Involvement of caspase-8 in apoptosis enhancement by cotreatment with retinoic acid-inducible gene-I-like receptor agonist and ionizing radiation in human non-small cell lung cancer

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Received January 20, 2018; Accepted September 7, 2018

DOI: 10.3892/mmr.2018.9536

Abstract. Retinoic acid-inducible gene-I-like receptors (RLRs) serve an important role in antiviral immune responses. Recent studies demonstrated that RLR activation exerts antitumor activity by inducing an anticancer immune response and apoptosis in various cancer cells. The authors' recent study demonstrated that the cytotoxic effects of the RLR agonist Poly(I:C)-HMW/LyoVec™ [Poly(I:C)-HMW] in human non-small cell lung cancer (NSCLC) were enhanced by cotreatment with ionizing radiation (IR). Furthermore, cotreatment with Poly(I:C)-HMW and IR effectively induced cell death, including apoptosis, in a caspase-dependent manner. However, the mechanisms by which cotreatment with Poly(I:C)-HMW and IR effectively induce apoptosis remains unclear. Therefore, the pathways involved in the increase in apoptosis elicited by cotreatment with Poly(I:C)-HMW and IR in the A549 human NSCLC cell line were investigated. Poly(I:C)-HMW induced the expression of active caspase-8 and -9, and the Poly(I:C)-HMW-induced increase in the cell cycle sub-G1 population, which is one of the hallmarks of apoptosis, was decreased by treatment with a caspase-8 inhibitor and caspase-9 inhibitor. When cells were treated with Poly(I:C)-HMW and IR, the sub-G1 population, and the active caspase-8 and caspase-9 expression were all increased compared with cells treated with Poly(I:C)-HMW or IR alone. Furthermore, expression of X-linked inhibitor of apoptosis protein, which negatively regulates caspase activation, was decreased in cells cotreated with Poly(I:C)-HMW and IR. Notably, treatment with an inhibitor for caspase-8, not caspase-9, partially reversed the net increase in the sub-G1 population induced by cotreatment with Poly(I:C)-HMW and IR. Collectively, these results suggested that Poly(I:C)-HMW induces apoptosis through caspase-8 and caspase-9 activation; however, the apoptotic pathway mediated by casapse-8, and not casapse-9, is involved in the enhancement of apoptosis caused by cotreatment with Poly(I:C)-HMW and IR.

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Key words: retinoic acid-inducible gene-I-like receptor, ionizing radiation, apoptosis, caspase-8, X-linked inhibitor of apoptosis

Introduction

Retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) are pattern-recognition receptors that recognize pathogen-associated molecular patterns. RLRs, which include RIG-I and melanoma differentiation-associated gene 5, function as cytosolic virus sensors and serve an important role in mitochondrial-mediated antiviral immune systems (1). Once these receptors sense an RNA virus invasion, they interact with mitochondrial antiviral-signaling (MAVS) protein, an adaptor protein located on the mitochondrial membrane, to induce antiviral immune responses, including the production of type I interferons (2,3). Furthermore, previous studies demonstrated that RLR activation induces apoptosis in various cancer cells, including in lung cancer (4-6).

Certain previous studies attempted to enhance RLR-mediated antitumor and cytotoxic effects against cancer cells (6-8). Yuan *et al* (6) examined the 5'end of ppp-RNA, which is an agonist of RIG-I, and vascular endothelial growth factor (VEGF), which promotes angiogenesis in cancer, and produced a 5'-triphosphate-small interfering (si)RNA targeting VEGF. Consequently, the VEGF-targeting 5'-triphosphate-siRNA produced multiple antitumor effects against human non-small cell lung cancer (NSCLC) cells through not only the induction of RIG-I-mediated apoptosis and antitumor immunity; however, additionally through inhibition of tumor angiogenesis via VEGF knockdown. In another approach, the cytotoxic effect of cotreatment with an RLR agonist and ionizing radiation (IR) in human NSCLC was recently investigated. RLR agonist-induced cell death was demonstrated to be enhanced by IR, and the RLR agonist exhibited a radiosensitizing effect (9). However, although it was demonstrated that this cell death, which included apoptosis, occurred in a caspase-dependent manner, the molecular mechanisms of the effect remain unclear.

Apoptotic mechanisms may be broadly divided into two pathways: The extrinsic pathway via death receptors and the

intrinsic pathway via mitochondria. In the extrinsic pathway, activation of death receptors, including Fas cell surface death receptor (Fas), induces apoptosis via activation of caspase-8/caspase-3 (10). In contrast, in the intrinsic pathway, cytochrome c is released from mitochondria by external stressors, including DNA injury and oxidative stress, resulting in the activation of caspase-9/caspase-3 and the subsequent induction of apoptosis (11). Furthermore, it is known that apoptosis inhibitor proteins, including X-linked inhibitor of apoptosis protein (XIAP), are involved in these pathways and negatively regulate apoptosis by inhibiting caspase activation (12).

It is additionally known that IR may activate the extrinsic and intrinsic apoptosis pathways. Takahashi *et al* (13) demonstrated that X-ray irradiation induces caspase-8-mediated apoptosis accompanied with Fas upregulation in a human leukemia cell line. Furthermore, Kim *et al* (14) observed that exposure of the HeLa cervical cancer cell line to radiation induced the loss of the mitochondrial membrane potential, and the release of cytochrome c and apoptosis-inducing factor from mitochondria, which resulted in apoptotic cell death. Therefore, it is possible that cotreatment with the RLR agonist and IR may effectively activate the extrinsic and/or intrinsic apoptotic pathways.

To clarify the mechanism by which cotreatment with an RLR agonist and IR induces apoptosis, the pathways involved in the enhancement of apoptosis by cotreatment with RLR agonist and IR in the A549 human NSCLC cell line were investigated.

