

The Effects of Low-dose Ionizing Radiation in the Activated Rat Basophilic Leukemia (RBL-2H3) Mast Cells*

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Background: The effect of ionizing radiation in mast cells is not well known.

Results: Low-dose ionizing radiation that did not induce cell toxicity inhibited mediator release through the suppression of receptor expression.

Conclusion: Low-dose ionizing radiation regulates mast cell activation.

Significance: This is the first evidence of the effect of low-dose radiation in the activated mast cell.

Mast cells play important roles in many biological responses, such as those during allergic diseases and inflammatory disorders. Although laser and UV irradiation have immunosuppressive effects on inflammatory diseases by suppressing mast cells, little is known about the effects of γ -ionizing radiation on mast cells. In this study, we investigated the effects of γ -ionizing radiation on RBL-2H3 cells, a convenient model system for studying regulated secretion by mast cells. Low-dose radiation (<0.1 gray (Gy)) did not induce cell death, but high-dose radiation (>0.5 Gy) induced apoptosis. Low-dose ionizing radiation significantly suppressed the release of mediators (histamine, β -hexosaminidase, IL-4, and tumor necrosis factor- α) from immunoglobulin E (IgE)-sensitized RBL-2H3 cells. To determine the mechanism of mediator release inhibition by ionizing radiation, we examined the activation of intracellular signaling molecules such as Lyn, Syk, phospholipase C γ , PKCs, and MAPK, and intracellular free calcium concentrations ($[Ca^{2+}]_i$). The phosphorylation of signaling molecules following stimulation of high-affinity IgE receptor I (Fc ϵ RI) was specifically inhibited by low-dose ionizing radiation (0.01 Gy). These results were due to the suppression of Fc ϵ RI expression by the low-dose ionizing radiation. Therefore, low-dose ionizing radiation (0.01 Gy) may function as a novel inhibitor of mast cell activation.

Perhaps the most controversial and disputed field of radiation biology is the biological effects of low-dose radiation. The immune-suppressing effects of high-dose radiation have been clearly demonstrated and confirmed both experimentally and in epidemiological studies (1, 2). However, the effects of low-dose ionizing radiation on the immune system remain controversial. The connection between UV radiation and the immune system was recognized ~ 30 years ago, resulting in the development of photoimmunology. A single or limited number of UV

radiation exposures can suppress cell-mediated immunity (3, 4). Specifically, the narrow band of UVB and UVA phototherapy is a mainstay of treatments; both natural and artificial UVB irradiations are commonly employed in the treatment of atopic dermatitis (5, 6). UVB radiation of skin also suppresses airway hyperresponsiveness and cellular responses of airways to respiratory allergens (7). Although laser and UV irradiation have immunosuppressive effects on inflammatory diseases by suppressing mast cells, little is known about the effects of ionizing radiation on mast cells.

For many years, mast cells have been considered to participate specifically in allergic reactions (8–10). Mast cells express high-affinity IgE receptors, Fc ϵ RI,² on their surfaces that mediate their immunological activities (11–13). Cross-linking of Fc ϵ RI-bound IgE with multivalent antigens initiates the activation of mast cells by promoting the aggregation of Fc ϵ RI (11). The Fc ϵ RI-dependent mast cell activation process has three outcomes: (i) degranulation: secretion of preformed mediators that are stored in cytoplasmic granules of the cell; (ii) the *de novo* synthesis of pro-inflammatory lipid mediators; and (iii) synthesis and secretion of cytokines and chemokines (11). This activation process constitutes an important step in the immediate hypersensitivity reaction that occurs during allergic diseases such as urticaria, bronchial asthma, and allergic rhinitis (12).

Low-dose ionizing radiation has positive biostimulation effects on living organisms both *in vitro* and *in vivo* and has various applications in the medical field (14). However, few studies have extensively studied the effects of low-dose ionizing radiation, unlike UV radiation, on allergic reactions resulting from mast cell activation. Therefore, we first examined whether low-dose ionizing radiation modulates allergic reaction by activated mast cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Rat basophilic leukemia RBL-2H3 cells were purchased from American Type Culture Collection (ATCC,

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² The abbreviations used are: Fc ϵ RI, high-affinity IgE receptor I; DNP, dinitrophenyl; Gy, gray; HSA, human serum albumin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RBL-2H3, rat basophilic leukemia.

Manassas, VA). Cells were cultured in Eagle's minimum essential medium (GIBCO) containing 15% FBS (GIBCO) and maintained at 37 °C in a humidified incubator containing 95% air and 5% CO₂.

