

Cell Cycle Perturbations and Genotoxic Effects in Human Primary Fibroblasts Induced by Low-energy Protons and X/γ-rays

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The effect of graded doses of high-linear energy transfer (LET) low-energy protons to induce cycle perturbations and genotoxic damage was investigated in normal human fibroblasts. Furthermore, such effects were compared with those produced by low-LET radiations. HFFF2, human primary fibroblasts were exposed to either protons (LET = 28.5 keV/μm) or X/γ-rays, and endpoints related to cell cycle kinetics and DNA damage analysed. Following both type of irradiations, unsynchronized cells suffered an inhibition to entry into S-phase for doses of 1–4 Gy and remained arrested in the G₁-phase for several days. The levels of induction of regulator proteins, such as TP53 and CDKN1A showed a clear LET-dependence. DSB induction and repair as measured by scoring for γ-H2AX foci indicated that protons, with respect to X-rays, yielded a lower number of DSBs per Gy, which showed a slower kinetics of disappearance. Such result was in agreement with the extent of MN induction in binucleated cells after X-irradiation. No significant differences between the two types of radiations were observed with the clonogenic assay, resulting anyway the slope of γ-ray curve higher than that the proton one. In conclusion, in normal human primary fibroblasts cell cycle arrest at the G₁/S transition can be triggered shortly after irradiation and maintained for several hours post-irradiation of both protons and X-rays. DNA damage produced by protons appears less amenable to be repaired and could be transformed in cytogenetic damage in the form of MN.

INTRODUCTION

High-LET radiations are characterized by an enhanced biological effectiveness compared to low-LET radiations for several cellular endpoints. Many authors point to the hypothesis that the final cellular effects induced by high-LET radiations are dominated by the more severe, clustered DNA damage produced by the microscopic pattern of energy deposition that appears to be less repairable compared to the damage of low-LET radiation.^{1,2} Among the high-LET radiations, 28.5 keV/μm protons appear to be of particular radiobiological interest, as stopping particles, due to their high radiobiological effectiveness: results previously obtained for cell inactivation and hprt mutation induction have shown

that the RBE of protons rises as a function of LET and reaches a maximum at LET values around 25–30 keV/μm.^{3–5} Various endpoints have been evaluated so far in rodent and human cells exposed to protons with these LET values, including chromosomal aberrations,⁶ micronuclei (MN),⁷ chromosomal non-disjunction,⁸ mutations,^{4,5} DNA fragmentation,^{9,10} mitotic catastrophe¹¹ and clonogenic survival.^{4–6,12,13}

However, for other relevant biological responses as those related to cell cycle kinetics and protein expression only preliminary results have been reported so far,¹⁴ whereas there is a lack of information for DNA repair kinetics in the low-dose range of low-energy proton-irradiated normal human cells.

Exposure of eukariotic cells to ionizing radiations is known to affect the normal progression through G₁, S and G₂ phases of cell cycle.^{15,16} In the recent years the major molecular players taking part in pathways responsible for causing cell cycle delay have been identified. In this respect, the pathway governed by a wild-type TP53 and its downstream effector CDKN1A (formerly known as p21/CIP1/WAF1), a well known inhibitor of G₁/S cyclin-dependent

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kinases, plays a crucial role in the G₁- to S-phase transition.^{17–19} In spite of this knowledge, open questions concerning magnitude, persistence and control of the cell cycle still remain, particularly for high-LET radiations. In particular, many of the published studies have been carried out in rodent cell lines or in immortal tumor cells after irradiation with α -particles or heavy ions making results not directly transferable to normal primary cells.^{16,20–23} Furthermore, the effects of DNA/cytogenetic lesions on the kinetics of expression of regulator proteins and extent of cell cycle modulation and vice versa have been only partly investigated for densely ionizing radiations. An effect of radiations in terms of transient delay into S-phase and mitosis has been reported for AG01522 normal fibroblasts following exposure to X-rays, 11 MeV carbon ions and 9.9 MeV Ni ions.^{24–26} Other results obtained with α -particles, showed a different or similar capability compared with γ -rays to arrest cells in the G₁-phase.^{27,28} Notably, irradiated-human fibroblasts suffer for a prolonged and irreversible arrest characterized by hallmarks of G₀-growth arrest, senescence or differentiation, like persistent expression of TP53, CDKN1A and CDKN2A (also known as p16).^{25,26,29–32}

In the past, the effect of high-LET radiation on the kinetics of DNA double-strand breaks (DSBs) repair, has been extensively investigated, but many of the studies have been carried out by using the Pulse Field Gel Electrophoresis (PFGE) which is an assay sensitive for doses higher than 5 Gy, whereas only recently a re-evaluation of DSB rejoining at doses as low as 0.5–1 Gy is in progress for various types of radiations.^{33,34} In this respect, the immunofluorescent staining with a γ -H2AX antibody, which exploits a very early step in the cellular response to DSBs, that is the rapid phosphorylation of the histone H2AX at serine 139 resulting in γ -H2AX foci at the damaged sites, was proven extremely promising.^{35,36} Such an assay has been successfully used to show the presence and persistence of severe, clustered DNA lesions, as in the case of the slower γ -H2AX foci decay in human fibroblasts exposed to 39 keV/ μ m carbon ions³⁷ or to 150 keV/ μ m ⁵⁶Fe ions.³⁸ compared with γ -rays. Regarding studies aimed at evaluating intrinsic cellular radiosensitivity it has been reported that there is less retention of γ -H2AX foci associated with radioresistant tumour cell lines compared with radiosensitive ones.³⁹

Another sensitive assay in radiobiological studies on DNA damage is the Micronucleus (MN) test in cytochalasin-B-arrested binucleated cells, which measures the cytogenetic damage in the form of whole chromosomes or acentric fragments arising from mis-repaired lesions that have not been incorporated into daughter nuclei at mitosis.⁴⁰

In the present paper we report on the comparative effects of high-LET (28.5 keV/ μ m) protons and X/ γ -rays on cell cycle kinetics and DNA damage evaluated at short and long times after irradiation.

MATERIALS AND METHODS

Cell and irradiation procedures

HFFF2 human fetal foreskin fibroblasts (ECACC, UK) were cultured in MEM medium supplemented with 10% of either fetal calf serum or fetal bovine serum, penicillin and streptomycin and 2 mM L-glutamine in 5% CO₂ atmosphere at 37°C. In these conditions, the cell doubling time, T_d, evaluated from the growth curves, was of 24 ± 1 hours.

