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Immunocytochemical Demonstration of S-Phase Cells by Anti-Bromodeoxyuridine Monoclonal Antibody in Human Brain Tumor Tissues*

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Summary. Five patients with various brain tumors received bromodeoxyuridine (BrdU), 150–200 mg/m^2 i.v., at the time of craniotomy. Biopsied materials were fixed in 70% ethanol, sectioned, denatured with hydrochloric acid, and reacted with monoclonal antibodies against BrdU. Immunofluorescence and immunocytochemical methods were used to visualize BrdU-labeled nuclei. Our results showed that both methods demonstrated BrdU-labeled nuclei satisfactorily in tissue sections. Thus, BrdU can be used to measure the proliferative potential of human tumors in situ.

Key words: Brain tumor – Glioma – Cell kinetics – Bromodeoxyuridine monoclonal antibody – Labeling index – Immunocytochemistry

Introduction

Cell kinetics studies have helped to elucidate the growth characteristics of various types of human brain tumors and to predict their biologic malignancy (Chigasaki 1963; Hoshino 1977, 1979; Hoshino et al. 1972, 1975, 1980; Hoshino and Wilson 1979; Kury and Carter 1965; Tym 1969). Most of these studies were performed by obtaining autoradiograms after exposing the tumor to a pulse of ^{3}H-thymidine. However, autoradiographic studies require several months to complete, and therefore the results are of limited value in supplementing the histopathologic diagnosis and designing treatment in individual patients. For this reason, and because of the radiation hazard of ^{3}H-thymidine to normal tissue, cell kinetics studies have never become popular (Tannock 1978).

Bromodeoxyuridine (BrdU) is a thymidine analogue that is also incorporated into nuclear DNA, but is neither radioactive nor myelotoxic at the doses used in cell kinetics studies (Szybalski 1974). Gratzner (1982) has developed a monoclonal antibody (MAb) that can identify BrdU-containing nuclei. Anti-BrdU MAb can be detected by direct conjugation of FITC to the antibody or by indirect conjugation of FITC using an FITC-tagged secondary antibody. These methods have been applied both to single cells and to tissues to estimate the labeling index of individual specimens (Gratzner 1982; Dolbeare et al. 1983; Dean et al. 1984; Morstyn et al. 1983; Nagashima and Hoshino 1985). However, immunofluorescence techniques have obvious disadvantages, and preparation similar to that for autoradiography is required to preserve histological details and to locate BrdU-labeled cells in tissue. In this report, we describe a method of detecting BrdU-containing nuclei in paraffin-embedded tissue sections by secondary peroxidase-conjugated antibodies.

Materials and Methods

The subjects of the study were five consecutive patients who underwent craniotomy for removal of a tumor at the University of California, San Francisco (USCF). Permission to administer BrdU was obtained from the Human Experimentation Committee at the University of California and from the National Cancer Institute. Informed consent was obtained from each patient.

All patients received a 1-h i.v. infusion of BrdU, 150–200 mg/m^2, at the time of craniotomy. Each biopsy specimen was fixed in chilled 70% ethanol (ETOH) for a minimum of 12 h and divided into two parts. One portion was sectioned in a cryostat; the other was embedded in paraffin, sectioned at 6–8 μm, deparaffinized with xylene, and rinsed three times (5 min each rinse) in 100% ETOH and once with distilled water.

