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Biology Contribution

High and Low LET Radiation Differentially Induce Normal Tissue Damage Signals

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Summary

Higher induction of apoptosis occurs in high linear energy transfer (LET)–irradiated cells than in low LET–irradiated cells. Similar induction of the profibrotic plasminogen activator inhibitor 1 gene occurs with high and low LET radiation. Phosphorylation of p53 at serine 315 is involved in plasminogen activator inhibitor 1 expression and is similar after high and low LET radiation. Phosphorylation of p53 at serine 37 is involved in apoptosis and is much higher after high LET irradiation than in low LET–irradiated cells. Normal tissue damage signals may be differentially affected

Purpose: Radiotherapy using high linear energy transfer (LET) radiation is aimed at efficiently killing tumor cells while minimizing dose (biological effective) to normal tissues to prevent toxicity. It is well established that high LET radiation results in lower cell survival per absorbed dose than low LET radiation. However, whether various mechanisms involved in the development of normal tissue damage may be regulated differentially is not known. Therefore the aim of this study was to investigate whether two actions related to normal tissue toxicity, p53-induced apoptosis and expression of the profibrotic gene PAI-1 (plasminogen activator inhibitor 1), are differentially induced by high and low LET radiation.

Methods and Materials: Cells were irradiated with high LET carbon ions or low LET photons. Cell survival assays were performed, profibrotic PAI-1 expression was monitored by quantitative polymerase chain reaction, and apoptosis was assayed by annexin V staining. Activation of p53 by phosphorylation at serine 315 and serine 37 was monitored by Western blotting. Transfections of plasmids expressing p53 mutated at serines 315 and 37 were used to test the requirement of these residues for apoptosis and expression of PAI-1.

Results: As expected, cell survival was lower and induction of apoptosis was higher in high -LET irradiated cells. Interestingly, induction of the profibrotic PAI-1 gene was similar with high and low LET radiation. In agreement with this finding, phosphorylation of p53 at serine 315 involved in PAI-1 expression was similar with high and low LET radiation, whereas phosphorylation of p53 at serine 37, involved in apoptosis induction, was much higher after high LET irradiation.

Conclusions: Our results indicate that diverse mechanisms involved in the development of normal tissue damage may be differentially affected by high and low LET radiation. This may have consequences for the development and manifestation of normal tissue damage.

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Conflict of interest: none.

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by high and low LET radiation.

Keywords: LET, Normal tissue damage, Apoptosis, PAI-1, p53

Introduction

The quality of life of cancer patients treated with radiotherapy may be severely hampered by radiation-induced side effects, which may even limit the treatment dose and thereby reduce tumor control probability (1–4). Because the risk of normal tissue complications depends on irradiated normal tissue volume and dose, developments in radiotherapy such as high linear energy transfer (LET) particle therapy are aimed at reducing the amount of normal tissue that is co-irradiated (5, 6). However, high LET particle therapy is more complex and expensive than conventional radiotherapy. Therefore treatment focuses on localized tumors in proximity to critical organs or tumors resistant to conventional treatments. Clinically, the use of such treatment is therefore currently limited to cancers such as uveal melanoma, pediatric tumors, skull base tumors, and head-and-neck tumors (6, 7). Given the limited number of applications so far, the risk of induction of late normal tissue damage and second malignancies with particle therapy cannot yet be predicted accurately (6–8).

