Biochemical and Biophysical Research Communications 423 (2012) 654–660

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Depression of *p53*-independent Akt survival signals in human oral cancer cells bearing mutated *p53* gene after exposure to high-LET radiation

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ARTICLE INFO

Article history:
Received 26 May 2012
Available online 10 June 2012

Keywords:
High LET
Akt
Caspase
Apoptosis
Cell cycle

ABSTRACT

Although mutations and deletions in the *p53* tumor suppressor gene lead to resistance to low linear energy transfer (LET) radiation, high-LET radiation efficiently induces cell lethality and apoptosis regardless of the *p53* gene status in cancer cells. Recently, it has been suggested that the induction of *p53*-independent apoptosis takes place through the activation of Caspase-9 which results in the cleavage of Caspase-3 and poly (ADP-ribose) polymerase (PARP). This study was designed to examine if high-LET radiation depresses serine/threonine protein kinase B (PKB, also known as Akt) and Akt-related proteins. Human gingival cancer cells (Ca9–22 cells) harboring a mutated *p53* (m*p53*) gene were irradiated with 2 Gy of X-rays or Fe-ion beams. The cellular contents of Akt-related proteins participating in cell survival signaling were analyzed with Western Blotting 1, 2, 3 and 6 h after irradiation. Cell cycle distributions after irradiation were assayed with flow cytometric analysis. Akt-related protein levels decreased when cells were irradiated with high-LET radiation. High-LET radiation increased G_2/M phase arrests and suppressed the progression of the cell cycle much more efficiently when compared to low-LET radiation. These results suggest that high-LET radiation enhances apoptosis through the activation of Caspase-3 and Caspase-9, and suppresses cell growth by suppressing Akt-related signaling, even in m*p53* bearing cancer cells.

1. Introduction

Oral cavity squamous cell carcinoma (OSCC) therapy utilizes surgical treatment, radiotherapy, chemotherapy, and hyperthermia. For advanced OSCC cases, combined therapies using these modalities are required. Many mutations have been reported in the *p53* tumor suppressor gene in advanced human cancers [1]. The *p53* gene plays an important role in the pathway which controls apoptosis, cell growth, and cell proliferation [2–4]. Consequently, mutations or deletions in the *p53* gene can lead to resistance to cancer therapies such as radiotherapy and chemotherapy [5]. The involvement of the *p53* gene in the sensitivity of many cell types toward low-LET radiation is well established [5,6]. High-LET radiation has several advantages in radiotherapy when compared to low-LET radiation. These include a higher relative biological effectiveness (RBE), a reduction in the oxygen enhancement ratio, less variation in cell cycle-related radiosensitivity, and the existence of less efficient repair mechanisms for cellular radiation injuries [7–9]. For these reasons, high-LET radiation induces highly lethal events, even in radio-resistant tumors [10]. Moreover, high-LET radiation possesses excellent dose distribution properties which are an advantage when compared to low-LET radiation. High-LET radiation can produce a high dose in a deep and narrow region when compared to the shallow and broad high dose region produced by low-LET radiation [7]. This means that heavy ion beams can severely damage a tumor, and simultaneously produce fewer deleterious effects, and lead to a reduction of damage to the surrounding normal tissues. This means that heavy ion beams can deliver a high dose to a tumor while minimizing the amount of radiation delivered to surrounding normal tissues. This property may permit the design of more effective therapeutic strategies when based on the genetic and molecular events involved in cell death. Caspases function as components in cell

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http://dx.doi.org/10.1016/j.bbrc.2012.06.004

signaling events such as apoptosis, cell growth and differentiation [11]. While Caspases serve as the main effectors of apoptosis, the mechanisms involved in the activation of the Caspase system after exposure to high-LET radiation (which are independent of cellular *p53* gene status) are still not well known. Recently, it was reported that the induction of *p53*-independent apoptosis was induced through the activation of Caspase-9 which results in the cleavage of Caspase-3 and poly (ADP-ribose) polymerase (PARP) [12–14]. The present study was designed to examine the depression of survival signaling such as Akt and the associated downstream proteins which are known to inhibit Caspase-9 and promote cell growth.

2. Materials and methods

2.1. Cells

The cell line used in this study is a human squamous cell carcinoma gingival-derived cell line (Ca9–22) bearing a point mutation at codon 248 [Arg (CGG) → Trp (TGG)] in the *p53* gene. Ca9–22 cells were obtained from the Japanese Collection of Research Bioresources (Health Science Research Resources Bank, Osaka, Japan). The cells were cultured at 37 °C in a humidified 5% CO_2 incubator, and were grown in Dulbecco's modified Eagle's medium containing 10%(v/v) fetal bovine serum, penicillin (50 U/mL), streptomycin (50 μg/mL) and kanamycin (50 μg/mL).

