▣ PAPER ▣

THE SHORT-TERM EFFECTS OF LOW-DOSE-RATE RADIATION ON EL4 LYMPHOMA CELL

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To determine the biological effects of low-dose-rate radiation (^{137}Cs, 2.95 mGy/h) on EL4 lymphoma cells during 24 h, we investigated the expression of genes related to apoptosis, cell cycle arrest, DNA repair, iron transport, and ribonucleotide reductase. EL4 cells were continuously exposed to low-dose-rate radiation (total dose: 70.8 mGy) for 24 h. We analyzed cell proliferation and apoptosis by trypan blue exclusion and flow cytometry, gene expression by real-time PCR, and protein levels with the apoptosis ELISA kit. Apoptosis increased in the low-dose-rate irradiated cells, but cell number did not differ between non- (Non-IR) and low-dose-rate irradiated (LDR-IR) cells. In concordance with apoptotic rate, the transcriptional activity of ATM, p53, p21, and Parp was upregulated in the LDR-IR cells. Similarly, Phospho-p53 (Ser15), cleaved caspase 3 (Asp175), and cleaved Parp (Asp214) expression was upregulated in the LDR-IR cells. No difference was observed in the mRNA expression of DNA repair-related genes (Msh2, Msh3, Wrn, Lig4, Neil3, ERCC8, and ERCC6) between Non-IR and LDR-IR cells. Interestingly, the mRNA of Trfc was upregulated in the LDR-IR cells. Therefore, we suggest that short-term low-dose-rate radiation activates apoptosis in EL4 lymphoma cells.

Keywords: Low-dose-rate irradiation, EL4 lymphoma cell, Apoptosis

1. INTRODUCTION

The effects of high-dose radiation on cell metabolism include damage to DNA resulting in irregular signal transduction that stimulates carcinogenesis. The mutagenic effects of irradiation increase the probability of oncogenic mutation, and irradiation can also work as a tumor promoter and induce mutations in Notch1, or disrupt mutations in p53 [1], a protein that acts as a signaling mediator for apoptosis and DNA repair.

A recent study reported that p53 plays an important role in suppressing cancer in low-dose irradiated cells and animals. For example, low-dose irradiated Trp53 heterozygous mice appeared to suffer from an increase in tumors such as osteosarcomas, sarcomas, and carcinomas, indicating that reduced Trp53 function raises the lower dose/dose-rate threshold for both detrimental and protective tumorigenic effects [2]. For p53, gain-of-function mutations have been found to promote tumorigenesis by impaired Ataxia-telangiectasia mutated (ATM) activation involving active disruption of critical DNA damage-response pathways [2]. Irradiated Trp53 heterozygous mice also showed a decrease in the number of lymphocytes in the bone marrow, while irradiation at a young age resulted in delayed p53 expression in splenocytes and reduced lifespan [3].

Irradiation may also interfere with the ability of p53 to mediate DNA repair and other functions. A study by Zhao reported that the orphan receptor TR3 enhanced p53 transactivation and repressed DNA double-strand break repair in hepatoma cells under ionizing radiation [4]. Similarly, Meador reported decreased histone gene expression that was dose- and time-dependent in the colon cancer cell line HCT116 and its p53-null derivative [5], while Squatrito suggested that loss of elements of the ATM/Chk2/p53 cascade accelerated tumor formation in a glioma mouse model [6].

In the present study, we investigated the short-term effects of low-dose-rate radiation on EL4 lymphoma cells, by examining the gene expression of proteins in-

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volved in apoptosis [Mdm2 (transformed mouse 3T3 cell double minute 2), p53, Bax (BCL2-associated X protein), and Parp (poly (ADP-ribose) polymerase 1)], cell cycle arrest [p21 (cyclin-dependent kinase inhibitor 1A)], DNA repair [Msh2 (mutS homolog 2), Msh3 (mutS homolog 3), Wrn (Werner syndrome homolog), Lig4 (ligase IV, DNA, ATP-dependent)), Neil3 (Nei-like 3), ERCC8 (excision repair cross-complementing rodent repair deficiency, complementation group 8), and ERCC6 (excision repair cross-complementing rodent repair deficiency, complementation group 6)], and iron transport [Trf (transferrin), Trfc (transferrin receptor), and FTH1 (ferritin heavy polypeptide 1)], and RRM2 (ribonucleotide reductase M2). To accomplish this, we exposed EL4 cells to low-dose-rate radiation (^{137}Cs, 2.95 mGy/h, a cumulative dose of 70.8 mGy) for 24 h. These conditions satisfied the criteria for the biological low-dose-rate (≤6 mGy/h) recommended by the United Nations Scientific of Atomic Radiation [7].

