

DNA damage caused by ionizing radiation in embryonic diploid fibroblasts WI-38 induces both apoptosis and senescence

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Short title: Irradiation induces in WI-38 both apoptosis and senescence

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Summary:

Cellular response to ionizing radiation-induced damage depends on the cell type and the ability to repair DNA damage. Some types of cells undergo apoptosis, whereas others induce a permanent cell cycle arrest and do not proliferate. Our study demonstrates two types of response of embryonic diploid fibroblasts WI-38 to ionizing radiation. In the WI-38 cells p53 is activated, protein p21 increases, but the cells are arrested in G2 phase of cell cycle. Some of the cells die by apoptosis, but in remaining viable cells p16 increases, senescence associated DNA-damage foci occur, and senescence-associated beta-galactosidase activity increases, which indicate stress-induced premature senescence.

Keywords: DNA damage, ionizing radiation, senescence, stem cells

Abbreviations:

ATM – ataxia telangiectasia mutated, BCA – bicinchoninic acid, Cdk – cyclin dependent kinase, CDKN1A - inhibitor of cyclin-dependent kinase 1A, DAPI - 4'-6-diamidino-2-phenylindole, EDTA - ethylenediaminetetraacetic acid, FITC - fluorescein isothiocyanate, Mdm2 – murine double minute protein, PBS - phosphate Buffered Saline, PI – propidium iodide, PVDF – polyvinylidene difluoride, Rb – retinoblastoma protein, SDS – PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Introduction:

Radiotherapy is a crucial method of treatment of solid tumors. The goal of radiotherapeutical irradiation is tumor eradication, while maintaining integrity of healthy tissue. Fibroblasts are relatively quiescent and long living cells in almost all adult tissues and their radiosensitivity determines a baseline for therapeutical irradiation at maximum biologically effective doses. While in hematopoietic, lymphoid and germ cells the main response to irradiation is induction of apoptosis, in many solid tumors, fibroblasts and epithelial cells radiation-induced apoptosis is not convincingly proved. It is possible that the main mechanism of response of these tissues to irradiation is not apoptosis, but induction of so-called stress-induced premature senescence, SIPS.

In reaction to DNA damage an interconnected network of signaling is activated, and results in damage repair, temporary or permanent cell cycle arrest or cell death. The classic DNA-damage response pathway is initiated by activation of kinase ATM, which phosphorylates many substrates, among them transcription factor protein p53. Tumor suppressor p53 is a protein, which responds to various types of genotoxic stress, and is involved in induction of cell cycle arrest, apoptosis, senescence, DNA repair or changes in metabolism. Among others, protein p53 controls the expression of protein p21^{Cip1/Waf1}, product of the *CDKN1A* (inhibitor of cyclin-dependent kinase 1A) gene, which blocks the activity of cyclin dependent kinases and regulates progression of cell cycle in G1/S checkpoint (Ljungman 2000, Bringhold and Serrano 2000) and G2/M checkpoint (Taylor and Stark 2001). The arrest of the cell-cycle allows time for DNA damage repair. If the repair is unsuccessful, the cells are removed by apoptosis, necrosis or their proliferation is permanently suppressed by initiation of stress-induced premature senescence (SIPS).

The SIPS can be induced by UV radiation, ionizing radiation, hydrogen peroxide or chemotherapeutic agents (e.g. doxorubicin, cisplatin, camptothecin) (Suzuki and Boothman 2008). It was found, that ionizing radiation and chemotherapeutics used for treatment of cancer cause SIPS both in normal cells and in tumour cells (Raffetto *et al.* 2001). It was also found that some oncogenes can induce oncogene-induced senescence (Campisi *et al.* 2007). Several characteristics are in use for identification of senescent cells – permanent cell cycle arrest, increased expression of cell-cycle inhibitors (p21^{Cip1/Waf1}, p16^{INK4a}, p15^{INK4b}), hypophosphorylation of Rb (retinoblastoma protein), activation of DNA-damage response, senescence-associated secretory phenotype, morphologic changes, increase in senescence-associated β -galactosidase and formation of senescence-associated DNA-damage foci containing proteins that are associated with DNA damage response, e.g. γ H2AX (Suzuki and Boothman 2008).

If the cells with not completely repaired DNA damage are not removed by apoptosis, but enter SIPS instead, it allows for selection of a resistant population, but it also has a significant downside as it creates a risk of developing a population of cells with dangerously damaged genome. Stem cells are nowadays intensively studied, because of their self-renewal capability, ability to produce a broad spectrum of cell types and high proliferation potential. Stem cells can be used in treatment of cancer, in bone marrow transplantation and peripheral blood stem cell transplantation for patients who had radiation treatment or high dose of chemotherapy. However, there is very little information about their reaction to DNA damage as a response to genotoxic stress, such as ionizing radiation used in the treatment of many cancer types.