2.2. Irradiation

Cells were grown exponentially in 25 cm^2 flasks (Nunc Roskilde, Denmark) before irradiation. The cells were irradiated with X-rays or Fe-ion beams. For X-irradiation (1.7 keV/μm), a 200 kVp X-ray generator (PANTAK-320S, Shimadzu, Kyoto, Japan) was used with total filtration of 0.5 mm aluminum plus 0.5 mm copper. The X-ray dose rate was measured with a thimble ionization chamber (PTW Freiburg, Freiburg, Germany) at the sample position, and the dose rate was about 1 Gy/min. Fe-ion beams (500 MeV/u, 200 keV/μm) were generated by a heavy ion medical accelerator at the National Institute of Radiological Sciences in Chiba, Japan. The flasks were placed in a special rack designed to position them to obtain a uniform exposure to the beams. The beams across each sample were uniform and varied by less than ±5% from the intensity at the center of the flask. Irradiation was conducted using horizontal heavy-ion beams with a dose rate of approximately 3 Gy/min, and no binary filter. Kanai et al [15] measured exposures under these conditions using a calibrated parallel plate ionization chamber and/or a plastic scintillation counter at the sample position.

2.3. Cell survival assay

Cell survival was measured using a standard colony forming assay as previously describe in detail [14]. Three flasks were used for each experiment, and two or more independent experiments were repeated for each survival point. Microscopic colonies containing approximately 50 cells were scored as having grown from single surviving cells.

2.4. Analysis of apoptosis

After irradiation, attached and floating cells were stained with 0.2 mM Hoechst 33342 (Sigma Chemical Co. St. Louis, MO, USA). Apoptotic events were scored as previously described in detail [14]. A minimum of 300 cells was counted in every sample, and the percentage of apoptotic cells was recorded.

2.5. Analysis of Caspase-3 activity

Cells were incubated for 47 h at 37 °C after irradiation. The cells were then incubated with fluorescein Caspase-3 (Millipore Co. Billerica, MA, USA) for 1 h at 37 °C, and were illuminated under a yellow light. Measurements were performed according to the manufacturer's protocol. The cell suspension was mixed with a propidium iodide (PI) solution for a final concentration of 1.25 μg/mL. After filtration through a 35 μm nylon mesh, the samples were measured for fluorescein using a flow cytometer (Becton-Dickinson, San Jose, CA, USA). Data were then analyzed from viable Caspase positive cells.

2.6. Caspase inhibitors

A Caspase-3 inhibitor (z-DEVD-FMK) and a Caspase-8 inhibitor (z-IETD-FMK) were purchased from Kamiya Biomedical Co., (Seattle, WA, USA). A Caspase-9 inhibitor (z-LEHD-FMK) was purchased from R&D systems (Abingdon, UK). Cells were pre-incubated for 2 h in different concentrations of Caspase inhibitors before irradiation.

2.7. Western Blotting

After irradiation, cells were harvested at the indicated time points. Western Blotting was carried out as previously described in detail [14].The membranes were then incubated for 1 h at room temperature with primary antibodies. Rabbit polyclonal anti-mTOR, anti-phospho-mTOR (Ser2448), anti-Akt, anti-phospho-Akt (Ser473), rabbit monoclonal anti-S6 Ribosomal Protein (rpS6), and anti-phospho-rpS6 (Ser240/244) were purchased from Cell Signaling Technology, Inc., (Beverly, MA, USA). Goat polyclonal anti-Actin antibody (I-19) and mouse monoclonal anti-Survivin were obtained from Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA). Mouse monoclonal anti-cyclin D1 antibody (Ab-3) was obtained from Calbiochem Inc., (San Diego, CA, USA). Appropriate horseradish peroxidase-conjugated secondary antibodies of anti-rabbit IgG antibody (Amersham Pharmacia Biotech Inc. Piscataway, NJ, USA) or anti-mouse IgG antibody (Zymed laboratories Inc. San Francisco, CA, USA) were incubated for 1 h at room temperature. For visualization of bands, an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech Inc.) was used according to the manufacture's protocol. The amounts of the proteins in the samples were determined with scanning profiles using the Scion imaging program (Scion Co. Frederick, MD, USA).

