Original Article

In vivo study of interferon-γ, transforming growth factor-β, and interleukin-4 gene expression induced by radioadaptive response

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ABSTRACT

Introduction: In the present study, the radioadaptive role of the immune system induced by low dose (LD) was investigated for its *in vivo* protective activity.

Materials and Methods: Quantitative analysis of cytokine gene expression was assessed for their *in vivo* activity in BALB/c mice. To evaluate the adaptive response induced by LD on the mice spleen lymphocyte, the cytokine interleukin (IL)-4, interferon (IFN)-γ, and transforming growth factor (TGF)-β expression was measured by a real-time quantitative polymerase chain reaction. To verify the radioadaptive effect of LD, animals were preirradiated at 10 cGy from a ^{60}Co source and then challenge dose at 200 cGy was delivered. Independent sample student's *t*-test was employed to compare cytokine gene expression in radioadaptive (10 + 200 cGy), LD (10 cGy), high-dose (HD, 200 cGy), and control groups of animals.

Results: Following the HD, the cytokine gene expression of IFN-γ, IL-4, and TGF-β was significantly decreased compared to the control group ($P = 0.0001$). However, TGF-β expression was also decreased significantly in the LD and adaptive groups compared to the control group ($P = 0.0001$). IFN-γ/IL-4 ratio in the adaptive group was significantly decreased compared to the HD group ($P = 0.0001$).

Conclusion: These results indicate that the immune system plays an important role for radioadaptive response induction by LD radiation to adjust the harmful effects of HD irradiation.

KEY WORDS: Gene expression profiling, immune system, low-dose ionizing radiation, radioadaptive response

INTRODUCTION

Adaptive response (AR) is an important biological effect following low-dose (LD) ionizing radiation (IR) which for the first time was confirmed by Olivieri *et al*.[1] This protective phenomenon has been observed in both normal and tumor cells.[2] This is interpreted as a resistive reaction of cells to a high dose (HD) when they initially are exposed to a LD. AR was approved by studying various endpoints including DNA damage, cellular damage, micronucleus formation, chromosomal aberration, neoplasm formation, or apoptosis induction. Some studies have linked AR to the immune system, repair mechanisms, and molecular pathways.[3-6] The value of LD and HD, dose rate, and the time interval between LD and HD have an effect on AR outcome.[7] Helper T-cells are possibly the most important cells in adaptive immunity, as they are required for almost all adaptive immune responses.[8,9] T helper (Th1) cells produce interleukin (IL)-2 and interferon (IFN)-γ that organize cell-mediated immunity against intracellular pathogens and tumors.[1,10] Th2 cells produce IL-4, IL-5, and IL-13. The two helper T-cell sessions also differ by the type of immune response they produce. While Th1 cells tend to produce responses against intracellular parasites such as bacteria and viruses, Th2 cells produce immune responses against helminths and other extracellular parasites and involving allergic reaction such as asthma and atopic dermatitis.[11,12]

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Moreover, the Th1/Th2 concept suggests that modulation of the relative contribution of Th1- or Th2-type cytokines makes possible to regulate the balance between protection and immunopathology, as well as the development and/or the severity of some immunologic disorders, for example, multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis.[8,13,14] Due to the sensitivity of the immune system to IR, it is a critical system that plays a functional and decisive role in the development of long-term effects, including leukemia and tumor formation.[15] Hence, several studies have investigated the effects of LD and HDIR on the immune system and the changes of gene expression levels both *in vivo* and *in vitro*.[5] Furthermore, some investigators have studied DNA damage of mouse spleen cells.[16,17] Immune response to IR depends on many factors such as dose and dose rate.[18] Suppression of the immune system and induction of inflammatory processes are common effects of HDIR;[19] however, the effects of LD radiation on the immune system response are not clear and are a controversial issue.

The aim of this study was to investigate the impact of adaptive radiation response on the immune system in mice. For this purpose, a number of important parameters in the immune system such as gene expression of Th1, Th2, and Treg cytokines were examined.

