



Inhibition of Crandell-Rees Feline Kidney cell proliferation by X-ray-induced senescence

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ABSTRACT. Radioresistance and radiotoxicity have been reported following cancer treatments in felines. Optimizing radiation doses to induce cytotoxic effects to only cancer cells and not normal cells is critical in achieving effective radiation therapy; however, the mechanisms of radiation resistance, radiotoxicity, and DNA damage response (DDR) in feline cells have not yet been elucidated. A DNA double-strand break (DSB) is the most toxic type of DNA damage induced by X-rays and heavy ion beams used in treating cancers. Crandell-Rees Feline Kidney (CRFK) cells is one of the most widely used cat cells in life science research. Here, we report that DSB-triggered senescence induced by X-rays is important in inhibiting the proliferation of CRFK cells. We demonstrated through cell proliferation assay that X-rays at doses 2 Gy and 10 Gy are toxic to CRFK cells that irradiating CRFK cells inhibits their proliferation. In X-irradiated CRFK cells, a dose-dependent increase in DSB-triggered senescence was detected according to morphological changes and using senescence-associated β galactosidase staining assay. Moreover, our data indicated that in CRFK cells, the major DDR pathway, which involves the phosphorylation of H2AX at Ser139, was normally activated by ATM kinases. Our findings are useful in the understanding of X-rays-induced cellular senescence and in elucidating biological effects of radiation, *e.g.*, toxicity, in feline cells. Furthermore, our findings suggest that the CRFK cell line is an excellent matrix for elucidating radioresistance and radiotoxicity in cat cells.

KEY WORDS: cat, companion animal, DNA double-strand break, radiation, senescence

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Companion animals assume an increasingly important role in human society. Owing to the extension of the lifespan of companion animals, the number of elderly dogs and cats have been increasing, and many dogs and cats are newly diagnosed with cancer every year [26, 27]. Radiation therapy, which is one of the three major treatments for cancer, has been increasingly used in veterinary hospitals as an alternative for treating cancer in dogs and cats [24, 26]. To achieve the most effective radiation therapy, it is important to balance between the toxicity between normal and cancer cells. Meanwhile, although radioresistance is observed in cats and cat tumors following cancer radiotherapy, the mechanism of such resistance has not yet been clarified [24].

Numerous studies on the effects of radiation to cells derived from human and rodents have been conducted [6, 10, 23, 28]. Genomic DNA is the main biological target of therapies that use radiation such as X-rays and heavy ion beams. Double-strand break (DSB) is the most critical type of radiation-induced DNA damage. Various DNA damage response (DDR) pathways, including DNA damage sensing, DDR signaling, and DNA repair, are activated when a DSB occurs. As a result, the cells recover from radiation injury, and the affected DNA is accurately and efficiently repaired [8, 25]. In dog and cat cells, DSBs may mainly be repaired by non-homologous end joining (NHEJ) repair mechanism, which induces the binding of DSB ends of the Ku heterodimer, consisting of Ku70 and Ku80 [14–17]; however, further studies are needed to clarify this. Additionally, in the early repair stage, kinases, such as DNA-PK and ATM kinases, are activated, and these phosphorylate Ser139 of the histone H2AX near DSB sites [5, 6]. It is likely that DSBs activate programmed cell death mechanisms, including apoptosis and senescence, when the cells cannot be repaired [9, 32]. The molecular mechanisms of various DDRs, such as DNA repair, in cat cells has been poorly studied.

The immortalized Crandell-Rees Feline Kidney (CRFK) cell line is one of the most widely used cat cell lines. The CRFK cell line was first established in 1964 from the kidney cortex of a 12-week-old female kitten [3]. Crandell *et al.* [3] described that CRFK cell line is useful in feline virus research and diagnostic medicine and has become of particular interest in cancer research. Currently, the CRFK cell line is indispensable in life science research, including cancer and virus research. Thus, it is important to understand the radiosensitivity of CRFK cells, whose various molecular pathways have been analyzed, for obtaining primary

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research information to elucidate the molecular mechanisms of the biological effects of radiation in cats. However, there currently are no reports on the effects and mechanisms of radiation on the proliferation of CRFK cells.

In this study, we investigated the biological effects of X-rays on the proliferation of CRFK cells. Our findings indicate that X-rays markedly suppressed the proliferation of CRFK cells. Additionally, our results suggested that the suppression of proliferation was due to increased cellular senescence triggered by the DSBs. Furthermore, we confirmed in CRFK cells that the major DDR pathway is activated normally by ATM kinases.