2.8. Cell cycle analysis

After irradiation, attached and floating cells were fixed with cold 70% methanol and stored at 4 °C for 3 days before analysis. For cell cycle analysis, the cells were incubated for 30 min at room temperature with 1 mg/mL RNase and 50 μg/mL propidium iodoide (PI), and were analyzed using a flow cytometer (Becton–Dickinson). The cell cycle distribution was assayed by determining the DNA content twice and calculating the average.

3. Results

3.1. Surviving fraction after irradiation

To investigate radiation sensitivity to X-rays and Fe-ion beams, Ca9–22 cells bearing m*p53* were exposed to both types of beams. The surviving fraction after exposure to 2 Gy of X-rays, 7 Gy of X-rays, and 2 Gy of Fe-ions beams were 58%, 8%, and 9% when compared with unirradiated controls (Fig. 1A). The D_{10} doses were

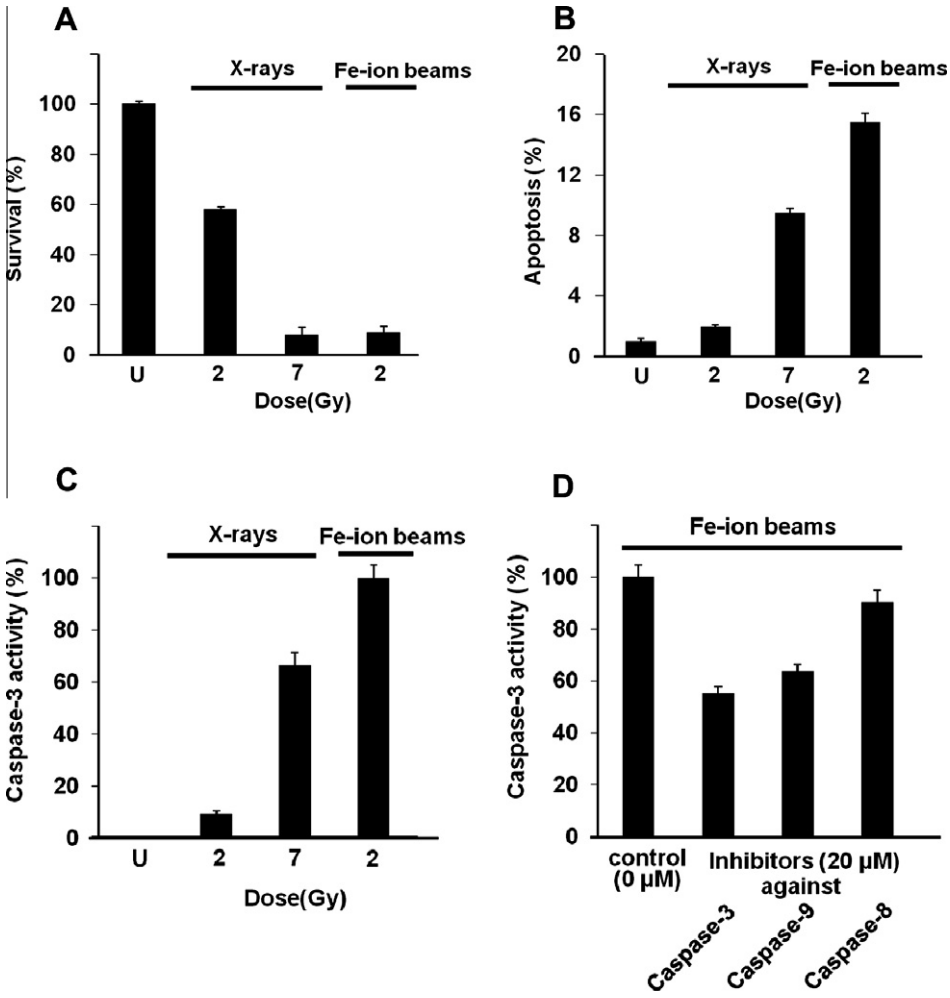

Fig. 1. Sensitivity to X-rays or Fe-ion beams. (A) Surviving rate; (B) Apoptosis frequency. (C and D) Caspase-3 activity after exposure to X-rays or Fe-beams. Caspase-3 activity was analyzed at 48 h after irradiation in the absence C or presence D of different Caspase inhibitors which act against Caspase-3, Caspase-9, and Caspase-8 at a concentration of 20 μM. U, non-irradiated cells in A, B and C.

about 2 Gy for the Fe-ion beams and 7 Gy for X-rays. The RBE value for the D_{10} dose of the Fe-ion beams was approximately 3.5.