Materials and methods

Reagents. Ca2+- and Mg2+-free PBS(-), propidium iodide (PI), RNaseA and dimethyl sulfoxide (DMSO) were all purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Poly(I:C)-HMW/LyoVec™ [Poly(I:C)-HMW], which is a complex of a synthetic double-stranded RNA analogue poly(I:C) and a transfection reagent (LyoVec™), was purchased from InvivoGen (San Diego, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-human cluster of differentiation (CD)95 (Fas) antibody (cat. no. 305606) was purchased from BioLegend, Inc. (San Diego, CA, USA). FITC-conjugated anti-mouse immunoglobulin (Ig)G1 antibody (cat. no. A07795) was purchased from Beckman Coulter, Inc. (Brea, CA, USA). Anti-rabbit horseradish peroxidase (HRP)-conjugated IgG and anti-mouse HRP-conjugated IgG secondary antibodies, anti-XIAP monoclonal (cat. no. 2045), anti-caspase-3 monoclonal (cat. no. 9661), anti-caspase-8 monoclonal (cat. no. 9746), anti-caspase-9 monoclonal (cat. no. 9502) and anti-β-actin monoclonal (cat. no. 4967) primary antibodies were purchased from Cell Signaling Technology Inc., (Danvers, MA, USA). Z-Val-Ala-Asp (OMe)-CH2F (Z-VAD-fmk), Ac-Ile-Glu-Thr-Asp-H (aldehyde; AC-IETD-CHO) and Ac-Leu-Glu-His-Asp-H (aldehyde; AC-LEHD-CHO) peptides were purchased from Peptide Institute, Inc. (Osaka, Japan). Ambion's Silencer® Select Pre-designed siRNA against the gene encoding Fas (cat. no. s1506) was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell culture and treatment. A549 cells were purchased from RIKEN BioResource Center (Tsukuba, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich; Merck KGaA) supplemented with 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and 10% heat-inactivated fetal bovine serum (Japan Bioserum Co., Ltd., Fukuyuma, Japan) at 37˚C in a humidified atmosphere of 5% CO_2.

Cells were seeded in 35-mm ($6x10^4$ cells) or 60-mm culture dishes ($1.2x10^5$ cells; IWAKI Glass Co. Ltd., Chiba, Japan) and cultured overnight to allow them to adhere to the dish. The subsequent day, cells were treated with 250 ng/ml RLR agonist Poly(I:C)-HMW for 72 h at 37˚C in a humidified atmosphere of 5% CO_2. The cultured cells were harvested using 0.1% trypsin-ethylenediaminetetraacetic acid (Gibco; Thermo Fisher Scientific, Inc.) for subsequent analyses.

In experiments investigating the involvement of each caspase in apoptosis induction, cells were pre-incubated with 50 μM Z-VAD-fmk (a pan-caspase inhibitor), 100 μM AC-IETD-CHO (a caspase-8 inhibitor) and 100 μM AC-LEHD-CHO (a caspase-9 inhibitor) for 1 h at 37˚C in a humidified atmosphere of 5% CO_2, prior to treatment with 250 ng/ml Poly(I:C)-HMW. As a vehicle control, cells treated with the same amount of DMSO (0.2%) were prepared.

siRNA transfection. A549 cells were transfected with siRNA targeting Fas (cat. no. s1506; Ambion; Thermo Fisher Scientific, Inc.) using Lipofectamine® RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The sense sequence for Fas was 5'-GGAAGA CUGUUACUACAGUTT-3'. Silencer® Select Negative No. 1 Control siRNA (cat. no. AM4611, Ambion; Thermo Fisher Scientific, Inc.) was used as a control (sequence not available). The final concentration of the siRNAs was 5 nM. Following incubation for 24 h, transfected cells were harvested and used for subsequent analyses.

In vitro irradiation. Cells were irradiated (150 kVp; 20 mA; 0.5-mm Al filter and 0.3-mm Cu filter) using an X-ray generator (MBR-1520R-3; Hitachi, Ltd., Tokyo, Japan) at a distance of 45 cm from focus and at a dose rate of 0.99-1.02 Gy/min, and the duration of exposure was ~4 min.

SDS-PAGE and western blotting. Harvested cells were lysed in 1XLaemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) containing 2.5% 2-mercaptoethanol and the resulting cell lysates were boiled for 10 min. Protein concentrations of the lysates were determined using the XL-Bradford assay kit (APRO Science Corporation, Tokushima, Japan) and a SmartSpec™ plus spectrophotometer (Bio-Rad Laboratories, Inc.). SDS-PAGE and western blotting were performed as previously described (15). Membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% non-fat skimmed milk for 1 h at room temperature. The following primary antibodies were used: Anti-XIAP (1:3,000), anti-caspase-3 (1:3,000), anti-caspase-8 (1:3,000), anti-caspase-9 (1:3,000) and anti-β-actin (1:4,000). The following secondary antibodies were used: HRP-conjugated anti-rabbit IgG (1:10,000) and HRP-conjugated anti-rabbit IgG (1:10,000). Antigens were visualized using the Enhanced Chemiluminescent (ECL) Prime Western Blotting Detection System (GE Healthcare Life Sciences, Little Chalfont, UK)

for the detection of caspase-3, caspase-8 and caspase-9, or Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Inc.) for the detection of XIAP and β-actin. Blots were stripped using a Stripping Solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Quantification of the bands was performed using ImageJ software ver.1.51K (National Institutes of Health, Bethesda, MD, USA) and the relative XIAP/β-actin ratio computed and presented.

Cell cycle analysis. Cell cycle analysis was performed, as previously described (16). Harvested cells were fixed overnight in ice-cold 70% ethanol at -20˚C. Fixed cells were washed with and subsequently suspended in PBS(-) and treated with 20 μg/ml RNase A for 30 min at 37˚C. Following treatment, cells were resuspended in PBS(-) containing 20 μg/ml PI and incubated in the dark for 30 min. Finally, cells were passed through a cell strainer (BD Falcon; BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using a flow cytometer (Cytomics FC500 with CXP software ver.2; Beckman-Coulter, Inc.).

