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RESEARCH ARTICLE

Stimulation of the endosomal TLR pathway enhances autophagy-induced cell death in radiotherapy of breast cancer

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Abstract

Toll-like receptors (TLRs), which are mainly expressed in antigen presenting cells, perform a critical role in innate immunity by recognizing the specific structural patterns of pathogens and transducing signals to induce an inflammatory reaction. Although it has been reported that various solid cancers express endosomal TLRs, TLR3, 7, 8, and 9, the cellular and molecular function of TLRs in tumorigenesis has not yet been elucidated. In this report, we identified the expression of TLR3 and TLR7 in the human breast cancer cell line MCF-7 and found that TLRs stimulated with their specific ligand induced an anti-tumoral effect in this cell line. Among four synthetic commercial agonists of TLR3 and 7, Poly(I:C) and imiquimod (IMQ) proved to have superior anti-tumoral activity over the other agonists. A decreased growth rate was observed in MCF-7 cells treated with either TLR agonist. The decreased growth rate was due to autophagy and autophagy-induced cell death because treatment with 3-methyladenine, inhibitor of autophagy rescued the growth rate and increased the expression levels of autophagy-related genes. Moreover, survival of MCF-7 cells significantly decreased when the cells were stimulated simultaneously with TLR agonists and radiation exposure. Therefore, this study can be applied to developing a therapeutic adjuvant of TLR agonists in radiotherapy for radio-resistant breast cancer treatment.

Keywords Toll-like receptor (TLR); Poly(I:C); Imiquimod; Autophagy; MCF-7; Radiotherapy

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Introduction

TLRs are known to recognize pathogen associated molecular patterns (PAMPs) in innate immunity and induce the adaptive immune reaction (Akira, 2006; Medzhitov and Janeway, 2000a; Medzhitov and Janeway, 2000b). TLR ligands have been considered as potential targets for the development of therapeutic drugs for infectious diseases, autoimmune diseases, and cancers (Suzuki et al., 2002; Tse and Horner, 2007). Although the anti-tumoral activities of TLR ligands have been previously demonstrated in various cancer cell lines and animal models, the main focus of previous studies was the anti-cancer immunity can be enhanced by Th1-mediated immunity via activation of dendritic cells (Schwartz et al., 2009; Smits et al., 2008; Vidal et al., 2004; Watts et al. 2010). Recently, it has been reported that Poly(I:C), a TLR3 ligand, and imiquimod (IMQ), a TLR7 agonist, have direct anti-tumoral activity, which is mediated by autophagy in melanoma and colon cancer (Mathew et al., 2007; Tormo et al., 2009; Yi et al., 2009). It has not been fully elucidated whether other TLR ligands have the same or a similar effect as Poly(I:C) or IMQ or whether these two TLR agonists induce autophagy-related cell death in other cancers.

Programmed cell death is an important process targeted by cancer therapies (Cormary et al., 2005; Reed, 2008). Apoptosis, the most thoroughly studied form of programmed cell death, is defined by the dependence on caspases (Gyrd-Hansen and Meier, 2010). The other programmed cell death mechanism that is clearly distinct from apoptosis is autophagy (Djavaheri-Mergny et al. 2010). Autophagy is one of the main defense mechanisms in phagocytic cells to eliminate pathogens by which autophagic vesicles fuse with lysosomes and degrade foreign organisms. Autophagy is not only involved in cleaning pathogens as a defense mechanism in the immune system but also in maintaining homeostasis by which damaged proteins or organelles are cleared from the cytosol.

During autophagy, cells exhibit endomembrane remodeling, engulfing a portion of the cytoplasm in double-membrane vesicles to form autophagosomes. The autophagosomes fuse with lysosomes, and the contents of the fusion vacuoles are eventually degraded finally (Xie and Klionsky, 2007). A group of *atg* genes, which are conserved from yeast to humans, has been shown to be associated with autophagy (Hanada et al., 2007).