3.2. Apoptosis after irradiation

Using Hoechst33342 staining, the kinetics of apoptosis induction was analyzed at 48 h after exposure to X-rays or Fe-ion beams. The apoptosis frequencies at equal survival doses (D_{10}) were 10% for X-rays (7 Gy), and about 16% for Fe-ion beams (2 Gy). Furthermore, the apoptosis frequency after 2 Gy of X-rays was approximately 2%. Thus, Fe-ion beams were eight times as effective as X-rays in inducing apoptosis at an equal dose (Fig. 1B).

3.3. Caspase-3 activity

The activity of Caspase-3 increased in viable cells at 48 h after irradiation (Fig. 1C). The activities of Caspase-3 after a 2 Gy and 7 Gy X-irradiation were about 10% and 70% of that after a 2 Gy exposure to Fe-ion beams. To examine how Caspase-3 was activated, inhibitors of Caspase-3, Caspase-9 and Caspase-8 were used (Fig. 1D). Cells were pre-incubated for 2 h before irradiation with a Caspase inhibitor. The activity of Caspase-3 was observed at 48 h after irradiation with Fe-ion beams in the presence of inhibitors for Caspase-3, Caspase-9 and Caspase-8 (each at a concentration of 20 μM). Caspase-3, Caspase-9 and Caspase-8 inhibitors suppressed the activities of Caspase-3–55%, 63% and 90% compared to cells grown in the absence of inhibitors, respectively (Fig. 1D). The Caspase-9 inhibitor suppressed Caspase-3 activation by Fe-ion beams to a greater extent than the Caspase-8 inhibitor.

3.4. Cellular content of Akt and related proteins after irradiation

The levels of Akt protein decreased to a larger extent after exposure to Fe-ion beams than after exposure to X-rays (Fig. 2A, B). Though Akt levels were reduced to about 75% of that in unirradiated cells at 3 h after X-irradiation, Fe-ion beams caused a reduction of about 45%. Furthermore, Fe-ion beams apparently reduced p-Akt more than X-rays (Fig. 2A, C). Moreover, Akt-related proteins were effectively depressed by Fe-ion beams. X-rays reduced the cellular contents of the mTOR protein, phospho-mTOR, rpS6, phospho-rpS6 and Survivin to 53%, 81%, 78%, 68% and 78%, respectively, of the value in unirradiated cells at 3 h after irradiation (Fig. 2D, E). Fe-ion beams induced a 44% reduction of the mTOR protein, 72% of phospho-mTOR, 49% of rpS6, 82% of phospho-rpS6 and 28% of Survivin when compared with X-rays at 3 h after irradiation. At 8 h after irradiation, cyclin D1 levels were apparently depressed in the cases of the irradiated cells with X-rays at 7 Gy and Fe-ion beams at 2 Gy (Fig. 3A), and their relative values were 49% and 37%, respectively, though the value in the irradiated with 2 Gy of X-rays was 80% (Fig. 3B).

