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A secretome analysis reveals that PPARα is upregulated by fractionated-dose γ-irradiation in three-dimensional keratinocyte cultures

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ABSTRACT

Studies have shown that γ-irradiation induces various biological responses, including oxidative stress and apoptosis, as well as cellular repair and immune system responses. However, most such studies have been performed using traditional two-dimensional cell culture systems, which are limited in their ability to faithfully represent *in vivo* conditions. A three-dimensional (3D) environment composed of properly interconnected and differentiated cells that allow communication and cooperation among cells via secreted molecules would be expected to more accurately reflect cellular responses. Here, we investigated γ-irradiation–induced changes in the secretome of 3D-cultured keratinocytes. An analysis of keratinocyte secretome profiles following fractionated-dose γ-irradiation revealed changes in genes involved in cell adhesion, angiogenesis, and the immune system. Notably, peroxisome proliferator-activated receptor-α (PPARα) was upregulated in response to fractionated-dose γ-irradiation. This upregulation was associated with an increase in the transcription of known PPARα target genes in secretome, including angiopoietin-like protein 4, dermokine and kallikrein-related peptide 12, which were differentially regulated by fractionated-dose γ-irradiation. Collectively, our data imply a mechanism linking γ-irradiation and secretome changes, and suggest that these changes could play a significant role in the coordinated cellular responses to harmful ionizing radiation, such as those associated with radiation therapy. This extension of our understanding of γ-irradiation-induced secretome changes has the potential to improve radiation therapy strategies.

1. Introduction

Energy from γ-radiation is transferred to target molecules, yielding highly reactive free radicals in a process referred to as ionization. The resulting free radicals damage macromolecules, causing oxidation of lipids and proteins, and—importantly—significantly damaging DNA double helixes [1]. Specifically, free radicals break double-stranded DNA and prevent replication, thereby inducing cell death. In addition, γ-irradiation induces oxidative stress, apoptosis, and activates DNA repair pathways [2]; it also elicits immune responses. The magnitude and extent of exposure determine the severity of γ-radiation damage, with the most radiosensitive organ systems being bone marrow, reproductive and gastrointestinal systems, skin, muscle, and the brain [3]. In an effort to reduce radiation exposure during radiation therapy, clinicians often use fractionated-dose irradiation for radiation therapy [4], applying an appropriate dose and schedule of γ-irradiation. However, many patients still suffer from side effect of radiation therapy, and once γ-irradiation–induced harm has been done, treatment options are very limited and rudimentary.

Among radiation-vulnerable organ systems, the skin, as the outermost tissue of the human body, is most easily exposed. Radiation therapy commonly results in exposure of the epidermis to radiation, and prolonged exposure to ionizing radiation is associated with skin diseases, such as dermatitis [5]. Considerable research effort has been devoted to studying the effects of radiation exposure to the skin. However, most such studies have used traditional two-dimensional culture systems, which typically fail to reproduce the conditions cells experience *in vivo*. Although skin is mainly composed of keratinocytes (~95%), the level of their

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differentiation varies. Undifferentiated keratinocytes proliferate in the basal layer, and are progressively differentiated in upper layers, where they reach terminal differentiation. The functional roles of keratinocytes differ according to their differentiation states. Different stages of keratinocytes may behave differently and exhibit substantial differences in their degree of cooperation. Therefore, considerable research effort has been devoted to developing more physiologically relevant cell culture methods. Three-dimensional (3D) culture systems employing primary human keratinocytes grown at an air/liquid interface, which leads to the differentiation of keratinocytes, have been optimized for the study of skin [6]. In these 3D cultures, keratinocytes are stratified and differentiated, reproducing a fully differentiated epithelium. Therefore, 3D culture systems are superior to traditional systems for studying cell-cell communication and cooperation in epithelium.

In this study, we investigated γ-irradiation–induced changes in the keratinocyte secretome using a 3D culture system and Affymetrix Primeview array analyses. The resulting extension of our understanding of γ-irradiation–induced secretome changes has the potential to offer better strategies for clinical applications of γ-irradiation.

2. Materials and methods

2.1. Cell culture and sample preparation

All human cells used our experiments were purchased from Biosolution., Ltd (Korea). 3D culture was performed as described previously [7]. Keratinocytes (passage #5) were seeded at a density of 2×10^5 cells per dermal equivalent and cultured first in a submerged state for 7 days and then in an air-liquid interface state for 7 days. In the air-liquid interface state, cultures were maintained in growth media consisting of Dulbecco's modified Eagle's medium (DMEM; Welgene Inc., South Korea) and Ham's nutrient mixture F12 (Welgene Inc.) at a 3:1 ratio, supplemented with 10% fetal bovine serum (FBS), 1×10^{-10} M cholera toxin, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, and 2×10^{-11} M triiodothyronine. Upon completion of differentiation, samples were exposed to fractionated doses (four doses at 2 Gy/dose) of γ-irradiation at 24-h intervals; a control, non-irradiated 3D culture was also prepared. RNA was isolated 6 h after the final γ-irradiation dose. The irradiations were performed with Gammacell 3000 (Atomic Energy, Canada) with [^{137}Cs].