In this study, agonists of TLR3 and TLR7 were tested to evaluate synergistic anti-cancer effects in the radiotherapy of breast cancer. Treatment of the human breast cancer cell line MCF-7 with Poly(I:C) and IMQ induced the cell death via autophagy, which was confirmed by increased expression levels of autophagy-related genes and the number of autophagosomes. In addition, Poly(I:C) and IMQ enhanced the radiosensitivity of MCF-7cells, which revealed reduced survival under γ-irradiation exposure. Therefore, TLR agonists could be developed into therapeutic drugs as synergistic adjuvants in cancer radiotherapy.

Materials and methods

Cell lines and reagents

The human breast cancer cell line MCF-7 was originally purchased from the American Type Culture Collection (Manassas, USA) and maintained in RPMI medium containing 10% fetal bovine serum (FBS; Lonza, Walkersville, USA), 10,000 U/L of penicillin, and 100 mg/ℓ of streptomycin (Gibco-BRL, Gaithersburg, USA). Polyinosinic : polycytidylic acid (Poly(I:C), 10 μg/ml) (TLR3 agonist), lipopolysaccharide (LPS, 1 μg/mℓ) (TLR4 agonist), gardiquimod (GDQ, 2.4 μg/mℓ) (TLR7 agonist), imiquimod (IMQ, 5 μg/mℓ) (TLR7 agonist), and loxoribine (LOX, 1 mM) (TLR7 agonist) were purchased from InvivoGen (San Diego, USA). 3-Methyladenine (3-MA, 10mM), and rapamycin (100 nM) were purchased from Sigma-Aldrich (St. Louis, USA). A pan-caspase inhibitor (Z-VAD-FMK, 10 μM) was purchased from Promega (Madison, USA).

RNA isolation and RT-PCR

Total cellular RNA was extracted using the RNeasy Mini column (Qiagen, Austin, USA) according to the manufacturer's instruction. The cDNA was reverse transcribed from 2 μg of total RNA with oligo dT using the M-MLV Reverse Transcriptase (Takara, Otsu, Japan) as recommended by the supplier. cDNA was subjected to PCR to amplify and was subsequently resolved on a 1.2% agarose gel to confirm the size of each amplicon. The primer sequences for amplifying TLR3 are 5'-CGCCAACTTCACAAGGTA-3', and 5'-GGA-AGCCAAGCAAAGGAA-3' TLR7 5'-AGTGTCTAAAGAA-CCTGG-3', and 5'-CCTGGCCTTACAGAAATG-3'. The primers for amplifying β-actin gene are 5'-GTG GGG CGC CCC AGG CAC CAG GGC-3', and 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'.

Cell viability test

Cell viability was assessed by cell counting and a colorimetric assay. Cell counting was conducted in 6-well plates after seeding 1 X 10^5 cells per well. At each indicated time point, cells were harvested, viable cells were counted, and dead cells were excluded by trypan blue staining. The results are expressed as the mean ± SE of triplicate measurements. The colorimetric assay was conducted with a cell viability and cytotoxicity assay kit, WST-1 (Takara, Otsu, Japan). Briefly, MCF-7 cells were seeded at 5 X 10^3 cells per well in 96-well plates and incubated for 2 days. After treatment with 3-MA or a pan-caspase inhibitor for 2 h, IMQ or Poly(I:C) was added to the media. At each indicated time point, 10 μℓ of the color reagent was added to each well, and incubated for another 4 hr. After the incubation, the mean absorbance at 450 nm in each set of samples was measured with an ELISA reader (Biotec, Winooski, USA)

Clonogenic assay

To determine MCF-7 cell survival after exposure to γ-irradiation, 200 to 1 X 10^3 cells were seeded in 35-mm dishes and exposed to 2 or 5 Gy of irradiation in the presence or absence of TLR agonists. After 14 days of incubation, the colonies were stained with trypan blue (0.4%) and counted under microscope.