Irradiation of Cells—RBL-2H3 cells were irradiated with 0.01–5 Gy using a ¹³⁷Cs γ-irradiator (IBL 437C; CIS Bio International, Bangnols sur Ceze, France) with a dose rate of 0.8 Gy/min for high-dose rate irradiation (acute irradiation). A low-dose rate irradiation facility equipped with a ¹³⁷Cs source and a dose rate of 0.01 Gy/h was used for low-dose rate irradiation (chronic irradiation).

Cell Survival Measurements—Cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye (Sigma) 48 h and 72 h after irradiation. Yellow MTT is reduced to purple formazan in the mitochondria of living cells. The absorbance of this colored solution was measured at 540 nm spectrophotometer (Labsystems, Helsinki, Finland) (15). For long-term cell survival determination, irradiated cells were seeded in methylcellulose complete medium (R&D Systems, Minneapolis, MN). After 14 days of incubation, colonies were stained with nitro blue tetrazolium (Sigma) and counted (>50 cells). Data were normalized to untreated control plating efficiencies.

Assays for Histamine and β-Hexosaminidase Secretion—RBL-2H3 cells were sensitized with 0.1 μg/ml monoclonal anti-dinitrophenyl (DNP) (IgE) Ab clone SPE-7 (Sigma). Cells were washed with modified Tyrode's buffer consisting of 137 mM NaCl, 0.42 mM NaH₂PO₄, 2.6 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 12 mM NaHCO₃, 5 mM dextrose, 1 g/liter glucose, 1 μg/liter gelatin, pH 7.4. Cells were irradiated with 0.01–2 Gy before stimulation with 0.01 μg/ml DNP-human serum albumin (HSA) (Sigma). After 1 h, histamine concentrations were detected using enzyme immunoassay kits (Oxford Biomedical Research, Rochester Hills, MI). The amount of released histamine was expressed as a percentage of the total histamine produced by unstimulated cells (16). To determine β-hexosaminidase release, supernatants and lysed pellets were aliquoted into 96-well plates. Samples were mixed with substrate solution (1 mM *p*-nitrophenyl *N*-acetyl-β-D-glucosamine in 0.05 M citrate buffer, pH 4.5) and incubated for 1 h at 37 °C. Reactions were terminated by the addition of 0.05 M sodium carbonate buffer, pH 10. Absorbance was measured with a spectrophotometer (Labsystems) at 405 nm (17).

Cytokine Measurements—IgE-sensitized RBL-2H3 cells were irradiated and stimulated with DNP-HSA for 5 h. IL-4 and tumor necrosis factor-α (TNF-α) concentrations in cell culture supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc.).

Measurement of Intracellular Ca²⁺ Levels—RBL-2H3 cells were sensitized, irradiated, and incubated with 5 μM Fluo-3/AM (Molecular Probes) in modified Tyrode's buffer (without CaCl₂) for 30 min at 37 °C. After washing, cells were stimulated with DNP-HSA. Fluo-3/AM fluorescence intensities were monitored using a microplate fluorometer (Berthold Technologies, Bad Wildbad, Germany). Cytosolic free calcium concentrations ([Ca²⁺]_i) were calculated as described previously (18).

Immunoblotting—Following treatment RBL-2H3 cells were harvested, washed, and lysed. Cell lysates of equal protein concentrations were subjected to immunoblotting.

Flow Cytometry—RBL-2H3 cells were sensitized and irradiated with 0.01–0.5 Gy. After 30 min incubation, cells were treated with FITC-conjugated rat anti-IgE (BD Pharmingen) and analyzed by flow cytometry (Beckman Coulter).

Immunofluorescence Microscopy—RBL-2H3 cells, cultured on 5 mm glass coverslips, were stimulated and irradiated. After 30 min incubation, cells were fixed with 4% paraformaldehyde and permeabilized for in PBS containing 0.1% saponin, 5% FBS, and 2% BSA. Coverslips were rinsed with PBS and incubated for 30 min with FITC-conjugated rat anti-IgE. After washing and mounting, fluorescence image were observed using a fluorescent microscope (Axiovert 200; Carl Zeiss, Jena, Germany) (19, 20).

Statistic Analysis—Experimental data are represented as means ± S.E. The analysis of significance between controls and experimental groups was performed using SAS software (version 8.1; SAS Institute Inc., Cary, NC) and *t* tests. *p* values <0.05 were considered significant.

RESULTS

Low-dose Ionizing Radiation Did Not Reduce Mast Cell Viability—The reduction of cell viability by ionizing radiation is commonly used as a criterion to determine irradiation-induced cytotoxicity. Therefore, we examined the cell viability following various doses of ionizing radiation before investigating the effects of ionizing radiation in the activated RBL-2H3 cells. We used the MTT assay to assess short-term cell viability (48 h and 72 h after irradiation) and the colony-forming assay to examine long-term viability (14 days after irradiation). Changes in cell viability following low-dose radiation (<0.1 Gy) were not detected. However, cell viability following high-dose radiation (>0.5 Gy) was significantly reduced in both short-term and long-term analyses compared with that of non-irradiated cells (Fig. 1).

