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Hyperfractionated and Hypofractionated Radiation Therapy for Human Malignant Glioma Xenograft in Nude Mice

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Xenografts of a human malignant glioma subcutaneously transplanted into nude mice were irradiated with graded single doses (2, 5, 10 or 20 Gy) or five types of fractionation schedules in two weeks: conventional [20 Gy in 10 fractions (fr)], hyperfractionated [24 Gy in 20 fr (two fractions per day)], and hypofractionated-1, 2, 3 [20 Gy, 18 Gy, 16 Gy in 4 fr]. All of the fractionated irradiation groups showed tumor regression. The hypofractionation-1 group (20 Gy in 4 fr) demonstrated the most prominent tumor regression, while the hyperfractionation group (24 Gy in 20 fr) showed the least effect. The hypofractionation-2 group (18 Gy in 4 fr) showed similar regression to the conventional fractionation group (20 Gy in 10 fr). Histologically, tumors in the control groups consisted of a homogenous population of small anaplastic cells, and only a small number of tumor cells were glial fibrillary acidic protein (GFAP)-positive. Following irradiation, the population of small anaplastic cells decreased and the percentage of GFAP-positive cells increased. Cellular pleomorphism became much more prominent after irradiation in all of the fractionated irradiation groups as compared with the graded single dose irradiation groups. In this study, hyperfractionation was not effective against human glioma xenografts compared with conventional fractionation and hypofractionation. This indicates that care is needed in applying hyperfractionation regimens to human malignant gliomas.

Key words: Hyperfractionation — Hypofractionation — Malignant glioma — Nude mice — Morphologic change

Malignant gliomas (including glioblastoma and anaplastic astrocytoma) are the most common primary brain tumors in adults, and the prognoses for them are extremely poor. Post-operative radiotherapy has resulted in significantly prolonged survival, but most tumors recur at the primary sites or close to them.[1,2] Recently, treatment trials of altered fractionation schedules such as hyperfractionation have been done to improve the therapeutic ratio, but statistically significant amelioration has not always been obtained.[3] The present study was designed to compare hyperfractionated and hypofractionated radiation therapy with conventional radiation therapy. A human malignant glioma transplanted into nude mice was irradiated with graded single doses and 5 different fractionation schedules. The early response was evaluated in terms of growth curves and the morphologic changes of tumor cells.

MATERIALS AND METHODS

Animals Male and female athymic nude mice with a BALB/c genetic background, were used. They were maintained in a specific-pathogen-free room. All food, water and bedding were sterilized.

Original tumor Brain tumor tissue was obtained at surgery from the left temporal lobe of a 67-year-old male. It was kept under sterile conditions at 4°C prior to implantation into mice.

Tumor implantation and passages in mice Tumor tissue was cut into small fragments (approximately 3 mm in diameter). Three grafts at a time were subcutaneously implanted into the right flank in serial passages, and into the right thigh in the irradiation experiments. The subcutaneous tumors were measured with calipers every three days. The width (a) and length (b) of the tumor nodules were measured, and tumor volume (V) was calculated by using the formula $V = 1/2a^2b$. This tumor has shown unchanged histological and growth characteristics for more than 8 years. Spontaneous regression was never found. In this experiment, tumors in passages 15–22 were used.

Tumor irradiation Transplanted tumors in the right thigh were irradiated with 200 kV X-rays at 0.86 Gy per minute (Siemens, Stabilipan 2: 1 mm Cu filter, 40 cm target distance) when the tumor volume exceeded 200 mm^3. The mice were not anesthetized and their bodies were shielded with lead plates. Five types of fractionated irradiation schedules were done as shown in Table I. Each group consisted of 6 or more mice. In the hyperfractionation, mice were treated with 1.2 Gy twice daily,

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Table I. Irradiation Schedules

