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Anti-inflammatory effects of low-dose rate ionizing radiation on cell lines derived from osteoarthritis patients

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► ***Original article***

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ABSTRACT

Background: The efficacy of low-dose rate ionizing radiation (LDRIR) for anti-inflammatory treatment has been confirmed in various clinical studies. However, the radiobiological mechanisms involved in the anti-inflammatory action of LDRIR have not been completely elucidated. ***Materials and Methods:*** This study investigated whether LDRIR at 0.5 or 1 Gy affected the regulation of cytokine messenger RNA expression and protein levels in inflamed synoviocytes and chondrocytes induced by lipopolysaccharide. Intercellular adhesion molecule-1, interleukin-6, interleukin-8, and growth-regulated alpha protein expression were analyzed in synoviocytes and chondrocytes at 24 h after lipopolysaccharide treatment using reverse transcription polymerase chain reaction, immunostaining, Western blotting, and enzyme-linked immunosorbent assays. ***Results:*** The messenger RNA expression levels of interleukin-6, interleukin-8, and growth-regulated alpha protein were lower in synoviocytes cotreated with lipopolysaccharide and 0.5 or 1 Gy radiation than in those treated with lipopolysaccharide alone. The immunostaining results showed that all target cytokines were downregulated after 0.5 and 1 Gy of radiation in inflamed synoviocytes and chondrocytes. In chondrocytes, all four cytokines were significantly downregulated at a dose rate of 0.053 Gy/min, and the extent of regulation was similar to that at 4.02 Gy/min. ***Conclusion:*** Our findings indicate that LDRIR may regulate pro-inflammatory cytokine expression in both synoviocytes and chondrocytes, regardless of the dose rate. Therefore, LDRIR can alleviate concerns of carcinogenesis and may be useful in clinical settings.

INTRODUCTION

Osteoarthritis (OA) is the most common joint disorder in the world and affects all tissues of the joint, although articular cartilage loss and adjacent bone changes are its most predominant features [1]. OA is a leading cause of disability, and the increasing prevalence of knee OA with the ageing population may have a substantial impact on public health [2].

The management of OA involves multiple components, such as joint replacement surgery, non-steroidal anti-inflammatory drugs, and physiotherapy. However, biological preparations are limited in terms of frequency and degree of use, and joint prostheses have a limited life span of 3–5 years [3].

Recently, several studies have shown that low-dose rate ionizing radiation (LDRIR) at 0.5–1.5 Gy is useful for benign, painful skeletal disorders such as arthritis, fasciitis, and epicondylitis for pain relief and functional improvement [4,5]. LDRIR is widely accepted and is frequently used to treat several benign diseases in Germany and other European countries [6,7] because it is non-invasive and cost-effective. However, exposure to ionizing radiation increases the incidence of some types of cancer such as leukemia and sarcoma. The possibility of secondary cancer after LDRIR for benign diseases is the biggest obstacle in treatment planning, although the carcinogenic risk of LDRIR is less than

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1% in older patients [8]. Increasing evidence suggests that radiation exposure at low dose-rates may be less effective in carcinogenesis than high dose rates [9]. Therefore, efforts to minimize the possibility of carcinogenesis are required.

The anti-inflammatory effects of LDRIR have been confirmed in various clinical studies. However, the radiobiological mechanisms involved in the anti-inflammatory action of LDRIR have not been fully elucidated. Furthermore, several *in vitro* and *in vivo* studies have suggested that radiation biological effects can vary according to dose rate [9-11].

Therefore, in the present study, we investigated whether LDRIR at different dose rates affects cytokine secretion in chondrocytes and synoviocytes of the knee joint. This study evaluated the radiobiological mechanisms involved in the anti-inflammatory action of LDRIR, which have not been reported previously.

MATERIALS AND METHODS

Ethics and cell culture

Primary chondrocytes and synoviocytes were harvested from human articular cartilage samples obtained from the Department of Orthopedic Surgery of Seoul National University Hospital (Seoul, Republic of Korea). Clinical information on the seven patients is presented in table 1, including patient age and sex and the cell passage number. All experiments using these cells were approved by the Institutional Review Boards (IRBs) of the Seoul National University Hospital, Seoul, Republic of Korea (IRB number H-1907-028-1045) and the Dongnam Institute of Radiological and Medical Science, Busan, Republic of Korea (IRB number D-2110-005-002). Written informed consent was obtained from all the patients. Cells were incubated in Dulbecco's modified Eagle medium (WelGENE, Daegu, Korea) supplemented with 10% heat-inactivated fetal bovine serum (Atlas Biologicals, Fort Collins, CO, USA) and 1% antibiotic-antimycotic (Gibco; Thermo Scientific, Waltham, MA, USA). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and grown until approximately 85–90% confluence was achieved. Only cells with a passage number of ≤ 15 were used in the experiments.

