Jpn. J. Cancer Res. 84, 1130–1135, November 1993

Alteration of Radiosensitivity of Quiescent Cell Populations in Solid Tumors Irradiated with X-Rays Twice at Various Intervals

Shin-ichiro Masunaga,[1,3] Koji Ono,[1] Michihide Mitsumori[2] and Mitsuyuki Abe[2]

[1]*Radiation Oncology Research Laboratory, Research Reactor Institute, Kyoto University, Noda, Kumatori-cho, Sennan-gun, Osaka 590-04 and* [2]*Department of Radiology, Faculty of Medicine, Kyoto University, 54 Shogoin-kawahara-cho, Sakyo-ku, Kyoto 606-01*

5-Bromo-2′-deoxyuridine (BUdR) was injected into SCC VII or EMT6/KU tumor-bearing mice intraperitoneally to label all the proliferating tumor cells. First, the mice were irradiated with X-rays at a dose of 10 Gy, followed by a dose of 0–20 Gy at 0, 12, 24 or 48 h later. During the interval, no BUdR was injected. Immediately after the second irradiation, the tumors were excised and trypsinized. The micronucleus (MN) frequency in cells without BUdR labeling was determined by means of incubation with cytochalasin-B (a cytokinesis-blocker) and immunofluorescence staining for BUdR. When the tumors were not pretreated with BUdR before the first irradiation, the MN frequency in all tumor cells was determined. To determine the labeling indices of SCC VII and EMT6/KU tumors at the time of the second irradiation, each group also included mice that were continuously administered BUdR until just before the second irradiation using mini-osmotic pumps which had been implanted subcutaneously 5 days before the first irradiation. The MN frequency of all tumor cell populations obtained immediately after the second irradiation decreased in proportion to the increase in interval time. However, in both tumor systems, the MN frequency of unlabeled cell populations, which could be regarded as quiescent cells in the tumors at the time of the first irradiation, was raised with increase in the interval time. In addition, the labeling index at the second irradiation was higher than that at the first irradiation. These findings support the occurrence of recruitment from quiescent to proliferating state during fractionated irradiation.

Key words: Fractionated irradiation — Quiescent cell — Recruitment — Micronucleus assay — Immunofluorescence staining

In the 1920s and 1930s, famous experiments were performed in Paris in which the testes of rams were irradiated with X-rays.[1)] In these experiments, when the dose was fractionated over a period of time, sterilization could be achieved with little apparent skin damage. It was argued that the testes were a model for the rapidly growing tumor, while the skin represented a normal tissue response. On this basis, fractionation was introduced into clinical radiotherapy.

Since techniques to culture single mammalian cells *in vitro* (in 1956) and to determine survival curves *in vivo* (in 1959) were developed, the biological basis of fractionated radiation therapy has been revealed.[2)] However, how the quiescent cell populations behave in solid tumors during fractionated radiotherapy remains unknown. Therefore, in this study, we investigated the quiescent cell populations in murine solid tumors *in situ* (SCC VII squamous carcinoma and EMT6/KU sarcoma), using our newly developed method for selectively detecting the irradiation response of quiescent cells in solid tumors,[3)] and we examined the behavior of quiescent cells in solid tumors irradiated twice at various intervals with X-rays.

[3] To whom correspondence and reprint requests should be addressed.

MATERIALS AND METHODS

Labeling with 5-bromo-2′-deoxyuridine (BUdR) SCC VII carcinoma derived from C3H mice and EMT6/KU sarcoma derived from Balb/c mice were maintained *in vitro* in Eagle's minimum essential medium containing 12.5% fetal bovine serum. Approximately 1.0×10^5 cells were inoculated subcutaneously into both hind legs of syngeneic female C3H/He or Balb/c mice aged 8–11 weeks. Fourteen days later, the tumor reached 1 cm in diameter. Nine days after the inoculation, 100 mg/kg of BUdR dissolved in physiological saline was administered intraperitoneally, 10 times at 12 h intervals, to label all proliferating cells in the tumor. The tumor was 1 cm in diameter at the first irradiation. The labeling index after 10 doses of BUdR was $55.3\pm4.5\%$ (mean$\pm$SD) for the SCC VII tumors and $74.6\pm5.4\%$ for EMT6/KU tumors, and the labeling index reached a plateau level at this stage. Therefore, in this study, tumor cells not incorporating BUdR after 10 doses were regarded as quiescent cells at the time of the first irradiation for all practical purposes, and the growth fraction of the respective tumors was considered to be $55.3\pm4.5\%$ and $74.6\pm5.4\%$. Administration of BUdR did not change the tumor growth rate (data not shown).

