Original Articles

Proteomic Analysis of Radiation-Induced Alterations in L929 Cells

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Abstract. In this study we examined the protein expression profiles in X-irradiated L929 cells to get insight into how mammalian cells respond to radiation-induced cell damage. L929 cells were irradiated with the dose of 6 Gy and cell lysates were collected at different time intervals (20 min, 12, 24, 36, 48 and 72 h). The extracted proteins were separated by 2-DE and quantified using computerized image analysis. Proteins exhibiting significant abundance alterations when comparing irradiated to unirradiated cells were identified by mass spectrometry. Using the proteomics approach we detected 47 proteins that exhibited a significant radiation-induced increase or decrease in the course of 72 h. From this group of spots 28 proteins were identified by mass spectrometry and of these 24 proteins exhibited minimally 2-fold differences in mean abundance values in comparison to controls. The identified proteins represent diverse sets of proteins participating either in protective and reparative cell responses or in induction of apoptosis and oncogenesis. The results document that proteomics is a useful method for unravelling the molecular mechanisms involved in cell reaction to ionizing radiation.

One of the crucial aims in radiobiological research is the search for biological parameters applicable for early detection of radiation-induced cell damage (biological indicators) and estimation of the absorbed radiation dose (biological dosimetry). Recently, it has been shown that the sum of different modes of cell death, namely micronucleation, apoptosis and abnormal cells (MAA) (e.g. necrotic cells; MAA-assay) correlates with the radiation-induced cell damage, but independently of the cell model in use (Abend et al., 2000). This suggested that MAA might be suitable as a biological indicator in tissues of different origin. However, the assay has its limitations (delayed onset of measurements, multiple measurements necessary and time consuming). From the clinical point of view, morphological changes after irradiation appear too late. In the case of radiation accidents it would also be desirable receiving dose estimates within a short period of time using a simple assay. Furthermore, there are indications that not only apoptosis, but also other modes of cell death like micronucleation (Abend et al., 1999) and even necrosis represent actively controlled processes of cell destruction induced by the damaged cells. It is very likely that those processes are controlled at the gene expression level.

Nowadays, monitoring of radiation-induced alterations in gene expression represents the most rapidly developing area of research aimed at identification of molecular markers for radiation exposure. Several methods have been developed for the comprehensive analyses of gene expression in complex biological systems. Until now the major effort was focused on application of procedures based on genomic and transcriptomic profiling. Ongoing experiments based

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Abbreviations: ALDR – aldose reductase, DTT – dithiothreitol, eIF 5A – initiation factor 5A, HINT – histidin triad nucleotide-binding protein, IMPDH-II – inosin-5'-monophosphate dehydrogenase 2, IPG – immobilized pH gradient, MAA – micronucleation, apoptosis, abnormal cells, MN – micronuclei, MTF2 – metal response element-binding transcription factor, NDK B – nucleoside diphosphate kinase B, PDI – protein disulphide isomerase, RhoGDI 1 – rho GDP-dissociation inhibitor 1, RhoGDI 2 – rho GDP-dissociation inhibitor 2, SDS-PAGE – sodium dodecylsulphate polyacrylamide gels, TCP-1 – T-complex protein 1, TCTP – translationally controlled tumour protein, VEGF-D – vascular endothelial growth factor D, 2-DE – two-dimensional gel electrophoresis.

on macro- or microarray hybridization protocols have been identifying large numbers of potential biomarkers (Admundson et al., 2001). However, these approaches exhibit various limitations (Celis et al., 1998); therefore, the ultimate profiling of gene expression necessitates incorporation of a third procedure, named proteomics, which encompasses qualitative and quantitative analyses of real biological effectors – proteins (Hanash et al., 2002). In the presented study we applied proteomics for the measurement of protein expression during the first three days after cell irradiation. Using a comparative proteomics procedure, the proteins exhibiting significant radiation-induced down- or upregulation were detected and their possible roles in radiation-induced cell response were suggested.

Material and Methods

Cells and cell culture

Mouse fibrosarcoma cells (L929) (Flow Laboratories, Meckenheim, Germany) were grown as a monolayer culture and subcultured twice per week. Cells were maintained in the MEM medium (Sigma, Deisenhofen, Germany) supplemented with 20% heat-inactivated foetal calf serum (Boehringer Mannheim, Mannheim, Germany). Culture conditions were 37˚C in a humidified atmosphere buffered by 5% CO_2 in air and hydrogen carbonate (Merck, Darmstadt, Germany), at a pH 7.4.

Radiation conditions

Twenty-four hours after passaging, exponentially growing cell cultures were irradiated at room temperature with single doses of 240 kV X-rays (Isovolt 320/10; Seifert, Ahrensberg, Germany) filtered with 3 mm Be. The absorbed dose was measured with a Duplex dosimeter (PTW; Freiburg, Germany). The dose rate was ~ 1 Gy/min at 13 mA: L929 cells were irradiated with 6 Gy.

