Up-regulation of Bcl-2 expression in cultured human lymphocytes after exposure to low doses of gamma radiation

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ABSTRACT

Lymphocytes have demonstrated complex molecular responses to induced stress by ionizing radiation. Many of these reactions are mediated through modifications in gene expressions, including the genes involved in apoptosis. The primary aim of this study was to assess the effects of low doses of ionizing radiation on the apoptotic genes, expression levels. The secondary goal was to estimate the time-effect on the modified gene expression caused by low doses of ionizing radiation. Mononuclear cells in culture were exposed to various dose values ranged from 20 to 100 mGy by gamma rays from a Cobalt-60 source. Samples were taken for gene expression analysis at hours 4, 24, 48, 72, and 168 following to exposure. Expression level of two apoptotic genes; BAX (pro-apoptotic) and Bcl-2 (anti-apoptotic) were examined by relative quantitative real-time polymerase chain reaction (PCR), at different time intervals. Radio-sensitivity of peripheral blood mononucleated cells (PBMCs) was measured by the Bcl-2/BAX ratio (as a predictive marker for radio-sensitivity). The non-parametric two independent samples Mann–Whitney U-test were performed to compare means of gene expression. The results of this study revealed that low doses of gamma radiation can induce early down-regulation of the BAX gene of freshly isolated human PBMCs; however, these changes were restored to near normal levels after 168 hours. In most cases, expression of the Bcl-2 anti-apoptotic gene was up-regulated. Four hours following to exposure to low doses of gamma radiation, apoptotic gene expression is modified, this is manifested as adaptive response. Modification of these gene expressions seems to be a principle pathway in the early radioresistance response. In our study, we found that these changes were temporary and faded completely within a week.

Key words: Apoptosis; deoxyribose nucleic acid damage; gene expression; ionizing radiation

Introduction

Nowadays human beings are exposed to various sources of ionizing radiation. The level of radiation exposure from environmental and man-made sources (particularly occupational exposure) is measured by various personal dosimeters such as thermoluminescent dosimeter (TLDs), films, and other devices or methods. However, physical dosimeters are not capable to assess unplanned or accidental exposures from occupational, therapeutic or environmental sources. In such circumstances, quantification of biological changes at cellular or molecular levels in the living media may be more appropriate techniques. It is generally believed that deoxyribonucleic acid (DNA) is the main target when ionizing radiation interactions occur within cells. Thus, principal cell injuries following interactions of ionizing radiation can be attributed to the DNA damages.[1] Cells’ response following DNA damage include cell cycle arrest,[2] DNA repair,[1] and programmed cell death (apoptosis).[4,5] Ionizing-radiation–induced apoptosis involves the B-cell lymphoma-2 (Bcl-2) family and is mediated by extrinsic and intrinsic pathways. The latter is the principal cause
of apoptosis, and the Bcl-2 family proteins are the most important component of this pathway. Pro-apoptotic members of Bcl-2 family proteins, such as Bcl-2–associated X protein (BAX) induce mitochondrial outer membrane permeabilization to cause cytochrome-c release, whereas anti-apoptotic members such as Bcl-2 act as protectors of the outer membrane and preserve its integrity by opposing BAX function. These cellular endpoints are considered as biological markers of radio-sensitivity by some researchers. Most of these reactions are mediated through modifications in gene expression, to prevent the conversion of abnormal DNA structures into inheritable mutations, and minimize survival of cells with irreparable damage. However, the available data are not consistent and molecular responses to low doses of radiation are poorly characterized. In recent years, variation of gene expression level has been suggested as a biological indicator of ionizing radiation exposure level. In some studies, performed on lymphocytes suggest gene expression level are time-dependent. A good knowledge of post-irradiation history of a biomarker would help to assess absorbed dose, regardless of the elapsed time between exposure and sampling. In vitro studies, mostly have been carried out on peripheral blood lymphocytes (PBL), and the elapsed time between irradiation and gene expression assessment varies from 15 minutes to 72 hours.

However, human health risks from exposure to low doses of ionizing radiation remains completely vague, and is the subject of intense debates. In the present study, we have used PBLs to investigate the effect of elapsed time on the modification of gene expression level up to one week following to low doses of ionizing radiation.