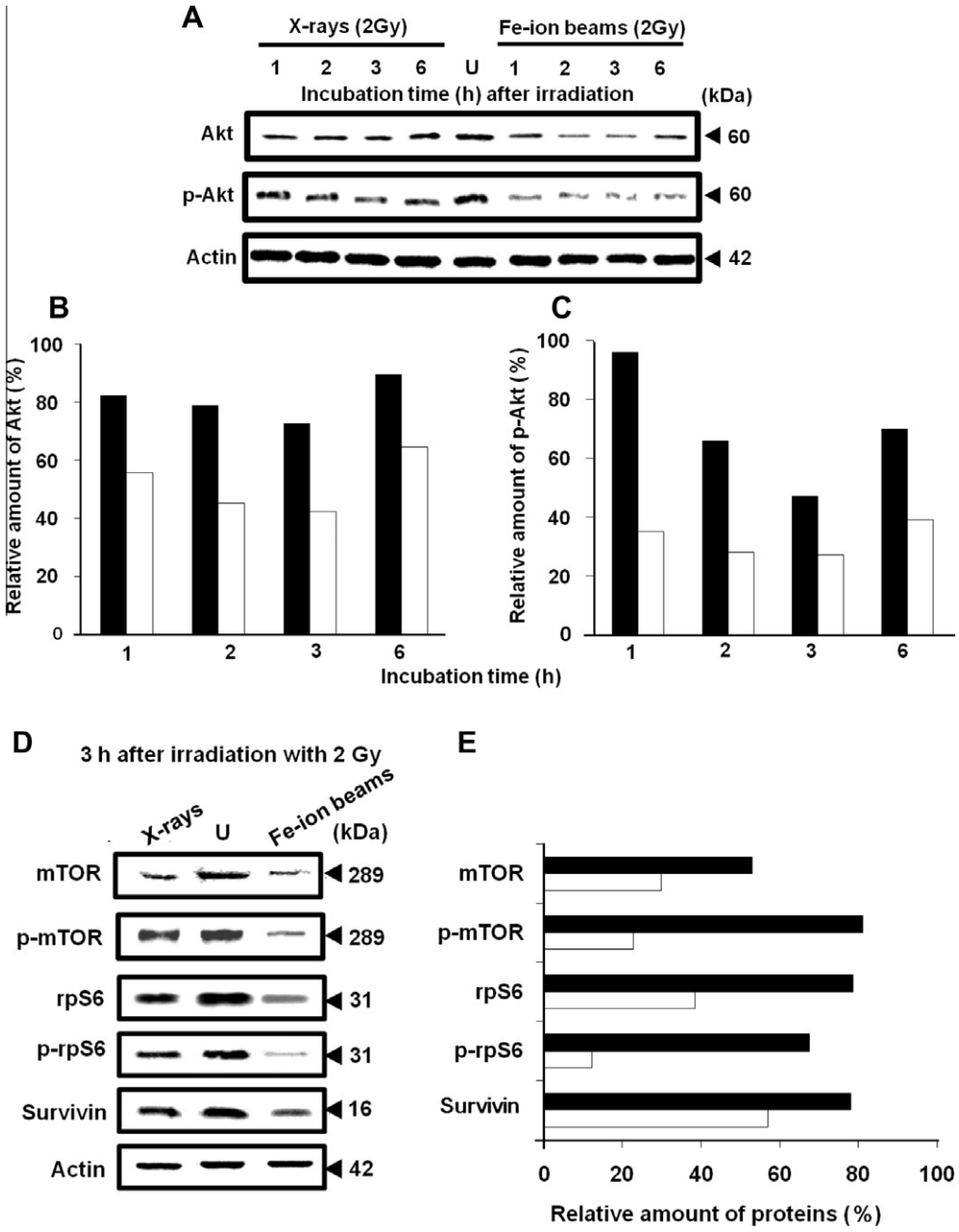

Fig. 2. Western Blotting of Akt and downstream proteins. (A) Time course for levels of Akt and Akt phosphorylated (p-) at ser473 after irradiation. (D) Protein levels at 3 h after irradiation at 2 Gy. (B, C and E) Each band is compared with the individual band density for Actin. The relative amounts of the proteins were calculated by comparing them with the density of the unirradiated samples (U) in A and D. Black columns indicate X-irradiation; white columns indicate Fe-ion beam irradiation. B, Akt; C, p-Akt; E, downstream Akt associated proteins.

3.5. Cell cycle distribution

The distribution of the G_1, S, and G_2/M phases in unirradiated cells was 43%, 17% and 40%, respectively (Fig. 3C). At 12 h after irradiation with 2 Gy of X-rays, 7 Gy of X-rays, or 2 Gy of Fe-ion beams, the G_2/M phase cell fraction was 47%, 56% and 56% (Fig. 3D, F, H), showing that there was a significant accumulation of cells in the G_2/M phase. At 24 h after irradiation with 2 Gy of X-rays, 7 Gy of X-rays, or 2 Gy of Fe-ion beams, the G_2/M phase cell fraction was 35%, 47% and 50% (Fig. 3E, G, I). A G_2/M phase arrest was not observed after 2 Gy of X-rays or in the unirradiated controls, but was observed 24 h after irradiation with 7 Gy of X-rays and 2 Gy of Fe-ion beams.

4. Discussion

The *p53* gene can play a pivotal role in pathways which control apoptosis, cell growth, and cell proliferation [2–4]. There is a broad consensus that the primary physiological role of *p53* in DNA damage-induced apoptosis is to act as a transcriptional activator for genes which encode apoptotic effectors [16]. Previously reported cellular sensitivities to radiation were dependent on cellular *p53* gene status in human tongue squamous cell carcinomas [2], human glioblastoma cells [17], and human non-small lung cancer cells [12]. It was observed that wt*p53* cells were about 1.6-fold more sensitive to X-rays than cells with a dysfunctional *p53 status* (carrying mt*p53* or deleted *p53*) in the H1299 human lung cancer cell

