A comparative study of apoptosis and proliferation in germinoma and glioblastoma¹

Masahiro Mizoguchi, Takanori Inamura,² Tadahisa Shono, Kiyonobu Ikezaki, Satoshi Inoha, Shoichiro Ohgami, and Masashi Fukui

Department of Neurosurgery, Neurological Institute, Faculty of Medicine, Kyushu University, Fukuoka, 812-8582 Japan (M.M., T.I., T.S., K.I., S.I., M.F.); and Department of Neurosurgery, Hamanomachi Hospital, Fukuoka, 810-8539 Japan (S.O.)

Intracranial germinoma has a relatively good prognosis when treated with radiotherapy and chemotherapy, whereas glioblastoma has a poor prognosis irrespective of these treatments. Cell proliferation and cell death are opposing processes in tumor growth, with tumor progression reflecting the balance between proliferating and apoptotic cells. We investigated cell proliferation and cell death using MIB-1 staining and nick-end labeling in 13 germinomas in comparison with 11 glioblastomas. Expression of BAX and Bcl-2, which regulate apoptosis, were studied by immunohistochemistry. Although germinomas showed strong MIB-1 immunostaining similar to that seen in glioblastomas, germinomas included significantly more apoptotic cells. The ratio of apoptotic ratio to MIB-1 labeling index for germinomas was 72.9 ± 36.9 (mean $\pm$ SD), a higher, statistically significant ratio as compared with glioblastomas (14.5 ± 11.2 ; $P < 0.01$). Furthermore, germinomas showed greater expression of BAX than did glioblastomas, while the expression of Bcl-2 was weak in both tumor types. A comparison of these apoptotic-related proteins showed that immunoreactivity for BAX was relatively higher in germinomas than in glioblastomas ($P < 0.01$), corresponding well to numerous apoptotic

cells identified in germinoma tissues. These findings may account for the prognostic difference between germinoma and glioblastoma in the face of a similar proliferation potential according to MIB-1 immunostaining. The balance between cell proliferation and death should be considered when predicting outcomes in patients with intracranial tumors. *Neuro-Oncology* 2, 96-102, 2000 (Posted to *Neuro-Oncology* [serial online], Doc. 99-35, March 3, 2000. URL <neuro-oncology.mc.duke.edu>)

Germ cell tumors are relatively rare among brain tumors. Germinoma stands out as being the most common type and as having a characteristically benign clinical course when treatment exploits its remarkable chemo- and radiosensitivity. Recently, chemotherapy and radiotherapy have achieved higher survival rates for patients with intracranial germinoma, with 92.7% and 80.6% of patients surviving for 10 and 20 years, respectively (Matsutani et al., 1997). In sharp contrast, the median survival of patients with malignant glioma is between 1 and 2 years, even in patients treated with cytoreduction surgery, radiotherapy, and chemotherapy (Shapiro et al., 1989).

Both cell proliferation and spontaneous cell death occur in tumor tissue. The extent of tumor growth is thought to reflect a balance of cell proliferation and cell death. We hypothesized that the different biologic behaviors of intracranial germinoma and glioblastoma result from different ratios of cell proliferation and death. Therefore, we compared cell proliferation, spontaneous cell death, and expression of apoptotic-related proteins (BAX and Bcl-2) in germinoma and glioblastoma using MIB-1 staining (Shibuya et al., 1993), nick-end labeling (Gavrieli et al., 1992), and immunohistochemical staining, respectively.

Received 19 July 1999, accepted 20 January 2000.

¹This work was supported by grant nos. 08407043 and 09771044 from the Ministry of Education, Science and Culture of Japan.

²Address correspondence and reprint requests to Takanori Inamura, Department of Neurosurgery, Neurological Institute, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan.

³Abbreviations used are as follows: AR, apoptotic ratio; LI, labeling index; TUNEL, TdT-mediated dUTP-biotin 3'-OH nick-end labeling.