Western blotting analysis

Total cell lysates were prepared for western blotting analysis. The cell lysates were extracted using a lysis buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.1% deoxycholate, 1% Triton X-100, 10 mM Tris–HCl (pH 8.0), 140 mM NaCl, 1 mM EDTA, and 1X protease inhibitor cocktail and boiled at 95 ℃ for 5 min. The protein concentration of each sample was determined by Bradford assay (Bio-Rad, Hercules, USA). Equal amounts of protein (30 μg) were analyzed by SDS–polyacrylamide gel electrophoresis. After transfer the proteins to a PVDF membrane (Promega, Madison, USA), protein expression levels were detected with specific antibodies to LC3, Beclin-1, Atg5-12 (Cell Signaling Technology, Danvers, USA),

puma, caspase3, and β-actin (Santa Cruz Biotechnology, Delaware Avenue, USA).

Immunofluoscence staining

For immunostaining, MCF-7 cells were seeded on 12-well culture dishes that contained 18-mm diameter round glass coverslips. The cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min, and permeabilized with 0.2% Triton X-100 in PBS for 10 min. The cultures were then stained with primary antibodies, including mouse or rabbit anti-human LC3 (MBL, Nagoya, Japan) for 2 hr and washed with PBS three times. After washing to remove excess primary antibodies, the cultures were incubated for 1 hr at RT with a FITC anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, USA) and washed with PBS three times. MCF-7 cells were incubated with 4'-6-diamidino-2-phenyl indole (DAPI, Sigma-Aldrich, St. Louis, USA) for the indicated time periods and washed five times with PBS before fixation for immunostaining.

Results

Anti-tumoral activity of TLR agonists in MCF-7 cells

We determined the expression level of TLR3 and TLR7 in the breast cancer cell line MCF-7 using RT-PCR. In MCF-7 cells, TLR3 and TLR7 were detected at sizes 688 bp and 544 bp, respectively (Fig. 1A). To examine if each TLR was stimulated with its specific ligand to induce cell death in MCF-7 cells, the cells were treated with commercial TLR agonists and the growth rate was observed. TLR agonists, TLR3 agonist Poly(I:C) and TLR7 agonists GDQ, LOX, and IMQ were added to MCF-7 cells, and the number of viable cells were counted under a microscope at the indicated time points (Fig. 1B and C). A growth suppressive effect was detected in all agonists-treated groups. IMQ seemed to have the strongest anti-tumoral activity among the three TLR7 agonists. Poly(I:C) also had some anti-tumoral activity, but the suppressive effect of Poly(I:C) was weaker than IMQ (Fig. 1C). After 4 days of treatment with the TLR agonists, the fraction of cells that survived was less than 30% in the IMQ-treated group, and less than 55% in the Poly(I:C)-treated group compared with the control group. These results indicate that TLR3 and TLR7 agonists exert direct anti-tumoral effects in MCF-7 cells through the activation of endosomal TLR pathways.

