Gamma-Radiation-Induced Phosphorylation of p53 on Serine 15 Is Dose-Dependent in MOLT-4 Leukaemia Cells

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Abstract. Molecular indicators of the absorbed dose of ionizing radiation are powerful tools in biodosimetry. The studies reported here were undertaken with the motivation to find such a marker among the molecules involved in ataxia-telangiectasia mutated kinase-dependent signalling induced by ionizing radiation (ATM-kinase, checkpoint kinase-2, protein p53, and oncoprotein Mdm2). In our previous work on T-lymphocyte leukaemia MOLT-4 cells we described the mentioned molecules of ATM-dependent pathway and none of them showed a pronounced dose-dependent response. Here we employed Western blotting and ELISA assay to investigate the response of post-translationally modified p53 (particularly phosphorylated on serine 15) after gamma-irradiation. We have found the amount of phosphorylated p53 to be homogenously increased after irradiation by the doses of 0.5 to 7.5 Gy. The dose-dependent response was pronounced especially after the doses up to 3.0 Gy. The presented data indicate that p53 phosphorylated on serine 15 might be used as a potential biodosimetric marker.

Introduction

Double-strand breaks (DSB) are the severest form of DNA damage induced by ionizing radiation (IR) in eukaryotic cells. Their reparation demands sensitive, rapid, and effective signalling machinery. A core unit of this apparatus is ataxia-telangiectasia mutated kinase (ATM), which is a key regulator of biological responses to DNA damage and controls the signal transduction pathway leading to activation of reparation proteins as well as to cell cycle arrest (Bartkova et al., 2005).

Activation of ATM involves rapid intermolecular autophosphorylation of Ser1981 that causes dissociation of the inactive dimer (Bakkenist and Kastan, 2003). Active ATM then affects numerous different targets via phosphorylation and some of them participate in the cell cycle arrest. These are for instance p53, murine double minute protein (Mdm2), and checkpoint kinase-2 (Chk-2) in the G1 checkpoint (Maki et al., 1997; Canman et al., 1998; Maya et al., 2001) and many others for the transient S-phase arrest and the G2/M checkpoint.

Protein p53 is a tumour suppressor and a transcriptional regulator that plays an important role in cellular responses to various stress signals including IR (Levine, 1997). More than 50% of all human tumours carry inactivating mutations in the p53 gene and its importance is undeniable since it suppresses tumorigenesis by influencing DNA repair, promoting cell cycle arrest or inducing apoptosis (Brooks and Gu, 2006). In unstressed cells wild-type p53 is typically a short-lived protein and its degradation is tightly regulated by E3 ubiquitin ligases, such as p53 negative regulator Mdm2, and proteasome (Brooks and Gu, 2006). The half-life of p53 increases in response to IR-induced DNA damage and it is accompa-
nied by up-regulation of its level and activity and induction of apoptosis as proved by many studies (Maki et al., 1997; Nakano et al., 2001; Szkanderová et al., 2003). ATM, ATM-Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) are responsible for this prolonged half-life since they phosphorylate p53 on Ser\textsuperscript{15} (Shieh et al., 1997; Tibbetts et al., 1999; Helt et al., 2005). This phosphorylation is important for activation of p53 after exposure to IR by impairing the ability of Mdm2 from inhibiting p53-dependent transactivation (Shieh et al., 1997). Taken together, phosphorylation on Ser\textsuperscript{15} is crucial for IR response, and therefore we focused on this modification in context of the received dose.

In our previous work (Tichý et al., 2007) we described some participants of the ATM-dependent signalling pathway and none of them (ATM, Chk-2, Mdm2, and p53) showed a pronounced dose-dependent response. The main objective of this work was to investigate phosphorylation of p53 on Ser\textsuperscript{15} induced by IR in MOLT-4 cells and its possible contribution to biodosimetry. Here we report this particular modification of p53 as a promising biomarker, since its phosphorylation on Ser\textsuperscript{15} is dose-dependent.

**Material and Methods**

**Cell cultures and culture conditions**

The human T-lymphocyte leukaemia MOLT-4 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). The cells were cultured in Iscove’s modified Dulbecco’s medium (Sigma, St. Louis, MO) supplemented with 20% foetal calf serum, 0.05% L-glutamine, 150 UI/ml penicillin, 50 μg/ml streptomycin in a humidified incubator at 37 °C and controlled 5% CO\textsubscript{2} atmosphere. The cultures were divided every second day by dilution to a concentration of 2 × 10\textsuperscript{5} cells/ml. The cell counts were performed with a haemocytometer; the cell membrane integrity was determined by using the Trypan blue exclusion technique. Cell lines in the maximal range of up to 20 passages were used for this study.