Hematopoietic stem cells react to irradiation predominantly by apoptosis induction, but some senescence induction was also detected (Vavrova *et al.* 2002; Meng *et al.* 2003). Mesenchymal stem cells isolated from bone marrow do not die by apoptosis after irradiation,

but they lose their ability to proliferate. The irradiation with the dose 2.5 – 15 Gy induces telomere shortening, cell division is stopped, but the cells are not destructed. The activity of senescence-associated β -galactosidase increases (Serakinci *et al.* 2007). Also mesenchymal stem cells isolated from dental pulp respond to irradiation primarily by induction of senescence (Muthna *et al.* 2010). It was found in the normal human embryonic diploid WI-38 fibroblasts, that busulfan (an alkylating agent leading to DNA damage) induces cellular senescence mainly associated with activation of extracellular signal-regulated kinase (Erk) and p38 MAPK (mitogen activated protein kinase). This cascade is independent on p53 activation (Probin *et al.* 2006).

The study of the reaction of stem cells to genotoxic stress is important for their safe usage, because they persist in organism for long time and can be the possible risk for the progression of population with damaged genome. The WI-38 human embryonic diploid fibroblasts are used as a model system in studies of stem cells. In this study we investigated the effect of ionizing radiation on WI-38 cells viability, proliferation, apoptosis and senescence induction, and changes in expression and phosphorylation status of proteins related to apoptosis and senescence.

Material and Methods:

Cell culture: WI-38 cells (human embryonic lung diploid fibroblasts) obtained from European Collection of Cell Cultures were cultured in 5% CO₂ atmosphere under 37° C in Dulbecco's modified Eagle's medium (Sigma, USA) supplemented with 10% FCS (PAA, USA), 2% glutamine (Gibco, UK), penicillin/streptomycin (Gibco, UK) and 1% non-essential amino acids. Cells were dissociated and reseeded by using trypsin-EDTA (Gibco, UK). In all experiments at early passages (<10 passages) were used cells and each of the passage reached

approximately 70% confluence. Trypan blue staining was used for counting viable and non-viable cells.

Irradiation procedure: Exponentially-growing WI38 cells were suspended to $2.5 \cdot 10^6$ cells/ml in complete medium. The aliquots were plated into 75 cm² flasks (Nunc) or in chamber slides (Thermo Fisher Scientific) and after 24 h irradiated at room temperature using ⁶⁰Co γ -ray source (Chisotron, Chirana, CZE) at the photon dose rate of 1 Gy/min in the distance 1 m from the source. Cells were irradiated with the doses of 2, 6 and 20 Gy. Dosimetry was performed with an ionization chamber (Dosemeter PTW Unidos 1001, serial No. 11057, with ionization chamber PTW TM 313, serial No. 0012; RPD Inc., USA); the set was validated by Czech Metrology Institute (CMI) - Inspectorate for Ionising Radiation (IIR), protocol No. 9011-OL-U4124/2005). After irradiation, the flasks/chambers were placed in a 37 °C incubator in a humidified atmosphere with 5% CO₂ and removed at various times following irradiation for analysis. Non irradiated controls were handled in parallel. The media was changed every 3 days after centrifugation, so the apoptotic floating cells were not discarded.

Cell cycle analysis: 1×10^6 of cells for each group were washed in cold PBS and fixed in 70% ethanol to permeabilize the cells. Then the cells were incubated for 5 min at room temperature in buffer (192 ml 0.2 mol/l Na₂HPO₄ + 8 ml 0.1 mol/l citric acid, pH 7.8) and then stained with PI in Vindelov's solution for 60 minutes at 37 °C. The DNA content was determined by flow cytometer Cytomics FC500 (Beckman Coulter) using 15 mW argon-ion laser with excitation capabilities at 488 nm, and the total emission above 560 nm was recorded. List mode data were analyzed using Multicycle AV software (Phoenix Flow Systems, Inc.) and CXP (Beckman Coulter).

Apoptosis detection: For apoptosis detection Apoptest-FITC kit (DakoCytomation, CZE) was used according to manufactures instructions. Annexin V binds to phosphatidylserine at the cell surface in the presence of calcium ions. Propidium iodide is a marker of cell membrane permeability. The measurement was performed on Cyan ADP (Beckman Coulter, USA); list mode data were analyzed using Summit 4.3v software. 1×10^6 of cells were used for each group.

Electrophoresis and western blotting: After irradiation adherent WI-38 cells were collected and whole cell lysates were prepared for the analysis according to manufactures instructions (Cell Lysis Buffer, Cell Signaling Technology, Inc, Boston, MA). The content of protein was determined by using bicinchoninic acid assay (Sigma-Aldrich, St. Louis, MO). For the SDS-PAGE electrophoresis an equal amount of protein (10 μ g) was loaded into the gel. The proteins were then transferred to a PVDF membrane (Bio-Rad Laboratories s.r.o., CZE), the membrane was blocked in TBS with 5% non-fat dry milk and incubated with primary antibody (p53, p53_S392-Exbio, CZE; β -actin, p21 – Sigma-Aldrich, St. Louis, MO; p53_S15-Calbiochem-Merck, CZE; mdm2_S166 - Cell Signaling Technology, Inc, Boston, MA) at 4 °C overnight. Then the membrane was incubated with polyclonal anti-mouse or anti-rabbit secondary antibody (DakoCytomation, CZE). For the signal detection a chemiluminescence detection kit (Roche, CZE) was used.

