J. Radiat. Res., 48, 281–288 (2007)

Regular Paper

Low Dose Radiation Increased the Therapeutic Efficacy of Cyclophosphamide on S_{180} Sarcoma Bearing Mice

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Low dose radiation/S_{180} sarcoma/Apoptosis/Cell cycle.

We examined whether low dose radiation (LDR) exposure (75 mGy) could increase the therapeutic efficacy of cyclophosphamide (CTX) by comparing the effects of tumor suppression, tumor cell apoptosis, cell cycle and proliferation of bone marrow *in vivo*. Kunming mice implanted with S_{180} sarcoma cells were given 75 mGy whole body γ-ray radiation exposure and CTX (300 mg/kg) by intraperitoneal injection 36 hours after LDR. Proliferation of bone marrow and tumor cells was analyzed by flow cytometry. Cytochrome c leakage from the tumor was measured by Western-blot. We discovered that tumor growth was significantly reduced in the group exposed to CTX add to LDR. The apoptosis of tumor cells increased significantly after LDR. The tumor cells were arrested in G_1 phase in the groups treated with CTX and CTX + LDR, but cell cycle was more significantly arrested in mice exposed to LDR followed by CTX than in mice exposed only to LDR or CTX chemotherapy. Concentration of bone marrow cells and proliferation index in CTX + LDR mice were higher than those in the untreated mice. LDR or CTX + LDR could induce greater cytochrome *c* levels and caspase-3 activity in tumors. These results suggest that low dose radiation can enhance the anti-tumor effect of the chemotherapy agent CTX markedly. Furthermore, LDR significantly protects hematopoetic function of the bone marrow, which is of practical significance on adjuvant chemotherapy.

INTRODUCTION

Experimental studies have shown that low doses of ionizing radiation induce various cancer types with different effects. The effects include radioadaptive response, activation of immune function, and enhancement of resistance to high-dose radiation in the initial slope of cell survival curve.[1,2] In recent years, studies on the effects of low dose radiation (LDR) have attracted the attention of scientists in radiation research.[3,4] Joiner and colleagues[5] have shown low doses of ionizing radiation lead to a unique radiobiologic phenomenon, which is an initial phase of hyper-radiosensitivity (HRS) to low doses radiation. The discovery that HRS does not stimulate cellular repair mechanisms, such as those seen at higher doses, provides a plausible explanation for why there is no induction of radio resistance with HRS, as measured *in vitro*.[6–8] To take advantage of the benefits of HRS in the clinical setting, one logical method to exploit the enhanced cell death at low doses of radiation is to combine it with systemic chemotherapy.

We recently reported that LDR could markedly improve the tumor therapeutic efficiency of chemotherapy via the reduction of chemotherapeutic damage on the immune system and stimulation of the antitumor immune reaction in tumor-bearing mice. It was found that the immune depression caused by a larger dose of X-rays could be reduced, to a certain extent, by low dose pre-irradiation.[9] The immune indices also improved in the tumor-bearing mice with LDR combined with chemotherapy. The tumor diameters in mice treated with CTX (Cytoxan, CTX) and LDR at different times after treatment were much smaller than those of the mice treated with CTX chemotherapy alone. The cytotoxic activities of NK, LAK and specifically CTL were significantly increased in LDR + CTX mice compared with mice treated with CTX chemotherapy alone. Swatee Dey *et al*[10] have also found that low-dose ionizing radiation delivered in fractionated form (ultrafractionation) acts synergistically with chemotherapy *in vitro*. Preclinical data indicates that

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This work was supported by National National Scentific Foundation of China (No: 30030781).

doi:10.1269/jrr.06093

low-dose ionizing radiation provides optimal cell killing *in vitro* when combined with chemotherapy.

There is growing evidence that factors that influence apoptotic cell death may contribute to the outcome of cancer therapy. Induction of apoptosis in tumor cells has an important role in the efficiency of radiation therapy and chemotherapy. Considerable evidence suggests that Cytochrome *c* release and caspases are essential components of the mammalian cell death machinery. Since the proteolytic cleavage of proteins is largely irreversible, activation of these enzymes may represent a rate-limiting step in apoptosis. Thus, elucidating the role and regulation of caspases is essential for a complete understanding of how apoptosis is induced by radiation.[11]

In light of the chemo-potentiating properties of LDR, as well as its documented effects on cell cycle and apoptosis, we designed this study to investigate the effect of chemotherapy using CTX in combination with single low dose radiation as a chemo-potentiator for CTX, and to compare the chemo-potentiating effects of single standard dose radiation with single chemotherapy and radiation. Furthermore, we studied the mechanism of chemo-potentiation by single dose radiation at 75 mGy by analyzing the kinetics of cell cycle, tumors and chemotherapy-sensitive marrows.