Schedule	Fraction size (Gy)	Number of fractions	Total dose (Gy)
Single dose fr	2, 5, 10, 20	1	2, 5, 10, 20
Conventional fr	2.0	10 (5 days/week)	20
Hyperfractionation	1.2[a)]	20 (5 days/week)	24
Hypofractionation-1	5.0	4 (2 days/week)	20
Hypofractionation-2	4.5	4 (2 days/week)	18
Hypofractionation-3	4.0	4 (2 days/week)	16

a) Twice daily, with an interval of 6 h.

with an interval of 6 h. Graded single dose irradiation (2, 5, 10, or 20 Gy) was also done for comparison. Two other groups of mice served as non-irradiated controls to get two different sizes of non-irradiated control tumors for histological investigations, because the histological findings differ with tumor size. Growth curves were established by plotting the relative tumor volume (V' = volume on the day/volume at initiation). Statistical comparisons of the volume among the groups were also made (Student's t test). Tumors in the fractionated irradiation groups were excised 21 days after the start of radiation therapy (10 or 11 days after the last irradiation). Tumors treated with graded single dose irradiation were excised 15 or 21 days after irradiation. Non-irradiated control tumors were excised when the volume exceeded 200 mm^3 (control group A) and 21 days after the volume reached 200 mm^3 (control group B).

Morphological examination The excised tumors were fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE) for light microscopic examination. Immunohistochemical stains were also performed by the peroxidase-anti-peroxidase method with polyclonal antibodies to glial fibrillary acidic protein (GFAP) and S-100 protein (DAKO, Denmark). These proteins are often expressed in gliomas, and GFAP especially is known to correlate with glial differentiation.

RESULTS

Morphologic characteristics of original human tumor and mouse-passaged tumors The original human tumor contained fibrillary astrocytes and fusiform anaplastic cells. Most of the tumor cells were GFAP- and S-100 protein-positive. Prominent vascular proliferation was seen, though no palisading necrosis was found. These findings were compatible with the features of anaplastic astrocytoma.

Mouse-passaged tumors predominantly consisted of small anaplastic cells. Only a few of the tumor cells were GFAP-positive, though most of them were S-100 protein-positive. Palisading necrosis was frequently seen, but no prominent vascular proliferation was evident. These findings were comparable to those for typical small cell glioblastoma except for the lack of vascular proliferation. These morphologic characteristics have been maintained in mice for more than 8 years.

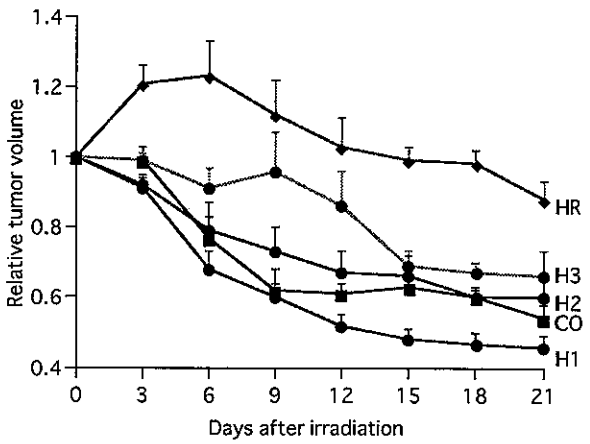

Fig. 1. Mean relative tumor volume of the fractionated irradiation groups. CO: conventional fractionation (20 Gy in 10 fr), HR: hyperfractionation (24 Gy in 20 fr), H-1, 2, 3: hypofractionation-1, 2, 3 (20, 18, 16 Gy in 4 fr). The volume of the HR group decreased significantly compared with that of the CO group ($P<0.001$).

Growth characteristics of control and treated groups Tumors in the control groups showed exponential growth, and the average tumor volume doubling time was approximately 9 days. There was a significant response to irradiation in all of the treated groups except for the group given a single dose of 2 Gy. All tumors in the fractionated irradiation groups showed regression, but the extent of response varied from group to group (Fig. 1). The hypofractionation-1 group (20 Gy in 4 fr) had the most prominent regression, while the hyperfractionation group (24 Gy in 20 fr) had the least. The mean relative tumor volume in the hyperfractionation group was significantly larger than that in the conventional group ($P<0.001$). The hypofractionation-2 group (18 Gy in 4 fr) showed the most similar regression to the conventional group (20 Gy in 10 fr).

