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Low Dose γ-Rays Activate Immune Functions via Induction of Glutathione and Delay Tumor Growth

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Low-dose gamma-rays/Glutathione (GSH)/Immune function Interferon-γ/Tumor suppression.

We examined whether the increase of glutathione level induced by low dose γ-ray irradiation is involved in the appearance of enhanced natural killer (NK) activity and antibody-dependent cellular cytotoxicity (ADCC), leading to delayed tumor growth in Ehrlich solid tumor-bearing mice. NK activity in ICR mouse splenocytes significantly increased from 4 h to 6 h after whole-body γ-ray irradiation at 0.5 Gy, and thereafter decreased almost to the zero-time level by 24 h post-irradiation. ADCC also increased significantly in a similar way. Reduced glutathione exogenously added to splenocytes obtained from normal mice enhanced both NK activity and ADCC in a dose-dependent manner. Since immune functions were enhanced through the induction of cellular glutathione after low-dose irradiation, the inhibitory effect of the radiation on tumor growth was then examined in Ehrlich solid tumor-bearing mice. Tumor growth after inoculation was significantly delayed by the radiation. These results suggest that low-dose γ-rays activate immune functions via an induction of glutathione, leading to a delay of tumor growth.

INTRODUCTION

Low doses of ionizing radiation induce various effects, including radioadaptive response,[1] an activation of immune function,[2] a stimulation of growth rate,[3] and an enhancement of resistance to high-dose radiation.[4] These phenomena have generally been called "radiation hormesis." Regarding the activation of immune function, Liu *et al.* reported that low-dose whole-body irradiation increased the proliferative reactivity of splenic and thymic lymphocytes to suboptimal concentrations of various mitogens in mice, and they showed that the immune enhancement was associated with an increased splenic catecholamine content and a lowering of serum corticosterone level.[5] Later they showed that low-dose irradiation induced these effects through the facilitation of signal transduction in lymphocytes.[6] More recently, the same group isolated a 10-kDa protein (RIP 10), which was induced in the cytoplasm of thymocytes after low-dose whole-body irradiation, and they suggested a role of this protein in modulating the proliferation of thymocytes and splenocytes.[7] Ibuki and Goto reported that low-dose whole-body irradiation enhanced the concanavalin A (Con A)-induced proliferation of splenocytes only when spleen macrophages were simultaneously stimulated by radiation.[8]

It has been well documented that the immune function is linked to the release of radical oxygen species (ROS). An excess amount of ROS is commonly eliminated by endogenous antioxidant systems, thereby preventing injury to DNA, intracellular protein and membrane lipids. Thus, immunocompetent cells also contain compounds that act to maintain the antioxidant/oxidant (redox) balance. Among them, reduced glutathione (GSH) has direct or indirect roles in many biological processes, including protein and DNA synthesis, amino acid transport, the activation of enzyme activities, the activation of metabolism, and the protection of cells from damage caused by ROS.[9] A depletion of GSH can lead to increased lipid peroxidation with concomitant changes in membrane permeability and cellular damage, but an increase in GSH level enhances antioxidant protection and cellular functions. It has been well established that optimal immune function requires proper levels of GSH, and exogenous provision of thiol-group donors is beneficial for immunocompetent cells.[10] In our previous studies, the changes of endogenous GSH were examined in mice exposed to whole-body γ-ray irradiation, and it was found that low doses of radiation significantly increased total glutathione levels in organs such as liver, pancreas and brain.[11] More recently, it has been shown that the induction of endogenous GSH following radiation exposure is responsible for the enhancement of Con A-induced proliferation of mouse splenocytes.[12]

In this study, we examined whether the increase of glutathione induced by low doses of γ-rays is involved in the

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appearance of the enhanced natural killer (NK) activity of splenocytes and antibody-dependent cellular cytotoxicity (ADCC) of lymphocytes. Furthermore, the effect of low-dose radiation on tumor-growth rate was investigated in Ehrlich solid tumor (EST)-bearing mice.

