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PROTEOMIC AND GENOMIC APPROACH TO UNDERSTANDING γ-RADIATION INDUCED EARLY CELLULAR RESPONSE: BIOTECHNOLOGY IN RADIATION COUNTERMEASURES

12

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Abstract The advent of post-genomic era has caused a radical paradigm shift in underdtanding of cellular response to radiation exposure wherein the overall manifestation of cellular damage appears to be mediated through a complex cascade of molecular events. A series of events are postulated to go through a complex sequence of largely unclear interactions and processes to eventually manifest itself as discrete biological end-points. It is becoming increasingly clear that each repair system or pathway, though independent and of varying complexity, cross-talk to each other for most optimal biological responses, which exhibit both temporal and spatial components of responses. For many well founded reasons, there exists urgent requirement to identify reliable indicators of absorbed radiation

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doses and suitable technology for quick and reliable bio-dosimetry in cases of radiation exposure of large segment of human populations for appropriate medical interventions. This study has been initiated to identify early response proteins (ERP) and early response genes (ERG) that are altered in human peripheral blood lymphocytes (PBL) exposed to γ-irradiation in a medically relevant dose range. Using 2D-PAGE and HPLC/MS, 20 ERPs, which were either quantitatively or qualitatively (extent of protein phosphorylation) modulated, have been identified. In parallel, the second screening of the global gene expression using DNA microarray technique has identified 44 ERGs, almost all of which, except the docking protein 7 (DOK7), exhibited at least 2-fold up-regulation of the gene expression following increasing doses of γ-radiation. From the preliminary observations made in this piece of investigation it can be postulated that γ-radiation induces immune system mediated responses in lymphocytes.

INTRODUCTION

In the conventional paradigm of radiation biology, the effect of radiation was thought to be exclusively due to deposition of radiation energy indirectly and/or directly into cells, particularly in the nuclei. It is well accepted that radiation causes different types of damage especially to the cellular chromatin. For obvious and argumentably sustainable reasons, the genome (DNA) is the critical target of radiation damage with serious biological consequences. However, other biomolecules, including proteins, and/or components like membrane, mitochondrion, etc. may also get damaged and, thereby, seriously influence the overall impact of radiation on a biological system (Kiefer, 1990; Devi et. al, 2000). The indirect effects of radiation are mediated through free radical (FR) and reactive oxygen species (ROS) in the overwhelmingly aqueous environment in cells producing a variety of damage appropriately grouped as oxidative damage (OD) to the genome (Beckman and Ames 1997; Meriyani and Sharan 2005; 2010). Direct effects result in direct deposition of radiant energy onto DNA usually leading to single strand breaks (SSB), double strand breaks (DSB) and cross-links between biomolecules (e. g., intra- and inter-strand DNA, DNA-protein, protein-protein, etc.), to name the most obvious. The quality and quantity of radiation are two parameters that are known to significantly influence the course of built-up of the radiation damage. At the same time, living systems are endowed by different cellular repair system(s) that essentially get triggered by radiation itself and/or radiation induced damage(s). These repair systems work in the opposite direction to restore normalcy in the damaged entity (Alberts *et al.* 1994, Beckman and Ames 1997, Klungland *et al.* 1999, Issacs and Spielmann 2004). In the post-genomic paradigm of radiation biology, the overall expression of cellualr damage appears to be mediated through a complex cascade of molecular events. It is now believed that a series of events starting immediately following the absorption of radiation energy go through a complex sequence of largely unclear interactions and processes to eventually manifest itself as discrete biological end-points. It is becoming increasingly clear that each repair system or pathway, though independent and of varying complexity, cross-talk to each other for most optimal biological responses. Therefore, the

ultimate cellular response to radiation and manifestation of radiation damage has a serious 'temporal' component, that is, at different points of time after exposure to radiation the cellular response might be different.

There are a number of biological end-points and assays which have been developed and standardized for monitoring radiation induced biological damage to the genome or DNA. Such dosimeters include quantification of radiation induced dicentric frequency in chromosomes; cytochalasin-B blocked micronucleus test, premature chromosome condensation (PCC), fluorescent *in situ* hybridization (FISH), etc. However, many of these parameters may not be specific as they are known to responds to several other genotoxins and stimuli besides radiation. Measurement of chromosome aberrations in peripheral blood lymphocytes is currently the most sensitive and reliable biomarkers of radiation exposure (gold standard) that is be used for biological dosimetry. In such studies, chromosome damage is measured in metaphase cells that are collected at one time point when the majority of cells reach first cell division after exposure. Number of dicentrics per cell is calibrated using linear array of increasing doses of radiation. Biological dose to the exposed cell or individual is measured by quantifying the number of dicentrics per cell. This reliable parameter, despite its potentials, remains underused for bio-dosimetry purposes simply because it takes a long time to score dicentrics due to methodological and technological limitation and dicentric frequency must be counted in large number of cells to obtain a statistically valid figure. In all, it is likely to take several days before the dosimetry is completed. This limits application of the technology on a mass scale and in short time. Therefore, there exists urgent requirement to identify reliable indicators of absorbed radiation doses and suitable technology for quick and reliable bio-dosimetry. It will be particularly needed in cases of exposure of large segment of human populations, which needs to be triaged and grouped on the basis of dose of exposure for appropriate medical interventions. Such accidental exposures may become more probable in the future due to increasing number of nuclear energy based industries being established in India and across the globe. There is also an increasing fear perception across the globe of nuclear holocaust or dirty bombing by rogue organization(s) or groups(s).