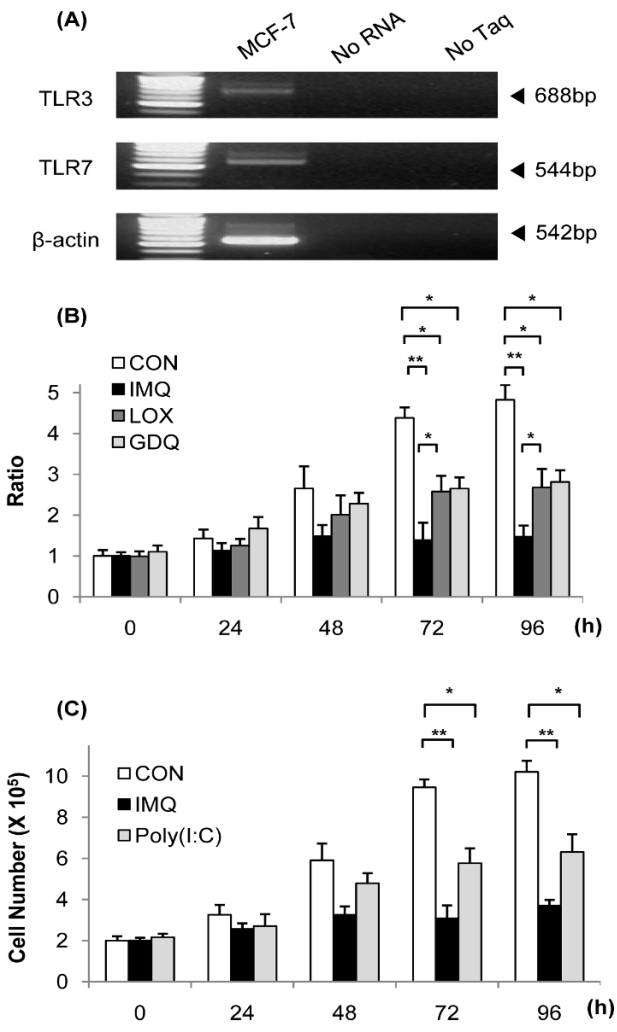

Figure 1. Detection of TLR3 and TLR7 expression and the growth inhibitory effect of TLR agonists in the MCF-7 human breast cancer cell line. (A) RT-PCR was performed to detect the expression levels of TLR3 and TLR7 in the MCF-7 breast cancer cell line. Total RNA was extracted, converted to cDNA, and then amplified with a pair of sequence specific primers. TLR3 and TLR7 transcripts were amplified at a size of 688 bp and 544 bp, respectively. β-actin was also amplified as an internal standard control. No RNA indicates a negative control without an RNA template in the reverse transcription reaction step, and No Taq is an amplified sample without Taq polymerase. (B) The decreased growth rate of MCF-7 cells was measured in the TLR7 agonist- treated groups. The concentration of each agonist was described in the material and method section. □ CON, control; ■ IMQ, imiquimod; ■ LOX, loxoribin; ■ GDQ, gardiquimod. (C) The TLR3 agonist Poly(I:C) suppressed the growth rate of MCF-7 cells. Compared with the IMQ-treated group, Poly(I:C) seemed to have less suppressive activity than IMQ in MCF-7 cells. Data are presented as the mean ± standard deviation of the results from triplicate wells per time point. Triplicate experiments yielded similar results. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; compared with control cells.

Poly(I:C) and IMQ activate autophagy-induced cell death in MCF-7 cells

Programmed cell death is a critical molecular event in the process of cancer treatment, and the mechanisms relevant to TLR-mediated anti-tumoral activity were examined. Cells were pretreated with well-known inhibitors of programmed cell death and then stimulated with Poly(I:C) or IMQ. Because the TLR7 agonists LOX and GDQ were less effective than IMQ (Fig. 1B), they were excluded in the following experiments. Pretreatment with a pancaspase inhibitor (Z-VAD-FMK) or 3-MA restored cell survival after treatment with Poly(I:C) or IMQ (Fig. 2A). 3-MA treatment rescued 42% of the cells from cell death in the IMQ-treated MCF-7 cells, and Z-VAD-FMK rescued 22% of the cells. Poly(I:C) treatment also had a similar result in this experiment. Rapamycin, which is a known autophagy-stimulating drug, was introduced as a positive control of autophagy-induced cell death. The percentage of viable cells in the IMQ- or rapamycin-treated group presented similar values (Fig. 2A). Cell death also seemed to be due to apoptosis, but the main death mechanism was autophagy. Therefore, it was confirmed that Poly(I:C)- and IMQ-treatment killed cells by autophagy rather than apoptosis.