Low-dose Ionizing Radiation Inhibited Mast Cell Degranulation—To examine the effects of ionizing radiation on mediator release following activation of IgE-mediated RBL-2H3 cells, we measured histamine and β-hexosaminidase levels in culture supernatants. Acute low-dose ionizing radiation decreased histamine release by RBL-2H3 cells with statistical significance in the range of 0.01–0.1 Gy (Fig. 2A), β-hexosaminidase release also decreased similarly (Fig. 2B). To examine the effects of chronic low-dose radiation, RBL-2H3 cells were continually irradiated at a dose rate of 0.01 Gy/h until the final accumulated dose reached 0.001–0.05 Gy. Both histamine and β-hexosaminidase release were most significantly inhibited by irradiation with 0.01 Gy (Fig. 2, C and D). This finding correlated with the results from the acute irradiation.

Low-dose Ionizing Radiation Inhibited Mast Cell Cytokine Production—As shown in Fig. 2, low-dose radiation, especially 0.01 Gy, inhibited mast cell degranulation. Thus, we examined whether low-dose radiation would alter the secretion of IL-4 and TNF-α by RBL-2H3 cells following IgE-mediated stimulation. Challenging IgE-sensitized mast cells with antigens led to the production of multiple cytokines. Because IL-4 and TNF-α

play important roles in allergic diseases induced by mast cell activation, effects of low-dose radiation on the production of these cytokines were examined. Low-dose radiation significantly inhibited IL-4 and TNF- α production (Fig. 3, A and B).

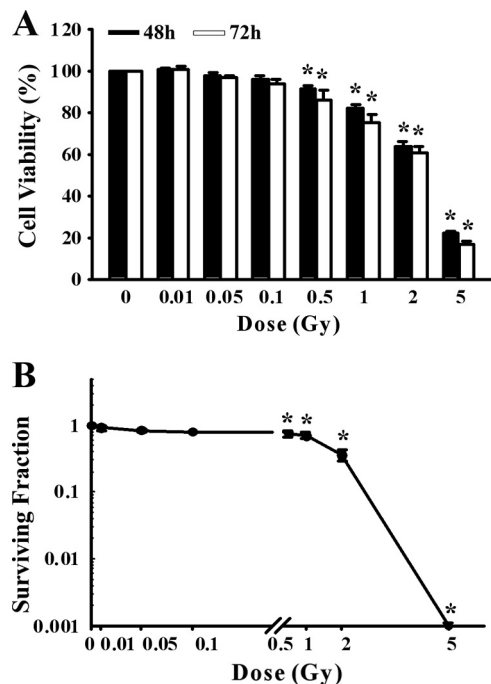


FIGURE 1. **Cell survival following ionizing radiation in RBL-2H3 cells.** Cells were exposed to ionizing radiation (0–5 Gy) and incubated for 48 h (filled bars), 72 h (open bars) (A), or 14 days (B). Cell survival was evaluated using MTT assays (A) and colony-forming assays (B). Data represent means $\pm$ S.E. (error bars) from three independent experiments performed in triplicate. *, $p < 0.05$.