The post-genomic era has made it possible to look at radiation induced early changes at molecular levels in humans. This can greatly facilitate deciphering the molecular intricacies of living process through which cellular radiation response is mounted. Recent additions of exceptionally sensitive and deep-probing tools of biotechnology and ever-expanding bioinformatics have laid solid foundations of two sub-disciplines of biotechnology that can potentially be exploited for obviating these molecular events. The sub-disciplines are 'Proteomics' and 'Genomics'. In the proteomic approach, two dimensional polyacrylamide gel electrophoresis (2D-PAGE), high performance liquid chromatography (HPLC) and mass spectrometry (MS) are abundantly used to discover the protein diversity, identification of new proteins or their variants, and finding out the numerous ways in which they interact with each other. In the genomic approach, DNA microarrays and real-time quantitative polymerase chain reaction (RT-qPCR), etc. are used which unravels the patterns, behaviours and interactions of array of genes at different time points of metabolic processes.

Bioinformatics are extensively used in both approaches for data mining and opening new vistas for better and deeper molecular understanding of the normal and patho-physiological status of the living process. Therefore, new and hitherto unknown wealth of information is being generated which have opened up new possibilities to understand cellular responses at molecular levels at any time scale that one may desire. Clearer understanding of molecular events following exposure to radiation has potentials of application in the welfare of mankind.

These tools have also lead to identification of some early response proteins (ERP) and early response genes (ERG) that can potentially give deeper insights into the radiation induced cellular injuries, which is of particular interest both in radiation therapy and protection programs. Several studies addressing these issues have produced a pool of potential protein biomarkers for different low LET radiations using non-human systems. For example, ten proteins have been identified in murine fibroblasts 20 min to 72 h after 240 kVp X-ray irradiation to a dose of 6 Gy (Szkanderova et al. 2003). The study points to the possibility of involvement of metabolic processes associated with cell proliferation, glycolysis, and protein folding and degradation in buildup of radiation response. Employing 2D-PAGE and MS analysis in human T-lymphocyte leukemia cells after exposure to 7.5 Gy of ^{60}Co γ-rays, a temporal response in fourteen proteins belonging to various cell signaling pathways, protein degradations, malignant transformations and detoxification processes have been deciphered. Such proteins can potentially be used in biodosimetry (Szkanderova et al. 2005; Marchetti et al. 2006). Similarly, cyclin dependent kinase inhibitor-1A (CDKN1A), interleukin-6 (IL6), salivary-amylase (SA) and c-reactive protein (CRP) levels in blood serum of non-human primates exhibited reasonably good response to whole body exposure of 250 kVp X-rays at a dose of 6 Gy *in vivo* (Ossetrova et al. 2007). However, extrapolation of these results from non-human experimental systems to human situation for the welfare of mankind is likely to encounter several scientific and ethical problems. One of the major scientific questions would relate to the specificity of the 'candidate' protein to radiation in human beings. Elevated levels of CDKN1A are also measured following exposure to cytotoxins, including UV radiations, chemicals and free radicals (Fotedar et al. 2004). Likewise, CRP and IL6 are also up-regulated in acute phase inflammatory reactions (Whiteley et al. 2009). Some efforts have been made in human system as well. Several studies using DNA microarray technology have profiled gene expression changes in various cell types, suggesting some potential biomarkers for human exposure to γ-radiations (Amundson et al. 2000, Kang et al. 2003, Amundson et al. 2004 and Dressman et al. 2007).

Our group has been making consistent efforts in this direction with the specific aim of identifying suitable biomarker from among the radiation responsive ERPs and ERGs in humans. We visualize a great potential of such 'candidate' ERG and ERP in radiation protection and biodosimetry. In our preliminary study, we exposed human blood *ex vivo* to 2 Gy of ^{137}Cs γ-rays, isolated peripheral blood lymphocytes (PBL) and looked for ERP 15 min post-irradiation using proteomic approach (Turtoi et al. 2007). Five proteins associated with the cytoskeleton (β-actin [ACTB], mutant β-actin [mACTB], talin [TLN] and zyxin [ZYX]) and glycolysis (phosphoglycerate kinase-1 [PGK1]) were found to be the most promising radiation responsive ERPs. In another

study employing genomic approach on human PBL, 102 ERGs have been identified (Turtoi et al. 2008). This study could identify five ERGs namely, early growth response gene-1 (EGR1), early growth response gene-4 (EGR4), gamma-interferon (IFN-γ), cytoplasmic jun oncogene (c-JUN) and tumour necrosis factor super-family member-9 (TNFSF9) as potential 'candidate' biomarkers suitable for biodosimetric and other biomedical applications. Looking at these groups of ERPs and ERGs, it becomes obvious that separate proteomic and genomic approaches have yielded different candidates for the same purpose. This will limit application of the candidate ERPs and ERGs to fully understand the early cellular responses and also its application in biodosimetry. In order to get a holistic picture, it would be necessary that full potentials of proteomics, genomics and bioinformatics are simultaneously employed in human system exposed to medically relevant radiation doses. Results of such studies may possibly give better insight for exploitation of the applied potentials of radiation.

