



ORIGINAL ARTICLE

Single Low-Dose Radiation Induced Regulation of Keratinocyte Differentiation in Calcium-Induced HaCaT Cells

Hyung Jin Hahn, Hae Jeong Youn¹, Hwa Jun Cha², Karam Kim², Sungkwan An^{2,*}, Kyu Joong Ahn^{1,*}

Department of Dermatology, Uijeongbu St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Uijeongbu,
¹Department of Dermatology, Konkuk University School of Medicine, ²Korea Institute for Skin and Clinical Sciences and
Molecular-Targeted Drug Research Center, Konkuk University, Seoul, Korea

Background: We are continually exposed to low-dose radiation (LDR) in the range 0.1 Gy from natural sources, medical devices, nuclear energy plants, and other industrial sources of ionizing radiation. There are three models for the biological mechanism of LDR: the linear no-threshold model, the hormetic model, and the threshold model. **Objective:** We used keratinocytes as a model system to investigate the molecular genetic effects of LDR on epidermal cell differentiation. **Methods:** To identify keratinocyte differentiation, we performed western blots using a specific antibody for involucrin, which is a precursor protein of the keratinocyte cornified envelope and a marker for keratinocyte terminal differentiation. We also performed quantitative polymerase chain reaction. We examined whether LDR induces changes in involucrin messenger RNA (mRNA) and protein levels in calcium-induced keratinocyte differentiation. **Results:** Exposure of HaCaT cells to LDR (0.1 Gy) induced p21 expression. p21 is a key regulator that induces growth arrest and represses stemness, which accelerates keratinocyte differentiation. We correlated involucrin expression with

keratinocyte differentiation, and examined the effects of LDR on involucrin levels and keratinocyte development. LDR significantly increased involucrin mRNA and protein levels during calcium-induced keratinocyte differentiation. **Conclusion:** These studies provide new evidence for the biological role of LDR, and identify the potential to utilize LDR to regulate or induce keratinocyte differentiation. (*Ann Dermatol* 28(4) 433 ~ 437, 2016)

-Keywords-

Cell differentiation, Keratinocytes, Low-dose radiation

INTRODUCTION

Low-dose radiation (LDR; ≤ 100 cGy) is used for diagnostic imaging and interventional radiology¹. We are continuously exposed to LDR from natural sources, medical devices, nuclear energy plants, and other industrial sources of ionizing radiation². Therefore, it is important to understand the biological effects of LDR. The biological effects of LDR have been explained by the linear no-threshold (LNT) model, which considers that LDR effects are similar to those of high-dose ionizing radiation (HDR). The LNT model suggests that, similar as HDR, even very low exposure to LDR can cause harmful DNA lesions, such as single-strand breaks, double-strand breaks, apurinic/apyrimidinic site (either apyrimidinic or apurinic), DNA-DNA and DNA-protein cross-linking, and a plethora of base modifications³. However, there is some debate whether LDR is harmful to living organisms. Some studies have shown that LDR induces beneficial effects on DNA stability, growth rate, survival, life span, and immune activa-

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*These authors equally contributed to this work.

Corresponding author: Kyu Joong Ahn, Department of Dermatology, Konkuk University School of Medicine, 120 Neungdong-ro, Gwangjin-gu, Seoul 05029, Korea. Tel: 82-2-2030-5172, Fax: 82-2-2030-5179, E-mail: kjahn@kuh.ac.kr

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tion, whereas other studies have shown that LDR has no biological effects^{4,8}. Repeated exposure to LDR induces adaptation to ionizing radiation. Therefore, understanding the biological effects of LDR is crucial, particularly with the increasing use of LDR in medical and therapeutic protocols.

The epidermis is the outermost layer of the body, which forms a physical and immunological barrier. Keratinocytes are the most abundant cells in the epidermis⁹. Differentiating keratinocytes migrate from the stratum basale to the stratum corneum; this migration is implicated in forming the physical and immunological barrier and helping to form a multilayered epidermis. Mouse skin keratinocytes exposed to HDR exhibit accelerated differentiation due to the activation of protein kinase C (PKC) signaling pathways¹⁰. HDR-mediated activation of PKC induces the G2/M arrest and reduces keratinocyte stemness, which accelerates the initiation of keratinocyte differentiation¹¹. In this study, we investigate whether LDR also accelerates keratinocyte differentiation.

