

Effect of autophagy on irradiation-induced damage in osteoblast-like MC3T3-E1 cells

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Abstract. Autophagy is activated under radiation stress, which serves an important role in maintaining bone homeostasis. However, the underlying mechanisms of irradiation-induced autophagy in bone homeostasis is not well understood. The present study aimed to determine the effects of radiation-activated autophagy on pre-osteoblastic MC3T3-E1 cells. X-ray irradiation activated autophagy in a dose-dependent manner, with an increased fluorescence intensity of monodansylcadaverine staining, increased ratio of microtubule-associated protein 1 light chain 3 β (LC3)-II/LC3-I, decreased p62 expression, and increased ATG5 and beclin-1 expression levels in MC3T3-E1 cells 72 h after irradiation compared with those in non-irradiated MC3T3-E1 cells. Irradiation reduced colony formation and mineralization in a dose-dependent manner in MC3T3-E1 cells at 2 and 3 weeks after irradiation, respectively. Decreased levels of alkaline phosphatase activity and runt-related transcription factor 2 expression were observed at 72 h post-irradiation. In addition, irradiation-induced apoptosis was accompanied by a decreased ratio of Bcl-2/BAX protein and increased the activity of caspase-3. By contrast, doxycycline (DOX)-inhibited autophagy attenuated the decreased colony formation and mineralization, and aggravated the increased cell apoptosis in irradiated MC3T3-E1

cells. Furthermore, the ratio of phosphorylated P38/P38 was observed to be higher following DOX treatment within 1 week of irradiation, which was reversed 2 weeks post-irradiation. In conclusion, DOX-inhibited autophagy aggravated X-ray irradiation-induced apoptosis at an early stage, but maintained cell proliferation and mineralization at a late stage in irradiated MC3T3-E1 cells.

Introduction

Radiation therapy serves a key role in the treatment of patients with head and neck cancer; however, it causes long-term side effects on the surrounding healthy tissues (1). The bone is one of the most commonly irradiated normal tissues, and osteoradionecrosis (ORN) of the jaw and temporal bone represents one of the most frequent radiation-induced head and neck cancer treatment complications (2,3). ORN, a type of post-radiation bone damage that includes devitalization and devascularization, is described as a chronic disease that, if left untreated, spontaneously results in disrupted bone homeostasis, with occurs in 5-15% of patients in the first 3 years after radiotherapy (4,5). Preventing radiation-induced bone tissue damage remains a challenge in clinical radiotherapy.

Macroautophagy, also referred to as autophagy, is a highly evolutionarily conserved catabolic process characterized by the formation of a double-membrane autophagosome with recruited lysosomes in the outer membrane, which degrades damaged molecules for recycling (6). Autophagy plays an important role in maintaining cellular homeostasis during environmental or intracellular stress (7). Previous studies have indicated that autophagy is associated with radiotherapy, *in vitro* and *in vivo* (8-11). In response to stress induced by radiation, autophagy is activated to promote cell survival by removing damaged cellular components (9). However, successive autophagy activation could induce cell death through the degradation of constitutive cellular components (10). Autophagic pathways behave as either cytoprotective or cytotoxic depending on the cellular context in normal and cancer cells (8-11).

Autophagy has been suggested to regulate bone homeostasis by modulating osteoblast differentiation and mineralization, as well as adjusting osteoclast differentiation and function (12,13).

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Abbreviations: DOX, doxycycline; ALP, alkaline phosphatase; MDC, monodansylcadaverine; RUNX2, runt-related transcription factor 2; LC3, microtubule-associated protein 1 light chain 3; ORN, osteoradionecrosis

Key words: irradiation, autophagy, mineralization, osteoblast, MC3T3-E1 cells

Recent studies have reported the cytoprotective effects of autophagy on osteoblast survival, differentiation and mineralization (13-15). However, it is currently unclear whether radiological stress-induced autophagy is actually cytoprotective and which molecular mechanisms are involved. The present study aimed to determine the role of autophagy in osteoblasts under radiotherapy stress using doxycycline (DOX) to inhibit autophagy by suppressing autophagy-related 5 (*Atg5*) expression in a pre-osteoblastic MC3T3-E1 cell line (16,17).

Materials and methods

Cell culture. The mouse pre-osteoblastic MC3T3-E1 cell line was obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Animal Blood Ware, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin. The complete medium was replaced every 2-3 days. The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. In MC3T3-E1 cells, *Atg5* expression was suppressed by 2.5 nM DOX for at least 48 h before subsequent experiments. An MTT cell viability assay confirmed that 2.5 nM DOX exerted no significant effects on cell viability. For mineralization, the cells were cultured in complete medium supplemented with dexamethasone (10 nM), β -glycerophosphate (10 mM) and ascorbic acid (50 mg/l) for 3 weeks in a humidified atmosphere with 5% CO₂ at 37°C (18).