Table 1. Clinical information of the patients

Case	Sex	Age (years)	Passage	
			Synoviocyte	Chondrocyte
1	Female	69	1	1
2	Female	65	2	2
3	Female	82	1	1
4	Female	70	1	1
5	Female	73	1	1
6	Female	83	1	1
7	Female	72	0	0

Inflammation induction and irradiation

Cells (5×10^4 cells/well) were seeded in a 60-mm tissue culture plate and cultured in a humidified atmosphere of 5% CO_2 at 37°C overnight. The following day, cells were exposed to 0.5 μg/mL of lipopolysaccharide (LPS) for 24 h. The cells were then exposed to gamma radiation in media with or without LPS on the next day. Cells were irradiated with either 0.5 or 1 Gy at 0.4 Gy/min using a ^{137}Cs ray source (LDIR system; Chiyoda Technology Corp., Tokyo, Japan) or linear accelerator (Infinity; Elekta AB, Stockholm, Sweden). After 24 hours of radiation exposure, the irradiated cells were collected. The non-irradiated cells were also collected at this time point. The harvested cells were washed with phosphate-buffered saline (PBS) and subjected to protein lysis and RNA extraction procedures, according to the manufacturer's instructions.

Cytokine assay

A human cytokine array kit (R&D Systems, Minneapolis, MN, USA) was used to detect 36 cytokines in chondrocyte and synoviocyte cell lysates according to the manufacturer's instructions. All samples had the same concentration of protein. The 36 cytokines included chemokine (C-C motif) ligand 1/2; macrophage inflammatory protein 1a; chemokine (C-C motif) ligand 5; CD40 ligand; complement component 5a; chemokine (C-X-C motif) ligands (CXCL) 1, 10, 11, and 12; granulocyte-colony stimulating factor; granulocyte macrophage colony-stimulating factor; intercellular adhesion molecule-1 (ICAM-1); interferon (IFN) gamma; interleukin (IL)-1 alpha; IL-1 beta; IL-1 receptor antagonist (IL-1ra); IL-2, -4, -5, -6, -8, -10, -12, -13, -16, -17A, -17E, -18, -21, and -32a; migration inhibitory factor (MIF); serpin family E member 1, tumor necrosis factor-alpha, and triggering receptor expressed on myeloid cells 1.

Immunostaining

For cell identification, synoviocytes or chondrocytes (1×10^4/well) were seeded in 24-well glass-bottom plates (Cellvis, Mountain View, CA, USA). For cytokine expression, the cells were pelleted at 1000 rpm for 10 min, fixed with 1:1 acetone/methanol, permeabilized in 0.1% Triton X in PBS for 20 min, and washed twice with PBS for 5 min. The cells were blocked in 1% bovine serum albumin in 0.1% Tween-20 in PBS for 30 min at 24°C and incubated overnight at 4°C with the primary antibody (hyaluronan and proteoglycan link protein 1 [HPLN1] and microfibril associated protein 5 [G-Biosciences, St. Louis, MO, USA]; ICAM-1, IL-8, IL-6, and growth-regulated alpha protein (GRO-α) [Abcam, Cambridge, UK]; ICAM-1 was diluted to 1:50, and IL-6, IL-8, and GRO-α were diluted to 1:100) and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich, St. Louis, MO, USA). After repeated washing, cells were incubated for 40 min at 24°C with the appropriate

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secondary antibodies (Alexa Fluor 488 goat anti-mouse, Alexa Fluor 594 goat anti-rabbit immunoglobulin G; Invitrogen, Carlsbad, CA, USA) and washed as previously described. The cells were washed repeatedly and then incubated in DAPI (Sigma Aldrich) diluted 1:100 in PBS for 10 min. Then, slides were mounted using an aqueous mounting solution (ProLong™ Gold Antifade Mountant; Invitrogen), and the well plates were directly observed with a confocal microscope (Zeiss LSM 700; Carl Zeiss, Jena, Germany).

Quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from cell cultures using Qiazol (Qiagen, Valencia, CA) following the manufacturer's instructions. RNA quantity was measured using a NanoDrop 2000/2000c instrument (Thermo Scientific).

Human IL-1α and human IL-6 messenger RNA (mRNA) levels were used to determine the LPS concentration, and human CD54, IL-6, IL-8, and CXCL1 mRNA levels were used to determine the anti-inflammatory effect of LDRIR.

Using the iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA), 1 μg mRNA was reverse-transcribed into complementary DNA (cDNA). The reaction for priming was performed for 5 min at 25°C and then reverse-transcribed for 20 min at 46°C, which was terminated by heating to 95°C for 1 min. All cDNA was stored at -20°C until further use. Transcripts of target genes were analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR), which was performed using a CFX96 Touch™ real-time PCR system (Bio-Rad Laboratories). qRT-PCR was performed for 5 min at 95°C, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 40 s. The results were calculated using the comparative method ($2^{-\Delta\Delta Ct}$) using the housekeeping gene ACTB (β-actin). All primers were designed using Primer3 [12]. The primers used for qRT-PCR were as follows: IL1A forward, 5′- TGT ATG TGA CTG CCC AAG ATG AAG -3′, reverse, 5′- AGA GGA GGT TGG TCT CAC TAC C -3′; CD54 (ICAM-1) forward, 5′- AGC GGC TGA CGT GTG CAG TAA T -3′, reverse, 5′- TCT GAG ACC TCT GGC TTC GTC A -3′; IL6 forward, 5′ - AGA CAG CCA CTC ACC TCT TCA G -3′, reverse, 5′- TTC TGC CAG TGC CTC TTT GCT G -3′; IL8 forward, 5′ - GAG AGT GAT TGA GAG TGG ACC AC -3′, reverse, 5′- CAC AAC CCT CTG CAC CCA GTT T -3′; and ACTB forward, 5′- CAC CAT TGG CAA TGA GCG GTT C -3′, reverse, 5′- AGG TCT TTG CGG ATG TCC ACG T -3′.