Materials and Methods

Tissue Samples

Thirteen intracranial germinoma specimens used in this study were resected at Kyushu University Hospital from 1980 to 1998 and were compared with 11 resected glioblastoma specimens. The outcomes of these patients are summarized in Table 1. The patients with germinoma were followed for more than 12 months (range 12 to 109 months, median 53.5 months). Only two patients died, at 48 months and 39 months after the operation; the others were still alive at last follow-up (median 59.3 months). In contrast, the patients with glioblastoma were followed for 1.4 to 35.8 months. Only two patients were alive at last follow-up; one was alive at 2.5 months and another at 7.5 months. The median overall survival of the other glioblastoma patients was 14.2 months (range 1.4 to 35.8 months). All germinomas showed characteristic histologic features: uniform cells with large vesicular nuclei, prominent nucleoli, and clear-to-pale, glycogen-rich cytoplasm admixed with infiltrating lymphocytes and delicate fibrovascular septa. No syncytiotrophoblastic giant cells positive for human chorionic gonadotropin were detected in any specimen. On the other hand, glioblastomas were composed of highly atypical glial cells and showed mitotic activity, vascular proliferation, and/or pseudopalisading necrosis. The tumors were classified histologically by expert neuropathologists. Formalin-fixed, paraffin-embedded materials were used for routine staining with hematoxylin and eosin, as well as by immunohistochemical techniques and the TUNEL³ method (Gavrieli et al., 1992).

Immunohistochemical Study

In all cases, immunohistochemical reactions were performed using a labeled biotin-streptavidin method. Serial sections were studied with the monoclonal antihuman Ki-67 antigen antibody MIB-1 (Immunotech, Marseille, France; dilution, 1:100), polyclonal antihuman BAX protein antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.; dilution, 1:50), and monoclonal antihuman Bcl-2 protein antibody Bcl-2/100 (Wako, Osaka, Japan; diluted 1:150).

The sections were cut at a thickness of 6 μ m and were then deparaffinized in xylene, hydrated in ethanol, and incubated with 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature to inhibit endogenous peroxidase. After being washed with Tris-HCl buffer (50 mmol/L Tris-HCl, pH 7.6), the sections were completely immersed in 0.01M citrate buffer (pH 6.0) and were autoclaved for 10 min to enhance the immunoreactivity. After pretreatment, the sections were incubated with diluted primary antibodies at 4°C overnight. Subsequent steps were carried out with species-specific biotinylated secondary antibodies and horseradish peroxidase-labeled streptavidin according to manufacturer's instructions (Amersham, Buckinghamshire, UK). The colored reaction product was developed with 3,3'-diaminobenzidine tetrahydrochloride. The sections were counterstained lightly with hema-

toxylin. For MIB-1 immunostaining, glioblastoma specimens for which the MIB-1 LI was already known were used for the control. Normal astrocytes contained only faint or no Bcl-2 immunoreactivity and no BAX immunoreactivity, consistent with previous reports (Krajewski et al., 1997; Krishna et al., 1995; Rieger et al., 1998). In this study, controls for internal comparison were provided by normal astrocytes for the Bcl-2 (+/-) and BAX (-) immunostaining.

TUNEL Method

Detection of cells undergoing apoptosis was performed by an in situ end-labeling method using the in situ Apoptosis Detection Kit (Takara Biomedicals, Shiga, Japan). The sections were deparaffinized in xylene, hydrated in ethanol, and then digested with proteinase K (20 μ g/ml) for 15 min at room temperature. After washing in phosphate-buffered saline, endogenous peroxidase was blocked with 3% hydrogen peroxide in phosphate-buffered saline for 5 min at room temperature. The sections were washed in phosphate-buffered saline and subsequently incubated with 50 μ l of working solution containing 5 μ l of TdT enzyme and 45 μ l of labeling-safe buffer in a humid chamber at 37°C for 90 min. After being washed in phosphate-buffered saline to stop the reaction, sections were incubated with 70 μ l of antiferrescein isothiocyanate-horseradish peroxidase conjugate at 37°C for 30 min before the colored reaction product was developed with 3,3'-diaminobenzidine tetrahydrochloride. The sections were counterstained lightly with hematoxylin.