MATERIALS AND METHODS

Materials

Reduced glutathione (GSH), oxidized glutathione (GSSG), cysteine (Cys), *N*-acetylcysteine (NAC), glycine (Gly), glutamine (Glu), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). β-NADPH was obtained from Oriental Yeast Co. Ltd. (Tokyo Japan). GSH reductase (120 U/ml), *concanavalin* A (Con A), and buthionine sulfoximine (BSO) were from Sigma (St. Louis, MO, USA). [^{51}Cr]Sodium chromate (17.0 GBq/mg) was purchased from New England Nuclear Life Science Products, Inc. (Boston, MA., USA). Lymphocyte separation medium was from Flow Laboratories (McLean VG., USA). Sheep red blood cells and rabbit anti-serum to sheep red blood cell were obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, CA., USA).

Animals and γ-ray irradiation

Male ICR strain mice, 7 weeks of age, were divided into irradiated and non-irradiated control groups. Irradiation was done with γ-rays from a ^{137}Cs source (GAMMACELL 40, Nordin International, Inc., Canada) at a dose of 0.5 Gy (1.01 Gy/min).

Preparation of splenocytes

The mice were killed by cervical dislocation and their spleens were removed aseptically. The spleens were minced with scissors, and single-cell suspensions were prepared. The cells were washed 3 times with PBS, and the numbers of splenocytes were counted under a microscope.

Assay of total glutathione contents

Total glutathione (GSH + GSSG) content in the splenocytes was measured by using a modified spectrophotometric technique. Splenocytes obtained from non- and γ-ray-irradiated animals were washed 3 times with ice-cold PBS, and suspended in 0.5 ml of RIPS (pH 7.4), sonicated for 15 seconds, and centrifuged at 15,000 rpm for 20 min. An aliquot of each sample was removed for protein assay. An equal volume of 10% TCA was added to another aliquot, and solutions were cleared by centrifugation at 15,000 rpm for 20 min to remove protein. The supernatant acid-soluble fraction was treated with 3 ml of ice-cooled diethyl ether, and the diethyl ether layer was removed with a pipette. This procedure was repeated 5 times to remove excess TCA.

The supernatant was diluted 1:25, and a 25 μl aliquot was mixed with 250 μl of 1 mM DTNB, 733 μl of 0.3 mM NADPH, and 10 μl of glutathione reductase (2 U/ml). The change in absorbance was measured at 412 nm. Authentic GSH (0–20 μg/ml) was analyzed in the same manner to obtain a standard curve. The total glutathione concentration of each sample was calculated as nmol/mg protein. The protein content was measured according to the method of Lowry *et al.*[13)]

Assay of NK activity

Splenocytes (5×10^6 cells/ml) were prepared in RPMI1640 medium containing 10% FBS. Target cells were YAC-1, a murine lymphoma cell line, in this assay. ^{51}Cr (37 MBq) was added to the cell pellet (1×10^7) and the mixture was incubated at 37°C for 60 min. After incubation, 10 ml of PBS was added to the cells. The cells were washed 3 times with PBS by centrifugation and resuspended in RPMI1640 medium containing 10% FBS with a final concentration of 1×10^5 cells/ml. Effector cells (splenocytes) and target cells (YAC-1) were distributed into 96-well U-bottomed plates at effector cell/target cell ratios of 50:1, centrifuged at 800 rpm for 6 min, and incubated for 4 h at 37°C in a 5% CO2/95% air atmosphere. One hundred microliters of supernatant obtained by centrifugation was subjected to radioactivity assay with a gamma-counter (Aloka ARC 300, Tokyo, Japan). The percentage lysis was calculated as follows:

% Lysis = $(E - S) \times 100/(T - S)$, where E is the mean experimental cpm released in the presence of effector cells; S, the mean cpm spontaneously released by target cells incubated with medium alone; and T, the mean total cpm released after the incubation of target cells with the same volume of 1 N HCl instead of effector cells at each effector cell/target cell ratio.

Preparation of lymphocytes

Lymphocytes were separated from mouse whole blood by using the lymphocyte separation medium according to the Ficoll-Conray method, and the numbers of lymphocytes were counted under a microscope.