Cell viability assay

The CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI, USA) is a method used for assaying cell proliferation and cytotoxicity based on the quantitation of adenosine 5-triphosphate (ATP) in living cells. Cells (5×10^4 cells/well) were seeded into six-well tissue culture plates (Falcon; Corning Inc., Corning, NY, USA), incubated for 24 h before LPS treatment, and then grown for 24 h with LPS (0.5 μg/mL). After 48 h, cells were irradiated with 0.5 or 1 Gy. At the indicated time, cell viability was tested using the luminescent CellTiter-Glo assay (Promega) according to the manufacturer's instructions. After the reaction was completed, 200 μl of the reaction solution was transferred to a 96-well plate, and luminescence was measured in luminometer mode (at 610 nm) using the Spectra Max Paradigm Microplate Reader (Multi-Mode Detection Platform; Molecular Devices, LLC, San Jose, CA, USA).

Western blot analysis

Cells were lysed in protein lysis buffer (Intron Biotechnology, Sungnam, Korea) with a commercial 25× protease inhibitor cocktail, centrifuged for 15 min at 13,000 rpm at 4°C, and the supernatant was collected. The protein concentration was determined using a bicinchoninic acid assay. Proteins (15 μg) were separated on 4–12% gels through sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to methanol-activated polyvinylidene fluoride membranes (GE Healthcare, Chicago, IL, USA). The membranes were blocked with 5% skim milk dissolved in Tris-buffered saline containing 0.01% Tween 20 (TBST) for 30 min and then incubated overnight at 4°C with the appropriate antibody (ICAM-1, IL-8, IL-6, GRO-α; Abcam) diluted to 1:1000 in TBST. The following day, the membranes were washed three times with TBST for 10 min each time. The resulting membranes were detected using peroxidase-conjugated secondary antibodies at a dilution of 1:10000 and washed three times with TBST for 10 min each, incubated with enhanced chemiluminescence-solution (Amersham Biosciences Corp., Piscataway, NJ, USA), and captured and analyzed using the Fusion FX5 system (Vilber, Marne-le-Vallée, France). To confirm equal loading in each sample, the membranes were blotted with an anti-β-actin antibody (Santa Cruz Biotechnology, Dallas, TX, USA).

Enzyme-linked immunosorbent assay

GRO-a, IL-6, and IL-8 protein levels in the cell culture medium or ICAM-1 protein levels in cell extracts were measured using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. All samples used in the experiments had the same concentration of protein. We measured absorbance at 450 nm using the Spectra Max Paradigm Microplate Reader (Molecular Devices). We used the following ELISA kits: human GRO-a (ab190805, Abcam), human IL-6 (ab46027, Abcam), human IL-8 (ab214030, Abcam), and human ICAM-1 (ab174445, Abcam) ELISA kits.

Statistical Analyses

The statistical significance of differences between the samples was determined using one-way analysis

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of variance followed by Tukey's multiple comparisons test using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). The results were expressed as means ± standard deviations (n = 3), and P-values < 0.05 were considered significant.

RESULTS

Identification of patient-derived synoviocytes and chondrocytes

Cultured synoviocytes and chondrocytes can be differentiated based on HPLN1 and MAGP2 expression [13]. Therefore, we first confirmed whether the cultured cells were synoviocytes and chondrocytes. The cells were co-stained with HPLN1 or microfibril-associated glycoprotein 2 (MAGP2) antibodies with DAPI distinguished by cell type (figure 1). Synoviocytes were only positive for MAGP2 and chondrocytes were mainly positive for HPLN1.

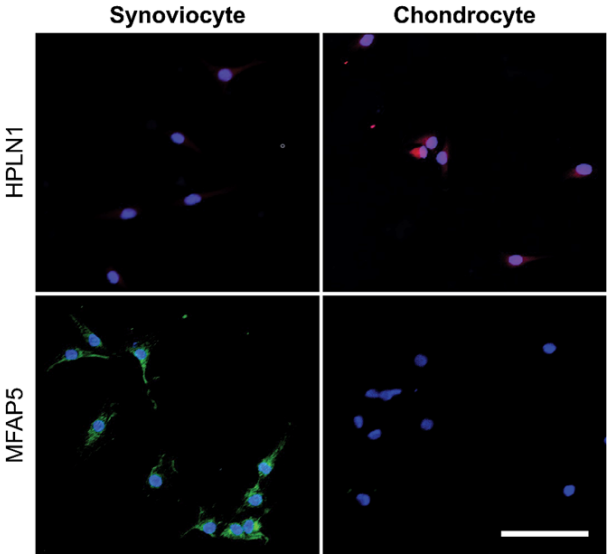

Figure 1. Identification of patient-derived synoviocytes and chondrocytes. The confocal image shows that MFAP5 (green) is only expressed in synoviocytes and HPLN1 (red) is expressed in chondrocytes. Cells were costained with 4',6-diamidino-2-phenylindole (blue), a nuclear marker. MFAP5, microfibril-associated protein 5; HPLN1, hyaluronan and proteoglycan link protein 1. Scale, 100 µm.