The results of the TUNEL method were recorded similarly to those of MIB-1 immunohistochemistry. Diffuse cytoplasmic TUNEL staining was recognized occasionally in necrotic areas of malignant gliomas (Kordek et al., 1996; Patsouris et al., 1996); such cytoplasmic staining does not represent apoptosis and instead reflects partial preservation of the structural integrity of chromatin until late in necrosis (Wyllie, 1987). We did not count cytoplasmically stained cells in necrotic areas of malignant gliomas as apoptotic. For the TUNEL method, rat mammary tissue was used for the positive control (Fig. 1F), and normal CNS neuronal tissue was used for the negative control.

Evaluation and Statistical Analysis

In the MIB-1 immunostaining and TUNEL methods, the tumor cells showed nuclear staining. More than 400 tumor cells from each specimen were counted under $\times 400$ magnification, and the MIB-1 LI and AR were calculated as percentages of positively stained nuclei. The vascular components, infiltrating lymphocytes, and necrotic areas of tumor were carefully excluded.

The difference in AR, MIB-1 LI, and AR/LI ratio values between the two groups of tumors was tested for statistical significance using the Mann-Whitney *U* test.

BAX expression was detected either in the nucleus and/or the cytoplasm, while Bcl-2 showed cytoplasmic staining. In widespread staining, tumor cells were considered as negative if none were stained (-), negative

Table 1. Summary of 24 cases

	Diagnosis	F/U ^a (months)	AR	AR/LI	MIB-1 LI	BAX ^b	Bcl-2 ^b	Bcl-2 (lymph.) ^b
1	Germinoma	41+	19.1	166.1	11.5	+/-	+/-	++
2	Germinoma	39D	20.0	119.8	16.7	++	-	+
3	Germinoma	101+	12.0	93.0	12.9	-	-	+/-
4	Germinoma	77+	13.8	83.6	16.5	++	-	+/-
5	Germinoma	19+	16.3	74.8	21.8	+/-	+/-	+/-
6	Germinoma	50+	21.3	68.1	31.3	+	-	++
7	Germinoma	45+	16.9	61.5	27.5	+/-	-	+
8	Germinoma	109+	13.0	60.7	21.4	++	+/-	+
9	Germinoma	77+	12.5	54.6	22.9	+	-	+
10	Germinoma	48D	10.7	53.0	20.2	+	+/-	+
11	Germinoma	101+	8.3	46.4	17.9	+/-	-	+/-
12	Germinoma	12+	14.8	34.5	42.9	+/-	-	++
13	Germinoma	20+	7.5	32.5	23.1	+	+	+
14	Glioblastoma	7.5+	5.1	35.9	14.2	-	+/-	
15	Glioblastoma	1.4D	3.3	28.6	11.5	+	+	
16	Glioblastoma	20.0D	8.6	27.5	31.3	+/-	+	
17	Glioblastoma	2.5+	5.8	17.5	33.2	+/-	+/-	
18	Glioblastoma	14.6D	2.8	10.7	26.1	++	+/-	
19	Glioblastoma	9.1D	2.1	9.9	21.2	+	+	
20	Glioblastoma	31.7D	2.5	7.8	32.1	+/-	++	
21	Glioblastoma	10.7D	1.8	6.1	29.5	-	+/-	
22	Glioblastoma	35.8D	1.4	5.2	27.1	+/-	+	
23	Glioblastoma	9.9D	1.9	5.1	37.2	-	+	
24	Glioblastoma	7.3D	2.2	4.8	46.3	-	+	

Abbreviations: F/U, follow-up period (months); AR, apoptotic ratio; LI, labeling index; lymph, infiltrating lymphocytes.