ELISA: For quantitative assay of p21 PathScan Sandwich ELISA kit by Cell Signaling Technology was used according to manufactures instructions. The optical density (OD) measured at 450 nm was plotted against dose (in Gy).

Reverse transcription and real-time polymerase chain reaction: Total cellular RNA was isolated using RNeasy mini kit (Qiagen, Austria). One microgram of total cellular RNA was transcribed to cDNA with Multiscribe RT enzyme and random primers contained in the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Gene expression was measured using TaqMan Gene Expression assay (Applied Biosystems), specifications are summarized in table 1. CDKN2A generates several transcript variants. At least three alternatively spliced variants (NM_058195 variant 4, NM_058197variant 3, NM_000077 variant 1) encoding distinct proteins have been reported. For any additional information see www.appliedbiosystems.com.

cDNAs were quantified with TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix, using the 7500 Fast (Applied Biosystems, USA). The results were normalized to 18S RNA.

Caspase activity assay: For detection of caspase 3/7, caspase 8 and caspase 9 activity Caspase-Glo Assays (Promega, USA) were used according to manufactures instruction. To measure the assays results Tecan Infinite 200 (Schoeller Instruments) was used.

Microscopy: The cells were irradiated in chamber slides (Thermo Fisher Scientific). Activity of beta-galactosidase was detected by Senescence beta-galactosidase Staining Kit (Cell Signaling) according to manufactures instructions. For detection of vimentin and γ H2AX 1×10^4 cells were irradiated, centrifugated, washed in PBS and fixed with paraformaldehyde for 10 min at room temperature, then washed in PBS. Cells were blocked in solution with 7% FCS and 2% BSA and immunostained with appropriate primary Ab (anti-Vimentin -DakoCytomation, CZE; γ H2AX - Cell Signaling Technology, Inc, Boston, MA; p16 – Sigma-Aldrich, St. Louis, MO,) overnight at 4 °C. The secondary antibody was applied

to each slide (after their pre-incubation with 5.5% donkey serum in PBS for 30 min at room temperature) and incubation for 1 h in the dark was succeeded by washing (3x5 min) in PBS. The nuclei were counterstained by DAPI solution (Sigma, St. Louis, MO, USA), incubated 8 minutes, washed and fixed. Images were obtained by Nikon Eclipse fluorescence microscope; the exposition time and dynamic range of camera in all channels were adjusted to the same values for all slides to obtain quantitatively comparable images.

Comet assay: DNA damage was measured using alkaline version of comet assay. Cells embedded in 1% agarose on microscope slides were lysed in 10 mM Tris-buffered 2.5 M NaCl (pH 10.0) containing 1% Triton X 100, 100 mM EDTA for 1 hour at 4 °C. After a 40 minutes period of unwinding the electrophoresis was carried out at 25 V, 300 mA, for 30 min at 4 °C. Before microscopy examination the cells were stained with 2% ethidium bromide. At least 100 cells were accounted. The damage was classified into 3 categories, intact cells, damaged cells (40-70% of DNA in the tail) and severely damaged cells (70-100% of DNA in the tail).

Results:

Cell viability: WI-38 cells (1×10^6) were irradiated with the doses of 2, 6 and 20 Gy and were counted after 3, 6 and 13 days. After 3 days cells in control group doubled, and kept proliferating during the whole experiment. The irradiation with the dose of 2 Gy inhibits proliferation until day 3, but the cells remain viable (5 % of dead cells) and start proliferating again, and until day 6 they doubled their amount and kept their proliferative activity till day 13. Irradiation by the doses of 6 and 20 Gy causes decrease in number of viable cells (day 3 7×10^5 in both groups, which deepened until day 13 (5.9×10^5 after 6 Gy, 5.3×10^5 after 20

Gy). After the irradiation with the doses 6 and 20 Gy the amount of dead cells increased in time, reaching 34 % (6 Gy), resp. 44 % (20 Gy) on day 13.

Comet assay: The ionizing radiation induces massive DNA fragmentation (Fig. 1). Our results show that in control 94 % of cells were intact. 1 hour post irradiation significant DNA damage was found in all irradiated samples: after the dose of 2 Gy 67 % of cells were damaged (40 – 70 % DNA in comet tail) and 6 % of cells severely damaged (70 – 100 % DNA in comet tail). In cells irradiated with the doses 6 and 20 Gy significant DNA damage also occurred 1 hour after the irradiation - 14 % resp. 33 % of cells were damaged and 84 % resp. 62 % were severely damaged. After 24 hours post irradiation the cells repaired most of the damage and the percentage of damaged cells was low: 10 % in 2 Gy, 10 % in 6 Gy, and 8 % in 20 Gy were damaged and 4 % in 2 Gy, 3 % in 6 Gy, and 6 % in 20 severely damaged (Fig. 1B).

Apoptosis induction, activation of caspases and morphologic changes: The irradiation induced moderate apoptosis after 6 days (10 % after 2 Gy, 14.4 % after 6 Gy and 10.2 % after 20 Gy; control 7.5 %) (Fig. 2A).