Sample preparation for two-dimensional gel electrophoresis

The L929 cells were lysed in 500 µl of lysis buffer (137 mM NaCl, 10% glycerol, 1% n-octylglucopyranosid, 50 mM NaF, 20 mM TRIS, pH 8, Complete ^{TM}Mini, 1 mM Na_3VO_4) at 20 min, 12, 24, 36, 48 and 72 h after irradiation. Extracted proteins were precipitated overnight in 20% trichloroacetic acid (TCA) in acetone (-18˚C) containing 0.2% dithiothreitol (DTT) (Görg et al., 1997) and then solubilized in a buffer for isoelectric focusing (IEF) (9 M urea, 4% CHAPS, 70 mM DTT and 2% carrier ampholytes pH 9–11). The lysates were centrifuged (5 min; 15 000 x *g*; 4˚C) and protein concentration in the IEF buffer was determined by the modified bicinchoninic acid (BCA) assay (Brown et al., 1989).

Two-dimensional gel electrophoresis and analysis of gel images

Immobilized pH 3–10 gradient (IPG) gels (Immobiline DryStrip 3–10 NL, Amersham Pharmacia Biotech, Uppsala, Sweden) were used for first dimension-isoelectric focusing. Commercial strips were swollen in rehydration buffer containing 2 M thiourea, 5 M urea, 2% CHAPS, 2% SB 3–10, 2 mM tributyl phosphine (TBP), 40 mM Tris base and 0.5% Ampholine, pH 3–10, overnight. For analytical two-dimensional gel electrophoresis (2-DE), 100 µg of proteins were loaded in the first dimension. The sample was applied at the cathodic side of the gel and the IPG separation was carried out using a Multiphor II apparatus (Amersham Pharmacia Biotech) overnight (100 kVh, 20˚C). Immediately after being focused, IPG gels were equilibrated for 15 min in 6 M urea, 2% sodium dodecylsulphate (SDS), 50 mM Tris-HCl, pH 6.8, 30% v/v glycerol and 1% DTT, then for 15 min in the same solution except that DTT was replaced by 5% w/v iodoacetamide. In the second dimension the vertical slab gradient 9–16% SDS polyacrylamide gels (SDS-PAGE) were used. These gels were cast in a Protean^{TM} multi-gel casting chamber and SDS-PAGE electrophoresis was performed using a Protean^{TM} 2-D multi-cell apparatus (Bio-Rad, Hercules, CA) at 40 mA per gel for 5 h. Immediately after electrophoresis, proteins were visualized by silver staining (Hochstrasser et al., 1988) and 12-bit monochromatic images at 50 µm resolution were obtained by scanning gels with a laser densitometer (Molecular Dynamics, Palo Alto, CA). Two gels were run for each sample. The computerized image analysis was carried out using a Melanie 3 software package (Bio-Rad). Proteins separated by 2-DE were quantitated in terms of their relative spot volumes (% vol), i.e., digitized staining intensity integrated over the area of an individual spot divided by the sum of integrated staining intensities of all spots and multiplied by 100. The quantitative data from two independently prepared samples were submitted to Student's *t*-test implemented in Melanie 3 software for determination of significant differences at the levels of $P < 0.05$. From this group of spots only proteins having a positive or a negative fold change with magnitude of ≥ 2 were further taken into account.

In-gel digestion and mass spectrometry

For the micropreparative 2-DE gels, 1 mg of protein was loaded on IPG strips. Selected spots stained with Coomassie blue R-250 were then excised and covered with 500 µl of 100 mM Tris-HCl, pH 8.5, in 50% acetonitrile for 20 min at 30˚C. Five hundred µl of equilibration buffer (50 mM ammonium bicarbonate, pH 7.8 in 5% acetonitrile) were added to the gel pieces. After that the gel pieces were vacuum-dried, covered with 0.1 µg of sequencing grade trypsin (Promega,

Madison, WI) in 30 µl of 50 mM ammonium bicarbonate, pH 7.8, and 5% acetonitrile and mildly shaken overnight at 37°C. The mass spectra were recorded in reflector mode in a MALDI mass spectrometer Voyager-DE STR (Perseptive Biosystems, Framingham, MA) equipped with delayed extraction. The peptide mixture samples were interfused with the same volume of the matrix solution α-cyano-4-hydroxycinnamic acid (in 50% acetonitrile with 0.5% trifluoroacetic acid (TFA)), 2 µl of the solution were applied to a plated sample holder and introduced into the mass spectrometer after drying. The instrument was calibrated externally with a two-point calibration using peptide standards. Proteins were identified by peptide mass fingerprinting (PMF) using ProFound and PeptIdent programs. Liquid chromatography-tandem mass spectrometry data were recorded in a LC-MS/MS mass spectrometer Q-TOF Ultima™ API (Micromass, Manchester, UK) fitted with a nanospray ionization source (Z-spray). The mass spectrometer combines the selectivity of a quadrupole (MS 1) with the ultra high efficiency of an orthogonal acceleration time of flight (TOF) mass analyzer (MS 2). The in-gel protein digest samples were separated by means of a Micromass modular CapLC system (Micromass) connected directly to the Z-spray source. The MS/MS spectra were acquired on the four most intense ions. All data were processed automatically by means of ProteinLynx software. Proteins were identified by searching the Non Redundant Data Base (NRDB) using the ProteinLynx Global Server engine.

