, NF-$\kappa$B and G9a in the $\gamma$-irradiation-induced cellular production of IL-6. Further, the delay of IL-6 production in response to $\gamma$-irradiation compared to other stimuli (IFN-$\gamma$, UV, silica nanoparticles, ATP, LPS, etc.) suggests that the mechanism of radiation-induced cytokine production is different from that of the response to the other stimuli. These findings should be helpful to understand the pathogenesis of radiation-induced inflammation, as well as the potential side effects of therapeutic irradiation on surrounding normal cells or cancer cells. Our data suggest that the treatment with antagonist of P2Y11 receptor might contribute to attenuation of the inflammation in irradiated tissues by suppressing pro-inflammatory cytokine production.
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Conflict of interest
The authors declare no conflict of interest.

Supplementary materials
The online version of this article contains supplementary materials.
References


9) Fillingham J, Keogh M-C, Krogan NJ. γ H2AX and its role in DNA double-strand break repairThis paper is one of a selection of papers published in this Special Issue, entitled 27th International West Coast Chromatin and Chromosome Conference, and has undergone the Journal’s usual peer review process. *Biochem. Cell Biol.*, 84, 568–577


19) Kawakami M, Kaneko N, Anada H, Terai C, Okada Y. Measurement of interleukin-6,


37) Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE.


Figure Legends

Fig. 1 IL-6 production after γ-irradiation in HaCaT cells

(A) Cells were irradiated with various doses of γ-irradiation (1-20 Gy) and incubated for 24 h or 48 h and the culture supernatant was harvested. (B) Cells were irradiated (5 Gy) and incubated for 24 h. The culture supernatant was harvested (day 0-1). Thereafter, culture medium was replaced with fresh medium and cells were incubated in fresh medium for another 24 h, and then the culture medium was harvested again (day 1-2). That action was repeated up to day 4-5 after γ-irradiation (5 Gy). The samples were collected at each day (day 2-3, day 3-4 and day 4-5). (C) Cells were irradiated (5 Gy) and incubated for the indicated period (24-48 h). The culture supernatant was harvested. (D) Cells were irradiated (5 Gy) and incubated for indicated period (24-30 h). IL-6 mRNA level was measured by real time RT-PCR. Each value represents the mean ±SE (n=3). (E) Cells were irradiated (5 Gy) and incubated for the indicated period (24-48 h). The cells were lysed and harvested. (A, B, C and E) The IL-6 concentration in culture supernatants was measured by means of ELISA. Each value represents the mean ±SE (n=4). (A) Significant differences from the control (non-irradiation 48 h) are indicated with asterisks (**P < 0.001) and significant differences between 24 h and 48 h are indicated with sharps (###P < 0.001). (B, C and E) Significant differences from γ-irradiation (5 Gy) 24 h are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001) and significant differences between non-irradiation and γ-irradiation (5 Gy) are indicated with sharps (#P < 0.05, ##P < 0.01, ###P < 0.001).

Fig. 2 Involvement of P2Y11 receptor in IL-6 production by γ-irradiation

(A) Total RNA was extracted from HaCaT cells, and the expression of P2 receptors mRNA was analyzed by RT-PCR. (B, F and G) Cells were γ-irradiated (5 Gy) and incubated for 48 h. Inhibitors (NF157 50 μM, apyrase 10 U/mL, PPADS 100 μM, NF449 10 μM, paroxetine 10 μM, BDBD 10 μM, oxATP 300 μM, AZ10606120 10 μM, BBG 50 μM, suramin 100 μM, MRS2179 100 μM, MRS2578 10 μM, clopidogrel 30 μM, MRS2211 100 μM or LY294002 10 μM) were added 1 h before irradiation (B and F). Cells were pretreated with NF157 at 1 h before irradiation, or post-treated with NF157 at 9, 12, 15 or 18 h after irradiation (G). (D) Cells were stimulated with ATP at the indicated concentration and incubated for 24 h. Cells were pre-incubated with NF157 (50 μM) for 1 h. IL-6 concentration in culture supernatants was measured by means of ELISA. Each value represents the mean ±SE (n=4). (C) HaCaT cells were γ-irradiated (5 Gy) and incubated for 24 h or 48 h. Relative P2Y11 receptor level was analyzed by immunoblotting. (E) The ATP concentration in culture supernatants was
measured as described in Materials and Methods. Each value represents the mean ±SE (n=12).
(B and E-G) Significant differences from control γ-irradiation (5 Gy) are indicated with asterisks (**P < 0.001) and significant differences between non-irradiation and γ-irradiation (5 Gy) are indicated with sharps (###P < 0.001). (D) Significant differences between control and NF157 (same stimulation conditions) are indicated with asterisks (***P < 0.001) and significant differences between control and ATP-treated cells are indicated with sharps (##P < 0.001).