In particle irradiations, both protons and heavy ions share an advantage in physical dose distribution, allowing a more conformal dose delivery to the tumor than irradiation with the conventionally used photons (6, 7). The density of ionization events, or LET, is higher for radiation with heavy ions than for photons and protons, giving heavy ions an additional potential biologic advantage because cell death increases with LET (5, 6). However, normal tissue effects are not exclusively caused by cell death but are caused by a combination of cell death and other processes such as cell differentiation, epithelial to mesenchymal transition, proliferation of specific cells, extracellular matrix deposition, and many other reactions (1–3, 9, 10). Large variations in the relative biologic effectiveness (RBE) of particles have been measured in different studies (11, 12). The observation that differences in RBE are endpoint specific and tissue specific (11, 12) suggests that high LET radiation may not only differ from low LET radiation in severity of cell death induction but also in the type of mechanisms it activates. Different organs respond to radiation by activating specific mechanisms, which results in tissue-specific manifestations of normal tissue toxicity (3, 13). Some organs, such as the lungs, can even show two distinct types of normal tissue toxicity after radiation—pneumonitis and fibrosis—that show different manifestations depending on irradiated dose and volume (4, 14). Fibrosis is induced by activation of cell signaling and cytokines, which regulate proteins inducing the tissue remodeling seen in fibrotic tissue (1–3, 9, 15, 16). One such protein, plasminogen activator inhibitor 1 (PAI-1), is the main inhibitor of the fibrinolytic system (10, 15). PAI-1 regulates the degradation of extracellular matrix components (10, 15). An increase of PAI-1 expression therefore disturbs the extracellular matrix balance, which results in fibrotic reactions as seen in medical conditions including radiation-induced fibrosis (10, 15). Because PAI-1 is regulated cooperatively by radiation [in a p53-dependent fashion (17)] and hypoxia (18) or transforming growth factor β (17, 19, 20), changes in radiation dose potentially lead to a particularly large deviation in PAI-1 expression in hypoxic or transforming growth factor β–rich areas. During radiotherapy, DNA is the primary target and specific DNA damage is required for cell killing (21, 22). It has been shown that the physical differences between high LET radiation and low LET radiation have a distinct impact on the induction of DNA damage, causing unambiguous differences in the properties of the damaged DNA (21, 23). Late radiation effects eventually leading to organ dysfunction involve signals such as those induced by the p53 pathway in response to DNA damage (9, 24). In response to genotoxic stress, the p53 protein is activated by phosphorylation at numerous sites, depending on the severity and type of DNA damage and on cell type (25, 26). In this way p53 phosphorylation patterns are generated that result in specific activation of the p53 protein that can consequently activate specific (albeit not exclusive) processes such as apoptotic cell death and induction of genes involved in tissue remodeling such as PAI-1 (10, 17, 27–29). However, those signals vary in type and severity according to the characteristics of the DNA damage (*e.g.*, the complexity of the DNA damage formed) (25, 26, 28). This introduces different responses to high and low LET radiation (7), potentially resulting in a different development and manifestation of normal tissue complications.

The aim of this study was therefore to investigate whether a difference in the induction of two completely different processes related to normal tissue damage, p53-induced apoptosis and p53-regulated expression of the profibrotic PAI-1 gene (2, 3, 9, 17, 20, 29), can be observed after high and low LET irradiation. We show that high and low LET radiation differentially induces these two actions in an unparalleled way. This finding indicates that the profile of biologic mechanisms contributing to normal tissue damage induced by high and low LET radiation may differ, thus potentially leading to different manifestations and development of normal tissue damage.

Methods and Materials

Cell culture and irradiation

Human alveolar basal epithelial cells (A549) and human embryonic kidney cells (HEK293) were maintained in DMEM Dulbecco's Modified Eagle Medium (DMEM) (complete medium) supplemented with 10% fetal bovine serum and irradiated as described previously (30) with (high LET) 90 mega-electronvolt per nucleon (MeV/u) carbon ions in a 1.3-mm-long spread-out Bragg peak with a diameter of 30 mm, allowing accurate irradiation of the cells. At the position of the sample, the track-averaged LET was 132 ± 10 keV/μm whereas the dose-averaged LET was 189 ± 15 keV/μm. Experiments were performed with a dose rate between 3 and 5 Gy/min (30). If this resulted in irradiation times shorter than 10 seconds, the dose rate was reduced by a factor of 10 by reducing the beam current injected into the cyclotron (30). We irradiated (low LET) photon-irradiated samples with a ^{137}Cs source (IBL637 γ-radiation machine, CIS Biointernational, Gif-Sur-Yvette, France) with a dose rate of 0.636 Gy/min. Survival assays were performed as described previously (30). Apoptosis was monitored with a microscope or a fluorescence-activated cell sorter after the cells were stained by use of the Annexin V–Cy3 Apoptosis Kit (BioVision, Mountain View, CA) according to the manufacturer's protocol.