Inducing inflammation using LPS and cytotoxicity of LPS and LDRIR

To determine the concentration of LPS that induces inflammation, both cell types were treated with various concentrations of LPS (0.5, 1, and 2 µg/mL). IL6 and IL1A expression levels were examined at 24 h after LPS treatment. Both IL6 and IL1A expression were upregulated in synoviocytes and chondrocytes in a concentration-dependent manner (figure 2A). In particular, 0.5 µg/mL LPS was sufficient to induce an inflammatory response, and this concentration was used for further study. Next, cell viability in response to LPS treatment and low-dose radiation exposure was evaluated using the ATP assay (figure 2B). Cells were irradiated with 0.5 or 1 Gy of radiation without LPS, treated with 0.5 µg/mL LPS without radiation, or cotreated with LPS and radiation for 24 h. Neither the LPS concentration nor the radiation dose used in this study induced cytotoxicity in synoviocytes or chondrocytes.

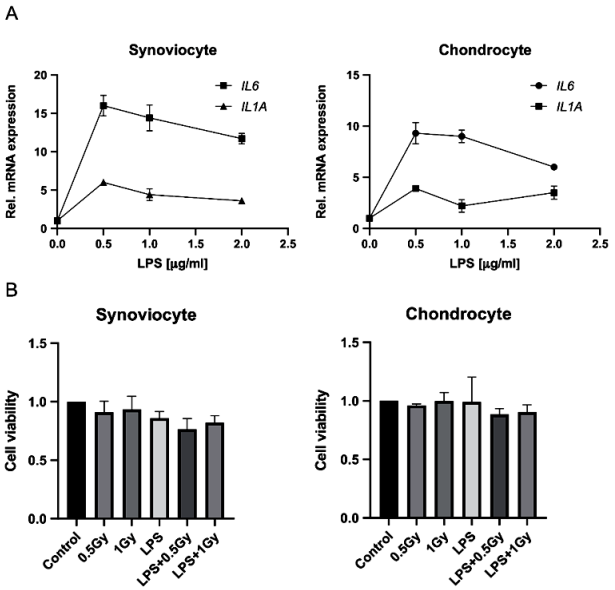

Figure 2. Inducing inflammation with LPS and cytotoxicity of LPS and low-dose radiation. **(A)** The messenger RNA expression of IL6 and IL1A was determined at 24 h after treating cells with the indicated LPS concentration using reverse transcription polymerase chain reaction in both synoviocytes and chondrocytes. The data are presented as means ± standard deviations (n = 3). **(B)** Cytotoxicity of LPS and low-dose rate ionizing radiation was evaluated using an adenosine 5-triphosphate (ATP) assay. Cells were treated with 0.5 µg/mL LPS or irradiated with 0.5 or 1 Gy of gamma radiation. The ATP assay was performed after 24 h. The data are presented as means ± standard deviations (n = 3). LPS, lipopolysaccharide; Rel, relative.

Effect of LDRIR on cytokine expression in inflamed synoviocytes and chondrocytes

First, we performed a human cytokine assay to identify cytokines expressed in response to LPS treatment. We used a membrane-based antibody array for the parallel determination of relative levels of selected human cytokines and chemokines and validated the results in both cell lysates treated with 0.5 µg/mL LPS for 24 h. The analytes are listed in figure 3A, and representative array blots of controls and LPS-treated synoviocytes and chondrocytes are shown in figure 3B. Six reference spots (a1-2, a19-20, and e1-2) expressed the highest spot signal density, whereas the negative control (e19-20) and all blank spots showed no background staining. Finally, the expression levels of 8 (CXCL1/GRO-a, CXCL12/stromal cell-derived factor 1, ICAM-1/CD54, IL-1ra/IL-1F3, IL-6, IL-8, MIF, and E1/plasminogen activator inhibitor-type-1) of the 35 examined cytokines were

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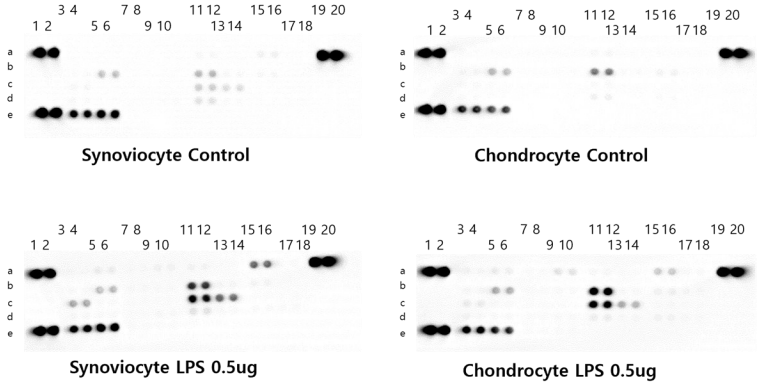
increased in LPS-treated synoviocytes and chondrocytes (Figure 3C). CXCL1/GRO-a, ICAM-1/CD54, IL-6, and IL-8 levels, which were increased by more than 1.5-fold compared with those in the control groups, were selected as the target cytokines in LPS-induced inflammation models. Next, we examined whether LDRIR abolished the increased expression of the target cytokines. CD54, IL6, IL8, and CXCL1 mRNA expression levels were analyzed in synoviocytes and chondrocytes at 24 h after LPS treatment using qRT-PCR. The mRNA expression levels of IL6, IL8, and CXCL1 were lower in synoviocytes cotreated with LPS and 0.5 or 1 Gy radiation than in those treated with LPS alone (figure 4A). In chondrocytes, the IL6 and IL8 mRNA expression levels were lower only at 1 Gy radiation, whereas that of CXCL1 remained unchanged (figure 4B). The CD54 mRNA expression levels were not affected by LDRIR in either synoviocytes (figure 4A) or chondrocytes (figure 4B).