Materials and Methods

Blood sampling and lymphocyte culture

Fifty milliliter whole blood samples were collected in individual tubes containing Ethylene Diamine Tetra-acetic acid (EDTA) from male volunteers. PBMCs were separated by Ficoll (Cedarlane) according to the recommendations of the manufacturer. The mononuclear cell layer was removed and washed in Phosphate Buffer Saline (PBS). The cells were counted and re-suspended in a 10 ml culture medium, including RPMI 1640 (Gibco) containing 20% FBS (Biosera), 1% of 200 mM L-glutamine (Biosera), 100 IU/ml penicillin, and washed in Phosphate Buffer Saline (PBS). The cells were transferred to four 25 cm² flasks (Spl) in a final cell concentration of 1 × 10⁶ cells/ml. Blood sampling and lymphocyte culture

Gamma irradiation

Dosimetry study was performed with Farmer-type 0.6 cm² ionization chamber with a Farmer 2581 electrometer. Each sample was divided into four flasks. Three flasks were exposed to a Co-60 (Phoenix TheraTron) source at SSD = 80 cm and 13.7 mGy/min. A lead attenuator was used to reduce the output dose rate of the source. This arrangement made it possible to deliver 20, 50, and 100 mGy to a selected flask in a practical time interval. The fourth flask containing the sham control group did not receive any radiation, which was treated exactly similar to the other three flasks. One percent Phytolhaemagglutinin (PHA) (Gibco) was added to the culture medium following to irradiation, and all irradiated and control samples were incubated at 37°C with 5% CO₂ for 1 week as suggested by Falt et al.

RNA preparation

To study patterns of apoptotic genes’ expression in human peripheral blood, lymphocytes were collected for ribonucleic acid (RNA) isolation from cultures 4 hours, 24 hours, 48 hours, 72 hours, and 168 hours following irradiation. The results are expected to help understanding early and delayed apoptotic response to ionizing radiation.

Approximately 3 × 10⁸ lymphocytes were taken for the subsequent isolation of RNA using the TriPure reagent (Roche Applied Science, Germany). The lymphocyte pellets were transferred into 750 µl TriPure reagent. Each sample was incubated for 5 minutes at room temperature and then 150 µl Chloroform (Merck, Darmstadt, Germany) was added to it. After vigorous manual shaking for a further 15 seconds, all samples were incubated at room temperature for 10 minutes. To separate the solution into three phases, the mixture was centrifuged at 12,000 g for 15 minutes at 4°C. The colorless upper aqueous phase was transferred to a new centrifuge tube and 350 µl Isopropanol (Merck, Darmstadt, Germany) was added to it, then samples were incubated at room temperature for 10 minutes to allow the RNA precipitate to form. The solution was centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was discarded, and the RNA pellets were washed with 75% Ethanol (Merck, Darmstadt, Germany), air-dried, and then resuspended in 20 µl DiEthylPyroCarbonate (DEPC)-treated RNase-free water.

cDNA synthesis

Complementary DNA (cDNA) synthesis was performed in a total volume of 20 µl, containing 200 U of M-MuLV Reverse Transcriptase, 4 µl of 5 × Reaction Buffer, 20 U of Ribolock™ RNase inhibitor, 2 µl of Deoxyribonucleotide triphosphate (final 1 mM), 1 µl of oligo (dt) 18 primer (0.5 µg), and 1 µg of total RNA. The prepared template with the control Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA and GAPDH control primers (1.3 kb) were used as a positive control, and no negative template control was prepared with total reagent for the reverse transcription reaction, except for the RNA template. cDNA was synthesized according to the manufacturer recommendations (RevertAid™ First Strand cDNA Synthesis Kit, Fermentas). Each cDNA was amplified by control polymerase chain reaction (PCR) reaction using primers for the GAPDH according to the manufacturer protocol (Prime Taq DNA polymerase, Genet

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Bio, South Korea). Five microliter of PCR product was loaded on 1% agarose gel and a distinct 496 bp was observed after Ethidium Bromide staining.