Plasmids and transfection

pcDNA−p53 (wild type) has been described previously (20). Plasmids expressing mutated p53 were constructed by site-directed mutagenesis with pcDNA p53 (wild type) as a template by use of the following primers: 5′-ctggccccttgccgtcccaagcaatg and 5′-caaggggccagaacgttgttttcaggaag for p53−S37A and 5′-gctccgctccccagccaaagaagaaaccac and 5′-ctggggagcggagctggtg ttgttgggc for p53−S315A. For the apoptosis assay, the pEGFP-N2 plasmid (Clontech, Mountain View, CA) was transfected in HEK293 cells in a ratio of 1:1, in combination with pcDNA3.1+ (Invitrogen, Paisley, Scotland) as empty vector or one of the p53 expression plasmids described earlier, with Lipofectamine (Invitrogen) according to the manufacturer's protocol. The luciferase assays were performed essentially as described previously (20); however, we used 0.5 μg of reporter plasmid and only 5 ng of (p53) expression plasmids per sample (this very low concentration is sufficient to induce the PAI-1−luc plasmid but too low to induce apoptosis in 24 h).

Western blotting and quantitative polymerase chain reaction

Western blotting was performed as described previously (17), and the primary antibodies were as follows: γ-tubulin (Sigma-Aldrich, St Louis, MO); (total) p53 DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA); and Phospho-p53−Ser20, Phospho-p53−Ser37, Phospho-p53−Ser315, and Phospho-p53−Ser392 (Cell Signaling Technology, Danvers, MA). Induction of p53 phosphorylation was determined by quantifying bands on Western blots with the appropriate antibody by use of ImageJ software (National Institutes of Health, Bethesda, MD) and normalizing the bands for γ-tubulin. Error bars in graphs are the standard error of the mean of quantification of three separate Western blots, with different protein samples. Quantitative polymerase chain reaction was performed by use of glyceraldehyde 3-phosphate dehydrogenase for normalization as described previously (20).

Results

Cell survival versus induction of profibrotic PAI-1

To investigate whether a difference in induction of two completely different biologic processes related to normal tissue damage can be observed after high and low LET irradiation, we compared cell survival and expression of the profibrotic PAI-1 gene in A549 lung epithelial cells (Fig. 1A) and HEK293 embryonic kidney cells (Fig. 1C). As expected, in a clonogenic survival assay, at all levels of cell survival, higher doses of low LET radiation were needed to induce the same effect (Figs. 1A and 1C). The observed RBE was about 3.4 for A549 cells and 2.3 in HEK293 cells for a level of 10% survival (Figs. 1A and 1C, respectively). However, the increase in expression of PAI-1 messenger RNA was virtually identical after irradiation with high or low LET radiation (Figs. 1B and 1D). Our results show an endpoint-specific difference in response to high and low LET irradiation of cell survival and PAI-1 induction. HEK293 cells showed the clearest response and are most suitable for subsequent experiments such as transfections. Therefore, from here on, the experiments were performed with this cell type only.