MATERIALS AND METHODS

Cell cultures, chemicals, and X-rays

CRFK cells (HSRRB, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum as previously described [17]. Adherent cells were irradiated with X-rays at 1, 2, or 10 Gy and at a dose rate of 0.71–0.77 Gy/min (200 kVp/20 mA with 0.5-mm Al and 0.5-mm Cu filters) using Pantak HF320S (Shimadzu, Kyoto, Japan) at room temperature, as described in previous studies [17]. KU-55933, an ATM kinase inhibitor, was purchased from Wako Pure Chemical (Osaka, Japan). The KU-55933, were diluted in DMSO (Sigma-Aldrich, St. Louis, MO, USA) and diluted in culture medium immediately before use.

Determination of viable cell number

Trypan blue exclusion test was used to determine the number of viable cells. Cells were seeded at a density of 2.2×10^4 cells per dish in 60-mm dishes. Next day, the cells were irradiated with X-rays and incubated for 5 days post-irradiation. Subsequently, the cells were washed with phosphate-buffered saline (PBS), suspended in Trypsin-EDTA solution (T3924, Sigma-Aldrich), collected, centrifuged, stained with 0.4 w/v% Trypan Blue Solution (Wako Pure Chemical) or 0.4% Trypan Blue (Bio-Rad, Hercules, CA, USA), and counted using TC10™ Automated Cell Counter (Bio-Rad). All irradiations were performed in triplicate.

Immunoblotting

Total protein extraction and western blot analysis were performed according to our previously described methods [11, 17] with the following modifications. The total proteins were electrophoresed on Extra PAGE One Precast Gel 5–20% (Nacalai Tesque, Kyoto, Japan) or Super Sep ACE Gel 5–20% (Wako Pure Chemical). The fractionated products were electrophoretically transferred onto Hybond-P membranes (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Then, the membranes were blocked in Blocking One (Nacalai Tesque) for 60 min at room temperature and incubated with mouse anti- γ H2AX monoclonal antibody (JBW301) (Upstate Biotechnology Inc., Charlottesville, VA, USA) or mouse β -actin monoclonal antibody (Sigma-Aldrich). After washing, the membranes were incubated with the anti-mouse IgG HRP-Linked Whole Ab (from sheep) (NA931) (GE Healthcare Bio-Sciences Corp.) for 60 min at room temperature. Immunoblotting was performed using Select Western Blotting Detection System (GE Healthcare Bio-Sciences Corp.). Protein bands were visualized using ChemiDoc XRS system (Bio-Rad).

Immunocytochemistry

Immunostaining was performed as previously described [12, 17] with the following modifications. Briefly, cultured cells were fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, blocked using a blocking solution, and incubated with mouse anti- γ H2AX monoclonal antibody (JBW301) (Upstate Biotechnology Inc.) for 60 min at room temperature. After washing the cells with PBS, detection was performed using an Alexa Fluor 568-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA) or a fluorescein isothiocyanate-conjugated secondary antibody (Cappel Laboratories, Durham, NC, USA). Then, nuclei were stained with 0.025 μ g/ml of 4,6-diamino-2-phenylindole (DAPI) fluorescent dye (Boehringer Mannheim, Mannheim, Germany). Images of cells were obtained using Olympus Fluorescence Microscope BX51 (Olympus, Tokyo, Japan) equipped with a digital camera (Olympus DP50, Olympus).

Senescence-associated β galactosidase (SA- β -gal) staining assay

For this assay, cells (5×10^3) were plated in 35-mm plates and stained using Senescence β -Galactosidase Staining Kit (Cell Signaling Technology Inc., Danvers, MA, USA) 5 days after irradiation according to the manufacturer's instructions with the following modifications. Nuclei were stained with 0.025 μ g/ml of DAPI fluorescent dye. Images of the stained cells after 18 hr were obtained using Olympus CKX41 Microscope equipped with a digital camera (Olympus DP12, Olympus) or using Olympus Fluorescence Microscope BX51 equipped with a digital camera (Olympus DP50). The percentage of positively stained cells was determined by analyzing three random fields of at least 100 cells each using the Image J software. The size of cell nuclear was determined by analyzing three random fields of 10 cells each using the Image J software. All irradiations were performed in triplicate.

