The Effect of Combining Fast Neutron and Photon Irradiation on the Human Osteosarcoma OS-732 Cell Line

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OBJECTIVE *To determine the lethal effect of combining fast neutron with* photon radiation on the OS-732 cell line.
METHODS We examined the effect of irradiation by fast neutrons, photons and a mixed beam (fast neutrons plus photons) on the lethality and colony forming ability of the OS-732 cell line at different times.
RESULTS Following a single irradiation close, the lethality was markedly strong at 24, 48 and 72 h in the group treated with fast neutrons alone and in the mixed beam group in which there was a high proportion of fast neutrons.
CONCLUSION The lethal effect of a fast neutron and mixed beam with a high proportion of fast neutrons on the OS-732 cell line is highly significant. These studies provide guidance for the clinical application of fast neutrons for osteosarcoma treatment.

KEYWORDS: human osteosarcoma, OS -732 cell line, fast neutron, photon, radiotherapy.

Osteosarcoma is one of the major malignancies threatening the life of adolescents. It has usually been treated by surgical amputation with a 5-year survival rate of 10% to 20%. With the wide development of comprehensive treatments, such as radiotherapy, immunotherapy, combined chemotherapy and local microwave thermotherapy etc., the 5-year survival rate has increased to 50% and even to 65%, sometimes.[1-7] In order to examine the sensitivity of osteosarcoma to the irradiation of fast neutrons, we conducted experiments testing the effect of a single fraction of irradiation by fast neutrons and photons, as well as by a mixed beam. We conductd these studies on the OS-732 cell line in order to gather information for potential clinical application.

MATERIALS AND METHODS

Materials

1. The human osteosarcoma cell line (OS-732, purchased from the Section of Immunology, Jishuitan Hospital, Beijing); 2. DMEM nutrient chemicals (GiBCo Co.); 3. fetal bovine serum (The Institute

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of Veterinary Medicine, Beijing Military Command); 4. trypsin, trypan-blue stain, tetrazole blue MTT, RNAase (Sigma Co.); 5. propyl pyridine iodide staining solution (PI, purchased from Huamei Co.); 6. dimethyl sulfoxide (DMSO, produced by Beijing Chemical Plant); 7. inverted microscope (Olympus 1× 70 type, made in Japan); 8. flow cytometer (FACS Calibur type, manufactured by Becton Dickinson Co.); 9. enzyme marker determinator (Wellscan MK3 type, made in Finland); 10. 96-well plastic plates; 11. CO_2 incubator (Hareus BB5060 type, made in Germany); 12. proton linear accelerator (Philips SL18 type); 13. 35 MeV proton linear accelerator (manufactured in the Institute of High-energy Physics, Chinese Academy of Science).

Methods

The recovery and culture of the cells

After recovery of the OS-732 cell line, the DMEM nutrient solution was added containing 15% fetal bovine serum. The pH value was adjusted to 7.2, and the cells transferred into 50-ml culture flasks; the cells were incubated under 5% CO_2 at 37℃ for seven days and passaged twice. The cells achieved logarithmic growth without contamination. The cells were digested using 0.25% trypsin, placed into cell suspension, counted, diluted and transferred into 96-well plates at 1×10^7 cells per well for culture.

Cell grouping

The cells were divided into 6 groups and were irradiated with fast neutrons and photons; group A was the control, with no irradiation (Table 1).

Table 1. Cell groups and radiation dose (cGy)

Groups	Fast neutrons	Photons
A	0	0
1	0	600
2	50	450
3	100	300
4	150	150
5	200	0

The methods of irradiation

The neutronic irradiation was performed using 18 MeV fast neutrons, produced by the 35MeV proton linear accelerator manufactured by the Institute of High-energy Physics, Chinese Academy of Science. The dose rate was from 16 to 25 cGy /min. The photon irradiation was produced using 6 MeV-X rays, generated from the Philps SL18 linear accelerator. The dose rate was 280 cGy /min.

The methods following irradiation of the cells

Determination of tetrazole blue for the MTT assay: The OS-732 cells were suspended, counted and innoculated in 96-well plates at 1×10^4 per well using 10 wells per group on a single plate. The plates were incubated under 5% CO_2 at 37℃ for 24 h. Under microscopic examination it was seen that the cells in each group had adhered to the wall with good proliferation. Then they were irradiated by fast neutrons and photons using different doses. The cells were examined for the enzyme marker at 24 h and 72 h after irradiation.

Assay of dead and living cells by the flow cytometry: Eight ml of culture solution containing 4×10^5 cells were added to each well. The cells then were transfered to a 6-well plate, with three samples in each group. They were kept in a 5% CO_2 incubator at 37℃ for a 24 h culture, then recultured for 24 h after irradiating with fast neutrons and photons, by the same means and same dose. Each group of cells was digested with 0.25% trypsin and a cell suspension prepared; then the dead cells were determined by trypan-blue staining. The cells in each well were counted to get an average value for each group. Each group of cells was centrifuged and the supernatant fluid was discarded. Four ml of cold alcohol (70%) were added to the precipitated cells with shaking. Then the cells were resuspended with a dropper into a single-cell suspension and maintained at 4℃ overnight. After centrifugation and discarding of the supernatant, 50 μg/ml RNAase was added with protection from light. After 30 min the cells were centrifuged at 300 rpm for 15 min. Afterwards 60 μg/ml of PI was added and the cells kept in the dark for 30 min of fluorescent

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staining. Relative DNA content by flow cytometry was used to analyze the percentage of cells in various phases of cell cycle (G1, G2M, S, G2/G0).