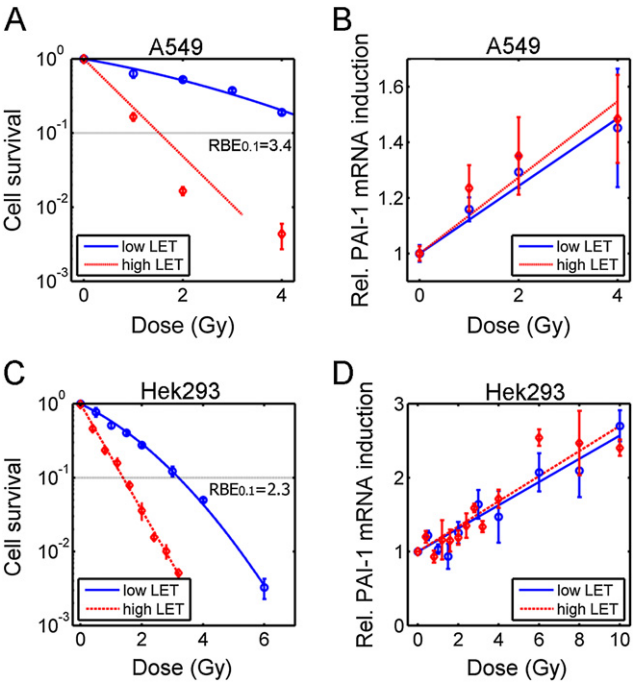

Fig. 1. Cell survival is lower for high linear energy transfer (LET) radiation than for low LET radiation, whereas plasminogen activator inhibitor 1 (PAI-1) expression is similar. (A) Colony-forming (survival) assay of A549 cells, irradiated with indicated doses of photons (cesium source [blue]) or with carbon ions (red). Cell cultures and treatments were performed identically and simultaneously in each experiment. (B) Quantitative polymerase chain reaction (qPCR) quantification of PAI-1 messenger RNA (mRNA) levels in A549 cells 24 h after irradiation with high (red) and low (blue) LET radiation. (C) Colony-forming (survival) assay of HEK293 cells, irradiated with indicated doses of photons (cesium source [blue]) or with carbon ions (red). Each data point is the mean of at least three individual experiments with similar results. Cell cultures and treatments were performed identically and simultaneously in each experiment. (D) qPCR quantification of PAI-1 mRNA levels in HEK293 cells 24 h after irradiation with high (red) and low (blue) LET radiation. Error bars represent standard error of the mean. RBE = relative biologic effectiveness.

High and low LET radiation induces different p53 activation

Interestingly, both apoptotic cell death (28, 29) and expression of PAI-1 (17, 20) are regulated by p53. To find a possible explanation for their differential regulation, we compared phosphorylation patterns of key p53 amino acid residues after high and low LET irradiation (Fig. 2). The p53 residues serine 315 and serine 37 were induced by radiation in HEK293 (Figs. 2A−2D), whereas the other residues did not show a quantifiable increase in phosphorylation (Figs. 2A and 2B). Dose response functions for high and low LET radiation were obtained. An increase in phosphorylation of p53−Ser315 was observed for both high and low LET radiation. At the same physical dose, the observed patterns were very similar for both types of radiation (Figs. 2A−2D), especially 24 h after radiation (Fig. 2D). On the other hand, a large difference between high and low LET irradiation was observed for p53−Ser37 (Figs. 2A, 2B, 2E, and 2F). For high LET radiation,