Radiation. The cells were divided into the control (without irradiation), DOX (2.5 nM DOX for 48 h immediately after irradiation), irradiation and irradiation + DOX (2.5 nM DOX for 48 h immediately after irradiation) groups. For irradiation treatment, MC3T3-E1 cells were irradiated using a PRIMUS linear accelerator radiotherapeutic machine (Siemens AG). The cells were treated with X-ray irradiation doses of 0.25, 0.5, 1, 2 or 4 Gy, with the final irradiation dose reaching 4 Gy by being successively superimposed at a rate of 6 MV/min and a distance of 100 cm. To ensure the irradiation efficiency of cells, before irradiation, the medium was replaced with 1.5 ml osteogenic medium (complete culture containing 10 nM dexamethasone, 10 mM β -glycerophosphate and 50 mg/l ascorbic acid) (18). After irradiation, the osteogenic medium was supplemented to 3 ml. The cells were incubated at 37°C with 5% CO₂ for up to 72 h. The medium was replaced every 24 h.

Clonogenic assay. A total of 1×10^3 cells were plated in 60-mm culture dishes in triplicate. Irradiation was performed as described above. Following irradiation, the cells were further incubated for 2 weeks to form visible colonies. The cells were fixed with 100% methanol at room temperature for 20 min, and 0.5% Giemsa staining (Merck KGaA) was performed at room temperature for 30 min. The culture dishes were observed under white light and photographed. All experiments were repeated three times. Colonies that contained >50 cells were counted. ImageJ software (version 1.52j; National Institutes of Health) was used for quantification of the colonies.

Alizarin red staining. MC3T3-E1 cells were cultured in 12-well plates. After cells reached 60% confluence, the medium was replaced with osteogenic medium for 3 weeks. Subsequently, mineralization was analyzed by alizarin red staining. Briefly, cells were washed with PBS and fixed with 95% ethanol at room temperature for 15 min. After washing with deionized water, 0.2% Alizarin Red S (Beijing Solarbio Science & Technology Co., Ltd.) was added to the wells, followed by incubation at room temperature for 30 min. Plates were rinsed three times with deionized water and allowed to air dry, and images were captured for analysis. All experiments were repeated three times. ImageJ software (version 1.52j) was used for quantification of the grey-scale values of Alizarin red staining.

Alkaline phosphatase (ALP) activity assay. The MC3T3-E1 cell culture supernatant was used to detect ALP activity, which is one of the earliest markers expressed during osteoblast differentiation (19). ALP activity was measured by colorimetric assay using a commercial ALP assay kit (cat. no. A059-1-1; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. All experiments were repeated three times.

Autophagic vesicle detection by monodansylcadaverine (MDC) staining. MC3T3-E1 cells (5,000) were seeded on coverslips in 24-well plates, with or without DOX after irradiation at 37°C with 5% CO₂ for 72 h. Cells were washed twice with PBS and incubated with 50 μ M MDC (cat. no. D4008; Sigma-Aldrich; Merck KGaA) for 15 min at 37°C. Following incubation, the cells were washed three times with PBS, and the coverslips were analyzed using fluorescence microscopy (IX-81; Olympus Corporation). ImageJ software (version 1.52j) was used to quantify fluorescence intensity.

Hoechst 33342 staining. MC3T3-E1 cells (5,000) were seeded on coverslips in 24-well plates, with or without DOX after irradiation at 37°C with 5% CO₂ for 72 h. For Hoechst 33342 staining, the cells of each group were washed with PBS twice and fixed with 4% paraformaldehyde for 15 min at room temperature, then stained with 1 mM Hoechst 33342 for 15 min at 37°C in the dark. Apoptotic cells with clear condensation and small bright nuclei were evaluated using a fluorescence microscope.

Caspase-3 activity assay. The Caspase-3 Activity Assay kit (cat. no. G015-1-3; Nanjing Jiancheng Bioengineering Institute) was used to detect cell apoptosis. As aforementioned, after 72 h of treatment with DOX or irradiation, proteins were extracted from each group. After operating according to the instructions of the kit, the absorbance was detected at 405 nm by using a microplate reader (Tecan Group, Ltd.).

Western blot analysis. Whole cell lysates were collected using RIPA lysis buffer (Beyotime Institute of Biotechnology). The protein concentration of the cell lysates was determined using the Bicinchoninic Acid Protein Assay kit (CWBio). In the irradiation and irradiation + DOX groups, 50 μ g protein from each sample was resolved via SDS-PAGE on a 12% gel for protein separation. Fractionated proteins were transferred

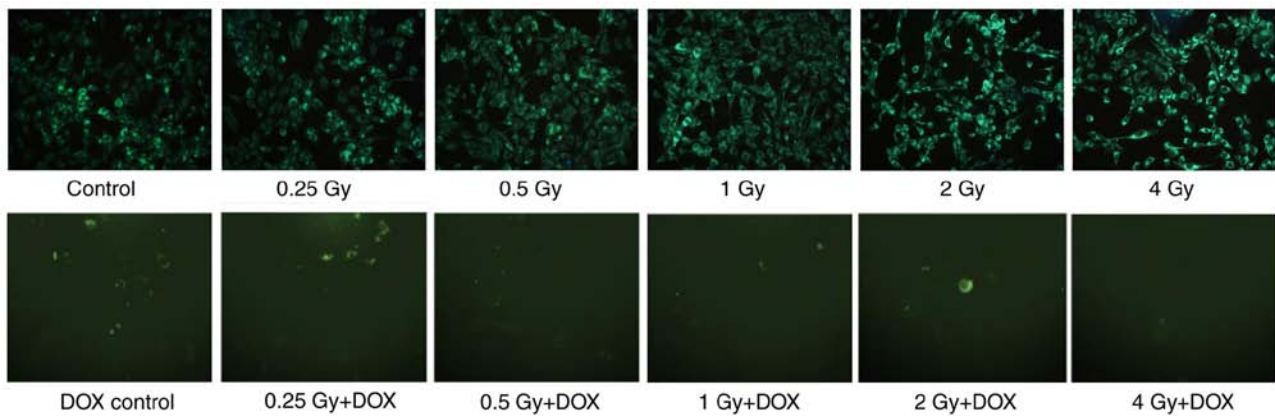


Figure 1. DOX inhibits the radiation-induced dose-dependent increase in autophagy in MC3T3-E1 cells. Monodansylcadaverine staining was used to observe the autophagy of MC3T3-E1 cells in the irradiation and irradiation + DOX groups. Magnification, x200. DOX, doxycycline.