Irradiation For tumor-bearing C3H/He and Balb/c mice, the first irradiation with a dose of 10 Gy was carried out 1 h after the last dose of BUdR. Mice received whole-body irradiation with 10 MV X-rays generated by a linear accelerator at a dose rate of 5.6 Gy/min, and 0, 12, 24, or 48 h after the first irradiation, the second irradiation with a dose of 0–20 Gy was performed. During the interval, no BUdR was administered. Each treatment group included both C3H/He and Balb/c mice with and without BUdR pretreatment.

To determine the labeling index of SCC VII and EMT6/KU tumors at the time of the second irradiation, each group also included mice that were continuously administered with BUdR until just before the second irradiation using mini-osmotic pumps (Alzet model 2001, USA) which had been implanted subcutaneously 5 days before the first irradiation.

Immunofluorescence staining and micronucleus assay Immediately after the second irradiation, tumors were excised and trypsinized (0.05% trypsin, 0.02% ethylenediaminetetraacetic acid (EDTA), 37°C, 15 min). Tumor cell suspensions were incubated in tissue culture dishes containing complete medium and 1.0 μg/ml of cytochalasin-B to inhibit cytokinesis while preserving nuclear division. The cells were trypsinized and single-cell suspension were fixed with 70% ethanol. After centrifugation, the cell pellet was resuspended with 0.4 ml of cold modified Carnoy's fluid (ethanol:acetic acid=3:1), and then placed on a microslide glass using a dropper and dried at room temperature.

After the denaturation treatment with 2 *M* hydrochloric acid for 30 min at room temperature and the neutralization with borax-borate buffer (pH 8.5), 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 phosphate-buffered saline (PBS). The antibody was applied to microslides for 30 min at room temperature in a humidified chamber. The microslides were washed three times in PBS, and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Becton Dickinson) was applied in the same manner as above.

To observe double-staining of tumor cells with FITC and propidium iodide (PI), cells on the microslides were treated with PI (1–5 μg/ml in PBS). In this manner, we could 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 obtain selectively 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. Although the effects of cytochalasin-B on chromosome damage in X-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 available as a tool for rapid assay of radiosensitivity of cells.[4,5]

When tumors were not pretreated with BUdR before the first irradiation, the ratio obtained indicated the MN frequency of all tumor cells inside the solid tumor.

Table I shows the MN frequencies for all tumor cells and for quiescent cells in both tumors.

Determination of labeling index at the time of second irradiation Tumors from mice that were continuously administered BUdR with mini-osmotic pumps until just before the second irradiation were also excised and trypsinized. Tumor cell suspensions were fixed, and then resuspended in cold modified Carnoy's fluid. This suspension was placed on a microslide glass using a dropper in the same manner as mentioned above. Thereafter, using indirect immunofluorescence staining with FITC to BUdR and nuclear staining with PI, the labeling index for each interval group after the first irradiation was obtained from the ratio of the number of cells radiating green fluorescence (FITC) to the total number of cells observed. Four mice were used for each set of conditions and each experiment was repeated 4 times.

Table I. Micronucleus Frequencies at 0 Gy and after 10 Gy Irradiation

	SCC VII	EMT6/KU
I) All tumor cells		
0 Gy	0.086 (0.084–0.088)[a]	0.050 (0.046–0.054)
Time after the first irradiation of 10 Gy		
0 h	0.510 (0.487–0.533)	1.21 (1.16–1.26)
12 h	0.399 (0.385–0.413)	1.09 (1.05–1.13)
24 h	0.373 (0.355–0.391)	1.03 (0.982–1.08)
48 h	0.341 (0.330–0.352)	0.931 (0.903–0.959)
II) Quiescent cells		
0 Gy	0.087 (0.082–0.092)	0.103 (0.100–0.106)
Time after the first irradiation of 10 Gy		
0 h	0.474 (0.422–0.526)	0.850 (0.776–0.924)
12 h	0.279 (0.269–0.289)	0.700 (0.693–0.707)
24 h	0.259 (0.248–0.270)	0.673 (0.638–0.708)
48 h	0.242 (0.232–0.252)	0.652 (0.602–0.702)

a) Numbers in parentheses are 95% confidence limits, which were determined using mean values, standard deviations and the number of observations on which the means and the standard deviations were based.