MATERIALS AND METHODS

Experimental animals and grouping

Protocols for animal care and experimental management were approved by the Affiliated Hospital of Qingdao University Scientific Committee, Qingdao Animal Research Institute. Sixty healthy Aseptic Kunming strain male mice, weighing 18–22 grams and aged 4–6 weeks, provided by Drug Control Institution of Qingdao, were randomly divided into five groups (15 animals/group): blank control group (BC group); direct tumor-bearing group (DT group); LDR tumor-bearing group (75 mGy, LDR group); CTX (Tianjin Jinshi Pharmaceutical Co,. Ltd. Tianjin, China) chemotherapy group (300mg/kg, CTX group) and LDR (75 mGy) tumor-exposure with CTX chemotherapy (300mg/kg) group (LDR + CTX group). The mice were cared for and handled according to the national regulations for experimental animals and raised conventionally without limits on water and food.

Implantation of tumor cells

S_{180} sarcoma cells, kindly provided by Shandong Medical Institute, were passaged twice in the abdominal cavity of the mice. Suspension containing 6.5×10^7/mL cells in logarithmic growth phase was conventionally prepared and 6.5×10^6 cells were implanted subcutaneously in the left inguen of the mice.

Xenograft growth measurement

The average tumor sizes were directly measured and calculated with the following standard formula every other day. Maximum horizontal diameter *a* (cm) and vertical diameter *b* (cm) were measured twice respectively with a slide gaud (Yu HS, *et al.* 2004). Mice with tumors that were either too large ($ab > 1.00\text{cm}^2$) or too small ($ab < 0.20\text{cm}^2$) were removed. The average tumor sizes were calculated according to the following formula: $V = (1/2)ab^2$.

Irradiation protocol followed by CTX chemotherapy

The mice were put in a carton, with a water mold in the middle to filter radiation, and irradiated at the desired dose 75 mGy using the FCC-7000 iso-centricity revolution ^{60}Co therapeutic machine (Shandong Xinhua Medical Apparatus Factory, Shandong, China). The radiation distance was 209.5 cm, and the dosage rate was16.404 mGy/min.

Five days after the implantation, if no difference was observed between the sizes of tumors among all the groups, the mice of the LDR and LDR + CTX groups were given 75mGy γ-irradiation, and 36 hours later 3 mg CTX were injected into the abdominal cavity of the CTX and LDR + CTX groups. We repeated the process of LDR and/or CTX injection on the 8^{th}–11^{th} days from first LDR exposure and measured the tumor diameters every other day.

Preparation of bone mallow cells

All the mice were sacrificed on the 12^{th} day by cervical dislocation. Tumor tissues were removed entirely at necropsy, exsanguinated via washing with PBS, and placed on dry ice immediately. BM cells were flushed from long bones with phosphate-buffered saline (PBS)/l mmol/L EDTA using a syringe with a 23-gauge needle. Cells were separated from the matrix core by manual pipetting, filtered through gauze, washed twice in PBS, and pelleted at 1,200 rpm for 7 minutes.

Observation of cell death under transmission electron microscopy

A piece of the tumor tissue was taken, fixed in 0.2 M cacodylate buffer/3% glutaraldehyde for 30 min, dehydrated, and embedded with Epon812 following fixation. The fixation was removed with three rinses in 0.2 M cacodylate. Samples were then postfixed in 1% OsO_4 in 0.1 M cacodylate for 1 hr, rinsed once in dH_2O, dehydrated through a graded ethanol series, and embedded in epon-araldite. Thin sections were cut on a Reichert Ultracut E, picked up on copper grids, and poststained with uranyl acetate and Reynold's lead citrate. Grids were examined and observed under JEM-1200EX transmission electron microscopy (TEM, JEM-1200EXII, Jed Ca, Japan).

