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Note

mRNA Expression Profiles in Human Lymphoid Progenitor Cell are Modified by High-dose Ionizing Radiation

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The treatment of lymphocytopenia induced by high-dose ionizing radiation (IR), a component of acute radiation syndrome (ARS), is an important issue in radiation emergency medicine. A limited stockpile of cytokines (hematopoietic stimulation factors) is recommended by the International Atomic Energy Agency in case of radiation disasters leading to high numbers of ARS cases. We hypothesized the presence of a radiosensitive target molecule specifically in lymphoid cells (the most radiosensitive lineage of hematopoietic cells) could improve treatment of ARS through pharmacological intervention. To examine this, human lymphoid progenitor mRNA from IM-9 cell was isolated and analyzed using cDNA microarray. The mRNA profiles demonstrated a strong correlation between IR and up-regulation of p53 signaling and 10 other molecules that together constituted a complete signaling pathway (*TNFRSF10B, FAS, PIK3CG, BBC3, TP53I3, CDKN1A, JUN, MDM2, RRM2B,* and *TP53INP1*). Only one member of this pathway, RB1, was found to be down-regulated. The induction of apoptosis and accumulation of G2/M cell cycle phase were observed following IR. These results identified that behavior of lymphoid progenitor cell following IR regulates apoptosis-related signaling and cell cycle-related signaling by eleven mRNAs. In our next study phase, we aim to verify whether the alternation of these gene expressions is similarly observed in each lymphoid lineage (from immature progenitors to mature cells) and other radiosensitive cell types following IR, and whether gene expression control is possible in lymphoid cells.

Key words: lymphoid progenitor cells, acute radiation syndrome, mRNA expression, ionizing radiation

1. Introduction

Acute radiation syndrome (ARS) occurs following whole, or partial, body exposure to ionizing radiation (IR) greater than 1 Gy and may be prevented by urgent intervention. As prophylaxis against the hematopoietic manifestation of ARS, the International Atomic Energy Agency recommends the administration of hematopoietic growth factors and cytokines, such as granulocyte colony-stimulating factor and granulocyte macrophage colony-stimulating factor[1, 2]. However, stockpiles are limited and many individuals may require treatment for ARS in the case of a substantial, unexpected radiation disaster. Therefore, it is important that new pharmacological

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agents and techniques for the treatment and prevention of ARS are developed. To address this issue, we studied lymphoid lineage cells, the most radiosensitive lineage of hematopoietic cells, and analyzed gene expression profiles following IR. In our previous studies, we clarified that *MDM2* and *CDKN1A*, known to be involved in p53-ubiquitination and cell cycle arrest, are up-regulated in lymphoid cells exposed to X-irradiation[3]. However, the mechanism underlying up-regulation of these genes in response to X-irradiation in lymphoid cells is currently unknown. We hypothesized the presence of a radiosensitive target molecule specific to lymphoid cells whose identification may provide a novel therapeutic target for ARS.

In this study, to clarify the network of mRNA expression in human lymphoid cell, cDNA microarray analysis was performed using IM-9 cell, which is lymphoid cell model, exposed to X-irradiation.

2. Materials and Methods

Cell culture

The human lymphoblast model IM-9 (JCRB0024) was purchased from the Health Science Research Resources Bank (Osaka, Japan). IM-9 cells were cultured in Roswell Park Memorial Institute-1640 medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Life technologies, Tokyo, Japan) and antibiotics, including 100 U/ml penicillin and 100 μg/ml streptomycin (Wako), at 37°C in a humidified atmosphere of 95% air/5% CO_2.

Irradiation

X-ray irradiation (150 kVp, 20 mA, 0.5-mm aluminum and 0.3-mm copper filters) was performed using an X-ray generator (MBR-1520R-3, Hitachi Medical Co. Ltd., Tokyo, Japan) with a distance of 45 cm between the focus and the target. The dose was monitored with a thimble ionization chamber placed next to the sample during the irradiation. The dose rate was approximately 0.8 Gy/min. IM-9 cells plated on the 6-well plates at 2×10^5 cells/well were cultured for 24 h prior to X-irradiation for collecting mRNAs that respond to IR sufficiently.