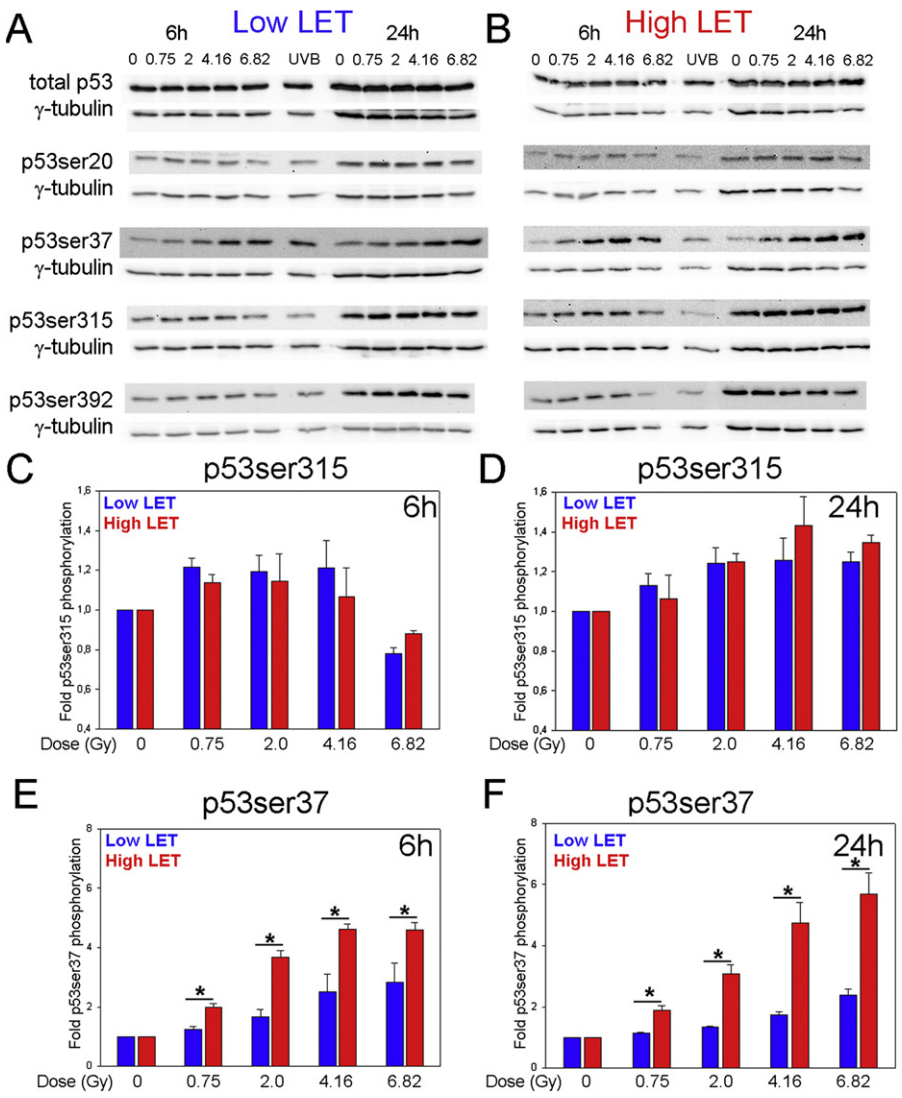

Fig. 2. p53 phosphorylation patterns after high linear energy transfer (LET), low LET, and UV-B radiation. Typical Western blot results of phosphorylation patterns of important p53−serine residues (serine 20, 37, 315, and 392) 6 or 24 h after low LET (photon) radiation (A) or high LET (carbon ion) radiation (B). UV-B radiation was used as internal control (6 h only) in A and B. Quantification of p53−Ser315 phosphorylation 6 h (C) and 24 h (D) after low or high LET radiation, showing high similarity of p53−Ser315 phosphorylation between low and high LET radiation. Quantification of p53−Ser37 phosphorylation 6 h (E) and 24 h (F) after low or high LET radiation, showing higher p53−Ser37 phosphorylation after high LET radiation. Error bars represent standard error. Asterisks, $p < 0.05$ by two-tailed t test.

phosphorylation was much higher than for low LET radiation at both 6 h (Figs. 2A and 2E) and 24 h (Figs. 2B and 2F) after radiation. Thus the activation of p53−Ser315 phosphorylation—like that of the increase in expression of PAI-1—is similar for the same physical dose of high and low LET radiation, whereas p53−Ser37 phosphorylation—like the induction of cell death—is much higher with high LET radiation.

Signals induced by high LET radiation induce relatively more apoptosis than expression of PAI-1

Because p53 is involved in the regulation of apoptotic cell death (26, 28, 29), we investigated whether there is a difference in apoptosis induction by high or low LET radiation. Indeed, consistent with the data for cell survival, the increase in the number of apoptotic cells was significantly higher in high LET−irradiated cells after both 4 and 8 Gy (Fig. 3). This finding indicates that apoptosis may be responsible (at least in part) for the difference in cell survival between high and low LET radiation.

To test whether the p53 phosphorylations monitored after irradiation with high and low LET radiation can differentially affect different processes, we tested whether plasmids expressing p53 mutants for specific phosphorylation sites differentially induce apoptosis (Figs. 4A and 4B) and PAI-1 expression (Fig. 4C). To identify cells expressing the p53 variants in an apoptosis assay, we co-transfected a Green Fluorescent Protein (GFP)-expressing plasmid together with an empty vector, a plasmid expressing wild-type p53, or a plasmid expressing p53 mutants deficient for phosphorylation at p53−serine 315 (p53−S315A) or p53−serine 37 (p53−S37A). Subsequently, the cells were stained with Cy3-labeled annexin V to identify apoptotic cells. After co-transfection with empty vector, green