Cell Cycle analysis of tumor cells and marrow cells

Pieces of the tumor tissue were made into fresh single cell suspensions mechanically. Marrow cells from double thigh bones of the mice were sucked out and made into single cell suspensions. Approximately 10^6 tumor cells or marrow cells

were harvested to determine cell cycle progression using a flow cytometric analysis. Cells were washed in phosphate-buffered saline and fixed in ice-cold 70% ethanol for 30 min. After an additional wash step, cells were stained overnight with propidium iodide. The stained cells were analyzed using a FACScalibur (BD Biosciences, NJ, USA) flow cytometer. The software packages CELLquest (BD Biosciences, NJ, USA) and Modfit LT (Verity) were used to calculate the proliferation index (PI) using the following formula: PI = (S + G2/M)/(G0/G1 + S + G2/M).The cell cycle analysis was repeated three or four times for each experimental setting, and a *t* test was performed to determine statistically significant differences between the various double knock-out cell lines and their controls.

Cell viability

Approximately 1×10^5 cells were collected for trypan blue exclusion analysis and scored within 1 hr of stopping treatment. At least 200 cells were counted for each point. The percentage of dead cells was also determined by flow cytometric analysis using annexin-propidium iodide (PI) and Annexin V-FITC costaining according to the manufacturer's (R & D Systems) instructions.

Cytochrome c release assay

Isolated tumor cells (5×10^7) were collected by centrifugation at 600 *g* for 5 minutes at 4°C and washed with ice-cold PBS. The cells were assayed with the cytochrome *c*–releasing apoptosis kit (BioVision, CA, USA). Briefly, they were homogenized with the cytosol extraction buffer provided in the kit and then centrifuged at 700 *g* for 10 minutes at 4°C to remove the debris. The supernatant was then centrifuged at 10,000 *g* for 30 minutes at 4°C. The pellet contained the mitochondrial fraction, and the supernatant was collected as the cytosolic fraction. These fractions were analyzed for cytochrome *c* by Western-blot, using the cytochrome *c* antibody provided in the kit.

Caspase-3 activity assay

Tumor cells were lysed in lysis buffer [1% Triton X-100, 150 mM NaCl, pH 7.7, supplemented with protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany)] for 30 minutes on ice. The protein concentration of the lysates was determined using the bicinchoninic acid (BCA) method (Pierce Biotechnology, Rockford, IL). Equivalents of 30 μg protein for each sample were diluted in caspase activity buffer (20 mM Hepes, 10 mM dithiothreitol, 10% glycerol, 100 mM NaCl, pH 7.5) to a total volume of 120 μl. Caspase-3 substrate was added to the samples to a final concentration of 2 μM. The efficiency of the substrate cleavage by active caspase-3 was analysed using a luminescence spectrometer LS50B (PerkinElmer, Norwalk, CT, USA) immediately after substrate addition and after 1 hour incubation at 37°C.

Statistical analysis

Results were expressed as mean ± SD. For multiple comparisons, continuous parametric data were subjected to analysis of variance (ANOVA) followed by the Student-Newman-Keuls post-hoc test for between-group differences. Results were considered statistically significant at $P < 0.05$.

RESULTS

Effect of radiation and CTX on tumor growth

Figure 1 shows the effects of LDR combined with CTX on the cell apoptosis and cell cycle of S_{180} sarcoma xenografts in Kunming strain male mice. Low dose radiation with abdominal cavity administration of CTX (300 mg/kg) suppressed tumor growth of the xenograft significantly. On *day 11*, the volume of the xenograft was significantly smaller in the groups treated with CTX than in the control group and the volume of the xenograft was significantly smaller in the LDR + CTX group than the CTX group. Suppression of tumor growth by LDR combined with CTX was observed even when the agent was given to the host animals 7 days after the cancer xenograft was established (Fig. 1).

Assay of tumor cell apoptosis and cell cycle

Several different batches of irradiated mice were analyzed for the number of cell cycle phase S stage cells over time. Apoptotic indices of tumor xenografts were also measured. The rate of apoptosis in mouse tumor cells and the number of S stage cells was more significantly increased in the LDR group than in the direct tumor-bearing group, while the number of G_2/M stage cells was more significantly decreased. Few apoptotic cells were observed in the CTX and LDR + CTX groups. In the LDR + CTX group, the number of G_1 stage cells increased, but the number of S stage cells decreased significantly (Table 1) (Fig. 3).