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Table 1. Histopathologic characteristics and BrdU positivity of tumors

Case no.	Pathology	Cellularity	Pleomorphism		Vascular proliferation	Mitoses	BrdU-positive nuclei (mean ± SD)
			Nuclear	Cytoplasmic			
1	Gliosarcoma	++	+++	++	+	0	7.8 ± 1.5%
2	Highly anaplastic astrocytoma	+++	+	+	++	2/HPF	18.1 ± 4.5%
3	Meningotheliomatous meningioma	++	0	0	0	1/10 HPF	< 1%
4	Moderately anaplastic astrocytoma	++	+	+	0	1/6 HPF	< 1%
5	Glioblastoma multiforme	+++	++	++	++	1/5 HPF	4.8 ± 0.3%

0, none; +, mild; ++, moderate; +++ high; HPF, high-power field

Fluorescence Immunocytochemistry

Slides prepared from cryostat or paraffin-block sections were incubated for 30 min in 2 N HCl to denature DNA and rinsed for 5 min with 0.1 *M* $Na_2B_4O_7$ (pH 8.5) to neutralize the acid. After three 5-min rinses in phosphate-buffered saline (PBS), the tissue sections were covered with a 1:100 dilution of FITC-conjugated anti-BrdU MAb (Becton Dickinson, Mt. View, CA, USA) in PBS containing 1% bovine serum albumin and 0.5% Tween 20 (Sigma, St. Louis, MO, USA) and left for 30 min at room temperature in a 100% humidified atmosphere. The slides were washed three times in PBS, mounted with Immumount (Shandon, Swickley, PA, USA), and examined under a fluorescence microscope.

Peroxidase Immunocytochemistry

Deparaffinized tissue sections were incubated for 30 min in 2 N HCl, neutralized with 0.1 *M* $Na_2B_4O_7$, and rinsed with PBS. Then, a 1:100 dilution of purified anti-BrdU MAb (Becton Dickinson, Mt. View, CA, USA) in PBS containing 1% bovine serum albumin and 0.5% Tween 20 was used to cover the tissue sections for 60 min at room temperature in a 100% humidified atmosphere. Next, the slides were rinsed three times with PBS, covered with a 1:40 dilution of peroxidase-conjugated anti-mouse IgG antibody (Zymed, So. San Francisco, CA, USA) in PBS and left for 60 min at toom temperature in a 100% humidified atmosphere. Finally, the slides were rinsed three times with PBS and reacted for 10–15 min with 12.5 mg of diaminobenzidine tetrahydrochloride and 5 µl of 30% H_2O_2 in 50 ml of TRIS buffer.

Case Reports

Case 1 is a 72-year-old woman who had "electric shock sensations" on the right side of her face and in her right hand in April 1983. A computerized tomography (CT) scan showed an isodense, contrast-enhancing, left parietal mass. On September 23, 1983, she underwent a left parietal craniotomy for removal of the tumor, which had the histological characteristics of a gliosarcoma. Postoperatively, she received radiation therapy and adjuvant chemotherapy with hydroxyurea, but the residual tumor increased in size and continued to grow despite further treatment with BCNU and 5-fluorouracil. After administration of BrdU, a second resection was performed on May 25, 1984.

Case 2, a 2-year-old boy with a 1-month history of gradually increasing right hemiparesis, was first examined at UCSF on July 22, 1984. A CT scan showed a large left hemispheric mass. The patient underwent a left frontoparietal craniotomy and subtotal resection of a highly anaplastic astrocytoma on July 23, 1984.

Case 3 is a 60-year-old man who came to UCSF on July 22, 1984, with a 6–7-month history of episodes in which he smelled a "foul odor" and experienced "unusual sensations" in his forehead; he also had a 10-year history of intermittent numbness in his right arm and leg. CT scanning and angiography demonstrated a right sphenoid wing tumor, which was partially embolized on July 23, 1984. The next day he underwent a right frontoparietal craniotomy.

Case 4 is a 54-year-old man who underwent subtotal resection of a left parietal grade II astrocytoma in 1976. Postoperatively, he received 5,400 rads of radiation therapy and was stable until April 1984, when he developed expressive dysphasia and right hemiparesis. He was found to have a recurrence of his left parietal tumor, which now contained a cystic component as well. On July 23, 1984, he underwent multiple biopsies and decompression of the cystic mass.

