Acta Oncologica Vol. 33, No. 7, pp. 813–817, 1994

THE RESPONSE OF QUIESCENT CELL POPULATIONS IN MURINE SOLID TUMORS TO IRRADIATION WITH FAST NEUTRONS

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5-bromo-2′-deoxyuridine (BUdR) was injected into SCC VII tumor-bearing mice intraperitoneally to label all proliferating tumor cells. The mice were irradiated with fast neutrons or x-rays. Immediately, or 24 h after irradiation, the tumors were excised, minced and trypsinized. The tumor cell suspensions were incubated with cytochalasin-B (a cytokinesis blocker). The micronucleus frequency was determined using immunofluorescence staining to BUdR. The cells that were not labeled with BUdR could be regarded as the quiescent cells. The micronucleus frequency in total tumor cells was determined from the irradiated tumors that were not pretreated with BUdR. The difference in radiosensitivity between total and quiescent cells was markedly reduced with fast neutrons, especially at higher doses of radiation. Potentially lethal damage repair by total and quiescent cells was inhibited more strongly with neutrons than with x-rays. When using fast neutrons, the radiosensitivity of solid tumors depends on their heterogeneity less critically than for x-rays.

Heavy-particle beam radiation therapy has been expected to offer a therapeutic gain, partly because fast neutrons have superior radiobiologic properties as compared to conventional x-rays and gamma-rays, partly because the dose distributions achieved with protons and helium ions are superior in many clinical situations to those obtained with photons or electrons, and partly because heavy ions and pions have both a potential biologic and a dose distribution advantage (1).

To improve radiation therapy, it is necessary to determine the response of quiescent (Q) cells in solid tumors to radiation, because many tumor cells are quiescent in situ (2) but are still clonogenic. These Q cells are thought to be resistant to radiation because they may have higher hypoxic fractions and greater potentially lethal damage repair (PLDR) capacities than proliferating (P) cells (3–5). Therefore, in this study, we examined the characteristics of radiosensitivity and PLDR in the Q cell populations of murine solid tumors irradiated with fast neutrons, and compared them with those irradiated with 10 MV x-rays, using our newly developed method for selectively detecting the response of Q cells in solid tumors (6).

Received 7 June 1993.
Accepted 25 March 1994.
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Material and Methods

Labeling with BUdR. SCC VII tumor cells were maintained in vitro in Eagle's minimal essential medium containing 12.5% fetal bovine serum. Cells were collected from monolayer cultures, and approximately 1.0×10^5 cells were inoculated subcutaneously into both hind legs of C_3H/He syngeneic female mice, aged 8–11 weeks. Tumors reached 1 cm in diameter 14 days after inoculation. Nine days after inoculation, 100 mg/kg of BUdR dissolved in physiological saline was administered intraperitoneally 10 times at 12-h intervals to label all P cells in the tumors. We had previously determined that regardless of whether BUdR was

 ISSN 0284-186X

injected intraperitoneally 10 times at 6-h or 12-h intervals, the profiles of the labeling indices and the number of injections of BUdR were almost the same. Moreover, when mini-osmotic pumps (Alzet model 2001, USA) were implanted subcutaneously for continuous labeling, the profiles were still almost the same (data not shown). Administration of BUdR did not change the tumor growth rate (data not shown). The tumor was 1 cm in diameter at treatment. The labeling index after 10 doses of BUdR reached a plateau level of $55 \pm 4.5\%$ (mean $\pm$ SD). Therefore, tumor cells not incorporating BUdR after 10 injections were regarded as Q cells in this study, and the growth fraction of the tumor was considered to be $55 \pm 4.5\%$.

Irradiation. Irradiation was carried out 1 h after the last dose of BUdR was given. Mice received whole-body irradiation with 10 MV x-rays, generated by a linear accelerator at a dose rate of 5.6 Gy/min, or fast neutrons, produced by bombarding a thick beryllium target with 30 MeV cyclotron deuterons at the National Institute of Radiological Science. The mean energy of the fast neutrons was 13 MeV and the gamma ray contamination was 3–5% of the total dose. Radiation was administered from a single portal. Mice with and without BUdR injection were irradiated with x-rays or neutrons. The tumors were then excised immediately or 24 h after irradiation.