Morphologic characteristics of tumors of the control and the treated groups Histologically, tumors in the non-irradiated control groups displayed a homogeneous population of small anaplastic cells, and mitoses were frequently seen (Fig. 2a). Massive confluent necrosis and small foci of necrosis with nuclear pseudopalisading were frequently found in the large tumors of the control group B (Fig. 2b), though these features were inconspicuous in the small tumors of control group A. No prominent

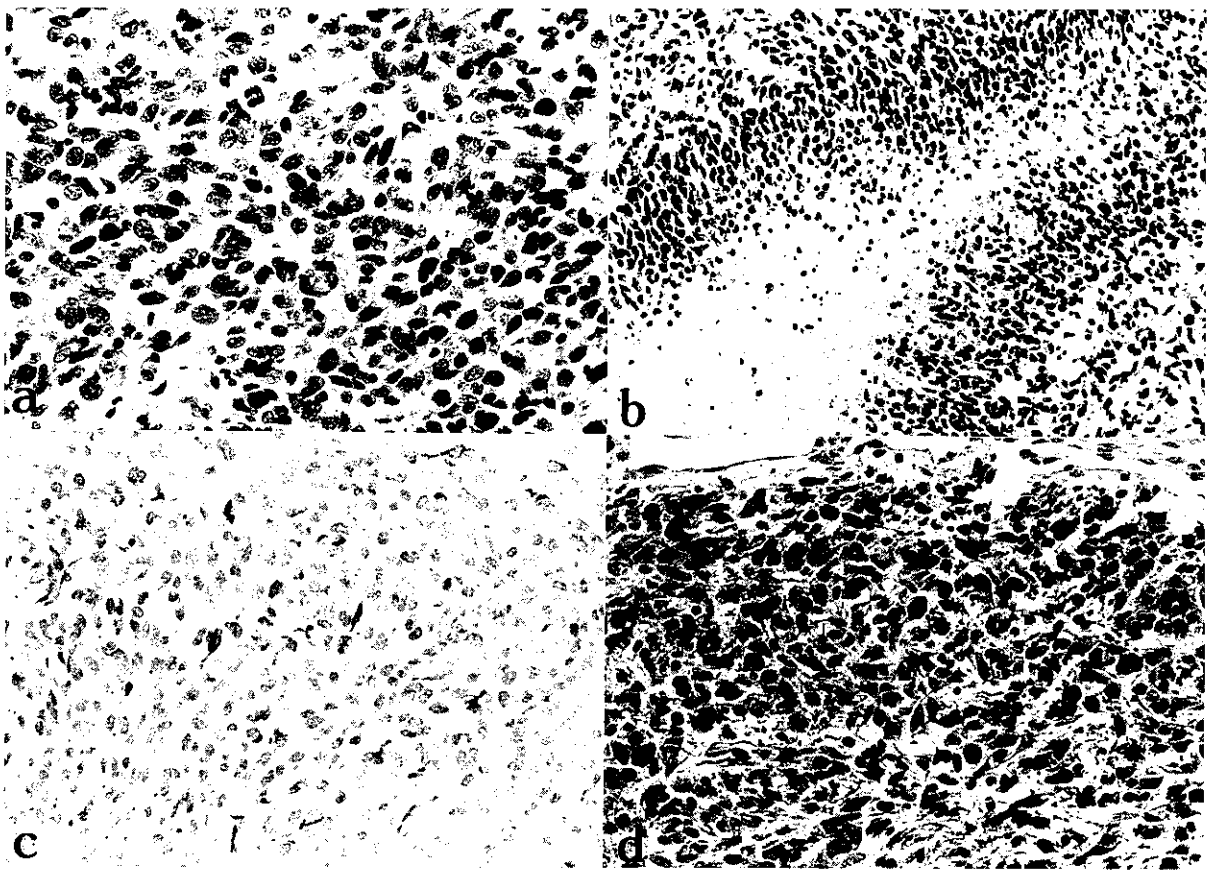

Fig. 2. Non-irradiated control tumors. a. Tumor consists mainly of small anaplastic cells. (HE, ×320). b. Pseudopalisading around necrosis is seen in the large tumor excised 21 days after the volume reached 200 mm^3. (HE, ×160). c. Only a few of the tumor cells are GFAP-positive. Darkly stained narrow cytoplasm is seen in a scattered fashion. (Immunoperoxidase-hematoxylin, ×320). d. Most of the tumor cells are S-100 protein-positive. Both the cyotoplasm and nuclei are stained darkly. (Immunoperoxidase-hematoxylin, ×320).

vascular proliferation was found in any tumor, but dilated vessels without endothelial proliferation were often recognized in some tumors of control group B. Immunohistochemically, only a few of the tumor cells were GFAP-positive (Fig. 2c), but most of the whole tumor cells were S-100 protein-positive (Fig. 2d).

On the other hand, the morphologic features of tumors in the treated groups were clearly different from those in the non-irradiated control groups. In all treated tumors except for those which had received a single dose of 2 Gy, small anaplastic cells decreased and the cellular pleomorphism became conspicuous (Fig. 3a–c). These tumors contained a mixed cell population of several types, including fibrillary, bizarre and giant cells. The number of mitotic figures was decreased. Neither necrosis nor nuclear pseudopalisading was found, though stromal fibrosis was more frequently seen than in the non-irradiated control tumors. No prominent vascular proliferation was seen, and no dilated vessels were found. Immunohistochemically, the percentage of GFAP-positive cells was markedly increased (Fig. 3d), though small anaplastic cells and multi-nucleated giant cells were often negative for the protein. However, most of the tumor cells of various types were S-100 protein-positive. All of these histological features were found in every tumor in the treated groups except for those which had received a single dose of 2 Gy. Histopathological changes in tumor cells were more prominent in the 5 groups given fractionated irradiation than in the groups given graded single dose irradiation, but there was little difference among the 5 groups.

DISCUSSION

Walker *et al.*[4] studied patients with malignant gliomas, who received 171–200 rad per day with 5 fractions per