Annexin V/propidium iodide (PI) apoptosis assay

Cells (2.2×10^4) were plated in 60-mm plates and irradiated with X-rays on the next day. Five days after the irradiation, the cells were harvested and stained with Annexin V Alexa Fluor 488-PI-solution (Tali™ Apoptosis Kit, Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. A 200-fold dilution of the PI staining solution was used for staining. In addition, Annexin V labeled-apoptotic cells were detected using the Tali Image-based Cytometer (Invitrogen). All irradiations were performed in triplicate.

Statistical analysis

Statistical results are presented as means \pm standard deviations ($n=3$). Statistical analysis excluding nuclear size was performed using ANOVA and Ryan's multiple comparison tests (ANOVA4 on the WEB, <https://www.hju.ac.jp/~kiriki/anova4/>). Nuclear size was evaluated by Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Inhibition of CRFK cell proliferation by X-rays

To investigate the effect of X-rays on the proliferation of CRFK cells, irradiation was performed at 2 and 10 Gy. As shown in Fig. 1, cell proliferation was significantly inhibited in a dose-dependent manner ($P<0.05$). These findings indicate that the proliferation was inhibited by X-rays at both doses.

Induction of DSBs in CRFK cells by X-rays

The phosphorylation of H2AX at Ser139 is one of the major and early cellular DDRs to DSBs [8, 20, 28]. γ H2AX is a gold standard biomarker for DSBs [8, 20, 28]. Previously, using western blotting, we determined the change in γ H2AX expression in CRFK cells between from 1 hr to 24 hr post-irradiation with X-rays at a single dose of 10 Gy [17]. As a result, the expression level of γ H2AX was the highest after 1 hr [17]. To examine whether H2AX phosphorylation was induced in a dose-dependent manner in the total extracts from CRFK cells 1 hr post-irradiation, western blot analysis was performed with the anti- γ H2AX antibody. As shown in Fig. 2A, western blot analysis indicated that the expression of γ H2AX increased depending on the dose of X-rays. Next, we examined whether γ H2AX foci were detected in irradiated cells by immunostaining assay using the anti- γ H2AX antibody. Our results indicated that γ H2AX foci were formed in the nuclei of CRFK cells at 1 hr post-irradiation (Fig. 2B).

ATM kinase dependent-phosphorylation of H2AX at Ser139 in CRFK cells by X-rays

One of the important roles of ATM kinase or DNA-PK in DDR triggered by X-rays is to phosphorylate H2AX surrounding the DSB sites in various human and rodent cells [18, 29]; however, such a role has not yet been investigated in cat cells, including CRFK cells. To confirm whether ATM kinase is responsible for X-ray-induced H2AX phosphorylation at Ser139 in CRFK cells, the cells were incubated in a medium containing the ATM kinase inhibitor KU-55933 (10 μ M) or solvent (DMSO) for 1 hr prior to irradiation (10 Gy). As shown in Fig. 3, the formation of γ H2AX foci was inhibited in cells irradiated in the presence of KU-55933. These results suggest that ATM is the main kinase for the phosphorylation of H2AX at Ser139 induced by irradiation in CRFK cells.

Induction of senescence in CRFK cells by X-rays

To investigate whether the inhibition of CRFK cell proliferation in response to irradiation is related to senescence and apoptosis, the cells were irradiated with X-rays at 2 and 10 Gy. Firstly, 5 days after the irradiation, cells were analyzed using SA- β -gal staining, a gold standard assay for senescence. We observed senescence-specific morphologies such as a large and flat cellular shape (Fig. 4A) and abnormal nuclei, including an enlarged- or multinucleated-nuclei (Fig. 4B), in irradiated cells. In addition, in irradiated CRFK cells, SA- β -gal-positive cells markedly increased in a dose-dependent manner (Fig. 4A–C). We also quantitatively analyzed the enlargement in the size of the cell nucleus, which is another marker for senescent cells. As shown in Fig. 4D, the size of the cell nucleus was significantly larger in irradiated CRFK cells ($P<0.05$). Altogether, these findings indicate that X-rays highly induce senescence in CRFK cells. Next, to examine whether X-rays induced apoptosis in the irradiated CRFK cells, apoptosis was quantified using Tali™ Image-based Cytometer. Dual staining with fluorescent Annexin V and PI was performed to detect apoptotic