MATERIALS AND METHODS

Cell culture and exposure to ionizing radiation

HaCaT cells were obtained from Cell Lines Service (Eppelheim, Germany) and cultured as described in a previous study¹² in serum-free medium (GIBCO; Invitrogen, Carlsbad, CA, USA). Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. For exposure to ionizing radiation, cells were seeded in a 60 mm culture dish (SPL Life Sciences, Seoul, Korea) and exposed to a ¹³⁷Cs γ -ray source (MDI-KIRAMS 137 irradiator; Korea Institute of Radiological and Medical Sciences, Seoul, Korea).

Cell viability analysis

Cell viability was determined by the tetrazolium salt (WST-1) assay (EZ-Cytox Cell Viability Assay kit; ITSbio, Seoul, Korea). HaCaT cells (1×10^4 cells) were seeded into the wells of 96-well plates and cultured for 24 h. Then, cells were irradiated with 0, 0.01, 0.05, and 0.1 Gy gamma radiation (MDI-KIRAMS 137) and incubated for 28 h. Next, WST-1 solution was added to LDR-irradiated cells for 30 min. After incubation for 30 min, the absorbance of the samples at 450 nm was measured spectrophotometrically using a plate reader (iMark plate reader; Bio-Rad Laboratories Inc., Hercules, CA, USA). The optical density of each well was determined to quantify cell viability.

Western blot analysis

Western blot assays were performed on cell pellet lysates in radioimmunoprecipitation assay buffer with protease in-

hibitors (Roche, Basel, Switzerland), as previously described¹³. The antibodies used were anti-involucrin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti- β -actin (Sigma-Aldrich, St. Louis, MO, USA). Western blots were scanned and band intensities were quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (PCR) was performed as described previously¹⁴. In brief, total RNA was isolated using TRIzol (Invitrogen; Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Then, cDNAs were synthesized from 1 μ g total RNA using reverse transcriptase (Bioneer, Daejeon, Korea). PCR was performed using SYBR Green I in a PCR premix and a LineGene K cycler (BioER, Hangzhou, China). All results shown are representative of at least three independent experiments. All primers used for PCR have been reported in a previous study and include: involucrin forward primer, 5-CAAAGAACCTGGAGCAGGAG-3; involucrin reverse primer, 5-CAGGGCTGGTTGAA TGTCTT3; β -actin forward primer, 5-CGACAGGATGCA GAAGGAG-3; and β -actin reverse primer, 5-ACATCTG CTGGAAGGTGGA-3. β -actin was used as the internal loading control. Changes in relative levels of involucrin messenger RNA (mRNA) were obtained by relating each PCR product to its internal loading control.

Statistical analysis

Statistical significance was determined by t-test with Microsoft Excel 2016 (Microsoft, Redmond, WA, USA). A *p*-value 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

LDR accelerates keratinocyte differentiation

LDR increased involucrin protein levels in calcium-activated developing keratinocytes (Fig. 1). The relative involucrin expression level was elevated from 2,649.03 to 17,053.31 at day 8. LDR also induced increases in involucrin mRNA levels (Fig. 2). Involucrin is a representative marker of keratinocyte differentiation, and accumulates according to the level of differentiation¹⁵. Therefore, LDR-induced increases in involucrin mRNA and protein levels indicate that ionizing radiation facilitates calcium-mediated keratinocyte differentiation. In previous studies, gamma radiation has diverse effects on proliferation, DNA repair, release of cytokines and etc. in *in vitro* cellular as well as *in vivo* models¹⁶⁻²⁰. Especially, high gamma radiation increases calcium-induced keratinocyte

differentiation through activating PKC signaling pathway in keratinocytes¹⁰. Thus, in our present study, we show