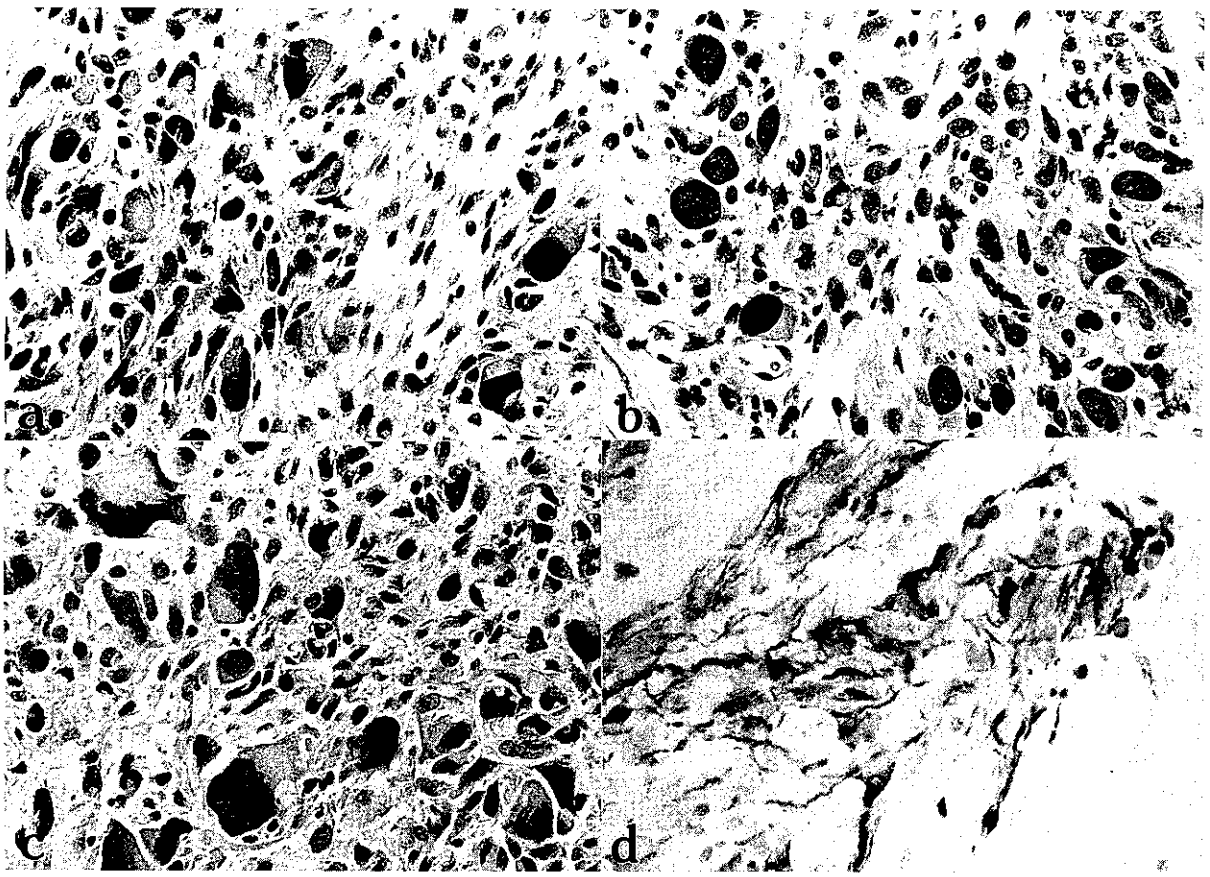

Fig. 3. Treated tumors of the fractionated irradiation groups. a. Conventional fr (20 Gy in 10 fr). b. Hyperfractionation (24 Gy in 20 fr). c. Hypofractionation-1 (20 Gy in 4 fr). Small anaplastic cells are markedly decreased and cellular pleomorphism is conspicuous in tumors of these groups. Bizarre and giant cells are often found, but necrosis is not. There is little difference of histological findings among the groups, though they differ in the extent of tumor volume regression. (HE, ×320). d. Hyperfractionation. Many of the tumor cells are GFAP-positive. Darkly stained elongated cytoplasm is seen. (Immunoperoxidase-hematoxylin, ×320).

week. They reported that post-operative conventional radiation therapy had a significant influence on the survival of patients who received more than 5000 cGy, and a clear-cut dose-effect relationship existed. However, the maximum median survival period among them was 42 weeks, and the poor prognosis was not greatly improved.

Compared to conventional fractionation, multiple daily fractionation or hyperfractionation improved the survival of patients with gliomas.[5–9] In patients with brain stem gliomas, hyperfractionated radiation therapy appeared to be especially effective. In other studies, however, significant differences in survival were not always found[10–15] though a trend toward improved survival was often suggested. However, such a comparison among studies is not always reliable, because details of the fractionation schedules, histological classification and patients' characteristics, such as age and performance status, were not always identical. In the present series of experiments, the hyperfractionation group was treated with 1.2 Gy twice daily to a total dose of 24 Gy, which was 20% higher than that in the conventional group, though the radiation response was not as effective as we had expected.

In addition, some authors have suggested that large dose fractionation or hypofractionation might offer a better prognosis than conventional fractionation for patients with glioblastoma.[16, 17] Hercbergs *et al.*[18] suggested that hypofractionated radiation in combination with cisplatinum might offer rapid palliation in severely compromised patients with malignant gliomas. But survival times were not always long enough to allow information to be obtained on the late effects on normal brain tissue.