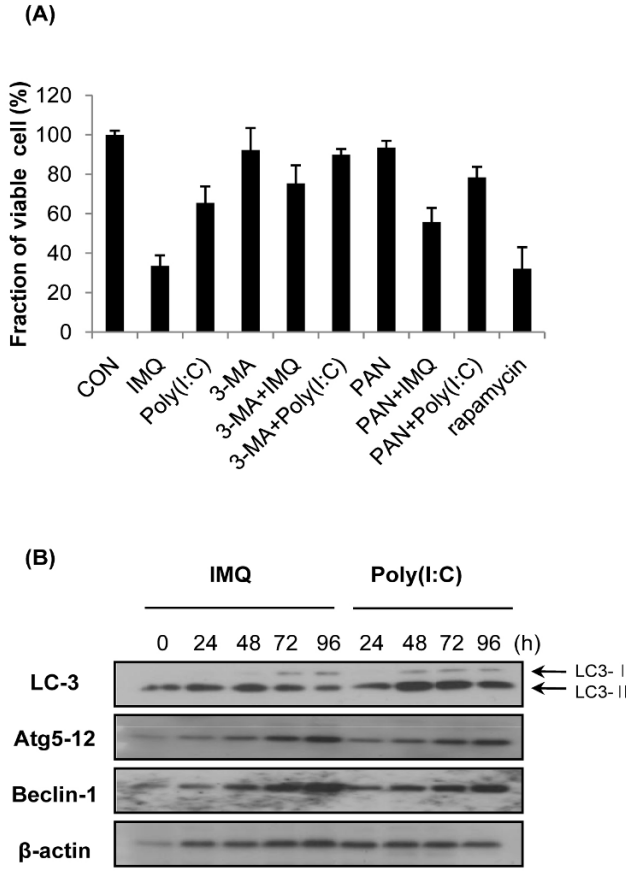

Figure 2. Inhibition of autophagy rescues cell survival in MCF-7 cells. (A) Treatment of MCF-7 cells with IMQ or Poly(I:C) decreased the fraction of viable cells, and inhibition of autophagy with 3-MA rescued the number of surviving cells to 80% of the control (CON) group. Inhibiting apoptosis with a pancaspase inhibitor (PAN) did not significantly affect cell survival. The autophagy activator rapamycin was introduced as a positive control. Data are presented as the mean ± standard deviation of the results from three independent experiments. (B) The changes in expression levels of autophagy-related genes were assessed with western blot analysis in IMQ- or Poly(I:C)-treated MCF-7 cells. Compared to the control (0 h), the mobilization shift of LC3-I to LC3-II was detected after 48 to 72 h of incubation in IMQ- or Poly(I:C)-treated cells, respectively. Moreover, the expression level of the Atg5-12 complex and beclin-1 was significantly increased at 48 h after incubation in both IMQ- and Poly(I:C)-treated cells. β-actin was utilized as a loading control.

To assess Poly(I:C)- or IMQ-induced autophagy, autophagy-related gene expression was analyzed by western blotting after treatment with each agonist in MCF-7 cells. Because the mobilization of microtubule-associated protein 1 light chain (LC3) to LC3-II is one of the critical molecular events in autophagy, LC3-II expression was measured in MCF-7 cells after incubation with Poly(I:C) or IMQ. During autophagy, LC3 is processed to soluble LC3-I and, in turn, is switched over to membrane-bound LC3-II (Kabeya et al., 2000; Mizushima and Yoshimori, 2007). The mobilization shift of LC3-I to LC3-II was elevated in both Poly(I:C)- and IMQ-treated cells beginning after 48 h of incubation with each agonist. Moreover, a significantly increased expression level of the Atg5-12 complex and beclin-1, which started after 48 h of incubation further indicated TLR agonist-induced autophagy in MCF-7 cells (Fig. 2B).

Poly(I:C) and IMQ enhance cell death induced by γ-irradiation exposure.

Because MCF-7 cells are known to be resistant to radiation-induced cell death (Wang et al., 2005), we tested whether TLR agonists could enhance the radiosensitivity of that cell line. The induction of cell death by 2 Gy of γ-irradiation, a relatively low dose of γ-irradiation, and by 5 Gy, a relatively high dose, in TLR agonist-treated MCF-7 cells was estimated by a clonogenic assay. Although there were significant differences in the survival fraction of TLR agonist-treated MCF-7 cells compared with the control cells without γ-irradiation exposure, the cell death rate was accelerated when cells were pretreated with each agonist for 3 h and then exposed to low dose of γ-irradiation (Fig. 3). Simultaneous stimulation of IMQ and 2 Gy of γ-irradiation reduced the survival fraction of MCF-7 cells to 53% of the cells treated with IMQ alone, or 35% of the cells that were exposed to 2 Gy of radiation alone.

Poly(I:C) treatment showed a similar result. The surviving fraction in the cells exposed to the 5 Gy of γ-irradiation was too low to discriminate influences of TLR agonists and γ -irradiation stimuli on cell death. Therefore, it is speculated that treatment with TLR agonists makes MCF-7 cells more sensitive to γ-irradiation exposure, and cell death was accelerated under this stimuli (Wang et al., 2009).