Assay of ADCC

Lymphocytes (2×10^6 cells/ml) were prepared in RPMI 1640 medium containing 10% FBS. Target cells were sheep red Blood cells (SRBC). ^{51}Cr (37 MBq) was added to the cell pellet (1×10^7 cells/ml) and the mixture was incubated at 37°C for 60 min. After incubation, the cells were washed 3 times with PBS by centrifugation, and re-suspended in RPMI1640 medium containing 10% FBS with a final concentration of 1×10^5 cells/ml. Effector cells (lymphocytes), target cells (SRBC) and rabbit anti-serum to sheep red blood cells (IgG) were distributed into 48-well U-bottomed plates, centrifuged at 800 rpm for 6 min, and incubated for 4 h at 37°C in a 5% CO_2/95% air atmosphere. One hundred microliters of supernatant obtained by centrifugation was subjected to radioactivity assay with a gamma-counter (Aloka ARC300, Tokyo, Japan). The percentage lysis was calculated as follows:

% Lysis = (E – S) × 100/(T – S), where E is the mean experimental cpm released in the presence of effector cells; S, the mean cpm released in the presence of effector cells without IgG antibody; and T, the mean total cpm released after incubation of target cells with the same volume of 1 N HCl instead of effector cells at each effector cell/target cell ratio.

Assay of interferon-γ production

Splenocytes obtained from γ-ray-irradiated mice were washed 3 times with ice-cold PBS. The cells (5×10^6 cells/ml) were prepared in RPMI 1640 medium containing 10% FBS, distributed into 48-well plates, and incubated for 24 h at 37°C in a 5% CO2/95% air atmosphere, in the presence of Co A (final concentration of 5 μg/ml). Fifty microliters of the supernatant obtained by centrifugation was subjected to ELISA assay with Endogen ELISA Assay Kit.

Effect of whole-body γ-ray irradiation on tumor growth in Ehrlich solid tumor-bearing ICR mice

The mice were irradiated with γ-rays at a dose of 0.5 Gy, and soon thereafter, Ehrlich ascites tumor cells (1×10^6) suspended in 0.2 ml of sterilized PBS were inoculated subcutaneously into the back on the right side. The mice were irradiated with γ-rays at a dose of 0.5 Gy twice a week for 2 weeks. The solid tumor size was measured with a caliper twice a week until day 30, and the volume (*V*) was calculated from the following equation:

$$V\ (\mathrm{mm}^3) = \text{length (mm)} \times (\text{width [mm]})^2 \times 0.5$$

Statistical analysis

The statistical significance of differences was determined by using Student's *t*-test for a comparison between two groups or two-way repeated measures analysis of variance (ANOVA) and Dunnett's tests for multiple comparison, where appropriate. *P* values of less than 0.05 were considered significant.

RESULTS

Effect of whole-body γ-ray irradiation on cellular glutathione content in splenocytes, NK activity, and ADCC

As shown in Fig. 1, the total glutathione content in mouse splenocytes increased significantly from 2 h to 6 h after whole-body γ-ray irradiation at 0.5 Gy, peaked at 4 h, and thereafter decreased almost to the zero time level by 24 h post irradiation. NK activity in mouse splenocytes after whole-body γ-ray irradiation with 0.5 Gy was assayed as a function of time at the constant effector cells/target cells ratio of 50:1, which was chosen on the basis of preliminary assays. The activity increased significantly from 4 h to 6 h post-irradiation and decreased to the non-irradiation control value by 24 h post-irradiation. ADCC also increased significantly after whole-body γ-ray irradiation in a similar way.