The objective of this study was to come up with a series of radiation dependent early response proteins and genes that are altered in human lymphocytes exposed to γ-irradiation in a medically relevant dose range. In a two-pronged approach, quantitative and qualitative modulations of cellular proteins were monitored using 2D-PAGE and HPLC/MS techniques. In parallel, the second screening was performed on the gene expression level using the whole human genome DNA microarray technique. This two-way approach - one starting from the protein levels itself and the other from the gene expression stage - should provide a more complete picture of the early alterations taking place in human lymphocytes exposed to increasing dose of γ-radiation in a clinically relevant dose range.

MATERIALS AND METHODS

Chemicals and human subject: All chemicals of highest purity grade were purchased from Sigma-Aldrich Co., USA and used as such unless otherwise mentioned. Blood from an informed and consenting donor (male, 30 years, healthy, non-smoker free of any drug usage) was used for the study in accordance with the ethical guidelines of the Research Centre Juelich, Germany.

Experimental protocol: Heparinized whole blood, drawn afresh on three successive days from the same donor, was irradiated *ex vivo*. While one half of the isolated peripheral blood lymphocytes (PBL) were subjected to proteomic analysis (2D-PAGE and MS identification of proteins), the other half was simultaneously processed for genomic (DNA Microarray) analysis to identify ERP and ERG, respectively.

Radiation source and irradiation: ^{137}Cs Gamma Cell 40 delivering γ-rays at a dose rate of 0.8 Gy/min was used. Whole blood sample (6 ml) was irradiated to accumulate doses of 1.0, 2.0 and 4.0 Gy at 37°C and immediately processed for further analysis.

Preparations of PBL, total protein and total RNA: PBL were isolated immediately after irradiation by density-gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, USA) as described earlier (Turtoi et al. 2008). Protein extract was prepared from one part of the PBL (3×10^6 cells) using the 2D Clean-Up kit (GE Healthcare, USA) as described (Turtoi et al. 2007). The second part of PBL was used for preparation of RNA using Trizol method as previously described (Turtoi et al. 2008). The RNA

pellet exhibited RNA integrity number (RIN; Bioanalyzer 2100; Agilent Technologies, USA) ranging from 8.0 to 10 indicating the highest quality RNA specimen.

Proteome analysis by 2D-PAGE: The method has been described in details in our previous publications (Turtoi et al. 2007, 2008). Briefly, protein sample (130 μg) was incubated overnight with the immobilized pH gradient (IPG) strips and subjected to isoelectric focussing (IEF) in a PROTEAN-IEF Cell (BioRad, USA) (20 min at 250 V followed by 2.5 h at 250 V to 10000 V linear gradient or until 40000 Vh had accumulated at 20°C). The second dimension 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was run using a PROTEAN-II cell (16 mA/gel for 30 min, followed by 24 mA/gel for ~5 h at 19°C,). The gels were stained and visualised using (a) fluorescence dye Pro-Q Diamond and (b) Sypro Ruby dye to visualize only phosphoproteins and total proteins, respectively. The 2D-PAGE gel was analysed using the FLA5000 laser scanner (Fuji Film, Japan) at 100 μm resolution and analyzed using Delta 2D software 3.3. Only spots showing significant changes of intensity (two-sided, unpaired Student's t-test, assuming equal variances with an error probability of $p \leq 0.05$, $n = 3$) and greater than two-fold increase or decrease in magnitude of relative concentration for both 2 and 4 Gy samples were selected for further analysis.

Protein identification by HPLC and MS: This was done as described earlier (Turtoi et al., 2007). Briefly, the identified gel spots were excised using Proteineer SP II spot-picking robot (Bruker, Germany), transferred to a 96-well plate and peptides were extracted using the Janus pipetting robot (Perkin Elmer, USA). The peptide containing samples were analyzed using the 1D-Nano-high performance liquid chromatography (HPLC) (Eksigent, USA) coupled with the ESI-QTrap-2000 MS (Applied Biosystems, USA) using the C18 pre-column (Acclaim PepMap, 300 μm i.d. × 5 mm; Dionex, USA) for 5 min at a flow rate of 20 μl/min (97.9% water, 2% acetonitrile and 0.1% formic acid). The separation of the peptides was performed using the analytical C18 column (Acclaim 75 μm × 150 mm; Dionex) and a 45 min solvent gradient (t = 0 min, 0% B [B: 97.9% acetonitrile, 2% water and 0.1% formic acid]; t = 45 min, 40% B) at the flow rate of 0.3 μl/min. The MS scanned the mass range from 400 to 1200 m/z where three most intense peptides were fragmented in MS/MS mode (m/z range: 100-2000, rolling collision energy default settings, linear ion trap fill time set at 250 ms). Protein identification was conducted using the Mascot search engine Version 2.1 (Matrix Sciences, USA) and the human non-redundant and non-identical protein database Swisprot (Swiss Institute for Bioinformatics, Switzerland, release 57.0; 20,334 entries). Following parameters were used: MS tolerance 0.6 Da, MS/MS tolerance 0.3 Da, 1 missed cleavage was allowed, carbamidomethylation was set as fixed whereas oxidation and phosphorylation were variable modifications.

Genome analysis by DNA-Microarray: The global gene expression analysis employed 4 × 44K DNA microarrays (Agilent Technologies) as detailed earlier (Turtoi et al. 2008). Briefly, starting with total RNA (0.4 μg), the purified Cy3-labelled cRNA (1.65 μg) was hybridized under rotation (10 rpm) at 65°C for 17 h on the 4 × 44K array using the Gene Expression Hybridization Kit (Agilent Technologies). The arrays were washed in GE wash buffer-1 and 2, and analyzed. The data accrued

on the Agilent Microarray Scanner and processed with the GeneSpring 7 software to generate ratios of relative gene expression (RE) of the irradiated vs. unirradiated samples. For statistical evaluation, a 1-way ANOVA test (parametric, unequal variances, including Benjamini-Hochberg false discovery rate multiple testing correction combined with Turkey post-hoc test at the false positive rate $P \leq 0.05$, $n = 3$) was employed for the initial screening. Only those genes showing a significant ($P \leq 0.05$), minimum two-fold up- or down-regulation in at least one of the irradiated samples with respect to the controls were considered for the final evaluation (see Table 12.2).