Treatment of data

The *t*-test was used for statistical analysis.

RESULTS

After fast neutron and photon irradiation, the biological characteristics of the cells in various groups changed

The results of the MTT assays and the percentage of dead and living cells are shown in Tables 2 and 3. The morphological changes of the cells were detected by flow cytometry, after irradiation. The OS-732 cells which were transferred into the wells in the multi-well plates for 4 h were observed under a microscope. The condition for the cells adhering to the walls and growing well could be seen. After 24 h, active proliferation of the cells and cells covering the wall of the wells could be found. After 24 h of irradiation, more round cells were seen under an inverted microscope, but the stereoperception was poor.

Table 2. The MTT assays after irradiation ($\bar{x}\pm s$)

Groups	The rate of living cells by MTT assays			
	24 h	%	72 h	%
A	0.709 ± 0.236	100	1.042 ± 0.124	100
1	0.606 ± 0.213	85.4	0.878 ± 0.089	84
2	0.572 ± 0.021	80.7	0.841 ± 0.068	81
3	0.503± 0.031	70.8	0.802 ± 0.077	77
4	0.391± 0.103	55.0	0.665 ± 0.053	64
5	0.320 ± 0.070	45.1	0.740 ± 0.029	71

Table 3. The percentage of dead cells by trypan-blue staining (48 h) %

Groups	Rate of dead cells in each well			($\bar{x}\pm s$)
A	5.0	6.0	4.0	5.0 ± 1.00
1	15.6	19.0	17.0	17.2 ± 1.71
2	19.0	20.7	20.0	19.9 ± 0.85
3	21.6	23.0	19.0	21.2 ± 2.03
4	24.5	22.0	23.1	23.2 ± 1.25
5	19.5	23.0	25.0	22.5 ± 1.78

After 48 h of irradiation, the cells groups 1 to 5 totalled 4.5, 5.4, 4.8, 4.7 and 3.86 million, respectively. There was no significant difference between the groups. The total number of the cells in the controls was 7.46×10^6.

DISCUSSION

It can be seen in Tables 2 and 3 that at 24 h and 72 h after irradiation, the lethality to the OS-732 cells in groups 4 and 5 was the greatest, while that in groups 1 and 2 it was the least. These differences were significant ($P < 0.05$). The results indicate that fast neutron irradiation has a greater lethal effect than that of the photon irradiation, after 24 h and 72 h. Furthermore, the lethality of mixed irradiation from fast neutrons plus photons in a high proportion had a definite synergic effect. In group 4 (exposed to 150 cGy of fast neutrons plus 150 cGy of photons), the lethality was slightly greater than that in group 5 (simple irradiation of 200 cGy photons), but there was no significant difference between the 2 groups ($P > 0.05$). These results were confirmed by the dead-cell rate of trypan-blue staining at 48 h and the DNA assays by flow cytometry.

The analysis of DNA content by flow cytometry assays showed that in the controls, 79.26% of the cells were in stage G1 and only of 2.96% in stage G2M. This finding indicated that because of the rapid cellular proliferation, contact-growth inhibition resulted in the cells of the controls in the 6-well plates growing in mass, so more cells were in the intermediate (G1) stage; whereas in the experimental group, because of the irradiation, the postirradiated cells were blocked in the G2M stage. Only a few of the cells in the G2M stage could move into the G1 stage, most being kept in G2M, thus inhibiting proliferation of the cells, or allowing the cells to die gradually. In group 4, the cells in the G2M stage accounted for 29.85%, the next was group 3 with 27.04% and group 2 with 26.8%. Group 5 and 1 were the lowest in percentage of G2M with 26.4% and 24.66%, respectively. It was shown that the lethality to the cell line of the mixed rays of fast neutrons, after 24 h of treatment, was greatest.

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Osteogenic sarcoma has a low radiosensitivity to conventional irradiation, but it is rather sensitive to fast neutrons. This is because the fast neutrons produce high linear eneregy transfer (LET). Its radiological charateristics for treatment of malignant tumors are as follows: 1. The relative biological effectiveness (RBE) of fast neutrons is higher compared to photons; 2. The lethality of fast neutrons to anoxic cells is extensive. The oxygen enhancement ratio (OER) of fast neutrons is approximately 1.6, whereas the limit of conventional irradiation is from 2.5 to 3.0; 3. The damage caused by fast neutrons is not easily recovered, including sublethal damage and latent lethal damage; 4. There is no major difference in radiosensitivity among the cell cycle phases.[8-10]

The objective of our study was to irradiate proliferating tumor cells adhering to the wall of the plates after 24 h of innoculation in order to simulate the status of an in vivo tumor with a normal blood supply. There is no major difference between the status of tumor cells in a culture solution compared to those in the human body.

The results of this adhering junction report indicate that in a short period of time after a single dose of irradiation (24 h, 48 h and 72 h), lethality of irradiation to OS-732 cells by simple fast neutrons and that of a mixed ray of fast neutrons in a high proportion, is significant. After 48 h and 72 h of irradiation and in the group of fast-neutron-mixed-ray with a high proportion of neutrons, the lethality of irradiation by fast neutrons and photons has a definite synergism, thus providing a meaningful guidance for clinical application of fast neutrons for treatment of osteosarcoma.

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