Ultrastructure observation under electron microscopy

Tumor cells were highly vacuolized, contained swollen mitochondria, and appeared incapable of completely breaking down various cellular organelles. Although some of these characteristics are suggestive of necrotic cell death, the terminal processes of apoptosis and phagocytosis of apoptotic bodies by macrophages were observed under transmission electron microscopy. It confirmed that LDR could induce tumor cell apoptosis that resulted in aberrant chromatin condensation (Fig. 2).

Effect of LDR and CP on Cytochrome c Leakage

The leakage of cytochrome *c* from mitochondria into the cytoplasm is known to activate caspases and initiate apoptosis. We therefore examined the leakage of cytochrome *c* from the tumor cells of the different groups. The cytochrome *c* in the cytoplasm was determined using methods described previously. As seen in Fig. 3, there was a marked increase

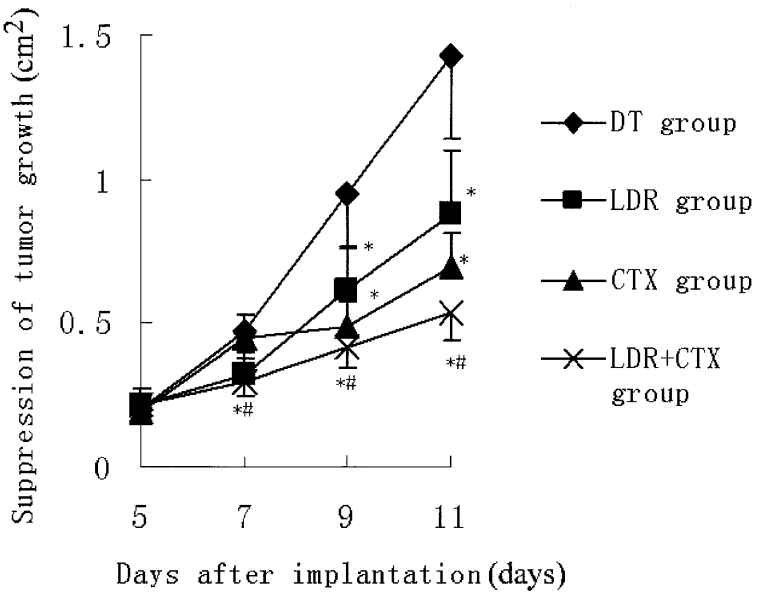

Fig. 1. The average sizes of tumors exposed to LDR and CTX. The tumors were directly measured and size was calculated using the following formula: $V = (1/2)ab^2$. Data show the mean ± S.E.M. for 10 animals in each group, analyzed in three separate experiments. Tumor sizes of mice exposed to low dose radiation combined with CTX are significantly less than that of direct tumor-bearing mice. Compared with DT group * $P < 0.05$; LDR + CTX group compared with CTX group # $P < 0.05$.