In our study, tumor regression in the hyperfractionation group (24 Gy in 20 fr), was significantly less than in

the conventional group (20 Gy in 10 fr). On the other hand, the hypofractionation-1 group (20 Gy in 4 fr) showed the most effective tumor regression, but we cannnot conclude that this fractionation is the best method, because the difference in the fraction size would lead to differences in radiation effects on various tissues, and also the effect on normal brain tissue was not studied.

Studies of the linear quadratic (LQ) model indicate that hyperfractionated irradiation should reduce late injury to normal brain tissue, and total dose escalation could achieve increased tumor injury, because the α/β ratios for tumors are usually higher than those of late-responding normal tissues.[19, 20] If the LQ model is appropriate for this tumor, the ratio can be determined from the dose per fraction and the number of fractions by varying the number of fractions in constant overall time.[21–23] In our irradiation schedules, conventional fractionation (20 Gy in 10 fr) and hypofractionation (18 Gy in 4 fr) achieved the most similar degrees of tumor regression. If these are considered to be iso-effective doses, the ratio is calculated to be approximately 20 Gy. However, this value might not be correct, because the effects of the mixed oxic-hypoxic population, reoxygenation, etc. were not excluded.[24] It is desirable that experimental tumors should be sensitized by misonidazole or clamped to minimize the influence of changing hypoxic fractions,[23, 24] though tumors in most patients are neither sensitized nor clamped in practice. Zietman *et al.*[20] reported a ratio of human high-grade glioma xenograft of 16–38 Gy. In their study, tumors were irradiated under hypoxic conditions and the ratios were corrected for oxic conditions with an assumed oxygen enhancement ratio (OER).

If the ratio for this tumor is as high as 20 Gy, multiple small dose fractionation (hyperfractionation) schedules should be available, and the calculated dose of 1.05 Gy twice daily (total dose 21 Gy) is predicted for equal early tumor injury compared with conventional fractionation of 2 Gy once daily (total dose 20 Gy). However, the tumors treated with 1.2 Gy twice daily (total dose 24 Gy) showed significantly less effective regression. These findings suggest that the LQ model does not hold for the early radiation responses of this tumor, and the hyperfractionated radiotherapy with a 20% total dose escalation was not more effective than the conventional fractionation, contrary to what we expected. During the course of fractionated irradiation, proliferation may have occurred. In addition, human tumors in nude mice are not always identical to the original tumors.

Histological changes were noted in every irradiated tumor except for those exposed to a single dose of 2 Gy. Small anaplastic cells which characterize small cell glioblastoma decreased markedly after irradiation. Cellular pleomorphism became conspicuous, and no nuclear pseudopalisading around micronecrosis was found. These histological features were more evident in tumors exposed to the five types of fractionated irradiation than in those given single dose irradiation, though there was little difference among the five types. Our previous study showed that small anaplastic cells became predominant again and pseudopalisading was frequently found at regrowth.[25] These findings in nude mice are comparable to those in histological studies of tumors in man.[26, 27]

Immunohistochemically, the percentage of GFAP-positive cells greatly increased, though small anaplastic cells and multi-nucleated giant cells were often negative. These changes appeared to correspond to the decrease of small anaplastic cells following irradiation. However, most of the tumor cells of various types were S-100 protein-positive both before and after irradiation.

These results obtained in the current study are not necessarily comparable to those for brain tumors in man, but most of the histological characteristics of this tumor have been maintained in long-term serial passages for more than 8 years. The volume doubling time was about 9 days, which was not as short as that of most murine tumors.

In conclusion, hyperfractionated radiation therapy for this human glioblastoma model was not as effective as we had expected theoretically. This suggests that we have to be careful in using altered fractionation schedules for practical clinical purposes.