TLR agonists trigger the formation of autophagosomes, and when combined with γ-irradiation, accelerate autophagosome formation in MCF-7 cells

The above results showed that combining a TLR agonist with γ-irradiation exposure stimulated the cell death machinery, which decreased cancer cell survival. Though treatment with TLR agonists provoked MCF-7 cells autophagy-induced death, it was not clear that simultaneous stimulation with a TLR agonist and γ-irradiation exposure accelerated autophagy.

Here, we examined whether γ-irradiation and pretreatment with TLR agonists induced an acceleration in the formation of autophagosomes in MCF-7 cells. Because LC3 is typically associated with autophagosomes (Kabeya et al., 2000), the number of endogenous LC3-positive vesicles was monitored in MCF-7 cells pretreated with Poly(I:C) or IMQ. As shown in Figure 4A, LC3 aggregates were formed in MCF-7 cells when they were treated with either Poly(I:C) or IMQ, or exposed to γ-irradiation under 2 Gy. The number of autophagosomes increased when cells were pretreated with TLR agonists for 3 h and then exposed to 2 Gy of γ-irradiation. Although a low dose of radiation induced over 44% of the MCF-7 cells to make autophagosomes, pretreatment with IMQ accelerated the number of LC3 positive cells to approximately 83%. Poly(I:C) treatment also increased the percentage of LC3-positive cells from 48% to 68% after γ-radiation exposure. Therefore, IMQ may be one of the most potent inducers of autophagy and autophagy-induced cell death in MCF-7 cells combined with cancer radiotherapy.

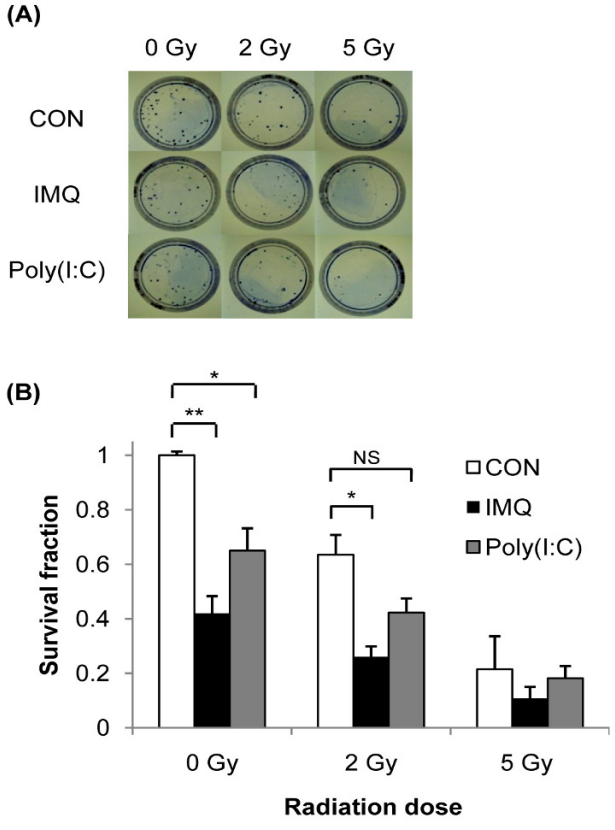

Figure 3. The synergistic cell-death effect of TLR agonists with γ -irradiation exposure in MCF-7 cells. (A) A clonogenic assay was performed to confirm the synergistic cell-death effect of Poly(I:C) or IMQ combined with radiation. Cells were treated with each agonist and then exposed to 2 or 5 Gy of γ-irradiation. Representative colony forming assay images of five independent replicates are shown. CON: control. (B) Stimulation of TLR agonists accelerated radiation-induced cell death after 2 a low (2 Gy) and a high (5 Gy) dose of γ-irradiation. Quantitative analysis of the ratio of colony number is shown. Combination of a TLR agonist and 2 Gy of γ-irradiation reduced the survival fraction of MCF-7 cells. Triplicate experiments yielded similar results. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; compared with control cells. NS: no significance