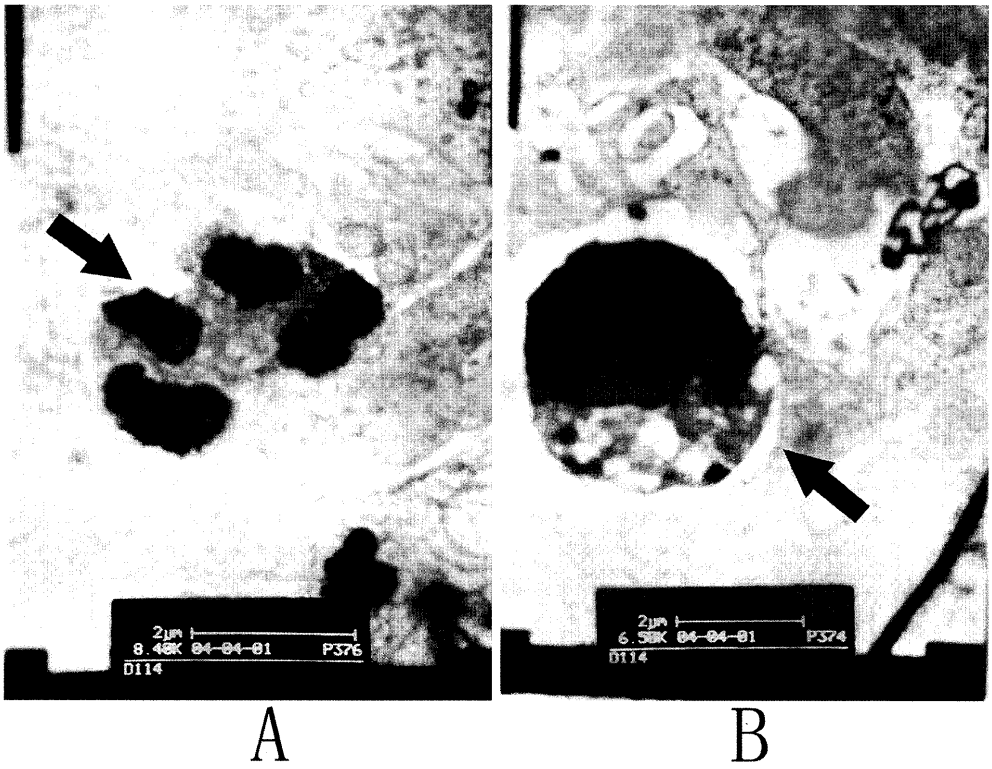

Fig. 2. Ultrastructure characteristic change of tumor cells at 48 h after LDR. Processing for electron microscopy visualization was described in Materials and Methods section. (A) phenomenon of the terminal process of apoptosis (original magnification × 15000). Note the aberrant chromatin condensation (*arrow* shown) and vacuolization of the tumor cells. (B) phenomenon of macrophages phagocytizing apoptotic bodies and forming secondary lysosome (× 15000) (*arrow*). Round apoptotic bodies were seen with intact nuclear and cytoplasmic membrane.

Table 1. Changes of tumor cell apoptosis and cell cycle at 12 days after first LDR exposure (n = 15)

Group	Apoptotic rate (%)	Cell cycle (%)		
		G_1/G_0	S	G_2/M
BC	6.66 ± 2.73	52.05 ± 5.83	34.57 ± 4.67	13.38 ± 4.39
LDR	15.45 ± 7.42	46.03 ± 5.34*	48.49 ± 6. 7*	5.47 ± 5.03*
CTX	16.52 ± 6.86	68.94 ± 5.40*	15.5 ± 4.92*	15.6 ± 3.71
LDR + CTX	25.21 ± 11.33*	73.57 ± 4.62*#	11.61 ± 4.80*#	14.56 ± 4.32

Note: compared with BC group * $P < 0.05$; LDR + CTX group compared with CTX group # $P < 0.05$