MATERIALS AND METHODS

Experimental animals and radiation

Seven-eight week-old male BALB/c mice, weighting 23–25 g, were purchased from Pasteur Institute of Iran for this study. The animals were acclimated for 2 weeks and then randomly divided into four groups. They were housed in a specific-pathogen-free environment of the laboratory. The mice were divided into four groups and were total-body irradiated to a total dose of 0, 0.1, 2, and 0.1 + 2 (Gy) by a ^{60}Co teletherapy unit (Theratron, Phoenix Model, Canada) at Source-Skin Distance (SSD) = 80 cm and field size = 7 × 18 cm^2. Dose rates were 449 mGy/min and 31 mGy/min for delivering HD and LD, respectively. Dosimetry was performed with a Farmer-type 0.6 cm ionization chamber and a Farmer 2581 electrometer.

Isolation of lymphocytes

Mice were killed 24 h after irradiation, and spleens were mechanically extracted; erythrocytes were lysed with 1.66% ammonium chloride solution. Cells were resuspended in RPMI-1640 culture medium supplemented with 5% Fetal Calf Serum (FCS), 10 mM Hepes, 20 mM beta-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2.5 mg/ml amphotericin B. The number of viable splenocytes was determined using a microscope with trypan blue exclusion of dead cells. Single-cell suspensions of splenocytes were used for subsequent immunological measurements.

Measuring cytokine expression of unfractionated total splenocytes by real-time reverse transcription-polymerase chain reaction

Mouse spleens were removed 24 h following irradiation. RNAs were isolated from the unfractionated lymphocytes. Total cellular RNA was prepared using the TriPure reagent (Roche Applied Science, Germany) followed by chloroform (Merck, Darmstadt, Germany) extraction and isopropanol (Merck, Darmstadt, Germany) precipitation. The concentration and purity of the RNA were determined by spectrophotometry and denaturing gel electrophoresis. A total of 10 µl RNA was subjected to cDNA synthesis using a kit (RevertAid™ First Strand cDNA Synthesis Kit, Fermentas, Waltham, MA, USA) according to the manufacturer's protocol and used for reverse transcription-polymerase chain reaction (RT-PCR). The RT-PCR reaction mix was as follows: 1.5 µl cDNA, 0.3 µl forward primer (10 pM), 0.3 µl reverse primer (10 pM), 0.3 µl of ROX™, and 7.5 µl of SYBR Premix® Ex Taq™ qPCR Master Mix (Takara, Burlington, Japan), and 5.1 µl water. Reactions were performed in a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

The cycling profile of the reaction mix was as follows: initial denaturation at 95°C for 10 s (for 40 cycles) and finally 60°C for 30 s. After each run, the result was analyzed automatically once, with StepOne software v. 2.1 (Applied Biosystems, Foster City, CA, USA). The relative standard curve method was applied for cDNA quantification. This approach gives rise to highly accurate quantitative results (quantity of an unknown sample is acquired from interpolation of the standard curves. The curve produced from the same samples for each plate). The quantity of the target genes was normalized by the quantity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Table 1: Sequence of primers used for real-time polymerase chain reaction

Mouse gene	Sequence of primers (5'-3')	PCR product size (bp)
IFN-γ	F: GAACTGGCAAAAGGATGGTGA R: GTGTGATTCAATGACGCTTATGTT	127
IL-4	F: ACCACAGAGAGTGAGCTCGTCT R: TGAATCCAGGCATCGAAAAG	147
TGF-β	F: CGGACTACTATGCTAAAGA R: CTGTGTGAGATGTCTTTG	88
GAPDH	F: AACTCCCATTCTTCCACCTTTG R: CTGTAGCCATATTCATTGTCATACCAG	98

IFN-γ=Interferon-gamma, IL-4=Interleukin-4, TGF-β=Transforming growth factor-beta, GAPDH=Glyceraldehyde 3-phosphate dehydrogenase, PCR=Polymerase chain reaction

as the endogenous control gene (GAPDH was used as the housekeeping gene). To obtain the final relative quantity, the normalized quantity of the treated samples was compared with the normalized quantity of the control sample. The sequences of primers used in the RT-PCR reactions are shown in Table 1.

Statistical analysis

The data were analyzed using the SPSS program version 16. (SPSS Inc., Chicago, IL, USA). Independent sample Student's *t*-test was employed to assess if the results obtained for different groups were statistically significant ($P < 0.05$).

Ethical considerations

The ethical issues about the maintenance and care of experimental animals comply with the National Institutes of Health guidelines for the humane use of laboratory animals.