Analysis of cell surface Fas expression. The analysis of cell surface Fas expression was performed as previously described (17). Harvested cells were washed once with PBS(-) and subsequently stained with FITC-conjugated anti-human CD95 (Fas) antibodies (1:10) or FITC-conjugated mouse IgG1 isotype control for 30 min at 4˚C in the dark. Following staining, cells were washed and analyzed using flow cytometry. Prior to analysis, 20 μg/ml PI was added to cell suspensions to discriminate dead cells from viable cells. Following gating PI negative cells, the fluorescence intensity of CD95 staining was analyzed.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from A549 cells using the RNeasy® Plus Mini kit (Qiagen, Inc., Valencia, CA, USA) and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). cDNA templates were synthesized from 1 μg RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc.), according to the manufacturer's protocol. The reaction conditions were 5 min at 25˚C, followed by 30 min at 42˚C and 5 min at 85˚C. RT-qPCR was performed, as previously described (18). Power SYBR®-Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and a Step One Plus™ system (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used with typical amplification parameters (95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min). Differences in gene expression relative to non-irradiated controls were determined using a standard curve based method (19). β-actin was used as a housekeeping gene. β-actin primer sequences were as previously described (20). Primers for XIAP were sense, 5'-TTTTGGGACATGGAT ATACTCAGGT-3' and antisense, 5'-TGAAAGCACTTTACT TTATCACCTTC-3'.

Statistical analysis. Data are presented as the mean ± standard error of the mean of at least three independent experiments. Comparisons between the control and experimental groups were performed using a two-sided Student's t-test or a two-sided Mann-Whitney's U-test depending on the data distribution. Multiple data were analyzed using one-way analysis of variance followed by the Tukey-Kramer test. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using Excel 2016 software (Microsoft Corporation, Redmond, WA, USA) and with Statcel 4 (OMS Publishing, Inc., Tokyo, Japan).

Results

Poly(I:C)-HMW induces apoptosis through caspase-8 and caspase-9 activation. The authors previously demonstrated that Poly(I:C)-HMW activates caspase-3 and that a pan-caspase inhibitor (Z-VAD-fmk) suppresses Poly(I:C)-HMW-induced cell death, including apoptosis in A549 cells (9). Although these results suggested that Poly(I:C)-HMW induces apoptosis in a caspase-dependent manner, the pathways involved in Poly(I:C)-HMW-induced apoptosis remained unclear and were investigated in the present study.

As demonstrated in Fig. 1A, Poly(I:C)-HMW activated caspase-8 and caspase-9 in addition to caspase-3. Whether caspase-8 and caspase-9 are involved in apoptosis induction by Poly(I:C)-HMW was examined using caspase inhibitors. The sub-G1 cell cycle peak, which contains cells with fragmented DNA and is a hallmark of apoptosis (21), was analyzed. As demonstrated in Fig. 1B, Poly(I:C)-HMW significantly increased the sub-G1 population compared with the DMSO control and treatment with Z-VAD-fmk significantly repressed this effect ($P<0.01$). Furthermore, caspase-8 inhibitor (IETD) and caspase-9 inhibitor (LEHD) significantly decreased Poly(I:C)-HMW-induced apoptosis ($P<0.05$; Fig. 1B). These results suggested that Poly(I:C)-HMW induces apoptosis in A549 cells through caspase-8 and caspase-9 activation. However, the inhibitory effect of the combination of caspase-8 inhibitor and caspase-9 on the Poly(I:C)-HMW-induced sub-G1 population was similar to that of each treatment alone (Fig. 1B).

Caspase-8 involvement in apoptosis enhancement by Poly(I:C)-HMW and IR cotreatment. The apoptotic pathway involved in the enhancement of apoptosis by cotreatment with Poly(I:C)-HMW and IR was investigated. As demonstrated in Fig. 2A, cotreatment with Poly(I:C)-HMW and 4 Gy X-ray irradiation markedly increased the sub-G1 population compared with treatment with Poly(I:C)-HMW or X-ray irradiation alone ($P<0.01$). Furthermore, the active caspase-8 and caspase-9 expression in cells treated with Poly(I:C)-HMW and IR increased compared with cells treated with Poly(I:C)-HMW alone (Fig. 2B). The net increases in the sub-G1 population following IR in the absence or presence of Poly(I:C)-HMW were ~2 and 20%, respectively (Fig. 2C). This elevated sub-G1 population in cotreated cells significantly decreased to ~4 and 12% in the presence of Z-VAD-fmk or the caspase-8 inhibitor IETD, respectively ($P<0.05$); however, there was no significant difference when treated with the caspase-9 inhibitor LEHD. Furthermore, the inhibitory effect of the combination of IETD and LEHD was similar to that of IETD alone (Fig. 2C). Taken together, these results suggested that caspase-8; however, not caspase-9, is involved in the enhancement of apoptosis by cotreatment with Poly(I:C)-HMW and IR.