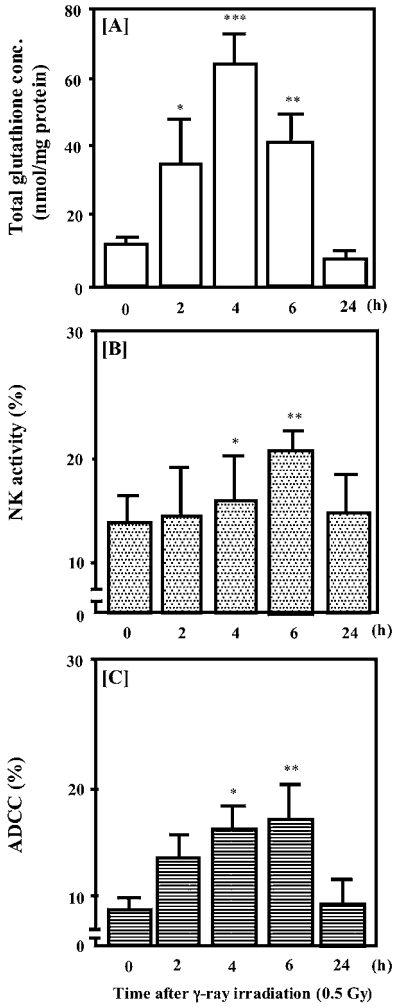

Fig. 1. Effect of whole-body γ-ray irradiation on total glutathione level in splenocytes, natural killer activity, and antibody-dependent cellular cytotoxicity (ADCC). [A] Splenocytes were obtained from male ICR mice after whole-body γ-ray irradiation at 0.5 Gy. Total glutathione levels (GSH + GSSG) in the splenocytes were measured by using a modified spectrophotometric technique. [B] Splenocytes were obtained from male ICR mice after whole- body γ-ray irradiation at 0.5 Gy. Splenocytes and YAC-1 cells labeled with ^{51}Cr were distributed into 96-well U-bottomed plates at ratio of 50:1, and incubated for 4 h at 37°C in 5% CO_2/95% air atmosphere. The percent of lysis was calculated. [C] Lymphocytes for ADCC assay were separated from ICR mouse blood at various time points after whole-body γ-ray irradiation at 0.5 Gy. Effector cells (lymphocytes), target cells (sheep red blood cells; SRBC) labeled with ^{51}Cr and rabbit antiserum to sheep red blood cell (IgG) were distributed into 48 well U-bottomed plates, and incubated for 4 h at 37°C in a 5% CO_2/95% air atmosphere. Percent lysis were calculated. Each point indicates the mean ± SD of 5 mice. *, **, and *** are significantly different from the 0 h group at $p < 0.05$, $p < 0.01$, and $p < 0.005$, respectively.

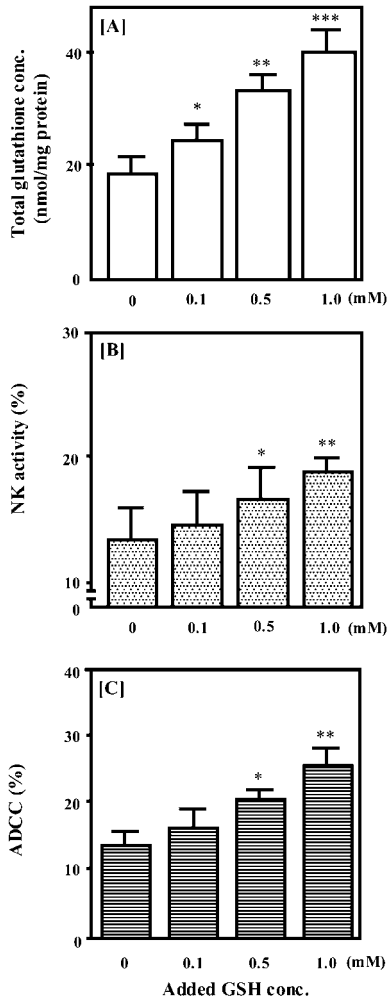

Fig. 2. Effect of exogenous GSH on total glutathione level in splenocytes, NK activity and ADCC. [A] Splenocytes were obtained from normal ICR mice, and incubated with different concentrations of GSH ranging from 0.1 mM to 1.0 mM for 3 h. Total glutathione levels (GSH + GSSG) in the splenocytes were measured by using a modified spectrophotometric technique. [B] Splenocytes were obtained from normal ICR mice, and incubated with different concentrations of GSH ranging from 0.1 mM to 1.0 mM for 3 h. Splenocytes and YAC-1 cells labeled with ^{51}Cr were distributed into 96-well U-bottomed plates at ratio of 50:1, and incubated for 4 h at 37°C in a 5% CO_2/95% air atmosphere. The percent of lysis was calculated. [C] Lymphocytes for ADCC assay were separated from normal ICR mouse blood, incubated with various concentrations of GSH ranging from 0.1 mM to 1.0 mM for 3 h. Effector cells (lymphocytes), target cells (sheep red blood cells; SRBC) labeled with ^{51}Cr and rabbit anti-serum to sheep red blood cell (IgG) were distributed into 48-well U-bottomed plates, and incubated for 4 h at 37°C in a 5% CO_2/95% air atmosphere. The percent of lysis was calculated. Each point indicates the mean ± SD of 5 mice. *, **, and *** Are significantly different from the 0 h group at $p < 0.05$, $p < 0.01$, and $p < 0.005$, respectively.