RESULTS

Figs. 1 and 2 show the dose-response relation between the second irradiation dose and the normalized MN frequency (MN frequency − C, where C is the MN frequency in unirradiated tumors at the time of second irradiation) obtained immediately after the second irradiation for SCC VII and EMT6/KU tumor cells, respectively. In both tumor systems, the normalized MN frequency of all tumor cell populations decreased with increase in the interval time, but that of quiescent cell populations, which could not be labeled with BUdR at the time of the first irradiation, was increased with increase in the interval between the two irradiations. The tendency in the case of normalized MN frequency was particularly significant immediately after and 48 h after (or long after) the first irradiation.

Table II shows the labeling indices at the time of the first and second irradiations. In both tumors, the longer the interval between the two irradiations, the higher was the labeling index. Furthermore, in both tumors, the labeling index 48 h after the first irradiation was significantly higher than that immediately after the first irradiation of 10 Gy.

Table II. The Labeling Indices at the Time of the First and Second Irradiations (%)

	SCC VII	EMT6/KU
At the first irradiation		
	55.3[a] (51.8–58.8)[b]	74.6[c] (70.4–78.8)
At the second irradiation		
Time after the first irradiation of 10 Gy		
12 h	56.7 (53.8–59.6)	75.6 (71.0–80.2)
24 h	59.0 (56.6–61.4)	77.9 (72.8–83.0)
48 h	61.8[d] (59.1–64.5)	82.1[e] (78.9–85.3)

a) $P<0.05$ compared with *d*).
b) Numbers in parentheses are 95% confidence limits, which were determined using mean values, standard deviations and the number of observations on which the means and the standard deviations were based.
c) $P<0.05$ compared with *e*).