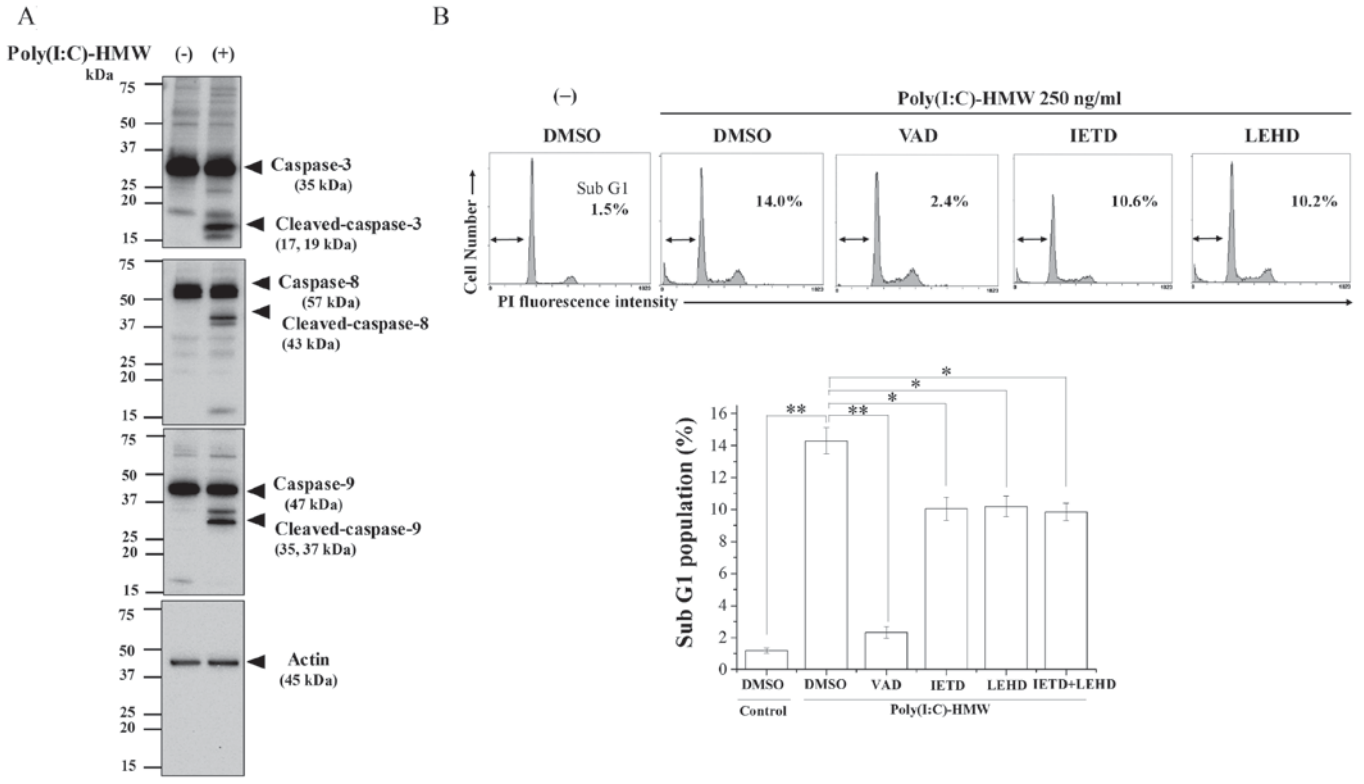

Figure 1. Involvement of caspase-8 and caspase-9 in Poly(I:C)-HMW-induced apoptosis. (A) A549 cells treated with Poly(I:C)-HMW for 72 h were harvested for western blot analyses of caspase-3, 8 and 9; β-actin was used as a loading control. Representative blots are presented. (B) A549 cells were pre-incubated with each caspase inhibitor for 1 h, followed by treatment with 250 ng/ml Poly(I:C)-HMW. Following culturing for 72 h, cells were harvested for cell cycle analysis. Upper panel, representative histograms and the data are presented. The double-headed arrows indicate the sub-G1 population and the inset numbers in the figure indicates the proportion of total cells in the sub-G1 population. Lower panel, sub-G1 populations are presented as the mean ± standard error of at least three independent experiments. *P<0.05 and **P<0.01. DMSO, dimethyl sulfoxide; VAD, Z-VAD-fmk; IETD, AC-IETD-CHO; LEHD, AC-LEHD-CHO; Poly(I:C)-HMW, Poly(I:C)-HMW/LyoVec™; PI, propidium iodide.

Effects of Poly(I:C)-HMW and IR cotreatment on XIAP expression. The expression of the apoptosis inhibitor protein XIAP was examined. As demonstrated in Fig. 3A, XIAP protein expression in cells treated with Poly(I:C)-HMW and/or IR 72 h following treatment was decreased compared with the control cells. Notably, XIAP protein expression levels were lowest in the cotreatment group. When the expression of XIAP mRNA was investigated, there was a significant difference in expression between the control group and the Poly(I:C)-HMW treatment and Poly(I:C)-HMW and IR cotreatment groups (Fig. 3B; P<0.05).

Involvement of Fas in apoptosis induction by Poly(I:C)-HMW and IR cotreatment. As caspase-8 is involved in the sub-G1 population increase following cotreatment with Poly(I:C)-HMW and IR, the involvement of death receptor Fas in cotreatment apoptosis induction was investigated. As demonstrated in Fig. 4A and B, Poly(I:C)-HMW and IR increased cell surface Fas expression. Furthermore, cell surface Fas expression in cells cotreated with Poly(I:C)-HMW and IR was significantly increased compared with Poly(I:C)-HMW or IR alone (P<0.05; Fig. 4B). Therefore, A549 cells were transfected with a Fas-targeting siRNA and apoptosis induction by Poly(I:C)-HMW and/or IR was analyzed in these cells. The knockdown of Fas decreased its cell surface expression (Fig. 4C); however, did not decrease apoptosis induction by Poly(I:C)-HMW and/or IR (Fig. 4D).

Discussion

The authors previously investigated the effect of IR on RLRs in human monocytic cells and demonstrated that it negligibly affected expression of RLRs and their response to their agonists (22), suggesting a potential for RLR agonists as effective immunostimulants during radiation therapy. Furthermore, the authors recently identified that RLR agonist Poly(I:C)-HMW exhibited cytotoxicity against human NSCLC cells and that its cytotoxicity was enhanced by cotreatment with IR (9). The authors additionally demonstrated that Poly(I:C)-HMW, or cotreatment with Poly(I:C)-HMW and IR, induced caspase-mediated apoptosis (9). In the present study, the involvement of caspase-8 and caspase-9 in apoptosis induction by Poly(I:C)-HMW or cotreatment with Poly(I:C)-HMW and IR was investigated. As a result, Poly(I:C)-HMW was demonstrated to induce apoptosis through caspase-8 and caspase-9. Furthermore, cotreatment with Poly(I:C)-HMW and IR was demonstrated to effectively activate caspase-8 and caspase-9 compared with each treatment alone, and that caspase-8 inhibitor IETD decreased the apoptosis increase induced by cotreatment. These results suggested that caspase-8; however, not caspase-9, mediates the enhancement of apoptosis induced by cotreatment with Poly(I:C)-HMW and IR.