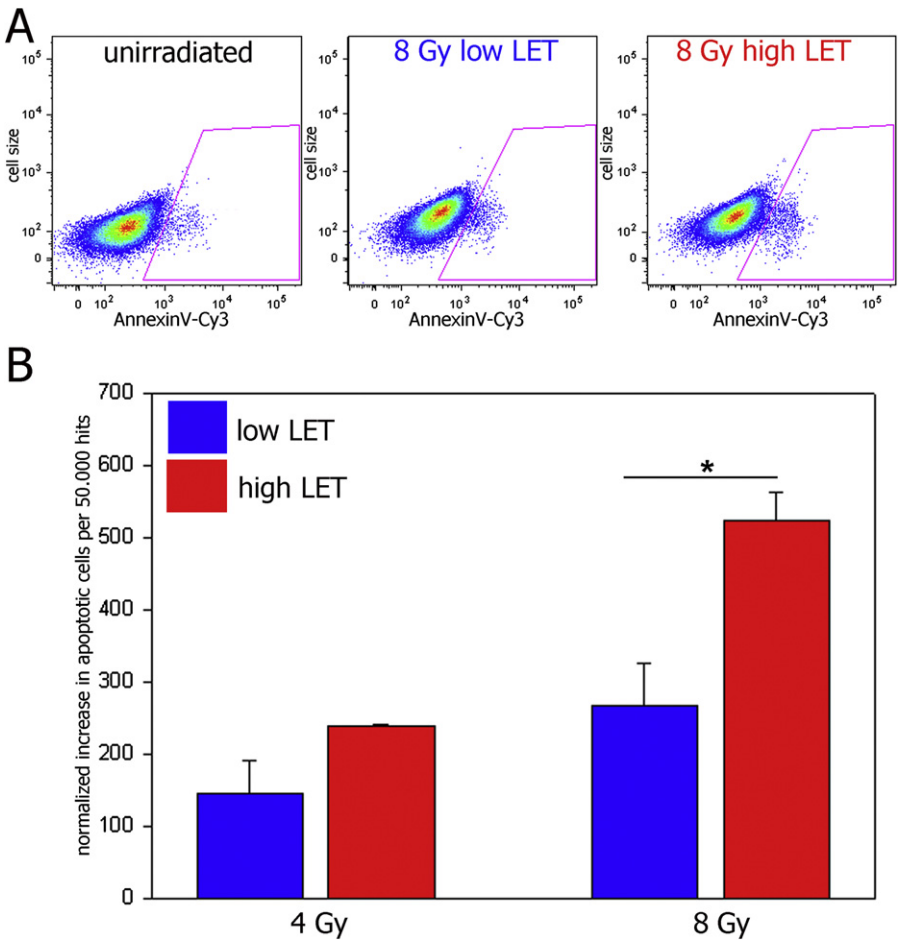

Fig. 3. High linear energy transfer (LET) radiation induces more apoptosis than low LET radiation. HEK293 cells were mock treated or irradiated with 4- or 8-Gy carbon ions or photons. Apoptosis was assayed by fluorescence-activated cell sorter (FACS) after staining with annexin V−Cy3 24 h after irradiation. (A) Representative FACS plots for unirradiated cells (left), cells irradiated with 8-Gy photons (middle), and cells irradiated with 8-Gy carbon ions (right). Apoptotic cells, showing a high Cy3 signal, were gated (pink area). (B) The increase in apoptotic cells per 50,000 hits (cells) normalized for experimental background levels (average unirradiated cells) is shown after radiation with 4- or 8-Gy photons (low LET [blue]) or carbon ions (high LET [red]). Error bars represent standard error. Asterisk, $p < 0.05$ by two-tailed t test.

cells appeared normal in morphology and showed minimal apoptotic cell death as judged by the (red) annexin V−Cy3 staining (Fig. 4A). However, green wild-type p53−co-transfected cells showed a much higher rate of apoptosis 24 h after transfection (Fig. 4A), showing that when p53 is overexpressed in HEK293 cells with the used concentrations, the amount of active p53 is sufficient to induce apoptosis and radiation is not required to further activate p53 in order to observe apoptotic cells. Green cells co-transfected with p53−S37A, however, showed only few apoptotic cells 24 h after transfection. Quantification shows that when the p53 phosphorylation site most affected by high LET radiation is mutated, p53-induced apoptosis is reduced by more than 75% in this experiment (Figs. 4A and 4B). In contrast, mutation of serine 315 did not reduce the capacity of p53 to induce apoptosis because annexin V staining was similar for wild-type p53− and p53−S315A−transfected cells (Figs. 4A and 4B). However, in contrast to the effect seen for apoptosis, the p53 phosphorylation site serine 37 appeared not to be involved in PAI-1 induction because mutation of this residue did not reduce the capacity of p53 to induce a PAI-1 luciferase reporter construct. Conversely, after mutation of p53−serine 315, a significant reduction in PAI-1 luciferase activity was observed (Fig. 4C). This finding indicates that p53−serine 315 is involved in expression of PAI-1 rather than in apoptosis.

