Combined ataxia telangiectasia mutated and DNA-dependent protein kinase inhibition radiosensitizes Madin–Darby canine kidney cells

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ABSTRACT

Uncovering radiation toxicity is critical for the adaptation and expansion of advanced radiation therapies and for the development of novel cancer radiotherapy. In the near future, advanced radiotherapies, including heavy ion beam treatment, are expected to be applied in the treatment of dogs, but further basic research on the effects of radiation using canine normal and cancer cells is necessary to actually apply these techniques and achieve high therapeutic efficacy. The radiation sensitivity is varied by the activities of DNA damage response (DDR) and DNA repair. The development of radiosensitizers that target DDR- and DNA repair-kinases, like ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK), is progressing and is expected to be introduced into canine radiotherapy. However, there are no cytotoxicity reports on using the combination of radiation and these sensitizers as treatment in canine cells. In this study, we examined the cytotoxic effects of X-rays and/or radiosensitizers on the Madin–Darby Canine Kidney (MDCK) cell line. Our results show that X-rays suppress MDCK cell colony formation and proliferation in a dose-dependent manner. Additionally, our observations imply that the combination treatment with ATM inhibitor KU-55933 and DNA-PK inhibitor NU7441 significantly increased X-ray cytotoxicity in MDCK cells compared with the drugs alone. Furthermore, our findings further suggest that MDCK cells might be useful in clarifying the cytotoxicity in canine epithelial cells due to radiation and/or radiosensitizers, such as molecule-targeted drugs.

KEY WORDS: ataxia telangiectasia mutated, canine, companion animal, DNA-dependent protein kinase, radiation
INTRODUCTION

Companion animals are playing a role of increasing importance in human life. The lifespan of dogs is prolonged owing to improved nutrition, hygiene and rearing conditions, and advances in veterinary medicine. As a result of the extended lifespan of companion animals, many dogs are newly diagnosed with cancer annually [28, 29]. Moreover, radiation therapy, which is one of the top three treatments for cancer, has been increasingly used for treating canine cancer in veterinary hospitals [26, 27]. Meanwhile, in canine tumors, radioresistance is observed and is an issue that needs to be overcome. However, the mechanism is not yet understood [26].

Understanding the cytotoxicity of radiation is important for the safe use of radiation in radiotherapy. In order to maximize its efficacy, it is important to balance the therapeutic effect on cancer cells with the toxicity on normal cells. The effects of radiation on human and rodent cells have been extensively investigated at the cellular and molecular levels, whereas little information is available on the effects on canine cells [5, 14, 25, 30].

The Madin–Darby canine kidney (MDCK) cell line is one of the most characterized and widely used canine cell lines in life science research. The cell line was established by Madin and Darby in 1958 from the kidney of a normal cocker spaniel [8]. This cell line was initially used in viral research but has since been used in basic biological epithelial cell research, including lumen formation, cell polarization, and cell differentiation, and in applied research including drug discovery and toxicology [31-33, 35].

Radiosensitivity is modulated by DNA damage response (DDR) and DNA repair activities in irradiated cells. The protein kinases ataxia telangiectasia mutated
(ATM) and DNA-dependent protein kinase (DNA-PK) play a key role in the mechanism of DDR and DNA repair, respectively [14, 25, 27]. Recently, molecule-targeted drugs targeting ATM and DNA-PK have gained attention as radiosensitizers, and the development of various new target drugs is underway [9, 12, 24, 27]. It is known that ATM and/or DNA-PK phosphorylate H2AX surrounding the X-ray-induced DNA double strand break (DSB) sites in human and rodent cells, and phosphorylation regulation might not only depend on the cell type, but also the tissue type [5, 16-18]. The highly specific inhibitors KU-55933 and NU7441, for ATM and DNA-PK, respectively, have been reported to inhibit kinase activity, and together show a synergistic effect on γH2AX in MDCK cells [6]. These findings suggest that these kinase inhibitors are also attractive as radiosensitizers to overcome the radioresistance in canine cells. However, there are no reports on the cytotoxicity of the combination of these two drugs with X-irradiation and the effects on MDCK cell colony formation and proliferation.

In the present study, we examined the cytotoxicity of X-rays on the colony formation and proliferation abilities of MDCK cells. Our results show that X-rays affect MDCK cells in a dose-dependent manner. In addition, our data suggest that the combination of KU-55933 and NU7441 enhances the cytotoxicity of X-rays in MDCK cells.