It has been demonstrated that RLR activation may induce apoptosis via the intrinsic (caspase-9) and/or extrinsic (caspase-8) apoptotic pathways (4,5,23). For example,

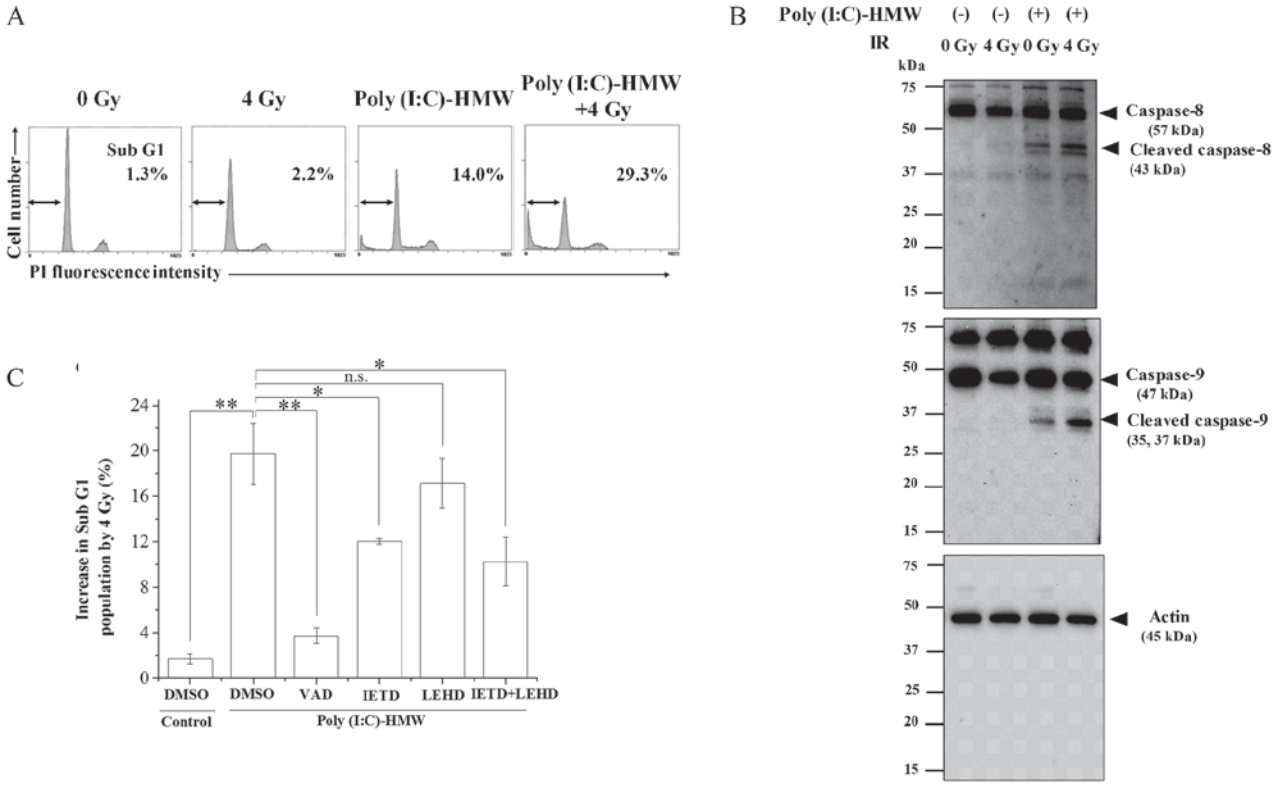

Figure 2. Involvement of caspase-8 and 9 in the enhancement of apoptosis by cotreatment with Poly(I:C)-HMW and IR. A549 cells were pre-incubated with 250 ng/ml Poly(I:C)-HMW. Following incubation for 1 h, the cells were irradiated with 4 Gy X-rays. Following culturing for 72 h, the cells were harvested for (A) cell cycle and (B) western blot analyses. Representative histograms of cell cycle analyses are presented. The double-headed arrows indicate the sub-G1 population and the inset numbers indicate the proportion of cells in the sub-G1 population. Representative blots of caspase-8 and caspase-9 are presented. β-actin was used as a loading control. (C) Cells were pre-incubated with each caspase inhibitor for 1 h, followed by treatment with 250 ng/ml Poly(I:C)-HMW. Following incubation for 1 h, the cells were irradiated with 4 Gy X-rays. Following culturing for 72 h, the cells were harvested for cell cycle analysis. Data of sub-G1 population are presented as the mean ± standard error of at least three independent experiments. *P<0.05 and **P<0.01. DMSO, dimethyl sulfoxide; VAD, Z-VAD-fmk; IETD, AC-IETD-CHO; LEHD, AC-LEHD-CHO; n.s., not significant; Poly(I:C)-HMW, Poly(I:C)-HMW/LyoVec™; PI, propidium iodide; IR, ionizing radiation.