Immunofluorescence staining and micronucleus assay. Tumors were excised, minced and dissociated into single cell suspensions by trypsin (0.05% trypsin, 0.02% ethylenediamine-tetraacetic acid (EDTA), 37°C, 15 min). Tumor cell suspensions were incubated in 60-mm tissue culture dishes containing 5 ml of complete medium and 1.0 μg/ml of cytochalasin-B to inhibit cytokinesis while preserving nuclear division. In a preliminary study, the cytokinesis-blocked cells were easily recognized as binucleate cells, and their percentage reached a maximum 48 h after the beginning of culture. During these procedures, the cultures were kept in the dark to avoid any damage to the DNA that had incorporated BUdR. Forty-eight h after the beginning of cytochalasin-B treatment, the cells were trypsinized and single-cell suspensions were fixed with 70% ethanol. After centrifugation, the cell pellet was resuspended with 0.4 ml of cold modified Carnoy's fluid (three volumes of ethanol and one volume of acetic acid). Thirty microliters of this suspension were then placed on a microslide glass using a dropper and dried at room temperature.

Thereafter, the microslides were treated with 2 M hydrochloric acid for 30 min at room temperature to dissociate histones and partially denature the DNA. Microslides were then immersed in borax-borate buffer (pH 8.5) to neutralize the acid. After three washes in phosphate-buffered saline (PBS, pH 7.6) at room temperature, BUdR-labeled cells were detected by indirect immunofluorescence. A monoclonal anti-BUdR antibody (Becton Dickinson, USA) was used as the primary antibody at a dilution of 1:50 in 0.5% bovine serum albumin and 0.5% Tween 20 in PBS. The antibody was applied to microslides for 30 min at room temperature in a humidified chamber. After the microslides were washed three times in PBS, fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Becton Dickinson, USA) was applied in the same manner. Then the microslides were washed three times in PBS.

To distinguish BUdR-labeled cells that were stained with FITC from BUdR-unlabeled cells that were not stained with FITC, cells on the microslides were treated with 30 μl of propidium iodide (PI) (1–5 μg/ml in PBS) while the fluorescence intensity was observed under the fluorescence microscope. When the intensity of the red fluorescence produced by PI became almost the same as that of the green fluorescence from nuclei pre-stained with FITC, treatment was stopped by rinsing the microslide with water. In this manner we were able to distinguish between cells incorporating BUdR, in which at least part of the nucleus or micronucleus (MN) showed green fluorescence, and cells not incorporating BUdR in which the nucleus and MN showed only red fluorescence. It was then possible to selectively obtain the MN frequency of nonincorporating cells by counting the micronuclei in binucleate cells that showed only red fluorescence. The MN frequency was defined as the ratio of the number of micronuclei in binucleate cells to the total number of binucleate cells observed. MN was also scored from irradiated tumors without BUdR injection to avoid the radiation sensitization effects of BUdR. MN frequency determined from these tumors was defined as the MN frequency of total tumor cells.

Although the effects of cytochalasin-B on chromosome damage in irradiated cells have not been completely elucidated, a close relationship between cell survival and MN frequency obtained with the cytochalasin-B method has been reported and the MN frequency assay using the cytokinesis-block method is accepted as a tool for rapid assay of radiosensitivity of cells (7, 8).

During the 10 injections of BUdR over 5 days, the shift of cells from proliferating to quiescent populations could result in labeled Q cells. These cells must be excluded when scoring micronuclei, because radiosensitization by BUdR might alter the response of these cells. With our technique, the cells could be stained with FITC. Therefore, the cells could be excluded when scoring micronuclei in binucleate cells showing only red fluorescence by PI.

Three mice were used to assess each set of conditions and each experiment was repeated three times.

Results

The MN frequencies in the unirradiated tumors were 0.056 ± 0.0020 and 0.081 ± 0.061 for total (P + Q) tumor cells and for Q cells alone respectively.

The dose–response curves of the normalized MN frequency (MN frequency–C, where C is the MN frequency