RESULTS AND DISCUSSION

The 2D-PAGE analysis revealed about 20 proteins which were either up- or down-regulated more than 2 folds immediately following the exposure of lymphocytes to γ-radiation (Figs. 12.1 and 12.2). While Figure 12.1 shows quantitative modulations at protein level (Sypro-Ruby dye), Figure 12.2 shows the same in terms

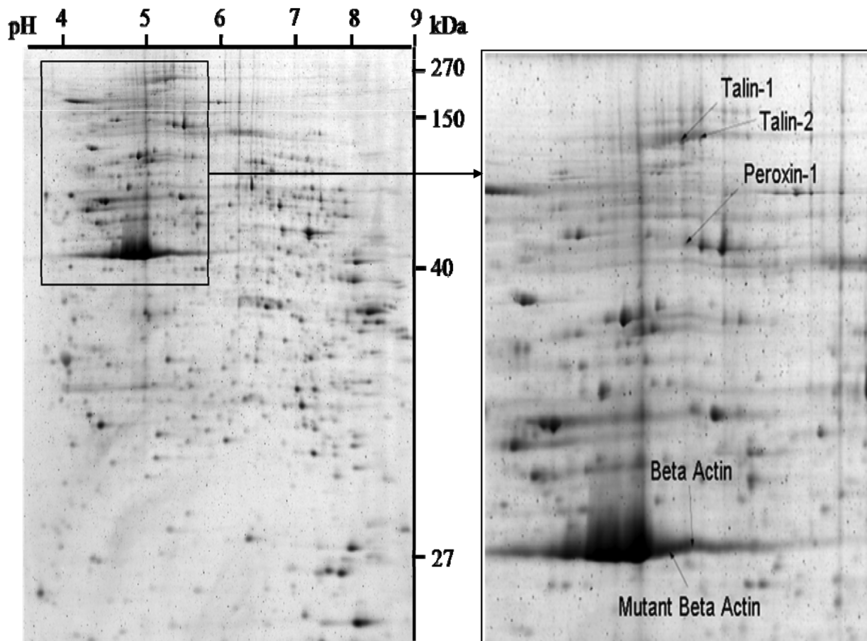

Fig. 12.1 *Quantitative proteomic analysis of cellular proteins by 2D-PAGE – A representative 12% SDS-PAGE gel (1D in the pH range of 3–10) showing the control PBL proteins after staining the gel with Sypro-Ruby stain to visualize all cellular proteins (left panel). The right panel shows the amplified picture of the selected region of the main gel, wherein those spots of proteins that displayed at least two-fold increase or decrease in the concentration ($p \leq 0.05$) following irradiation have been identified (see details in the text; also Table 12.1).*

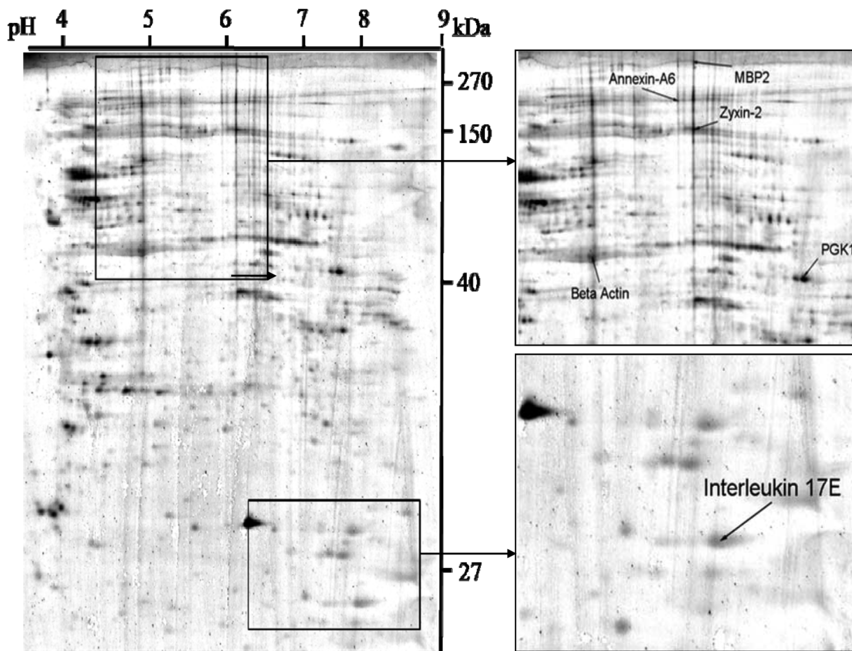

Fig. 12.2 ***Qualitative proteomic analysis of cellular proteins by 2D-PAGE – A representative 12% SDS-PAGE gel (1D in the pH range of 3–10) showing the control PBL proteins after staining the gel with Pro-Q-Diamond™ dye to selectively visualize the phosphorylated cellular proteins (left panel). The right panels show the amplified picture of the selected regions of the main gel, wherein those spots of proteins that displayed at least two-fold increase or decrease in the level of protein phosphorylation ($p \leq 0.05$) following irradiation have been identified (see details in text; also Table 12.1.***