In experiments with protons, 48 hours before irradiation, 1 × 10⁵ cells were seeded on specially designed stainless steel Petri dishes, incubated at 37°C in a humidified atmosphere of 5% CO₂ in air and grown as a monolayer attached to a Mylar foil (52 μ m thick; area 133 mm²) used as bottom of the Petri dishes. For each dose point, at least 4 different dishes were irradiated at the radiobiological facility of the 7 MV Van de Graff CN accelerator at the Laboratori Nazionali di Legnaro (LNL) of the Istituto Nazionale di Fisica Nucleare (INFN), with a dose-rate of 1 Gy/min.^{41,42} Proton beams with energy of 0.8 MeV on the cell surface, which corresponds to 28.5 keV/ μ m LET, were used.^{41,42} All proton irradiation experiments were performed in the so-called track segment conditions. Irradiation facility, beam dosimetry and irradiation modalities have been described in detail elsewhere.^{41,42}

For X-rays (Gilardoni apparatus 250 kV, 6 mA; dose-rate of 0.70 Gy/min) 48 hours before the treatment 3 × 10⁵ cells were seeded in plastic Petri dishes containing (H2AX, MN and CDKN1A assays) or not containing glass cover slips (proteins and cell cycle analysis).

For gamma irradiations, 9 × 10⁵ cells were seeded on T25 flasks 48 hours before the treatment. The irradiations have been carried out at the ⁶⁰Co “gammabeam” of CNR-FRAE (at the INFN-LNL), with a dose-rate of 1 Gy/min.

Sham irradiated cells were used in all the experiments as control (unirradiated) cells.

Protein extraction and immunoblotting

Cells were washed twice on ice in cold PBS, collected using a scraper, centrifuged for 6 min at 4000 RPM, and then the pellet was lysed for 30 minutes on ice in 40 μ l of extraction buffer solution (20 mM Tris HCl Ph 8.0, 137 mM NaCl, 10% glicerol, 1% NP-40, 10 mM EDTA, 1 μ g/ml of aprotinin, pepstatin and leupeptin, 1 mM orthovanadate and 2 mM of PMSF). Protein concentration was evaluated with a Micro BCA protein assay (Pierce). 25 μ g of soluble proteins were separated by electrophoresis on a 10% SDS polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon). The efficiency homogeneity of running and transfer were evaluated using Coomassie blue and Ponceau Red, respectively. Blotted filters were blocked for 30 minutes in 5% skimmed milk at RT and then incubated over-night with 1 μ g/ml mouse monoclonal TP53 (Dako)

and CDKN1A antibodies (Signal Transduction). Filters were then incubated for 1 hour with secondary anti-mouse antibody (Amersham) diluted 1:2000 in skimmed milk. The antibody reaction was checked by the enhanced chemiluminescence detection procedure according to the manufacturer's instructions (Amersham). The densitometric analysis of signals was carried out by using a Fluor-S Multimager (Biorad). Three independent experiments have been carried out.

Immunofluorescence staining with CDKN1A and γ -H2AX antibodies

At various times after irradiation cells were fixed 30 minutes in absolute methanol at -20°C . Then, samples were rinsed in PBS and incubated overnight at $+4^{\circ}\text{C}$ with CDKN1A anti-mouse monoclonal antibody (Signal Transduction) diluted 1:20 in PBS/2%BSA. Slides were washed 4×5 min in PBS/BSA 1% and incubated 1 hour at 37°C with a FITC-conjugated secondary antibody (Vector) diluted 1:50 in PBS/BSA 1%. After extensive washing in PBS/BSA 1% and once in PBS, cells were stained with $0.2 \mu\text{g/ml}$ DAPI, mounted with anti-fade solution (Vector) and analyzed. Nuclei with a bright and uniform CDKN1A staining, as those reported in Fig. 2, were considered as positive and scored in a blind eye-analysis. For each experiments, 1000 cells were scored in three independent experiments.

For γ -H2AX foci, cells were irradiated and fixed at different times after irradiation in 2% paraformaldehyde, permeabilized on ice for 5 minutes with 0.2% Triton X-100 and blocked in PBS/BSA 1% for 30 min at room temperature. Slides were incubated with $1 \mu\text{g/ml}$ γ -H2AX mouse monoclonal antibody (Upstate), and detected with an anti-mouse FITC-conjugated antibody (Vector). Images were captured using the Zeiss Axiophot imaging epifluorescent microscope equipped with a charge-coupled device CCD camera and ISIS software. Quantitative analysis was carried out by counting by eye foci in at least 50 cells/experiment in experiments repeated at least twice. Overlapping foci were scored as a single focus.

Flow cytometric analysis of cell cycle progression

At 8, 24 and 32 hours after irradiation with 2 and 4 Gy, cells were harvested by trypsinisation, washed twice in cold PBS and gently resuspended in 1 ml of fluorochromic solution containing $50 \mu\text{g/ml}$ propidium iodide, 0.1% sodium citrate and 0.1% Triton X-100, and then placed at 4°C overnight in the dark. The fluorescence of the nuclei was measured with a Galaxy (DAKO) flow cytometer and the percentage of G_1 , S and G_2/M distribution was calculated using FloMax v2.3 (Partec) in three or four repeated experiments.

Immunofluorescence staining with anti-BrdU antibody

Cells were irradiated and one hour before harvesting, 30

μM BrdU was added to the cultures and cells were fixed and maintained in absolute ethanol at -20°C . Coverslips were washed twice with PBS and treated with 1.5 N HCl to denature the DNA. Thirty minutes later, they were washed twice with $\text{Na}_2\text{B}_4\text{O}_7$ for neutralization. Coverslips were incubated for 1 hour at 37°C with 1:10 diluted anti-BrdU antibody (Dako) in a wet chamber. Then cells were washed twice with PBS and incubated 1 h with an appropriate FITC-conjugated secondary antibody. DNA was counterstained with $20 \mu\text{g/ml}$ propidium iodide, slides were mounted with anti-fade solution (Vector) and then analyzed with a Zeiss Axiophot microscope. Experiments were repeated 3 times and the statistical analysis was performed using the χ^2 -test. For the evaluation of inhibition of the G_1/S -transition, three and two independent experiments have been carried out for short term and long term evaluation, respectively.