We also determined the effect of irradiation of WI-38 with the doses 2, 6 and 20 Gy on the activity of caspases 3/7, 8 and 9 6 days post irradiation. There were no significant changes in caspases activity observed in day 1 and 3 (data not shown). In 6 days the activity of caspases 3/7, 8 and 9 was slightly but significantly increased after the irradiation with the doses of 6 and 20 Gy ($p < 0,05$) (Fig. 2B). When compared to caspase activation in T-leukemic MOLT-4 cells 24 h after irradiation with the dose of 3 Gy (100% apoptosis induction), the caspase activity in irradiated WI-38 cells was approximately 10 times lower.

For detection of morphologic changes which are contributed to apoptosis the cell nuclei were stained with DAPI. Our results show no changes in nuclear morphology of adherent cells irradiated with the dose of 20 Gy during the experiment (Fig. 5B).

Ionizing radiation induces cell cycle arrest: We used cell cycle analysis by flow cytometry to determine possible cell cycle arrest after irradiation. The irradiation with the dose of 2 Gy has only small effect on cell cycle distribution with slight accumulation of cells in G2 phase (26 % in day 1, 22 % in day 3, 21 % in day 6; control 19 % in G2 phase) and decrease in number of cells in S phase (4 % in day 1, 6 % in day 3, 9 % in day 6; control 13 % in S phase), both manifesting mainly on day 1 (Fig. 3). After the irradiation with the doses of 6 as well as 20 Gy significant number of cells are arrested in G2 phase as early as first day after irradiation, the number of arrested cells decreases in time (40 % resp. 42 % in day 1, 30 % resp. 36 % in day 3, 11 % resp. 28 % in day 6; control 19 %) (Fig. 3).

Protein p53 and its phosphorylations: Ionizing radiation caused significant upregulation of p53. 4 hours after the irradiation the level of protein p53 increases and reaches its maximum. First day post irradiation the level is decreased, than increases in day 3 again and is rapidly decreasing in time. Phosphorylation of p53 at serine 15 occurs also already 4 hours after irradiation and its level is decreased back to control level in 6 days. Maximal phosphorylation at serine 392 reaches its maximum 4 hours after irradiation as well and is decreased back to control level in 1 day. The negative regulator of the protein p53 mdm2 is upregulated and activated by phosphorylation of serin 166. Mdm2 protein and its phosphorylation on serin 166 are increased 4 hours after the irradiation (Fig. 4A).

Changes in expression of proteins p21 and senescence induction: Ionizing radiation also induces the expression of p21 protein (inhibitor of cyclin-dependent kinase 1A). Its level is increased 1 and 3 days after irradiation and then it goes back to the control level (Fig. 4A). These results correlate with upregulated expression of its gene CDKN1A, which has the maximum in 3 days after irradiation (Fig. 4B). By using quantitative ELISA method we found that increase in p21 protein is dose-dependent (Fig. 4C).

In the cells irradiated by 20 Gy senescence associated DNA-damage foci of γ H2AX persisted in nuclei for 13 days (Fig. 5B). After p16 immunostaining we found out that the expression of p16 (inhibitor of cyclin-dependent kinase 2A), which is associated with cellular senescence, increases in day 13 post irradiation (Fig. 5D).

We detected enhanced activity of senescence-associated beta-galactosidase already on the first day after the irradiation with the dose of 20 Gy and its activity progressively increasing till day 13 (Fig. 5C).

Discussion:

It is known that cellular response to ionizing radiation-induced damage depends on the cell type and the ability to repair DNA damage. In normal human skin fibroblasts irradiated by 6 Gy apoptosis induction is minimal, but 3 or more DNA damage foci persist 48 h and cell cycle is arrested, primarily in G1 phase, and the cells stop proliferation (Toussaint *et al.* 2000; Dimitrijevic-Bussod *et al.* 1999; Tsuboi *et al.* 2007). On the other hand, 48 h after the irradiation most of the cells of human leukemia cell line MOLT-4 die by apoptosis, but 80% of the surviving cells repaired the damage and do not contain DNA-damage foci (Rezacova *et al.* 2008).

WI-38 cell line is derived from embryonic lung fibroblasts, and their response to irradiation differs from the response of normal skin fibroblasts. By using a comet assay we confirmed high amount of DNA damage induced by the irradiation, however the cells were able to repair single-strand breaks proficiently within 24 hours post irradiation and the percentage of DNA in comet tail quickly decreased. After irradiation a decrease in cell viability was observed in WI-38 cells, as well as weak induction of apoptosis, but more than half of the cells remained viable even after irradiation by the dose of 20 Gy. Through the use of cell cycle analysis we detected significant amount of cells arrested in G2 phase (approx. 40 % in day 1 and 30 % in day 3). Our findings are in agreement with the work of Filion *et al.* (2009), who found in human embryonic stem cells that these cells lack a traditional restriction point in G1 and with work of Muthna *et al.* (2010), who detected similar cell cycle arrest in irradiated stem cells isolated from dental pulp. These findings are distinct from that observed in normal somatic cells (Filion *et al.* 2009).