^a+, still alive; D, dead (overall survival).

^b-, negative; +/-, negative and/or positive (0%–10%); +, positive (10%–25%); ++, highly positive (25%).

and/or positive if <10% were stained (+/-), positive if 10% to 25% were stained (+), or highly positive if over 25% (++) were stained, as in previous reports (Krajewski et al., 1997; Rieger et al., 1997). The comparison of the degrees between Bcl-2 and BAX expression of the tumor was emphasized in this study. In germinoma, only tumor cells were included in counts of immunoreactivity excluding infiltrating lymphocytes.

The association between Bcl-2 and BAX expression in both tumors was analyzed with the χ^2 test of independence with Yates' correlation for continuity.

Results

Results of all cases are summarized in Table 1. Results for proliferation and apoptosis are shown in Table 2. In Fig. 1A, both apoptotic bodies, which have small shrunken nuclei and mitotic figures, were evident in the germinoma by hematoxylin and eosin staining. MIB-1 LIs of germinomas and glioblastomas were 22.1 ± 8.3 and 28.2 ± 9.9 (mean $\pm$ SD), respectively, indicating high levels of cell proliferation in both tumor types (Fig. 1B and C, Table 2). There was no statistically significant difference in the MIB-1 LI values between the two groups of tumors. In contrast to MIB-1 immunostaining, TUNEL staining differed greatly between germinomas and glioblastomas. ARs of germinomas and glioblastomas were 14.3 ± 4.3

and 3.4 ± 2.2 , respectively (Fig. 1D and E, Table 2). Furthermore, with respect to the relation between apoptotic and proliferating cells, the ratio of AR/MIB-1 LI of germinomas was 72.9 ± 36.9 , which was significantly greater than that of glioblastomas (14.5 ± 11.2). Statistical analysis revealed significant differences in AR and AR/LI ratio between the two groups of tumors (Table 2; $P < 0.01$).

Seven of 13 germinomas showed immunoreactivity for BAX in >10% of the tumor cells, while only 3 of 11 glioblastomas showed the same immunoreactivity. In contrast, only one germinoma showed immunoreactivity for Bcl-2 in >10% of the cells. Bcl-2 expression was identified mainly in infiltrating lymphocytes of germinomas. A comparison of these apoptotic-related proteins showed that the degrees of BAX immunoreactivity uniformly predominated in germinomas, while the degrees of Bcl-2 immunoreactivity were stronger in 7 of 11 glioblastomas (Fig. 2, Table 3; $P < 0.01$).

Discussion

Cell proliferation and cell death are opposing processes in tumor growth, and progression of a tumor is thought to be determined by the balance between them. Tumor progression is observed when the former exceeds the latter (Reed et al., 1994; Williams, 1991). Furthermore,

Table 2. Comparison of apoptotic ratio and MIB-1 LI

	AR*	AR/MIB-1 LI*	MIB-1 LI**
Germinoma	14.3 ± 4.3	72.9 ± 36.9	22.1 ± 8.3
Glioblastoma	3.4 ± 2.2	14.5 ± 11.2	28.2 ± 9.9

Mean values are expressed as the mean ± SD.

Abbreviations: AR, apoptotic ratio; LI, labeling index.

* $P < 0.01$.

**Not significant (Mann-Whitney U test).

increased malignancy of a tumor occasionally may be associated with an inability of cells to undergo apoptosis rather than their increased ability to proliferate (Allan et al., 1992).

