

# Involvement of reactive oxygen species in ionizing radiation–induced upregulation of cell surface Toll-like receptor 2 and 4 expression in human monocytic cells

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## ABSTRACT

Toll-like receptors (TLRs) are pattern recognition receptors that recognize pathogen-associated molecular patterns and are indispensable for antibacterial and antiviral immunity. Our previous report showed that ionizing radiation increases the cell surface expressions of TLR2 and TLR4 and enhances their responses to agonists in human monocytic THP1 cells. The present study investigated how ionizing radiation increases the cell surface expressions of TLR2 and TLR4 in THP1 cells. The THP1 cells treated or not treated with pharmaceutical agents such as cycloheximide and N-acetyl-L-cysteine (NAC) were exposed to X-ray irradiation, following which the expressions of TLRs and mitogen-activated protein kinase were analyzed. X-ray irradiation increased the mRNA expressions of TLR2 and TLR4, and treatment with a protein synthesis inhibitor cycloheximide abolished the radiation-induced upregulation of their cell surface expressions. These results indicate that radiation increased those receptors through *de novo* protein synthesis. Furthermore, treatment with an antioxidant NAC suppressed not only the radiation-induced upregulation of cell surface expressions of TLR2 and TLR4, but also the radiation-induced activation of the c-Jun N-terminal kinase (JNK) pathway. Since it has been shown that the inhibitor for JNK can suppress the radiation-induced upregulation of TLR expression, the present results suggest that ionizing radiation increased the cell surface expressions of TLR2 and TLR4 through reactive oxygen species–mediated JNK activation.

**KEYWORDS:** Toll-like receptor, ionizing radiation, reactive oxygen species, c-Jun N-terminal kinase

## INTRODUCTION

Toll-like receptors (TLRs) are pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs). TLRs are indispensable for antibacterial and antiviral immunity [1, 2]. TLRs are receptive to various components of bacterial cell walls. For example, TLR2 and TLR4 recognize peptidoglycan from gram-positive bacteria and lipopolysaccharide from gram-negative bacteria, subsequently initiating host defense responses against bacteria. In contrast, TLR3 and TLR9 recognize genes of single-strand RNA viruses as well as DNA viruses such as herpes simplex virus, and initiate the production of antiviral cytokines such as type I interferon.

Many reports have shown the link between TLRs and radiation response, e.g. the radioprotective and/or radiomitigative effects of

TLR agonists [3–7]. Burdelya *et al.* reported that injection of CBLB502 (a TLR5 agonist), before lethal total-body irradiation, can improve the survival of irradiated rhesus monkeys as well as mice [3]. Furthermore, it has been reported that TLR2–/– mice are more susceptible to ionizing radiation–induced mortality because of severe bone marrow cell loss, and wild-type mice pre-treated with TLR2 agonist show resistance to ionizing-induced motility [6]. In addition to the exogenous danger molecules PAMPs, TLRs recognize endogenous danger molecules, the so-called damage-associated molecular patterns (DAMPs) [8, 9]. It has been shown that the responses of TLRs to DAMPs such as host RNA and high-mobility group box 1 (HMGB1), which are released from damaged cells, also cause biological responses, including the radiation response [8–12].

Takemura *et al.* reported the involvement of TLR3 in the pathogenesis of gastrointestinal syndrome induced by ionizing radiation [11]. They showed that radiation-induced crypt cell death causes leakage of cellular RNA, which in turn induces extensive crypt cell death via TLR3, leading to gastrointestinal syndrome. Furthermore, Apetoh *et al.* reported that HMGB1 secreted from dying tumor cells as a result of radiotherapy or chemotherapy activates TLR4 on dendritic cells, which results in the induction of antitumor effects through processing and cross-presentation of antigen from dying tumor cells [12]. Collectively, these reports indicate that TLRs play important roles in radiation response, including radiation-induced tissue damages and the efficacy of cancer radiotherapy.

We recently investigated the effects of ionizing radiation on TLR2 and TLR4 by using human monocytic THP1 cells and THP1-derived macrophage-like cells, and we showed that ionizing radiation affects the cell surface expression levels of those receptors and the response to their agonist depending on the cell differentiation state [13]. In undifferentiated THP1 cells, the cell surface expressions of TLR2 and TLR4 were shown to increase after X-irradiation, which was accompanied by the enhancement of the proinflammatory response induced by their agonists. Therefore, it is possible that ionizing radiation enhances the inflammatory responses at least by upregulating the cell surface expressions of TLR2 and TLR4. However, the mechanism responsible for the increases in the cell surface expressions of TLR2 and TLR4 due to ionizing radiation remains unknown. Therefore, in the present study, we investigated the mechanisms by which ionizing radiation increases the cell surface expressions of TLR2 and TLR4 in human monocytic THP1 cells.