**Gene expression analyses by real-time PCR**

Real-time RT-PCR reactions were carried out in a MicroAmp™ Fast Optical 48-well reaction plate with Optical Adhesive Film (Applied Biosystems) as duplicates in a total volume of 15 μl, containing cDNA (1.5 μl), forward and reverse primers (300 nM), SYBR® Premix Ex Taq™ (7.5 μl) and ROX™ Reference Dye II (0.3 μl) (Takara, Japan) and dH2O 5.1 μl. Gene expression assessments were performed on a StepOne (48-well) Real-Time PCR system (Applied Biosystems). The samples were thermally cycled under the following conditions: First holds at 95°C for 60 seconds, followed by cycling at 95°C for 10 seconds (for 40 cycles) and finally 60°C for 50 seconds. After each run, the result was analyzed automatically once, with Step One software v. 2.1 (Applied Biosystems). The relative standard curve method was applied for cDNA quantification. This approach gives rise to a 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)109. The quantity of the target genes was normalized by quantity of Beta-2 Microglobulin (β2M) as the endogenous control gene (β2M was used as the housekeeping or “stably expressed” gene). To obtain final relative quantity (RQ); the normalized quantity of the treated samples were compared with the normalized quantity of the control sample. Primers were purchased from Metabion (Martinsried, Germany). Primer sequences were: β2M forward 5'-GTA TGC CTG CCG TGT GAA C-3', reverse 5'-AAC CTC CAT GAT GCT CCT TAC-3'; Bel-2 forward 5'-TAC TTA AAA AAT ACA ACA ACA TCA CAG-3', reverse 5'-CGA ACA CCTT GAT CCT CGT G-3'; BAX forward 5'-GGT GGG TTT CAT CCT CCA G-3', reverse 5'-GCG GGC AAT CAT CCT CTG-3'.

**Results**

Human whole blood samples were collected from four healthy volunteers, without any history of radiation therapy and they received no medication during this study [Table 1]. Relative quantitative RT-PCR method was employed to compare gene expression levels of treated and control groups. The results were expressed as the means and standard errors of the means (S.E.). All data were analyzed by Statistical Package for the Social Sciences (SPSS) 11.5 software. Mann–Whitney U-test was performed to compare average of gene expression level for irradiated and control groups, a P value ≤ 0.05 was considered statistically significant.

**Effects of low-dose gamma radiation on Bel-2 and BAX genes**

The results indicate that when lymphocytes are exposed to 20, 50, and 100 mGy of gamma radiation; down-regulation is induced for BAX pro-apoptotic gene 4 hours, 24 hours, 48 hours, and 72 hours following irradiation [Figure 1a-d]. Expression of Bel-2 anti-apoptotic gene was found to be up-regulated at doses as low as 20 mGy after 48 hours, 72 hours, and 168 hours following irradiation [Figure 1c-e]. The results also show that the Bel-2 expression was induced by 50 mGy at 24 hours [Figure 1b], 72 hours [Figure 1d], and 168 hours [Figure 1c] following irradiation. In addition, up-regulation of Bel-2 expression was induced by 100 mGy at 4 hours, 24 hours, 48 hours, 72 hours, and 168 hours after irradiation [Figure 1a-e].

**Effect of elapsed time on Bel-2 and BAX gene expression following exposure**

In this study, the effect of elapsed time following exposure was also examined. Early down-regulation of BAX gene expression induced by 20, 50, and 100 mGy; 4 hours following irradiation is shown in Figure 2a-c. This phenomenon was terminated 168 hours after delivery of 20 and 50 mGy. Maximum reduction in BAX gene expression was observed 4 hours following to 20 and 100 mGy [Figure 2a and b], similar result was observed 24 hours after 50 mGy [Figure 2c]. Low doses of gamma gave rise to up-regulation of Bel-2 expression. The highest increase was noticed at 72 hours following to 50 mGy [Figure 2b], this was also true for delivery of 100 mGy at 24 hours after irradiation [Figure 2c].