to polyvinylidene difluoride membranes (EMD Millipore). Following blocking using 5% non-fat milk (BD Biosciences) for 1 h at room temperature, the membranes were incubated with the following rabbit polyclonal primary antibodies: β -actin (1:1,000; cat. no. 20536-1-AP), ATG5 (1:1,000; cat. no. 10181-2-AP), Beclin-1 (1:1,000; cat. no. 11306-1-AP), P62 (1:1,000; cat. no. 18420-1-AP), microtubule-associated protein 1 light chain 3 β (LC3; 1:500; cat. no. 14600-1-AP), Bcl-2 (1:1,000; cat. no. 26593-1-AP), BAX (1:1,000; cat. no. 50599-2-Ig), caspase-3 (1:1,000; cat. no. 19677-1-AP), P38 (1:1,000; cat. no. 14064-1-AP) (all from ProteinTech Group, Inc.), runt-related transcription factor 2 (RUNX2; 1:1,000; cat. no. ab76956; Abcam) and p-P38 (1:1,000; cat. no. 4631; Cell Signaling Technology, Inc.) at 4°C overnight. Goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (1:2,500; cat. no. TA130023; OriGene Technologies, Inc.) for 1 h at 37°C. Enhanced chemiluminescent reagents (EMD Millipore) were used for chemiluminescence development. β -actin was used as an internal control. All experiments were repeated three times. The band intensities were determined by gray value using Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc.).

Statistical analysis. Data are presented as the mean \pm standard deviation. Calculations and statistical tests were performed using SPSS 22.0 (IBM Corp). One-way analysis of variance with Bonferroni post hoc test was conducted to evaluate differences between the irradiation and control groups; between the irradiation + DOX and DOX control groups; and between the irradiation + DOX and irradiation groups under same irradiation dose. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

DOX inhibits autophagy in irradiated MC3T3-E1 cells. Autophagy was observed in irradiated MC3T3-E1 cells by MDC fluorescence staining at 72 h post-irradiation. The results demonstrated that the visible fluorescence intensity of the irradiation groups increased as the irradiation dose increased. In the irradiation + DOX groups, only a limited number of MDC-stained cells were observed (Fig. 1).

DOX-mediated inhibition of autophagy attenuates the irradiation-induced dose-dependent decrease in MC3T3-E1 cell colony formation and mineralization. The results of the clonogenic assay demonstrated that the colony formation rate of the irradiation groups decreased gradually in a dose-dependent manner at 2 weeks post-irradiation ($P < 0.01$; Fig. 2A and B). In the irradiation with DOX treatment groups, the clone formation rates of MC3T3-E1 cells were significantly higher compared with those of the corresponding irradiation groups ($P < 0.05$), with the exception of the 0.25 Gy-treated groups (Fig. 2A and B).

Alizarin red staining was used to observe the effects of irradiation on the mineralization ability of MC3T3-E1 osteoblasts at 3 weeks post-irradiation. The results demonstrated that the number of mineralized nodules decreased significantly with the increase in radiation dose ($P < 0.01$; Fig. 2C and D). In the irradiation + DOX groups, the number of mineralized nodules significantly increased in the 2 and 4 Gy-treated groups compared with the respective irradiation groups ($P < 0.05$; Fig. 2C and D). The ALP activity of MC3T3-E1 osteoblasts was detected by the ALP kit 72 h post-irradiation. The results demonstrated that the ALP activity was significantly decreased in the irradiation group compared with that in the control group at a radiation dose of 0.5-4 Gy ($P < 0.01$). In the irradiation with DOX treatment groups, the ALP activity levels were higher compared with those in the irradiation groups at all radiation doses ($P < 0.05$; Fig. 2E).

DOX-mediated inhibition of autophagy significantly enhances the dose-dependent increase in cell apoptosis in irradiated MC3T3-E1 cells. Hoechst 33342 staining and a caspase-3 activity assay were used to detect apoptosis 72 h post-irradiation. Hoechst 33342 staining demonstrated that irradiation of MC3T3-E1 osteoblasts increased the number of nuclear agglomerations and apoptotic bodies, which increased as the irradiation dose increased. Compared with the irradiation groups, an increased number of apoptotic bodies was observed in the irradiation + DOX groups under the same radiation dose treatment conditions (Fig. 3A). The results of the caspase-3 activity assay revealed that caspase-3 activity was significantly increased in a dose-dependent manner in the irradiation groups; in the irradiation + DOX groups, caspase-3 activity levels were