Case 5 is a 60-year-old man with a 3-week history of expressive dysphasia. A right parietotemporal mass was found, and he underwent a left temporal lobectomy and subtotal resection of the tumor on July 20, 1984.

Results

The histological characteristics of the tumor and the diagnosis in each case are presented in Table 1. Sections of alcohol-fixed biopsy specimens, stained with hematoxylin-eosin (HE), from patients 2, 3, and 5 are shown in Figs. 1A, 2A, and 3A, respectively. The glial neoplasms were classified according to the criteria currently in use at UCSF. Astrocytomas were classified as non-, mildly, moderately, and highly anaplastic based on the degree of cellularity, nuclear and cytoplasmic pleomorphism, vascular proliferation, and the number of mitotic figures. The diagnosis of glioblastoma multiforme was based on the presence of dense cellularity, moderate to severe nuclear and

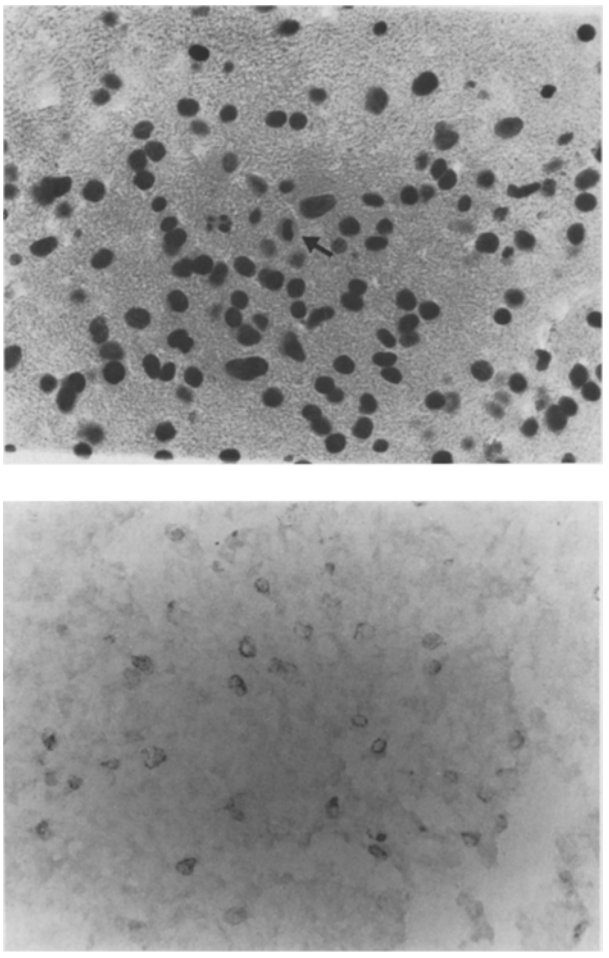

Fig. 1 A,B. Highly anaplastic astrocytoma (case 2). **A** Alcohol-fixed tissue section stained with HE shows nuclear pleomorphism and a mitotic figure (*arrow*). ×210. **B** Immunoperoxidase stain for BrdU shows many labeled tumor nuclei. ×210