Together, these findings show that signals that are regulated at least partially via the same key regulator (p53 protein) of radiation-induced biologic processes can be differentially induced by high and low LET radiation.

Discussion

To be able to optimally exploit the benefits of irradiation of patients with heavy ions such as carbon ions, it is essential to adequately predict the effect of the irradiation on the treatment outcome in comparison to the conventionally used photons. This should take into account the difference in the response of tumors but also of normal tissues. In cell survival experiments similar to ours, carbon ions were found to be more effective in killing cells in culture than photons (7, 31), which is consistent with our results. However, we also show in two cell lines that induction of profibrotic PAI-1 is the same after high and low LET irradiation. In line

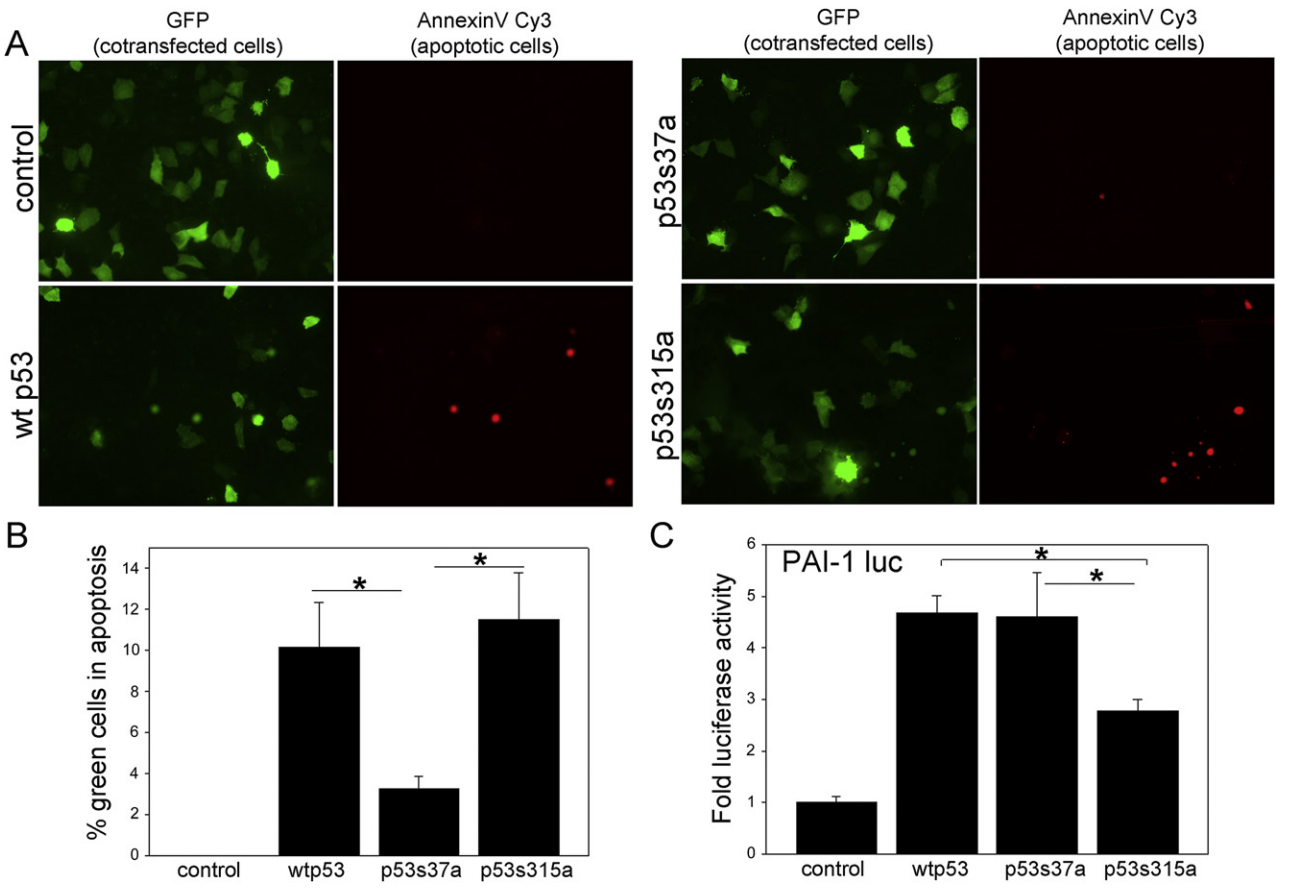

Fig. 4. Mutation of p53−Ser37 reduces apoptotic potential of p53, whereas mutation of p53−Ser315 reduces plasminogen activator inhibitor 1 (PAI-1) activation. (A) Microscopic pictures of apoptotic cells (annexin V−Cy3 [red]) after co-transfection of GFP (green) with empty vector (control) or wild-type (wt) p53, p53−S37A, or p53−S315A plasmids. (B) Quantification of apoptosis after co-transfection of GFP with empty vector (control) or wild-type (wt) p53, p53−S37A, or p53−S315A plasmids. (C) Induction of a PAI-1 luciferase reporter construct by empty vector (control) or wild-type (wt) p53, p53−S37A, or p53−S315A constructs., AnnexinV-Cy3 staining: original magnification 400× Error bars represent standard error. Asterisks, $p < 0.05$ by two-tailed t test.

with the observation on cell survival, we show that high LET radiation induces more apoptosis than low LET radiation. p53−serine 37 was phosphorylated more extensively by high LET radiation, and our observation that mutation of this residue reduces the capacity of p53 to induce apoptosis in HEK293 cells suggests its importance for apoptosis. On the other hand, p53−serine 315 phosphorylation, which was induced to a similar extent by high and low LET radiation, evidently plays a more important role in PAI-1 expression than in apoptosis because mutation of p53−Ser315 reduces the capacity of p53 to induce expression of the profibrotic PAI-1 gene (a schematic overview of these results is shown in Fig. 5).