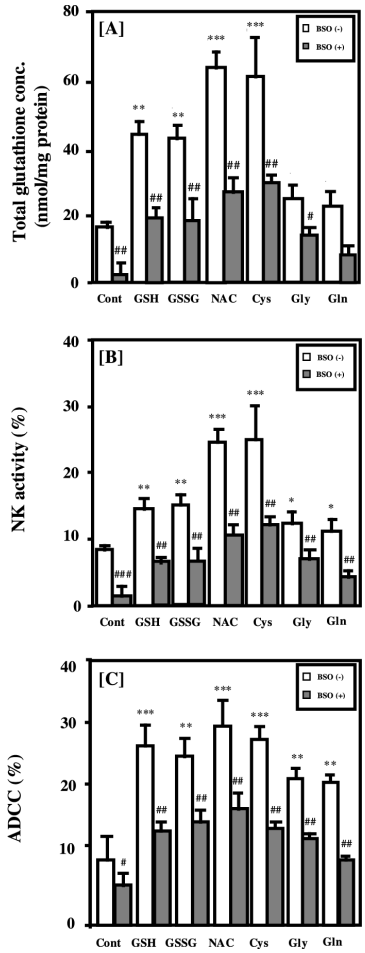

Fig. 3. Effect of exogenous precursors of GSH *de novo* synthesis on total glutathione (GSH + GSSG) levels in splenocytes, NK activity and ADCC in the presence and absence of buthionine sulfoxime (BSO). [A] Splenocytes were obtained from normal ICR mice and incubated with 1 mM GSH, GSSG, NAC, Cys, Gly or Gln in the absence and the presence of BSO (0.5 mM) for 3 h. Total glutathione levels in the splenocytes were measured by using a modified spectrophotometric technique. [B] Splenocytes were obtained from normal ICR mice, incubated with 1 mM GSH, GSSG, NAC, Cys, Gly or Gln in the absence or presence of BSO for 3 h, and washed 3 times with ice-cold PBS. Splenocytes and YAC-1 cells labeled with ^{51}Cr were distributed into 96-well U- bottomed plates at a ratio of 50:1, and incubated for 4 h at 37°C in a 5% CO_2/95% air atmosphere. The percent of lysis was calculated. [C] Lymphocytes for ADCC assay were separated from normal ICR mouse blood, incubated with 1 mM GSH, GSSG, NAC, Cys, Gly or Gln in the absence or presence of BSO for 3 h and washed 3 times with ice-cold PBS. Effector cells (lymphocytes), target cells (sheep red blood cells; SRBC) labeled with ^{51}Cr and rabbit anti-serum to sheep red blood cell (IgG) were distributed into 48-well U-bottomed plates, and incubated for 4 h at 37°C in a 5% CO_2/95% air atmosphere. The percent of lysis was calculated. Each point indicates the mean ± SD of 5 mice. *, **, and *** Are significantly different from the corresponding control group at $p < 0.05$, $p < 0.01$ and $p < 0.005$, respectively. #, ##, and ### Are significantly different from the corresponding BSO-non-treated group at $p < 0.05$, $p < 0.01$, and $p < 0.005$, respectively.

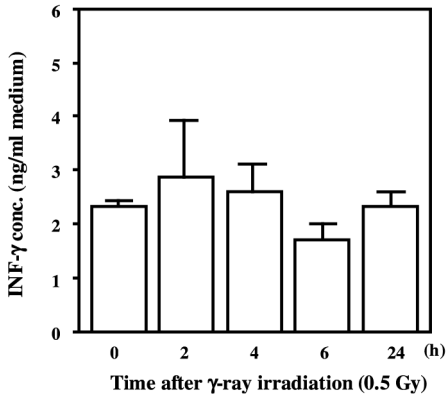

Fig. 4. Effect of whole-body γ-ray irradiation on INF-γ production of splenic cells in ICR mice. The mice were irradiated with γ-rays (0.5 Gy). Splenic cells (5×10^6 cells/ml) were prepared in RPMI 1640 medium containing 10% FBS, distributed into 48-well plates and incubated for 24 h at 37°C in a 5% CO_2/95% air atmosphere, in the presence of Co A (final concentration of 5 μg/ml). Fifty microliters of the supernatant obtained by centrifugation was subjected to INF-γ assay.