RESULTS

This study examined the effect of LD (0.1 Gy), HD (2 Gy), and AR (0.1 + 2 Gy) on some immune system parameters, as shown in Figure 1.

Four groups of mice received 0, 0.1, 2, and 0.1 + 2 Gy of gamma-radiation from a cobalt-60 radiation source, respectively. Then, the spleens of mice were removed; increased dose reduced the number of lymphocytes in mouse spleen [Figure 2]. In all irradiated groups, the reduction in lymphocyte number was significant compared to the control group ($P < 0.001$). However, a significant increase was observed in cell number in the AR group (0.1 + 2 Gy) relative to the HD group.

Effects of low-dose and high-dose gamma-radiation and adaptive response on expression of interleukin-4, interferon-γ, and transforming growth factor-β genes

First, lymphocytes were counted, RNAs of mouse lymphocytes were isolated, cDNA was synthesized, and then, relative values of IL-4, IFN-γ, and transforming growth factor (TGF)-β were obtained by quantitative RT-PCR.

There were no significant differences in expression of IL-4, IFN-γ, and TGF-β between the LD and control groups [Figure 3].

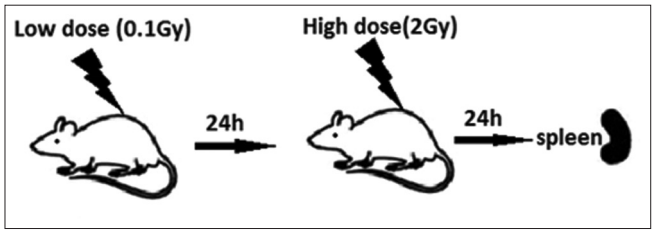

Figure 1: Experimental design: BALB/c mouse lymphocyte was examined for radioadaptive response after exposures. Radioadaptive response experiments, low-dose radiation was delivered as a low dose to mice before a subsequent high-dose exposure, and mice were euthanized 24 h after the high dose

However, in the HD radiation group, IL-4 and IFN-γ gene expression was significantly reduced ($P < 0.0001$) and also TGF-β was significantly decreased ($P < 0.001$) [Figure 3a-c]. Interestingly, AR effect induced significantly different expression between the HD and AR groups for all genes ($P < 0.0001$). The results, as shown in Figure 3d, indicate that IFN-γ/IL-4 ratio in the LD group showed no significant increase relative to the control group. In the HD group, IFN-γ/IL-4 ratio showed a significant increase compared to the control group ($P < 0.001$). In the AR group, increased expression ratio was not significant relative to the control group and significantly decreased compared to the HD group ($P < 0.0001$) [Figure 3d].

DISCUSSION

Over the past two decades in addition to previously well-known effects of LD (cancer and genetic effects), it has been revealed that LDs may initiate molecular changes in irradiated and nonirradiated cells. The endpoints of these changes are known as increasing radiation resistance, AR, bystander effect, hypersensitivity, and death-inducing factor activation.[8,9,20-22] The principle of AR is the biological process that pretreatment by LD radiation can make an organism adapt to subsequent HD radiation and reduce the damage caused by HD irradiation in normal tissue.[21] The underlying molecular mechanisms of protective biological effects of LD radiation mainly involve enhanced DNA repair, stimulated immune regulation, and changing expression of some genes.[23-26] AR indicates reduced harmful effects of HD exposure followed by a LD, confirming the usefulness of LDs. In the present study, we have examined the consequence of AR on immune system activity in mice by figuring out the expression levels of IFN-γ, IL-4, and TGF-β cytokines.

First, the number of lymphocytes in the spleen of irradiated mice was counted; it revealed reduction in number of cells