of level of protein phosphorylation (Pro-Q-Diamond™ dye). The limited number of radioresponsive proteins out of the whole array of cellular proteins (Figs. 12.1 and 12.2) may indicate limitation of 2D-PAGE technique for such study. On the other hand, it may also suggest that only limited cellular response could be mounted in the small time period that lapsed between irradiation and beginning of isolation of PBL and preparation of cell lysate and mRNA (about 15 min). In the latter case, it may be possible that post-irradiation incubation may influence the total number of different proteins that may be identified as ERP using 2D-PAGE approach. This aspect is under active investigation. The radioresponsive proteins were subjected to HPLC and data-dependent MS analysis for their identification. Figure 3 shows a typical total ion chromatogram of a protein. The MS-generated amino acid sequence was matched with the amino acid sequences available at the Swissprot databank to finally identify the proteins. From among these, 11 radiosensitive 'candidate' ERP have been identified as shown in Table 12.1. Five of these 11 ERPs exhibited significant ($0.5 \geq RE \geq 2.0$ and $p \leq 0.05$, in at least two out of three irradiated samples) down-regulation

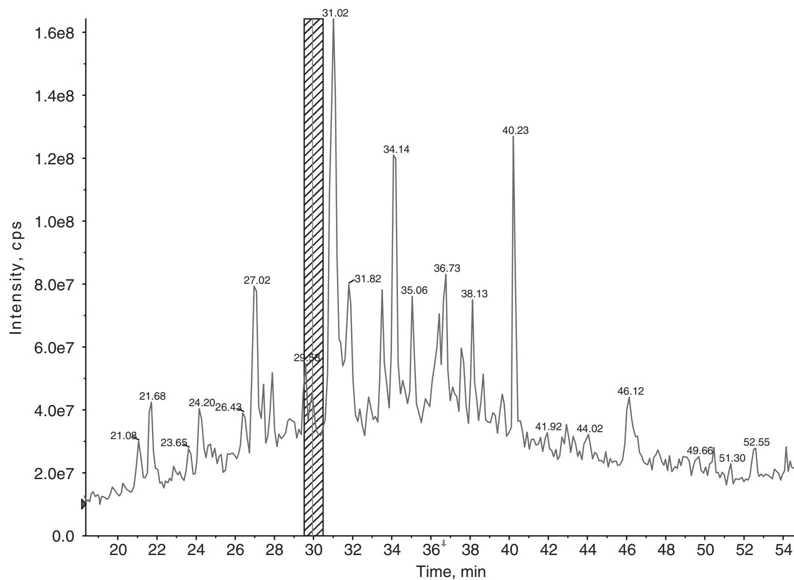

Fig. 12.3 **MS analysis of selected proteins for identification** - ***Representative results of MS analysis of phosphoglycerate kinase-1 (PGK1) showing the total ion chromatogram of the protein. From this profile, an enhanced MS spectrum at selected time was prepared based on which amino acid sequence was deciphered. The deciphered amino acid sequence was compared with the protein sequence available in the Swissprot® database for protein identification (see text for details).***

either in terms of total protein quantity (quantitative modulation) or in terms of level of protein phosphorylation (qualitative modulation) (Table 12.1). Of these 5 ERPs exhibiting quantitative modulation, four (TLN1 & 2, ACTB and mutant ACTB [mACTB]) are essentially structural proteins while the fifth, Peroxin–1 (PEX1), is involved in protein transport and degradation. The remaining 6 ERPs (MBP2, PGK1, ANXA6, zyxin–2 (ZYX2), interleukin-17E (IL17E) and phosphorylated ACTB) exhibited significant qualitative up-regulation in the level of phosphorylation of the proteins (Table 12.1). Four of these proteins (MBP2, PGK1, ANXA6 and IL17E) are associated with the cell cycle control and immune system. So, it appears that the total quantum of proteins associated with structure or architecture of cell went down as a response to increasing dose of γ-irradiation. On the other hand, most proteins associated with cell cycle and immune system exhibited no quantitative but qualitative change wherein their level of phosphorylation exhibited a radiation dose dependent up-regulation. It is important to note that the quantitative or qualitative alterations in protein observed in this study are the result of a cumulative effect of γ-radiation on human PBL *ex vivo*. Therefore, the observed alterations in the protein concentration could be the result of both the alteration of gene expression and possibly an altered biological half-life of the protein. γ-radiation is known to generate ROS in the cells, which can react with a variety of molecules, including proteins, to

Table 12.1 ***Early Response Proteins (ERPs):*** **Human peripheral blood lymphocyte (PBL) proteins identified by the proteomic approach as the 'candidates' ERPs to γ-irradiation. The table also shows their approximate molecular weights, metabolic functions and responses (0.5 ≥ RE ≥ 2.0 and p ≤ 0.05, in at least two out of three irradiated samples).**

Protein	Molecular weight (≈kDa)	Metabolic association	Response to irradiation
Talin-1 (TLN1)	269.6	Structural proteins	**Quantitative modulation** - down-regulated total quantity of protein
Talin-2 (TLN2)	271.4		
β-Actin (ACTB)	42.0		
Mutant β-Actin (mACTB)	42.1		
Peroxin-1 (PEX1)	142.8	Protein transport & degradation	
Major histocompatibility banding protein-2 (MBP2)	268.9	Cell cycle control & Immune response	**Qualitative modulation** - up-regulated **protein phosphorylation**
Phosphoglycerate kinase-1 (PGK1)	44.8		
Annexin-6 (ANXA6)	75.7		
Interleukin-17E (IL17E)	20.3		
Zyxin-2 (ZYX2)	62.4	?	
Phosphorylated β-Actin (pACTB)	42.3	Protein transport & degradation ?	

potentially build toxic complexes (Stadtman & Levine 2003, Nyström 2005). The accumulation of toxins might influence biological half life of biomolecules.