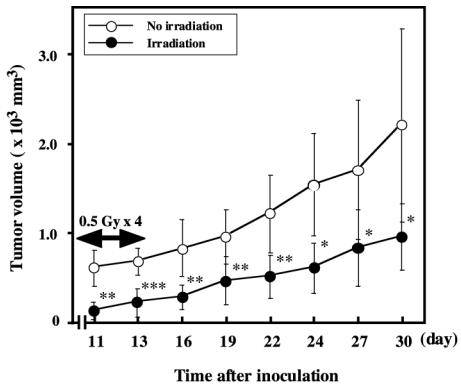

Fig. 5. Effect of whole-body γ-ray irradiation on tumor volume of Ehrlich solid tumor (EST)-bearing ICR mice. The mice were irradiated with γ-rays (0.5 Gy), then immediately inoculated with Ehrlich ascites tumor cells (1×10^6 cells) suspended in 0.2 ml of sterilized PBS subcutaneously into the back of the right side. They were irradiated twice a week for 2 weeks at a single dose of 0.5 Gy per time. The solid tumor size was measured with a caliper twice a week until day 30. *, ** and *** are significantly different from the corresponding non-irradiated control group at $p < 0.05$, $p < 0.01$, and $p < 0.005$, respectively.

Effect of exogenously added GSH on total glutathione content in splenocytes, NK activity, and ADCC

Since the involvement of glutathione induced by low-dose γ-ray irradiation in the enhancement of the immune function was suggested, we examined the effect of exogenous GSH on the total glutathione content in splenocytes, NK activity, and ADCC. As shown in Fig. 2, total glutathione content, NK activity, and ADCC were all enhanced by exogenously added GSH in a dose-dependent manner.

Effect of exogenously added precursors of GSH de novo synthesis on glutathione contents and NK activity in splenocytes, and ADCC with and without buthionine sulfoxime (BSO)

As shown in Fig. 3, the precursors of *de novo* GSH synthesis, Cys and NAC, elevated the cellular total glutathione more effectively than GSH did, and GSSG, the oxidized form of GSH, was as effective as GSH. Gly and Glu also increased total glutathione, but without statistical significance. The increases were inhibited by BSO, a specific inhibitor of γ-glutamylcysteinyl synthetase (γ-GCS), which is the rate-limiting enzyme of the *de novo* GSH synthetic pathway. GSH and its precursors all enhanced NK activity and ADCC to extents that roughly paralleled their effect on total glutathione. Furthermore, the effect of BSO on the elevated NK activity and ADCC induced by GSH precursors was examined. The actions of GSH precursors were abolished in the presence of BSO. The levels of inhibition by this reagent were in good accordance with the effect on the cellular total glutathione level.

Effect of low-dose γ-ray irradiation on IFN-γ productivity in normal ICR mice

To investigate the mechanisms of augmentation of immune functions by low-dose radiation, we examined the effect of the radiation on INF-γ productivity of splenic lymphocytes, including T-cells and NK cells. As shown in Fig. 4, the production of IFN-γ was slightly, but not significantly, increased from 2 h to 4 h after whole-body γ-ray irradiation.

Effect of low-dose γ-ray irradiation on tumor volume in Ehrlich solid tumor bearing mouse

We then examined whether the enhancement of NK activity and ADCC would result in an inhibition of tumor growth. As shown in Fig. 5, the tumor growth at each time point examined after the inoculation was significantly inhibited by radiation within an early time, and a delay of the tumor growth by the radiation was indeed observed.

DISCUSSION

Ionizing radiation at medium to high doses is well known to be carcinogenic and to induce a suppression of immune function. Low-dose radiation, however, may have different effects; it may not always increase the cancer risk according to the so-called "linear hypothesis," but instead it may decrease the relative risk in exposed populations.[14)]

Immune function is a key component of the mammalian defense systems against infection and cancer. Immune responses have been found to be up-regulated in the inhabitants of high-background-radiation areas.[5)] Thus there is evidence

that low-dose radiation stimulates the immune functions and that it can induce some degree of tumor regression and suppression of metastases, though it is still uncertain whether these inhibitory effects are caused by the immune stimulation. Consequently, an elucidation of the mechanisms of the up-regulation of immune responses by low-dose radiation may shed light on the nature of hormesis at low levels of ionizing radiation exposure.

The cytotoxic capacity of immune cells, NK activity and ADCC, plays a key role in immune surveillance against viral, bacterial, fungal and protozoal infections, and these cells also produce a variety of regulatory cytokines,[15,16] which have inhibitory effects on neoplastic growth, such as cancer.