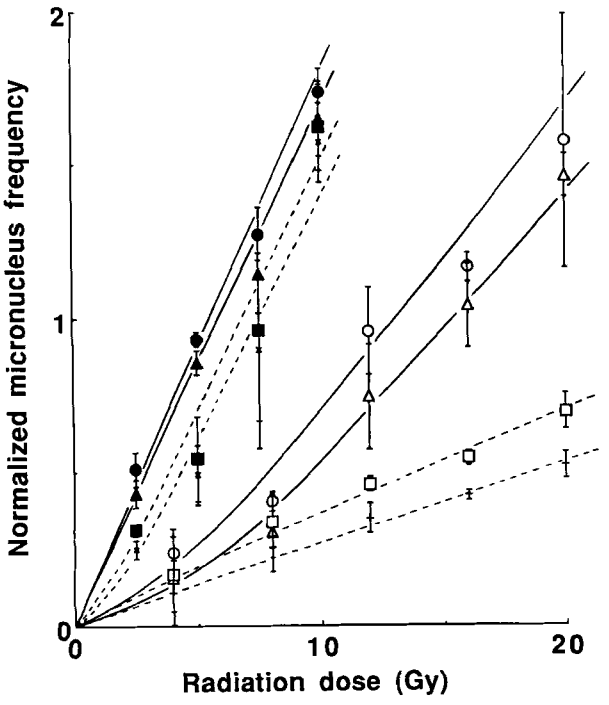

Figure. Dose–response curves of the normalized micronucleus frequency for each cell population in tumors irradiated with fast neutrons (solid symbols and X) and x-rays (open symbols and +). All tumor cell populations in tumors excised immediately and 24 h after irradiation are shown as circles and triangles respectively. Q cell populations in tumors excised immediately and 24 h after irradiation are shown as squares and crosses respectively. Bar represent the standard diviations. Fast neutrons: ● all cells (I.A.); ▲ all cells (delayed); ■ Q cells (I.A.); X Q cells (delayed); X-rays: ○ all cells (I.A.); △ all cells (delayed); □ Q cells (I.A.); + Q cells (delayed).

in unirradiated tumors) for total tumor cells and Q cells in tumors irradiated with x-rays and fast neutrons are presented in the Figure. The normalized MN frequencies for tumors irradiated with neutrons were much higher than those for tumors irradiated with x-rays. The difference in normalized MN frequency between total tumor cells and Q cells was reduced more markedly in tumors irradiated with neutrons than in those irradiated with x-rays. The decrease in the normalized MN frequency by delaying the excision of the irradiated tumors was considerably suppressed by using neutrons.

Dose-modifying factors (DMFs) under each irradiation condition, which compare the radiation doses necessary to obtain various normalized MN frequencies in Q cells with those in total tumor cells, were calculated using the mean values of the data in the Figure (Table 1). The DMFs for x-rays were greater than 1.50. On the contrary, at both excision times, the DMFs for neutrons were smaller than those for x-rays, and as the normalized MN frequency became larger, i.e., at higher doses of radiation, the DMFs decreased even more towards 1.00.

To compare PLDR by total tumor cells with that by Q cells under both irradiation conditions, DMFs after PLDR were calculated at various normalized MN frequencies using the mean values of the data in the Figure (Table 2). These DMFs, especially for Q cells, in tumors irradiated with neutrons were smaller than those in tumors irradiated with x-rays. Furthermore, as the normalized MN frequency became larger, the DMFs of both cell types in tumors irradiated with neutrons became smaller and nearer to 1.00.

Finally, to look into the relative biological effectiveness (RBE) of irradiation with fast neutrons, DMFs, which compare the radiation doses necessary to obtain various normalized MN frequencies in tumors irradiated with x-rays with those in tumors irradiated with neutrons in each cell type at each excision timing, were calculated using the

Table 1

Dose-modifying factors[a] for quiescent cell relative to total tumor cells

Normalized MN frequency[b]	Fast neutrons		X-rays	
	I. A.[c]	Delayed[d]	I. A.	Delayed
0.4	1.48	1.60	1.60	1.72
0.5	1.42	1.48	1.73	1.91
0.75	1.30	1.36	2.00	-
1.0	1.25	1.28	-	-
1.25	1.22	1.23	-	-
1.5	1.19	1.20	-	-

[a] DMF, radiation dose necessary to obtain each normalized micronucleus frequency in quiescent cells/radiation dose necessary to obtain each normalized micronucleus frequency in all tumor cells.
[b] Micronucleus frequency–C, where C is the micronucleus frequency in unirradiated tumors
[c] Tumors were excised immediately after irradiation.
[d] Tumors were excised 24 h after irradiation.

Table 2

Dose-modifying factors[a] after repair of PLD[b]

Normalized micronucleus frequency[c]	Fast neutrons		X-rays	
	Total tumor cells	Quiescent cells	Total tumor cells	Quiescent cells
0.4	1.07	1.15	1.25	1.35
0.5	1.08	1.12	1.23	1.37
0.75	1.07	1.12	1.19	-
1.0	1.07	1.09	1.18	-
1.25	1.06	1.08	1,17	-
1.5	1.06	1.07	1.17	-

[a] DMF, radiation dose necessary to obtain each normalized micronucleus frequency with PLD repair/radiation dose necessary to obtain each normalized micronucleus frequency without PLD repair.
[b] Potentially lethal damage.
[c] Micronucleus frequency–C, where C is the micronucleus frequency in unirradiated tumors

Table 3

Relative biological effectiveness[a] in total tumor and quiescent cell populations

Normalized MN frequency [b]	Total tumor cells		Quiescent cells	
	I. A.[c]	Delayed[d]	I. A.	Delayed
0.4	3.14	3.69	3.29	3.94
0.5	2.91	3.29	3.54	4.22
0.75	2.56	2.83	3.92	–
1.0	2.38	2.61	–	–
1.25	2.25	2.44	–	–
1.5	2.18	2.35	–	–