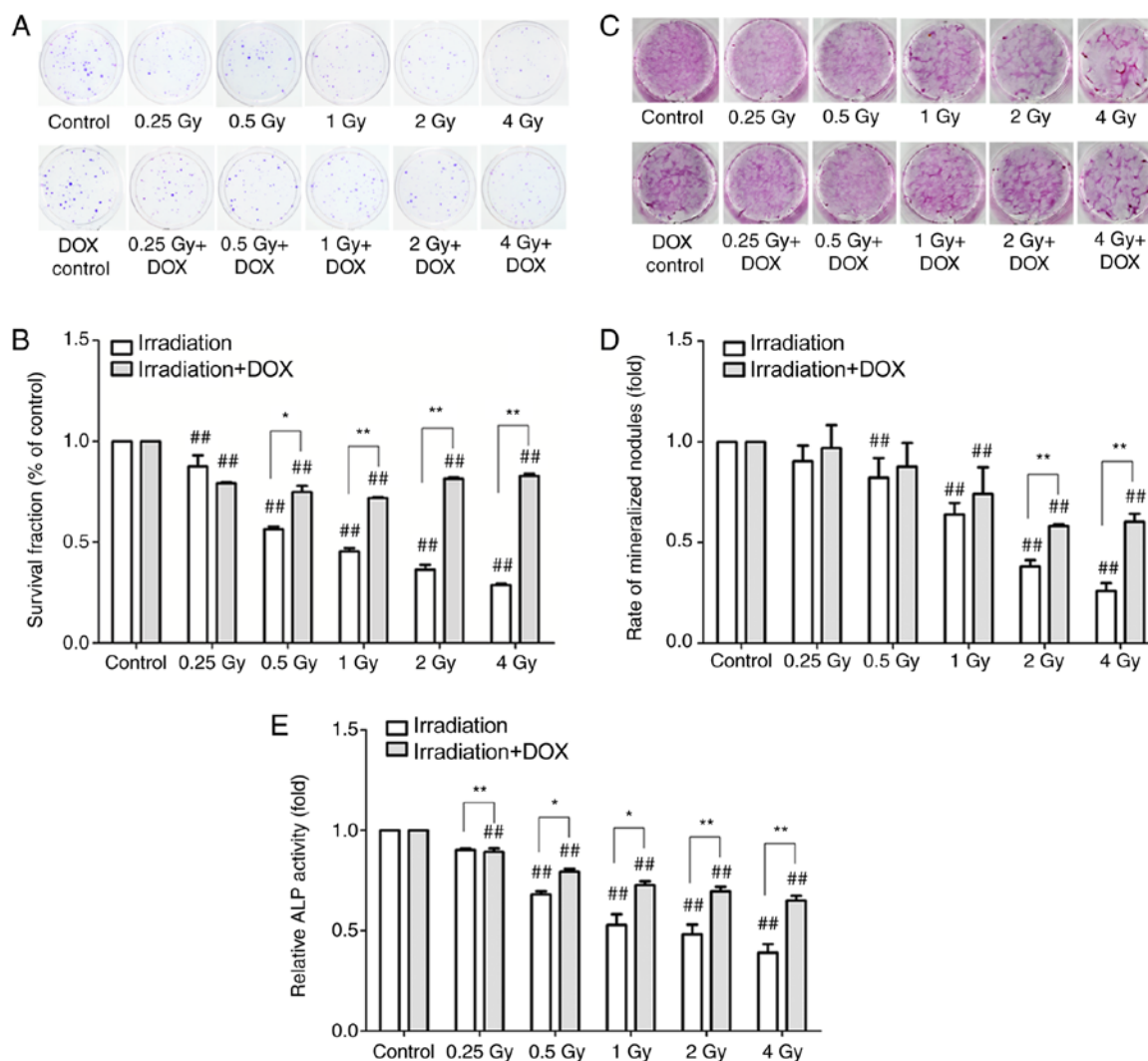


Figure 2. DOX attenuates the radiation-induced dose-dependent decrease in MC3T3-E1 cell colony formation and mineralization. (A) Representative images of the MC3T3-E1 cell colony formation assay. (B) Quantitative analysis of the colony formation rate in the irradiation and irradiation + DOX groups. (C) Alizarin red staining was used to observe mineralized nodules formed in the irradiation and irradiation + DOX groups. (D) Quantitative analysis of the mineralized nodule formation rate. (E) ALP activity of MC3T3-E1 cells in the irradiation and irradiation + DOX groups. $n=3$. $^{##}P<0.01$ vs. control or control + DOX; $^{*}P<0.05$ and $^{**}P<0.01$ vs. the irradiation group. DOX, doxycycline; ALP, alkaline phosphatase.

higher compared with those in the irradiation groups under the same radiation dose treatment conditions ($P<0.05$; Fig. 3B).