The simultaneous DNA microarray analysis identified 44 ERGs, almost all of which, except the docking protein 7 (DOK7), exhibited at least 2-fold up-regulation of the gene expression following increasing doses of γ-radiation (Table 12.2). The table shows GenBank accession number, gene symbol, biological process, molecular function and relative expression (RE) along with standard deviation of means (SD) and probability of error (P) at 1, 2 and 4 Gy doses of γ-rays for the identified genes. The RE of the unirradiated control has been taken as 1.00. This outcome, on the face value, demonstrates the sensitivity of genomic approach vis-à-vis proteomic approach in finding out ERGs. DNA microarray technique being more sensitive was able to pick up a larger number of radioresponsive genes immediately following irradiation. Even though the metabolic or biological functions of the identified genes were varied but essentially comprised the functions enlisted in Table 12.1, the ERPs (Table 12.1) and ERGs (Table 12.2) show almost no parity.

Table 12.2 ***Early Response Genes (ERGs):*** **Human peripheral blood lymphocyte (PBL) genes identified by the genomic approach as the ERGs to γ-irradiation. After the whole blood γ-irradiation (1, 2 and 4 Gy), DNA Microarray was performed on lymphocyte mRNA. Genes showing significant ($0.5 \geq RE \geq 2.0$ and $p \leq 0.05$, in at least two out of three irradiated samples) up- or down-regulation have been listed. All identified ERGs were up-regulated in radiation dose dependent manner, except docking protein-7 (DOC7). The table also shows relative gene expression (RE), SD and the p-values (one way ANOVA) with respect to non-irradiated control samples which were set to 1.000 (n = 3).**

Genbank	Gene Symbol	Biological Process	Molecular Function	1 Gy			2 Gy			4 Gy		
				RE	SD	P	RE	SD	P	RE	SD	P
NM_004024	ATF3	*regulation of transcription*	*transcription factor*	**1.724**	0.201	0.001	**2.801**	0.285	0.000	**3.404**	0.352	0.000
BC007549	BAT2D1	*unknown*	*Unknown*	**2.470**	1.375	0.024	**3.751**	0.749	0.000	**5.086**	0.627	0.000
NM_014417	BBC3	*apoptosis, caspase activation*	*protein binding*	**3.865**	0.391	0.000	**4.184**	0.709	0.000	**4.617**	0.543	0.000
NM_006763	BTG2	*DNA repair, transcription regulation*	*transcription factor*	**1.404**	0.141	0.002	**1.873**	0.189	0.000	**2.312**	0.233	0.000
NM_001781	CD69	*defense response*	*transmembrane receptor*	**2.851**	0.343	0.000	**3.873**	0.294	0.000	**5.154**	0.444	0.000
NM_001252	CD70	*apoptosis, immune response*	*TNF receptor binding*	**3.272**	0.322	0.000	**3.224**	0.677	0.000	**3.987**	0.441	0.000
NM_004233	CD83	*defense and immune response*	*unknown*	**2.548**	0.254	0.000	**3.573**	0.356	0.000	**4.121**	0.411	0.000
NM_000389	CDKN1A	*cell cycle arrest*	*cyclin-dependent protein kinase*	**2.427**	0.140	0.000	**2.699**	0.425	0.001	**3.014**	0.469	0.000
NM_018947	CYCS	*electron transport, caspase activation*	*cytochrome c oxidase complex*	**1.439**	0.145	0.002	**1.752**	0.176	0.000	**2.547**	0.271	0.000

(Continued)

Table 12.2 ***(cont'd) Early Response Genes (ERGs):*** **Human peripheral blood lymphocyte (PBL) genes identified by the genomic approach as the ERGs to γ-irradiation. After the whole blood γ-irradiation (1, 2 and 4 Gy), DNA Microarray was performed on lymphocyte mRNA. Genes showing significant ($0.5 \geq RE \geq 2.0$ and $p \leq 0.05$, in at least two out of three irradiated samples) up- or down-regulation have been listed. All identified ERGs were up-regulated in radiation dose dependent manner, except docking protein-7 (DOC7). The table also shows relative gene expression (RE), SD and the p-values (one way ANOVA) with respect to non-irradiated control samples which were set to 1.000 (n = 3).**