[a] RBE, radiation dose necessary to obtain each normalized micronucleus frequency with x-rays/radiation dose necessary to obtain each normalized micronucleus frequency with fast neutrons.
[b] Micronucleus frequency-C, where C is the micronucleus frequency in unirradiated tumors
[c] Tumors were excised immediately after irradiation.
[d] Tumors were excised 24 h after irradiation.

mean values of the data in the Figure (Table 3). These values of DMFs are equivalent to the values of RBE. On the whole, all of them were greater than 1.00, and the values for Q cells were larger than those for total tumor cells. Moreover, in Q cells populations, the values for delayed assay were larger than those for immediately-after assay.

Discussion

Based on laboratory investigations, high linear energy transfer (LET) particle irradiation is capable of more efficient cell kill than that associated with conventional or low LET irradiation such as x-rays or gamma-rays (1). The advantages of high LET irradiation include: 1) a greater ability to damage hypoxic cells; 2) a lesser ability for repair of sublethal and potentially lethal radiation-induced damage; 3) less variation in radiation sensitivity relative to the cell cycle; and 4) a greater ability to deposit the radiation dose in the region of the tumor as opposed to the normal surrounding tissue (neutrons do not have this advantage compared with other particle therapy). The presence of quiescent cell populations in a solid tumor has been considered important in determining the response of tumors to treatment (2, 9). However, a comparatively simple assay for assessing the radiosensitivity of quiescent cells in solid tumors has not been available in the past. In the current study, we examined the characterisitics of radiosensitivity and PLDR in the Q cell population and the total tumor cell population within SCC VII solid tumors, comparing irradiation with fast neutrons and 10 MV x-rays, using our newly developed method for the selective determination of the radiosensitivity of Q cells in solid tumors. As far as we know, this is the first work to make an in vivo analysis of the sensitivity of Q cells to fast neutron irradiation, compared with that of total tumor cells.

Our previous report showed that the Q population has a larger percentage of hypoxic cells than total tumor cells (6). In this study, the difference in radiosensitivity between Q cells and total tumor cells was markedly reduced by using fast neutrons, especially when high doses of radiation were given. In other words, it follows that hypoxic and oxygenated cells in the SCC VII solid tumor have almost the same radiosensitivity to fast neutrons, especially when high doses were delivered. In the case of irradiation of x-rays, PLDR capacities by Q cells were much greater than those by total tumor cells. In contrast, the PLDR abilities of Q cells were suppressed with neutron irradiation, especially at high doses. Because there was less difference in radiation sensitivity as a function of position in the cell cycle and a diminished PLDR capacity when fast neutrons were used, the RBE values in Q tumor cells that were excised after a time delay were maximal, as shown by the data in Table 3. It is largely on the basis of these purported biological advantages that fast neutron therapy was applied in the clinic over the last two decades (10).

Incidentally, it is well known that there exists a heterogeneity of response of tumor tissue to radiation (11). Factors which cause heterogeneity of radiation response in laboratory systems include: distributions of inherent cellular radiation sensitivities, in vivo radiation sensitivity of cells as affected by distributions of pO_2, and other metabolites, cellular capacity to repair radiation damage, cell proliferation kinetics, immune rejection reaction by host against tumor, extent of loss of tumor clonogens by exfoliation from the surface or into the vascular spaces, among others. Furthermore, it is thought that the quiescent state of cells in solid tumors arises because of oxygen and nutrient deprivation caused by the heterogeneity in vivo within the solid tumors (9). In this study, the radiation with fast neutrons remarkably diminished the difference in radiosensitivity between total tumor cells and Q cells in solid tumors. That is to say, fast neutron therapy has the ability to minimize the heterogeneity in radiation sensitivity in solid tumors. Fast neutron beams produce radiation damage relatively independent of the phase of the cell cycle and, as a result, they are more efficient irradiators of slowly proliferating, relatively hypoxic bulky tumor masses (12).

Our method for selectively detecting the response of Q cells in solid tumors revealed the biological superiority of fast neutron irradiation to conventional x-irradiation. Using this method, we plan to investigate the responses of Q cells to treatment with radiation plus chemotherapeutic agents and/or hypoxic cell sensitizers, as well as the responses to low dose rate radiation. We also have a plan to determine the changes of the radiosensitivity of Q cells during fractionated irradiation.

ACKNOWLEDGEMENTS

This work was supported in part by Grants-in-aid for Cancer Research (05807076) from the Ministry of Education, Science and Culture, Japan. The authors are grateful to Dr Peter C. Keng of University of Rochester (Rochester, New York, USA) for his critical reading of the manuscript.

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