Effects of DOX-mediated inhibition of autophagy on protein expression in irradiated MC3T3-E1 cells. Western blot analysis was performed to detect autophagy- and apoptosis-associated protein expression levels in all groups at 72 h post-irradiation (Fig. 4A-B). Compared with control group, the expression levels of autophagy pathway-associated proteins in the irradiation groups were significantly altered; increases in the LC3-II/LC3-I ratio, ATG5 and beclin-1 expression levels, and decreases in P62 expression levels were observed (Fig. 4C-F). In addition, the expression levels of mitochondrial apoptosis pathway-associated proteins in the irradiation groups were significantly different compared with those in the control group, with the ratio of Bcl-2/BAX decreased and the expression of pro-caspase-3 increased (Fig. 4G and H). The expression of the bone development-related transcription factor RUNX2 was significantly decreased in the irradiation groups compared with that in the control group (Fig. 4I). Compared with the irradiation

groups, the changes in the expression levels of the autophagy pathway-associated proteins were attenuated, with the exception of P62, and the radiation-induced alterations in the expression of the mitochondrial apoptosis pathway-associated proteins were enhanced in the irradiation with DOX treatment groups (Fig. 4C-H). However, the expression of RUNX2 in the irradiation with DOX treatment groups was higher compared with that in the respective irradiation groups. Compared with the DOX control group, the expression of RUNX2 increased significantly at 0.25 Gy and then gradually decreased with increasing radiation doses in the irradiation with DOX treatment groups (Fig. 4I). These results indicated that DOX inhibited autophagy in irradiated MC3T3-E1 cells, which may have contributed to the increased apoptosis and cell mineralization.

Effects of DOX-mediated autophagy inhibition on MC3T3-E1 cells at different time points. The aforementioned results showed that cell apoptosis was increased at 72 h, whereas mineralization and clone formation were also increased at 2 and 3 weeks after DOX inhibited autophagy.

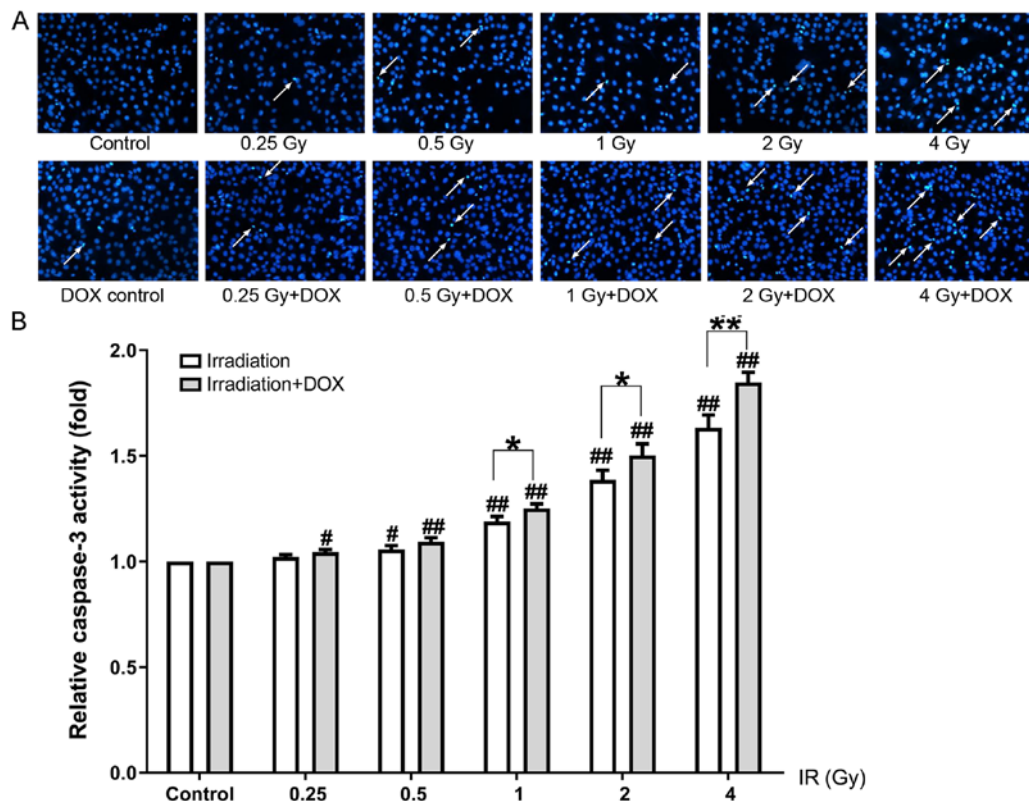


Figure 3. DOX enhances the levels of apoptosis in irradiated MC3T3-E1 cells. (A) Hoechst 33342 staining was used to observe the morphological changes associated with apoptosis in MC3T3-E1 cells in the irradiation and irradiation + DOX groups. White arrowheads indicate the nuclei of apoptotic cells. Magnification, $\times 100$. (B) Caspase-3 activity in the irradiation and irradiation + DOX groups. $n=3$. $^{\#}P<0.05$ and $^{##}P<0.01$ vs. control or control + DOX; $^{*}P<0.05$ and $^{**}P<0.01$ vs. the irradiation group. DOX, doxycycline; IR, irradiation.

These results seem to be contradictory, but perhaps this phenomenon can be explained when considering the different detection time points of each experiment. The P38 and p-P38 expression, ALP and caspase-3 activity levels were further examined at 72 h, 1 and 2 weeks post-4 Gy irradiation with or without DOX, and the results demonstrated that the ratio of p-P38/P38 and caspase-3 activity were increased, whereas ALP activity was decreased in the irradiation with DOX treatment group compared with those in the irradiation group at 72 h. This trend was maintained at 1 week post-irradiation. However, the ratio of p-P38/P38, caspase-3 and ALP activity levels were reversed at 2 weeks post-irradiation, with lower levels observed in the irradiation + DOX group compared with those in the irradiation group (Fig. 5A-D). These results may explain why DOX-inhibited autophagy promoted apoptosis at 72 h post-irradiation, and cell mineralization and clone formation at 2 and 3 weeks post-irradiation, respectively.