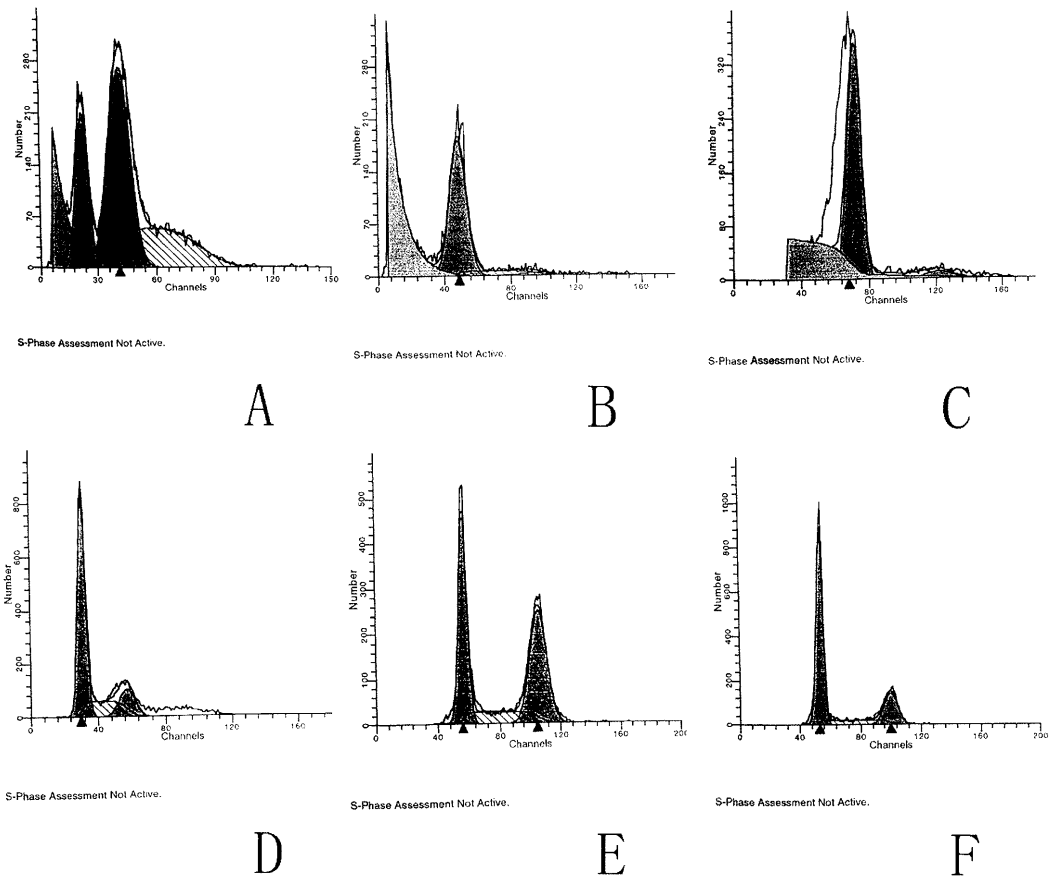

Fig. 3. FACS analysis of tumor cell apoptosis and cell cycle. Tumor cells and marrow cells of thighbone of the mice were collected and made into single cell suspension. Flow cytometric measurements were carried out on a FACScan flow cytometer according to Meterials and Methods. Tumor cells from: (A) DT group, (B) CTX group, (C) CTX + LDR group, and marrow cells from (D) DT group, (E) CTX group, (F) CTX + LDR group were compared respectively. LDR can increase apoptosis in tumor cells and number of cell cycle phase S stage cells.

of cytochrome *c* leakage in LDR-treated tumor cells compared with that in the untreated control cells. Moreover, there was a greater cytochrome *c* leakage in S_{180} sarcoma cells derived from the mice treated with LDR followed with CTX compared to the cells of mice treated with CTX only. Thus, the leakage of cytochrome *c* correlated with the exposure of the tumor cells to LDR and CTX.

Activity of caspase-3

The induction of apoptosis strongly correlates with the activation of effector caspases. To show that enhancement of caspase-3 activity was due to cytochrome *c* leakage, and induction of apoptosis, we performed caspase-3 activity assays to determine the effect of the LDR and CP on the caspase activity. The results of these experiments were fully consistent with the observations obtained for the cytochrome

Table 2. Proliferation of mouse bone marrow at 12 days after first LDR exposure (n = 15)

Group	Concentration of cells (10^7/ml)	Cell cycle (%)			
		G_1/G_0	S	G_2/M	PI
BC	$(3.26 \pm 1.89) \times 10^2$	72.82 ± 4.78	22.60 ± 3.62	5.56 ± 4.12	28.16 ± 4.78
DT	$(2.70 \pm 1.42) \times 10^2$	67.89 ± 5.72*	25.72 ± 5.71	5.33 ± 4.39	32.11 ± 5.72*
LDR	$(2.83 \pm 1.67) \times 10^2$	69.23 ± 4.46	24.91 ± 5.06	5.85 ± 4.72	30.77 ± 4.46
CTX	5.17 ± 2.49*	39.27 ± 5.02*	17.44 ± 4.30*	43.28 ± 3.21*	60.73 ± 5.02*
LDR + CTX	45.6 ± 2.17*#	33.33 ± 5.13*#	20.80 ± 4.19#	45.86 ± 5.1*	66.67 ± 5.13*#

Note: compared with BC group * $P < 0.05$; LDR + CTX group compared with CTX group # $P < 0.05$.

c leakage assay. The caspase-3 activity is influenced by the LDR and/or CP induction of cytochrome *c* leakage and apoptosis. The caspase-3 activity also seems to be associated with LDR and/or CP induced apoptosis.

Evaluation of bone marrow proliferation

Studies were performed to evaluate the effects of single doses of CTX in mice, either alone or preceded by a LDR. Table 2 shows that the concentration of marrow cells in the DC and LDR groups did not differ significantly from that in the BC group, while the concentration of cells in the CTX and LDR + CTX groups decreased significantly. The number of cell cycle phase G_1/G_0 and S stage cells in the CTX and LDR + CTX group decreased significantly compared with that of the BC, DT, and LDR groups, while the number of G_2/M stage cells in the CTX and LDR + CTX groups increased significantly. The proliferation index (IP) had also a significant increase (Table 2).