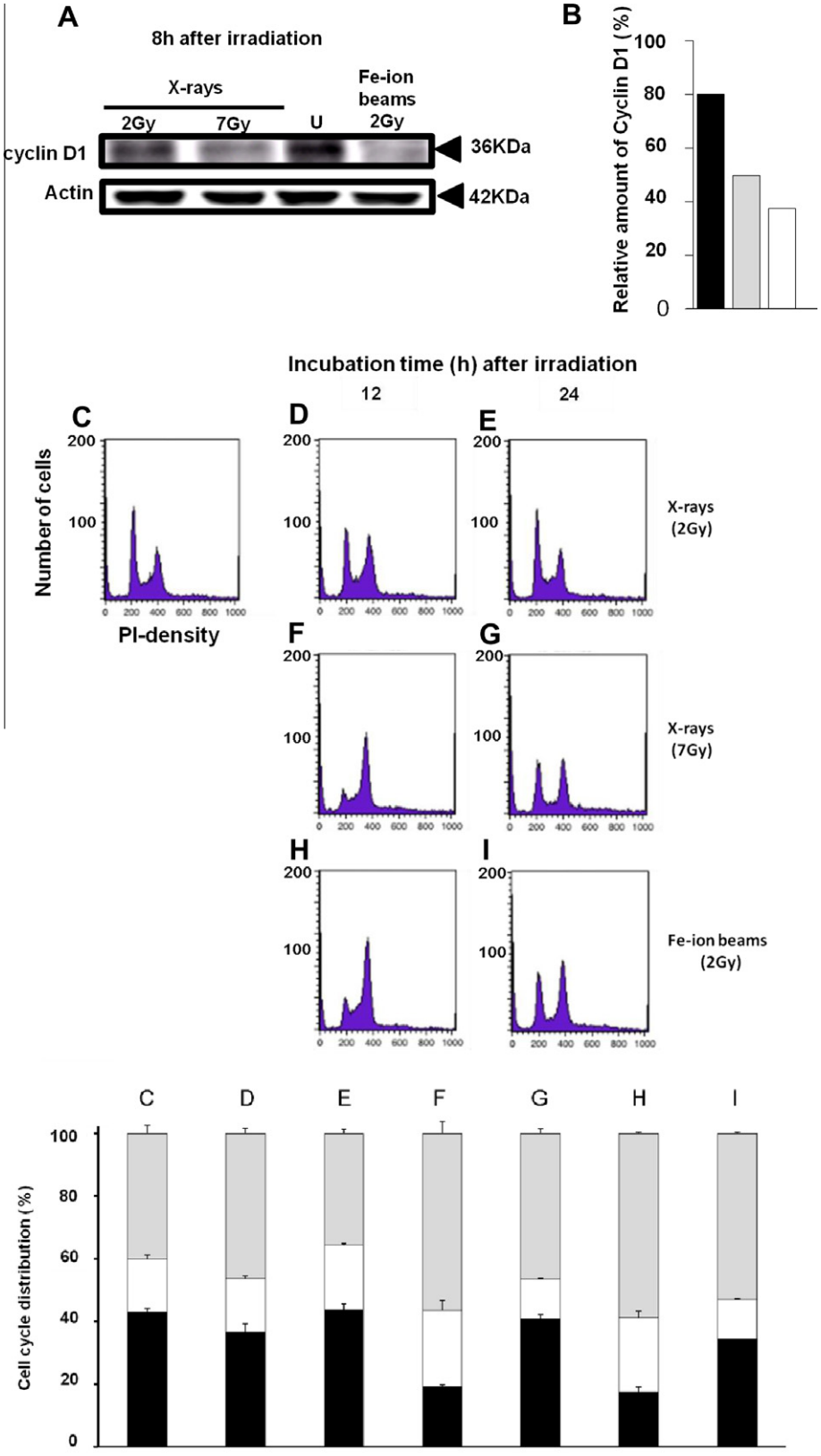

Fig. 3. Western Blotting of cyclin D1 and cell cycle analysis. (A) Protein levels of cyclin D1 at 8 h after irradiation. (B) Each band is compared with the individual band density for Actin. The relative amounts of the proteins were calculated by comparing them with the density of the unirradiated samples (U) in A. Black columns indicate X-irradiation at 2 Gy; gray columns indicate X-irradiation at 7 Gy, white columns indicate Fe-ion beam irradiation at 2 Gy. (C) Unirradiated controls. (D–G) Cells irradiated with X-rays. (H and I) Cells irradiated with Fe-ion beams; D, E, H and I, 2 Gy; F and G, 7 Gy. D, F and H, 12 h after irradiation; E, G and I, 24 h after irradiation. In the bottom figures (distribution); black columns indicate G_1; white columns indicate S; gray columns indicate G_2/M. C–I show the same samples between in the middle figures (histogram) and bottom figures.