Discussion

ORN of the jaw is a common late complication of radiotherapy in patients with head and neck malignancies (2,3). A radiation dose of >70 Gy is the main cause of ORN (4,5). Previous studies have suggested that radiation-induced vascular injury and fibrosis may comprise the pathophysiology of ORN, but the mechanism is still being explored (2,20). In addition, radiation-induced autophagy plays an important role in cell survival and bone homeostasis (12,13). In the present study,

the effects of autophagy in irradiated MC3T3-E1 cells were explored.

Autophagy is considered to be a protective cell response to stress conditions, such as ionizing radiation, toxic stimulation and chemotherapy (9,11). However, autophagy is also the mechanism of cell death in certain contexts (10). According to Kim *et al* (10), concurrent induction of apoptosis and autophagy leads to a lower cell survival rate and higher radiosensitivity compared with activation of apoptosis alone in H460 lung cancer cells and transplanted tumor models. The positive effect of autophagy on radiosensitization may be the primary mechanism underlying the promotion of cell death (11). Therefore, it is important to distinguish between the autophagy that protects cells and the autophagy that accelerates cell death (11). Beclin-1 induces autophagy initiation and nucleation (6,11). The combination of ATG5 complexes and the autophagosome membrane subsequently promotes the recruitment of LC3 to autophagy vacuoles (6,11). LC3 is an autophagy precursor and a protein marker of autophagy (6). P62 is a ubiquitin-binding protein, and aggregation of ubiquitinated protein is used as a marker for autolysosome degradation and autophagy flux. P62 expression is negatively associated with autophagy, and damaged autophagy is often accompanied by P62 accumulation (6,7). In the present study, the autophagy level was observed by MDC staining to be increased in irradiated MC3T3-E1 cells compared with the non-irradiated control group. The results demonstrated that the fluorescence intensity increased as the irradiation dose increased. DOX was added to inhibit autophagy, and the fluorescence intensity appeared