Discussion

Cancer immunotherapy has developed with the knowledge that it can have several benefits. However, despite decades of research and clinical trials, immunotherapy has been shown to be less effective than conventional cancer therapy (Flavell et al. 2010). Recent reports recognize the importance of tumor immune evasion mechanisms (Elpek et al., 2007), which resulted in the clinical use of anti-CTLA4 (Egen et al., 2002) and anti-PD1 antibodies (Dotti, 2009), which indicates the importance of costimulatory signals and innate immune activation, such as TLRs (Cormary et al., 2005; Dotti, 2009; Egen et al., 2002; Thompson et al., 2006). Defining of the role played by immune adjuvants and the influence of booster doses has represented important additions to the development of anti-tumor immunology (Celis, 2007; Melief et al., 2002; Peng et al., 2006). Many researchers would expect to eradicate cancer with a combination therapy in which cancer patients are treated with one of conventional therapy, surgical operation, chemotherapy, or radiotherapy, before a novel therapeutic approach (Zois and Koukourakis, 2009)(Roses et al., 2008). In our report, MCF-7 cells, a relatively chemo-resistant and radio-resistant breast cancer cell lines (Kumar et al., 2007; Mougel et al., 2004), was provoked into autophagy-induced

cell death by TLR agonist treatment, which indicates that the addition of TLR agonists could be another strategy to treat apoptosis-resistant cancer cells.

Although many solid tumors express endosomal TLRs, the molecular and cellular function of tumor-expressing TLRs in tumorigenesis and metastasis is not clear. Recently, several studies have been published that show tumor-expressing TLR stimulation could be a realistic therapeutic target for cancer immunotherapy in melanoma, glioma, and ovarian cancer (Huang et al., 2008; Tormo et al., 2009). Among synthetic TLR agonists, the CpG motif for TLR9 is the most popular because it is easily designed, has a low burden of synthesization, and strong immune modulatory benefits (Choi et al., 2010; Krieg, 2007). Another TLR agonist has been tested for therapeutic application in cancer treatment. In this study, we evaluated endosomal TLR agonists, Poly(I:C) for TLR3 and IMQ, LOX, and GDQ for TLR7, for induction of induced autophagy and autophagy-induced cell death in the MCF-7 human breast cancer cell line. Two agonists, Poly(I:C) and IMQ accelerated autophagy-induced cell death under low-dose γ -irradiation exposure. We first determined that autophagy was involved in Poly(I:C)- and IMQ-induced cell death. Apoptosis also seemed to be induced by TLR agonist treatment, but inhibition of apoptosis did not rescue the same level of cell survival of the autophagy-inhibiting groups. IMQ treatment induced autophagy, and cell death by Poly(I:C) treatment was likely balanced between apoptosis and autophagy. Agonist treatment of MCF-7 cells generated increased expression levels of autophagy-related genes, LC-3, Atg5-12 complexes, and beclin-1, which is an essential molecular event in autophagosome formation in the cytosol of MCF-7 cells (Fig. 3A). Moreover, pretreatment with TLR agonists enhanced the radio-