A

	1 2	3 4	5 6	7 8	9 10	11 12	13 14	15 16	17 18	19 20	
a	Ref	CCL1/I-309	CCL2/MCP-1	MIP-1α/MIP-1β	CCL5/RANTES	TNFSF5	C5a	CXCL1/GROα	CXCL10/IP-10	Ref	a
b	Blank	CXCL11/I-TAC	CXCL12/SDF-1	G-CSF	GM-CSF	ICAM-1/CD54	IFN-γ	IL-1α/IL-1F1	IL-1β/IL-1F2	Blank	b
c	Blank	IL-1ra/IL-1F3	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12 p70	Blank	c
d	Blank	IL-13	IL-16	IL-17A	IL-17E	IL-18/IL-1F4	IL-21	IL-27	IL-32α	Blank	d
e	Ref	MIF	Serpin E1/PAI-1	TNF-α	Blank	Blank	Blank	Blank	Blank	Negative control	e

B



C

		CXCL1/GROa	CXCL12/SDF-1	ICAM-1/CD54	IL-1ra/IL-1F3	IL-6	IL-8	MIF	Serpin E1/PAI-1
Control		1	1	1	1	1	1	1	1
LPS	Synoviocyte	3	1.1	2.2	2.4	3.2	2.3	1.1	1.2
	Chondrocyte	2.1	1.2	2.4	ND	6.2	2.7	1.2	1.1

Figure 3. Cytokine expression analysis with protein array in LPS-treated synoviocytes or chondrocytes. **(A)** The cytokine array contained antibodies against 35 cytokines in the membrane. The red value emphasizes a high value of the respective cytokine compared to the control group. **(B)** Cytokine protein arrays detected the presence of 8/35 cytokines in LPS-treated or control synoviocytes or chondrocytes. **(C)** Eight cytokines showed differential expression levels between the control and LPS-treated cells. LPS, lipopolysaccharide; Ref, references.

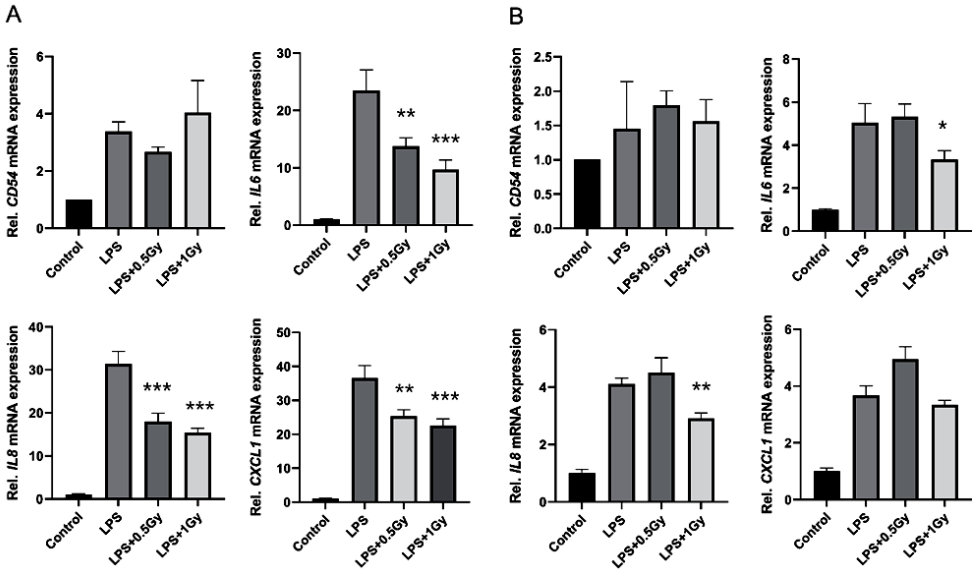


Figure 4. Analysis of CD54, IL6, IL8, and CXCL1 mRNA expression in inflamed synoviocytes and chondrocytes. Cells were irradiated with 0.5 or 1 Gy in the presence of 0.5 μg/mL LPS. After 24 h, mRNA expression levels of the target cytokines were examined using reverse transcription polymerase chain reaction in **(A)** synoviocytes and **(B)** chondrocytes. The data are presented as means ± standard deviations (n = 3). *P < 0.05, **P < 0.01 and ***P < 0.001 compared with treatment with LPS only. mRNA, messenger RNA; LPS, lipopolysaccharide; Rel, relative.