MIB-1 immunostaining is a reliable marker for cell proliferation, and it is likely to be prognostically informative (Sallinen et al., 1994). A high MIB-1 index has been associated with a poor prognosis for patients with brain tumors (Cunningham et al., 1997; Onda et al.,

Fig. 1. MIB-1 immunostaining and TdT-mediated dUTP-biotin 3'-OH nick-end labeling (TUNEL) of germinomas and glioblastomas. A. Both apoptotic bodies, which have small shrunken nuclei, and mitotic figures are evident in the germinoma by hematoxylin and eosin staining. B and C. Germinoma (B) shows high labeling ratio similar to that in glioblastoma (C) for MIB-1 staining. D and E. More apoptotic cells are identified in germinoma (D) than in glioblastoma (E) by TUNEL. F. Rat mammary tissue is used for positive control of the TUNEL method. Original magnification $\times 100$.

Fig. 2. Bcl-2 and BAX immunostaining of germinomas and glioblastomas. A and B. In germinomas, BAX-positive cells (B) predominate over Bcl-2-positive cells (A), and only infiltrate lymphocytes show immunoreactivity for Bcl-2. C and D. In contrast, in glioblastomas, Bcl-2-positive cells (C) are predominant over BAX-positive cells (D). Original magnification $\times 100$.

1996; Shibuya et al., 1993). In our study, high proliferative activities were evident in germinoma tissues and were similar to those in glioblastomas. The good prognosis of germinoma is not a result of low proliferation.

Typical apoptotic cells generally show small pyknotic nuclei and eosinophilic, hyalinized cytoplasm with hematoxylin and eosin staining. The more recently developed TUNEL staining method results in *in situ* labeling of DNA fragmentation within the nucleus, permitting detection of apoptotic cells in the early stages before evident morphologic change (Gavrieli et al., 1992). We found apoptotic cells to be diffusely present in germinoma by the TUNEL method, whereas few apoptotic cells were detected in glioblastomas. Although both germinoma and glioblastoma showed similar proliferative activity, the presence of apoptotic cells in germinoma was a crucial difference. Generally, spontaneous cell death predominated in germinomas, although cell proliferation was similar between both tumor types. This result suggested that differences in progression of these tumors resulted from a balance of proliferation and apoptosis. We speculate that the ratio of apoptotic to proliferative cells in tumor tissue might predict the course of a variety of brain tumors.

Possible mechanisms leading to apoptosis in tumors are varied and include a state of hypoxia, the lack of growth

factors in sufficient amounts, regulation by cytokines (Belloma et al., 1992), and the presence of oncogenes (Alderson et al., 1995; Clarke et al., 1993; Symonds et al., 1994). Bcl-2 and BAX are members of a large family of proteins that includes both apoptotic antagonists and agonists. These proteins regulate apoptosis by competing through dimerization. The balance of Bcl-2 and BAX is recognized as important in inducing apoptosis (Oltvai et al., 1993); Bcl-2 inhibits this form of cell death and promotes cell survival, while BAX is the main inhibitor of Bcl-2 and can enhance apoptotic cell death (Oltvai et al., 1993; Yin et al., 1997). Apoptosis, then, should accompany strong BAX expression. In this study, expression of BAX in germinomas was significantly higher than in glioblastomas, whereas expression of Bcl-2 was greater in glioblastomas, corresponding well to a high number of apoptotic cells in germinoma presumably resulting from relatively higher BAX expression than Bcl-2 expression. The principal

Table 3. Comparison of BAX and Bcl-2 staining

	BAX $\geq$ Bcl-2	BAX < Bcl-2	Total
Germinoma	13	0	13
Glioblastoma	4	7	11

$P < 0.01$ (χ^2 test of independence with Yates' correlation).

mechanism of apoptosis after exposure to chemotherapeutic agents or irradiation has been reported to involve apoptosis-related proteins (Reeve et al., 1996). Sakakura et al. (1996) found that human breast carcinoma cells transfected with a BAX expression vector show increased radiosensitivity, resulting in apoptosis after irradiation. Similarly, high BAX expression in germinomas might correlate with clinical radiosensitivity of germinoma because it induces apoptosis. On the other hand, a relatively low BAX expression in glioblastomas corresponded to small numbers of apoptotic cells as previously reported (Krajewski et al., 1997; Rieger et al., 1998). Recently, several proteins with homology to Bcl-2, such as Bak and Bcl-X, whose expressions display either positive or negative regulation for apoptosis, have been identified. Further studies will be required to evaluate the role of the Bcl-2 family, including interaction among these proteins in apoptotic cells of germinomas.