## MATERIALS AND METHODS

### Reagents

Dimethyl sulfoxide (DMSO), cycloheximide (CHX), N-acetyl-L-cysteine (NAC), fumonisins B1, desipramine and GW4869 were purchased from Sigma-Aldrich (St Louis, MO, USA). The fluorescence-labeled monoclonal antibody (mAb) anti-human TLR2-phycoerythrin (TLR2-PE) and TLR4-PE were purchased from eBioscience (San Diego, CA, USA). The mouse IgG<sub>2a</sub>-PE was purchased from Becton Dickinson (San Jose, CA, USA). Stress-activated protein kinases (SAPK)/c-Jun N-terminal kinases (JNK) rabbit Ab (#9252), phospho-SAPK/JNK (Thr183/Tyr185) mouse mAb (#9255), p44/42 mitogen-activated protein kinase (MAPK) (extracellular signal-regulated kinase (ERK)1/2) rabbit mAb (#4695), phospho-p44/42 MAPK (Thr202/Tyr204) rabbit mAb (#4370), apoptosis signal-regulating kinase 1 (ASK1) rabbit Ab (#3762), phospho-ASK1 (Thr845) rabbit Ab, phospho-c-Jun (Ser63) rabbit mAb (#2361), phospho-MAPK kinase 7 (MKK7) (Ser271/Thr275) rabbit Ab (#4171),  $\beta$ -actin Ab (#4967), anti-rabbit IgG horseradish peroxidase (HRP)-linked Ab (#7074) and anti-mouse IgG HRP-linked Ab (#7076) were purchased from Cell Signaling Technology Japan, K.K. (Tokyo, Japan). The fluorescent probe 3'-(p-hydroxyphenyl) fluorescein (HPF) was obtained from Molecular Probes (Eugene, OR, USA).

### Cell culture and treatment

THP1 human acute monocytic leukemia cells were obtained from the RIKEN Bio-Resource Center (Tsukuba, Japan). The cells were

cultured in RPMI1640 that was supplemented with 1% penicillin and streptomycin (Gibco, Grand Island, NY, USA) and 10% heat-inactivated fetal bovine serum (Japan Bioserum Co., Ltd, Japan) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. THP1 cells ( $1.0 \times 10^5$  cells/ml) were plated in 35 mm or 60 mm dishes (Iwaki, Tokyo, Japan) and irradiated with X-rays.

In some experiments, DMSO, 10  $\mu$ g/ml cycloheximide (a protein synthesis inhibitor), 20 mM NAC (a precursor to the critical antioxidant glutathione), 50  $\mu$ M fumonisins B1 (inhibitor for ceramide synthase), 10  $\mu$ M desipramine (inhibitor for acid sphingomyelinase) or 1.4  $\mu$ M GW4869 (inhibitor for neutral sphingomyelinase) were added to the culture medium at 1 h before X-ray irradiation. The pH of NAC was adjusted to 7.4 with NaOH.

### In vitro X-ray irradiation

X-ray irradiation (150 kVp, 20 mA, 0.5 mm Al and 0.3 mm Cu filters) was performed using an X-ray generator (MBR-1520R-3; Hitachi Medical Corporation, Tokyo, Japan) at a distance of 45 cm from the focus, with a dose rate of 1.01–1.03 Gy/min.

### Cell surface staining

THP1 cells were exposed to X-ray irradiation and were harvested after 24 h for the analysis of cell surface antigen expression. Cells were stained with TLR2-PE or TLR4-PE mAbs for 30 min at 4°C in the dark. Cells were also stained with corresponding PE-conjugated isotype control mouse IgG. After 30 min, the cells were washed with cold Ca<sup>2+</sup>- and Mg<sup>2+</sup>- free phosphate-buffered saline (PBS(-)) and were analyzed using a flow cytometer (Cytomics FC500; Beckman Coulter).

### Measurement of intracellular reactive oxygen species

The measurement of intracellular reactive oxygen species (ROS) levels was performed using the fluorescent probe HPF. In brief, treated cells were harvested, washed with PBS(-), and incubated for 15 min at 37°C with 5  $\mu$ M HPF in PBS(-). After incubation, the cells were washed with PBS(-) and analyzed by a flow cytometer (Cytomics FC500).

In some experiments, cells pretreated with vehicle (H<sub>2</sub>O) or 20 mM NAC for 1 h were harvested and washed with PBS(-). The cells were incubated for 15 min at 37°C with 5  $\mu$ M HPF in PBS(-), and then were exposed to X-rays in the presence of HPF. The cells were washed with PBS(-) immediately after irradiation and analyzed by a flow cytometer.

### Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting

Harvested cells were lysed in 1  $\times$  Laemmli sample buffer (Bio-Rad Laboratories, Inc.) containing 2.5% 2-mercaptoethanol by sonication and then boiled for 10 min. The protein concentration was determined using the XL-Bradford assay kit (APRO Science, Tokushima, Japan) and a SmartSpec<sup>TM</sup> plus spectrophotometer (Bio-Rad Laboratories, Inc.). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed as previously reported [14]. The following primary antibodies were

used: anti-JNK Ab (1:3000), anti-phospho-SAPK/JNK Ab (1:3000), anti-ERK Ab (1:3000), anti-phospho-ERK Ab (1:3000), anti-ASK1 Ab (1:3000), anti-phospho-ASK1 Ab (1:3000), anti-phospho-MKK7 Ab (1:3000), anti-phospho-c-Jun Ab (1:3000), or anti-actin Ab (1:4000). The following secondary antibodies were used: HRP-linked anti-rabbit IgG Ab (1:10 000) or HRP-linked anti-mouse IgG Ab (1:10 000). The antigens were visualized by the ECL Prime Western Blotting Detection System (GE Healthcare). Blot stripping was performed using Stripping Solution (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

### Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA extraction and the synthesis of complementary DNA templates were performed as previously reported [15]. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using Power SYBR® Green (Applied Biosystems Inc., Carlsbad, CA, USA) and a StepOnePlus™ system (Applied Biosystems Inc.) with typical amplification parameters (95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). Relative differences were calculated by the  $\Delta\Delta C_t$  method.  $\beta$ -actin was used as the house-keeping gene. Primers for TLR2, TLR4 and  $\beta$ -actin are shown in Table 1.