Results

Comparative proteomics analysis of unirradiated and X-irradiated L929 cells

Proteome analysis was performed with cells 20 min, 12, 24, 36, 48 and 72 h after irradiation with the dose of 6 Gy. As demonstrated previously (Abend et al., 2000), this dose induces the formation of micronuclei (MN), whose level starts to accrue at 18 h and peaks from 30 to 42 h after irradiation. Approximately 2000 spots were resolved with Melanie 3 software in gels of both control and irradiated cells. Globally, computer-assisted comparative analysis of 2-DE protein patterns of unirradiated and irradiated L929 cells revealed 47 proteins exhibiting significant radiation-induced alterations. All these differentially expressed proteins were submitted to peptide mass fingerprinting or LC-MS/MS analysis resulting in successful identification of 28 proteins (60%). The positions of all identified protein spots are indicated in the reference silver-stained gels of unirradiated and X-irradiated L929 cell

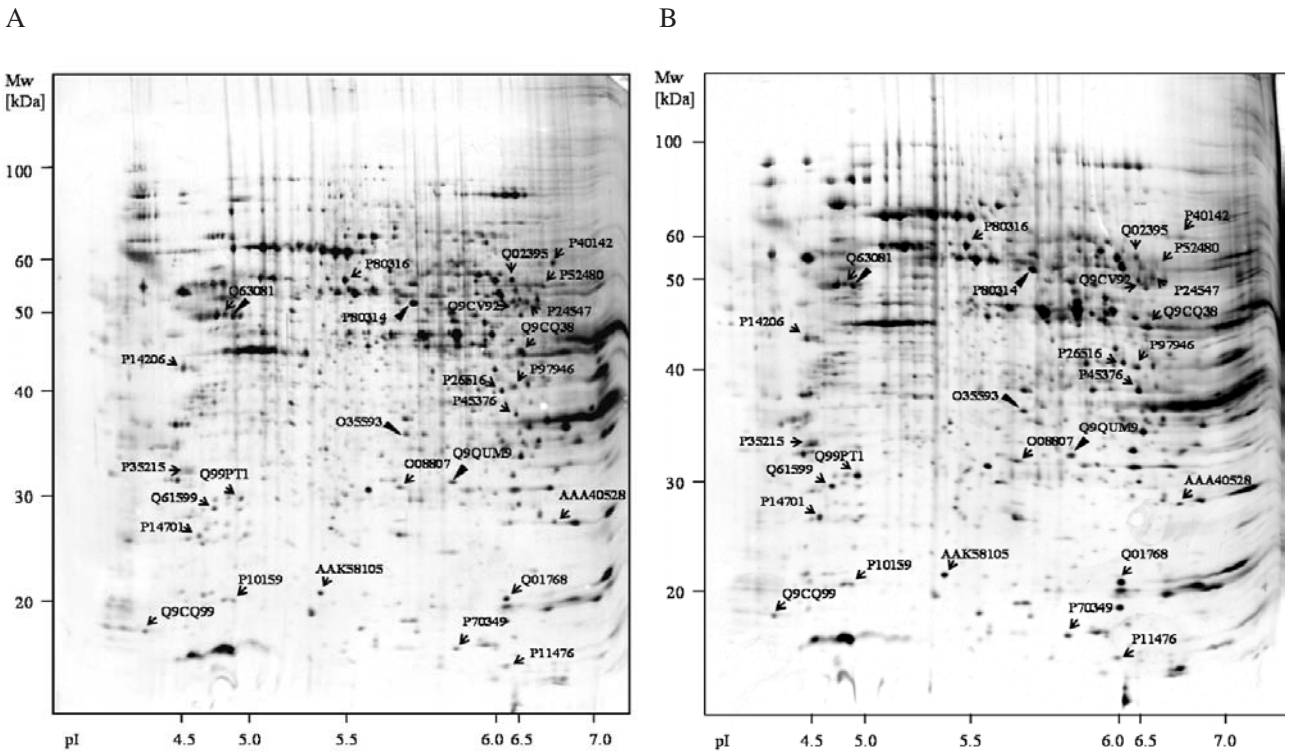

Fig. 1. Silver-stained 2-DE protein pattern of unirradiated (A) and X-irradiated (B) murine fibroblast L929 cell line. All identified spots are labelled with their accession numbers. Arrows mark the proteins whose levels exhibited a minimally 2-fold change comparing to controls; arrowheads then denote the proteins whose radiation-induced abundance alterations were also found to be statistically significant, but the magnitude of the mean abundance change was less than 2.