Micronuclei induction

Growing cells were irradiated and thereafter fresh medium containing $3 \mu\text{g/ml}$ Cytochalasin-B (Sigma) was added. Twenty-four hours later cells were washed once with PBS, detached by trypsinisation and collected onto glasses at 800 rpm for 4 minutes using a cytocentrifuge (Cytospin, Shandon). Then cells were fixed 30 minutes in absolute methanol at -20°C , air dried, stained with $0.2 \mu\text{g/ml}$ DAPI and mounted with anti-fade solution (Vector). The induction of MN was evaluated in at least 500 cells per experimental point in repeated experiments with a microscope under a blue-violet illumination. Five independent experiments have been carried out.

Cell inactivation

After proton- or γ -irradiation, the cells were trypsinised, diluted and plated at appropriate densities in Petri dishes ($\varnothing 100$ mm). The growth of cells seeded at low densities was optimized using feeder layers irradiated with 40 Gy ^{60}Co γ -rays. After 14 days of incubation at 37°C , the cells were fixed and the macroscopic colonies (i.e., colonies with more than 50 cells) were scored for cell surviving fraction determination, in agreement with the cell colony forming assay.

Six or seven independent experiments have been carried out with protons and γ -rays, respectively.

RESULTS

Radiation-induced TP53 and CDKN1A protein accumulation

The accumulation of TP53 and CDKN1A proteins was evaluated by means of immunoblotting as shown in Fig. 1, for representative experiments. TP53 was only slightly induced by X-rays and no modulation was observed between the two harvesting times; whereas, for $28.5 \text{ keV}/\mu\text{m}$ protons, an increase was only observed at 8 hours. In proton-treated cells, the accumulation of CDKN1A was about two times

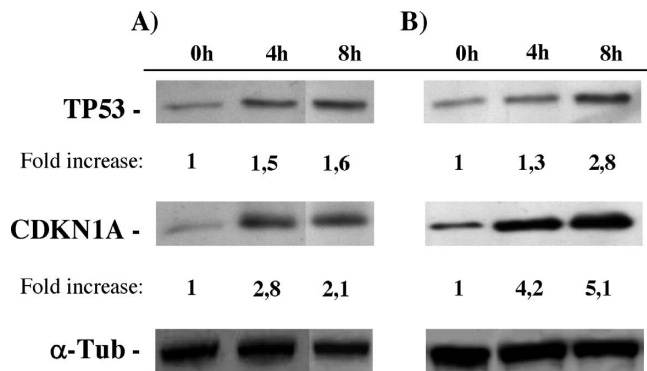


Fig. 1. Representative immunoblot showing the effect of X-rays (A) and 28.5 keV/μm protons (B) on p53 and CDKN1A expression. HFFF2 fibroblasts were irradiated with 2 Gy and harvested 4 and 8 hours later for immunoblotting assay. Equal loading of proteins was ascertained by probing filters with α-tubulin antibody. Three independent experiments have been carried out.

higher than that obtained in X-ray-treated cells. In contrast to that observed or X-rays, in proton-irradiated cells the CDKN1A accumulation was slightly higher at 8 than 4 hours.

In Fig. 2 a representative image of HFFF2 fibroblasts exposed to 1 Gy of 28.5 keV/μm protons, harvested at 8 hours and immunostained with a CDKN1A monoclonal antibody-FITC conjugated is shown (A). Two cells show bright nuclear localization for CDKN1A. Cells were counterstained with DAPI (B). The frequencies of CDKN1A positive nuclei are shown in Fig. 2C and D. The frequency of labeled cells in sham irradiated controls did not change as a function of time (not shown). In X-ray-treated cells, a significant increase in the fraction of CDKN1A positive cells was observed only for the highest dose shortly after irradiation ($p = 0.05$ and $p < 0.05$ t-test, respectively for 1 and 2 Gy vs sham irradiated); additionally, no differences could