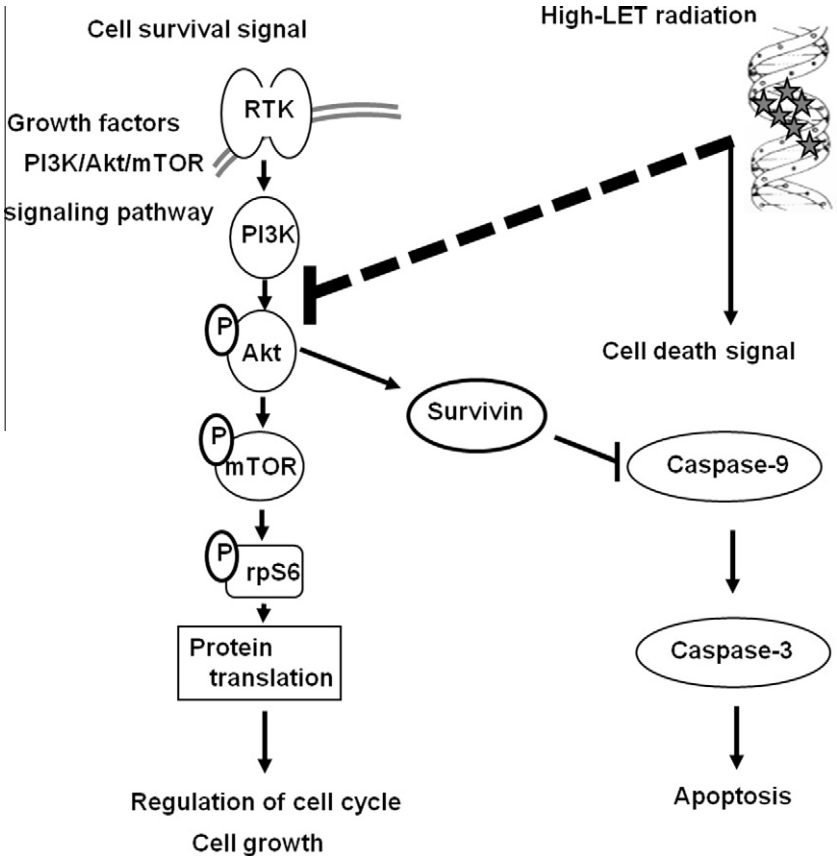

Fig. 4. A model for high LET radiation-induced *p53*-independent apoptosis through the enhancement of death signals and the depression of survival signals. An arrow → indicates enhancement; a sidewise "T" indicates depression.

line [13]. X-irradiation primarily induced apoptosis and necrosis. Although there were no significant differences between wt*p53* and m*p53* cells in the H1299 cell line with respect to the frequency of X-rays induced necrotic cells, wt*p53* cells had a higher proportion of apoptotic cells after X-rays than the m*p53* cells. On the other hand, there was almost no significant difference in the surviving fraction or in the proportion of apoptotic cells observed after exposure to high-LET radiation, regardless of cellular *p53* gene status in cancer cells [12]. These facts suggest that high-LET radiation induces apoptosis in wt*p53* cells and m*p53* cells at similar levels. These findings indicate that the RBE values for Fe-ion beams were about 5.5 and 2.1 in *p53* dysfunctional cells and wt*p53* cells, respectively [13]. In the present paper, it is shown that radiation sensitivity and apoptosis in Ca9–22 cells apparently increased after exposure to Fe-ion beams when compared to an equal dose of X-rays (2 Gy) (Fig. 1A, B and Fig. 4). It has been reported that RBE curves for both reached a peak at 100 keV/μm [14]. It is often not practical in the clinic to determine the status of a *p53* gene or other relevant genetic markers. Thus, therapies using high-LET radiation are expected to result in a higher efficacy in treatments, especially for malignant tumors bearing a m*p53* or a deleted *p53* gene status. This is because these cell types have a high possibility of being radioresistant [10], and high-LET radiation can induce apoptosis effectively in tumors regardless of *p53* gene status [12,13]. Tumors responsive to C-ion beams have been reported to include head and neck cancer, lung cancer, liver cancer, prostate cancer, bone and soft-tissue sarcomas, pelvic recurrences of rectal cancer, skull base tumors, and choroidal melanoma [18].

