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Dynamic changes in the proteome of human peripheral blood mononuclear cells with low dose ionizing radiation

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ABSTRACT

Humans are continually exposed to ionizing radiation from natural as well as anthropogenic sources. Though biological effects of high dose radiation exposures have been well accepted, studies on low-to-moderate dose exposures (in the range of 50–500 mGy) have been strongly debated even as researchers continue to search for elusive 'radiation signatures' in humans. Proteins are considered as dynamic functional players that drive cellular responses. However, there is little proteomic information available in context of human exposure to ionizing radiation. In this study, we determined differential expressed proteins in G_0 peripheral blood mononuclear cells (PBMCs) from healthy individuals 1 h and 4 h after '*ex vivo*' exposure with two radiation doses (300 mGy and 1 Gy). Twenty-three proteins were found to be significantly altered in irradiated cells when compared to sham irradiated cells with fold change ±1.5-fold ($p \leq 0.05$), with only three proteins showing ≥2.5-fold change, either with dose or with time. Mass spectrometry analyses identified redox sensor protein, chloride intracellular channel protein 1 (CLIC-1), the antioxidant protein, peroxiredoxin-6 and the pro-survival molecular chaperone 78 KDa glucose regulated protein (GRP78) among the 23 modulated proteins. The mean coefficient of variation (CV) for the twenty-three radiation responsive protein spots was found to be 33.7% for 300 mGy and 48.3% for 1 Gy. We thus, conclude that the radiation proteomic response of G_0 human PBMCs, which are in the resting stage of the cell cycle, involves moderate upregulation of protective mechanisms, with low inter-individual variability. This study will help further our understanding of cellular effects of low dose acute radiation in humans and contribute toward differential biomarker discovery.

1. Introduction

For the past several decades, understanding cellular and biological effects of low dose radiation exposures, and to quantify risks from such exposures, has remained a scientific challenge. Biological effects at high doses of ionizing radiation, which are well above the low dose range for environmental or therapeutic radiation exposures (>1 Gy) have been clearly documented [1–3]. In recent times, due to more and more technological advances, the human genome has been increasingly threatened by low dose low-linear energy transfer radiation from environmental, medical, and in many cases, occupational sources. Understanding biological effects of low-to-moderate dose ionizing radiation (50–500 mGy), directly on humans, is important to address key radiation protection concerns. As several recent studies have suggested, for many biological end points, the responses at low doses might be different from that of high doses [4]. The 2011 Fukushima nuclear accident of Japan has again returned the spotlight on linear no-threshold model and on the importance of validating data obtained from cell-lines or animal models, directly on humans.

Proteins are considered to be key effector molecules through which a cell enacts cellular changes and fine tunes its response to micro environmental signals. The differences found in the proteome may thus, better reflect, the global responses of cells following radiation stress with no '*a priori*' hypothesis about biological mechanisms. Over the years, many studies have been published which identify radiation responsive proteins using traditional single protein approach. However, very few examine whole proteome changes in human cells exposed to IR, either *in vivo* or *in vitro* [5–7]. Moreover, most such investigations have been performed either on the biofluids of patients undergoing radiotherapy [8–11] or with human immortalized cells in culture, which may differ from

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responses in primary human cells [12–15]. In the recent published map of the human proteome from 32 different tissues and organs, extreme caution has been advocated while extending conclusions derived from cell line studies to the corresponding tissues. The authors reported down-regulation or complete "turn off" of many of the tissue-enriched genes in the corresponding cell lines of the normal tissues [16]. The variation in the experimental conditions and methods used for analysis in multiple studies, further limits this extrapolation [2]. Application of proteome profiling for radiation research has also been limited by lack of data on the time- and dose-dependent variation of protein expression. In addition, any attempt to identify potential biomarker in response to radiation that can be tested in molecular epidemiological studies has been constrained by inadequate information on the inherent genetic and physiological variability between individuals leading to differences in radiosensitivity which makes the interpretation of these results more challenging [17].

In this paper, we attempt to bridge this information gap by undertaking a study on human peripheral blood mononuclear cells (PBMCs), which are considered to be highly radiosensitive. Also, since these cells are in the G_0 resting stage of the cell cycle, they may effectively mimic the *in vivo* conditions. Human PBMCs are easy to collect through semi-invasive means and as some reports indicate, may hold the additional advantage of low inter-individual variation as compared to other biofluids [18]. Differential protein expression changes in G_0 PBMCs from healthy individuals were investigated after acute gamma irradiation using two dimensional gel based proteomics (2-DE) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The analysis were performed after irradiation of cells with two radiation doses (300 mGy and 1 Gy) to understand relatively low and moderately high dose effects and at two time points (1 h and 4 h) to discern the early and late responses, post irradiation. The use of 2-DE method allowed direct visualization and robust detection of intact proteins, especially for the comparative experiments [19]. In addition, we determined inter- individual variations of differentially expressed proteins in the healthy volunteers which will be useful for quantitative expression studies in large epidemiological datasets.