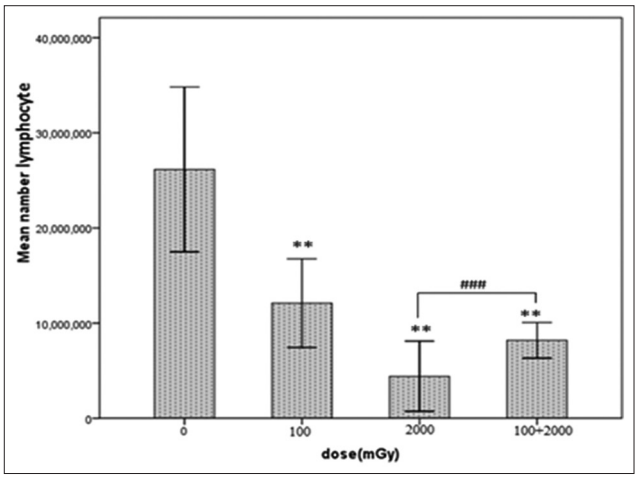

Figure 2: Alterations in the cell number of lymphocyte populations following γ-irradiation. Error bars represent standard deviations. Significance of differences from 0 Gy for the corresponding dose is indicated by **($P < 0.001$) and significance of difference between the adaptive and high-dose groups is indicated by ###($P < 0.0001$)

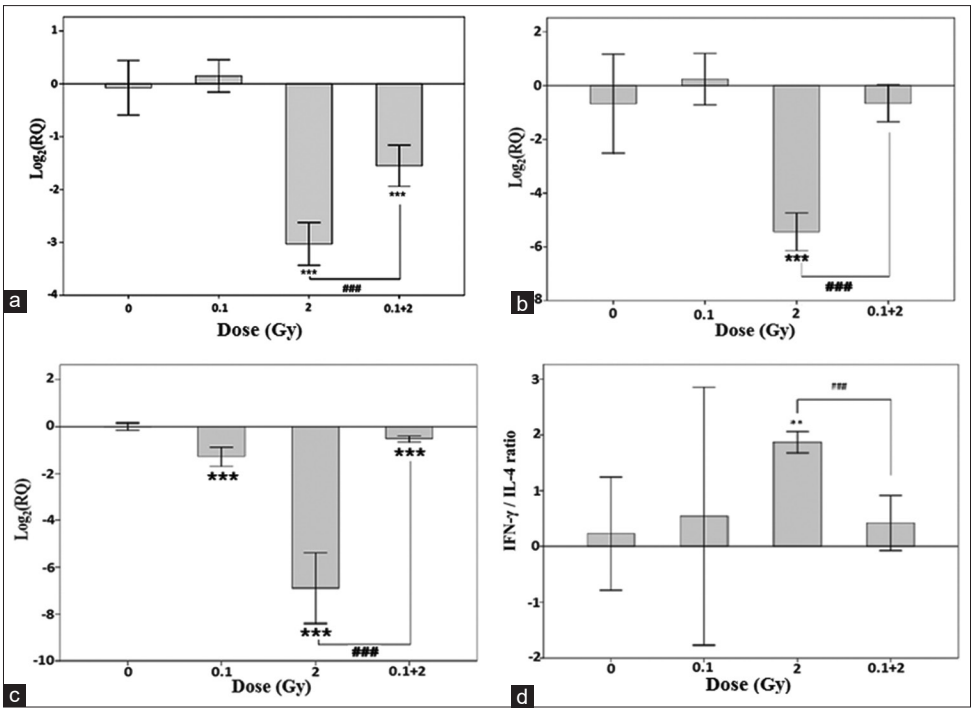

Figure 3: Changes in the cytokine expression profile of interleukin-4 (a), interferon-γ (b), transforming growth factor-β (c), and interferon-γ/interleukin-4 (d) in mice after irradiation by different doses. RNA was isolated and alterations in the cytokine expression patterns were studied by quantitative reverse transcription-polymerase chain reaction. The error bars represent standard deviations. Significance of differences from 0 Gy for the corresponding cytokine is indicated by ***($P < 0.0001$) and significance of differences between the adaptive and high-dose groups is indicated by ###($P < 0.0001$)

in all three irradiated groups following exposure, as shown in Figure 2. However, According to our observations in the AR group, delivery of a HD followed by a LD of IR caused a significant increase in number of cells relative to the HD group. The decreased number of dead cells observed per spleen of the AR group mice compared to those of the HD group is an indication of potential radioadaptive effect, although it is not clear whether the decrease in the number of cells in the BALB/c strain in our study was due to a decrease in lymphocyte apoptosis or necrosis. It may be noted that exposure to LDs can cause adaptation that these results are in agreement with previous studies.[22,27-29] In addition, in this study, a reduction in BALB/c mouse spleen cellularity was also observed following 2 Gy irradiation, which was also consistent with previous reports.[30,31]