Analysis of apoptosis induction revealed that there is no apoptosis induction till day 6, when small increase in apoptotic cells is observed (10 % after 2 Gy, 14.5 % after 6 Gy and 10 % after 20 Gy). This finding correlates with slight increase in activity of caspase 3/7, 8 and 9 6 days after irradiation. In our previous study (Rezacova *et al.* 2008) a typical response of proliferating somatic cells to massive DNA damage caused by high doses of ionizing radiation was induction of apoptosis. Based on this investigation we used leukemic cell line MOLT-4 as a positive control, where all of cells die by apoptosis 24 h after the irradiation with the dose of 3 Gy. In comparison with this leukemic cell line the increase of caspases activity in WI-38 cells is not so marked (activity of caspases in MOLT_4 cell line are approximately ten times higher).

In this study we have shown that p53 is quickly activated and phosphorylated on serine 15 and serine 392 in WI-38 embryonic diploid fibroblasts in response to ionizing

radiation. One of the most serious types of radiation-induced DNA damage are double strand breaks of DNA. The first response of the cell to this type of DNA damage is the activation of ATM kinase by its autophosphorylation (Bakkenist and Kastan 2003). An important substrate of ATM kinase is the protein p53, which is phosphorylated and activated by ATM. ATM kinase phosphorylates protein p53 in the area, where mdm2 protein is bound, most often on serine 15. That is why the affinity of p53 to mdm2 is decreased and p53 is not degraded. Phosphorylation on serine 392 increases the association constant for tetramer formation of protein p53 and can support the interaction with specific DNA sequences (Canman *et al.* 1998).

One downstream target of the p53 pathway is *CDKN1A*, gene encoding protein p21^{Cip1/Waf1}. This protein belongs to the Cdk2 and Cdk4 inhibitors and induces cell cycle arrest. We found in our work that increased expression of p53 is followed by increase of protein p21, which is the typical response to genotoxic stress (Ju *et al.* 2007).

What happened with WI-38 cells surviving the irradiation? In our study we have found increased expression of protein p16^{INK4a} in surviving cells 13 days post irradiation by the dose of 20 Gy. Tumor suppressor protein p16^{INK4a} is the crucial protein for induction of senescence (Suzuki and Boothman 2008). Ionizing radiation-triggered accelerated senescence strongly correlates with expression of p16^{INK4a} and p16^{INK4a} protects human fibroblasts from undergoing apoptosis in response to DNA damage (Mirzayans *et al.* 2009). Protein p16^{INK4a} inhibits the activity of Cdk4 and Cdk6 kinases and induces hypophosphorylation of Rb protein. When Rb is in a hypophosphorylated state it is fully active and can block cell cycle progression by interacting with E2F to repress genes involved in cell cycle progression (Delston and Harbour 2006). One of others known markers of senescence is the presence of senescence-associated DNA-damage foci containing proteins that are associated with DNA damage recognition and repair, such as phosphorylated histone H2AX - γ H2AX (Campisi *et*

al. 2007) in the nucleus. After γ H2AX staining we observed these foci in nuclei 13 days after the irradiation with 20 Gy. Other important marker of senescence is increased beta-galactosidase activity (Eriksson and Stigbrand 2010). Beta-galactosidase is a hydrolase enzyme, which is highly expressed and accumulated in lysosomes in senescent cells. We detected increased activity of senescence-associated beta-galactosidase from the first day after irradiation, with maximum after 13 days. More recent studies have reported a similar observation. Suzuki *et al.* 2008 observed senescence-associated beta-galactosidase positive staining 3 days after the irradiation with the dose of 4 Gy in normal human diploid cells. The irradiation with the dose 2,5 – 15 Gy induces positive beta-galactosidase staining in human mesenchymal stem cells in 10 days as well (Serakinci *et al.* 2007). Taken together our results indicate that the WI-38 human embryonic fibroblasts surviving irradiation by the dose of 20 Gy enter stress-induced premature senescence.

Human embryonic diploid fibroblasts are frequently used for the study of cellular senescence (e.g. Lawless *et al.* 2010 used MRC-5 cell line, Raffetto *et al.* 2001 used WI-38 cell line). WI-38 also react by stress-induced premature senescence after exposing cells at early passages to a single sub-cytotoxic dose of UVB (Straface *et al.* 2007) and to busulfan (an alkylating agent leading to DNA damage) (Probin *et al.* 2006).

In summary, our study demonstrates two types of response of embryonic diploid fibroblasts WI-38 to ionizing radiation. In the WI-38 cells p53 is activated, protein p21 increases, but the cells are arrested in G2 phase of cell cycle. Some of the cells die by apoptosis, but in remaining viable cells p16 increases, senescence associated DNA-damage foci occur, and senescence-associated beta-galactosidase activity increases, which indicate stress-induced premature senescence.

Acknowledgements:

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Figures and tables:

Fig. 1

DNA damage 1h after the irradiation of WI-38 by comet assay

Sample results from the comet analysis of control cells and in cells 1 h after the irradiation with the dose of 6 Gy.