2. MATERIALS AND METHODS

Apoptosis assays. Exponentially growing EL4 cells that had been seeded 24–48 h before they were irradiated, and at the time points indicated, were stained with Annexin V and propidium iodide (PI) by using an Annexin V-FITC Kit (Milteniy Biotec, Bergisch Gladbach, Germany). Apoptosis was measured by flow cytometry on a Cytomics FC 500 instrument (Beckman Coulter, California, USA).

Apoptosis ELISA kit. The total protein was quantified using a BCA™ protein assay kit (PIERCE, Rockford, USA). The relative protein expression of p53, phospho-p53, cleaved caspase 3, cleaved Parp, Bad (BCL2-associated agonist of cell death), and phospho-Bad in EL4 cells was determined using an ELISA kit (Cell Signaling, Danvers, USA) according to the manufacturer's instructions.

Cell culture. The C57BL/6 mice-derived EL4 lymphoma cell line was purchased from ATCC. EL4 cell lines were grown as cell suspensions in RPMI 1640 medium, supplemented with 10% (v/v) fetal bovine serum and 290 μg/ml l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in the logarithmic growth phase at a concentration of $1-5\times10^5$ cells/ml at 37°C in an atmosphere containing 5% CO_2 under aseptic conditions.

Cell growth and viability assay. An aliquot of cell suspension was mixed with trypan blue solution (0.4% in PBS; Sigma), and the number of live and dead cells (i.e., viable cells were not stained, while nonviable cells were stained blue) was counted under a microscope.

Low-dose-rate γ-irradiation. Low-dose-rate radiation (^{137}Cs, 2.95 mGy/h, a cumulative dose of 70.8 mGy) was exposed to EL4 lymphoma cell for 24 h at a Low-dose-rate Irradiation Facility in Radiation Health Research Institute.

Quantitative reverse transcription. PCR Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany), and 2 μg RNA was reverse transcribed using the Iscript™ cDNA synthesis kit (Bio-Rad, Hercules, USA). PCR was performed in duplicate using SYBR green (Qiagen, Hilden. Germany) and a 7500 real-time PCR machine (Applied Biosystems, Foster city, USA). The relative abundance of specific mRNA levels was calculated by normalizing to β2-microtubulin expression by the $2-\delta\delta$ct method. The primers are listed in Table 1.

Table 1. The Primer List of Apoptosis-, Cell Cycle Arrest-, DNA Repair-, Iron Transport-, and Ribonucleotide Reductase-related Genes.