Radiation-induced changes in gene expression are usually considered to be early events. Gene expression profiling of immune system cytokines indicates that exposure to low doses of ionizing radiation causes a significant reduction in TGF-β, which is in agreement with the results of Bogdándi *et al*.[5] TGF-β acts as a potent immunosuppressive cytokine, and it influences on both cell differentiation and cell proliferation. In this study, following HD radiation after 24 h of exposure, the expression of TGF-β was significantly decreased and LD before HD reduced this effect and thus increased the immunosuppressive effects. On the other hand, exposure to HD increased apoptosis[32] and caused a substantial decrease in IL-4 and IFN-γ which, in turn, can confirm the immune system suppression as a result of receiving HDs. Our results about gene expression alteration caused by a HD are in agreement with the study of Han *et al*.[33] Similar gene expression for TGF-β and IL-4 was reported by Bogdándi *et al*.[5] In the AR group after 24 h, the expression of IL-4 was significantly decreased compared to the control group. Eventually, it can be concluded that a LD before HD decreased the enhancement of immunosuppressive effects which can be occurred due to high dosage. Treg cells have indispensable roles in immunological tolerance and protect the host from autoimmune disease and allergies as well as insignificant increase in the expression of IL-4 and IFN-γ [Figure 3a and b]. The balance between inflammatory and anti-inflammatory signals is reflected by the expression patterns of IFN-γ and IL-4. Immune system responses play an important role in balance function of the human body and are divided into two groups: Th1 and Th2. Th cells represent a functionally heterogeneous population, comprising distinct subsets termed Th1 and Th2 defined by their cytokine secretion profiles. In general, cytokines produced by Th1 cells (e.g., IFN-γ and IL-2) promote both production of complement-fixing and opsonizing antibodies and macrophage activation. Cytokines produced by Th2 cells (e.g., IL-4, IL-5, IL-6, IL-10, and IL-13) stimulate antibody production and promote mast cell and eosinophil granulocyte differentiation and activation. Th1 immune responses improve the cellular immune response, but Th2 immune responses lead to enhancement of humoral immunity, production of antibodies as well as allergic

reactions. Disrupted balance between Th1/Th2 (i.e., IFN-γ/IL-4 ratio) with immune-regulatory cytokines predisposes the body to diseases such as autoimmunity, chronic infections, severe depression, and atherosclerosis and can cause allergy as well as increasing its severity. Changing balance of the immune system plays an important role in pathophysiology of some diseases, including asthma, autoimmunity, and cancer.[4] In this study, the expression ratio of IFN-γ/IL-4 genes was significantly increased after a HD. According to previous descriptions, the immune response shifts toward higher production of Th1 lymphocytes and enhances the cellular immune response, which may deduction that the patient will be susceptible to autoimmune diseases after receiving the HDs.[34] Fang *et al.* observed reduced IFN-γ/IL-4 ratio by studying the effect of a 0.5 Gy of gamma-radiation on cytokine levels in mice.[35] Chronic exposure to LDs with a total dose of 0.2 Gy also reduced the ratio of IFN-γ/IL-4 compared to corresponding values in the control group.[36] On the other hand, our result showed that LD did not cause a significant change in this ratio. In the AR group, the IFN-γ/IL-4 ratio was significantly reduced compared to the HD group. This is indicating that the LDs delivered before the HD have neutralized the harmful effects of challenge dose. The immune response has not shifted toward Th1 and/or Th2 responses. Consistent with these results, in our previous study, it was shown that LD can cause radioresistancy in lymphocytes.[29] As some of the previous studies that were conducted to investigate the adaptive effect and showed that this phenomenon can occur,[2,16,37] our study can confirm the induction of such response in the immune system.

CONCLUSION

In this study, we have investigated the *in vivo* AR of the immune system. For this purpose, some of the important cytokines in the immune response that are representing immune system functions were measured. The results of the present study have shown that LD causes resistance in lymphocytes. Induction of AR by LD was previously seen in Th1 and Th2 cells and causes changes in the expression of genes affecting these two main cell types of the immune system. Adaptive dose leads to reduced level of inflammatory cytokines and prevents the shift of immune response, minimizing the possibility of autoimmune and inflammatory diseases as well as allergic reactions after receiving the HD. The results of this work further confirm that AR does happen.

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Conflicts of interest

There are no conflicts of interest.

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