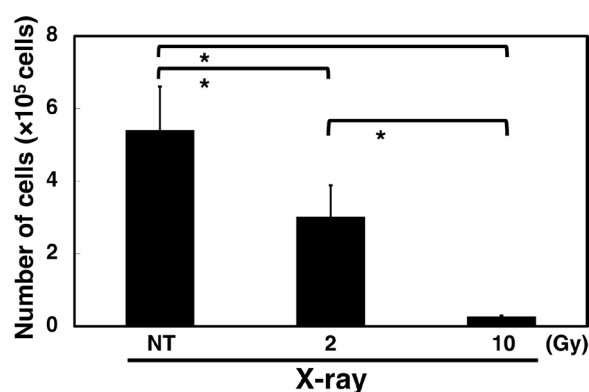


Fig. 1. Inhibition of the proliferation of Crandell-Rees Feline Kidney (CRFK) cells by X-rays. The cells were irradiated with X-rays at a dose of 2 or 10 Gy. The number of cells was counted 5 days after irradiation. NT: No treatment. Error bars denote standard deviation ($n=3$). The asterisk indicates $P<0.05$.

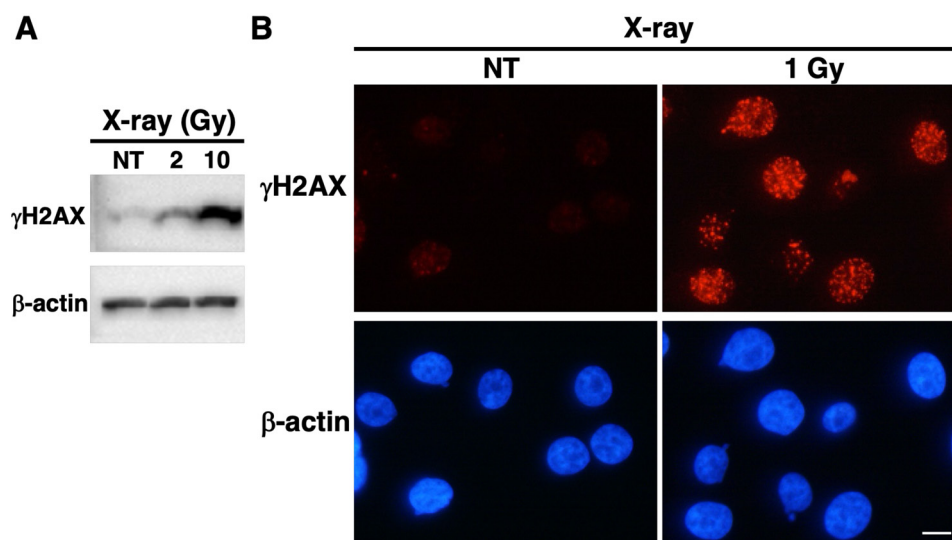


Fig. 2. Induction of DNA double-strand breaks (DSBs) in Crandell-Rees Feline Kidney (CRFK) cells by X-rays. (A) Western blot analysis of γ H2AX expression after irradiation. The cells were irradiated with 2 or 10 Gy X-rays. The extracts from the cells were prepared 1 hr after irradiation and were subjected to western blotting using an anti- γ H2AX antibody or anti- β -actin antibody. (B) Immunofluorescence analysis of γ H2AX expression and focus formation after irradiation. Cells were irradiated with 1 Gy X-rays. The cells were fixed 1 hr after irradiation and stained for immunofluorescence analysis using an anti- γ H2AX antibody. The DNA was stained with 4,6-diamino-2-phenylindole (DAPI). NT: No treatment. Scale bar represents 10 μ m.