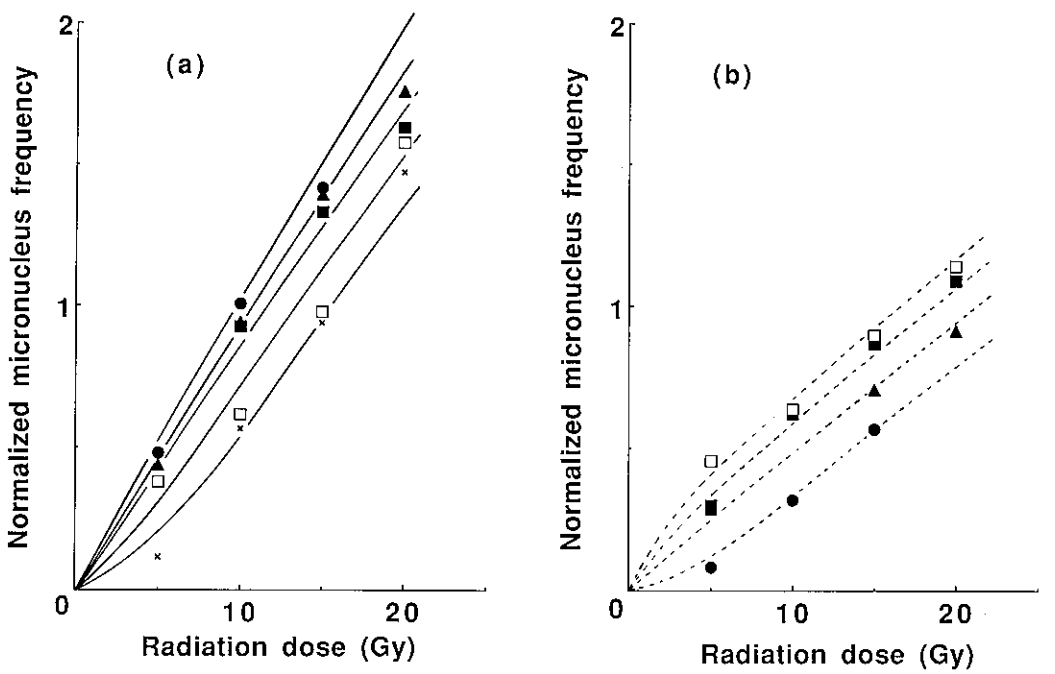

Fig. 1. The dose-response relation between the second irradiation dose and the normalized MN frequency (MN frequency − C, where C is the MN frequency in unirradiated tumors at the time of second irradiation) obtained immediately after the second irradiation for SCC VII tumor cells. All tumor cells (a) and quiescent cells (b) at the time of the first irradiation were given the second irradiation immediately after (●), 12 h after (▲), 24 h after (■) and 48 h after (□) the first irradiation. The data at 4 days after the first irradiation are included on (a) as the data long after (×) the first irradiation. There was a significant difference in normalized MN frequency between immediately after and 48 h after or long after with a $P<0.05$. Only mean values are shown to avoid confusion.

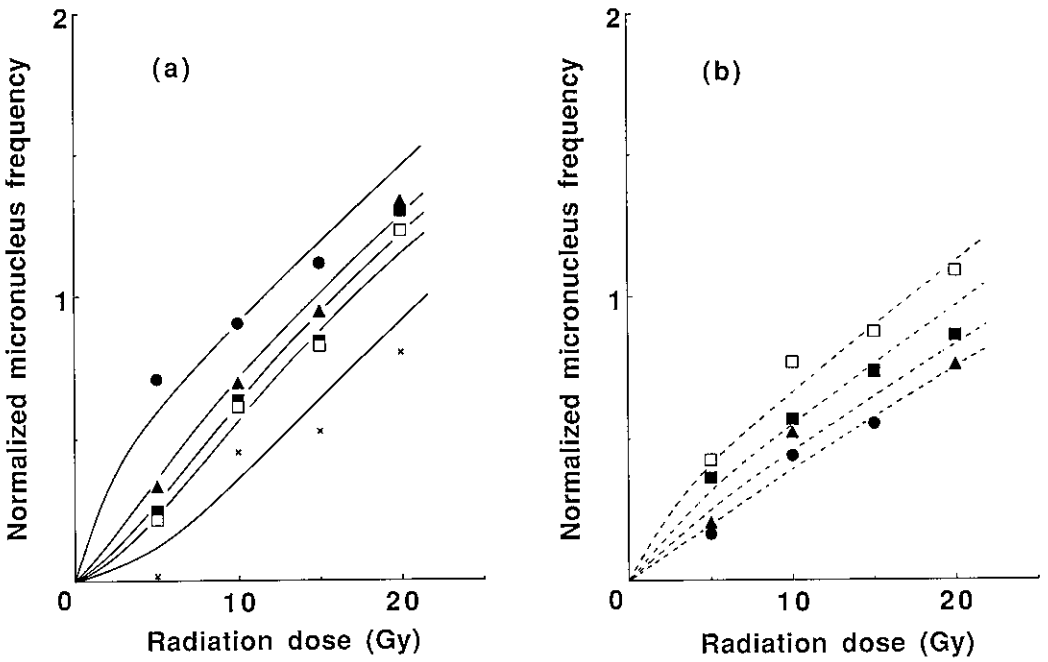

Fig. 2. The dose-response relation between the second irradiation dose and the normalized MN frequency obtained immediately after the second irradiation for EMT6/KU tumor cells. All tumor cells (a) and quiescent cells (b) at the time of the first irradiation were given the second irradiation immediately after (●), 12 h after (▲), 24 h after (■) and 48 h after (□) the first irradiation. The data at 4 days after the first irradiation are included on (a) as the data long after (×) the first irradiation. There was a significant difference in normalized MN frequency between immediately after and 48 h after or long after with a $P<0.05$. Only mean values are shown to avoid confusion.