**MATERIALS AND METHODS**

*Cell cultures, drugs, and irradiation*

MDCK cells (HSRRB, Osaka, Japan) were cultured in Dulbecco’s modified
Eagle’s medium supplemented with 10% fetal bovine serum at 37°C with 5% CO₂ as previously described [19-22]. Irradiation was carried out using a Pantak HF320S (Shimadzu, Kyoto, Japan) at room temperature. Cells were irradiated with X-rays at 0.63, 1.25, 2.5, 5, or 10 Gy and at a dose rate of 0.81–0.88 Gy/min (200 kVp/20 mA with 0.5-mm Al and 0.5-mm Cu filters), as described in previous studies [19]. Images of the cells were obtained using an Olympus CKX41 Microscope equipped with a digital camera (Olympus DP12) (Olympus, Tokyo, Japan). NU7441 and KU-55933 were purchased from Wako Pure Chemical (Osaka, Japan). NU7441 and KU-55933 were diluted in DMSO (Sigma-Aldrich, St Louis, MO, USA) and stored at −20°C. Both drugs were diluted in culture medium immediately before use.

**Clonogenic cell assay**

Cells were seeded at 100 cells per 60-mm dish in triplicate. The next day, the cells were irradiated with X-rays with the indicated doses. After 5 days, colonies were stained in 2% methylene blue stain solution (Wako Pure Chemical), and colonies containing more than 50 cells were counted manually. The survival rates were calculated as a ratio with the number of colonies in the untreated controls.

**Cytotoxicity assay**

Trypan blue exclusion test was used to determine the number of viable cells in accordance with our previously described methods [23] with the following modifications. For the radiation toxicity assay, cells were seeded at a density of 5.0 × 10⁴ cells per 60-mm dish. The next day, the cells were irradiated with X-rays and incubated for 72 hr 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 a hemocytometer. For the radiation-drug combination toxicity assay, cells were seeded at a density of \(2.5 \times 10^4\) cells per 35-mm dish. The following day, KU-55933 and/or NU7441 were added at the indicated concentrations 1 hr before irradiation. At 24 hr from irradiation, drugs were removed and cells were returned to the incubator. After 72 hr, the trypan blue exclusion test was performed. All assays were performed in triplicate.

**DAPI staining**

Nuclei were stained with 0.025 \(\mu g/mL\) of 4,6-diamino-2-phenylindole (DAPI) fluorescent dye (Boehringer Mannheim, Mannheim, Germany), in accordance with our previously described methods [15, 23]. Images of cells were obtained using an Olympus Fluorescence Microscope BX51 equipped with a digital camera Olympus DP50.

**Statistical analysis**

The mean values and standard deviations of each assay were calculated from the mean of three independent experiments. Statistical analysis was performed using the analysis of variance (ANOVA) and Ryan’s multiple comparison tests (ANOVA4 on the web, https://www.hju.ac.jp/~kiriki/anova4/) as previously described [23]. Statistical significance was set at a \(p\)-value of less than 0.05.

**RESULTS**
X-ray-induced cytotoxicity on MDCK cells

To examine the effect of X-rays on the clonogenic survival of MDCK cells, irradiation was performed at 0.63, 1.25, 2.5, and 5 Gy. Our results showed that the size and number of colonies formed by MDCK cells at 5 days from X-irradiation were dose dependent (Fig. 1A). As shown in Fig. 1B, clonogenic cell survival rates were significantly inhibited in a dose-dependent manner ($p < 0.05$). These results indicate that the colony formation ability was inhibited by X-rays in a dose-dependent manner.

X-ray-induced suppression of MDCK cell proliferation

To examine the effect of X-rays on the proliferation of MDCK cells, irradiation was performed at 1.25, 2.5, 5, and 10 Gy. As expected, non-irradiated MDCK cells showed a typical paving stone-like morphology as the one found in normal epithelial cells (Fig. 2A, left panel). Each of these cells also had a single, distinct, and relatively homogeneous nucleus (Fig. 2A and 2B, left panels). After irradiation, we observed abnormal morphologies, including large and flat cellular shapes (Fig. 2A, right panel), and abnormal nuclei, including enlarged and multi-nuclei (Fig. 2B, right panel). As shown in Fig. 2C, the cell proliferation was significantly suppressed in a dose-dependent manner ($p < 0.05$). These results indicate that the cell proliferation was suppressed by X-rays at least between 1.25 and 10 Gy in response to dose.

