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Transitional Increase in Growth Fraction Estimated by Ki-67 Index After Irradiation to Human Tumor in Xenograft

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Abstract. *The cell kinetics of human tumor cells (HSG) in vivo were estimated by Ki-67 index to analyze the biological behavior of quiescent cells after irradiation. The increment of Ki-67 index was dose-dependent up to 15 Gy irradiation. The Ki-67 index of tumor cells increased gradually from 35±5.0% before irradiation to 56±5.0% at day 5 after single irradiation of 15 Gy. Flow-cytometric study showed that the G1 population was decreased and the G_2M population was elevated at day 5 after the irradiation. In fractionated irradiation, the time-course in the change of the Ki-67 index was similar to that of single irradiation. It was likely that tumor cells were recruited into the cell cycle from quiescent phase after irradiation. We consider the Ki-67 index to be a reliable indicator of growth fraction and useful to estimate the cell kinetics of irradiated tumors in vivo. The growth fraction estimated by Ki-67 index during radiotherapy is expected to be useful in predicting tumor response to radiation therapy.*

Extensive efforts have been made to predict radiation effect on human tumors. Tumor cell kinetics have been studied by various methods to determine the effect of radiotherapy. Ki-67 is a nuclear protein, DNA replicate complex similar to DNA topoisomerase II, detected by a monoclonal antibody, which is expressed in the nuclei of cells in the cell cycle but not in resting G_0- and early G_1- phases (1,2). Several reports (3-7) showed Ki-67 to be useful for predicting the biological behavior and prognosis of human tumors. We have found that Ki-67-positive tumor cells increase during radiation therapy in patients with cervical squamous cell carcinoma, and proposed that quiescent cells might have been recruited into the cycling phase as a consequence of irradiation (8). However, little is known about the recruitment phenomenon induced by irradiation in experimental animals, and radiation biology on tissues based on histopathological research has not been fully evaluated. The present study was designed to analyze the time- and dose-dependence of Ki-67 indices after radiation to a human tumor cell line growing in nude mice *in vivo*.

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Key Words: Growth fraction, human tumor, Ki-67, radiation, recruitment.

Materials and Methods

Mice and tumor. A human salivary gland tumor cell line, HSG (9), was used for the present study. A single cell suspension was obtained by enzymic digestion of solid tumors with trypsin and hyalinase and 1 x 10^6 cells were subcutaneously transplanted into the hind legs of Balb/c nude mice. Fifteen to twenty days after transplantation, when tumors had reached 8 to 10 mm in diameter, the tumors in the leg received local irradiation with ^{137}Cs gamma rays from a ^{137}Cs unit at a dose rate of 1.9 Gy / min at 21 cm FSD. The time-course experiments were performed after irradiation. Tumors were treated with a dose of 15 Gy in the single-dose irradiation study, or with 5 daily fractions of 3 Gy giving a total dose of 15 Gy in the fractionated irradiation study. Then, tumors were surgically removed and cell samples immediately prepared for immunohistochemical staining before treatment and at 1, 3, 5, 7 and 9 days after irradiation. For the dose-dependence study, single doses of 5 Gy, 10 Gy, 15 Gy, 20 Gy and 25 Gy were administered to tumors. The Ki-67 index was estimated at 5 days after single irradiation. Data were determined from three independent experiments.

Immunohistochemical staining. All the fresh biopsy specimens were divided into two groups; one specimen was fixed with 10% formaldehyde solution for conventional hematoxylin and eosin (H-E) staining and the other specimen was digested by enzyme to make a single cell suspension for Ki-67 immunostaining. The cells were fixed with 4% cold paraformaldehyde solution at 4˚C for 30

min and then reacted with anti Ki-67 monoclonal antibody (DAKO-PC, Copenhagen, Denmark) for 1 h at room temperature. The samples were followed by reaction with biotinylated anti-mouse IgG for 30 min and avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA) for 30 min. They were reacted with 3,3,-diaminobenzibine tetrahydrochloride (DAB, Dojin Chemicals, Tokyo, Japan) solution with 0.01%(W/V) hydrogen peroxide for 2 to 5 min at room temperature and counterstained with hematoxylin. Control staining was performed by incubating with PBS instead of anti Ki-67 antiserum.

Assessment of Ki-67 index. Over 1000 tumor cells per specimen were counted on three x 200 colored photographs for calculation of the Ki-67 index. The Ki-67 index was estimated by the percentage of Ki-67-positive cancer cells in all the counted tumor cells.