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REFERENCES

1) Hochberg, F. H. and Pruitt, A. Assumptions in the radiotherapy of glioblastoma. *Neurology*, **30**, 907–911 (1980).
2) Wallner, K. E., Galicich, J. H., Krol, G., Arbit, E. and Malkin, M. G. Patterns of failure following treatment for glioblastoma multiforme and anaplastic astrocytoma. *Int. J. Radiat. Oncol. Biol. Phys.*, **16**, 1405–1409 (1989).
3) Sheline, G. E. Radiotherapy for high grade gliomas. *Int. J. Radiat. Oncol. Biol. Phys.*, **18**, 793–803 (1990).
4) Walker, M. D., Strike, T. A. and Sheline, G. E. An analysis of dose-effect relationship in the radiotherapy of malignant gliomas. *Int. J. Radiat. Oncol. Biol. Phys.*, **5**, 1725–1731 (1979).
5) Douglas, B. G. and Worth, A. J. Superfractionation in glioblastoma multiforme: results of a phase II study. *Int. J. Radiat. Oncol. Biol. Phys.*, **8**, 1787–1794 (1982).

6) Fulton, D. S., Urtasun, R. C., Shin, K. H., Geggie, P. H. S., Thomas, H., Muller, P. J., Moody, J., Tanasichuck, H., Mielke, B., Johnson, E. and Curry, B. Misonidazole combined with hyperfractionation in the management of malignant gliomas. *Int. J. Radiat. Oncol. Biol. Phys.*, **10**, 1709–1712 (1984).
7) Edwards, M. S. B., Wara, W. M., Urtasun, R. C., Prados, M., Levin, V. A., Fulton, D., Wilson, C. B., Hannigan, J. and Silver, P. Hyperfractionated radiation therapy for brain-stem glioma: a phase I-II trial. *J. Neurosurg.*, **70**, 691–700 (1989).
8) Linstadt, D. E., Edwards, M. S. B., Prados, M., Larson, D. A. and Wara, W. M. Hyperfractionated irradiation for adults with brainstem gliomas. *Int. J. Radiat. Oncol. Biol. Phys.*, **20**, 757–760 (1991).
9) Nelson, D. F., Curren, W. J., Jr., Scott, C., Nelson, J. S., Weinstein, A. S., Ahmad, K., Constine, L. S., Murray, K., Powlis, W. D., Mohiuddin, M. and Fishbach, J. Hyperfractionated radiation therapy and bis-chlorethyl nitrosourea in the treatment of malignant glioma — possible advantage observed at 72.0 Gy in 1.2 Gy B. I. D. fractions: report of the radiation therapy oncology group protocol 8302. *Int. J. Radiat. Oncol. Biol. Phys.*, **25**, 193–207 (1993).
10) Ludgate, C. M., Douglas, B. G., Dixon, P. F., Steinbok, P., Jackson, S. M. and Goodman, G. B. Superfractionated radiotherapy in grade III, IV intracranial gliomas. *Int. J. Radiat. Oncol. Biol. Phys.*, **15**, 1091–1095 (1988).
11) Deutsch, M., Green, S. B., Strike, T. A., Burger, P. C., Robertson, J. T., Selker, R. G., Shapiro, W. R., Mealey, J., Jr., Ransohoff, J., II, Paoletti, P., Smith, K. R., Jr., Odom, G. L., Hunt, W. E., Young, B., Alexander, E., Jr., Walker, M. D. and Pistenmaa, D. A. Results of a randomized trial comparing BCNU plus radiotherapy, streptozotocin plus radiotherapy, BCNU plus hyperfractionated radiotherapy, and BCNU following misonidazole plus radiotherapy in the post-operative treatment of malignant gliomas. *Int. J. Radiat. Oncol. Biol. Phys.*, **16**, 1389–1396 (1989).
12) Shrieve, D. C., Wara, W. M., Edwards, M. S. B., Sneed, P. K., Prados, M. D., Cogen, P. H., Larson, D. A. and Levin, V. A. Hyperfractionated radiation therapy for gliomas of the brainstem in children and adults. *Int. J. Radiat. Oncol. Biol. Phys.*, **24**, 599–610 (1992).