Category	Gene Symbol	Genbank Accession#	Forward seq.(5′→3′)	Reverse seq.(5′→3′)
Apoptosis	ATM	NM_007499	ctctgcgatgcctacatcat	ggctggaatattgatgcctt
	Mdm2	NM_010786	catcaggatcttgacgatgg	ttccgagtccagagactcaa
	p53	NM_001127233	gccgacctatccttaccatc	cttcttctgtacggcggtct
	Bax	NM_007527	actaaagtgcccgagctgat	tcttggatccagacaagcag
	Parp	NM_007415	attgacctccagaagatgcc	ggacctcgctgaggatagag
Cell cycle arrest	p21	NM_007669	caagaggcccagtacttcct	acaccagagtgcaagacagc
DNA repair	Msh2	NM_008628	tccacattcatggctgaaat	gttcctcttcccagctcatc
	Msh3	NM_010829	agcagcagcataaagacgca	ttgagttcccgggcagcaattt
	Wrn	NM_001122822	aaatgggaaccaatgggcgt	ttgcgttgggttccggcaataa
	Lig4	NM_176953	agcgcatgcagatgcacaaa	acatctgtcccgaacgcattgt
	Neil3	NM_146208	tgccagctgccaacaagaaaca	acaaaccacacaggaccact
	ERCC8	NM_028042	tcgacattgagcccgttgaa	tgccaacggaacacactgcttt
	ERCC6	NM_001081221	agccgagcagcaattgaaacca	aatcccactttgccctggaact
Iron transport	Trf	NM_133977	tcaaccactgcaaattcgat	ggccaatacacaggtcacag
	Tfrc	NM_011638	cagaccttgcactctttgga	gaaagaaggaaagccaggtg
	FTH1	NM_010239	aagaaaccagaccgtgatga	tagccagtttgtgcagttcc
Ribonucleotide reductase	RRM2	NM_009104	attcagcactgggaagctct	aatcgctccaccaagttctc
Housekeeping	B2M	NM_009735	agactgatacatacgcctgcag	gcaggttcaaatgaatcttcag

Statistical analyses. All data are presented as mean±SD and were analyzed by Student's *t* test, two-tailed, with unequal variance. P<0.05 was considered significant.

3. RESULTS AND DISCUSSION

Low-dose-rate radiation increased apoptosis in EL4 cell. EL4 cells were subjected to the short-term low-dose-rate radiation (2.95 mGy/h, a cumulative dose of 70.8 mGy) for 24 h. We determined cell number and apoptotic rate (%) in the low-dose-rate irradiated EL4 cells at 24 h (Fig. 1). No difference in cell number was observed between non-(Non-IR) and low-dose-rate irradiated (LDR-IR) EL4 cells (p=0.43). In spite of no difference, apoptotic rate (%) was increased in the LDR-IR (5.1±1.51) compared to the Non-IR (3.2±0.58) cells at 24 h (p=0.1). In accordance with our result, Fortress reported that low-dose of gamma ray (2 mGy) increased transformed cell apoptosis via stimulation of irradiated non-transformed cells [8]. Thus, we hypothesize that low-dose-rate radiation may boost anticancer system.

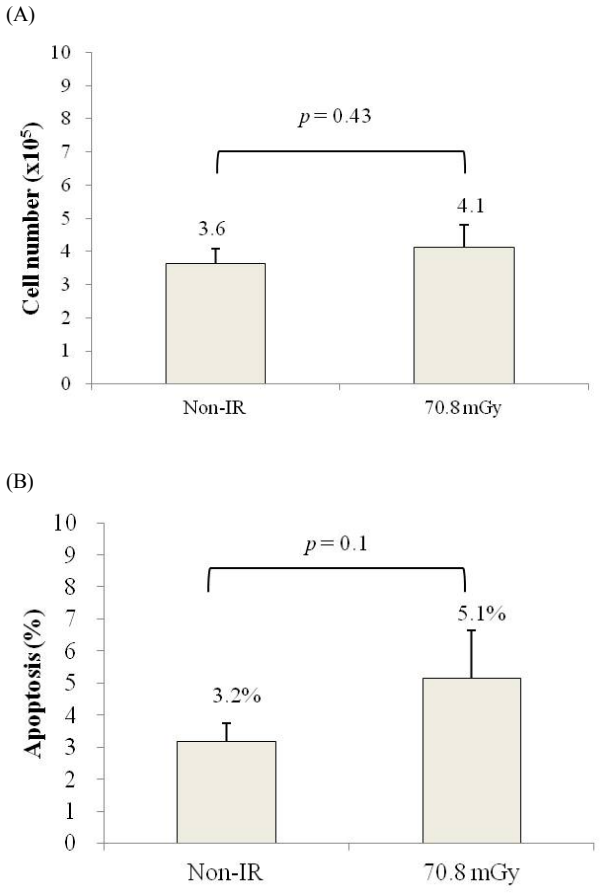

Fig. 1. Comparison of cell number and apoptosis in the non- and low-dose-rate irradiated EL4 cells. (A) Cell number was determined by trypan blue exclusion. Starting cell number=1×10^5 cells. (B) Apoptosis was measured by flow cytometry. Time points indicated for staining with Annexin V and propidium iodide (PI) using an Annexin V-FITC Kit. Non-IR: non-irradiated EL4. 70.8 mGy: low-dose-rate irradiated EL4. Data from a representative experiment are shown and 2 such independent experiments with 3 replicates in each group were carried out. $^*p$<0.05, as compared to non-irradiated EL4 cells.