### Statistical analysis

Data are presented as mean  $\pm$  standard error (SE). Comparisons between the control and experimental groups were performed using the two-sided Student's *t*-test or two-sided Mann-Whitney's *U*-test depending on normality of data distributions. Differences were considered significant when  $P < 0.05$ . The Excel 2010 software (Microsoft, USA) with the add-in software Statcel 3 (The Publisher OMS Ltd, Tokyo, Japan) was used to perform these statistical analyses.

## RESULTS

### Ionizing radiation increased cell surface TLR2 and TLR4 expressions through *de novo* protein synthesis

We first investigated whether the radiation-induced upregulation of cell surface expressions of TLR2 and TLR4 results from *de novo* protein synthesis. In line with our previous report [13], the

upregulation of cell surface expressions of TLR2 and TLR4 was observed in the 5 Gy-irradiated THP1 cells (Fig. 1A upper panels and Fig. 1B), and it was sustained at least until 48 h after irradiation (Supplementary Fig. 1). Since 5 Gy irradiation significantly induced apoptosis in THP1 cells at 48 h after irradiation (our unpublished data), we analyzed the cell surface expressions of TLRs at 24 h after irradiation in the present study. When the THP1 cells were treated with a protein synthesis inhibitor (cycloheximide), the radiation-induced upregulation of cell surface expressions of TLR2 and TLR4 was diminished (Fig. 1A lower panels and Fig. 1B). Furthermore, analysis of the qRT-PCR showed that the mRNA expressions of TLR2 and TLR4 were significantly higher in 5 Gy-irradiated THP1 cells compared with non-irradiated cells, though there was a temporal difference between TLR2 and TLR4 mRNA induction (Fig. 1C). Considered together, these results suggest that radiation increased the cell surface expressions of TLR2 and TLR4 through *de novo* protein synthesis.

### Involvement of ROS in radiation-induced upregulation of cell surface TLR2 and TLR4 expressions

Since low-energy transfer radiation such as X-ray irradiation mainly induces biological effects through ROS [16, 17], we next investigated the involvement of ROS in the radiation-induced upregulation of cell surface expressions of TLR2 and TLR4. We first examined the intracellular ROS levels using ROS-detecting fluorescence probe HPF. In line with our previous report [18], ionizing radiation increased the intracellular ROS levels during irradiation and 24 h after irradiation (Fig. 2A upper and lower panels, respectively). As shown in Fig. 2A, treatment with a precursor of critical antioxidant glutathione NAC decreased the intracellular ROS levels of 5 Gy-irradiated cells, thus suggesting the ROS-scavenging effect of NAC. Similar results were observed when we used another ROS-detecting fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (Supplementary Fig. 2). We next examined the effects of NAC on the cell surface expressions of TLR2 and TLR4. As shown in Fig. 2B, treatment with NAC dramatically decreased the cell surface TLR2 and TLR4 expressions of non- or X-irradiated cells. Furthermore, in the cells treated with NAC, no significant difference in the cell surface expressions of TLRs was observed between non-irradiated and 5 Gy-irradiated cells (Fig. 2B). Considered together, these results suggest that ionizing radiation increased the cell surface expressions of TLR2 and TLR4 in a ROS-dependent manner.