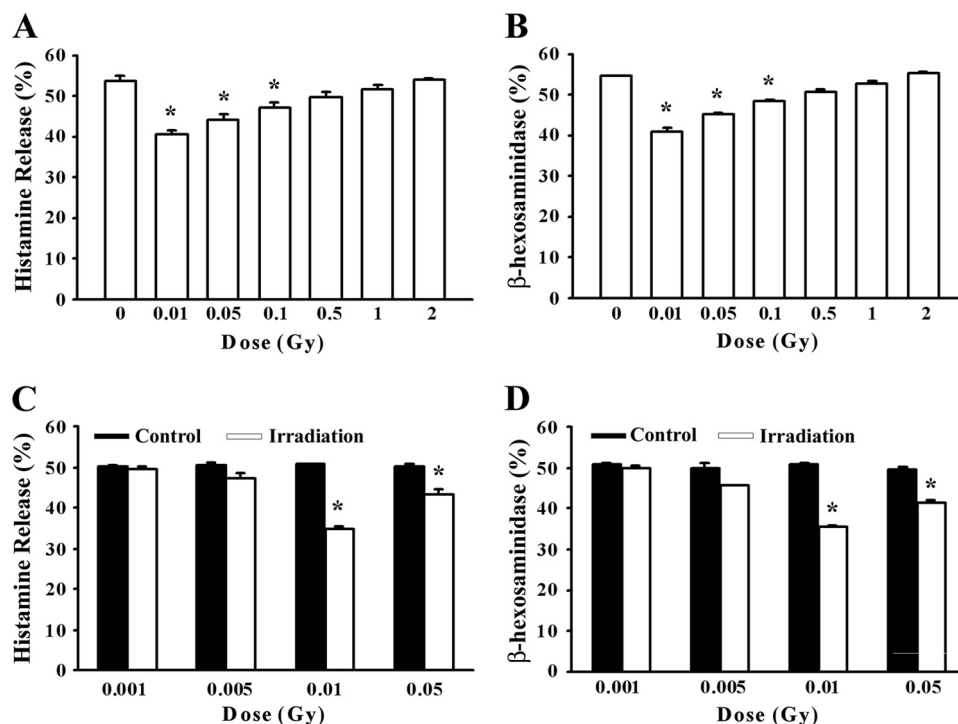


FIGURE 2. **Inhibitory effects of acute or chronic ionizing radiation on mediator released by RBL-2H3 cells.** IgE-sensitized cells were exposed to ionizing radiation (0.01–2 Gy) in an acute (A and B) or chronic (C and D) manner before the addition of DNP-HSA. After 1 h, histamine (A and C) and β -hexosaminidase (B and D) concentrations were determined in supernatants. For chronic conditions (C and D), we used a negative control (filled bars) because radiation exposure times varied due to the accumulated dose (open bars). Data represent means $\pm$ S.E. (error bars) from three independent experiments performed in triplicate. *, $p < 0.05$.

To observe the effects of chronic radiation, we measured cytokine production after cells were irradiated with 0.001–0.05 Gy at 0.01 Gy/h dose rate. We used a specific negative control for each condition because radiation exposure times varied due to the accumulated doses. However, there is no difference in cytokine production between the negative controls. Chronic low-dose irradiation with 0.01 Gy and 0.05 Gy significantly inhibited IL-4 and TNF- α production (Fig. 3, C and D).

Low-dose Ionizing Radiation Suppressed Intracellular Ca^{2+} Influx—Fc ϵ RI-mediated degranulation is dependent on calcium influx (12). Therefore, to learn more about the mechanism of degranulation suppression following low-dose radiation, we examined intracellular free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) in RBL-2H3 cells. In IgE-sensitized mast cells, DNP-HSA stimulation enhanced calcium mobilization within a few seconds and maximized mobilization after ~ 6 min, and maintained mobilization through 15 min (Fig. 4). RBL-2H3 cells that were exposed to ionizing radiation in the range of 0.01–0.5 Gy showed decreased levels of calcium influx in a dose-dependent manner compared with the IgE-mediated activation group (Fig. 4).

Inhibitory Effects of Low-dose Ionizing Radiation Were Dependent on Fc ϵ RI Signaling—Low-dose radiation decreased mast cell degranulation and cytokine production. Therefore, we asked whether the inhibitory effect of low-dose radiation occurs only during IgE-mediated signaling. To examine whether the inhibitory effects on degranulation and cytokine production by low-dose radiation were dependent on the Fc ϵ RI-mediated signaling, we compared IgE-mediated reactions with reactions mediated by agonists, such as the calcium