2. Materials and methods

2.1. Ethics statement

The blood samples were collected from healthy adult volunteers with informed consent. The project has been approved by the institutional ethics committee.

2.2. PBMC isolation and irradiation

Venous blood was collected from eight random healthy individuals in the age group of 25–45 years; and a gender ratio of 4/4 (M/F) in sterile EDTA tubes (BD^{TM} vacutainers, NJ, USA) and processed within 30 min of blood withdrawal. PBMCs were separated using Histopaque-1077 (Sigma–Aldrich Corp., MO, USA) density gradient media according to manufacturer's instructions. Cells were then counted and their viability was assessed by trypan blue exclusion. The isolated PBMCs were resuspended in RPMI-1640 media (Sigma–Aldrich Corp. MO, USA) and irradiated at room temperature using Co^{60} γ-rays (Blood irradiator, 2000, BRIT, India) at a dose rate of 0.4 Gy/min. Two radiation doses (300 mGy and 1 Gy) were used and the sham irradiated cells served as control. The irradiated cells were incubated in RPMI-1640 media at 37 °C in a humidified, 5% CO_2 atmosphere for the required time (1 h or 4 h) before analysis.

2.3. Sample preparation for proteome analysis

Cells were homogenized by sonication in 10 mM Tris Buffer, pH 7.0 containing 1X protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The cell extract was centrifuged at 20,000 × *g* for 40 min at 4 °C and the clear supernatant was collected. Protein concentration of the supernatant was determined in triplicate by bicinchoninic acid (BCA) method as recommended by the manufacturer (Bangalore Genei, India). Bovine serum albumin (BSA) was used as standard. The cell lysate was treated with benzonase endonuclease (Sigma–Aldrich MO, USA) at a final concentration of 0.5 U/μl of protein extract and purified using a Ready Prep 2D clean up kit (Bio-Rad, CA, USA) before loading on immobilized pH gradient (IPG) strips (Bio-Rad, CA, USA). All eight samples were singularly used for 2DE.

2.4. Two-dimensional polyacrylamide gel electrophoresis (2DE)

Isoelectric focusing (IEF) was performed on 17 cm ready made IPG strips on PROTEAN IEF system (Bio-Rad, CA, USA). The strips were rehydrated with pre-estimated protein samples dissolved in rehydration buffer (7 M urea, 20 mM dithiothreitol (DTT), 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 0.2% carrier ampholytes, 0.0002% bromophenol blue) by passive method. A three step program was used: 250 V for 20 min, 10,000 V for 4 h, and finally 60,000 Volt-h. After IEF, the strips were equilibrated first in an equilibration buffer I [6 M urea, 2% SDS, 0.05 M Tris-Cl (pH 6.8), 20% Glycerol and 2% DTT], then in equilibration buffer II containing 2.5% iodoacetamide instead of DTT. The second dimension electrophoresis was conducted using a PROTEAN-II vertical gel electrophoresis system on 10% SDS PAGE gels at 85 V. All the chemicals used for 2-DE were procured from Bio-Rad. CA, USA. For molecular weight range determination, molecular weight markers (Bangalore Genei, India) were applied during SDS-PAGE. After electrophoresis, gels were stained with coomassie blue R-250 and the images were acquired using a gel documentation system (Syngene, UK). Two gels were run for each sample preparation.

2.5. Image processing and analysis

2-D gel image analysis was performed with PDQuest software (ver 8, Bio-Rad, CA, USA). Each protein spot on the gel was marked by a standard spot number (SSP#), automatically assigned by the software. Manual editing was also done to correct ambiguous protein spots. Spot height (also known as peak value) of the Gaussian spot was employed to quantitate the level of each protein spot. To compensate for subtle differences in sample loading and inconsistencies in staining, sixteen gels from all the eight samples (with two gels for each sample) were normalized together. For normalization, the raw quantity of each spot in a member gel, divided by the total quantity of valid spots in the gel was used. Only the protein spot with a fold change ±1.5-fold change, and $p < 0.05$ were considered to be differentially expressed proteins. The fold change of protein expression between the two groups (irradiated *versus* sham-irradiated) was calculated by taking mean of spot intensity (measured as the relative volumes of spots) of all the gels in each group. A positive value indicates an increase in expression, and a negative value indicates a decrease in expression.

2.6. MALDI-TOF-Mass spectrometry and protein identification

Coordinates of protein spots of interest was matched and the spots were excised manually. The gel was destained by repeated washings with 50 mM NH_4HCO_3/acetonitrile (ACN) (v/v). An enzymatic in-gel digestion was performed with trypsin (25 ng/μl)