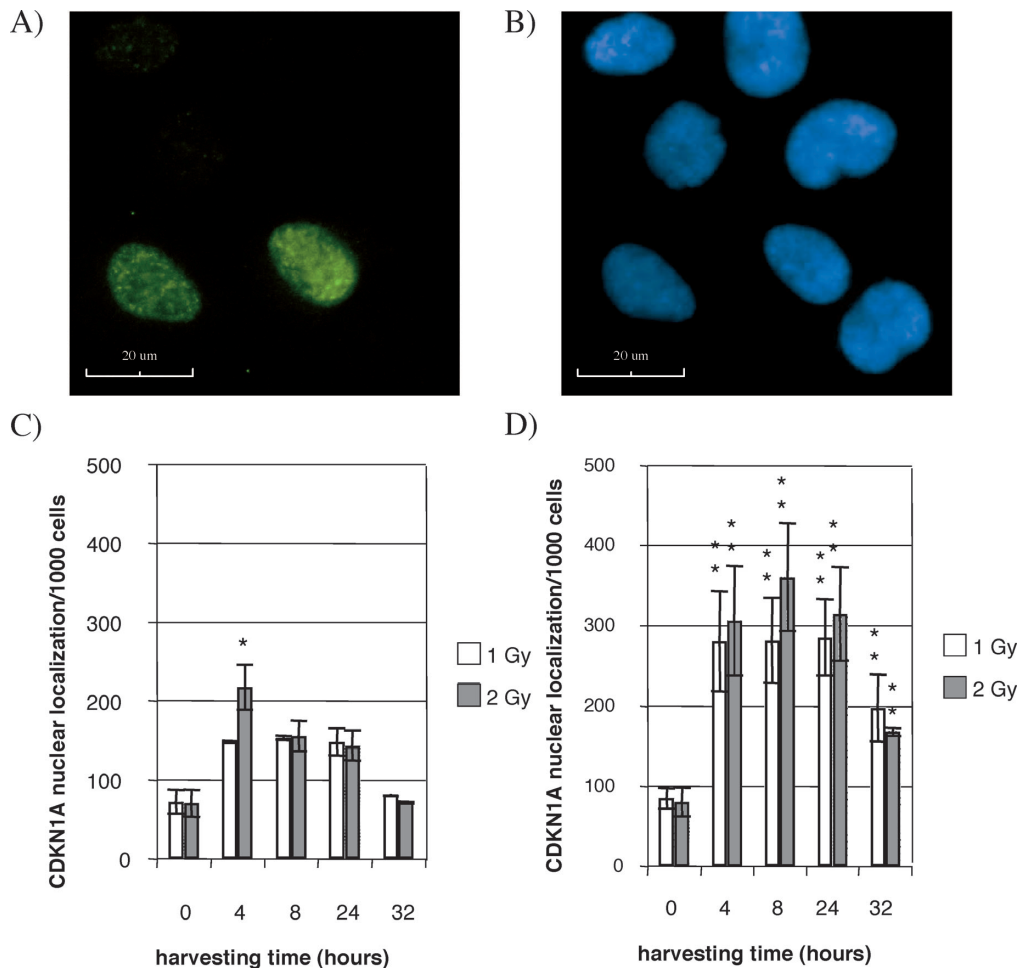


Fig. 2. An example of cells showing CDKN1A nuclear accumulation after 1 Gy of protons and harvested at 8 hours as stained with FITC (A) and DNA counterstained with DAPI (B). Kinetics of nuclear accumulation of CDKN1A in HFFF2 fibroblasts after exposure to 1 or 2 Gy of X-rays (C) and protons (D). The error bar denotes one standard error of the mean. Significant according to Student's T-test * $p < 0.05$, ** $p < 0.01$. For each experiments, 1000 cells were scored in three independent experiments.

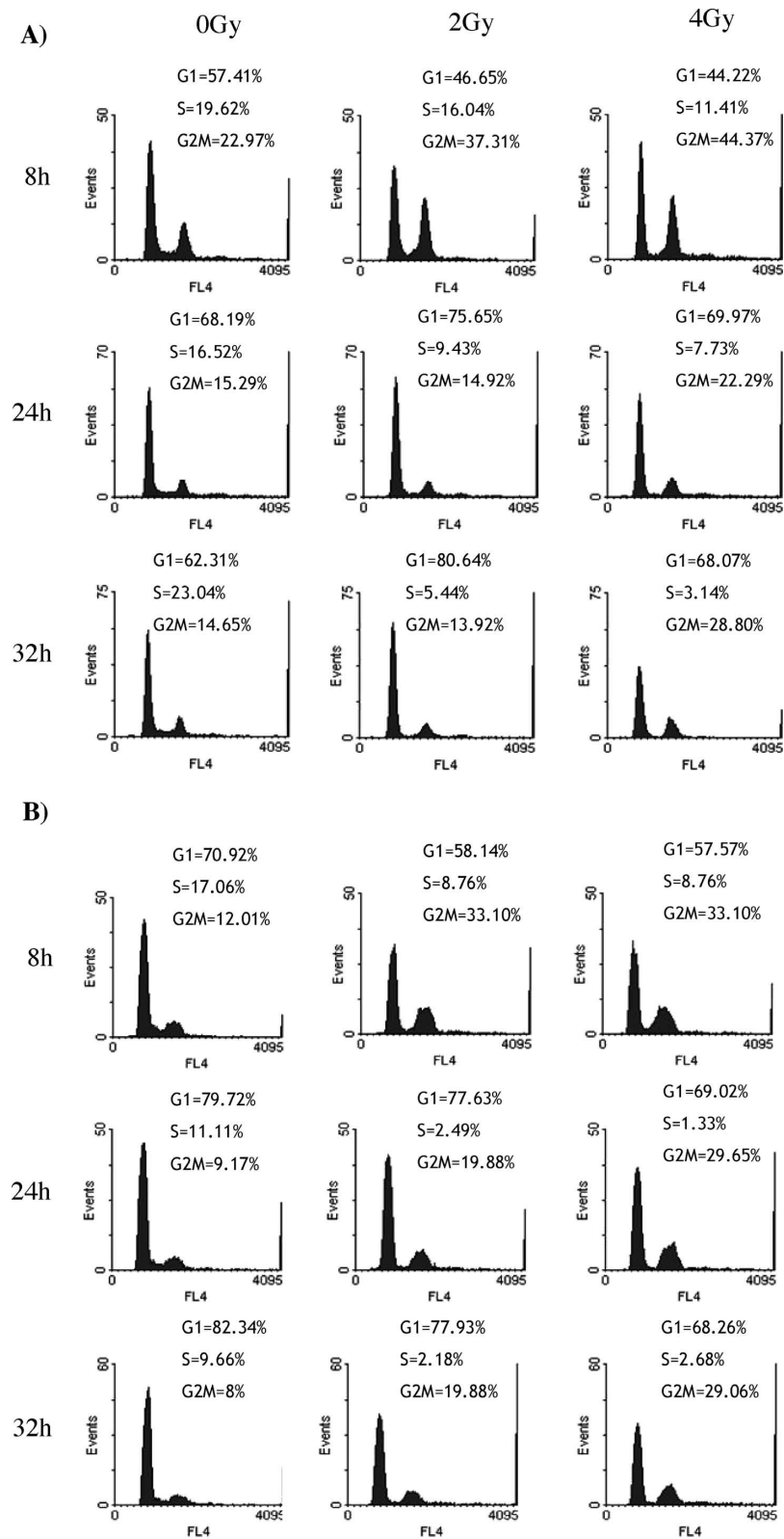


Fig. 3. X-ray- (A) and protons-induced (B) cycle perturbations in HFFF2 fibroblasts irradiated with 2 and 4 Gy and harvested at different times. Images are representative of one experiment. The cell cycle distribution as evaluated in untreated cells harvested at the 0 hours, did not differ from the 8 hour point (not shown). In x-axis is reported the DNA content. The percentage of G₁, S and G₂/M distribution was calculated in three or four repeated experiments.

be appreciated at late harvesting times (C). In particular, at 32 hours the values completely resembled those observed in untreated cells. On the contrary, CDKN1A was upregulated at both 1 and 2 Gy of protons with respect to untreated cells, irrespective of the harvesting times (t-test, p values ranging from $p = 0.001$ to $p = 0.006$). At 32 hours from proton exposure, the frequency of CDKN1A-positive cells did not reach control values ($p < 0,01$) (D).

Cell cycle effects

Cell cycle effects were evaluated by means of cytofluorimetric analysis in samples harvested at various times from treatment (Fig. 3). Starting from 24 hours after irradiation with both types of radiation, cells became markedly arrested in G_1 -phase, though no clear LET-dependence could be detected by comparing the fraction of accumulated cells (A, B). The G_1 -block was also consistently maintained in 32 hour-harvested samples.