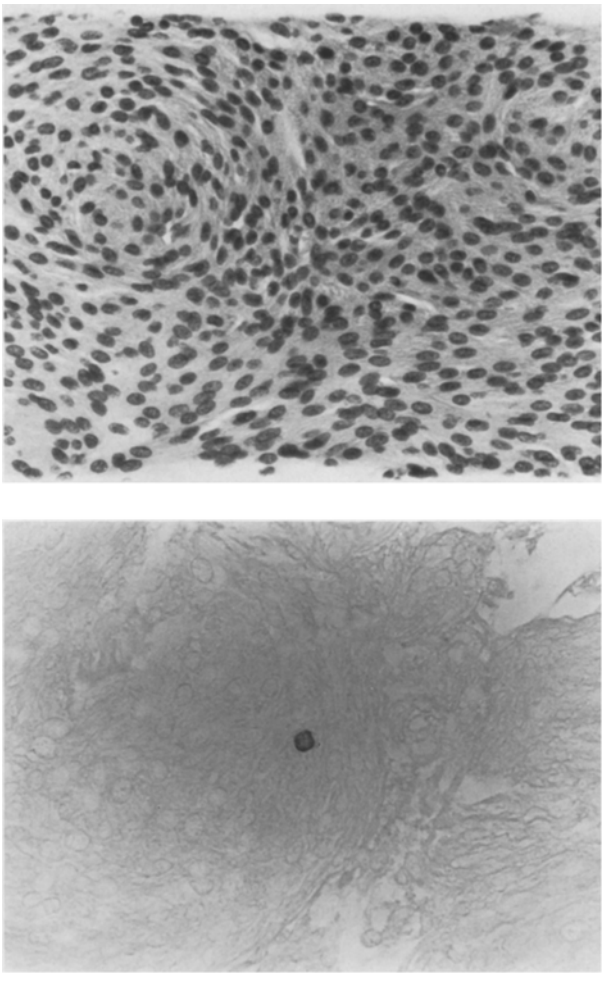

Fig. 2 A,B. Meningotheliomatous meningioma (case 3). **A** Alcohol-fixed tissue section stained with HE. ×210. **B** Immunoperoxidase stain for BrdU shows only an occasional labeled nucleus. ×210

cytoplasmic pleomorphism, and vascular endothelial proliferation.

The immunoperoxidase staining for BrdU was confined to the nucleus and consisted of both punctate and diffuse patterns (Figs. 1 B, 2 B, and 3 B). Similarly, immunofluorescence staining resulted in punctate or diffuse green fluorescence over the nucleus on both cryostat and paraffin-embedded sections (data not shown). The number of BrdU-positive nuclei did not correlate with the number of mitotic figures, but appeared to correlate well with the proliferative potential of the neoplasm based on its clinical behavior. The meningioma and the well-differentiated glioma, which are very slow growing tumors, contained very few labeled cells (less than 1%), whereas the gliosarcoma and the highly anaplastic astrocytoma contained many labeled cells (Table 1).

Discussion

The development of MAbs to identify nuclei that contain BrdU is an important breakthrough for studies of cell kinetics. Like ^{3}H-thymidine, BrdU is incorporated into nuclear DNA (Szybalski 1974), but it is not radioactive and is not myelotoxic at the doses used. Therefore, a wider variety of human tumors, including benign tumors, can be investigated. Since the immunoperoxidase method of detecting anti-BrdU MAb requires only 2–3 h, these studies can be completed in only 1 or 2 days after biopsy.

In our study of rat 9L brain tumor cells in vitro, S-phase cells could be identified by FITC-conjugated anti-BrdU MAb after a 30-min exposure to only 0.625 μ*M* BrdU (Nagashima and Hoshino 1985). We also found that BrdU-labeled cells could be detected