DISCUSSION

Recent studies suggest that induction of apoptosis in tumor cells has an important role in the efficacy of radiation therapy and chemotherapy. Our experiment showed that treatment with CTX combined with LDR significantly inhibited tumor growth. As shown in Fig. 1, tumor volumes of the LDR group, CTX group and LDR + CTX group decreased in turn. This indicates that the anti-tumor effect increased one by one, and indicates that LDR has a good adjuvant effect on chemotherapy and can significantly potentiate the effects of chemotherapy. Some researchers[12,13] in China have shown that LDR combined with radiotherapy reduces the side effects of radiation. It has been shown that LDR combined with CTX significantly reduces the growth of the tumors in tumor-bearing mice, and the pulmonary metastases of Lewis cells.[9] Our experiment was consistent with these findings, since we too observed the chemo-potentiating effects of single 75 mGy dose radiation on CTX. We used a novel protocol not only to achieve greater radio-sensitization effects of chemotherapy but also to enhance the chemo-potentiating effects of LDR. Low doses of radiation were found to induce the HRS phenomenon.[5]

Our experiment also showed apoptosis in a proportion of tumor cells was significantly increased in mice exposed to LDR compared with direct tumor bearing mice, with S phase cells increasing in a large proportion. CTX could not induce significant apoptosis of tumor cells in mice, but could induce G_1 phase arrest, S phase decrease, and the trend to synchronization of cell cycles. From Table 1, we showed that exposing mice to LDR followed by CTX increased tumor cell apoptosis, significantly arrested the G1 phase of the cell cycle, and decreased cell proliferation. It is evident that the cells apoptoses from the phase of cell cycle, in which they were irradiated.[14,1] Regardless of the cell cycle phase at the time of irradiation, cells with sub-Gl-phase DNA content accumulated in all populations after irradiation by dose of 75 mGy, indicating that apoptosis is most likely independent of cell cycle arrest. Cells irradiated in G1 phase underwent apoptosis more slowly than cells irradiated in other phases.[15] This type of death could be characterized as interphase death.[16] Our previous experiment[9] showed that, within 24 h after LDR, G_1 phase arrest increased in the tumor cells of the mice, and S phase arrest decreased, but no change was observed in G_2/M phase. At 48h after LDR, G1 phase arrest of the tumor cells disappeared, cells and apoptosis in S phase increased while cells in G_2/M phase decreased significantly. This observation is in good agreement with the findings of Joiner *et al.*[5] that in some cell lines, which are highly sensitive to low doses (under 0.1 Gy/h), the increase of the dose to 0.3 Gy/h increases radioresistance.

It has been suggested that induction of apoptosis by LDR is regulated through the cytochrome *c* leakage and activation of caspase-3.[11] To elucidate the mechanism regulating apoptosis of tumor cells exposed to LDR + CTX treatment, we analyzed the molecular alterations in signal transduction cascades using a cytochrome c release assay and a caspase-3 activity assay. Our research showed (Figs. 4 and 5) that LDR combined with CTX chemotherapy can more efficiently induce leakage of cytochrome *c*, followed by caspase-3 activation in S_{180} cancer cells. This data is consistent with the result of increased tumor cell shown in Fig. 3. Thus, this

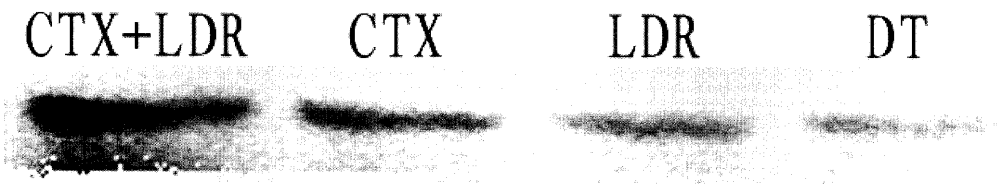

Fig. 4. Cytochrome *c* leakage in tumor cells exposed to LDR alone or in combination with CTX. Cytosolic fractions containing 10 μg protein of DT, LDR, CTX, and combination of LDR plus CTX were subjected to SDS-PAGE followed by Western-blot analysis with antibody to cytochrome c. One of the representative results from three independent samples is shown.