2.2. RNA isolation

In order to isolate keratinocytes from dermal equivalent, upper parts of 3D cultures were separated and subjected to further processing. Total RNA was extracted from 3D culture of keratinocytes using the TRI Reagent (Molecular Research Center, OH, USA) according to the manufacturer's instructions. Briefly, the isolated keratinocytes were processed in 1 ml of TRI Reagent, mixed with 0.2 ml of chloroform, and centrifuged at 12,000 × g for 15 min at 4 °C. The aqueous phase containing RNA was transferred to a new tube, and RNA was precipitated by mixing 0.5 ml of isopropyl alcohol and recovered by centrifuging at 12,000 × g for 10 min at 4 °C. The RNA pellet was washed briefly in 1 ml of 75% ethanol and centrifuged at 7500 × for 5 min at 4 °C. The resulting total RNA pellet was dissolved in 0.1% diethylpyrocarbonate (DEPC)-treated water, and its quality and quantity were assessed.

2.3. Microarray

Gene expression was analyzed using GeneChip Affymetrix Primeview arrays (Affymetrix, CA, USA). The data were analyzed with Robust Multi-array Analysis software using Affymetrix default analysis settings and global scaling as the normalization method. The trimmed mean target intensity of each array was arbitrarily set to 100. Normalized and log-transformed intensity values were then analyzed using GeneSpring GX 12.5 (Agilent technologies, CA, USA). Upregulated genes were defined as those whose expression was increased by ≥ 150% compared with controls, and downregulated genes were those expressed at levels ≤66% of controls. Hierarchically clustered data corresponded to clustered groups that behaved similarly across experiments using GeneSpring GX 12.5 (Agilent Technologies). The clustering algorithm used provided Euclidean distance and average linkage.

2.4. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

cDNA was prepared from total RNA extracted for microarray analysis by reverse transcription using Superscript II Reverse Transcriptase (Invitrogen, MA, USA). The resulting cDNA samples were diluted to 20 ng/μl. All real-time PCR reactions were performed using the BioRad CFX96 system (BioRad, CA, USA), and amplifications were performed using the SYBR Green PCR Master Mix (Enzo Life Science, MI, USA). Thermal cycling conditions consisted of a denaturation step at 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 30 s. Experiments were carried out in triplicate for each data point. Relative expression of target genes was quantified using the $2^{-\Delta\Delta Ct}$ method [8].

2.5. Immunohistochemistry

For immunohistochemical analyses, 9 h after the final γ-irradiation dose, 3D-cultured human keratinocyte were fixed in Carnoy's solution (ethanol:chloroform:acetic acid; 6:3:1) for 30 min at 4 °C. Fixed samples were embedded in paraffin, sectioned (3 μm), and placed on slide glass. For antigen unmasking, samples were heated in 10 mM sodium citrate buffer (pH 6.0), cooled for 20 min, and incubated in 0.5% H_2O_2 for 30 min. The samples were blocked with normal horse serum (20% in PBS) for 1 h, washed three times with PBS, and incubated overnight with anti-PPARα antibody (Santa Cruz Inc., CA, USA). Sections were then washed in PBS and immunoreactivity was detected using the Vectorstain ABC kit (Vector Labs.; Peterborough, England) for 30 min. After washing twice in PBS, positive antibody binding was visualized using the DAB Kit. The samples were counter-stained with hematoxylin and examined using a light microscope.

2.6. Statistical analysis

All data were analyzed using Microsoft Office Excel (Microsoft Corp., WA, USA) and are presented as means ± standard deviations (SDs). *p*-values less than 0.05, determined by Student's t-test, were considered significant.

3. Results

3.1. Fractionated-dose ionizing radiation induces differential expression of various genes in 3D-cultured keratinocytes

To understand the cellular effects of repeated γ-irradiation, we exposed keratinocytes, cultured in 3D as described in Materials and Methods, to fractionated-dose γ-irradiation (four doses of 2 Gy at 24-h intervals). Since the time required for newly-made proteins is about 6 h, we designed the experimental schedule with 6 h intervals. Numerous studies have reported that cells grown in 3D exhibit different gene expression patterns than those grown in 2D