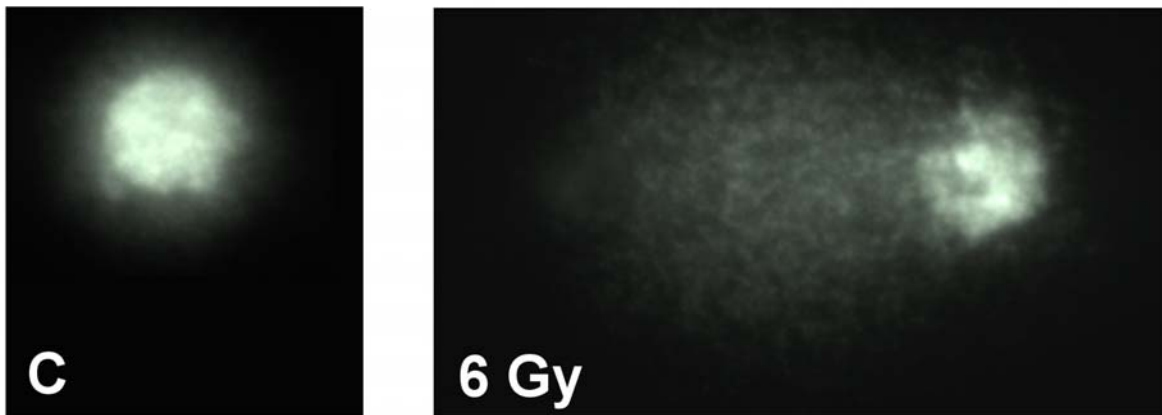


Fig. 2

The effect of irradiation of WI-38 cells with the doses of 2, 6 and 20 Gy on the apoptosis during 6 days after irradiation.

A: Apoptosis detection was determined by Annexin V and propidium iodide staining. Apoptosis was measured 1, 3 and 6 days after the irradiation with the doses of 2, 6 and 13 Gy.

B: Activity of caspases 3, 8 and 9 was determined 6 days after irradiation with the doses of 2, 6 and 20 Gy in WI-38 cells and after the irradiation with the dose of 3 Gy in MOLT-4 cells as a positive control.

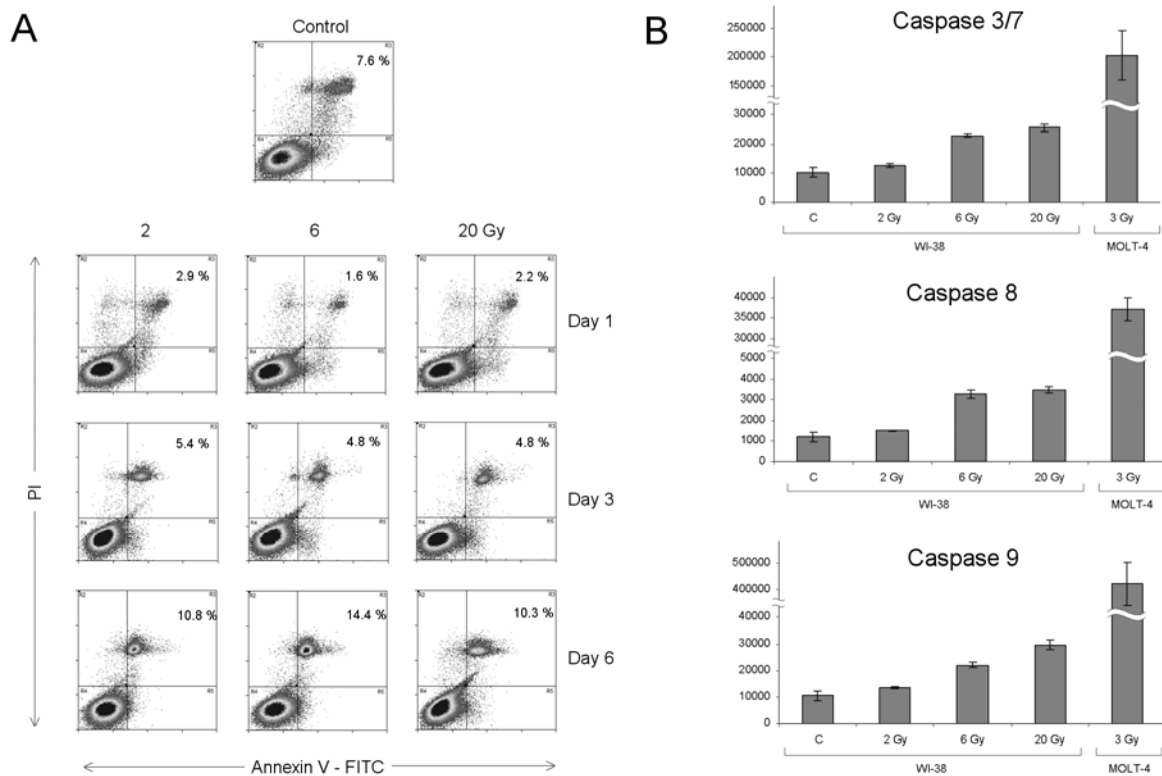


Fig. 3

The effects of irradiation on the distribution of WI-38 cells in the cell cycle.

Cell cycle distribution was measured using flow-cytometric detection of DNA content in the cells. Cell cycle was analysed 1, 3 and 6 days after the irradiation with the doses of 2, 6, and 20 Gy.