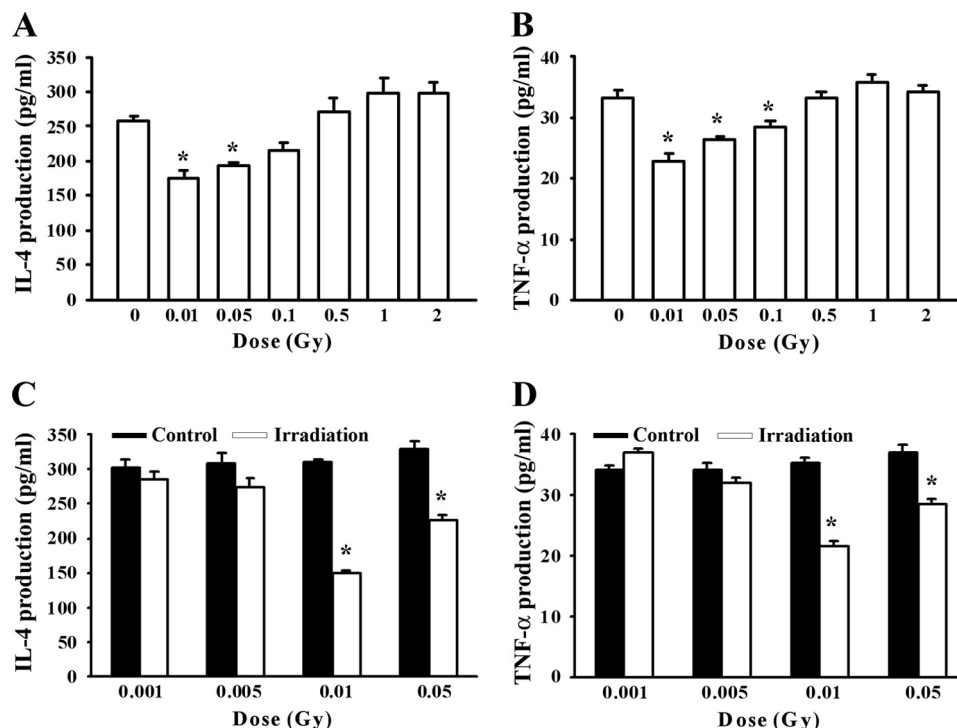


FIGURE 3. **Inhibitory effects of acute or chronic ionizing radiation on IL-4 and TNF- α production by RBL-2H3 cells.** IgE-sensitized cells were exposed to ionizing radiation (0.01–2 Gy) in an acute (A and B) or chronic (C and D) manner before the addition of DNP-HSA. After 5 h, IL-4 (A and C) and TNF- α (B and D) concentrations were determined in supernatants. For chronic conditions (C and D), we used a negative control (filled bars) because radiation exposure times varied due to the accumulated dose (open bars). Data represent the means $\pm$ S.E. (error bars) from three independent experiments performed in triplicate. *, $p < 0.05$.

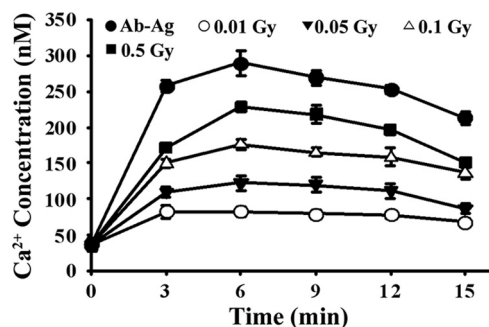


FIGURE 4. **Effects of ionizing radiation on intracellular Ca²⁺ mobilization in activated RBL-2H3 cells.** IgE-sensitized cells were exposed to ionizing radiation (0.01–0.5 Gy), and incubated with 5 μ M Fluo-3/AM fluorescence dye for 30 min. Fluo-3/AM-loaded cells were stimulated with DNP-HSA and monitored at 15 s intervals for 15 min. Data represent the means $\pm$ S.E. (error bars) from three independent experiments performed in triplicate.

ionophore A23187 and LPS, which are unrelated to Fc ϵ RI-mediated signaling in RBL-2H3 cells. Low-dose radiation did not inhibit mast cell degranulation by A23187 and cytokine production by LPS. The inhibitory effect of low-dose radiation was only observed during IgE-mediated mast cell activation (Fig. 5).

To investigate the signaling mechanism of the inhibitory effect on degranulation by low-dose ionizing radiation, we examined early intracellular signaling events. DNP-HSA treatment of IgE-sensitized cells induced phosphorylation of Lyn, Syk, PKCs, and phospholipase C γ at early time, this phosphorylation was reduced following low-dose radiation (Fig. 6, A and B). Specifically, RBL-2H3 cells irradiated with 0.01 Gy had the lowest phosphorylation levels as compared with cells irradiated with the other doses (Fig. 6B).

During IgE-mediated activation of mast cells, three major subfamilies of MAPKs (ERK, JNK, and p38) were activated, which resulted in cytokine production (21). To further identify relevant downstream effectors that directly modulated cytokine production following low-dose ionizing irradiation, we examined the levels of phosphorylated ERK, JNK, and p38. The phosphorylation of ERK, JNK, and p38 were most inhibited by low-dose ionizing radiation (Fig. 6D).

Low-dose Ionizing Radiation Altered Fc ϵ RI Expression—We examined whether low-dose radiation altered Fc ϵ RI expression because low-dose radiation suppressed Fc ϵ RI-mediated early stage signaling (Lyn and Syk). Low-dose ionizing radiation reduced the expression of Fc ϵ RI in a dose-dependent manner (Fig. 7A). As seen with our previous data, 0.01 Gy decreased the expression of Fc ϵ RI most dramatically. Additionally, we observed the expression of Fc ϵ RI using fluorescence microscopy (Fig. 7B). Fc ϵ RI expression was remarkably reduced following low-dose ionizing radiation (0.01 Gy). However, the reduction was nearly absent at the dose of 0.5 Gy.