Correlation of the AR/MIB-1 LI and comparison of BAX and Bcl-2 immunoreactivity with outcomes in the

present series of germinomas would clearly have been of great interest, but treatments of the 13 patients were not uniform, and follow-up periods varied from 3 months to 15 years. Therefore, statistically significant correlation could not be made. Intracranial germinomas are not common tumors, even in Japan, where incidence is higher than it is worldwide (Matsutani et al., 1997). Also, many patients with this tumor are treated successfully with chemotherapy and radiotherapy without histologic confirmation. While such factors limited the number of cases that we were able to study, our observations are important in understanding the biology of this rare tumor.

Acknowledgments

The authors thank Mayumi Terasaki, Kaori Imanara, and Yoshie Hirokawa for their excellent technical assistance.

References

- Alderson, L.M., Castleberg, R.L., Harsh, G.R., IV, Louis, D.N., and Henson, J.W. (1995) Human gliomas with wild-type p53 express bcl-2. *Cancer Res.* **55**, 999–1001.
- Allan, D.J., Howell, A., Roberts, S.A., Williams, G.T., Watson, R.J., Coyne, J.D., Clarke, R.B., Laidlaw, I.J., and Potten, C.S. (1992) Reduction in apoptosis relative to mitosis in histologically normal epithelium accompanies fibrocytic change and carcinoma of the premenopausal human breast. *J. Pathol.* **167**, 25–32.
- Belloma, G., Perotti, M., Taddei, F., Mirabelli, F., Finardi, G., Nicotera, P., and Orrenius, S. (1992) Tumor necrosis factor alpha induces apoptosis in mammary adenocarcinoma cells by an increase in intracellular free Ca^{2+} concentration and DNA fragmentation. *Cancer Res.* **52**, 1342–1346.
- Clarke, A.R., Purdie, C.A., Harrison, D.J., Morris, R.G., Bird, C.C., Hooper, M.L., and Wyllie, A.H. (1993) Thymocyte apoptosis induced by p53 dependent and independent pathways. *Nature* **362**, 849–852.
- Cunningham, J.M., Kimmel, D.W., Scheithauer, B.W., O'Fallon, J.R., Novotny, P.J., and Jenkins, R.B. (1997) Analysis of proliferation markers and p53 expression in gliomas of astrocytic origin: Relationships and prognostic value. *J. Neurosurg.* **86**, 121–130.
- Gavrieli, Y., Sherman, Y., and Ben-Sasson, S.A. (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**, 493–501.
- Kordek, R., Hironishi, M., Liberski, P.P., Yanagihara, R., and Gajdusek, D.C. (1996) Apoptosis in glial tumors as determined by in situ nonradioactive labeling of DNA breaks. *Acta Neuropathol. (Berl.)* **91**, 112–116.
- Krajewski, S., Krajewska, M., Ehrmann, J., Sikorska, M., Lach, B., Chatten, J., and Reed, J.C. (1997) Immunohistochemical analysis of Bcl-2, Bcl-X, Mcl-1, and Bax in tumors of central and peripheral nervous system origin. *Am. J. Pathol.* **150**, 805–814.
- Krishna, M., Smith, T.W., and Recht, L.D. (1995) Expression of bcl-2 in reactive and neoplastic astrocytes: Lack of correlation with presence or degree of malignancy. *J. Neurosurg.* **83**, 1017–1022.
- Matsutani, M., Sano, K., Takakura, K., Fujimaki, T., Nakamura, O., Funata, N., and Seto, T. (1997) Primary intracranial germ cell tumors: A clinical analysis of 153 histologically verified cases. *J. Neurosurg.* **86**, 446–455.