GSH is found at high concentrations in most living organisms, and has a multifunctional role, ranging from antioxidant defense to modulation of immune function. The immune function is generally lowered concomitantly with elevations of ROS formation and lipid peroxidation. For instance, it has been suggested that age-related immune dysfunction may be due to the senescent decline in antioxidant defenses, since the oxidation of cell-surface thiol groups of lymphocytes can abate the mitogen-induced proliferative response, and an exogenous supply of GSH and other thiol-group donors can reverse this.[17,18] It is also well known that intracellular GSH levels are key factors for lymphocyte proliferation. Since GSH is a strong scavenger of ROS, its immunostimulatory effect might be due to its inhibition of ROS formation, which has been suggested as an underlying factor in the reduced immuno responsiveness of aged animals.

We have recently found that low doses of radiation, unlike higher doses, do not always cause a decrease of cellular glutathione, but they can increase the level, leading to an elevation of Con A-induced proliferation of splenocytes.[12] In this study, we examined the relation between GSH induction and immune functions (NK activity and ADCC) in mice exposed to low-dose γ-ray irradiation, and we further investigated the inhibitory effect of the radiation on the growth of implanted solid tumor.

First, it was confirmed that the total glutathione level of splenocytes increased after low-dose whole-body γ-ray irradiation (0.5 Gy). As shown in Fig. 1, the level transiently increased soon after irradiation and reached a maximum at 4 h post-irradiation. Thereafter, the level reverted to the 0 h value by 24 h post-irradiation. A significantly increased NK activity of splenocytes was observed from 4 to 6 h post-irradiation. Moreover, ADCC also increased significantly in a similar way.

Next, the effects of exogenous GSH and its precursors on NK activity and ADCC were examined. As shown in Figs. 2 and 3, exogenously added GSH elevated both immune functions in a dose-dependent manner. Other thiol-containing compounds such as Cys and NAC, which act as precursors for *de novo* GSH synthesis in the cells, also enhanced the immune functions concomitantly with an elevation of the cellular glutathione levels. The effect of BSO, a specific inhibitor of γ-GCS, which is the rate-limiting enzyme of the *de novo* GSH synthetic pathway, on the elevated NK activity and ADCC induced by GSH precursors was further examined. As shown in Fig. 3, NK activity and ADCC were elevated to different extents by various exogenously added GSH precursors. These elevations induced by GSH and its precursors, however, were all completely abolished in the presence of BSO. Since it is also expected that immune functions such as NK activity and ADCC would also be activated via the enhanced production of INF-γ, which is generally considered not only to increase the cytotoxic capacity of immune cells such as macrophages and NK cells, but also to possess anti-viral activity itself, changes in the level of this cytokine were examined. As shown in Fig. 4, IFN-γ production of the splenic cells was slightly, but not significantly, increased from 4 to 6 h after whole-body γ-ray irradiation.

It is not surprising that, as the cumulative dose of radiation increases with the repetition of irradiation over the later experimental period, there should be an enhancement of tumor growth. Thus the mice were irradiated with 0.5 Gy of γ-rays twice a week for only 2 weeks at an early time after the inoculation of Ehrlich ascites tumor cells, and tumor growth was compared between the non-irradiated control and the irradiated group. As shown in Fig. 5, a significant inhibition of tumor growth at all time points was observed. Our results are consistent with other reports showing that GSH can improve immune functions. For example, GSH protects cell membranes from ROS, and increases IL-2 synthesis in lymphocytes, resulting in an enhancement of immune functions, such as antibody-dependent cellular cytotoxicity.[19] Low-dose ionizing irradiation has already been reported to exert carcinostatic effects: A delay of tumor growth by low-dose irradiation was seen in mouse models.[20] The same group has further reported a suppression of artificial and spontaneous lung metastases in mice following low-dose (0.15–0.2 Gy) irradiation.[21] Previous mechanistic studies on these effects have revealed that low-dose radiation enhances immune responses such as mitogenic response, IL-1 production, IL-2 response, and plaque-forming cell reaction.[22,23] Studies to determine whether the inhibitory effects of the radiation occur through the release of other cytokines such as IL-2 and IL-12, which are involved in the inhibition of neoplastic growth, are under way.

In summary, it has been shown that low-dose exposure to ionizing radiation enhances immune function through the induction of an increase of cellular GSH, resulting in delayed tumor growth.

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