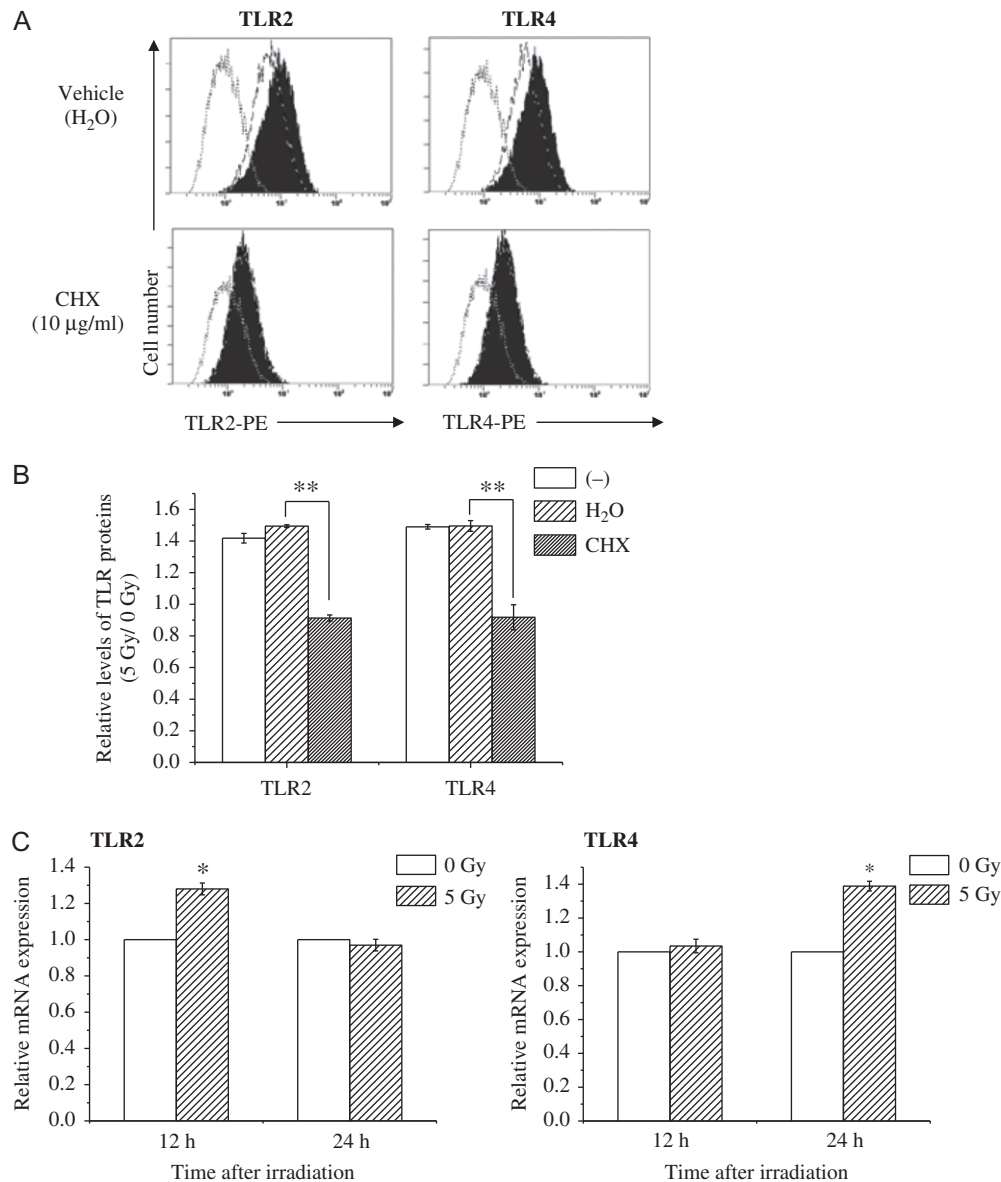
### Involvement of ceramide generation in cell surface TLR2 and TLR4 expressions

Ceramide is an important molecule as the precursor for all major sphingolipids and serves as a secondary messenger in several signaling pathways [19]. It is known that genotoxic stimuli and cellular stress, including ionizing radiation, increase cellular ceramide, which then causes various cellular responses such as apoptosis [19–21]. Recently, oxidative stress was reported to increase cell surface expression of TLR4 in murine macrophages through ceramide generation [22]. Therefore, we next investigated the involvement of ceramide in the radiation-induced upregulation of cell surface expressions of TLR2 and TLR4 using certain ceramide generation inhibitors. As shown

**Table 1. Primer sequences for quantitative reverse transcription polymerase chain reaction**

	Sequence (5'→3')
TLR2 F	CGTTCTCTCAGGTGACTGCTC
TLR2 R	CCTTTGGATCCTGCTTGC
TLR4 F	CCTGCGTGAGACCAGAAAG
TLR4 R	TTCAGCTCCATGCATTGATAA
$\beta$ -actin F	TGGCACCCAGCACAAATGAA
$\beta$ -actin R	CTAAGTCATAGTCCGCCTAGAAGCA

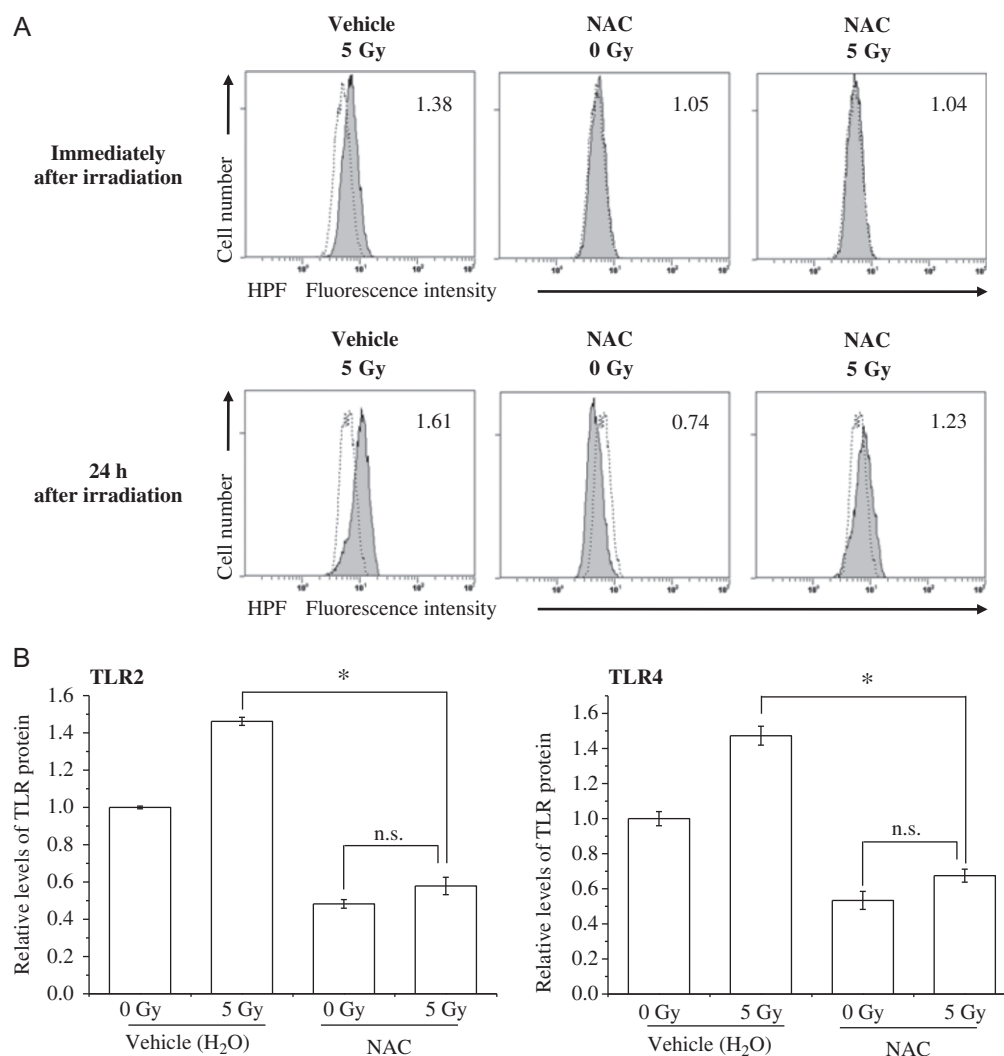
TLR = Toll-like receptor.