To further evaluate the effect of ionizing radiation on receptor internalization, we performed fluorescence microscopy analysis. Sensitized cells were stimulated with DNP-HSA for 30 min with or without radiation pretreatment. Fixed and permeabilized cells were stained with FITC-conjugated rat anti-IgE. After 30 min, Fc ϵ RI was internalized and present in endocytic vesicles dispersed throughout the cytoplasm of RBL-2H3 cells. At this same time point, there were reduced numbers of endocytic vesicles in the cytoplasm of cells treated with low-dose ionizing radiation (0.01 Gy), whereas the endocytic vesicles in cells treated with 0.5 Gy were sim-

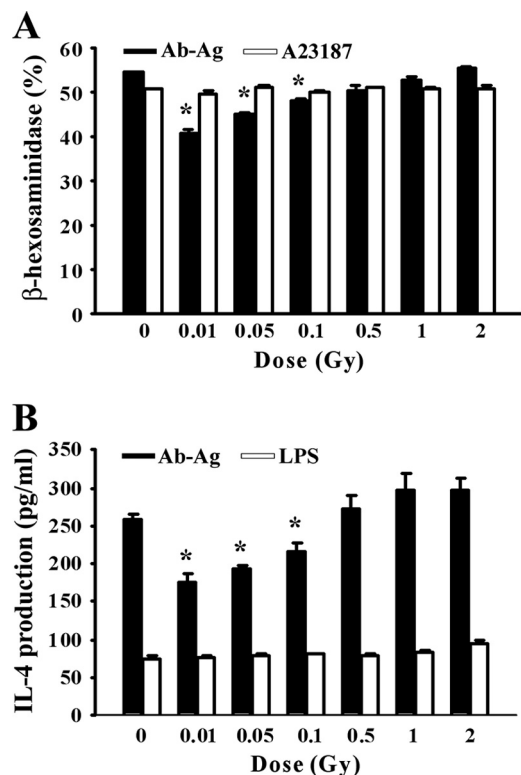


FIGURE 5. Ionizing radiation differentially affects activated mast cells based on the mode of activation. Cells were exposed to ionizing radiation (0.01–2 Gy) before the addition of 5 μ M A23187 (open bars) or 0.1 μ g/ml LPS (open bars). After a 30 min (for A23187) or 24 h (for LPS) incubation, β -hexosaminidase (A) and IL-4 (B) concentrations were determined in supernatants, respectively. For Ab-Ag reaction (filled bars), cells were treated as mentioned above (Figs. 2 and 3). Data present means $\pm$ S.E. (error bars) from three independent experiments performed in triplicate. *, $p < 0.05$.

ilar to those in the positive control cells without radiation treatments (Fig. 8).

DISCUSSION

Although high-dose ionizing radiation is carcinogenic, ionizing radiation can have different effects on immune responses depending on doses and dose rates. Therefore, we examined the immunomodulatory effects of low-dose ionizing on mast cells that contribute to allergic responses, one type of hypersensitivity. Because IgE-dependent release of mediators can begin within minutes of antigen challenge, the crucial roles of mast cells in acute allergic reactions are now widely accepted (8–14). RBL-2H3 cells are a histamine-releasing cell line commonly used in inflammation, allergy, and immunological research (22). RBL-2H3 cells have been extensively to investigate the interactions of IgE with Fc ϵ RI because RBL-2H3 cells possess some typical characteristics of mast cells (22). Therefore, we examined the effects of low-dose ionizing radiation on mast cell activation using RBL-2H3 cells.

Following exposure to low-dose ionizing radiation, RBL-2H3 cells activated through IgE cross-linking significantly reduced production levels of IL-4 and TNF- α and decreased release of mediators through degranulation. These results suggest that 0.01 Gy of low-dose radiation is an effective approach for inhibiting host hypersensitivity responses. There are two types of radiation exposure, acute and chronic. Acute exposure is a sudden dose that occurs over a short time, and chronic exposure is a dose that occurs for long time with at a low-dose rate. Therefore, we examined whether the biological effects of acute and chronic irradiation are different. Activated RBL-2H3 cells were