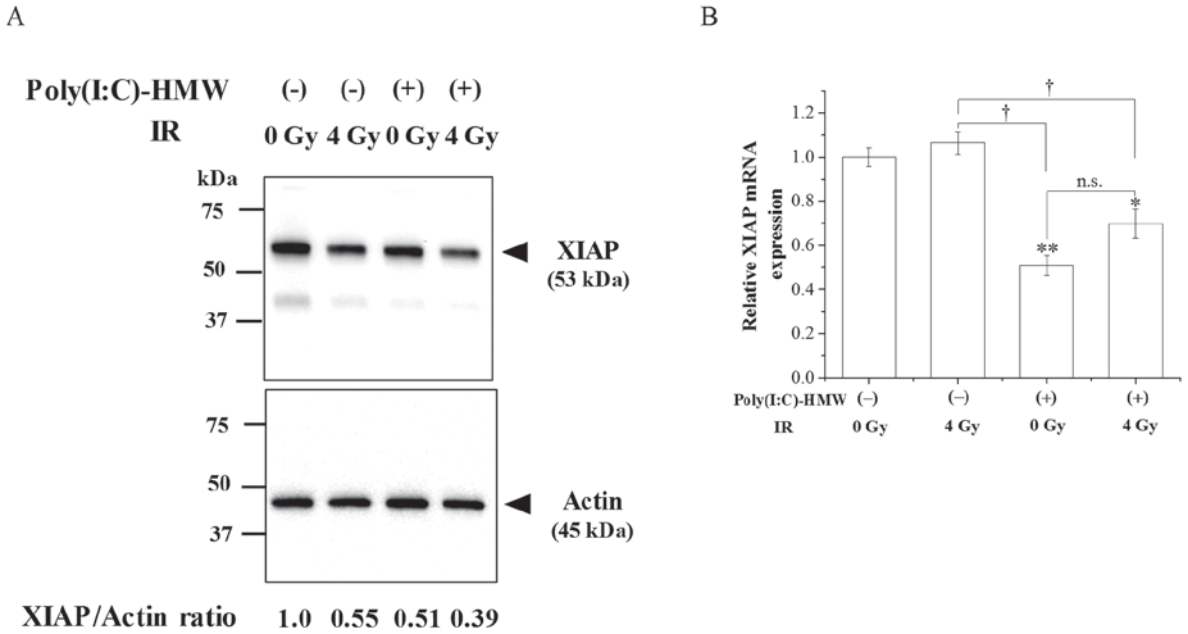

Figure 3. Effect of treatment with Poly(I:C)-HMW and/or IR on XIAP expression. A549 cells were pre-incubated with 250 ng/ml Poly(I:C)-HMW. Following incubation for 1 h, the cells were irradiated with 4 Gy X-rays. Following culturing for 72 h, the cells were harvested for (A) western blot analysis and (B) reverse transcription-quantitative polymerase chain reaction. Representative blots of XIAP are presented. β-actin was used as a loading control. The quantification of bands was performed using ImageJ software and the relative XIAP/actin ratio computed and presented. Data are presented as the mean ± standard error of three independent experiments. *P<0.05 and **P<0.01 vs. the control group; †P<0.01. n.s., not significant. Poly(I:C)-HMW, Poly(I:C)-HMW/LyoVec™; IR, ionizing radiation; XIAP, X-linked inhibitor of apoptosis protein.