**Fig. 1.** Involvement of *de novo* protein synthesis in the radiation-induced upregulation of Toll-like receptor (TLR) 2 and TLR4 expressions. (A, B) THP1 cells treated with or without vehicle (H<sub>2</sub>O) or cycloheximide (CHX) were exposed to 5 Gy. After 24 h of culture, the cells were harvested for analysis of cell surface expressions of TLR2 and TLR4. (A) Representative histograms of TLR2 and TLR4 expressions are shown. The dotted line and broken line indicate the isotype control and the result for non-irradiated cells, respectively. The black-filled histograms indicate the results for 5 Gy-irradiated cells. (B) Results are shown as the relative value of mean fluorescence intensity of irradiated cells compared with non-irradiated cells (5 Gy/0 Gy). Data are presented as the mean  $\pm$  standard error (SE) of three independent experiments. (C) Non-irradiated or 5 Gy-irradiated THP1 cells were cultured for 12 h or 24 h, and total RNA was harvested for the analysis of the quantitative reverse transcription polymerase chain reaction. Data are presented as the mean  $\pm$  SE of four independent experiments. \* and \*\* indicate  $P < 0.05$  and  $P < 0.01$ , respectively.

in Fig. 3A, fumonisins B1 and GW4869 decreased the cell surface expressions of TLR2 and/or TLR4, whereas desipramine had no effects on the cell surface expression of either TLR2 or TLR4. These results suggest that certain ceramide generation pathways were involved in the cell

surface expressions of TLR2 and TLR4 of non-irradiated THP1 cells. However, neither ceramide generation inhibitors decreased the radiation-induced upregulation of cell surface expressions of TLR2 and TLR4 (Fig. 3B).



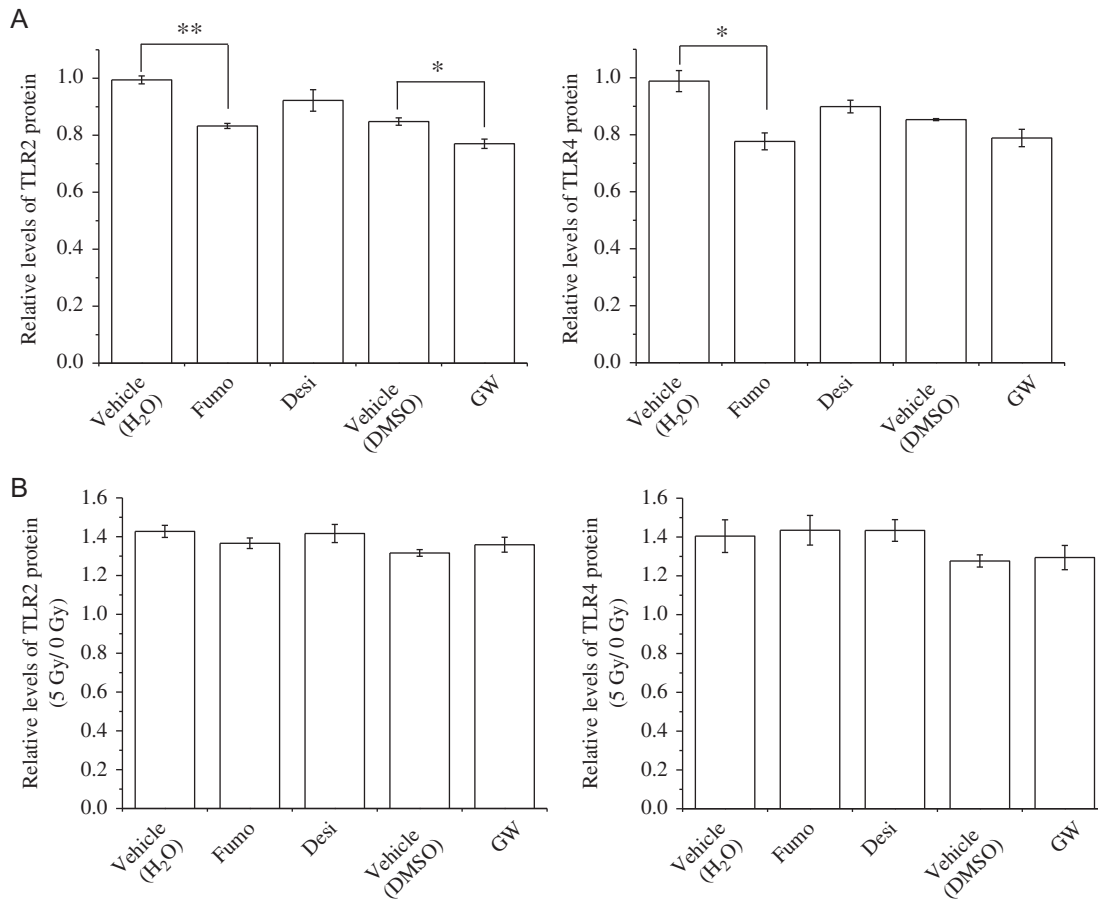
**Fig. 2.** Effects of N-acetyl-L-cysteine (NAC) on the radiation-induced upregulation of Toll-like receptor (TLR) 2 and TLR4 expressions. (A) Upper panel: THP1 cells pretreated with vehicle (H<sub>2</sub>O) or NAC for 1 h were harvested and washed with PBS (–). The cells were stained with 3′-(*p*-hydroxyphenyl) fluorescein (HPF), and then were exposed to 5 Gy in the presence of HPF. The cells were washed with PBS(–) immediately after irradiation and analyzed by a flow cytometer. Lower panel: NAC was added to the culture medium at 1 h before X-ray irradiation, following which the cells were exposed to 5 Gy. After 24 h of culture, the cells were harvested for the measurement of intracellular ROS levels. The dotted line indicates the result of the vehicle-treated non-irradiated control. Inset numbers indicate a relative value of mean fluorescence intensity compared with that of the vehicle-treated non-irradiated control. Representative results from two independent experiments are shown. (B) NAC was added to the culture medium at 1 h before X-ray irradiation, following which the cells were exposed to 5 Gy. After 24 h of culture, the cell surface expressions of TLR2 and TLR4 were analyzed. Results are shown as the relative value of mean fluorescence intensity compared with the vehicle-treated non-irradiated control. Data are presented as the mean  $\pm$  standard error (SE) of three independent experiments. \* indicates  $P < 0.01$ .