In contrast with that obtained for the G_1 -block, differences in the pattern, extent and persistence of the G_2 -phase accumulation were observed. In fact, G_2 -phase accumulation was markedly higher after proton exposure (B) than X-rays (A), and this was visible at 2 Gy as well as at 4 Gy. In fact, the G_2 ratio treated/untreated at 2 Gy was 2.7, 2.1 and 2.4 fold for protons and 1.6, 0.9 and 0.9 for X-rays at 8, 24 and 32 hours, respectively. At 4 Gy the G_2 ratio treated/untreated was 2.7, 3.2 and 3.6 fold for protons and 1.9, 1.4 and 1.9 for X-rays at 8, 24 and 32 hours, respectively. At 2 Gy of X-rays, in contrast to that obtained with protons, such G_2 arrest was not persistent and at 24 hours, the G_2 reflected that of untreated cultures (A).

For a more detailed analysis of the impairment in the G_1 to S-phase transition, we also monitored the fraction of BrdU incorporation before harvesting (Fig. 4). Protons were more effective than X-rays at 8 hours after treatment with 1 and 2 Gy, showing about 20% and 40%, respectively, in the depletion of S-phase cells (for 2 Gy of protons, χ^2 -test $p < 0.05$). At longer time from exposure, the percentage of S-phase cells showed strongly reduced values for both protons and X-rays (χ^2 -test $p < 0.01$) and no strong differences could be appreciated between the two types of radiations.

A permanent inhibition of cells to enter S-phase was observed up to 120 hours after irradiation with 4 Gy (Fig. 5) showing only 17.6% and 16.8% of cells resuming BrdU incorporation, for protons and X-rays, respectively (calculated as % BrdU labeled-treated cells/ % BrdU labeled-untreated cells). At 12 days from exposure to X-rays, the G_1 -block was still maintained in that only 12% of cells displayed BrdU staining (not shown).

γ H2AX foci assay

In Fig. 6, representative images of γ -H2AX focus formation at several harvesting times from irradiation of HFFF2 fibroblasts with 1 Gy of either X-rays or 28.5 keV/ μ m pro-

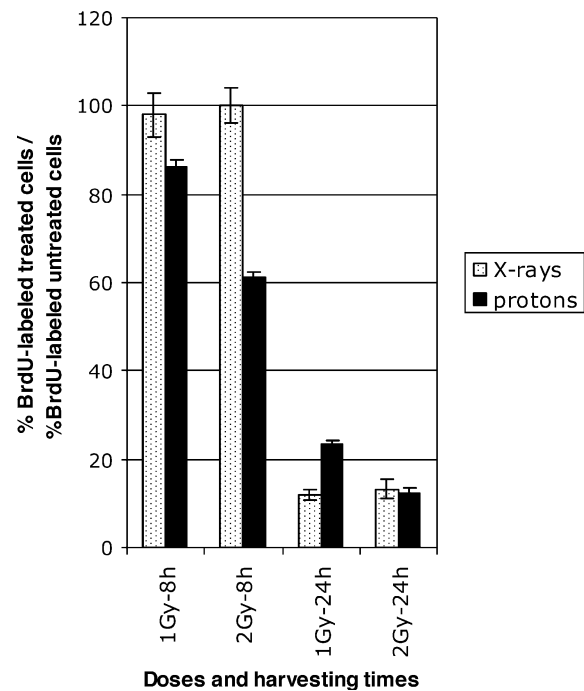


Fig. 4. Inhibition of G_1 /S-transition after irradiation. HFFF2 fibroblasts were irradiated with 1 or 2 Gy and harvested 8 or 24 hours later, including the last hour in the presence of 30 μ M BrdU. Three independent experiments have been carried out. The error bar denotes one standard error of the mean.

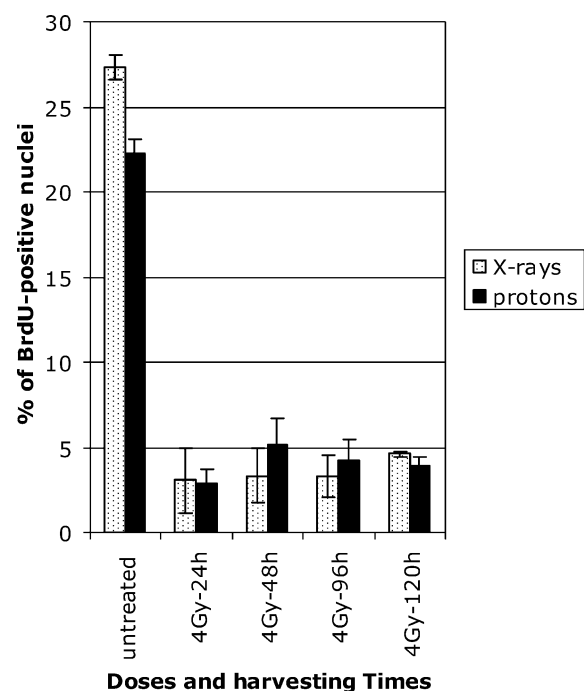


Fig. 5. Fraction of BrdU-labelled S-phase cells as a function of time after irradiation with 4 Gy X-rays or protons. Two independent experiments have been carried out. The error bar denotes one standard error of the mean.