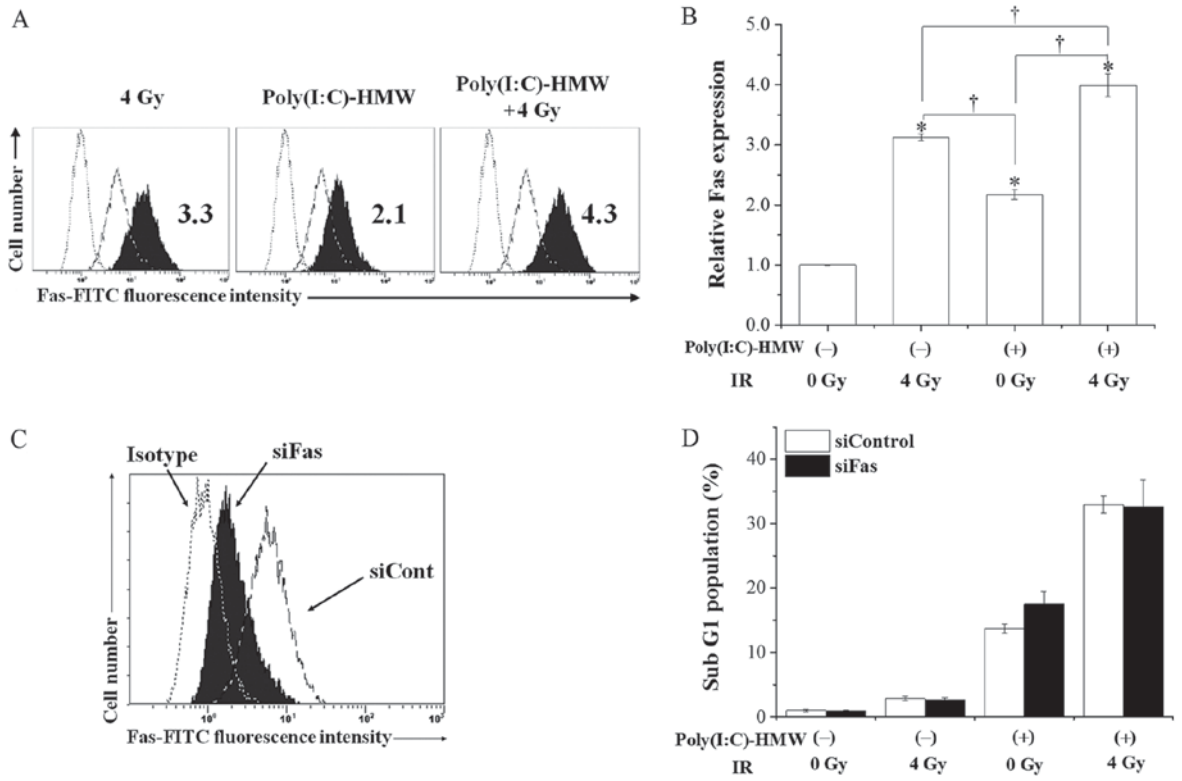

Figure 4. Involvement of Fas in apoptosis induction by Poly(I:C)-HMW and/or IR. A549 cells were pre-incubated with 250 ng/ml Poly(I:C)-HMW. Following incubation for 1 h, the cells were irradiated with 4 Gy X-rays. After culturing for 72 h, the cells were harvested for analysis of cell surface Fas expression. (A) Representative histograms are presented. The dotted line indicates the isotype control. The broken line and filled black histograms indicate the results from untreated cells and treated cells, respectively. The inset numbers indicate the relative values of median fluorescence intensity of Fas compared with non-treated control group. (B) Relative Fas expression in the control, Poly(I:C)-HMW, IR and cotreatment groups. Data are presented as the mean ± standard error of three independent experiments; $^{*}P<0.05$ vs. the control group. $^{\dagger}P<0.05$. (C) A549 cells treated with an siRNA against Fas were harvested for the analysis of cell surface Fas expression. The dotted line indicates the isotype control. The broken line and filled black histogram indicate the results of cells treated with control siRNA and Fas-targeting siRNA, respectively. (D) A549 cells treated with siRNA against Fas were incubated with 250 ng/ml Poly(I:C)-HMW, followed by 4 Gy X-ray irradiation. Following culturing for 72 h, the cells were harvested for cell cycle analysis. Data of sub-G1 population are presented as the mean ± standard error of four independent experiments. IR, ionizing radiation; Poly(I:C)-HMW, Poly(I:C)-HMW/LyoVec™; si, small interfering; Cont, control; FITC, fluorescein isothiocyanate; Fas, Fas cell surface death receptor.

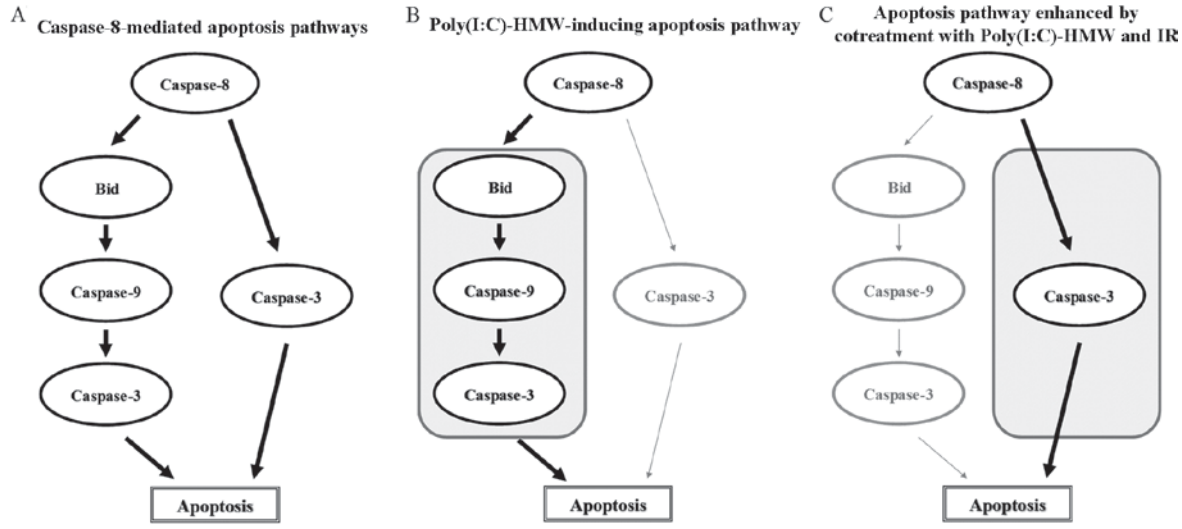

Figure 5. Apoptosis pathways activated by Poly(I:C)-HMW or cotreatment with Poly(I:C)-HMW and IR. (A) Caspase-8-mediated apoptosis pathways. (B) Poly(I:C)-HMW-inducing apoptotic pathway. (C) Apoptotic pathway enhanced by cotreatment with Poly(I:C)-HMW and IR. IR, ionizing radiation; Poly(I:C)-HMW, Poly(I:C)-HMW/LyoVec™; Bid, BH3-interacting domain death agonist.

Besch *et al* (5) demonstrated that the RIG-I agonist 5'-triphosphate RNA activates caspase-8 and caspase-9 in human melanoma cells; however, caspase-9, not caspase-8, serves an important role in 5'-triphosphate RNA-induced apoptosis.

However, El Maadidi *et al* (23) observed that the activation of melanoma differentiation-associated gene 5 by Semliki Forest Virus (SFV) induces caspase-9 and caspase-8-mediated apoptosis. These previous studies suggested that the apoptotic pathway induced by RLR activation depends on the type of cell or on the RLR agonists.

In the present study, it was demonstrated that Poly(I:C)-HMW induced apoptosis in A549 cells through caspase-8 and caspae-9 activation. It is known that caspase-8 may activate the mitochondrial-mediated apoptotic pathway by regulating mitochondrial outer membrane permeabilization (MOMP) (24). The activated caspase-8 cleaves a pro-apoptotic Bcl-2 family member, BH3-interacting domain death agonist (Bid), which subsequently induces MOMP and results in caspase-9 activation followed by cytochrome c release (Fig. 5A) (24). When the combination of caspase-8 inhibitor and caspase-9 inhibitor was examined, the inhibitory effect of it on the Poly(I:C)-HMW-induced sub-G1 population was similar to that of caspase-8 or caspase-9 inhibitor alone. Therefore, it is thought that Poly(I:C)-HMW induces apoptosis thorough caspase-8-mediated caspase-9 activation; however, the present study did not investigate whether Bid is involved in caspase-8-mediated apoptosis pathways (Fig. 5B). Notably, it was demonstrated that caspase-8 mediates the apoptosis enhancement from cotreatment with Poly(I:C)-HMW and IR, whereas caspase-9 does not, even though cotreatment with Poly(I:C)-HMW and IR effectively activated caspase-8 and caspase-9 compared with Poly(I:C)-HMW alone. Similar to the results of the present study, Liu *et al* (25) identified that the combination of measles virus virotherapy and radiation therapy effectively induced cleavage of poly(ADP-ribose) polymerase (PARP), a substrate of capsase-3, in glioma cells, and that the increase in PARP cleavage by the combination of them was inhibited by a pan-caspase inhibitor and a caspase-8 inhibitor; however, not a caspase-9 inhibitor. As caspase-3 may process pro-caspase-9 (26), it is possible that caspase-9 was cleaved following the caspase-8/caspase-3 apoptotic pathway. El Maadidi *et al* (23) demonstrated that the apoptotic pathway induced by SFV is independent of the MOMP induced by the pro-apoptotic Bcl-2 family member protein apoptosis regulator BAX/Bcl-2 homologous antagonist/killer and involves an SFV-induced recruitment of caspase-8 to MAVS on the mitochondrial membrane where caspase-8 is activated and required to cleave and activate caspase-3. Furthermore, considering that the inhibitory effects of a combination of caspase-8 inhibitor and caspase-9 inhibitor on the increase in sub-G1 population by IR in the presence of Poly(I:C)-HMW was similar to that of caspase-8 alone, it is likely that the caspase-9-independent (or MOMP-independent) and caspase-8-mediated apoptotic pathway is involved in the enhancement of apoptosis by cotreatment with Poly(I:C)-HMW and IR (Fig. 5C).