### Ionizing radiation activated JNK through ROS

Our previous report showed that treatment with inhibitor for JNK or ERK suppressed the upregulations of cell surface expressions of TLR2 and TLR4 by X-ray irradiation [13]. Since it is known that ROS activates MAPKs, including JNK and ERK [23, 24], we next investigated whether radiation-induced ROS in THP1 cells were

involved in the activation of JNK and/or ERK. The upregulation of phosphorylated JNK expression was observed in 5 Gy-irradiated cells, especially at 0.5 h and 1 h after irradiation, and it was sustained until 6 h after irradiation (Fig. 4A and Supplementary Fig. 3). As shown in Fig. 4A, treatment with NAC partly suppressed the upregulation of phosphorylated JNK expression in 5 Gy-irradiated cells.





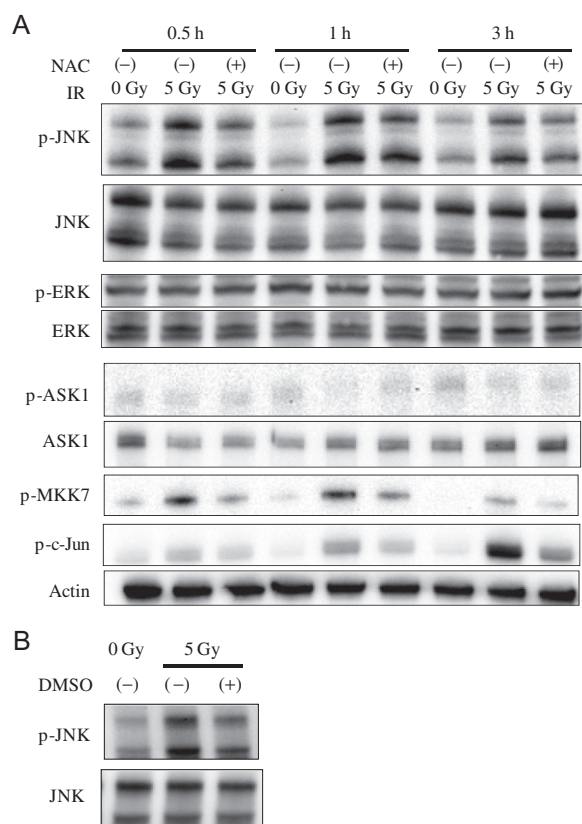
**Fig. 3.** Effects of ceramide generation inhibitors on cell surface Toll-like receptor (TLR) 2 and TLR4 expressions. (A) THP1 cells were cultured in the presence of each ceramide generation inhibitor for 24 h and the cell surface TLR2 (left) and TLR4 (right) expressions were analyzed. As vehicle controls, cells treated with H<sub>2</sub>O or 0.2% (v/v) dimethyl sulfoxide (DMSO) were prepared. Results are shown as a relative value of mean fluorescence intensity compared with that of the vehicle (H<sub>2</sub>O) control. Data are presented as the mean  $\pm$  standard error (SE) of three independent experiments. \* and \*\* indicate  $P < 0.05$  and  $P < 0.01$ , respectively. (B) Each ceramide generation inhibitor was added to the culture medium 1 h before X-ray irradiation, following which the cells were exposed to 5 Gy. After 24 h of culture, the cell surface expressions of TLR2 and TLR4 were analyzed. Results are shown as the relative value of mean fluorescence intensity compared with that of non-irradiated cells (5 Gy/0 Gy). Data are presented as the mean  $\pm$  SE of three independent experiments.