				1 Gy			2 Gy			4 Gy		
Genbank	**Gene Symbol**	**Biological Process**	**Molecular Function**	**RE**	**SD**	**P**	**RE**	**SD**	**P**	**RE**	**SD**	**P**
AK024926	DDAH1	*arginine catabolic process*	*dimethylargininase*	**1.646**	0.168	0.001	**2.016**	0.287	0.000	**2.100**	0.213	0.000
NM_173660	DOK7	*neuromuscular junction development*	*insulin receptor binding*	**0.585**	0.118	0.003	**0.458**	0.051	0.000	**0.440**	0.045	0.000
NM_004418	DUSP2	*inactivation of MAPK activity*	*tyrosine phosphatase*	**1.336**	0.134	0.004	**1.842**	0.335	0.001	**2.619**	0.262	0.000
NM_004419	DUSP5	*protein amino acid dephosphorylation*	*tyrosine phosphatase*	**1.609**	0.159	0.001	**1.715**	0.169	0.000	**2.178**	0.215	0.000
NM_004420	DUSP8	*inactivation of MAPK activity*	*tyrosine phosphatase*	**1.816**	0.185	0.000	**2.558**	0.529	0.000	**3.719**	0.819	0.000
NM_001964	EGR1	*regulation of transcription*	*transcription factor*	**3.339**	0.621	0.000	**7.129**	2.692	0.001	**8.794**	2.395	0.000
NM_153606	FAM71A	*unknown*	*unknown*	**1.318**	0.208	0.055	**2.303**	0.557	0.002	**2.403**	0.276	0.000
NM_001924	GADD45A	*regulation of cyclin dependent PKA*	*protein binding*	**2.778**	0.279	0.000	**2.747**	0.275	0.000	**2.826**	0.526	0.000
NM_005524	HES1	*regulation of transcription*	*transcription regulator*	**6.217**	0.632	0.000	**10.525**	1.705	0.000	**8.508**	0.863	0.000

NM_003538	HIST1H4A	*chromatin organization*	*DNA binding*	**1.241**	0.137	0.008	**1.965**	0.461	0.002	**2.300**	0.250	0.000
AK097297	HLA-DQB1	*antigen presentation via MHC class II*	*GTP binding, MHC class II receptor*	**1.389**	0.160	0.005	**2.087**	0.216	0.000	**2.304**	0.241	0.000
NM_002127	HLA-G	*antigen presentation via MHC class I*	*MHC class I receptor activity*	**1.285**	0.130	0.007	**1.538**	0.171	0.000	**2.032**	0.204	0.000
NM_021979	HSPA2	*protein folding*	*unfolded protein binding*	**1.645**	0.180	0.001	**2.421**	0.671	0.003	**2.911**	0.716	0.001
NM_016545	IER5	*unknown*	*unkown*	**1.625**	0.164	0.001	**1.953**	0.305	0.000	**2.203**	0.275	0.000
NM_000619	IFNG	*cell-cell signaling, response to virus*	*cytokine avtivity*	**2.611**	0.646	0.006	**3.038**	1.463	0.024	**6.683**	0.362	0.000
NM_022767	ISG20L1	*apoptosis, response to DNA damage*	*exonuclease acivity*	**3.984**	0.639	0.000	**3.314**	0.357	0.000	**3.581**	0.354	0.000
NM_002228	JUN	*regulation of transcription*	*RNA polymerase II transcription factor*	**1.611**	0.071	0.000	**3.367**	0.508	0.000	**3.851**	0.414	0.000
NM_006879	MDM2	*regulation cell cycle progression*	*ubiquitin-protein ligase*	**5.473**	0.545	0.000	**5.791**	0.576	0.000	**5.708**	1.065	0.000
NM_173198	NR4A3	*regulation of transcription*	*transcription factor*	**1.133**	0.113	0.079	**1.707**	0.169	0.000	**2.268**	0.238	0.000
NM_002592	PCNA	*regulation of DNA replication and repair*	*DNA polymerase processivity factor*	**2.961**	0.292	0.000	**2.983**	0.250	0.000	**3.711**	0.546	0.000
NM_015900	PLA1A	*phosphatidylserine metabolism*	*phospholipase A1 activity*	**1.842**	0.220	0.001	**3.015**	1.610	0.019	**3.482**	0.649	0.000
NM_006622	PLK2	*positive regulation of I-kB / NF-kB*	*serine/threonine kinase*	**2.948**	0.292	0.000	**3.572**	0.690	0.000	**4.396**	0.436	0.000
NM_021127	PMAIP1	*induction of apoptosis*	*protein binding*	**2.123**	0.077	0.000	**2.251**	0.127	0.000	**2.911**	0.444	0.000
NM_001198	PRDM1	*negative regulation of transcription*	*transcription factor activity*	**1.329**	0.135	0.004	**1.570**	0.437	0.027	**2.184**	0.222	0.000

(Continued)

Table 12.2 ***(cont'd) Early Response Genes (ERGs):*** **Human peripheral blood lymphocyte (PBL) genes identified by the genomic approach as the ERGs to γ-irradiation. After the whole blood γ-irradiation (1, 2 and 4 Gy), DNA Microarray was performed on lymphocyte mRNA. Genes showing significant ($0.5 \geq RE \geq 2.0$ and $p \leq 0.05$, in at least two out of three irradiated samples) up- or down-regulation have been listed. All identified ERGs were up-regulated in radiation dose dependent manner, except docking protein-7 (DOC7). The table also shows relative gene expression (RE), SD and the p-values (one way ANOVA) with respect to non-irradiated control samples which were set to 1.000 (n = 3).**