**Low-dose irradiation alters radio-sensitivity**

The Bel-2/BAX ratio has been introduced as a predictive marker for therapeutic response to radiotherapy and radio-sensitivity.20,22 Particularly, the high ratio is considered as a crucial factor of cell resistance to apoptosis. This means that the sensitive cells are characterized by low Bel-2/BAX ratio and resistant cells by high ratio.23 In other words, low doses of gamma radiation reduces radio-sensitivity of human peripheral blood lymphocytes. This is shown in Figure 3 (a-c). Maximum rise in the Bel-2/BAX ratio was observed 4 hours following to 20 and 100 mGy. Nevertheless, this phenomenon changed course and was almost eliminated 168 hours after 20 mGy gamma irradiation. However, this result may reflect the inter-individual variability between the donors [Table 1].

**Conclusion**

Bel-2 family proteins control the cell response to radiation and regulate apoptosis.24,25 Anti-apoptotic members of Bel-2–family proteins are Bel-2, Bel-xl, Bel-w, Mcl-1, Bfl1/A-1, and Bel-B. Also, some pro-apoptotic members of the Bel-2–family proteins are BAX, Bak, and Bok.26 The intrinsic pathway of apoptosis by adjusting the release of mitochondrial proteins, including cytochrome-c (cyt. c) controls the occurrence of apoptosis.

The maximal induction appeared between 2–3 hours post-exposure for BAX in the human myeloid leukemia cell
line ML-1 that was irradiated at approximately 51 mGy/min (total doses of 20–500 mGy of gamma rays), then rapidly declined to near control levels within 24 hours. While our study demonstrated that the expression peak of BAX is decreased at lower dose rates (13.7 mGy/min) (total doses of 20 and 100 mGy) at 4 hours post-exposure, also for 50 mGy at 24 hours post-exposure. On the other hand, a significant decrease in the expression level of BAX was observed in human PBMCs 4 hours post-exposure to 100 mGy. Although these changes faded with elapsed time, but a significant reduction in the expression level of the BAX gene was still noticed 72 hours following to 100 mGy dose.

In previous studies, the induction kinetics of apoptosis in vitro indicated that a maximum is reached approximately 72 hours after irradiation; in another study, a significant induction of apoptosis in T-cells was observed 44 hours after delivery of 300 mGy.

### Table 1: Characterization of donors, gene expression level (RQ) and Bcl-2/BAX ratio

| Age (years), sex, Smoking habit | Dose (mGy) | BAX 4 h | BAX 24 h | BAX 48 h | BAX 72 h | BAX 168 h | Bcl-2 4 h | Bcl-2 24 h | Bcl-2 48 h | Bcl-2 72 h | Bcl-2 168 h |
|--------------------------------|------------|---------|----------|----------|----------|-----------|----------|-----------|----------|-----------|-----------|
| 25, M No                       | 20         | 0.46    | 0.41     | 0.34     | 0.93     | 0.48      | 1.35     | 1.01      | 1.66     | 1.08      | 1.31      |
|                               | 50         | 0.86    | 0.60     | 0.41     | 0.72     | 0.38      | 1.47     | 1.80      | 1.07     | 2.06      | 1.00      |
|                               | 100        | 0.40    | 0.39     | 0.68     | 0.66     | 0.55      | 1.10     | 2.14      | 1.74     | 1.20      | 1.11      |
| 24, M No                       | 20         | 0.13    | 0.32     | 0.79     | 1.01     | 3.70      | 0.50     | 0.87      | 0.74     | 1.03      | 1.09      |
|                               | 50         | 0.32    | 1.12     | 0.34     | 0.69     | 1.92      | 0.37     | 1.04      | 0.66     | 2.10      | 0.85      |
|                               | 100        | 0.51    | 0.99     | 0.34     | 0.55     | 1.24      | 0.77     | 1.35      | 0.85     | 0.88      | 1.07      |
| 25, M No                       | 20         | 1.13    | 1.06     | 0.61     | 0.37     | 0.64      | 1.85     | 0.56      | 0.96     | 1.14      | 0.92      |
|                               | 50         | 1.29    | 0.65     | 1.01     | 2.26     | 2.56      | 1.40     | 0.94      | 1.08     | 1.18      | 1.56      |
|                               | 100        | 0.55    | 1.96     | 0.74     | 0.99     | 0.27      | 1.59     | 2.14      | 1.09     | 1.04      | 1.01      |
| 25, M No                       | 20         | 0.57    | 0.62     | 1.03     | 1.13     | 0.93      | 0.69     | 1.37      | 1.17     | 1.27      | 1.15      |
|                               | 50         | 1.23    | 0.20     | 2.64     | 0.62     | 1.31      | 1.03     | 0.93      | 1.07     | 1.66      | 0.98      |
|                               | 100        | 0.95    | 0.52     | 2.67     | 0.99     | 1.72      | 1.27     | 0.68      | 0.95     | 1.26      | 1.27      |