On the other hand, no large change in the phosphorylated ERK expression was observed in 5 Gy-irradiated THP1 cells with or without NAC (Fig. 4A and Supplementary Fig. 3). These results suggest that radiation-induced ROS activated JNK, but not ERK, in THP1 cells.

We next investigated the radiation-activated JNK pathway. As shown in Fig. 4A, X-irradiation increased the phosphorylated MKK7 expression, which is an upstream kinase of JNK. Furthermore, the phosphorylated c-Jun expression, which is a downstream target of JNK, was observed in 5 Gy-irradiated cells (Fig. 4A). Similar to the results of JNK expression, the radiation-induced phosphorylation of MKK7 and c-Jun was suppressed by treatment with NAC (Fig. 4A), thus suggesting that ionizing radiation activated the JNK signaling pathway in a ROS-dependent manner. ASK-1 is one of the MAP kinase kinase kinase (MAP3K) superfamily for JNK, and is known

as a ROS-responsive kinase [25, 26]. Therefore, we investigated the phosphorylated ASK1 expression of X-irradiated THP1 cells. However, no large increase in the phosphorylated ASK1 expression was observed in 5 Gy-irradiated cells (Fig. 4A), thus suggesting that ASK1 was not mainly responsible for the radiation-induced JNK activation.

Ionizing radiation rapidly caused the phosphorylation of JNK in a ROS-dependent manner, thus suggesting a possibility that ROS instantaneously generated by ionizing radiation were involved in the radiation-induced JNK activation. Since the fluorescence intensity of HPF, which mainly detects hydroxyl radicals, was higher in 5 Gy-irradiated cells compared with non-irradiated cells, we finally investigated the involvement of hydroxyl radicals on the radiation-induced phosphorylation of JNK using a quencher of hydroxyl radical DMSO [27]. As shown in Fig. 4B, treatment with 2% (v/v) DMSO



**Fig. 4. Expression of mitogen-activated protein kinase (MAPK)-related proteins in X-ray-irradiated THP1 cells. (A)** N-acetyl-L-cysteine (NAC) was added to the culture medium 1 h before X-ray irradiation, following which the cells were exposed to 5 Gy. After the indicated time-points, the cells were harvested for western blot analyses of phosphorylated c-Jun N-terminal kinase (p-JNK), phosphorylated extracellular signal-regulated kinase (p-ERK), phosphorylated apoptosis signal-regulating kinase 1 (p-ASK1), ASK1, phosphorylated MAPK kinase 7 (p-MKK7) and phosphorylated c-Jun (p-c-Jun). As a loading control, the expressions of JNK, ERK or  $\beta$ -actin were analyzed. Representative data from at least two independent experiments are shown. **(B)** Two percent (v/v) dimethyl sulfoxide (DMSO) was added to the culture medium 1 h before X-ray irradiation, following which the cells were exposed to 5 Gy. After 1 h of culture, the cells were harvested for western blot analyses of phosphorylated JNK. As a loading control, the expression of JNK was analyzed. Representative data from two independent experiments are shown.

decreased the phosphorylated JNK expression in 5 Gy-irradiated cells, thus suggesting that hydroxyl radicals were involved in the radiation-induced JNK activation.

## DISCUSSION

TLRs play major roles in antibacterial and antiviral immunity. Furthermore, recent studies have shown the involvement of TLRs in radiation response, including radiation-induced tissue damage and the efficacy of cancer radiotherapy [6, 9, 11, 12]. We have previously reported that ionizing radiation affects the expressions and responses of TLR2 and TLR4 in human monocytic THP1 cells [13]. In the present study, we demonstrated that ionizing radiation increased the cell surface expressions of TLR2 and TLR4 through *de novo* protein synthesis. Furthermore, the present study showed that ionizing radiation-induced ROS mediated the increase in cell surface expressions of TLR2 and TLR4 and the activation of JNK in THP1 cells.

Tawadros *et al.* reported that oxidative stress increases cell surface TLR4 expression in murine macrophages [22]. In their report, treatment with  $H_2O_2$  resulted in the translocation of TLR4 to the plasma membrane through ceramide generation. Here, we showed that radiation-induced upregulation of the cell surface expressions of TLR2 and TLR4 was not suppressed by the inhibitor for ceramide generation. Furthermore, in our preliminary analysis, treatment with ceramide analogs (C2- and C6-ceramide) hardly increased the cell surface expressions of TLR2 and TLR4 in THP1 cells (data not shown). Therefore, although ceramide generation induced by oxidative stress may regulate the cell surface expression of TLR4 through translocation to the plasma membrane depending on cell types, it did not appear likely that ceramides are involved in the radiation-induced *de novo* protein synthesis of TLR2 and TLR4 in THP1 cells.