Low-dose-rate radiation upregulated the transcriptional levels of ATM, Mdm2, p53, p21, and Parp in EL4 cell. LDR-IR cells reduced gene expression of Mdm2 compared with Non-IR cells. However, the transcriptional levels of ATM, p53, p21, and Parp exhibited a higher increase in the LDR-IR cells compared with the Non-IR cells. But, mRNA level of Mdm2 was decreased in LDR-IR cells compared to the Non-IR cells (Fig. 2A). In real-time PCR analysis, low-dose-rate radiation altered the transcriptional levels of apoptosis-related genes such as ATM, Mdm2, p53, p21, and Parp in EL4 cells. The p53 tumor suppressor protein plays a major role in cellular response to DNA damage and other genomic aberrations. Activation of p53 can lead to either cell cycle arrest and DNA repair or apoptosis [9]. DNA damage induces phosphorylation of p53 at Ser15 and Ser37 and leads to a reduced interaction between p53 and its negative regulator, the oncoprotein Mdm2 [10]. Mdm2 inhibits p53 accumulation by targeting it for ubiquitination and proteasomal degradation [11,12]. p53 can be phosphorylated by ATM, ATR, and DNA-PK at Ser15 and Ser37. Phosphorylation impairs the ability of Mdm2 to bind p53, promoting both the accumulation and activation of p53 in response to DNA damage [10,13]. Also, our study revealed that mRNA expression of Mdm2 in EL4 cells was suppressed following low-dose-rate irradiation. Similar results were reported by Mendrysa, indicating that Cre-mediated deletion of Mdm2 resulted in increased accumulation of p53, increased radiosensitivity, increased apoptosis, and inhibition of carcinogenesis [14]. Also, the spleens of mice showed downregulation of Mdm2 in both T and B lymphocytes by low-dose-rate radiation (0.0167 Gy/h) for 1-40 days [15]. However, the expression of Mdm2 for low-dose-rate ^{60}Co gamma radiation (from 0.1-10 cGy/h) was higher than for the high-dose-rate in immortalized murine NIH/PG13Luc cells stably transfected with a Trp53-dependent luciferase reporter plasmid. However, low-dose-rate radiation increased mRNA levels of p53, p21, and Parp. Solozobova previously reported that nuclear accumulation of p53 increased after exposing p53 target genes Mdm2, p21 and PUMA, transcribed in proliferating embryonic stem cells, to ionizing radiation [16]. Additionally, Slee reported that mutation of Ser312 in p53 interferes with cell cycle progression,