KU-55933 and NU7441 combination enhances X-ray cytotoxicity in MDCK cells

DNA-PK and ATM quickly phosphorylates H2AX following DNA damage in MDCK cells [6]. Flassig et al. [6] reported that in γ-irradiated MDCK cells KU-55933 (10 μM) strongly inhibits the phosphorylation of H2AX by ATM, whereas
NU7441 (1μM) mildly inhibits it by DNA-PK. Therefore, studies in radiation-drug combination toxicity were performed using KU-55933 (10μM) and NU7441 (2μM) concentrations. We evaluated the effects on the proliferation of MDCK cells of KU-55933 (10 μM) and/or NU7441 (2 μM) together with the cytotoxic effects of X-rays at 1.25 Gy. As shown in Fig. 3, there were no significant differences between the non-treated and DMSO-treated control groups under the conditions with or without irradiation. The treatment with either drug had no significant effect on cell proliferation in the absence of X-irradiation (Fig. 3A). Interestingly, the viable cell numbers were significantly decreased when the cells were treated with the combination of the two inhibitors (p < 0.01, compared with DMSO-treated control; p < 0.05, compared with KU-55933 or NU7441 alone). When cells were irradiated in the presence of KU-55933 alone, X-ray-induced suppression of cell proliferation was significantly enhanced (p < 0.05, compared with DMSO-treated control) (Fig. 3B). In contrast, NU7441 alone did not show a significant effect. Intriguingly, combination of KU-55933 and NU7441 led to a stronger suppression of cell proliferation than KU-55933 alone (p < 0.001, compared with DMSO-treated control; p < 0.05, compared with KU-55933 alone). These results suggest that KU-55933 and NU7441 combination significantly increases X-ray-induced cytotoxicity in MDCK cells, more than KU-55933 alone.

DISCUSSION

Uncovering radiation toxicity in canine normal and cancer cells is critical for the adaptation and expansion of advanced radiation therapies to dogs and for the
developing of novel cancer radiotherapy [27, 28]. The development of radiosensitizers that target molecules working as vital transducers in the DDR and DNA repair pathways is underway [9, 12, 27] and is expected to be introduced into canine radiotherapy. However, there are no reports on the cytotoxicity due to the combination of radiation and these sensitizers in canine cells. In this research, we examined the effects of X-irradiation on the abilities of colony formation and proliferation of MDCK cells to gather basic information on the radiotoxic effects on canine cells. Our findings might provide fundamental information for a better understanding of canine cell cytotoxicity by X-radiation and for the development of molecularly targeted drugs aimed at ATM and DNA-PK.

The MDCK cell line retains the common physiological features of normal canine renal epithelial cells, including cell morphology, cell function and cell society [31, 33]. In fact, this cell line has contributed to the elucidation of general biological phenomena such as cell–cell interactions and cell polarity in mammalian epithelial cells as well as renal epithelial cell functions [31, 33]. In this study, we confirmed that MDCK cells show a typical paving stone-like morphology, demonstrating that the MDCK cells used maintain, at least in part, characters derived from normal renal epithelial cells. Moreover, the culture of established MDCK cell lines is easier and less expensive than that of primary cell cultures. The cells are commonly used throughout the world, so the data obtained can be used for comparative studies with various published data. In fact, this cell line has contributed to clarify the carcinogenic mechanism of oncogenes, the tumor suppressor mechanisms (e.g., cell competition, DDR signaling and DNA repair), and the malignant transformation due to oncoviruses [1, 2, 19-22]. Thus, we consider that the MDCK cells may be useful for pre-assessing the cytotoxicity of X-radiation and
anticancer drugs on normal canine renal epithelial cells.

Recent studies demonstrated that many human and canine cancer cells have abnormalities in certain DNA repair mechanisms [10, 13]. The development of drugs that target the DNA repair mechanisms, including synthetic lethal drugs, is underway in human medicine [7, 12, 34]. However, these drugs are thought to have cytotoxic effects on normal cells in addition to their therapeutic effects on cancer cells. ATM and DNA-PK, which have crucial functions in DDR and DNA repair, are promising targets not only as single-cancer treatment drugs but also as multi-treatment drugs, as sensitizers for radiation or other genotoxic anticancer drugs [7, 12, 24, 34]. The candidate molecule-targeted drugs for these two proteins have been developed, and clinical investigation on the anticancer effects of some candidates have already begun [12, 34]. KU-55933 and NU7441 are well-known inhibitors with high specificity for ATM and DNA-PK, respectively, in cells derived from various mammalian species, and have also been reported to show effects on MDCK cells [3, 6, 36]. In the present study, treatment with either KU-55933 (10 \( \mu M \)) or NU7441 (2 \( \mu M \)) did not affect MDCK cell proliferation, whereas their combination inhibited proliferation. Previous works reported that single mutant mice of either Atm or Prkdc genes are born alive, whereas double knockout mice are embryonic lethal [11]. These findings suggest that the combination of ATM- and DNA-PK-targeting drugs may be toxic to normal canine cells, even at concentrations that are not toxic in single-drug treatment. Our findings suggest that, when treating tumors in dogs, toxicity to normal cells should be kept in consideration when developing drug combinations to obtain anticancer effects, even if the drugs have no side-effect when administered alone.