Ionizing radiation induces biological effects by instantaneously generating ROS such as hydroxyl radicals [28, 29]. However, recent reports show that ionizing radiation generates secondary ROS, which seem to be partly generated in mitochondria in irradiated cells [28, 30–32]. Our previous report shows that the secondary intracellular ROS generation in 5 Gy-irradiated THP1 cells occurs 12–24 h after irradiation [18]. However, in the present study, the activation of JNK in 5 Gy-irradiated THP1 cells was observed even at 0.5 h after irradiation. Therefore, we concluded that the activation of JNK by X-ray irradiation is mediated by instantaneously generated ROS such as hydroxyl radicals, but not by secondarily generated ROS. Interestingly, the phosphorylated JNK expression in 5 Gy-irradiated cells returned to the control levels at 24 h after irradiation (Supplementary Fig. 1), whereas the ROS levels in 5 Gy-irradiated cells were higher than those of non-irradiated cells. Therefore, although it is shown that secondarily generated ROS in irradiated cells are involved in various cellular responses to ionizing radiation, such as cell death induction, genomic instability, and antioxidant responses [18, 33, 34], the effects of secondarily ROS, which are generated through physiological processes after irradiation, may be different from those of the ROS generated by ionization.

Our previous study showed that both JNK and ERK inhibitors suppressed the radiation-induced upregulation of TLR2 and TLR4 expressions as well as the constitutive expression of those receptors [13]. The present study demonstrated that radiation activated JNK, but not ERK, in a ROS-dependent manner. Although it is well-

documented that ROS activates MAPK pathways, the mechanisms by which ROS activates MAPK pathways remain unknown. However, recent studies suggest the importance of MAP3K in ROS-induced MAPK activation [23]. Among the MAP3K family, ASK1 is the best-characterized kinase as a ROS-responsive kinase [35]. ASK1 is suppressed by the endogenous inhibitor thioredoxin under normal conditions. However, under oxidative conditions, ASK1 is activated due to the inactivation of thioredoxin by oxidative modification, and it activates the downstream kinases MKK4/MKK7 [35]. In the present study, ionizing radiation did not increase phosphorylated ASK1 expression, although the activation of the MKK7/JNK axis was observed in X-irradiated cells. Mitsutake *et al.* reported that ionizing radiation activates MKK7/JNK through the serine/threonine kinase protein kinase C (PKC)  $\delta$  in human thyroid cells [36]. Since PKCs are a highly susceptible direct target of ROS [35], it is possible that PKC  $\delta$  mediates the activation of the MKK7/JNK axis in X-irradiated human monocytic THP1 cells. Of course, there is a possibility that other MAP3Ks, which are activated by oxidative stress [35, 37], mediate the radiation-induced MKK7/JNK activation. Further studies are needed to clarify these possibilities.

c-jun is well known and studied among the transcription factors activated by JNK. The activated c-Jun through its phosphorylation forms the transcription factor activator protein-1 (AP-1) complex [38], and AP-1 regulates the transcription of various genes involved in proliferation, apoptosis and inflammation [39, 40]. Furthermore, it is reported that AP-1 plays critical roles in regulating *TLR4* gene expression in mice macrophages [41, 42]. Since phosphorylated c-Jun expression was observed in X-irradiated THP1 cells, it is possible that ionizing radiation increases *de novo* *TLR4* expression through the ROS/JNK/AP-1 axis. On the other hand, to our best knowledge there is no report showing the involvement of AP-1 in the induction of *TLR2* expression, though Tarang *et al.* [43] and we [13] showed the involvement of JNK in cisplatin- and radiation-induced *TLR2* expression, respectively. Haehnel *et al.* reported that specificity protein 1 (Sp1) family transcription factors play an important role in the activation of the proximal *TLR2* promoter in human monocytic cells, including THP1 cells [44]. Since JNK transduction pathways can phosphorylate Sp1 [45, 46], there is a possibility that Sp1 is involved in radiation-induced *TLR2* expression. In the present study, we observed a temporal difference between the increases in *TLR2* and *TLR4* mRNA expression after X-irradiation (Fig. 1C). Therefore, although further studies are required, it is likely that the transcription factors involved in radiation-induced *TLR2* expression are not identical to that of *TLR4*.

In the present study, a glutathione precursor NAC dramatically decreased the cell surface expressions of *TLR2* and *TLR4* (Fig. 2B), whereas the inhibition of JNK activation by NAC was partial (Fig. 4A). It is known that the intracellular redox state modulates gene expression by activating the transcription factors and/or the binding of the transcription factor to the promoter region of the target gene [47, 48]. Vayalil *et al.* reported that glutathione blocks transforming growth factor- $\beta$ -induced plasminogen activator inhibitor type 1 (*PAI-1*) expression by blocking *PAI-1* gene transcription through inhibition of JNK and p38 MAPK phosphorylation and the binding of transcription factors to AP-1, Sp-1 and Smad *cis* elements

in the promoter of the *PAI-1* gene [49]. Therefore, in addition to the ROS-scavenging effects of NAC, the suppressive effects of glutathione from NAC on the binding of the transcription factors to the promoter region of *TLR* genes may underlie the suppression of cell surface *TLRs* expression by NAC.

In conclusion, the present study suggests that ionizing radiation increased cell surface *TLR2* and *TLR4* expressions through ROS-mediated JNK activation in human monocytic THP1 cells. Further studies to clarify the effects of the upregulation of radiation-induced ROS-mediated *TLRs* on the responses to endogenous DAMPs as well as exogenous PAMPs may lead to the amelioration of radiation-induced tissue damages and effective cancer radiotherapy.

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## CONFLICT OF INTEREST

There are no conflicts of interest to declare.

## SUPPLEMENTARY DATA

Supplementary data is available at *Journal of Radiation Research* online.

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