During the induction of apoptosis, Caspases act as effectors, and their activities depend on *p53* gene status. Two distinct pathways upstream of the Caspase cascade have been identified: death receptor-induced apoptosis and mitochondrial stress-induced apoptosis [19,20]. Death receptors trigger Caspase-8, while mitochondria release apoptogenic factors leading to the activation of Caspase-9. Fe-ion beams apparently increase Caspase-3 activity in viable cells (Fig. 1C). The activity of Caspase-3 in cells irradiated with Fe-ion beams was higher than the activity in X-irradiated cells, not only at equal doses (2 Gy), but also at a D_{10} dose (7 Gy). Moreover, apoptosis induced by high-LET radiation appears to be dependent on Caspases, and Caspase-9 may contribute to Caspase dependent apoptosis after exposure to high-LET radiation (Fig. 1D and Fig. 4).

On the other hand, Akt survival signals are a ubiquitous and evolutionarily conserved signaling cascade that is involved in numerous cellular functions including apoptosis, cell proliferation, differentiation, migration, and metabolism [21,22]. Activation of Akt survival signaling is associated with a poor prognosis in multiple tumor types [23,24]. There are three closely related Akt isoforms in mammalian cells, consisting of Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ) [21]. All Akt isoforms bind to phosphatidylinositol(3,4,5)-triphosphate (PIP_3) through pleckstrin-homology (PH) domains, and translocate to the plasma membrane where they are activated *via* phosphorylation at residues Ser473 and Thr308. When Akt is activated through phosphorylation, Akt promotes cellular proliferation and inhibits apoptosis through the phosphorylation of multiple substrates, or the downstream proteins. These include Caspase-9, Bad, GSK3, and forkhead transcription factors such as FKHR (FOX1), FKHRL (FOXO3), and AFX (FOXO4) [22,25]. Recently it was suggested that C-ion beam irradiation effectively suppresses the metastatic potential of non-small cell lung cancer A549 cells by suppressing the PI3 K/Akt

signaling pathway [26]. The work described here was designed to examine whether high-LET radiation depresses survival signals from Akt and Akt-related genes which inhibit Caspase-9. The levels of Akt (Fig. 2A–C and Fig. 4) and the associated downstream proteins (Fig. 2D, E and Fig. 4) were effectively depressed by high-LET radiation when compared with low-LET radiation. The lowest level of Akt proteins was observed at 3 h after irradiation (Fig. 2B).

It has been reported that an mTOR inhibitor, rapamycin, in conjunction with radiation efficiently suppressed progression of the cell cycle [27]. After exposure to 7 Gy of X-rays and 2 Gy of Fe-ion beams, the cell cycle distributions were almost the same at 12 h and 24 h after irradiation (Fig. 3F–I). Moreover, it has been well known that Akt-related signals control expression of cyclin D1 and decreased expression of cyclin D1 induced G_2/M arrests [28]. The levels of cyclin D1 (Fig. 3A, B) was effectively depressed by high-LET radiation when compared with low-LET radiation. From these results, it was suggested that high-LET radiation increased G_2/M arrests and then, the progression of the cell cycle was stopped through the depression of Akt-related signaling (Fig. 4).

Clustered DNA lesions at multiple sites are thought to be critical for radiation-induced cell death. High-LET radiation is thought to produce high yields of clustered DNA damage, including DNA double-strand breaks (DSBs) and base alterations [29]. DSBs were repaired slowly when compared to the repair of DSBs induced by X-rays [30–32]. Consequently, cell multiplication was disturbed at the G_2/M stage [33] after exposure to high-LET radiation. In particular, Fe-ion beam-induced lesions are repaired more slowly than X-ray-induced lesions, because Fe-ion beam-induced lesions are reported to be more complex or extensive [32]. There are two major pathways in mammalian cells to repair DSBs: non-homologous end-joining (NHEJ) repair and homologous recombination (HR) repair [34]. Recently it has been reported that exposure to high-LET radiation induces cell lethality more efficiently than the same dose of low-LET radiation because NHEJ plays large role in the repair for low-LET radiation-induced DSBs [32].

In conclusion, the present study suggests that high-LET radiation such as Fe-ion beams enhance apoptosis through the activation of Caspase-3 and Caspase-9 *via* the depression of Akt-related signaling, even in m*p53* cancer cells (Fig. 4). These results clearly show that high-LET radiation is able to induce cell death efficiently with a higher level of apoptosis than X-rays, regardless of *p53* gene status. More detailed studies are still necessary to define the initial events occurring during the depression of Akt survival signaling and of the effect of *p53* gene status after exposure to high-LET radiation.

5. Conflict of interest statement

The authors declare that they have no conflicts of interest associated with this work.

Acknowledgments

This work was supported, in part, by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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