Analysis of viable cells

The cell viability of cell culture was assessed using an annexin V and propidium iodide (PI) staining kit (Biolegend; Tomy Digital Biology, Tokyo, Japan). The cell cycle distribution was analyzed by Hoechst 33342 staining. Fluorescence data were collected using a flow cytometry system (Aria SORP; BD Biosciences, Franklin Lakes, NJ, USA).

RNA extraction and microarray analysis

cDNA microarray scanning was performed on a single sample containing a mixture of three separately cultured cells. Collected cells were frozen in liquid nitrogen and stored at −80°C until further use. RNA was extracted using Isogen II (Nippon Gene, Tokyo, Japan) according to manufacturer's instructions. The concentration of quality of isolated RNA was determined using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA integrity was examined using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's instructions. Cyamine 3 (Cy3)-labeled cDNA was synthesized from 10 μg of RNA from each irradiated cell sample using a LabelStar Array Kit (Qiagen, Valencia, CA, USA), Cy3-dUTP (GE Healthcare, Buckinghamshire, UK), and a random monomer primer according to manufacturer's instructions. Cy3-labeled cDNA was purified using MinElute kits (Qiagen). Measurement of the concentration and quality of Cy3-labeled cDNA was performed using an Agilent Bioanalyzer. Agilent 44 K×4 human custom microarray slides (Agilent eArray Design ID = 19052, produced by Tsukuba GeneTech Laboratory, Ibaraki, Japan) were hybridized with Cy3-labeled cDNA (2μg) in hybridization solution and blocking Agent (Agilent Technologies) at 65°C for 16 h using a hybridization oven. Microarray slides were washed in wash Buffer 1 and 2 (Agilent Technologies) including 0.005% Triton X-102. Washed microarray slides were dried using nitrogen gas. Cy3 fluorescence images of array slides were obtained using a DNA microarray scanner (Agilent Technologies) and were processed using Feature Extraction version 8.1 software based on instructions from Agilent Technologies. The validation of the molecules that we focused on in this study was performed by quantitative RT-PCR (SmartCycler® II, TAKARA Bio Inc., Japan).

Statistics

Gene expression data from cDNA microarrays were processed using Microsoft Office Excel 2010 software (Microsoft Corporation, Redmond, WA) and perform global normalization between the respective microarrays. Up-regulated and down-regulated mRNAs transcripts were selected based on signal values (>50) of respective genes and fold change (>1.5-fold) of irradiated samples (1 Gy) versus non-irradiated samples (0 Gy). Functional analysis of each transcript was performed using Ingenuity® Pathway Analysis tools (QIAGEN Silicon Valley, Redwood City, CA, USA).

Table 1. Top 10 categories of significant pathways

Name of the pathway	*P*-value	Number of molecules
p53 signaling	2.84×10^{-8}	12
Agrin interactions at the neuromuscular junction	7.37×10^{-6}	8
GDNF family ligand-receptor interactions	7.33×10^{-5}	7
Inhibition of angiogenesis	1.09×10^{-4}	5
GNRH signaling	1.34×10^{-4}	9
HGF signaling	1.80×10^{-4}	8
Pancreatic adenocarcinoma signaling	2.05×10^{-4}	8
Activation of IRF by cytosolic pattern recognition receptors	2.84×10^{-4}	6
Ga12/13 Signaling	4.03×10^{-4}	8
Production of nitric oxide and reactive oxygen species in macrophages	4.03×10^{-4}	10

3. Results and Discussion

Collection of cDNA microarray data

Analysis of intracellular mRNA transcripts from IM-9 cells exposed to 1 Gy X-irradiation was performed using a cDNA microarray system. A total of 45220 target probes were exported. A total 356 entities were differentially expressed (greater or less than 1.5-fold difference between 1 Gy X-irradiated samples vs. non-irradiated samples) and exported to the Ingenuity® analysis system. Of these, 303 molecules (or genes) were used for pathway analysis. In canonical pathway analysis, a strong relationship between the top 10 gene expression pathways altered following exposure to 1 Gy X-irradiation was observed (Table 1). In particular, p53 signaling ($P = 2.84 \times 10^{-8}$), agrin interactions at neuromuscular junction ($P = 7.37 \times 10^{-6}$), and GDNF family ligand-receptor interactions ($P = 7.33 \times 10^{-5}$) were significantly altered. 12 genes within the p53 signaling pathway, the most significantly changed pathway, were found to be differentially expressed between groups.