DNA analysis. Irradiated tumors (HSG) were surgically removed and cell suspensions were prepared by enzymic digestion. Cell cycle analysis was performed after staining of 70% ethanol-fixed cells in a propidium iodide (PI, 1 x 10^{-6} g/ml) solution containing RNase (200 x10^{-6} g/ml). Flow cytometric measurement was performed with a FACScan (Becton-Dickinson).

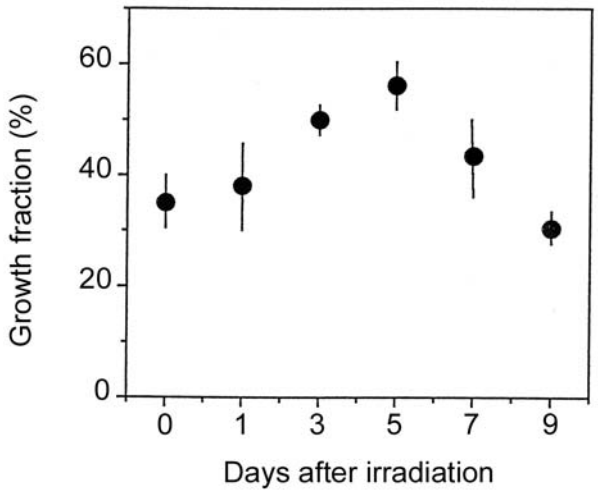

Figure 1. *Time-course study of Ki-67 index of HSG tumors before and after irradiation. Tumors were irradiated with a single dose of 15 Gy. Bars show ±standard error where these exceed the size of symbol.*

Results

The volume doubling time of the HSG tumors was 4.5 days *in vivo*. Ki-67 immunoreactive products were located only in the nuclei of the tumor cells, and Ki-67- positive features were diffuse nuclear-staining and dot-staining in the immunohistochemical studies. The Ki-67 index of unirradiated tumors was 35±5.0%. The time-course study after a single dose of 15 Gy showed that the Ki-67 index increased continuously up to 5 days, reaching 56 ± 5.0% at the peak, which was significantly higher than that of the unirradiated control ($p<0.05$) (Figure 1). The Ki-67 index then gradually decreased and returned to a control level of about 30% by day 9. Daily fractions of 3 Gy with a total dose of 15 Gy also induced increments of Ki-67 index as estimated on days 3 and 5 (Figure 2). The relationship between radiation dose and Ki-67 index was estimated on day 5 after single doses of 5 Gy, 10 Gy, 15 Gy, 20 Gy or 25 Gy. The change of Ki-67 index was dose-dependent up to 15 Gy, however the Ki-67 index after irradiation was not over about 60 % (Figure 3). The Ki-67 index at 15 Gy was 55 %, which was significantly greater than that of the control.

Flow-cytometric study showed that the G_1 population of the tumor cells had decreased to 43±11% at day 5 from 72±14% of non-irradiated cells, whereas the G_2M population increased from 8 ± 4 % before irradiation to 34±10% on day 5 after 15 Gy irradiation ($p<0.05$).

Discussion

The present study demonstrated that the Ki-67 index after a single dose irradiation (15 Gy) increased from 35±5.0% before irradiation and reached a peak value of 56±5.0% on day 5. A significant correlation between rising DNA content and increasing Ki-67 index was noted (10). The results of the flow-cytometric study showed that the G_2M population increased from 8% before irradiation to 34% on day 5 after 15 Gy irradiation. This phenomenon may be mainly due to division delay or G_2 block which caused an increased G_2M population. However, G_2 block could not solely explain the significant increase in growth fraction. Furthermore, this increment of G_2M population could not solely be due to a radiation-induced G_2 block whose length was linearly dependent on radiation in cultured cells (11). Higuchi observed a decrease in the G_0/G_1 fraction and an increase in the S fraction in cervical cancers during radiotherapy (12). Non-cycling cells (resting cells) in the tumor can be recruited into the cell cycle under certain conditions (13). Some reports (14,15) indicated that the growth fraction of tumors increased after irradiation *in vivo*, although their assessments, which were indirect methods, did not measure the growth fraction directly. Hermans demonstrated an increase in growth fraction of a rat rhabdomyosarcoma after a single dose of 20 Gy by measurement of cell cycle parameters *in vitro* (16). Szczepanski and Trott reported the increase in growth fraction of murine adenocarcinoma after a single dose of 6 Gy or 12 Gy by measurement of the labeling index and suggested a recruitment phenomenon. Therefore, as Nakano (8) discussed, it is likely that radiation stimulated quiescent cells to enter the cycling phase.