- Oltvai, Z.N., Millman, C.L., and Korsmeyer, S.J. (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**, 609–619.
- Onda, K., Davis, R.L., and Edwards, M.S. (1996) Comparison of bromodeoxyuridine uptake and MIB 1 immunoreactivity in medulloblastomas determined with single and double immunohistochemical staining methods. *J. Neurooncol.* **29**, 129–136.
- Patsouris, E., Davaki, P., Kapranos, N., Davaris, P., and Papageorgiou, K. (1996) A study of apoptosis in brain tumors by in situ end-labeling method. *Clin. Neuropathol.* **15**, 337–341.
- Reed, J.C. (1994) Mini-Review: Cellular mechanisms of disease series: Bcl-2 and the regulation of programmed cell death. *J. Cell Biol.* **124**, 1–6.
- Reeve, J.G., Xiong, J., Morgan, J., and Bleehen, N.M. (1996) Expression of apoptosis-regulatory genes in lung tumour cell lines: Relationship to p53 expression and relevance to acquired drug resistance. *Br. J. Cancer* **73**, 1193–1200.
- Rieger, L., Weller, M., Bornemann, A., Schabet, M., Dichgans, J., and Meyermann, R. (1998) BCL-2 family protein expression in human malignant glioma: A clinical-pathological correlative study. *J. Neurol. Sci.* **155**, 68–75.
- Sakakura, C., Sweeney, E.A., Shirahama, T., Igarashi, Y., Hakomori, S., Nakatani, H., Tsugimoto, H., Imanishi, T., Ohgaki, M., Ohyama, T., Yamazaki, J., Hagiwara, A., Yamaguchi, T., Sawai, K., and Takahashi, T. (1996) Overexpression of Bax sensitizes human breast cancer MCF-7 cells to radiation-induced apoptosis. *Int. J. Cancer* **67**, 101–105.
- Sallinen, P.K., Haapasalo, H.K., Visakorpi, T., Helen, P.T., Rantala, I.S., Isola, J.J., and Helin, H.J. (1994) Prognostication of astrocytoma patient survival by Ki-67 (MIB-1), PCNA, and S-phase fraction using archival paraffin-embedded samples. *J. Pathol.* **174**, 275–282.
- Shapiro, W.R., Green, S.B., Burger, P.C., Mahaley, M.S., Jr., Selker, R.G., VanGilder, J.C., Robertson, J.T., Ransohoff, J., Mealey, J., Jr., Strike, T.A., and Pistenmaa, D.A. (1989) Randomized trial of three chemotherapy regimens and two radiotherapy regimens in postoperative treatment of malignant glioma: Brain Tumor Cooperative Group Trial 8001. *J. Neurosurg.* **71**, 1–9.

- Shibuya, M., Ito, S., Miwa, T., Davis, R.L., Wilson, C.B., and Hoshino, T. (1993) Proliferative potential of brain tumors: Analyses with Ki-67 and anti-DNA polymerase alpha monoclonal antibodies, bromodeoxyuridine labeling, and nuclear organizer region counts. *Cancer* **71**, 199–206.
- Symonds, H., Krall, L., Remington, L., Saenz-Robles, M., Lowe, S., Jacks, T., and Van Dyke, T. (1994) p53-dependent apoptosis suppresses tumor growth and progression in vivo. *Cell* **78**, 703–711.
- Williams, G.T. (1991) Programmed cell death: Apoptosis and oncogenesis. *Cell* **65**, 1097–1098.
- Wyllie, A. (1987) Cell death. In: Bourne, G.H. (Ed.), *International Review of Cytology*, supplement 17. San Diego, Calif.: Academic Press, pp. 755–785.
- Yin, C., Knudson, C.M., Korsmeyer, S.J., and Van Dyke, T. (1997) Bax suppresses tumorigenesis and stimulates apoptosis in vivo. *Nature* **385**, 637–640.