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To investigate whether the protein levels of the target cytokines were altered in response to LDRIR, we performed immunostaining and Western blot analysis. Unlike the results for mRNA expression, the immunostaining results showed that the increased protein levels of all target cytokines were downregulated with 0.5 and 1 Gy radiation in inflamed synoviocytes (figure 5A) and chondrocytes (figure 5B). In addition, Western blot analysis showed that the ICAM-1 and IL-8 protein levels were downregulated by irradiation in both synoviocytes (figure 5C) and chondrocytes (figure 5D), whereas the other two targets (IL-6 and GRO-a) were not detected.

Effect of different LDRIR on cytokine expression in inflamed synoviocytes and chondrocytes

To determine the effect of radiation dose rate on the regulation of cytokine secretion, we examined cytokine expression at two different dose rates (0.053 and 4.02 Gy/min). IL-6, IL-8, and GRO-a levels were examined in the cell culture supernatants, and ICAM-1 was detected in cell extracts. The results showed that a dose rate of 0.053 Gy/min had little effect on regulating ICAM-1, IL-6, and IL-8 expression and had no effect on GRO-an expression in synoviocytes (figure 6A). However, all four cytokines were downregulated at a dose rate of 0.053 Gy/min, and the extent of regulation was similar to that at a dose rate of 4.02 Gy/min in chondrocytes (figure 6B). These results indicate that the anti-inflammatory effects of LDRIR at lower dose rates regulate cytokine expression in chondrocytes although not in synoviocytes.

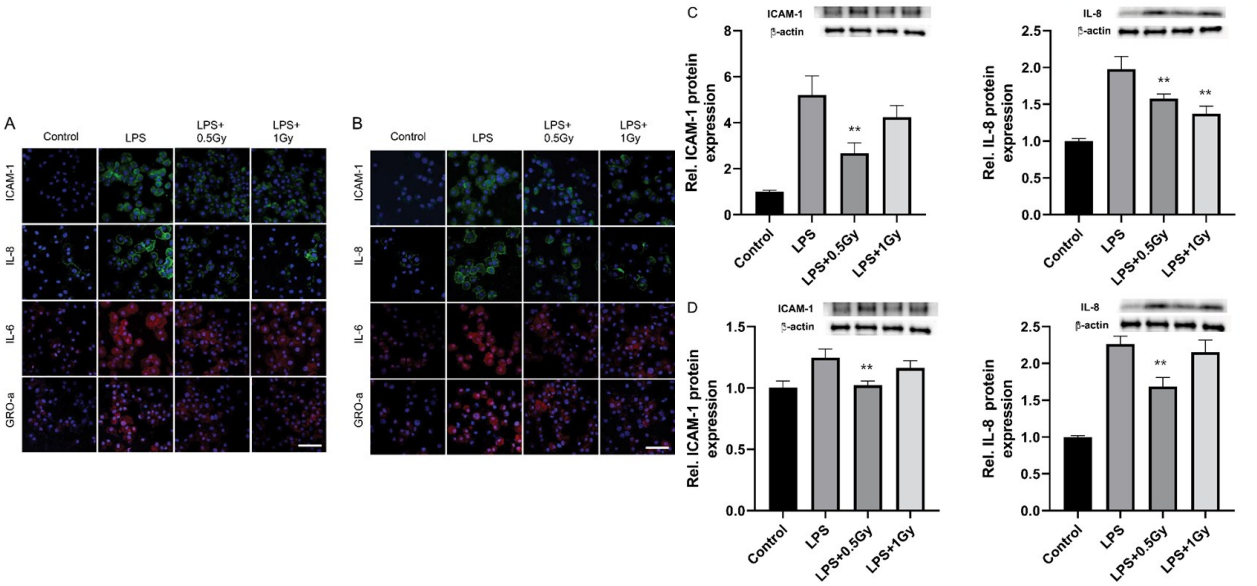

Figure 5. Analysis of ICAM-1, IL-6, IL-8, and GRO-α protein levels in inflamed synoviocytes and chondrocytes. Cells were treated with 0.5 µg/mL lipopolysaccharide (LPS) or irradiated with 0.5 or 1 Gy of radiation. After 24 h, immunostaining was performed to analyze the target protein levels in the cells. Positive ICAM-1 and IL-8 expression are shown in green and positive IL-6 and GRO-α expression in red in **(A)** synoviocytes and (B) chondrocytes. Western blotting was used to detect ICAM-1 and IL-8 protein levels in **(C)** synoviocytes and **(D)** chondrocytes. ß-actin was used as an internal standard. These are representative images from three independent experiments. The data are presented as means ± standard deviations (n = 3). **P<0.01 compared with treatment with LPS only. Scale, 100 µm. GRO-α, growth-regulated protein alpha; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; Rel, relative.