Analysis of p53 signal networks

To fully elucidate the alteration of p53 signaling in IM-9 cells exposed to 1 Gy X-irradiation, network analysis was performed using the 12 genes referred to above. Eleven genes were mapped by network analysis (Fig. 1A). The genes encoding plasma membrane proteins, *TNFRSF10B* and *FAS*, were up-regulated 1.61 and 1.52-fold, respectively. Genes encoding cytoplasmic proteins, *TP53I3*, *BBC3*, and *PIK3CG*, were up-regulated 1.62, 1.59, and 1.69-fold, respectively. Genes encoding nuclear proteins, *JUN, CDKN1A, MDM2, TP53INP1, and RRM2B*, were up-regulated 1.73, 1.81, 1.90, 1.74, and 1.73-fold, respectively. Only *RB1*, encoding a cytoplasmic protein, was down-regulated 0.53-fold. The reproducibility of similar expressions of these molecules was verified by RT-PCR (Fig. 1B). There are previous reports of relationships between these genes. The protein P21, encoded by *CDKN1A*, is induced by stimulation of FAS/CD95 signaling in T lymphocytes[4, 5]. Praveen *et al*. reported JNK and Fas pathway-mediated apoptosis in blood lymphocytes was induced by 1 and 2 Gy radiation exposure[6]. The phosphorylation of RB1 is mediated by the activation of CDK4/Cyclin D1 and CDK2 and contributes to cell cycle progression[7]. In a model of acute T cell leukemia, CD95 has been shown to inactivate Rb (encoded by *RB1*)[8]. Therefore, these studies demonstrate a common signaling pathway in lymphoid progenitor cells upstream of p53 and centered around FAS up-regulation in response to IR.

Further downstream, up-regulation of *CDKN1A* and *MDM2* and related genes was identified. In general, these molecules activate p53 signaling and result in apoptosis induction. We also verified these cells after IR (Fig. 1C). Conversely, only *RB1*, a regulator of cell-cycle progression, was down-regulated by IR, resulting in its accumulation during the G2/M phase (Fig. 1D). Similar results have been reported in non-lymphoid cells. Sdek *et al*. reported that *MDM2* is a critical negative regulator of Rb protein through promotion of proteasome-dependent ubiquitin-independent degradation[9]. In addition, up-regulation of c-jun and CD95 suppressed the expression of Rb protein[8, 10, 11]. Upon exposure to IR, mutation of the *RB1* gene in osteoblastic cells induces cell cycle arrest and polyploidy[12]. These reports and our results suggest IR exposure in lymphoid progenitor cells simultaneously results in the induction of apoptosis and suppression of cell-cycle activity. Our data regarding this detailed signaling network is a first study of lymphoid cells exposed to high-dose IR. In our next study phase, we aim to verify whether the alternation of these gene expressions is similarly observed in each lymphoid lineage (from immature progenitors to mature cells) and other radiosensitive cell types following IR, and whether gene expression control is possible in lymphoid cells. These clarifications will hopefully lead to the development of drugs for ARS prophylaxis.