In the present study, treatment with a pan-caspase inhibitor, Z-VAD-fmk, inhibited apoptosis induction by Poly(I:C)-HMW or by cotreatment with Poly(I:C)-HMW and IR. In contrast, a caspase-8 inhibitor only partially decreased their effects. These results suggested that other factors, other than caspase-8, are involved in apoptosis induction by Poly(I:C)-HMW or by cotreatment with Poly(I:C)-HMW and IR. It was demonstrated that the inhibitor of apoptosis protein, XIAP, was downregulated in cells treated with Poly(I:C)-HMW and/or IR. Nakhaei *et al* (27) observed that the downregulation of XIAP protein expression sensitizes cells to virus-induced apoptosis. Furthermore, it has been demonstrated that the suppression of XIAP overexpression enhances the radiation sensitivity of human lung cancer (28), suggesting an association between the radiation sensitivity of malignant tumors and XIAP expression. As XIAP is known to be a potent inhibitor of caspases 3, 7 and 9 (29), it is likely that the downregulation of XIAP by cotreatment with Poly(I:C)-HMW and IR enhanced caspase-mediated apoptosis. There is a possibility that other apoptotic mechanisms, including the endoplasmic reticulum-mediated apoptotic pathway, are involved in apoptosis induction by Poly(I:C)-HMW or cotreatment with Poly(I:C)-HMW and IR. Further studies are required to investigate other apoptotic mechanisms.

Downregulation of XIAP protein expression was observed in the cells treated with Poly(I:C)-HMW and/or IR. Notably, Poly(I:C)-HMW alone and cotreatment decreased XIAP mRNA expression, whereas IR alone did not. These results suggested that Poly(I:C)-HMW downregulated XIAP protein expression by decreasing the transcription of XIAP mRNA; whereas, X-ray irradiation affects XIAP expression through a transcription-independent manner. It is known that XIAP protein expression is tightly regulated not only at the transcriptional level; however, additionally post-transcriptionally (30,31). Yang *et al* (32) demonstrated that DNA damaging agents, including etoposide, may degrade XIAP through the ubiquitin-proteasome pathway. Therefore, it is possible that IR decreases XIAP protein expression through proteasome-mediated degradation. The proteasomal degradation of XIAP may additionally be involved in the downregulation of XIAP protein by Poly(I:C)-HMW, as it was identified that Sendai virus infection induces the proteasomal degradation of XIAP by phosphorylating XIAP at Ser430 through Iκ B kinase ε- or Tank-binding kinase 1, which function downstream of MAVS (27). Further studies are required to elucidate the mechanisms by which Poly(I:C)-HMW and/or IR regulate XIAP protein expression.

As IR increases Fas expression, which causes caspase-8-mediated apoptosis (13,33), Fas was predicted to be involved in apoptosis induction by cotreatment with Poly(I:C)-HMW and IR. However, although the upregulation of cell surface Fas expression in cotreated cells was observed (Fig. 4A and B), Fas knockdown did not decrease apoptosis induction (Fig. 4C). Therefore, it is unlikely that Fas upregulation is associated with apoptosis enhancement by cotreatment. El Maadidi *et al* (23) observed that the RLR adaptor protein MAVS directly associates with caspase-8 and subsequently activates caspase-3, leading to apoptosis. Therefore, it is possible that MAVS-mediated caspase-8 activation is involved in the apoptosis enhancement observed upon cotreatment with Poly(I:C)-HMW and IR. As the expression of other death receptors, including death receptor 5, is known to be enhanced by X-ray irradiation (14,34), there is a possibility that other death receptors are involved in the cotreatment-induced apoptosis enhancement of Poly(I:C)-HMW and IR. In a future study, the authors aim to investigate the mechanisms by which cotreatment with Poly(I:C)-HMW and IR effectively activate caspase-8.

In conclusion, in the present study it was demonstrated that caspase-8 is involved in the enhancement of apoptosis by

cotreatment with Poly(I:C)-HMW and IR. Furthermore, it was demonstrated that cotreatment effectively activates caspase-8 independently of the upregulation of cell surface Fas expression. As it has been demonstrated that RLR agonists increase Fas expression in pancreatic cells and that cells treated with RLR agonists become sensitive to Fas-mediated cell killing effects (35), it is hypothesized that combinations of RLR agonists, X-ray irradiation and Fas agonists may be effective in cancer treatments. This hypothesis requires further verification in a future study.

Acknowledgements

Not applicable.

Funding

The present study was supported by JSPS KAKENHI (grant no. JP25861053) and was partially supported by JSPS KAKENHI (grant no. JP15K09985) and the Takeda Science Foundation (for HY; Osaka, Japan).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HY and IK contributed to the conception of the study. HY, YS and YK performed experiments, collected data, and analyzed data. YS, HY and IK wrote, reviewed and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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