13) Freeman, C. R., Krischer, J. P., Sanford, R. A., Cohen, M. E., Burger, P. C., Carpio, R., Halperin, E. C., Munoz, L., Friedman, H. S. and Kun, L. E. Final results of a study of escalating doses of hyperfractionated radiotherapy in brain stem tumors in children: a pediatric oncology group study. *Int. J. Radiat. Oncol. Biol. Phys.*, **27**, 197–206 (1993).
14) Packer, R. J., Boyett, J. M., Zimmerman, R. A., Rorke, L. B., Kaplan, A. M., Albright, A. L., Selch, M. T., Finlay, J. L., Hammond, G. D. and Wara, W. M. Hyperfractionated radiation therapy (72 Gy) for children with brain stem gliomas. *Cancer*, **72**, 1414–1421 (1993).
15) Packer, R. J., Boyett, J. M., Zimmerman, R. A., Albright, A. L., Kaplan, A. M., Rorke, L. B., Selch, M. T., Cherlow, J. M., Finlay, J. L. and Wara, W. M. Outcome of children with brain stem gliomas after treatment with 7800 cGy of hyperfractionated radiotherapy. *Cancer*, **74**, 1827–1834 (1994).
16) Kapp, D. S., Wagner, F. C. and Lawrence, R. Glioblastoma multiforme: treatment by large dose fraction irradiation and metronidazole. *Int. J. Radiat. Oncol. Biol. Phys.*, **8**, 351–355 (1982).
17) Tamura, M., Nakamura, M., Kunimine, H., Ono, N., Zama, A., Hayakawa, K. and Niibe, H. Large dose fraction radiotherapy in the treatment of glioblastoma. *J. Neuro-Oncol.*, **7**, 113–119 (1989).
18) Hercbergs, A. A., Tadmor, R., Findler, G., Sahar, A. and Brenner, H. Hypofractionated radiation therapy and concurrent cisplatin in malignant cerebral gliomas: rapid palliation in low performance status patients. *Cancer*, **64**, 816–820 (1989).
19) Malaise, E. P., Fertil, B., Chavaudra, N. and Guichard, M. Distribution of radiation sensitivities for human tumor cells of specific histological types: comparison of *in vitro* to *in vivo* data. *Int. J. Radiat. Oncol. Biol. Phys.*, **12**, 617–624 (1986).
20) Zietman, A. L., Suit, H. D., Tomkinson, K. N., Thames, H. D. and Sedlacek, R. S. The response of two human tumor xenografts to fractionated irradiation. The derivation of ratios from growth delay, tumor control, and *in vitro* cell survival assays. *Int. J. Radiat. Oncol. Biol. Phys.*, **18**, 569–575 (1990).
21) Douglas, B. G. and Fowler, J. F. The effect of multiple small doses of X rays on skin reactions in the mouse and a basic interpretation. *Radiat. Res.*, **66**, 401–426 (1976).
22) Fowler, J. F. What next in fractionated radiotherapy? *Br. J. Cancer*, **49** (Suppl. VI), 285–300 (1984).
23) Lindenberger, J., Hermeking, H., Kummermehr, J. and Denekamp, J. Response of human tumour xenografts to fractionated X-irradiation. *Radiother. Oncol.*, **6**, 15–27 (1986).
24) Williams, M. V., Denekamp, J. and Fowler, J. F. A review of ratios for experimental tumors: implications for clinical studies of altered fractionation. *Int. J. Radiat. Oncol. Biol. Phys.*, **11**, 87–96 (1985).
25) Hasegawa, M., Niibe, H., Yamakawa, M., Kato, S., Furuta, M., Kiryu, Y., Ito, J., Nakanishi, Y. and Nakazato, Y. Morphological changes of irradiated human gliomas in nude mice. *Brain Tumor Pathol.*, **8**, 223–225 (1991).
26) Schiffer, D., Giordana, M. T., Soffietti, R. and Sciolla , R. Histological observations on the regrowth of malignant gliomas after radiotherapy and chemotherapy. *Acta Neuropathol.*, **58**, 291–299 (1982).
27) Giangaspero, F. and Burger, P. C. Correlations between cytologic composition and biologic behavior in the glioblastoma multiforme: a postmortem study of 50 cases. *Cancer*, **52**, 2320–2333 (1983).