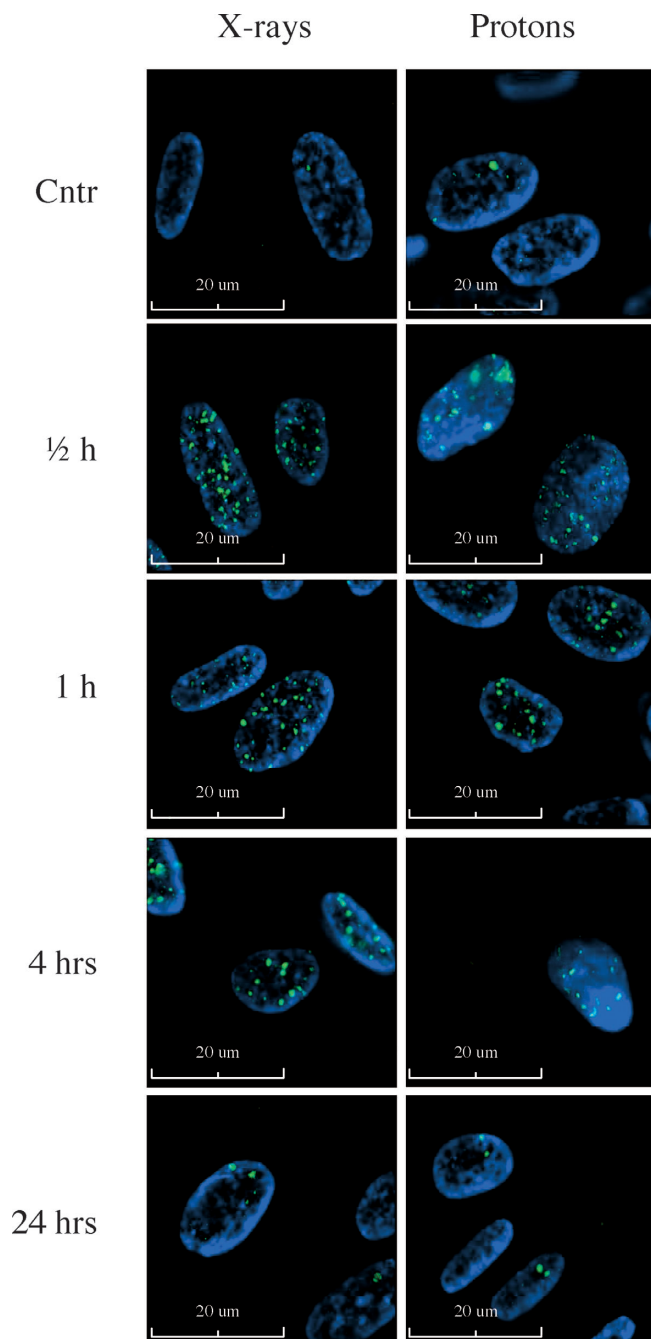


Fig. 6. An example of γ -H2AX foci induced by 1 Gy of either X-rays or 0,8 MeV protons and harvested at different times. γ -H2AX was stained with FITC whereas DNA was counterstained with DAPI.

tons are shown.

In Fig. 7A it is reported the number of γ -H2AX foci in cells exposed to 0.5–2 Gy X-rays or protons and harvested 30 min later. It should be noted that such values were significantly higher in protons than in X-ray-irradiated samples irrespective of the dose delivered. Figures. 7B–D show the

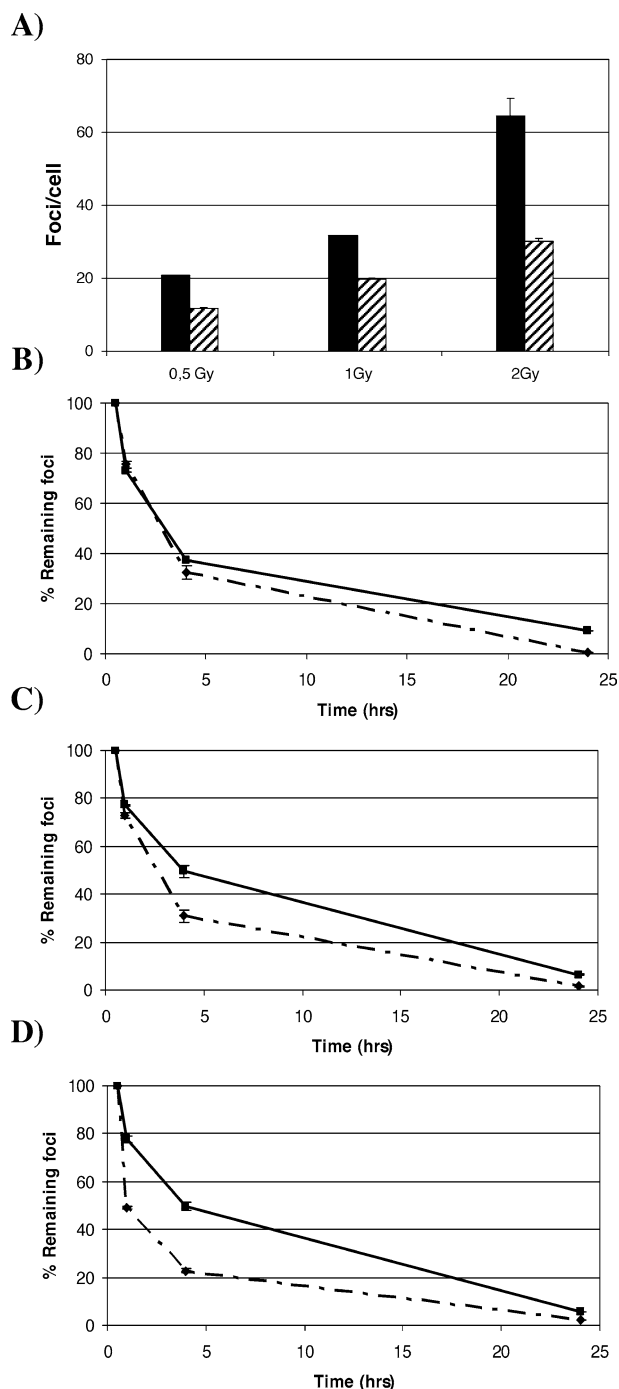


Fig. 7. Induction of γ -H2AX foci in HFFF2 human fibroblasts evaluated after 30 minutes from the exposure to 0, 5, 1 and 2 Gy of X-rays (black columns) and protons (striped columns) (A). Kinetics of loss of γ -H2AX foci after exposure to 0.5 (B), 1 (C) and 2 Gy (D) of X-rays (dotted line) or protons (solid line). The number of γ -H2AX foci was determined for each time point and, after subtracting background, the percentage foci remaining was plotted against time to obtain γ -H2AX foci loss. Analysis was carried out by counting by eye foci in at least 50 cells in two independent experiments. The error bar denotes one standard error of the mean.

kinetics of γ -H2AX foci loss in cells irradiated with graded doses of radiation and harvested for up to 24 hours expressed as % remaining foci. The number of γ -H2AX foci was determined for each time point and, after subtracting the background, the percentage foci remaining was plotted against time. Consistent with data obtained with other sources of high-LET radiations, the number of γ -H2AX foci induced shortly after treatment (30 minutes) by protons was significantly lower than for X-rays at each tested dose (A, B and C) (Student's t-test $p < 0.0001$). Foci were more persistent in proton-irradiated samples than in X-rays, this was particularly visible for the 4 hours harvesting point at both 1 and 2 Gy (B and C): around 49% of the initial number of foci persisted for protons versus 22–30% for X-rays. In addition, at 24 hours from protons, we found 1.1, 1.2, and 1.7 of residual foci per cell, at 0.5, 1 and 2 Gy, respectively, which correspond to 9.4, 6.4 and 5.7% of the number induced at 30 minutes. Such percentage, when scored in X-ray-treated cells, corresponded to 0.45, 1.4 and 2.2%, of those induced at 30 minutes.