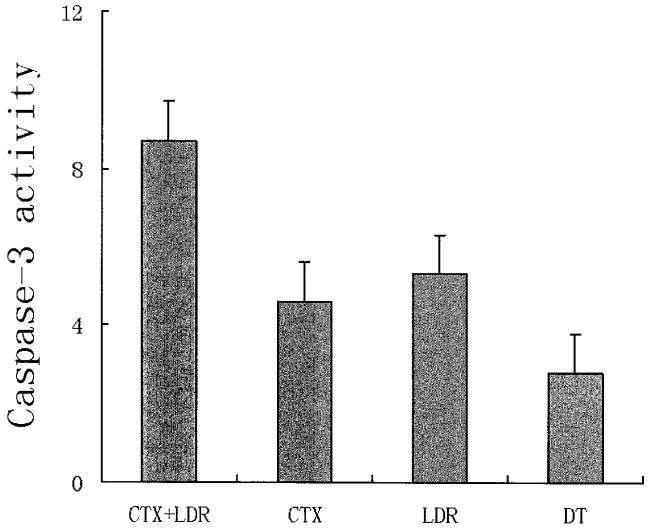

Fig. 5. LDR and/or CP-mediated caspase-3 activation. Tumor cells of different groups were washed and lysed. Caspase-3 activity was determined in cell lysates using a fluorogenic caspase-3 substrate. The caspase-3 activity in the different groups was fully consistent with their cytochrome *c* leakage data. Error bars represent the standard deviation of the mean of three independent experiments.

provides a biologically relevant mechanism in tumor suppression via the chemo-potentiating properties of LDR. In accordance with previously reported data, our results have important implications in the mechanism and therapeutic usefulness of the apoptosis cascade in treatment of tumor by radiation. It is clear from the previous reports that the cells irradiated in G1 phase are less sensitive to this type of apoptosis[17] and this is corroborated by our molecular analysis where cells exposed to LDR caused G_1 phase arrest. It still remains to be seen whether G2 phase is one of the most radiosensitive phases of the cell cycle and whether LDR of cells in this phase of the cell cycle has a radiosensitizing effect.[18]

One of the difficulties of tumor radiotherapy and chemotherapy lies in how to avoid the damage to the normal tissues caused by radiation and chemotherapy agents. Thus, reduction of the secondary effects and germ plasm damage caused by severe suppression of immune system in conventional radio-chemotherapy is a priority of research.[19,18] Our results in Table 2 showed that the concentration of marrow cells decreased and a proliferating proportion of marrow cells increased with the subcutaneous transplantation of tumor cells with CTX treatment, while the concentration increased and the proportion decreased with LDR treatment. For mice treated with LDR + CTX, the concentration of marrow cells increased 9 times as much as in the CTX group, and the proliferating proportion had a further increase. These results indicate that LDR could significantly decrease the damage to bone marrow caused by CTX. Concurrent research of immune response has shown that LDR could improve the immune function of tumor-bearing mice whose immune systems are depressed by chemotherapy and enhance the activity of NK and CTL cells, the phagocytic function of the macrophagocytes, and the reaction of IL-2 to splenic cells.[20,21]

This also supports our findings. Low dose radiation may have many different effects. It could decrease the relative cancer risk in exposed populations.[4,22] LDR could also decrease chromosome damage, gene mutation, cell death and tumor incidence.[23,24] LDR differs from the routine high dose radiation in that its target might be contained in the karyon such as cytoplasm, membrane of cell and some of conductive systems, instead of on DNA.[25] Consequently, all elucidation of the mechanisms of the up-regulation of immune responses by LDR may shed light on the nature of hormesis at low levels of ionizing radiation.[26] These findings provide us a framework for *in vivo* trials using LDR as a chemo-protective agent against the side effect of myelosuppression with chemotherapy.

From this study, we conclude that low dose exposure to ionizing radiation enhances chemotherapy, and combined with CTX chemotherapy significantly delays tumor growth. It suggests that the use of such low doses of radiation with a chemotherapeutic agent is a novel approach to achieve significant chemo-potentiation. If this strategy is successful, further investigation into the benefits of this approaching clinical setting could follow.

ACKNOWLEDGMENTS

We thank Prof. Zhong Jinyi for providing technical assis-

tance and insightful discussions during the preparation of the manuscript.

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Received on October 10, 2006
1st Revision received on January 28, 2007
2nd Revision received on March 5, 2007
3rd Revision received on March 13, 2007
Accepted on March 19, 2007
J-STAGE Advance Publication Date: June 5, 2007