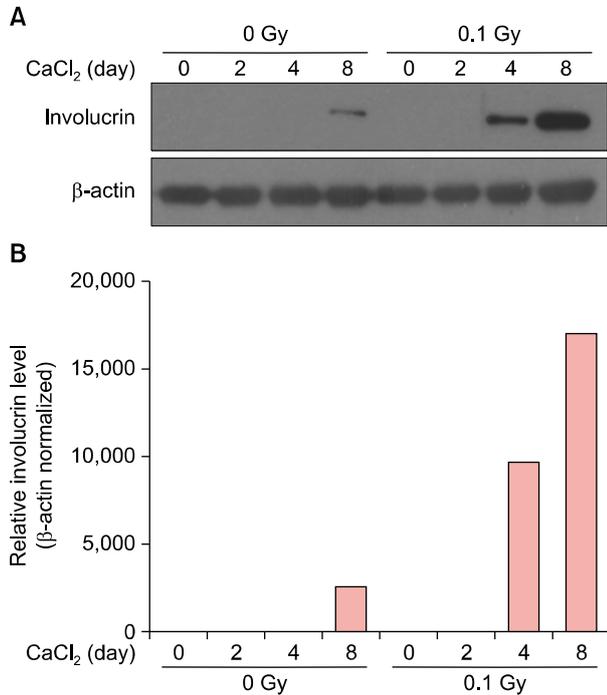


Fig. 1. Low-dose radiation stimulates involucrin accumulation in calcium-induced differentiating keratinocytes. (A) Western blot analysis of involucrin protein levels in control HaCaT cells (no exposure to ionizing radiation) and HaCaT cells exposed to 0.1 Gy gamma irradiation. In each experiment, equal loading was demonstrated by immunostaining for β -actin. (B) The band densities of interest were further analyzed by ImageJ. Involucrin levels were normalized against β -actin.

that effects of LDR on keratinocyte differentiation.

LDR induces growth arrest by elevating p21 (Cip1/Waf1) expression

Song et al.¹⁰ show that HDR-induced acceleration of calcium-mediated keratinocyte differentiation is caused by a reduction in stemness and growth arrest due to PKC activation. Therefore, we tested whether LDR could in-

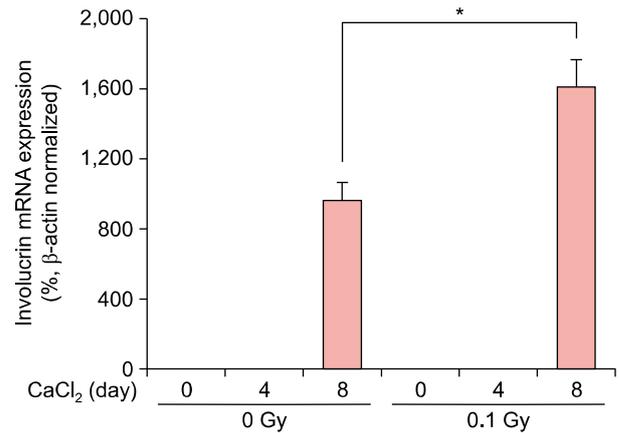


Fig. 2. Low-dose radiation elevates involucrin messenger RNA (mRNA) expression in calcium-induced differentiating keratinocytes. HaCaT cells were exposed to gamma radiation and control cells were untreated. After 4 h, keratinocyte differentiation was induced in HaCaT cells using 2 mM calcium for 4 and 8 days. Total RNA, isolated using TRIzol, was used to synthesize cDNA by reverse transcription, and was amplified by polymerase chain reaction. β -actin was amplified as a normalization control. Results are expressed as the mean percentage \pm standard error of the experiments performed in triplicate. * $p < 0.01$.