**Fig. 3 Involvement of p38 MAPK and NF-κB in IL-6 production induced by γ-irradiation**

(A) Cells were γ-irradiated (5 Gy) and incubated for 48 h. Inhibitor (SB203580 10 μM) was added 1 h before irradiation. IL-6 concentration in culture supernatants was measured by means of ELISA. Each value represents the mean ±SE (n=4). Significant differences from control γ-irradiation (5 Gy) are indicated with asterisks (**P < 0.01) and significant differences between non-irradiation and γ-irradiation (5 Gy) are indicated with sharps (###P < 0.001). (B and C) HaCaT cells were γ-irradiated (5 Gy) and incubated for 24 h or 48 h. (D-F) Inhibitors (NF157 50 μM or SB203580 10 μM) were added 1 h before irradiation. Cells were incubated for 24 h after irradiation. Relative phospho-p38 MAPK (B and E) or IκB level (C, D and F) was analyzed by immunoblotting (% of non-irradiation). Representative images are shown and each value represents the mean ±SE. (B, C) n=3, (D-F) n=4. Significant differences from control γ-irradiation (5 Gy) are indicated with asterisks (*P < 0.05, **P < 0.01).

**Fig. 4 Induction of DNA damage by γ-irradiation**

(A) HaCaT cells were γ-irradiated (5 Gy) and incubated for 24 h or 48 h. Involvement of cellular senescence was analyzed by SA-β-gal staining. Representative fluorescence microscope images are shown (scale bar 60 μm). (B-D) HaCaT cells were γ-irradiated (5 Gy) and incubated for the indicated periods. γH2AX (FITC; green) and ATM (Alexa 594; red) (B) or γH2AX (Alexa 594; red) and 53BP1 (FITC; green) (C and D) were detected by immunostaining. Representative laser-scanning confocal microscope images are shown (scale bar 20 μm). Arrowheads indicate micronucleation by irradiation and accumulation of γH2AX.

**Fig. 5 Cell cycle changes in response to γ-irradiation**

HaCaT cells were γ-irradiated (5 Gy) and incubated for 24 h or 48 h. The cell cycle was
analyzed by fluorescence-activated cell sorting (FACS). Amounts of cells in the G0/G1, S and G2/M phases of the cell cycle are indicated. The left panels are representative histograms. The right panel shows cell ratios in the G0/G1, S or G2/M phases. Each value represents the mean ±SE (n=3). Significant differences between non-irradiation and γ-irradiation (5 Gy) are indicated with asterisks (***P < 0.001).

Fig. 6 Involvement of G9a in IL-6 production induced by γ-irradiation
(A and B) HaCaT cells were γ-irradiated (5 Gy) and incubated for 24 h or 48 h. Relative G9a level (% of non-irradiation) was analyzed by immunoblotting. The inhibitor (MG132 1 μM) was added 1 h before irradiation (B). Each value represents the mean ±SE (n=3). (C) HaCaT cells were γ-irradiated (5 Gy) and incubated for 48 h. The inhibitor (MG132 1 μM) was added 1 h before irradiation. IL-6 concentration in culture supernatants was measured by means of ELISA. Each value represents the mean ±SE (n=4). Significant differences from control γ-irradiation (5 Gy) are indicated with asterisks (**P < 0.01).
Fig. 1

A

B

C

D

E
Fig. 2

A. P2X and P2Y receptor expression with GAPDH.

B. Comparison of IL-6 expression under non-irradiation and irradiation (5 Gy).

C. Western blot analysis of P2Y11 and GAPDH expression.

D. Graph showing IL-6 levels under different ATP concentrations.

E. Graph showing ATP levels under different treatment groups.

F. IL-6 levels after treatment with LY294002.

G. IL-6 levels after post-treatment with various time points.
**Fig. 3**

**A**

![Graph showing IL-6 levels](image)

**B**

![Western blots showing phosphorylated p38 MAPK](image)

**C**

![Western blots showing iκB/GAPDH levels](image)

**D**

![Western blots showing iκB/GAPDH levels](image)

**E**

![Western blots showing phosphorylated p38 MAPK and p38 MAPK levels](image)

**F**

![Western blots showing iκB/GAPDH levels](image)
Fig. 4

A. non-irradiation vs irradiation (5 Gy)

24 h

48 h

B. Hoechst, γH2AX, ATM, merge

non-irradiation

30 min

24 h

48 h

C. Hoechst, 53BP1, γH2AX, merge

non-irradiation

30 min

24 h

48 h

D. Hoechst, 53BP1, γH2AX, merge

non-irradiation

24 h

27 h

30 h

33 h

48 h

60 μm
Fig. 5

The figure shows cell cycle distribution in non-irradiation and irradiation (5 Gy) over 24 and 48 hours. The graphs on the left represent the dot plots for FL2-H and # Cells, while the right side displays the bar graph indicating cell cycle distribution in G0/G1, S, and G2/M phases.

The bar graph at 48 hours shows significant differences between non-irradiation and irradiation (5 Gy) with p-values marked as *** indicating statistical significance.
Fig. 6

A

irradiation (5 Gy)  
24 h 48 h

G9a
GAPDH

irradiation (5 Gy)

MG132

G9a
GAPDH

B

MG132
irradiation (5 Gy)
- - + +

C

non-irradiation
irradiation (5 Gy)

IL-6 (pg/mL)

control  MG132

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