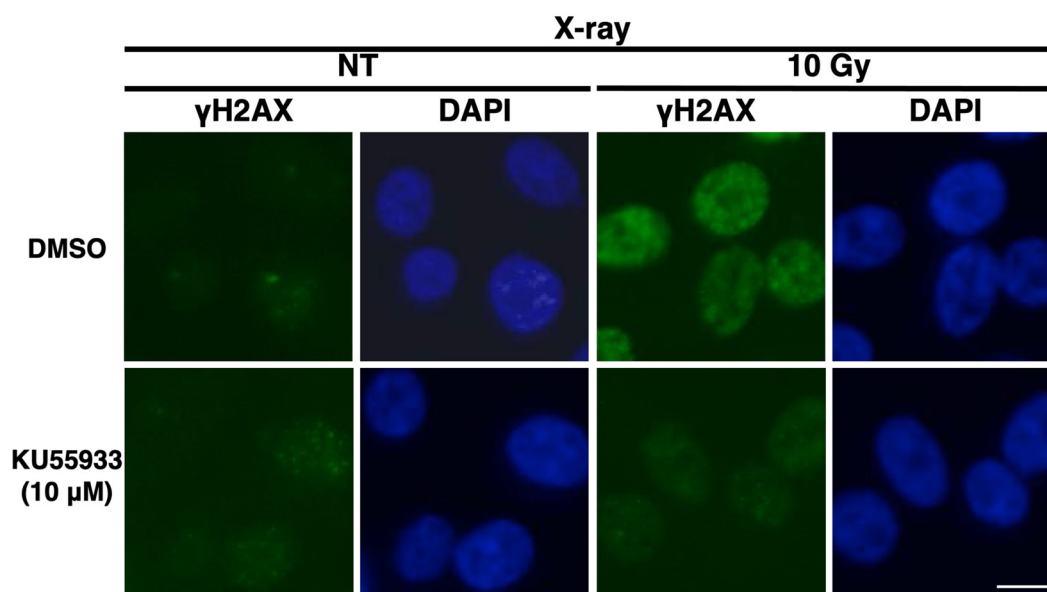


Fig. 3. Effects of KU-55933 on X-ray-induced H2AX phosphorylation at Ser139 in Crandell-Rees Feline Kidney (CRFK) cells. The cells were incubated in medium containing KU-55933 (10 μ M) or solvent (DMSO) for 1 hr prior to irradiation (10 Gy). The cells were fixed 1 hr after irradiation and stained for immunofluorescence analysis using an anti- γ H2AX antibody. The DNA was stained with 4,6-diamino-2-phenylindole (DAPI). NT: No treatment. Scale bar represents 10 μ m.

cells. Cells stained positive for Annexin V are considered apoptotic cells. As shown in Fig. 5, the proportion of apoptotic cells was significantly higher in the 10-Gy group than in the non-irradiated group and the 2-Gy group but was lower (approximately 6%) in the 10-Gy group. In addition, no significant increase in apoptosis was observed in the 2-Gy group compared with the non-irradiated group.