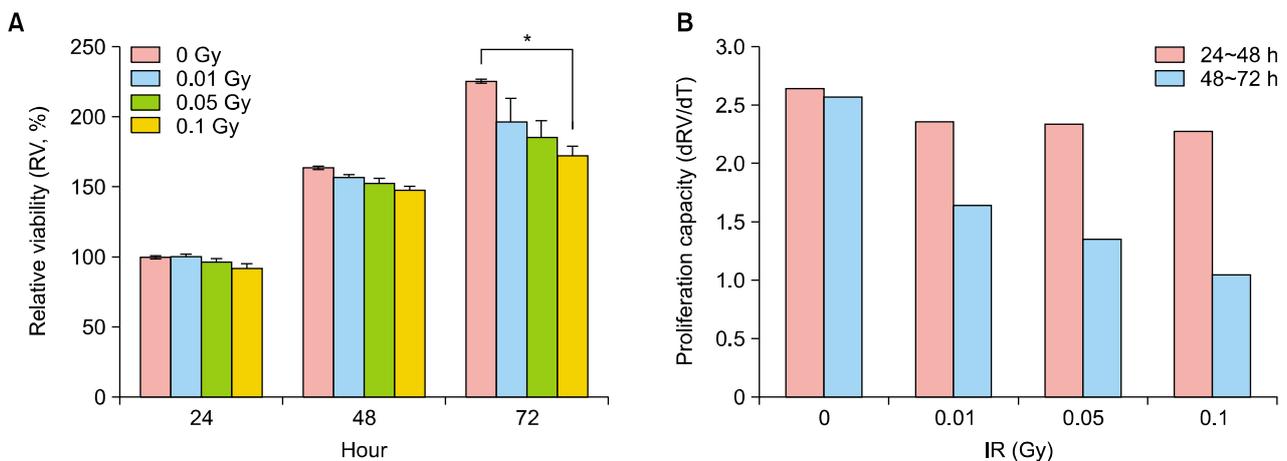


Fig. 3. Low-dose radiation reduced cell viability and growth capacity in HaCaT cells. (A) HaCaT cells (1×10^4 cells) were plated in 96-well plates and cultured for 24, 48, and 72 h after the exposure to the indicated dose of gamma radiation. Cell viability was measured by WST-1 as described in Materials and Methods. Results are expressed as the mean optical density \pm standard error, as a percentage of the control for the experiments performed in triplicate. * $p < 0.01$. (B) Growth capacity was calculated by calculating the dRV/dT as derivative relative viability (%) / derivative time (h). IR: gamma irradiation.

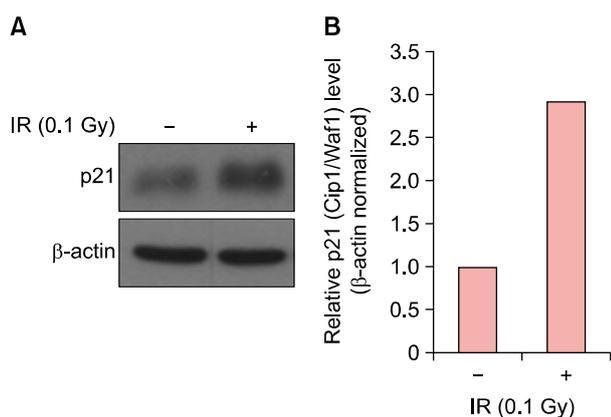


Fig. 4. Low-dose radiation induced p21 (Cip1/Waf1) expression in HaCaT cells. (A) Western blot analysis of p21 (Cip1/Waf1) protein levels in control HaCaT cells and HaCaT cells exposed to 0.1 Gy gamma irradiation. Equal loading in each experiment was demonstrated by immunostaining for β -actin. (B) Band densities of interest were further analyzed by ImageJ; p21 (Cip1/Waf1) levels were normalized against β -actin. IR: gamma irradiation.

duce keratinocyte growth arrest (Fig. 3). The results showed that LDR reduced keratinocyte viability in a time- and dose-dependent manner (Fig. 3), which is similar to the effects of HDR. Cell proliferation capacity was reduced by the exposure to 0.1 Gy LDR from 2.58 to 1.05 at 48~72 h, which suggested that LDR induced growth arrest. We also found that LDR elevated p21 (Cip1/Waf1) expression in the HaCaT cells (Fig. 4).

In general, increase of p21 (Cip1/Waf1) suppresses broad range of cyclin/CDK complexes, which leads to G1, G2 and S-phase arrest. Thus, p21 (Cip1/Waf1) represses stemness through the regulation of the cell cycle²¹, and is a well-characterized radiation marker that is activated by PKC^{11,22}. In keratinocytes, p21 (Cip1/Waf1) is increased by calcium-induced keratinocyte differentiation²³. And over-expression of p21 (Cip1/Waf1) induces acceleration of keratinocyte differentiation²³. Therefore, since keratinocytes initiates differentiation through halting cell proliferation and decreasing stemness, LDR-mediated elevation of p21 (Cip1/Waf1) facilitated calcium-mediated keratinocyte differentiation.

Ionizing radiation activates the PKC signaling pathway, which elevates the p21 expression levels. The PKC signaling pathway activation and elevation of p21 expression levels induces cell growth arrest and reduces stemness, which accelerates keratinocyte differentiation in HaCaT cells exposed to LDR. These results are consistent with previous results showing that HDR accelerates keratinocyte differentiation. Our results provide new evidence for the biological role of LDR, and suggest the potential uti-

lization of LDR as an inducer of keratinocyte differentiation on *in vitro* model.

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