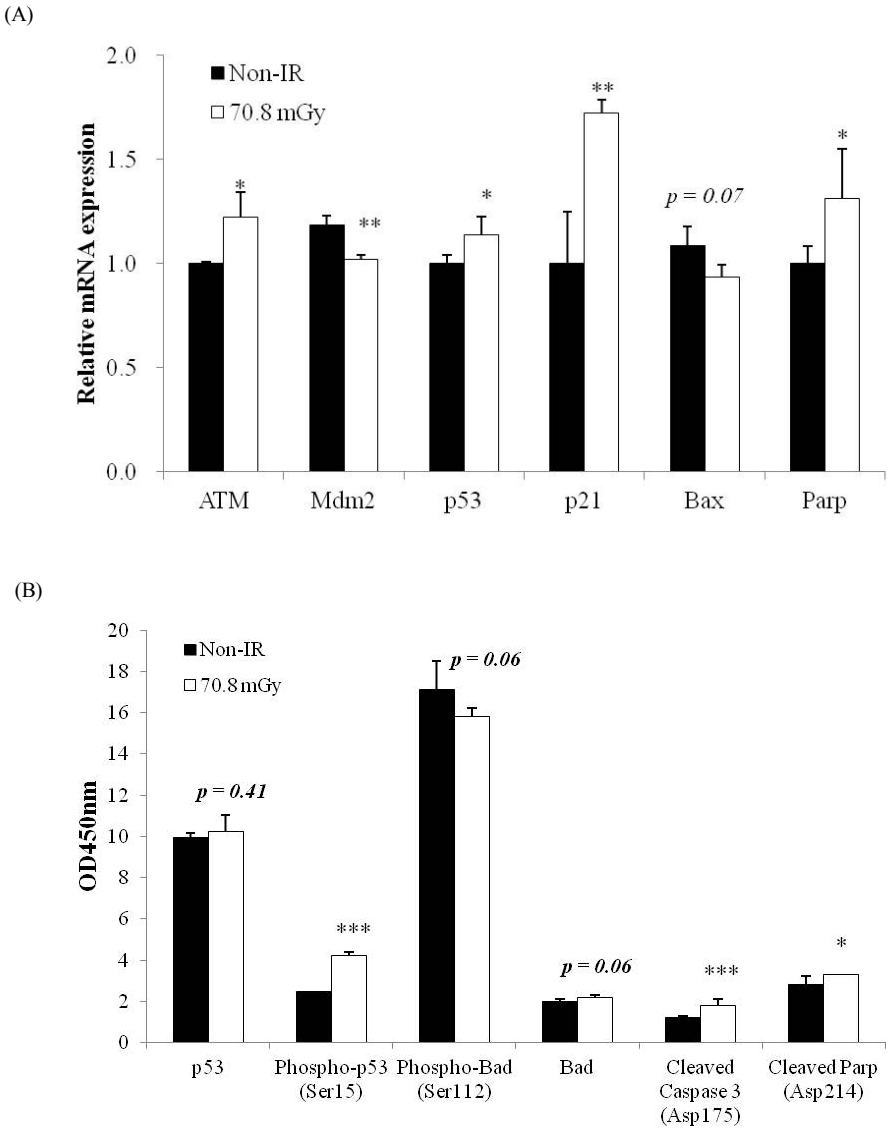

Fig. 2. Comparison of the mRNA and protein levels of apoptosis-related genes in the non- and low-dose-rate irradiated EL4 cells. Non-IR: non-irradiated cells. 70.8 mGy: low-dose-rate irradiated cells. (A) 70.8 mGy activated gene expression of ATM, Mdm2, p53, p21, Bax, and Parp. ATM: ataxia telangiectasia mutated, Mdm2: transformed mouse 3T3 cell double minute 2, p53: tumor protein p53, p21: cyclin-dependent kinase inhibitor 1A, Bax: BCL2-associated X protein, Parp: poly (ADP-ribose) polymerase 1. (B) Comparison of protein levels of p53, phospho-p53, cleaved caspase 3, cleaved Parp, Bad, and phospho-Bad in the non- and low-dose-rate irradiated EL4 cells. Non-IR: non-irradiated cells. 70.8 mGy: low-dose-rate irradiated cells. Each value represents mean ±SD, and the experiments were carried out in triplicate. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ as compared to non-irradiated EL4 cells.

and predisposes mice to develop thymic lymphomas and liver tumors following a sub-lethal dose of ionizing radiation [17]. In LDR-IR cells showed the upregulation of p21 mRNA, which indicates that p21 is involved in cell cycle arrest. Evidence for this was provided by Limesand, who reported that expression of myr-Akt1 in the salivary glands resulted in significant reduction in phosphorylation of p53 at serine 18, coupled with reduced accumulation of total p53, and p21 or Bax mRNA, following treatment with etoposide or gamma irradiation of primary salivary acinar cells [18]. The parotid glands of mice receiving pretreatment with IGF1 increased binding of p63 to the p21 promoter after irradiation, which coincides with increased p53 binding and p21 transcription [19].