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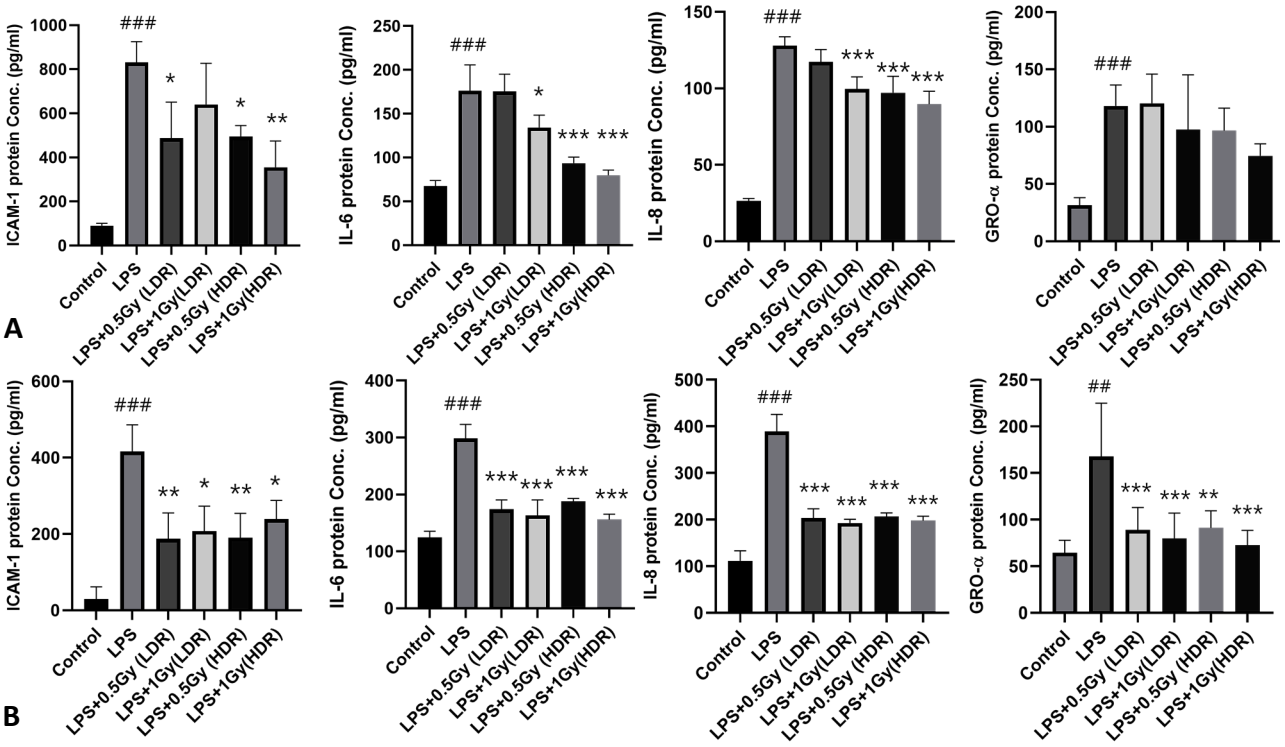

Figure 6. Effect of dose rate on ICAM-1, IL-6, IL-8, and GRO-α protein level regulation in inflamed synoviocytes and chondrocytes. Cells were treated with 0.5 μg/mL lipopolysaccharide (LPS) or irradiated with 0.5 or 1 Gy of radiation with a low dose rate (LDR) or high dose rate (HDR). After 24 h, ICAM-1 protein levels in the cell lysate or IL-6, IL-8, and GRO-α protein levels in the culture media were analyzed using enzyme-linked immunosorbent assays in **(A)** synoviocytes and **(B)** chondrocytes. The data are presented as means ± standard deviations (n = 3). *P< 0.05, **P < 0.01 and ***P < 0.001 compared with treatment with LPS only. Conc., concentration; GRO-α, growth-regulated protein alpha; ICAM-1, intercellular adhesion molecule-1; IL, interleukin.

DISCUSSION

Several retrospective studies have shown the advantages of LDRIR in the management of OA in terms of pain relief, mobility and functional improvements, and minimal side effects [14-16]. LDRIR at 0.5 Gy can improve pain relief in patients with degenerative joint disease of the fingers with no differences observed according to age, sex, number of cycles, or cumulative dosage [14,15]. However, the long -term effect of LDRIR was limited. A decrease of two points on the numeric pain rating scale is defined as clinical relevance. Clinical relevance was observed in 50% of the osteoarthritic joints (hip and knee joints) at 6 weeks' post-radiation; this difference decreased to 25% at 52 weeks' post-radiation [16].

Various hypotheses have been suggested to explain the mechanism of LDRIR, such as decreased adhesion molecule expression, production of pro-inflammatory cytokines, anti-inflammatory cytokines, nitric oxide synthase, and reactive oxygen species and increased nuclear factor-kappa B activation [5,17,18]. In this study, ICAM-1, a type of intercellular adhesion molecule that facilitates leukocyte-endothelial transmigration [19], and IL-8, a neutrophil chemotactic factor [20], were decreased when 0.5 Gy of LDRIR was used in both chondrocytes and synoviocytes. Both proteins play a role in attracting leukocytes to damaged sites, thus the recruitment of inflammatory cells to damaged sites in the cartilage and bones of the heel may be inhibited [21,22]. Other mechanistic considerations involve positive subcellular effects mediated by the activation of the nuclear factor erythroid-2-related transcription factor (NRF2)-mediated antioxidant response [23]. In our previous study, 0.5 Gy induced both nuclear respiratory factor 1 (NRF1) and NRF2 activation accompanied by the generation of reactive oxygen species and calcium flux [22].

Arenas et al. reported that LPS-induced leukocyte adhesions were successfully reduced by 0.1, 0.3, and 0.6 Gy of radiation in them *in vivo* experiments [23]. The study investigated cytokine levels in digestive organs and found that LPS-induced ICAM-1 upregulation was not decreased after LDRIR, although transforming growth factor beta-1 was neutralized after LDRIR.