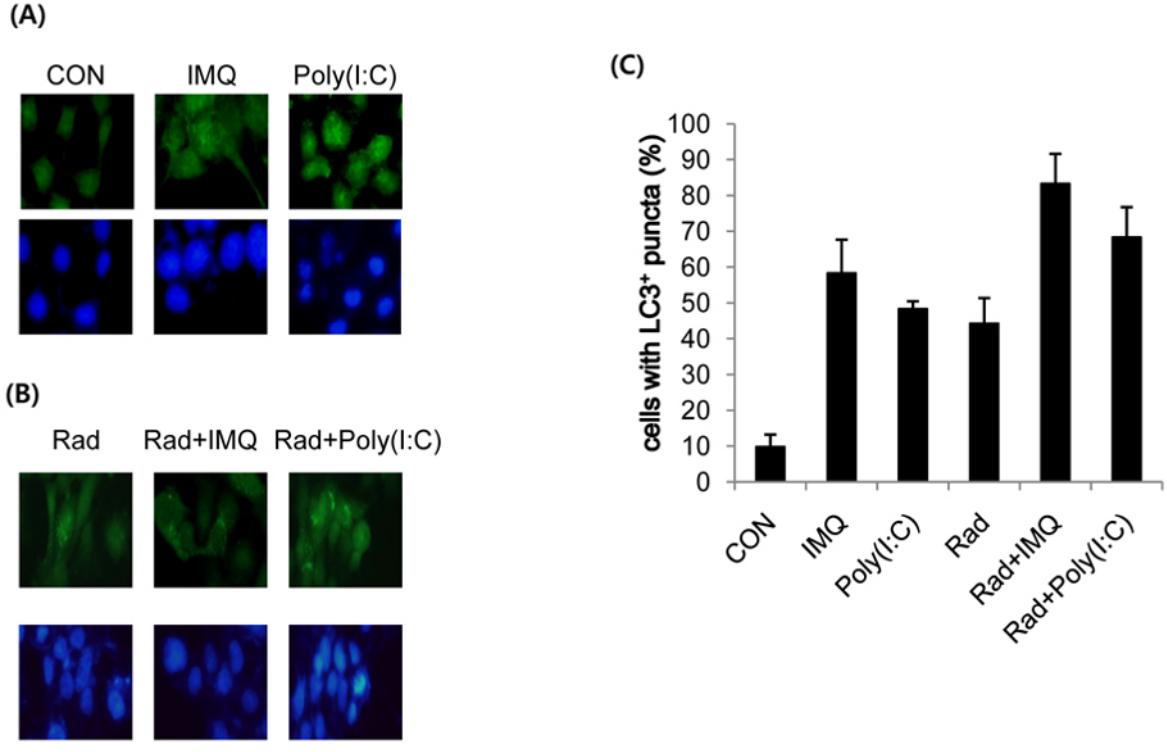

Figure 4. Treatment of MCF-7 cells with IMQ or Poly(I:C) triggers and accelerates the formation of autophagosomes in MCF-7 cells under γ-irradiation exposure. (A) MCF-7 cells were incubated with or without IMQ, or Poly(I:C) for 72 h, and then endogenous LC3 was detected with using an immunofluorescence assay. Cells were fixed, stained with DAPI to visualize the nuclei (blue), and immunolabeled with the anti-LC3 antibody, with the addition of FITC-conjugated goat anti rabbit IgG (green). The upper panel represents immunofluorescence images of three independent replicates. Treatment with a TLR agonist induced the formation of LC3 aggregation on the surface of autophagosomes. (B) Cells were pretreated with either Poly(I:C) or IMQ for 3 h and then exposed 2 Gy of γ-irradiation. After 72 h of incubation, endogenous LC3 and DAPI were stained. A strong aggregation of LC3 was observed in the group that had simultaneous treatment with agonist and radiation exposure compared to the single stimulus groups in A. (C) The number of cells with LC3-positive puncta was counted under a microscope, and the ratio was calculated as the number of cells with LC3-positive puncta divided by the total cell number. An increased ratio of cells with LC3 puncta was measured upon stimulation with IMQ or Poly(I:C) alone. More than 40% of the increased ratio was detected in the group stimulated simultaneously with a TLR agonist and γ-irradiation exposure. The mean percentages of triplicate measurements ± SE are shown.

sensitivity of MCF-7 cells, which caused cell death, as shown by the reduced survival and the increased number of autophagosome-bearing cells that were stimulated simultaneously with agonist and γ-irradiation.

Microbial components, including TLR agonists, are able to activate the host immune system as adjuvants and have emerged as an exciting new class of molecules for boosting immunity in cancer vaccine development. It is expected that Poly(I:C) or IMQ introduced locally to a tumor-bearing animal model for radiosensitizing drugs could induce the innate immune response by activating DC maturation, the inflammatory response, and stimulating costimulatory molecules that help antigen presentation. The enhanced immunity could be helpful to the host for eradicating cancer. Therefore, treatment with TLR agonists as an anti-cancer drug could promote autophagy and radio- or chemotherapy sensitivity in cancer cells. Therefore, this study could be applied to development of therapeutic adjuvants of TLR agonists in radiotherapy for radio-resistant breast cancer treatment.

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