The MN assay in cytochalasin-B arrested cells

The induction of micronuclei in binucleated-cells is reported in Fig. 8. As can be easily seen, 28.5 keV/ μ m protons were far more efficient than X-rays, at least in the range of 0.25–1.5 Gy. At the highest dose, the frequency of MN deeply declined for protons, whereas for X-rays, a linear trend was observed up to 2 Gy. Confirming what was already detected in Cl-1 cells⁷⁾ 28.5 keV/ μ m protons displayed a higher frequency of binucleated cells carrying more than 1

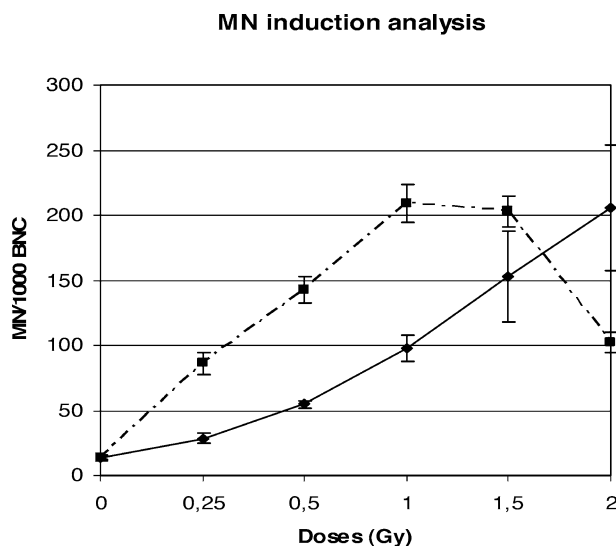


Fig. 8. Frequency of Micronuclei (MN) in cytokinesis-blocked binucleated cells (BNC) after exposure to graded doses of X-rays (dotted line) or protons (solid line). Five independent experiments have been carried out. The error bar denotes one standard error of the mean.

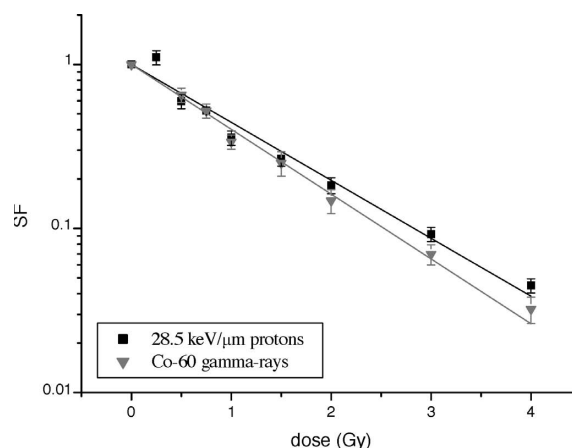


Fig. 9. Dose-response curves for cell inactivation in HFFF2 human fibroblasts after exposure to graded doses of γ -rays or protons. Each data point is the mean of 6 (proton) or 7 (gamma) independent experiments. The error bar denotes one standard error of the mean. The best-fitted linear curve parameters being: $\alpha = 0.91 \pm 0.03 \text{ Gy}^{-1}$, for gamma-rays; $\alpha = 0.81 \pm 0.02 \text{ Gy}^{-1}$, for protons.

MN (not shown).

Clonogenic assay

Dose-response curves for surviving fraction (SF) in HFFF2 cells after 28.5 keV/ μ m proton and ^{60}Co γ irradiations are reported in Fig. 9. Each data point is the mean of 6 (proton) or 7 (gamma) independent experiments. Error bars represent the standard error. In both cases, the experimental data show a linear relationship with the dose, confirming a behaviour previously found in the same laboratory by using a different fetal fibroblast cell line (HF19 cells).^{12,43)} The linear parameter, α , was determined from the best fit of the relationship $\text{SF}(D) = \exp(-\alpha D)$. The slope of the γ -ray curve results higher than that of the proton one.

DISCUSSION

We report here on cell cycle perturbations and genotoxic effects of low-energy (high-LET) protons in human primary skin fibroblasts. Though in the recent years various studies have been carried out on high-LET radiation-induced changes in the level of TP53 and CDKN1A regulator proteins and cell cycle effects of fibroblasts, it remains to fill a gap of knowledge for radiations in a LET range comparable to that of protons used in the present study. The relevance of cell cycle proteins in determining radiation response has been clarified in the last two decades. Transactivation of CDKN1A mediated by DNA-damage induced posttranslational modifications of TP53 is a well known mechanism responsible for the G_1 -phase arrest, through the inhibition of cyclin-dependent kinases 2, 4 and 6, which phosphorylate the RB protein.¹⁸⁾ Here, we have shown that, consistent with

an impaired transition at the G₁/S burden, TP53 was induced for both types of radiations, although time-course experiments indicated a delayed accumulation of the protein in proton- compared to X-ray-irradiated samples. Though only slight differences could be appreciated in the amount of TP53 comparing sparsely- to densely-ionizing radiations, our data are in agreement with those of Fournier *et al.*,⁴⁴ which shown a LET-dependence in TP53 accumulation in normal primary fibroblasts. Consistent with this finding, we detected an enhanced, delayed and persistent induction of CDKN1A in low-energy proton-treated cells compared to the ones exposed to X-rays, which was more pronounced than for TP53. Results obtained at single cell level, fit well with the immunoblot analysis, showing that CDKN1A nuclear accumulation peaked at 8 and 4 hours for protons and X-rays, respectively. In addition, a significant proportion of CDKN1A-positive nuclei was still present at 32 hours after irradiation to doses as low as 1 Gy of low-energy protons. Taking into account that for doses of 1 and 2 Gy the proportion of cells displaying CDKN1A staining, under the criteria of scoring adopted, is restricted to a maximum of 30% labeled cells, information gained at cellular level may serve as useful support to results obtained at whole cell population. Based on the cellular role of regulator proteins, one would have expected different cell cycle effects for X-rays or 28.5 keV/μm protons, particularly with respect to G₁/S transition. On the contrary irrespective of LET, we found that both types of radiations were similarly effective in causing a G₁/S block and consequently, a persistent depletion of S-phase cells particularly visible at 24–32 hours. Overall the finding seem to indicate that short term-transient modulation of TP53 and CDKN1A may be more representative of DNA damage accumulation rather than of cell cycle-effects.