DISCUSSION

To perform radiation therapy more efficiently, it is indispensable to clarify the response of nonproliferating cells (quiescent cells) in solid tumors to radiation, because many tumor cells are quiescent *in situ*[6] but still have clonogenicity. As shown in Table I, the MN frequencies at 0 h after the first irradiation of 10 Gy show the tendency for a lower radiosensitivity of the quiescent cell population than that of the whole tumor cell population in both tumors. In EMT6/KU tumors, the difference was significant. Additionally, when the magnitude of the decrease in MN frequency was expressed as the MN ratio, in which the MN frequency, which was normalized with the MN frequency at 0 Gy, immediately after 10 Gy irradiation was taken to be 1.0, the MN ratio values 48 h after 10 Gy irradiation for all cells and quiescent cells were 0.60 and 0.40 in SCC VII and 0.76 and 0.73 in EMT6/KU tumors, respectively. Namely, the potentially lethal damage repair (PLDR) capacity of quiescent cells is greater than that of all tumor cells, and PLDR capacity in the SCC VII tumor is greater than that in the EMT6/KU tumor. We have already discussed the PLDR capacity and radiosensitivity of quiescent cells in solid tumors after a single irradiation, and concluded that quiescent cell populations in solid tumors are more radioresistant and have greater PLDR capacities than all cells as a whole in the solid tumors.[3,7]

Since fractionation was introduced into clinical radiotherapy, the efficacy of fractionation has been understood in terms of well established radiobiologic principles,[2] i.e., reoxygenation, redistribution (reassortment), repair and repopulation. However, how the quiescent cell populations in solid tumors behave during fractionated irradiation using X-rays remains unknown. As shown in Figs. 1(b) and 2(b) and Table II, the radiosensitivity of quiescent cells at the time of the first irradiation was elevated and the labeling index at the time of the second irradiation increased with increase in the interval between the first and second irradiations. In other words, during the interval, recruitment from the quiescent to the proliferating state had occurred in the solid tumors, because the first irradiation caused cell loss. In fact, as mentioned in Tubiana's report,[8] in normal tissues, such as bone marrow, treated cells release stimulating factors which are able to recruit quiescent cells into proliferation. Similar mechanisms have been observed in experimental tumors. This recruitment was considered to be one of the

reasons why the radiosensitivity of quiescent cells in the tumors increased with increase in the interval in both tumor systems. Needless to say, during the interval, sublethal damage repair could develop in the quiescent cells in the same way as in all tumor cells shown in Figs. 1(a) and 2(a).[9] During the interval, in the quiescent cell population of each solid tumor at the time of the first irradiation, a part of the cells would be transformed to proliferating cells, which have higher radiosensitivity than the quiescent cells,[3] and the remaining quiescent cells and the newly developed proliferating cells seemed to repair sublethal damage brought about by the first irradiation. Consequently, the increase in radiosensitivity due to recruitment surpassed the decrease due to sublethal damage repair and the recruitment continued longer than the repair of sublethal damage. Therefore, the quiescent cells at the first irradiation became more sensitive to irradiation as the interval became longer. As to recruitment of quiescent cells, some *in-vitro* studies have been reported. Kallman *et al.*[10] observed a rise in quiescent-cell-derived colonies starting at two or three days and overshooting at approximately five or six days after 6 Gy, and suggested that quiescent cells are recruited into proliferating *in vitro*. Wallen *et al.*[11] have shown that the length of the quiescent-to-proliferating transition varied among the cell lines and that the depth of the quiescent state depended on the amount of time the cells had been quiescent in the *in-vitro* study. In some spheroid studies,[12] recruitment has been discussed. Dertinger[13] demonstrated quiescent cell recruitment to the proliferating state as a response to the production of additional extracellular space in the spheroid, due to cell death after irradiation. Durand[14] reported that during a multifraction irradiation regimen, rapid increases in the surviving fraction occurred when delivery of daily fractions was interrupted over the weekend, suggesting repopulation of the spheroids by the surviving cells. An increase in the number of cells with S-phase DNA contents was also reported, indicating that the repopulating cells were derived from the quiescent cell fraction. However, few *in-vivo* studies have been performed so far. In addition, few *in-vivo* studies on sublethal damage of quiescent cells have been reported. Our *in-vivo* study was performed using two fractionated irradiations to simplify the conditions, but by using multifraction irradiation the change of the size of the quiescent cell fraction in solid tumors during fractionated irradiations may be clarified.

Quiescent cells are operationally defined as those cells that are not in active proliferation during the course of the time when the measurements are obtained.[15] We consider the term "quiescent" to include all cells out of cycle, irrespective of the reason. On the other hand, the G_0 state is confined to viable cells that are out of cycle under normal physiological conditions (i.e., not nutrient deprivation *per se*) and can be recruited into active proliferation by a proper stimulus.[16] The best examples of these cells come from normal intact tissues (liver, salivary gland, etc.). Recently the flow cytometric analysis of tumor cells has been developed. Therefore, we also plan to examine the relationship between the response of quiescent cell populations and the change of the cell cycle using flow cytometric analysis.