Low-dose-rate radiation upregulated the endogenous protein levels of p53, phospho-p53, cleaved caspase-3, Bad, and cleaved Parp in EL4 cell. The transcriptional levels of apoptosis-related genes were increased in the LDR-IR cells. Thus, we determined the relative yield of apoptosis-related proteins by using the apoptosis ELISA assay. Protein levels of phospho-p53 (Ser15), cleaved caspase 3 (Asp175), and cleaved Parp (Asp214) exhibited increases in the LDR-IR cells (Fig. 2B). The

protein levels of p53 (p=0.41), phospho-Bad (Ser112) (p=0.06), and Bad (p=0.06) did not significantly increase in the LDR-IR cells. Therefore, low-dose-rate radiation (70.8 mGy) upregulated the mRNA and protein levels of apoptosis-related genes. The results of our apoptosis ELISA assays indicated increased protein levels of phospho-p53 (Ser15), cleaved caspase 3 (Asp175), and cleaved Parp (Asp214). Tsai reported that p53 was phosphorylated and stabilized in response to inhibition of DNA repair, and that the p53-targeted proteins PUMA and Bax were up-regulated and activated in chronic lymphocytic leukemia cells [20]. Our results showed the upregulation of caspase-3 protein in the LDR-IR cells compared to Non-IR cells. Caspase-3 (CPP-32, Apoptain, Yama, SCA-1) is a critical executioner of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly (ADP-ribose) polymerase (Parp) [21]. Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated p17 and p12 fragments. Cleavage of caspase-3 requires aspartic acid at the P1 position [22]. Low dose radiation exposure (75 mGy) could induce greater cytochrome c levels and caspase-3 activity in Kunming mice implanted with S(180) sarcoma cells [23]. Additionally, the protein levels of cleaved Parp (Asp214) were upregulated in the LDR-IR cells compared to Non-IR cells. Parp, a 116 kDa nuclear poly (ADP-ribose) polymerase, appears to be involved in DNA repair in response to environmental stress. For example, the interaction of HES1 (hairy and enhancer of split 1) and Parp in B-cell acute lymphoblastic leukemia was found to modulate the function of the HES1 transcriptional complex and signals through Parp to induce apoptosis [24]. Therefore, low-dose-rate irradiation-altered gene expression increased apoptosis of lymphoma cells.