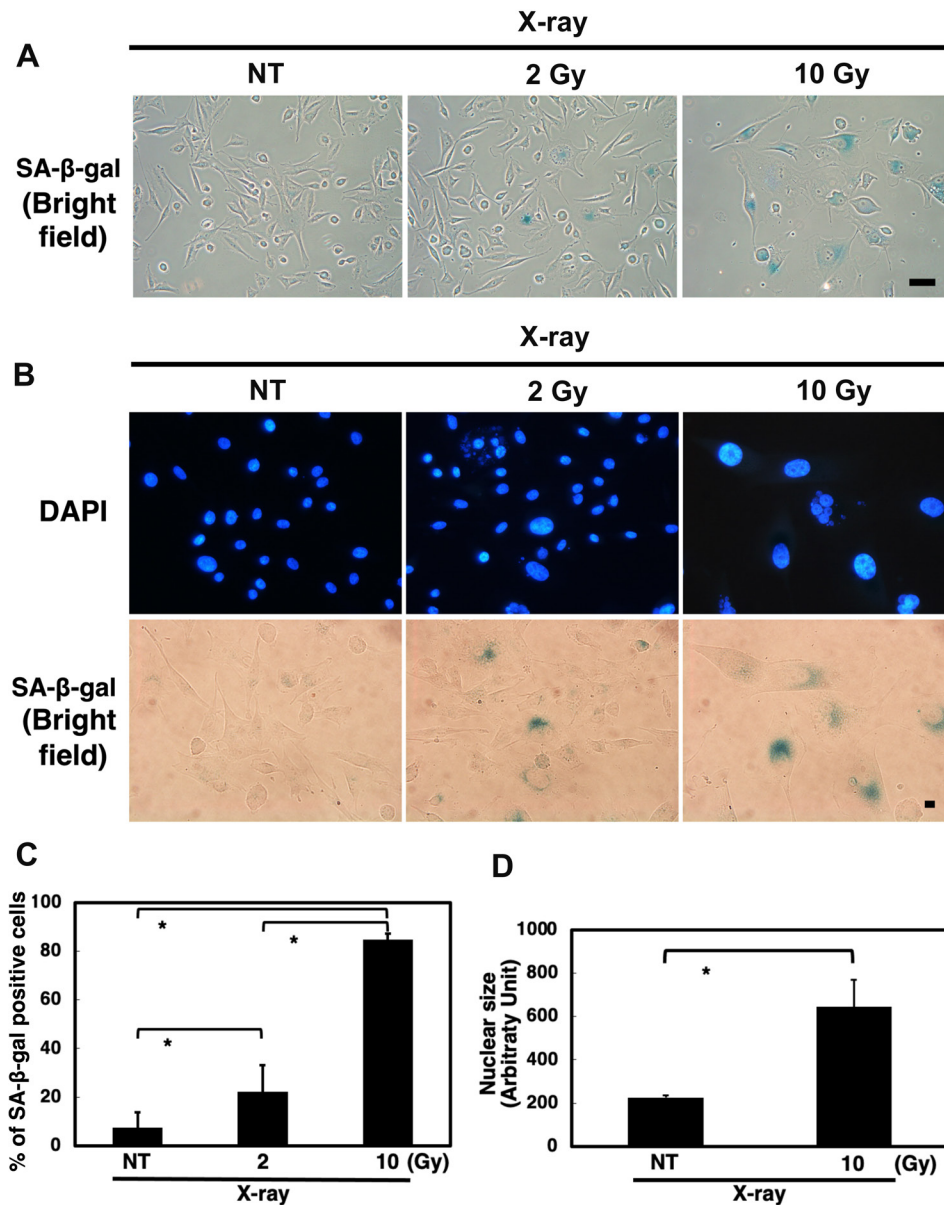


Fig. 4. Induction of senescence in Crandell-Rees Feline Kidney (CRFK) cells by X-rays. Five days after irradiation (2 or 10 Gy), SA-β-gal staining were performed to detect senescence. Morphology of cells with senescence-specific shape (A) and with abnormal nuclei (B) in irradiated cells. Scale bar represents 60 μm (A) and 10 μm (B). The DNA was stained with 4,6-diamino-2-phenylindole (DAPI). (C) Irradiation with X-rays resulted in dose-dependent increase in SA-β-gal-positive cells. (D) Irradiation with X-rays resulted in increasing the size of cell nucleus. NT: No treatment. Error bars denote standard deviation (n=3). The asterisk indicates $P < 0.05$.

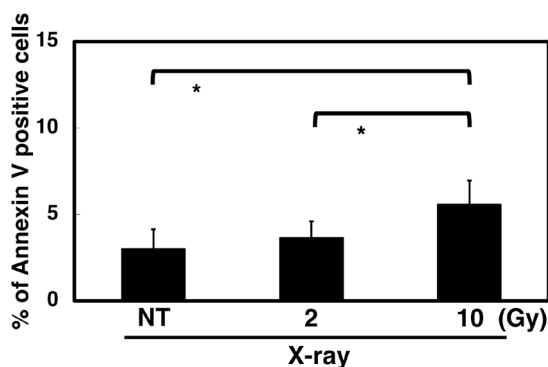


Fig. 5. Induction of apoptosis in Crandell-Rees Feline Kidney (CRFK) cells by X-rays. Five days after irradiation (2 or 10 Gy), the cells were double-stained with fluorescent Annexin V and propidium iodide to detect apoptotic cells. Apoptosis was quantified using Tali™ Image-based Cytometer. NT: No treatment. Error bars denote standard deviation (n=3). The asterisk indicates $P < 0.05$.

DISCUSSION

Understanding the biological effect of radiation on cat cells is important elucidating radiotoxicity and for developing novel therapeutic protocols for cancer treatment. In the present study, we found that the proliferation of the cat kidney cell line CRFK was significantly suppressed by X-rays at doses 2 and 10 Gy. These findings reveal that X-rays are toxic to the growth of CRFK cells under the conditions used in this study. Moreover, senescent cells markedly increased in irradiated CRFK cells. Meanwhile, our findings revealed in CRFK cells, ATM kinase induces the phosphorylation of H2AX at Ser139, which is one of the major DDRs to DSBs produced by X-rays. Radiation-induced DSBs reportedly can induce senescence and apoptosis in human and rodent cells [4, 22]. Our findings suggest that the senescence mechanism activated by DSBs contributed in suppressing the proliferation of CRFK cells by X-rays.