When tumors were irradiated with a fractionated schedule, the time interval from initiation of radiation to peak value of Ki-67 index was similar to that of single

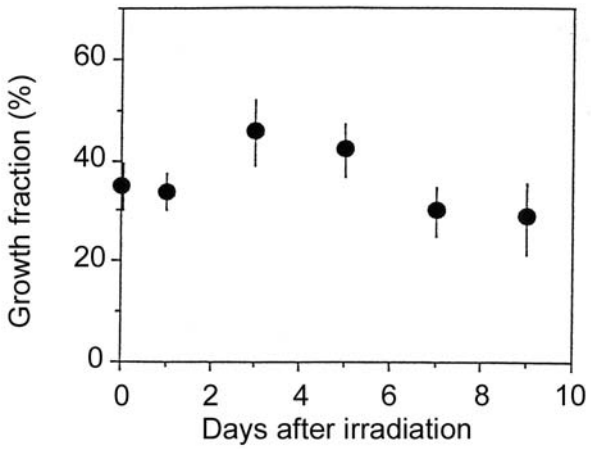

Figure 2. *Time-course study of Ki-67 index of HSG tumors before and during irradiation. Tumors were irradiated with daily fraction dose of 3 Gy giving a total dose of 15 Gy over 5 days. Bars show ±standard error where these exceed the size of symbol.*

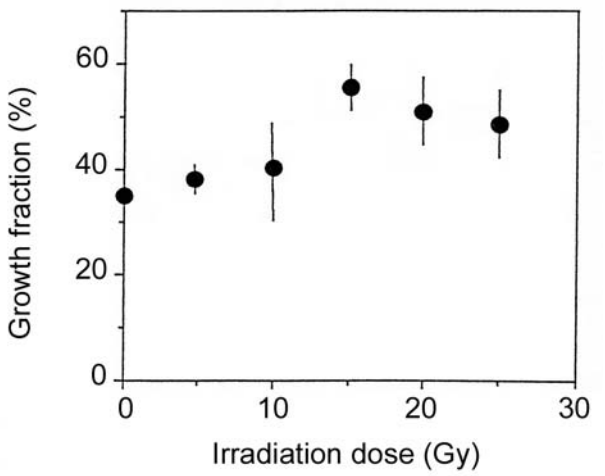

Figure 3. *Irradiated dose-response of Ki-67 index of HSG tumors at 5 days after single dose irradiation. Bars show ±standard error where these exceed the size of symbol.*

irradiation. Additionally, the interval was quite consistent and not dose-dependent. Therefore, recruitment may maximize, fairly constantly, at about 5 days after initiation of irradiation. Nakono and Oka found that the Ki-67 index of cervical cancer initially increased with fractionated radiation dose and reached a peak value at 1 week (900 cGy), decreasing when the dose was increased further (8).

The transition of Ki-67 index of the present study was coincident with the clinical results, reported by Oka and Nakano (8), in terms of time-course. Fowler suggests that as soon as a significant proportion of cells has been killed, the size of the tumor cord shrinks, meaning that enhanced nutrition is available to all surviving cells (17). The increment of Ki-67 index followed a dose-dependent manner below 15 Gy in our dose-effect study. The Ki-67 index of irradiated tumors could not increase more than 60%. It is thus reasonable to consider that recruitment is partly induced by changes of the nutritional conditions, since reoxygenation and recruitment of resting cells may be expected to appear in a dose-dependent manner and require about one week to reach a peak value of this phenomena.

The growth fractions measured for a variety of solid tumors in experimental animals are between 30% and 50% (18), as with our results. This suggests that more than half of tumor cells are in the quiescent phase and not in the cell cycle, at least before treatment. However, radiation biological studies have dealt mainly with cycling cells *in vitro*, while the radiobiological nature of resting cells has not been fully studied. The quiescent cells were considered to be resistant to radiation. Moreover, the growth fraction of human tumors is expected to be a useful indicator in predicting tumor response to radiation therapy. Valente described that the Ki-67 index after 10 Gy of radiotherapy provided an independent measure of responsiveness to radiotherapy (19). Nakano reported that the Ki-67 index was associated with early radiation response and local control in cervical cancer (20). Oka and Nakano suggested that a high growth fraction at 9 Gy was a predictive factor for good prognosis in patients with cervical squamous cell carcinoma who underwent radiotherapy alone (21). Further extensive study is required to clarify the radiobiological behavior of the quiescent cell population of tumors as well as normal tissues.

In conclusion, we suggest that the Ki-67 index is a useful indicator of the growth fraction and the cell kinetics of irradiated tumors *in vivo*.

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