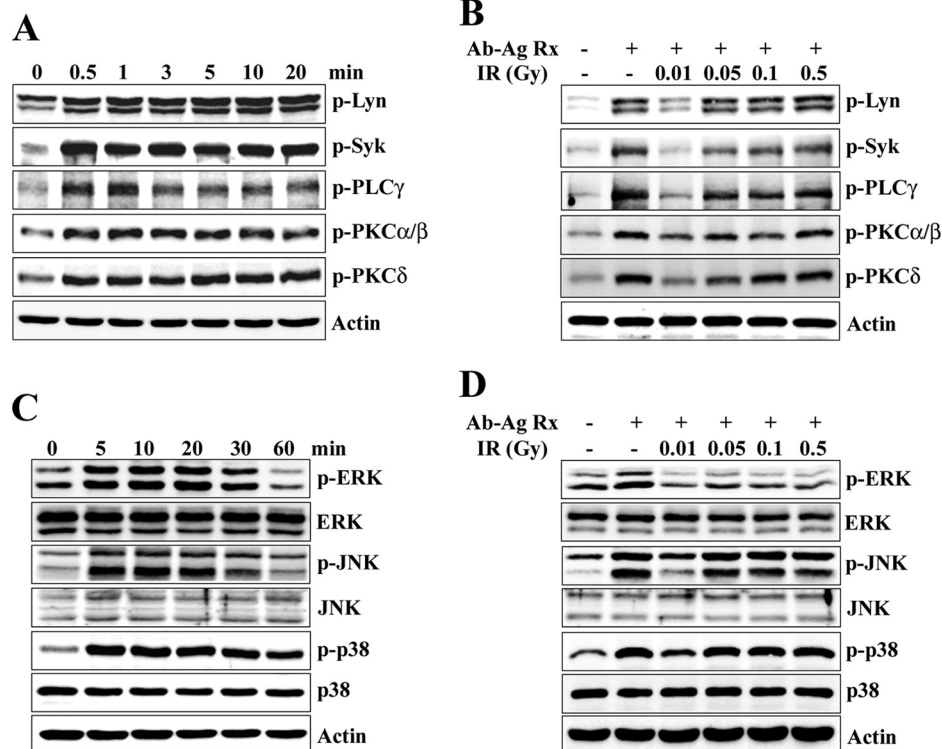


FIGURE 6. Effects of ionizing radiation on IgE-mediated signal transduction in RBL-2H3 cells. IgE-sensitized cells were exposed to ionizing radiation (0.01–0.5 Gy) and stimulated with DNP-HSA. Phosphorylation of signaling molecules was measured by immunoblotting at 1 min (A) or 10 min (B) after the addition of DNP-HSA.

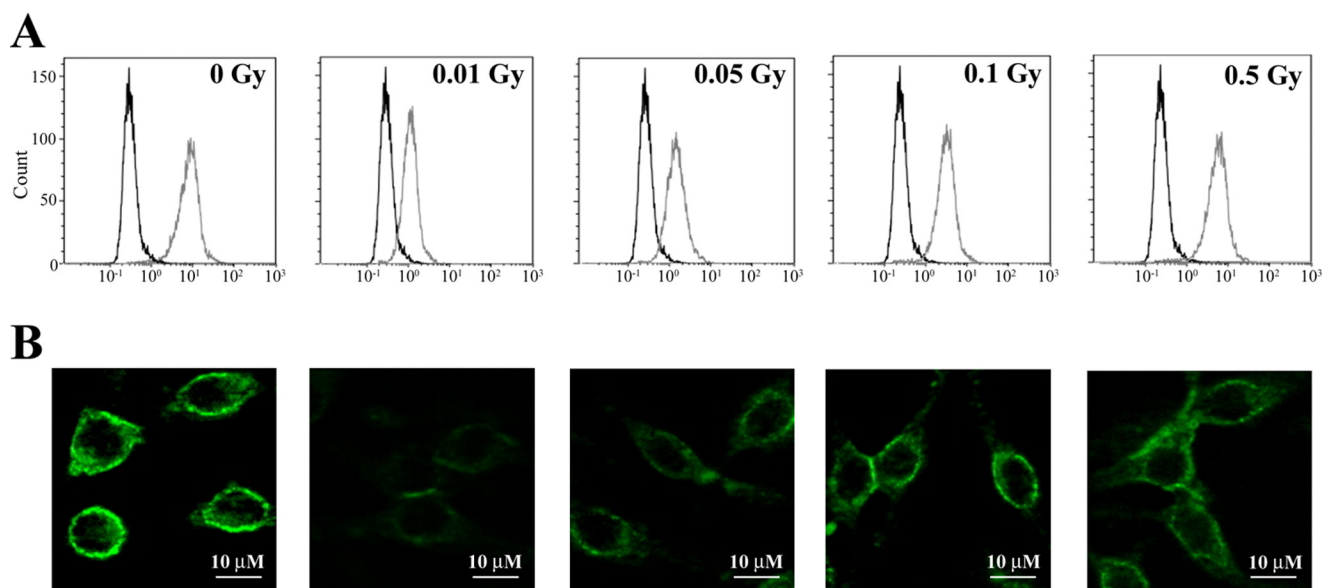


FIGURE 7. Effects of ionizing radiation on FcεRI expression of RBL-2H3 cells. A, IgE-sensitized cells were exposed to ionizing radiation (0.01–0.5 Gy). Thirty minutes after irradiation, cells were incubated with FITC-conjugated rat anti-mouse IgE for 30 min (gray line) and evaluated by flow cytometry. Black line is negative control. B, FcεRI expression was visualized with an Apotome microscope. Scale bars indicate 10 μm.