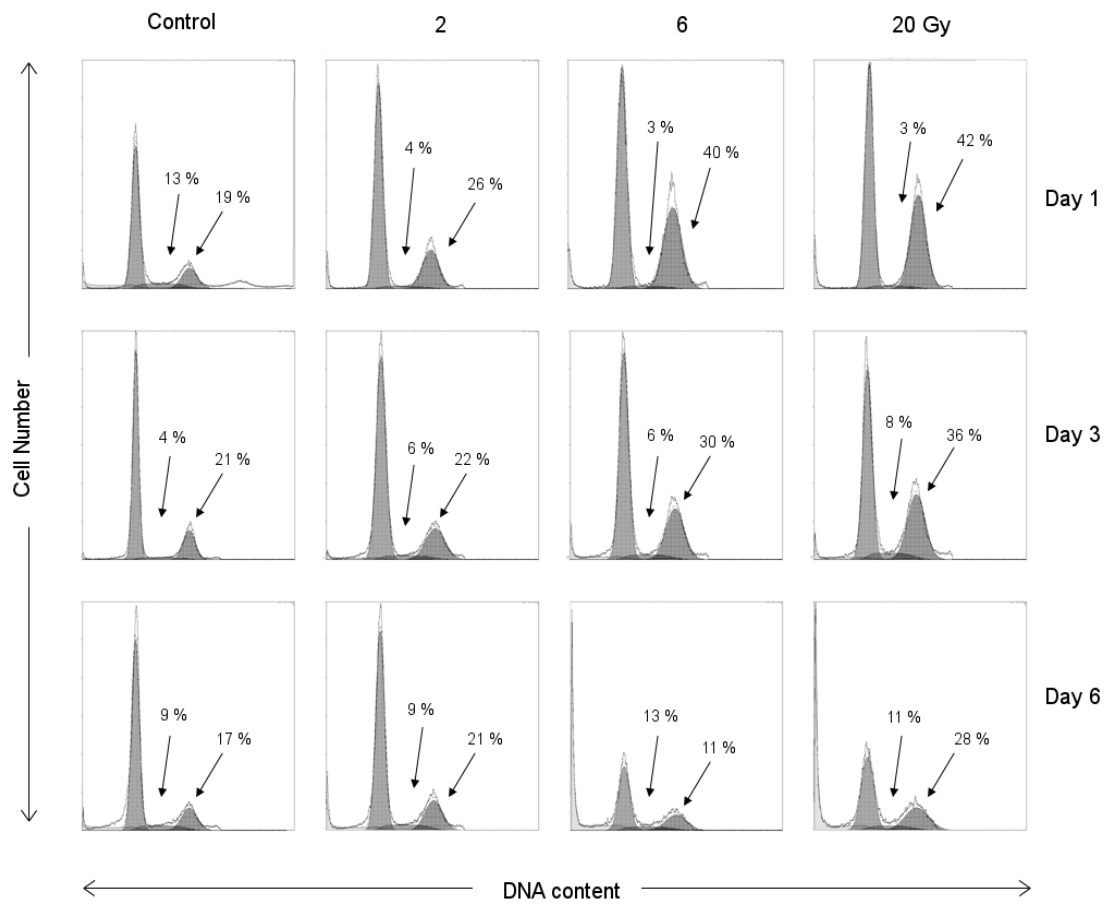


Fig. 4

Induction and activation of proteins in WI-38 cells after irradiation

A: WI-38 cells were irradiated with the dose of 20 Gy. Using electrophoresis and western blotting we evaluated changes in p53 and its phosphorylations at serines 15 and 392, mdm2 protein and its phosphorylation at serine 166 and p21 protein. To confirm equal protein loading, membranes were reincubated with β -actin.

B: The expression of genes for p21 was measured by using quantitative RT-PCR after the irradiation with the dose of 20 Gy during the interval of 13 days. The results of RT-PCR are in arbitrary units.

C: Dose dependence of changes in p21 protein over the doses 2 – 20 Gy after 3 days post irradiation in WI-38 cells.

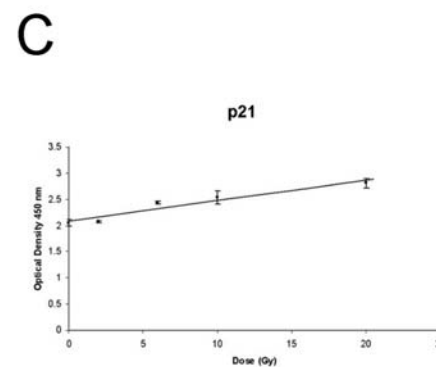
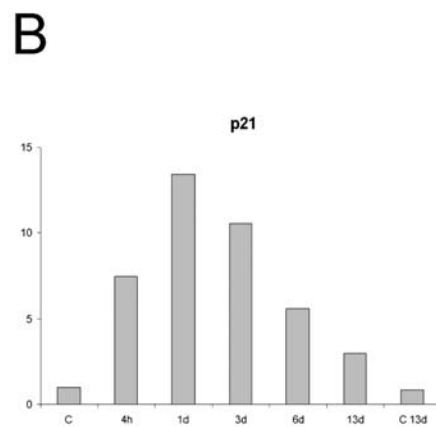
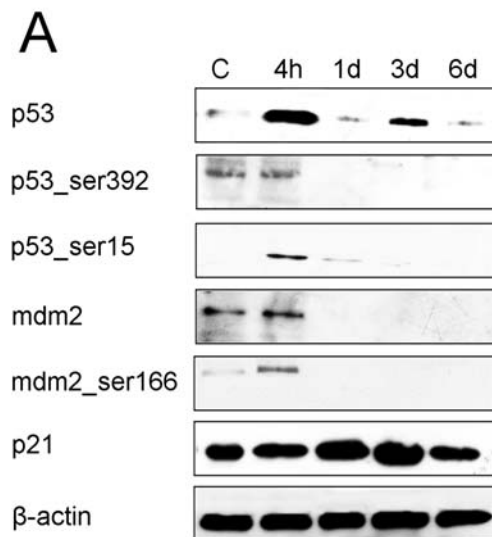