Our new method for examining the response of quiescent cells in solid tumors revealed that recruitment from quiescent to proliferating status was certainly brought about during the interval between two fractionated irradiations. Using this method, we plan to investigate the response of quiescent cells to treatment with radiation plus chemotherapy agents and/or hypoxic cell sensitizers, as well as the responses to high linear energy transfer radiation or low dose rate radiation.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for Cancer Research (04857111) from the Ministry of Education, Science and Culture, Japan.

(Received May 13, 1993/Accepted August 13, 1993)

REFERENCES

1) Regaud, C. and Nogier, T. Sterilization roentgenienne totale et definitive, sans radiodermite, des testicules du Belier adulte; conditions de sa realisation. *C. R. Soc. Biol.*, **70**, 202–203 (1911).
2) Hall, E. J. and Cox, J. D. Physical and biologic basis of radiation therapy. *In* "Radiation Oncology," ed. W. T. Mass and J. D. Cox, pp. 1–57 (1989). The C. W. Mosby Company, St. Louis, Baltimore, Toronto.
3) Masunaga, S., Ono, K. and Abe, M. A method for selective measurement of the radiosensitivity of quiescent cells in solid tumors — combination of immunofluorescence staining to BrdU and micronucleus assay. *Radiat. Res.*, **125**, 243–247 (1991).
4) Fenech, M. and Morley, A. Solutions to the kinetic problem in the micronucleus assay. *Cytobios*, **43**, 233–246 (1985).
5) Ono, K., Wandl, E. O., Tsutsui, K., Sasai, K. and Abe, M. The correlation between cell survival curve and dose response curve of micronucleus (MN) frequency. *Strahlenther. Onkol.*, **165**, 824–827 (1989).
6) Steel, G. G. Cell population kinetics of tumors in experimental animals. *In* "Growth Kinetics of Tumours," ed.

G. G. Steel, pp. 146–216 (1977). Clarendon Press, Inc., Oxford.
7) Masunaga, S., Ono, K. and Abe, M. Potentially lethal damage repair by quiescent cells in murine solid tumors. *Int. J. Radiat. Oncol. Biol. Phys.*, **22**, 973–978 (1992).
8) Tubiana, M. Repopulation in human tumors. A biological background for fractionation in radiotherapy. *Acta Oncol.*, **27**, 83–88 (1988).
9) Elkind, M. M. and Sutton, H. X-ray damage and recovery in mammalian cells in culture. *Nature*, **184**, 1293 (1959).
10) Kallman, R. F., Combs, C. A., Franko, A. J., Furlong, B. M., Kelly, S. D., Kemper, H. L., Miller, R. G., Rapacchietta, D., Schoenfeld, D. and Takahashi, M. Evidence for the recruitment of noncycling clonogenic tumor cells. *In* "Radiation Biology in Cancer Research," ed. R. E. Meyn and H. R. Withers, pp. 397–414 (1980). Raven Press, New York.
11) Wallen, C. A., Higashikubo, R. and Dethlefsen, L. A. Murine manmmary tumor cells *in vitro*. II. Recruitment of quiescent cells. *Cell Tissue Kinet.*, **17**, 79–89 (1984).
12) Freyer, J. P. Spheroids in radiobiology research. *In* "Spheroid Culture in Cancer Research," ed. R. Bjerkvig, pp. 217–275 (1992). CRC Press, London.
13) Dertinger, H. Kinetics of regrowth of V79 spheroids after γ- and π-irradiation. *Strahlenther. Onkol.*, **155**, 722–725 (1979).
14) Durand, R. E. Repair and proliferation: major determinants of the multifraction radiation response. *Int. J. Radiat. Oncol. Biol. Phys.*, **15**, 1147–1152 (1988).
15) Dethlefsen, L. A. In quest of the quaint quiescent cells. *In* "Radiation Biology in Cancer Research," ed. E. Meyn and H. Withers, pp. 415–435 (1980). Raven Press, New York.
16) Lajtha, L. G. On the concepts of the cell cycle. *Cell Comp. Physiol.*, **62**, 143–145 (1963).