Parenchymal cells, endothelial cells, and fibroblasts all play a prominent but specific role in the induction of normal tissue damage after irradiation, and signaling between these cell types, as well as the activation of an inflammatory response in response to the radiation-induced changes in these cells, is the trigger for induction of normal tissue damage (1−3). A difference in the initial reaction to radiation in any one of these cell types could already have an impact on manifestations of normal tissue damage because the interaction with other cell types could also change. We used lung epithelial and embryonic kidney cells to show proof of principle that differential activation of mechanisms involved in normal tissue damage can be induced by high and low LET radiation.

Because high and/or low LET radiation induces many responses in addition to expression of PAI-1 and p53-induced apoptosis, this study urges investigations of other mechanisms that

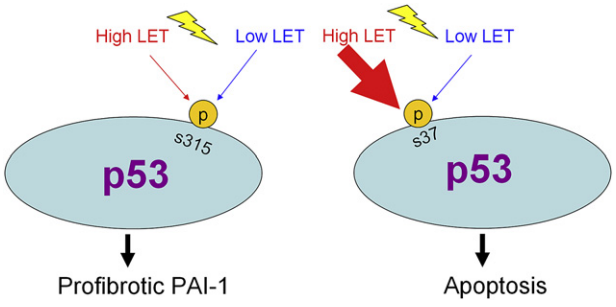

Fig. 5. Schematic overview. High linear energy transfer (LET) radiation has a stronger impact on cell survival and apoptosis than low LET radiation, whereas high LET radiation and low LET radiation similarly induce the profibrotic plasminogen activator inhibitor 1 (PAI-1) gene. p53−serine 37 is phosphorylated more extensively by high LET radiation, and this residue is more important for cell death than for expression of PAI-1. On the other hand, p53−serine 315 phosphorylation is induced similarly by high and low LET radiation, and this residue is more involved in PAI-1 expression than in cell death.

might be induced differentially, for example, p53-independent apoptosis (32, 33) as induced by high LET radiation through caspases 3 and 9 and Chk2 (34). These and other processes, in concert with p53-dependent processes such as those described in this study, could illuminate differential normal tissue responses to and between high and low LET radiation.

Our study implies that the comprehensive effect of high LET radiation on normal tissue cannot be determined by the measurement of one single mechanism. These findings may have implications for the development of new high LET radiation treatments.

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