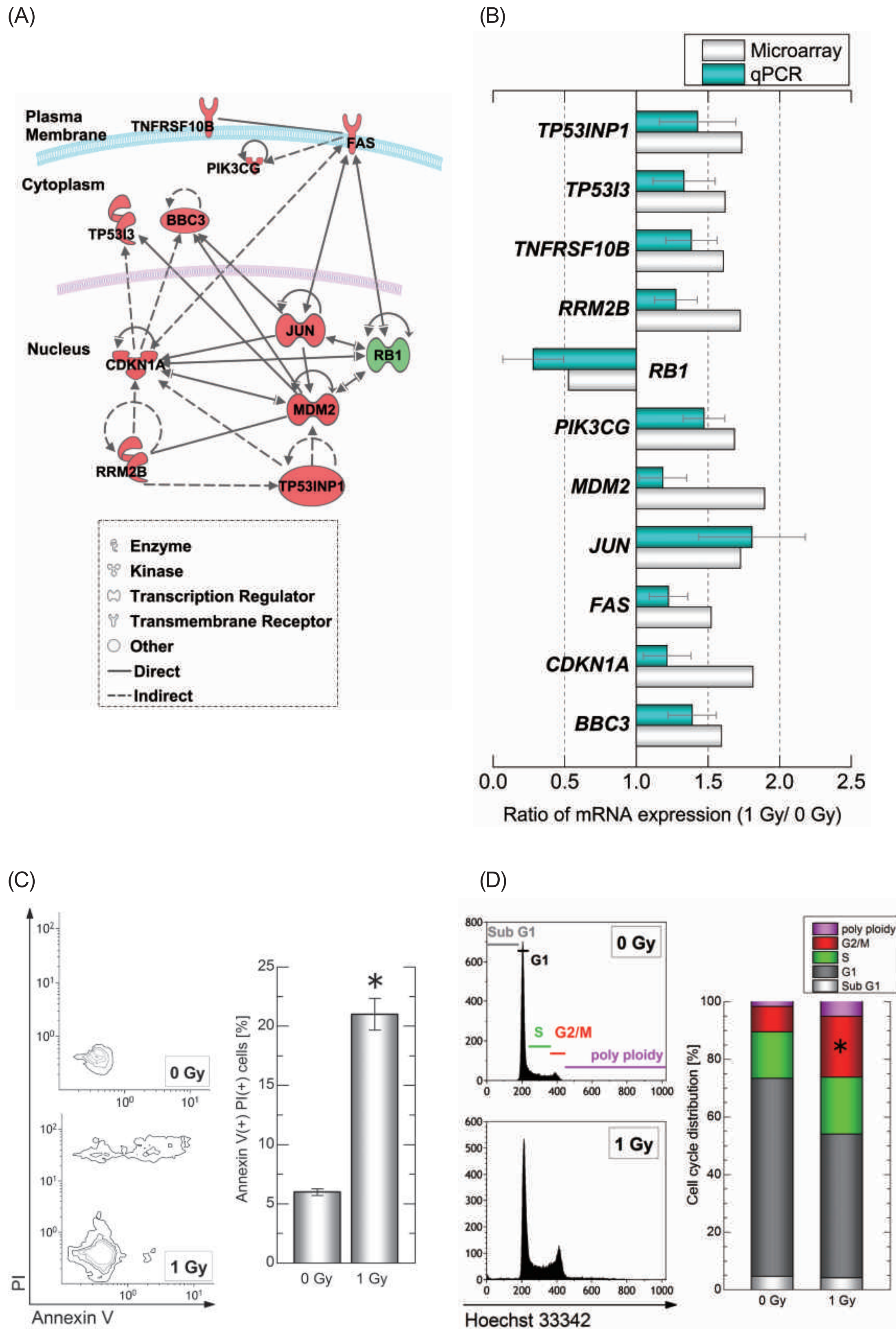

Fig. 1. Network analysis of the 11 molecules that showed significantly differential expression in response to IR (A, B) and cell viability (C, D). The expression analysis of up-regulated and down-regulated molecules by cDNA microarray mRNAs is shown in red and green, respectively (A). Using IPA database that reported in previous studies, a direct and indirect interaction between molecules has been depicted as continuous and dotted lines, respectively. The 11 mRNAs were verified by quantitative RT-PCR (B). The analysis of cell viability was performed by flow cytometry using annexin-V and PI fluorescence staining (C). Annexin-V/PI double-positive cells represent apoptotic cells. Cell-cycle distribution was analyzed using Hoechst 33342 staining (D). $^*P < 0.05$ vs. 0 Gy control.

In conclusion, we demonstrated that exposure of high-dose IR to lymphoid progenitor cell modifies the behavior of both apoptotic induction and cell-cycle progression through the differential expression of eleven identified genetic signaling pathways.

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