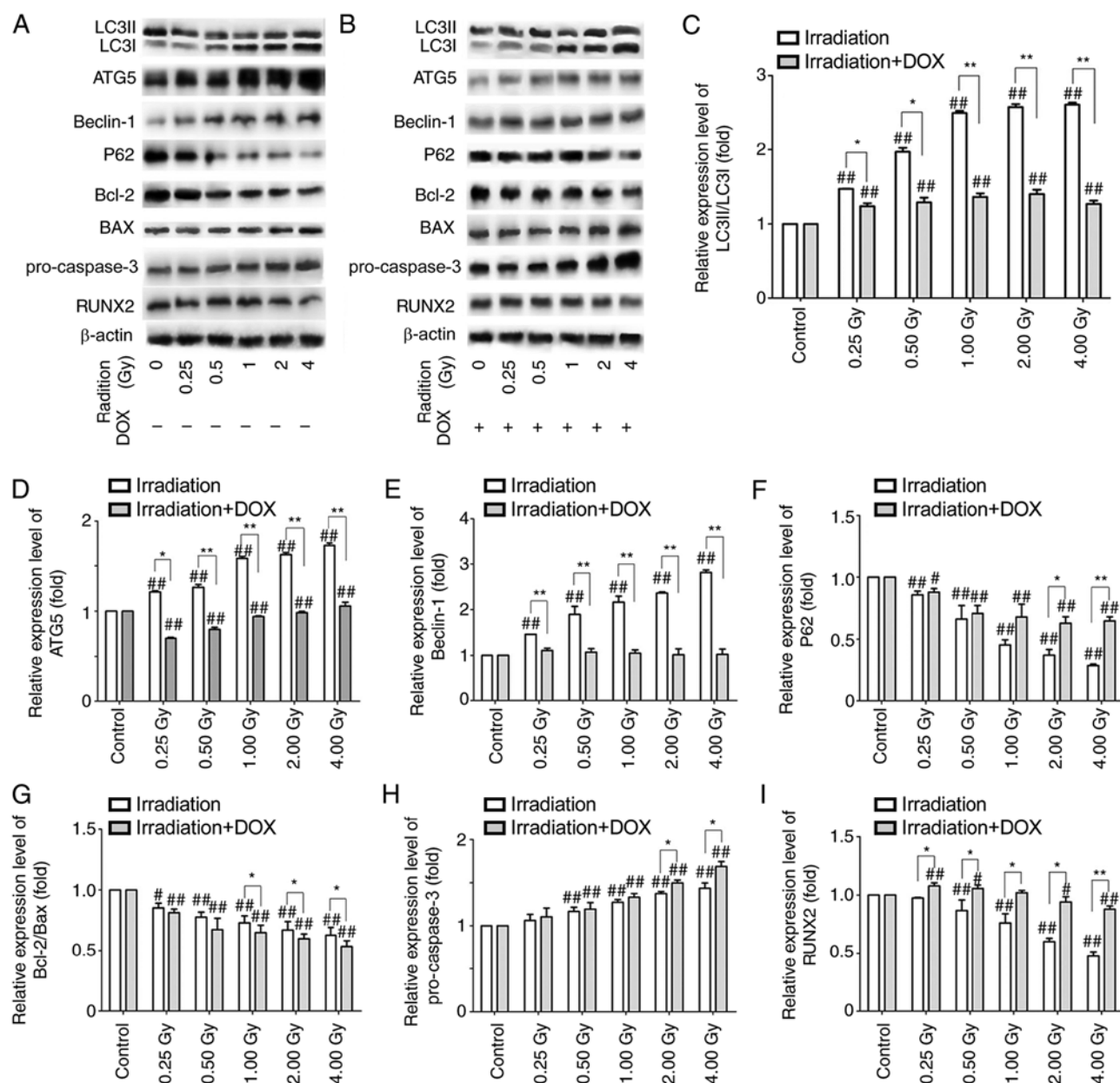


Figure 4. Western blot detection of the expression levels of proteins in the irradiation and irradiation + DOX groups. (A and B) Representative blots of the (A) irradiation and (B) irradiation + DOX group. (C-I) Protein expression levels of (C) LC3II/LC3I, (D) ATG5, (E) beclin-1, (F) P62, (G) Bcl-2/BAX, (H) pro-caspase-3 and (I) RUNX2, as assessed by western blot analysis in the irradiation and irradiation + DOX groups. β -actin was used as an internal control. $n=3$. * $P<0.05$ and ** $P<0.01$ vs. control or control + DOX; # $P<0.05$ and ## $P<0.01$ vs. the irradiation group. LC3, microtubule-associated protein 1 light chain 3 β ; ATG5, autophagy related 5; Bcl-2, B-cell lymphoma 2; BAX, Bcl-2 associated X protein; RUNX2, Runt-related transcription factor 2; DOX, doxycycline.

to be decreased in each group compared with the respective irradiation group. The results of western blot analysis revealed that the ratio of LC3 II/LC3 I as well as the levels of ATG5 and beclin-1 were increased, and those of P62 protein expression were decreased with the increase in the radiation dose in the irradiation groups compared with those in the control group. In addition, compared with the irradiation groups, the changes in the ratio of LC3-II/LC3-I, ATG5 and P62 expression were attenuated, and P62 exhibited a certain degree of accumulation in the irradiation with DOX treatment groups. Beclin-1 acts as a core protein in the phosphatidylinositol-3-kinase complex, which is required for vesicle nucleation (6,11). The expression of beclin-1 did not change with increasing radiation doses in the irradiation with DOX treatment groups, which

may have occurred due to DOX suppressing the expression of ATG5 rather than beclin-1. These results suggested that DOX inhibited the radiation-induced increases in autophagy and obstructed the autophagy process in irradiated MC3T3-E1 cells.

The results of the present study also demonstrated that the clone formation rate, mineralization ability and ALP secretion decreased in the irradiation groups with the increase in the radiation dose. However, those trends were attenuated following autophagy inhibition by DOX. In addition, the expression levels of RUNX2, which is a key transcription factor in osteoblast differentiation (12,21), were downregulated following irradiation compared with those in the control group, which may have impaired cell mineralization. However, DOX-inhibited

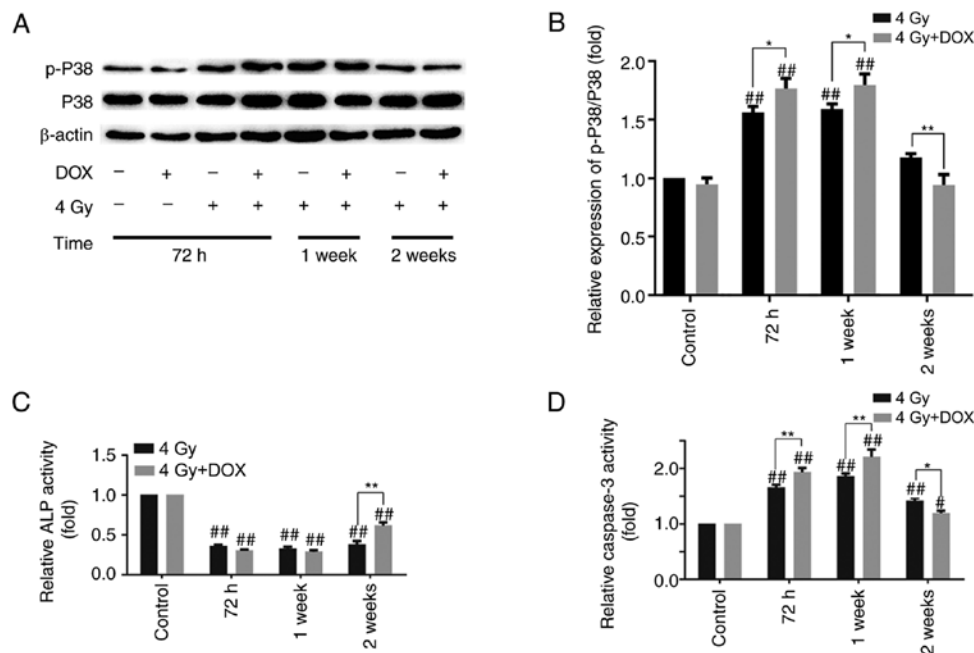


Figure 5. Effects of DOX-mediated autophagy inhibition on MC3T3-E1 cells at different time points. (A) Representative images and (B) relative expression levels of p-P38/P38, (C) ALP activity and (D) caspase-3 activity in the irradiation and irradiation + DOX groups at 72 h, 1 week and 2 weeks after 4-Gy irradiation. β -actin was used as an internal control. $n=3$. [#] $P<0.05$ and ^{##} $P<0.01$ vs. control or control + DOX; * $P<0.05$ and ** $P<0.01$ vs. the irradiation group. ALP, alkaline phosphatase; DOX, doxycycline; p-P38, phosphorylated P38.