In this study, our findings suggest that KU-55933 and NU7441 combination
significantly increased X-ray-induced cytotoxicity in MDCK cells, greater than the combination of irradiation with KU-55933 alone. These results suggest that both ATM and DNA-PK play a key role in the DDR and DNA repair activated by X-irradiation in canine epithelial cells, as in MDCK cells. Previously, Flässig et al. (2014) showed that phosphorylation at serine139 on H2AX after γ-irradiation was synergistically inhibited by pre-treatment with KU-55933 and/or NU7441 either alone or together in MDCK cells [6]. The suppression of cell proliferation we observed may have been caused by DSBs that could not be repaired due to suppression of the DDR and DSB repair mechanism in MDCK cells, although further studies are needed to confirm this. On the other hand, three-dimensional (3D) culture models with MDCK cells are an excellent model for lumen formation and also widely used as a kidney cyst growth model *in vitro* [31]. Recently, it has been reported that pharmacological inhibition of only ATM or DNA-PK reduced kidney cyst growth using MDCK cells [3, 36]. Altogether, MDCK cells might be a suitable cell line for evaluating radiotoxicity and toxicity of the combination treatment with radiation and novel radiosensitizers in canine cells, not only in monolayer cultures, but also in 3D cultures, although further studies are needed to clarify this.

Generally, radiation therapies have been used for the treatments of malignant tumors, but recently, radiation therapy for human ventricular tachycardia, a non-cancerous disease, has been used and is highly effective [4]. Radiotherapy for non-cancerous diseases is also expected to be used in the veterinary setting, allowing to gain further clinical insight that might improve human treatment and help develop new therapies. Thus, it will become increasingly important to accurately understand radiation toxicity in canine cells. In this study, we confirmed that the toxicity of radiation and/or drugs in MDCK cells can be detected by the simple assay used in the
present study. This simple and rapid experimental protocol can allow a pre-assessment of both the therapeutic efficacy and toxicity of various candidate compounds, including radiation sensitizers. Taken together, this and further basic studies using MDCK cells are likely to greatly contribute to gaining basic information for the advancement and expansion of various radiotherapy treatment in both dogs and human.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES


dorsal root ganglion and mitochondria through selectively inhibiting the transporter-mediated uptake thereby attenuates peripheral neurotoxicity. 

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**Figure legends**

**Fig. 1.** Clonogenic survival of Madin–Darby canine kidney (MDCK) cells treated with X-rays. The cells were irradiated with X-rays at 0.63, 1.25, 2.5 and 5 Gy. The number of colonies was counted 5 days after irradiation. (A) Representative images of the colonies formed by MDCK cells 5 days after irradiation. (B) Survival curves of MDCK cells. The experiments were performed in triplicate and data are presented as the mean ± standard deviation of three separate experiments (N=3). *p* < 0.05.

**Fig. 2.** Inhibition of the proliferation of Madin–Darby canine kidney (MDCK) cells caused by X-rays. (A) Representative cellular images of MDCK cells at 72 hr, with or without X-irradiation (10 Gy). (B) Representative nuclear morphology of MDCK cells at 72 hr, with or without X-irradiation (10 Gy). The nuclear DNA was stained with 4,6-diamino-2-phenylindole (DAPI). (C) Inhibition of the proliferation of MDCK cells after X-irradiation. The cells were irradiated with X-rays at 1.25, 2.5, 5 and 10 Gy, and the number of cells was counted 72 hr after irradiation. The
experiments were performed in triplicate and data are presented as the mean ± standard deviation of three separate experiments (N=3). * p < 0.05. Scale bar represents 200 µm (A) and 10 µm (B).

**Fig. 3.** Effects of KU-55933 and NU7441 on X-ray-induced proliferation suppression in Madin–Darby canine kidney (MDCK) cells. The drugs (KU-55933 (10 µM); NU7441 (2 µM); KU-55933 (10 µM) and NU7441 (2 µM); or solvent (DMSO)) were added 1 hr before X-irradiation and removed 24 hr after (1.25 Gy). The number of cells counted 72 hr after X-irradiation. (A) Cytotoxicity effects due to the combination of KU-55933 and NU7441 on the proliferation of MDCK cells. (B) KU-55933 and NU7441 combination enhanced the effects of proliferation suppression induced by X-irradiation, more than irradiated with KU-55933 alone. The experiments were performed in triplicate and data are presented as the mean ± standard deviation of three separate experiments (N=3). There were no statistically significant differences between the non-treated (NT) and DMSO-treated control (DMSO) groups (A, B). * p < 0.05, ** p < 0.01, and *** p < 0.001.
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