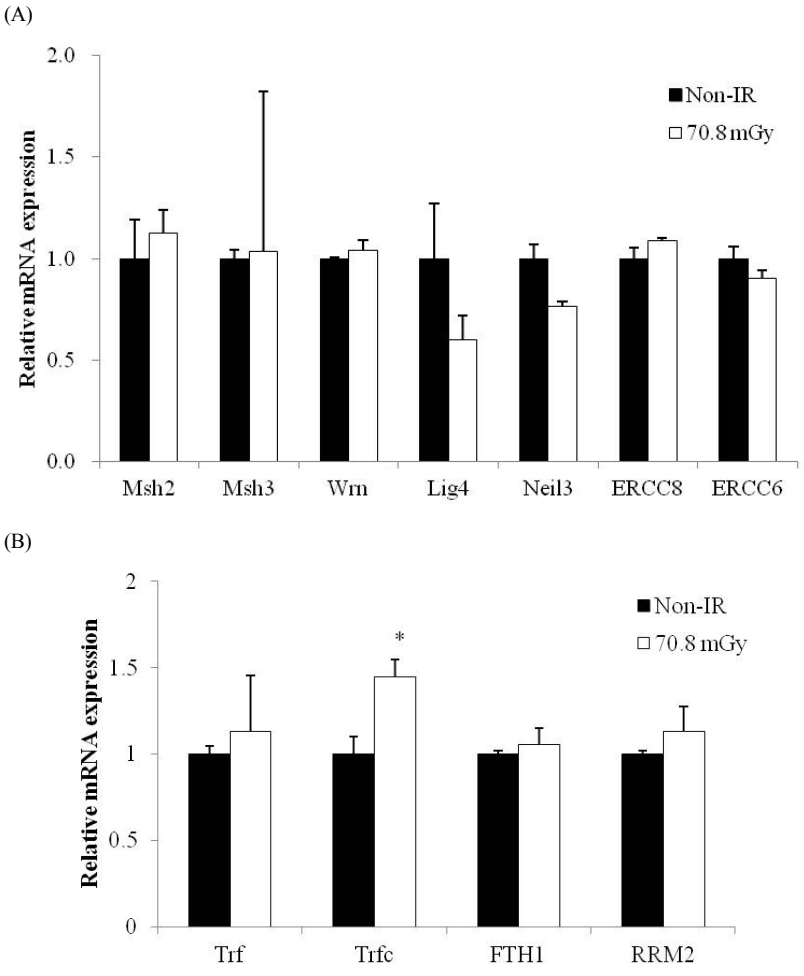

Fig. 3. Comparison of mRNA levels of DNA repair, iron transport- and ribonucleotide reductase-related genes in the non- and low-dose-rate irradiated EL4 cells. Non-IR: non-irradiated cells. 70.8 mGy: low-dose-rate irradiated cells. (A) Low-dose-rate radiation did not influence expression of DNA repair-related genes in EL4 cells. Msh2: mutS homolog 2, Msh3: mutS homolog 3, Wrn: Werner syndrome homolog, Lig4: ligase IV, DNA, ATP-dependent, Neil3: Nei-like 3, ERCC8: excision repair cross-complementing rodent repair deficiency, complementation group 8, ERCC6: excision repair cross-complementing rodent repair deficiency, complementation group 6. Each value represents the mean±SD. (B) Low-dose-rate radiation up-regulated gene expression of Trfc. Trf: transferrin, Tfrc: transferrin receptor, FTH1: ferritin, heavy polypeptide 1, RRM2: ribonucleotide reductase M2. $^{*}p < 0.05$. Each value represents mean±SD.

Low-dose-rate radiation did not influence expression of DNA repair-related genes in the EL4 lymphoma cell. We investigated the effects of DNA repair-related genes in the LDR-IR cells. In Real-time PCR analysis, no differences were observed between Non-IR and LDR-IR cells for Msh2, Msh3, Wrn, Lig4, Neil3, ERCC8, and ERCC6. Therefore, low-dose-rate radiation (70.8 mGy) did not influence expression of DNA repair-related genes in EL4 cells (Fig. 3A).

Low-dose-rate radiation up-regulated gene expression of Trf, Trfc, and RRM2 in EL4 cell. We further determined gene expression for iron transport and RRM2 in the LDR-IR EL4 cells. The mRNA levels of Trfc increased in the LDR-IR EL4 cells. However, the mRNA level of Trf, FTH1, and RRM2 was no different at 24 h between Non-IR and LDR-IR EL4 cells (Fig. 3B). Several recent studies have reported that ionizing radiation can influence iron transport and ribonucleotide reductase by modulation of p53. For example, radiation may improve transferrin (Tf)-cationic liposome-DNA complex gene delivery selectively to tumor cells both in vitro and in vivo [25]. Transferrin receptor expression appears to be involved in the regulation of UV-resistance, possibly via modulation of the concentrations of p53 and Bax proteins [26]. Additionally, Kunos suggested that blockage of ribonucleotide reductase activity by 3-AP (3-aminopyridine-2-carboxaldehyde thiosemicarbazone) impaired DNA damage responses that relied on deoxyribonucleotide production and thereby might substantially increase the chemoradiosensitivity of human cervical cancers [27]. This study suggests that low-dose-rate radiation could activate gene expression of iron transport and ribonucleotide reductase in EL4 cells. We suppose that iron transport and ribonucleotide reductase may be involved in apoptosis of LDR-IR EL4 cells.

4. CONCLUSION

Our results suggest that short-term low-dose-rate radiation activates expression of apoptosis and transferrin receptor-related gene in EL4 lymphoma cells, and that gene expression of transferrin receptor may be involved in activating apoptosis. Our study confirmed partial apoptosis signaling pathway responses to the short-term low-dose-rate radiation. Accordingly, future studies will investigate the upstream molecular signaling pathways which are influenced by the low-dose-rate irradiation. This study will contribute toward research on the molecular mechanisms response to low-dose-rate irradiation.

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