Even if a LET-dependent G₂-block has been largely reported in the literature,^{20,21,45} and is in agreement with the present results, regarding the G₁ arrest in primary fibroblasts, controversial results are available in the literature for α-particles (100 keV/μm), showing either an extent of G₁ arrest lower²⁷ or comparable to that obtained by γ-irradiation.²⁸ In normal human diploid fibroblasts, cell cycle arrest can be triggered by one or a few unrepaired breaks, a condition easily achieved taking into account the number of DSBs/cell induced by 1 Gy of either X-rays or protons. The molecular pathway responsible for this type of arrest seems to be rather sensitive and could be activated and maintained as active for a long time postirradiation. In fact, even low doses of radiation have been shown to cause long-term withdrawal from cell cycle progression in a TP53-dependent manner.^{29,30} In this respect, the primary role of TP53-dependent G₀/G₁ arrest pathway seems to be related to the maintenance of genetic stability through elimination of damaged cells from the reproductively viable population by permanent arrest.³⁰ In fact, it has been shown that irradiated untransformed fibroblasts suffer an immediate cell cycle

arrest followed by a long-term irreversible block with signs of either G₁-like senescence,⁴⁶ G₀-like growth arrest⁴⁷ or premature differentiation and senescence.^{25,26,32,48–50} Fournier and coworkers²³ showed that at 6 days after 4 Gy X-ray irradiation about 50% of cells resumed BrdU-incorporation, whereas such value was less than 5% for 150 keV/μm carbon ions. In our experiments, about 17% of either X-ray- or proton-irradiated cells, entered S-phase as measured by BrdU incorporation at five days from irradiation. The G₁- to S-phase block was maintained up to 14 days in X-ray-irradiated cells (not shown). Based on these observations, one should take into account that in fetal primary fibroblasts long-term cell cycle effects may contribute to the extent of cell survival, whereas the cytotoxic effect caused by DNA damage remained to be better investigated. For this reason, beside cell cycle perturbation effects, we investigated short- and long-term genotoxic effects. First, we evaluated whether cellular differences in the capability to handle lesions characterized by different complexity may be reflected in the amount and kinetics of disappearance of γ-H2AX-induced foci. We found that high-LET radiations induce per Gy a lower number of foci compared with low-LET ones, a result consistent with recent data,³⁷ that may be due to the induction of multiple DSBs within radiation tracks. In addition, in the comparison of the yield of γ-H2AX foci as we observed after the two type of radiations, we cannot rule out the contribution of confounding factors as those recently showed by Kegel *et al.*⁵¹ and related to the specific support to grow cells (in our case, mylar foil for protons and glass cover slips for X-rays). In fact, the number of γ-H2AX foci per unit dose of X-rays was two-fold higher in cells irradiated on glass cover slips compared to cells attached to plastic, suggesting an overestimation of foci number due to secondary radiation.⁵¹

Recently, on the basis of the different kinetics of DSB repair as obtained by PFGE and γ-H2AX antibody, the γ-H2AX foci assay has been questioned,⁵² still representing, however, a useful tool in cases where no fine resolution in the repair kinetics is needed or in assessment of residual DSBs. In particular at 4 and 24 hours from exposure to 1 and 2 Gy of protons we still observed a fraction of remaining γ-H2AX foci, suggesting that a subclass of DNA lesions characterized by high complexity is left unrepaired up after high-LET irradiation. A longer persistence of γ-H2AX foci has been also reported in MRC-5 fibroblasts exposed to 39 keV/μm carbon ions compared to those induced by γ-rays.³⁷

Consistent with delayed DSB repair and the clastogenic effect of protons detected in Cl-1 Chinese hamster cells,⁷ we found a LET-dependence in the induction of MN in cytochalasin-arrested binucleated cells, with the effectiveness of 28.5 keV/μm protons at least two times greater than that observed in X-ray-treated samples. The MN assay discriminated between the two types of radiations at doses as low as 0.25 Gy. Conversely, at the highest dose, the frequency of

scored MN deeply decreased to values lower than X-rays, probably as an effect of a higher G₂-phase block elicited in proton damaged cells. This is supported by the observation that at 24 hours from proton exposure the mitotic index dropped to 0.6% (data not shown), whereas the fraction of G₂-phase arrested cells was higher than in untreated samples as shown by the cytofluorimetric analysis. These results confirm the findings that as a function of dose and radiation quality, heavily damaged cells do not divide and will be not scored for MN as recently reported by Wu *et al.*⁵³) In terms of cell inactivation, the γ -rays result more efficient than the protons paralleling the results obtained in the same laboratory using HF19 fetal lung human fibroblast cell lines.¹²) In contrast, the survival fractions of γ -irradiated-non fetal diploid fibroblasts were higher than those obtained irradiating them with α -particles²⁸) or 39 keV/ μ m carbon ions (F. Antonelli personal communication). We hypothesised that the lack of difference we observed between low- and high-LET radiations reflect a characteristic of irradiated fetal primary fibroblasts on cell cycle arrest which masks the LET-dependent effect on cell survival resulting from DNA damage. This cell-type effect is also supported by the observation that in cells other than fetal primary fibroblasts as rodent cell lines^{3,5,7}) or epithelial tumour cells¹²) the clonogenic assay showed a higher effectiveness for 28.5 keV/ μ m protons than for X-rays.

In conclusion, we found a LET-dependence in the extent of accumulation and kinetics of cell cycle regulator proteins TP53, and particularly CDKN1A, in human primary fibroblasts irradiated with either 28.5 keV/ μ m protons or sparsely ionizing radiations, whereas no differences were observed in the extent of G₁/S transition and depletion of the S-phase compartment up to 5 days after irradiation comparing the two types of radiation. Furthermore, a delayed kinetic of loss of γ -H2AX foci and a higher induction of MN was observed in proton-irradiated samples. In this respect, radiation-induced modulation in the accumulation of TP53 and CDKN1A seems to be more representative of DNA damage accumulation rather than of cell cycle-effects.

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