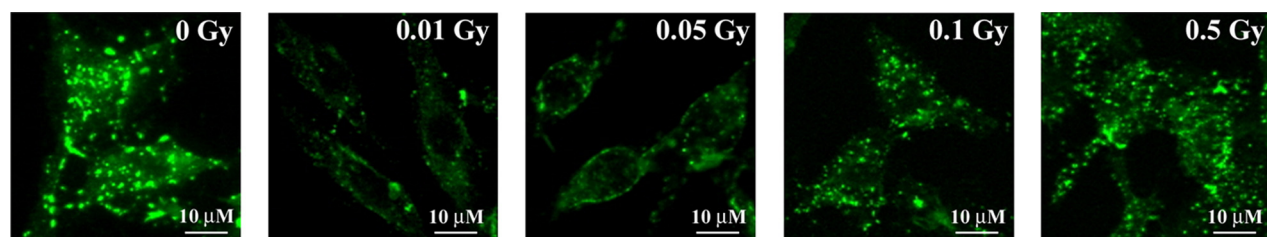


FIGURE 8. Effects of ionizing radiation on FcεRI internalization of activated RBL-2H3 cells. IgE-sensitized cells were exposed to ionizing radiation (0.01–0.5 Gy). Thirty minutes after irradiation, cells were stimulated with DNP-HSA for 30 min, permeabilized, and incubated with FITC-conjugated rat anti-mouse IgE. Fluorescent images of internalized FcεRI were obtained using an Apotome microscope. Scale bars indicate 10 μm.

suppressed by both acute and chronic irradiation (Figs. 2 and 3). To determine whether the inhibition of mast cell activation by low-dose radiation only occurred during FcεRI-mediated activation, we examined the effect of ionizing radiation on calcium ionophore (A23187)-induced degranulation and LPS-induced cytokine production. A23187 induces artificial activation of mast cells and is routinely used to study exocytosis by bypassing the molecular events that are associated with FcεRI excitation (23). LPS from most species of Gram-negative bacteria activates cells via TLRs and can activate mast cells to produce cytokines without degranulation (24, 25). As shown in Fig. 5, the inhibitory effects of low-dose ionizing radiation on mast cells occurred only during activation through FcεRIs.

Based on these results, the inhibitory effect of low-dose radiation was likely caused by suppressing the initial steps of mast cell activation. Therefore, we examined whether low-dose radiation inhibited the expression of FcεRI because the resulting mast cell activation was induced by the crossing of FcεRI and IgE. Although mast cells were irradiated for only a short time, FcεRI expression was dramatically reduced (Fig. 7).

According to a recent report, lipid rafts are specialized microdomains of the plasma membrane that play an important role in FcεRI signaling (26). These microdomains are thought to be important sites for protein tyrosine kinase-mediated protein-protein interactions that are involved in the initiation of

receptor signaling pathways (27). Therefore, FcεRI is loosely dispersed throughout the plasma membranes of unstimulated cells, but upon activation, FcεRI rapidly aggregates and can be found in lipid rafts in association with gangliosides. Following aggregation, FcεRI is internalized in coated vesicles (26). Receptor-mediated endocytosis, including endocytosis of FcεRI, is a temporally and spatially organized process. Following the binding of a ligand to its receptor, the receptor clusters in lipid rafts of the plasma membrane. Full activation of FcεRI requires receptor redistribution into lipid rafts. Upon FcεRI engagement, membrane rafts coalesce into larger and more stable structures where engaged receptors are concentrated and more easily interact with signaling molecules such as activated Lyn (28). Therefore, the reduction of FcεRI expression following low-dose radiation resulted in reduced receptor internalization and, therefore, decreased release of mediators and cytokines.

In conclusion, both acute and chronic low-dose radiation did not induce mast cell death and inhibited mast cell degranulation and cytokine production by FcεRI-dependent signaling suppression via the reduction of FcεRI expression in mast cell. Therefore, we suggest that 0.01 Gy of low-dose radiation is an effective approach for inhibiting host inflammatory disease because inflammatory disease, like allergic disease, is related to mast cell activation. So why did higher doses radiation cause less inhibition of activation than lower doses? We suggest that it

is the hormesis effect, which is the term for generally favorable biological responses to low exposure to toxins. Although ionizing radiation has toxicity in large doses, including cell death and damage, low-dose ionizing radiation has the opposite effect and positively regulates the hypersensitivity by mast cell activation. However, additional research is required using human mast cell lines and animal system to confirm these findings from the RBL-2H3 cells. We are currently working to confirm the effect of low-dose radiation in the human cell lines, HMC-1(5C6) and LAD2.

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