				1 Gy			2 Gy			4 Gy		
Genbank	**Gene Symbol**	**Biological Process**	**Molecular Function**	**RE**	**SD**	**P**	**RE**	**SD**	**P**	**RE**	**SD**	**P**
NM_002922	RGS1	*immune response, B cell activation*	*GTPase activator, calmodulin binding*	**1.138**	0.114	0.055	**2.318**	0.566	0.002	**2.780**	0.335	0.000
NM_014470	RND1	*actin filament organization*	*GTPase activity*	**2.483**	0.269	0.000	**2.355**	1.324	0.055	**3.794**	0.775	0.000
NM_080860	RSPH1	*meiosis*	*unknown*	**1.974**	0.246	0.000	**2.668**	0.359	0.000	**3.721**	0.758	0.000
NM_138356	SHF	*apoptosis*	*protein binding*	**1.640**	0.259	0.000	**1.810**	0.430	0.004	**2.478**	0.271	0.000
NM_138810	TAGAP	*signal transduction*	*guanyl-nucleotide exchange factor*	**1.887**	0.187	0.000	**2.042**	0.202	0.000	**2.256**	0.223	0.000
NM_031272	TEX14	*protein amino acid phosphorylation*	*protein kinase*	**1.300**	0.215	0.020	**1.697**	0.252	0.000	**2.134**	0.223	0.000
NM_003811	TNFSF9	*apoptosis, immune response*	*TNF receptor binding*	**2.644**	0.257	0.000	**2.834**	0.272	0.000	**3.028**	0.291	0.000
NM_004295	TRAF4	*apoptosis, protein ubiquitination*	*DNA binding, ubiquitin-protein ligase*	**2.514**	0.254	0.000	**3.095**	0.312	0.000	**3.688**	0.372	0.000
AK097045	WDR79	*unknown*	*unknown*	**1.540**	0.221	0.004	**1.794**	0.209	0.001	**2.579**	0.293	0.000
NM_023929	ZBTB10	*regulation of transcription*	*DNA and protein binding*	**1.701**	0.180	0.000	**1.968**	0.203	0.000	**2.532**	0.261	0.000

Among the ERGs lasted in Table 12.2, RND1 is known to phosphorylate several structural proteins, including ACTB. Its gene expression is proposed to be controlled by c-JUN that was up-regulated in DNA microarray studies (see Table 12.2). It can be postulated that γ-radiation activated the transcription factor AP-1 whose major part is c-JUN, which, in turn, activated the expression of the RND1 gene. Most probably, the RND1 protein leads to the depolymerization and rearrangements of structural proteins, including ACTB and ZYX1. This might, at least in part, explain our observations on modulations of structural proteins and the level of protein phosphorylation. Chen et al. (2005) have also shown radiation induced modulation of ACTB in the bone marrow macrophages. Zhang et al. (2005) have reported in IEC-6 rat epithelial cells a significant reduction of vimentin und β-tubulin protein concentrations 24 h after γ-irradiation (25 Gy) highlighting possible important role(s) of structural proteins in cellular radiation response.

Another group of genes and proteins that appears to be affected at an early stage after exposure to γ-radiation are those involved in cell cycle controls. ANXA6 is members of the RAS/RAF signaling pathway, which activates mitogen-activated protein kinase (MAPK) to induce cell cycle progression. It is a calcium dependent protein that induces the RAS-p120GAP complex formation. Its phosphorylation was found to increase with increasing γ-dose (Table 12.1). Though ambiguity persists, it is generally assumed that ANXA6 is activated through phosphorylation. This would induce the RAS-p120GAP complex formation which, in turn, inactivates RAS. This would halt the cell cycle progression (King and Sartorelli 1986). Our results from DNA microarray analysis (Table 12.2) support the assumption as it shows up-regulation of dual specificity phosphatase-2, 5 and 8 (DUSP2, DUSP5 and DUSP8) genes, which code for phosphatases that dephosphorylate their target kinases. These genes negatively regulate MAPK super-family proteins, which are associated with cellular proliferation. Several proteins and genes that are directly involved in the cell cycle control were also found to be altered after the exposure to γ-radiation. One of the most prominent candidates belonging to this group is MDM2 protein whose gene expression was up-regulated (see Table 12.2). We also measured increased gene expression of CDKN1A (see Table 12.2). Bae et al. (1995) have shown p53 regulation of CDKN1A after γ-irradiation, which caused p53 mediated G_1-arrest in Burkitt's lymphoma wild-type cells. They demonstrated that both protein concentration and gene expression of CDKN1A changed rapidly after irradiation reaching the highest level within 4 h. In addition, either as a direct consequence of alteration in the RAS/RAF or p53 signaling pathways, another cell cycle regulatory gene, GADD45A, was also up-regulated (Table 12.2). GADD45A is a gene whose expression is reported to increase following treatment with DNA damaging agents, including ionizing radiation (Smith et al. 2000).

From the preliminary observations made in this piece of investigation it can be postulated that γ-radiation induces immune system mediated responses in lymphocytes. Both DNA microarray and 2D-PAGE analyses showed a series of altered genes and proteins that are involved in immunity and defense reactions. Two of the first identified γ-radiation modulated proteins belonging to this group are MBP2 and IL17E (see Table 12.1). Both proteins were found to be increasingly phosphorylated in response to increasing γ-doses. The dose–response trend appeared to exist for

MBP2 and IL17E as well. MBP2 interacts with the DNA enhancer elements of genes coding for MHC-I, interleukin-2 and interferon proteins (Van't Veer et al. 1992). These proteins are mainly responsible for cellular defense processes. The subsequent DNA microarray analysis could confirm the up-regulation of the IFNG gene (Table 12.2). IL17E is involved in the activation of the nuclear factor of kappa light polypeptide enhancer in B cells (NFκβ), and it enhances the production of interleukin-8. Both proteins are related to apoptosis and immune processes. Further work is in progress to fill the gaps in information to fully clarify the point.

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