In our study, LPS-induced ICAM-1 in chondrocytes was decreased by 0.5 Gy of LDRIR both with low and high dose rates, although the mRNA level showed no significant change. According to the central dogma theory, protein changes inevitably follow changes in mRNA expression; however, recent evidence suggests that a causal relationship between mRNA and protein levels may not necessarily exist, and the strong contribution of post-transcriptional regulation levels is an emerging important issue [24,25]. Therefore, in this study, the fact that LDRIR lowered

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the ICAM-1 protein level itself has important significance. However, it can also be a technical or timepoint problem and will be discussed herein with other limitations of this study.

Although the anti-inflammatory effects of LDRIR have been well studied, inconsistent results have been reported. Two recently published randomized controlled trials by a group from the Netherlands demonstrated no significant benefits of LDRIR in terms of Outcome Measures in Rheumatology-Osteoarthritis Research Society International response rates for symptomatic hand [26] and knee OA [27]. The trial on knee OA included 55 patients, and no significant difference was observed in response rates between the LDRIR and sham groups (44% vs 43% at 3 months, P=0.9). Both studies were criticized for their small sample sizes, which were designed to detect a 40% difference. Furthermore, LDRIR was proven effective in the treatment of other benign inflammatory diseases, such as plantar fasciitis and humeral epicondylitis [28,29]. Therefore, LDRIR seems to be a valuable secondary option to delay the timing of aggressive treatment.

Anti-inflammatory effects of LDRIR were also elucidated in patients with severe pneumonia and acute respiratory distress syndrome, which are important causes of death among patients with coronavirus disease 2019. Meziani *et al.* [30] found that 0.5 or 1 Gy LDRIR reduced lung tissue damage and immune cell infiltration in a viral infected murine model. In addition, when 0.5-Gy LDRIR was targeted at human lung macrophages, IFN-gamma secretion decreased and the number of IL-6-secreting macrophages decreased compared with that in their nonirradiated counterparts.

The main concern impeding the acceptance of radiation therapy for benign diseases is the risk of carcinogenesis. Epidemiological studies on atomic bomb and nuclear plant accident survivors and patients irradiated for benign diseases demonstrated an increased incidence of radiation-induced cancer over the years since the exposure [31]. Nevertheless, epidemiological studies indicated a reduced occurrence of solid cancers, despite the low levels of radiation exposure. Moreover, several in vitro and in vivo studies have suggested that DNA damage and chromosomal aberrations are significantly decreased at lower radiation doses [32].

In this study, the anti-inflammatory effects of LDRIR were also observed with lower dose rates of LDRIR. A lower dose rate of LDRIR could reduce the risk of carcinogenesis while maintaining the anti-inflammatory effect.

Despite the novel findings, our study has some limitations. We assessed the regulation of candidate cytokine expression only at 24 h after irradiation. However, the period for which this regulation was maintained remains unclear. Moreover, we cannot exclude the possibility of variations in mRNA expression levels or cytokine concentrations by time point. Thus, future research on changes in mRNA expression and cytokine levels over time and the long-term effects of LDRIR is required. In addition, whether the effect of LDRIR is mediated by the NRF1/2 pathway through the regulation of cytokine expression must be investigated (figure 7). Moreover, we performed three repetitive experiments for each experimental group. Since the cell line from one patient was considerably unstable, conducting multiple experiments was impossible; therefore, we could not perform further experiments for each treatment. Furthermore, we used samples obtained from only female patients, and detailed drug histories were not obtained. We included only female patients because knee OA cases requiring total knee replacement surgery were much more prevalent in women; therefore, obtaining tissue samples from women was easier. Moreover, we selected only women to facilitate a consistent study design. However, the possibility that radiation responses may differ according to drug history or sex cannot be excluded.

Figure 7. Schematic representation of the crosstalk between LDRIR and inflammation pathways. GRO-α, growth-regulated protein alpha; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; LDRIR, low-dose rate ionizing radiation; NF-κB, nuclear factor kappa light chain enhancer of activated B cells; Nrf2, nuclear factor erythroid 2-related factor 2.

CONCLUSION

In conclusion, we demonstrated that LDRIR with a low dose rate can compromise LPS-induced inflammation in human knee joint-derived cells by decreasing ICAM-1, IL-6, IL-8, and GRO-an expression. Because the extent of DNA double-stranded breaks by radiation therapy is decreased at lower dose rates, LDRIR at a low dose rate can relieve concerns of carcinogenesis and may be useful in clinical settings.

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Data Availability: The data used to support the findings of this study are included within the article.

Author Contribution: S.S. and T.G.S. designed the experiments. T.Y. and Y.S.L. conducted the *in vitro* irradiation experiments and performed cell viability, Western blot, reverse transcription polymerase chain reaction, cytokine and enzyme-linked immunosorbent assay analyses. W.T.K. and M.Y.C. performed immunostaining. S.H.K., H.C.B., and H.S.H interpretated the data. H.C.B. and H.S.H obtained adjacent human normal samples. T.G.S. and T.Y. conducted the statistical and statistical learning analyses. S.S, T.G.S, and T.Y wrote the manuscript. All authors reviewed and edited the manuscript. T.G.S. supervised the study.

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