autophagy reversed the expression of RUNX2 in irradiated MC3T3-E1 cells. Taken together, these results suggested that irradiation inhibited osteoblast differentiation and mineralization in MC3T3-E1 cells, and that DOX-inhibited autophagy appeared to protect MC3T3-E1 cells from radiation damage. Kook *et al* (21) have suggested that irradiation-mediated ALP levels are decreased in MC3T3-E1 cells compared with non-irradiated cells, and that the oxidative stress-mediated nuclear factor erythroid 2-related factor 2/heme oxygenase 1 pathway under irradiation is responsible for the inhibition of mineralization and differentiation. However, previous studies have reported that low-dose irradiation promotes mineralization (22), and autophagy promotes the differentiation and mineralization of osteoblasts (13), which contradicts the results observed in the present study. This contradiction may be associated with the different response of osteoblasts to radiation stimulation in different environments. For example, RUNX2 mRNA expression was increased in osteoblast precursors after 2 or 4 Gy radiation treatment, whereas its expression was decreased in osteoblasts under the same treatment conditions (23). In osteoblastic MC3T3-E1 cells, reactive oxygen species production was decreased by low dose radiation (1.5 mGy), but increased by high dose radiation (15 mGy) (24). Thus, the role of radiation-induced autophagy in osteoblasts should be further studied.

The mitochondrial apoptosis pathway has been reported to be mediated by oxidative stress under irradiation in osteoblasts (25,26). In the present study, increased caspase-3 activity levels and numbers of apoptotic bodies were observed in irradiated MC3T3-E1 cells, as demonstrated by Hoechst 33342 staining and the caspase-3 activity assay. Western blot analysis revealed that irradiation decreased the ratio of Bcl-2/BAX but increased the expression levels of caspase-3 compared with those in the control group, which suggested that

irradiation promoted MC3T3-E1 apoptosis. Additionally, the treatment of irradiated MC3T3-E1 cells with DOX enhanced the increased number of apoptotic bodies, decreased the ratio of Bcl-2/BAX and upregulated caspase-3. Collectively, these findings suggested that irradiation-induced apoptosis in MC3T3-E1 cells was aggravated by the autophagy inhibitor DOX.

The aforementioned results suggested that autophagy was activated, mineralization and proliferation were decreased, and the apoptotic rate was increased in irradiated MC3T3-E1 cells compared with the control group, which was reversed by DOX-inhibited autophagy with the exception of apoptosis. The apoptotic, mineralization and clonogenic assays were performed at 72 h, 2 and 3 weeks post-cell treatment, respectively; the different detection time points may be responsible for the different results regarding the increases in cell mineralization, proliferation and apoptosis in the irradiation with DOX treatment groups compared with the irradiation groups.

Alwood *et al* (27) have reported that the expression of osteogenic genes in bone marrow stem cells or the bone is higher at the early compared with the late stage of irradiation. Liu *et al* (13) have revealed that the effects of autophagy on osteoblast differentiation are observed at a later stage, but not at the early stage. Different stages of autophagy have also exhibited different effects on MC3T3-E1 apoptosis; autophagy inhibits apoptosis during intracellular autophagosome formation, but promotes apoptosis after autophagosomes are fully formed (28). Furthermore, oxidative stress has been reported to serve a crucial role in irradiation-induced cell damage (29,30). P38 MAPK can be activated in response to oxidative stress and is associated with apoptosis (29-31). p-P38 MAPK serves important roles in MC3T3-E1 cell function (29-31). The results of the present study demonstrated that irradiation

increased the ratio of p-P38/P38 in MC3T3-E1 cells 1 week post-irradiation, and the ratio of p-P38/P38 was higher in the irradiation + DOX groups compared with that in the respective irradiation groups. However, at 2 weeks post-irradiation, the ratio of p-P38/P38 was observed to be lower in the irradiation with DOX treatment group compared with that in the irradiation group. The fluctuation in the p-P38/P38 ratio may indicate that the DOX-mediated inhibition of autophagy promoted apoptosis in the early stage of irradiation. Further induction of DOX reversed the radiation-induced apoptosis, promoting cell proliferation and maintaining the proliferation and mineralization of MC3T3-E1 cells at a later stage after irradiation.

In conclusion, the results of the present study indicated that autophagy was activated, cell mineralization and proliferation were decreased, and apoptosis was increased in irradiated MC3T3-E1 cells compared with the non-irradiated controls. Following the inhibition of autophagy by DOX, the decreases in cell mineralization and proliferation were alleviated, whereas the increase in the apoptosis rate was aggravated. p-P38 MAPK may be involved in the effects of DOX-inhibited autophagy at different time points after irradiation in MC3T3-E1 cells. The above molecular mechanisms may help understand the pathogenesis of ORN and provide a new target for the treatment of ORN.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YL and BL designed the experiments and revised the manuscript. RL, WY and XH performed the experiments and analyzed the data. RL and WY drafted the manuscript. DZ, KH and CW analyzed the data and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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