The mechanism underlying the difference in radiosensitivity among humans, dogs and cats and the repair mechanism of cat cells have not yet been elucidated. As mentioned above, our data indicated that the ATM kinase-dependent DDR is activated by X-rays in CRFK cells. Additionally, this study and previous studies demonstrate that X-ray-induced DSBs in CRFK cells can be detected by western blot analysis and immunocytochemistry using the commercially available anti- γ H2AX antibody [17]. These findings suggest that CRFK cells is useful in clarifying the molecular mechanisms of DDR in cat cells. Moor [24] described that radiation therapy for cats is different from that for dogs in terms of tumor responses and normal tissue toxicity. Fujii *et al.* [7] showed that the fibroblasts in cats are more radioresistant than those in humans in a colony-forming analysis and radiation-induced γ H2AX foci may be repaired faster and more effectively in feline fibroblasts. In addition, they suggested the possibility that DNA and chromosome damage induced by X-rays is more effectively repaired in feline lymphocytes than in human or dog lymphocytes. These findings suggest that the differences in radioresistance among humans, dogs and cats may be related to the difference in DNA repair ability. Recently, we demonstrated using CRFK cells that the core NHEJ repair proteins, *i.e.*, cat Ku70, Ku80, and XLF, accumulate at DSB sites induced by the 405-nm microlaser [17]. We have also revealed that the recruitment of cat XLF, human XLF, or dog XLF is dependent on the presence of Ku [13, 14, 17]; these findings strongly support that the spatiotemporal regulation of core NHEJ factors, including Ku70, Ku80, and XLF, is important in the regulation of NHEJ repair [10, 19]. Collectively, CRFK cells may be useful in uncovering the molecular mechanisms underlying not only the differences of radioresistance among cat, dog, and human cells but also DDRs, including Ku-dependent NHEJ repair conserved in them.

The epithelial mesenchymal transition (EMT) is an important mechanism for malignant transformation, *e.g.*, changing the resistance of cancer cells to radiation and anticancer drugs. Recently, CRFK cells have been reported to have the characteristics of fibroblast, particularly regarding morphology, biochemistry, and molecular biology, although CRFK cells have been established as an epithelial cell line [21]. Moreover, van Beusekom *et al.* [31] reported that TGF- β 1 changed the morphology of CRFK cells from an epithelial phenotype to a fibroblastic phenotype and that it significantly induced the expression of some EMT marker genes in CRFK cells, suggesting that CRFK cells can undergo EMT. In the present study, senescence appeared more important than apoptosis for growth suppression in irradiated CRFK cells. Altogether, CRFK cell line may be an excellent modality for not only elucidating the interrelationship between or among X-ray-induced senescence, radioresistance, and/or radiotoxicity in cat kidney epithelial cells but also for elucidating the effects of EMT against radiation and chemotherapeutics.

Pro-senescence therapy is a novel anti-cancer strategy on the basis of inducing cancer cell senescence. In order to apply this strategy to the treatment of cats, it is necessary to elucidate the molecular mechanism of senescence induction in feline cells. On the other hand, Li *et al.* [22] have described that senolytic agents, a class of small molecules that can selectively kill senescent cells, hold great potential to substantially reduce the side effects caused by radiotherapy while reasonably steer clear of carcinogenesis. Recently, it was shown that a senolytic agent ABT-737 eliminates ionizing radiation induced senescent cells from lung of irradiated mice [35]. As mentioned above, our data showed that senescent cells markedly increased in irradiated CRFK cells. Our findings and further studies using CRFK cells may be available for basic research for development not only of feline pro-senescence therapy, but also of senolytic agents in order to reduce the side effects caused by radiotherapy.

CRFK is one of the most important mammalian cell lines and is widely used in experiments including those on viruses, such as severe acute respiratory syndrome coronavirus (SARS-CoV) or SARS-CoV-2; DNA repair and cancer research in cats; chronic kidney disease; and the production of vaccines [1, 2, 17, 30, 33, 34]. CRFK cells can be infected with various viruses, including oncoviruses [1–3, 34]. Based on these, we speculate that the CRFK cell line may be suitable for basic research on changes in radiosensitivity induced by viral infection and on the effect of radiotherapy on tumors induced by oncoviral infection. CRFK cells can be used to elucidate the mechanisms underlying radioresistance and radiotoxicity in cat kidney epithelial cells and the effect of viral infection on radiosensitivity. Conclusively, CRFK cells may be excellent cell lines for conducting research on radiotoxicity in cats and basic research for developing new cancer treatment methods.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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