Fig. 5

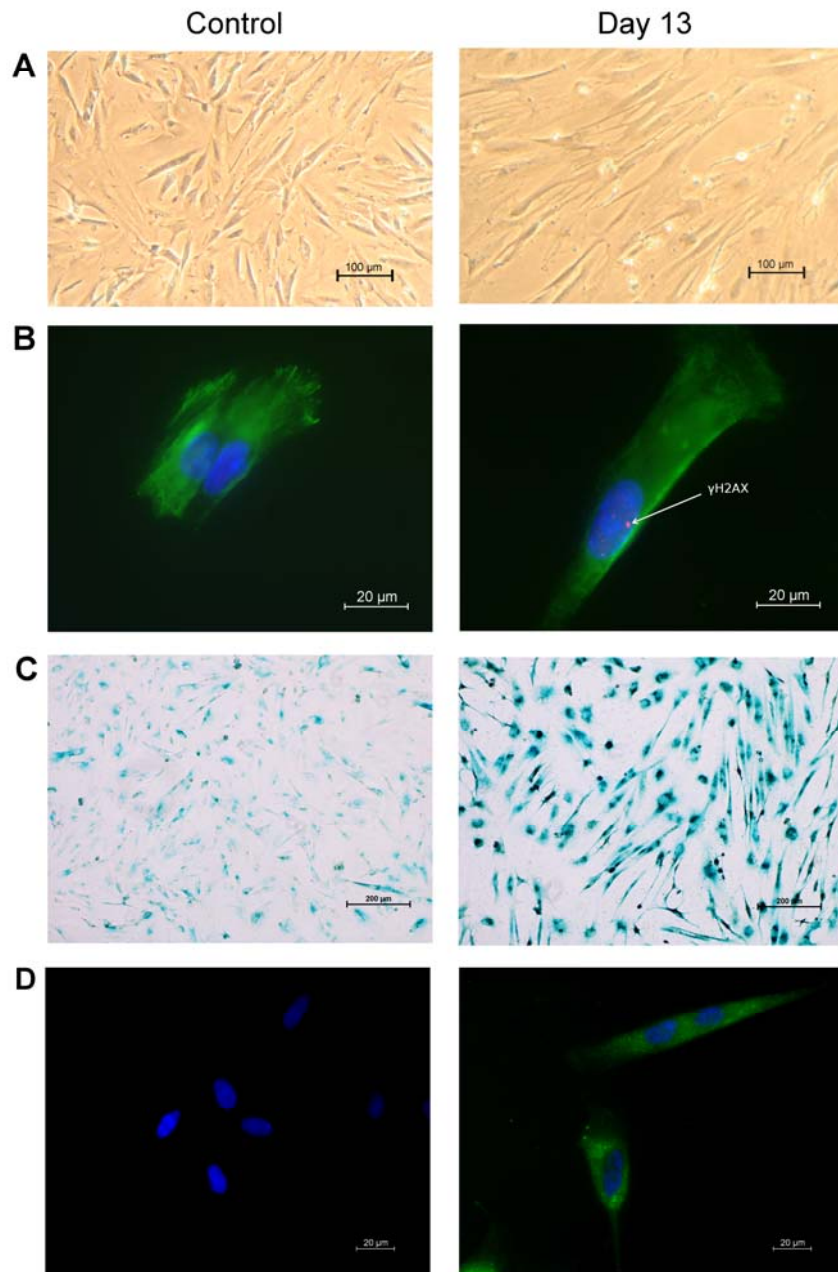
The effect of irradiation in cell morphology and induction of senescence in WI-38 cells irradiated with the dose of 20 Gy.

A: Changes in cell morphology of WI-38 cells after the irradiation with the dose of 20 Gy after 13 days.

B: Morphological changes and changes in nuclei were determined after 13 days after the irradiation with the dose 20 Gy. DAPI + γ H2AX (red) + vimentin (green) staining.

C: The cells were irradiated with the dose of 20 Gy in chamber slides and activity of senescence associated beta-galactosidase was detected histochemically after 13 days.

D: Changes in expression p16 protein were determined after 13 days after the irradiation with the dose 20 Gy.



Tab. 1**TaqMan Gene Expression assay**

Gene symbol	Accession number	Assay ID
TP53	NM_000546	Hs00153349_m1
CDKN1A (p21)	NM_078467	Hs00153349_m1
CDKN2A (p16)	NM_058195, NM_058197, NM_000077	Hs00233365_m1