**Gamma irradiation**

Exponentially growing cells were suspended at a concentration of 2 × 10\textsuperscript{5}/ml. Aliquots of 10 ml of cell suspension were plated into 25 cm\textsuperscript{2} flasks (Nunc, Wiesbaden, Germany) and irradiated at room temperature using \textsuperscript{60}Co γ-ray source with a dose-rate of 0.4–0.5 Gy/min, at a distance of 1 m from the source. After the irradiation the flasks were placed in a 37 °C incubator with 5% CO\textsubscript{2} and aliquots of the cells were removed at various times after irradiation for analysis. The cells were counted and cell viability was determined with the Trypan blue exclusion assay.

**Electrophoresis and Western blotting**

Two hours after irradiation by the doses 0.5, 1.0, 1.5, 3.0, and 7.5 Gy, the cells were washed with PBS and lysed. Whole-cell extracts were prepared by lysis in 500 μl of lysis buffer (137 mM NaCl, 10% glycerol, 1% n-octyl-β-glucopyranoside, 50 mM NaF, 20 mM Tris, pH 8, 1 mM Na\textsubscript{3}VO\textsubscript{4} (all from Sigma, St. Louis, MO) and 1 tablet of Complete\textsuperscript{TM} Mini (Roche, Mannheim, Germany). The lysates containing equal amounts of protein (30 μg) were washed onto a 12% sodium dodecyl sulphate (SDS) polyacrylamide gel. After electrophoresis (200 V; 50 min), proteins were transferred (100 V; 120 min) to a polyvinylidene difluoride (PVDF) membrane (BioRad, Hercules, CA), and hybridized with an appropriate antibody: anti-p53 1 : 1,000 (Exbio, Prague, Czech Republic); anti-p53 (Ser\textsuperscript{15}) – 1 : 1,000 (Calbiochem, San Diego, CA) and anti-β-actin – 1 : 10,000 from Sigma. After washing, the blots were incubated with secondary peroxidase-conjugated antibody (1 : 1,000–1 : 10,000 from Dako, High Wycombe, UK) and the signal was developed with ECL detection kit (BM Chem, San Diego, CA). The films were scanned and integrated optical density (IOD) was measured by ImagePro 1.0 software (Microsoft, Seattle, WA).

**ELISA**

For analysis of phosphorylation of protein p53 on Ser\textsuperscript{15} we used the PathScan ELISA Sandwich kit (Upstate, Chicago, IL). The cells were irradiated by the doses of 0.5, 1, 1.5, 3 or 7.5 Gy, washed with PBS and lysed 2 h after irradiation. The whole-cell lysates were prepared by adding 500 μl of lysis buffer (137 mM NaCl, 10% glycerol, 1% n-octyl-β-glucopyranoside, 50 mM NaF, 20 mM Tris pH 8, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 1 mM N\textsubscript{a}F, 1 mM N\textsubscript{a}F, 1 mM n-fenylmethylsulphonyl fluoride). The samples were analysed in a 96-microwell plate according to the manufacturer’s guide. Three independent measurements were carried out with antibody against unphosphorylated or phosphorylated p53 on Ser\textsuperscript{15}.

**Results and Discussion**

**Proliferative activity**

Figure 1 shows the effect of IR on the ability of MOLT-4 cells to proliferate. After irradiation by the doses up to 0.5 Gy we observed only a negligible effect on the growth of the cells. The dose of 0.2 Gy even stimulated the cells to proliferate 72 h after irradiation. The doses of 0.7 and 1.0 Gy caused a loss of proliferative activity (48 and 72 h) and the dose of 1.5 Gy had a cytostatic effect.

The doses of 3.0, 5.0 and 7.5 Gy were lethal (and supralethal) and resulted in cell death of the whole population within 48 to 72 h after irradiation.

**Phosphorylation of p53 on Ser\textsuperscript{15}**

In the Western blotting experiments we detected a low amount of p53 in sham-irradiated cells. Two hours after irradiation we observed a substantial increase in p53 and its amount slightly increased with the dose.
the other hand, the form of p53 phosphorylated on Ser15 was not detected in the control cells. A low amount of phosphorylated p53 was found 2 h after irradiation by the dose of 0.5 Gy and it increased in a dose-dependent manner (Fig. 2).

By the ELISA experiments we obtained similar results as by Western blotting. We detected a very low amount of unphosphorylated p53 in sham-irradiated cells. p53 was not significantly up-regulated 2 h after irradiation, but the amount of this protein was increased after all the doses when compared to non-irradiated cells. Phosphorylation of p53 on Ser15 was not induced in sham-irradiated cells, but we observed a dose-dependent increase especially in the dose range up to 3.0 Gy.

**Conclusions**

Conventional robust cytogenetic methods combined with analysis of molecular markers, molecules involved in IR-induced signalling pathways, might enhance biological dose assessment. Protein p53 phosphorylation on Ser15 is such a candidate for radiation biomarker. This particular modification of p53 is dose-dependent upon irradiation mainly after the doses up to 3 Gy. We recommend using a battery of protein biomarkers, each for dif-
different range of doses and time, combined with determination of clinical symptoms.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