The values shown are normalized relative to the untreated controls. RQ: Relative quantification, M: Male, h: Hour
Figure 2: Effect of elapsed time on relative quantification (RQ) after irradiation to (a) 20 mGy, (b) 50 mGy, and (c) 100 mGy. Gene expression results are presented in terms of the base-2 logarithm of the RQ; positive and negative values represent increased and decreased gene expression level, respectively. Each point represents the mean of four individual experiments and error bars show the S.E.

Figure 3: Effect of elapsed time on Bcl-2/BAX ratio (a) 20 mGy, (b) 50 mGy, and (c) 100 mGy post-irradiation. Each point represents the mean of four individual experiments and error bars show the S.E.
BAX as a pro-apoptotic member of Bcl-2–family proteins, induces mitochondrial outer membrane permeabilization, to cause cytochrome-c release and the occurrence of apoptosis. These results show that low doses of ionizing radiation lead to reduction of mitochondrial cytochrome-c release and prevent apoptosis. However, in already studies, the lowest dose level at which the radiation-induced apoptosis frequency was still significantly above control was 50 mGy.[28,31]

Some studies have demonstrated down-regulation of Bcl-2 expression and up-regulation of BAX expression following high doses of ionizing radiation.[22] However, the result of this study indicates that the expression of BAX was found to be down-regulated at low doses of ionizing radiation, and Bcl-2 was up-regulated by 20 mGy at 48 hours, 72 hours, and 168 hours, by 50 mGy at 24 hours, and also by 100 mGy, until a week after irradiation. Bcl-2 works as a protector of the outer membrane and preserves its integrity by opposing BAX.[30] Bcl-2 expression is appreciably increased 72 hours following irradiation to 20 and 50 mGy, same effect is observed 168 hours after PBL samples is irradiated by 100 mGy (P < 0.05). Furthermore, there were no more statistically significant difference between BAX and Bcl-2 expression levels of irradiated and control samples. On the other hand, over expression of the Bcl-2 protein has been reported for many types of human cancers, including leukemia, lymphoma, and carcinoma.[31]

The Bcl-2/BAX ratio was significantly increased 4 and 72 hours following irradiation 20 mGy, and 4 hours, 24 hours, and 72 hours following irradiation 100 mGy (P < 0.05).

The results of the present study have shown that low doses of gamma radiation can increase the Bcl-2/BAX ratio. This means that, although high doses of gamma radiation can cause apoptosis, the observed increase of the Bcl-2/BAX ratio is an indication that low doses of gamma radiation cause reduction of lymphocyte radio-sensitivity. Several researchers assessed cell radio-sensitivity by examining the level of apoptosis, they realized that it depends not only on dose value, but also on post-exposure elapsed time.[20,34] Some studies have suggested time-dependent expression,[36] particularly in the first 5 hours and a return to the base-line within 20 hours.[35] But, in our study, these changes remained for a much long time and gradually faded over a week. Although, biological responses to low-dose ionizing radiation are exhibited in several ways, adaptive response is considered one of them.[30] Induction of radio-adaptive response by low-level ionizing radiation was previously reported for protection against radiation induced chromosomal aberrations in human lymphocytes.[37] Our results could support the hypothesis that the radioresistance effects of low dose exposure may be due to the up-regulated expression of Bcl-2 genes that may provide a survival advantage. Although other cellular radio-protection mechanisms may be involved, such as alterations in the levels of some cytoplasmic and nuclear proteins, DNA repair, and other processes. There are some limitations in this study, the main one being the interindividual variability between the donors. It has to be emphasized that the results presented in this article are preliminary results of an ongoing research work.

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