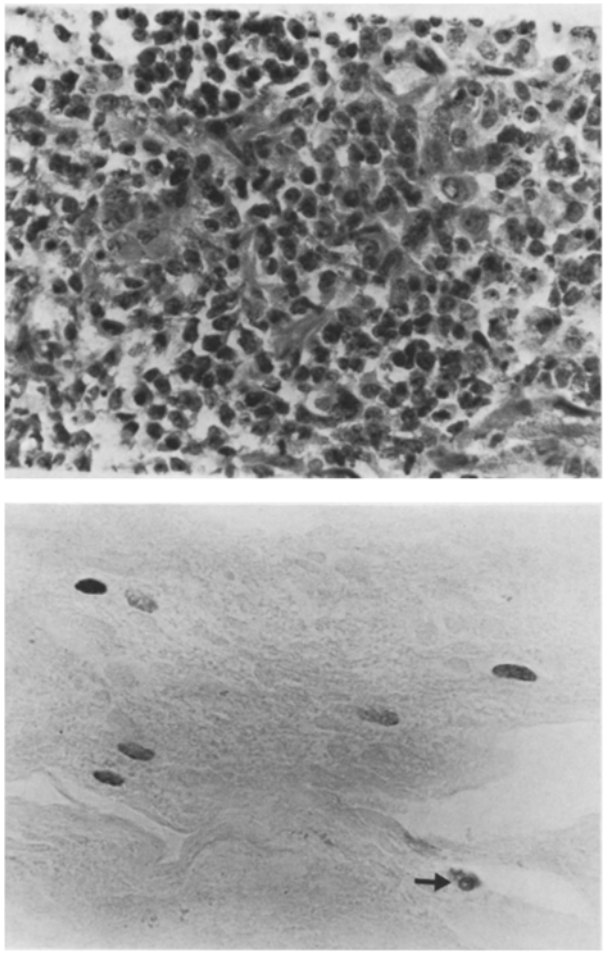

Fig. 3 A,B. Glioblastoma multiforme (case 5). **A** Alcohol-fixed tissue section stained with HE shows a region of high cellularity with nuclear and cytoplasmic pleomorphism. **B** Immunoperoxidase stain for BrdU shows multiple tumor nuclei as well as an endothelial cell nucleus (*arrow*). ×210

in brain tumor biopsy specimens from rats that had received BrdU in doses as small as 1 mg/kg (7 mg/m^2) by i.p. injection.

BrdU has been administered as a radiosensitizing agent to patients with malignant brain tumors or cancers of the head and neck at an i.a. dose of 500–1,000 mg/day for 4–6 weeks without any serious side effects (Bagshaw et al. 1967; Hoshino and Sano 1969; Sano et al. 1968). Daily infusions of BrdU, 600–700 mg/m^2 i.v., for several weeks can be tolerated without any severe myelosuppression (Mitchell et al. 1983; Kinsella et al. 1984; Russo et al. 1984). Russo et al. (1984) measured serum BrdU levels achieved by continuous i.v. administration of BrdU (up to 700 mg/m^2 per day) for 14 days. An infusion rate of 108 mg/m^2 per hour yielded a serum level of 3.9 μ*M*. Intravenous administration of BrdU should be limited to less than 700 mg/m^2 per day to prevent myelosuppression (Kinsella et al. 1984). If a serum BrdU level of 5 μ*M* for 1 h is sufficient to label S-phase cells, a 1-h BrdU infusion of 150–250 mg/m^2, which is well below a therapeutic dose, would produce adequate labeling.

We therefore administered BrdU at this dose to the five patients in the present study. BrdU-tagged nuclei in tumor specimens were detected by both immunofluorescence and immunoperoxidase methods. The labeling results were similar to those obtained autoradiographically after a pulse of ^{3}H-thymidine. The paraffin-embedded sections gave the same results as the cryostat sections; thus, the antigenicity of BrdU-tagged DNA fixed with 70% ETOH was quite stable. Although the use of FITC-conjugated anti-BrdU MAbs to scan labeled cells in tissues requires less work to stain, the slides do not last long and must be photographed each time; moreover, counter staining is difficult because most dyes are fluorescent. In contrast, the indirect peroxidase method of detecting anti-BrdU MAbs is equally reliable and has the advantage that tissue sections can be counter stained easily. When we tested the peroxidase-antiperoxidase method on the same slides, the reaction products were denser, but with variable background; therefore, this method may be used to detect low levels of anti-BrdU MAb in tissue.

These preliminary studies suggest that flow cytometric analysis (Van Dilla et al. 1969) and histochemical study of tissue exposed to a nontoxic amount of BrdU may allow more extensive cell kinetics studies of human tumors in situ. Our results indicate that cell kinetics studies using anti-BrdU MAbs can supplement the histopathological diagnosis by suggesting the prognosis and may be useful in designing treatment for individual patients.

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