Yonsei Medical Journal
Vol. 33, No. 3, 1992

Comparison of Brain Tumor Growth Kinetics by Proliferating Cell Nuclear Antigen (PCNA) and Bromodeoxyuridine (BrdU) Labeling

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The bromodeoxyuridine (BrdU) labeling study provides valuable cell kinetic information for individual tumors that could suggest the prognosis of each patient who had a tumor. Recently, a monoclonal antibody against the proliferating cell nuclear antigen (PCNA or cyclin), a nuclear protein expressed in proliferating cells, was developed which could be used on formalin fixed, paraffin embedded tissue. The purpose of this study was to compare the cell kinetic data obtained by the BrdU labeling study and the PCNA method in the same patient. The relationship between labeling indices of BrdU incorporated into S-phase and PCNA expressed by cycling cells was investigated in 31 patients with brain tumors. Both of the labeling indices showed good correlation with histological grade of the tumor. The values of the PCNA labeling index (LI) were parallel but higher than those of the BrdU LI, and the relation PCNA LI=2.2×BrdU LI+0.8 (r^2=0.86) was obtained. The results of this study show that PCNA could replace the BrdU method for identifying the proliferating cells, and the major advantages of PCNA method is that it could be done without any pretreatment and avoid injection of the teratogenic agent for diagnostic purpose.

Key Words: Brain neoplasm, cell kinetics, bromodeoxyuridine, nuclear proteins

The evaluation of the proliferative potential of tumors is of major interest for predicting their biological behavior, for defining the most appropriate therapy and for predicting the prognosis of each patient (Hoshino *et al.* 1972, 1989; Hall and Levison 1990). Traditionally, the rate of tumor growth has been estimated from histopathological features, especially the presence of focal necrosis, mitoses, nucleocytoplasmic pleomorphism, and cellularity. However, these do not always correlate well with biological behavior of a tumor or with the prognosis of the patient, and this has spurred the development of cell kinetic studies (Burger and Vollmer 1980; Murovic *et al.* 1986; Russell and Rubinstein 1989).

Thymidine labeling has been used as the gold standard of cell kinetic study, and most cell kinetic studies in the last three decades have been performed by autoradiography utilizing radioactive thymidine (Kury and Carter 1965). More recently, immunohistochemical techniques have been used to detect BrdU, a thymidine analogue, infused intravenously before operation (Gratzner 1982; Hoshino *et al.* 1986a). Time and labor consuming procedures involved in autoradiography and inherent potential hazard to the patients and environment of tritiated thymidine could be avoided by the BrdU method. Although BrdU labels proliferating cells in S phase, it requires preoperative selection and preparation, and still has the risk of DNA mdoulation. If a new technique could detect the substance produced naturally by proliferating cells, it will replace the BrdU labeling method which identifies artificially incorporated BrdU.

Proliferating cell nuclear antigen (PCNA), also

Received April 23, 1992
Accepted September 8, 1992
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This study was supported by a Faculty Research Grant from the Yonsei University College of Medicine (1990-1991)
Presented in part at the 8th Asian-Australasian Congress or Neurological Surgery, October 11, 1991.
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known as cyclin (Mathews *et al.* 1984), is a nonhistone nuclear protein with molecular weight of 36 kD.

The expression of PCNA appears during late G1 phase, reaches maximum during the S phase of the cell cycle (Celis and Celis 1985), and declines during G2 and M phase (Kurki *et al.* 1986). PCNA has recently been identified as the polymerase delta accessory protein which is essential for cellular DNA synthesis (Bravo *et al.* 1987, Lee and Hurvitz 1990). As the entire gene for human PCNA has been isolated and sequenced (Travali *et al.* 1989), a monoclonal antibody against human PCNA has also developed. However, investigation of human malignancies with PCNA has been limited (Robbins *et al.* 1987; Garcia *et al.* 1989).

The purpose of the present study was:

1) to analyze the state of proliferation in a series of brain tumors by immunohistochemical evaluation of BrdU incorporated into S-phase and of PCNA expressed by cycling cells,

2) to determine the relationship between these two labeling indices, and

3) to find the possibility of replacing the BrdU study with the more simple and safe PCNA method.

MATERIALS AND METHODS

The brain tumor specimens were obtained from 31 patients (13 women and 18 men) who had had operations at the yonsei University Hospital between April and December 1990 (Table 1). The patients' ages ranged from 1 to 75 years, with an average of 37.8years. The patients were given a 30 minutes intravenous infusion of BrdU (200 mg/m^2) after induction of anesthesia. The time interval between the infusion of BrdU and removal of tumor specimens varied from one hour to six and a half hours (mean two hours and 21 minutes). Each specimen was fixed in formalin and paraffin embedded for further processing.

BrdU Immunohistochemistry

Tissue sections 6-micron-thick were cut from the paraffin blocks, and deparaffinized with xylene. The tissue slides were incubated for 30 minutes in methanol with 0.3% hydrogen peroxide to avoid nonspecific tissue reaction of peroxidase. To denature the double strand of DNA, the slides were incubated for 30 minutes in 2N hydrochloric acid, neutralized with 0.1M borax, then rinsed with phosphate-buffered saline (PBS). A 1 : 20 dilution of purified anti-BrdU monoclonal antibody (DAKO Corp., Santa Barbara, Califrnia) in PBS containing 1 % bovine serum albumin and 0.5% tween 20 was used to cover the tissue section for 45 minutes at 37°C, and washed in PBS for 10 minutes. Immunoperoxidase staining was done with a labeled streptavidine-biotin (LSAB, DAKO Corp., Santa Barbara, California) kit with diaminobenziden (DAB) as a chromogen and counterstaining with 10% Harris

Table 1. Summary of cases and labeling indices of BrdU and PCNA

Diagnosis	No.	Ranges (Mean) of LI (%)	
		BrdU	PCNA
Medulloblastoma	1	10.2	32.1
Glioblastoma multiforme	7	1.9-17.9 (9.6)	1.6-48.7 (22.2)
High grade astrocytoma	3	0.4-18.0 (6.8)	2.7-38.1 (15.7)
Low grade astrocytoma	4	0.0- 2.2 (1.1)	1.1- 9.1 (4.2)
Meningioma	9	0.1- 3.9 (1.0)	1.1- 5.1 (2.3)
Pituitary adenoma	4	0.2- 0.7 (0.5)	1.0- 2.2 (1.8)
Acoustic neuroma	2	0.4- 0.6 (0.5)	0.4- 1.8 (1.1)
Metastatic adenocarcinoma	1	5.7	19.6
Total	31	0.0-18.0 (4.3)	0.1-48.7 (10.5)

BrdU: Bromodeoxyuridine
PCNA: Proliferating cell nuclear antigen
LI: Labeling index

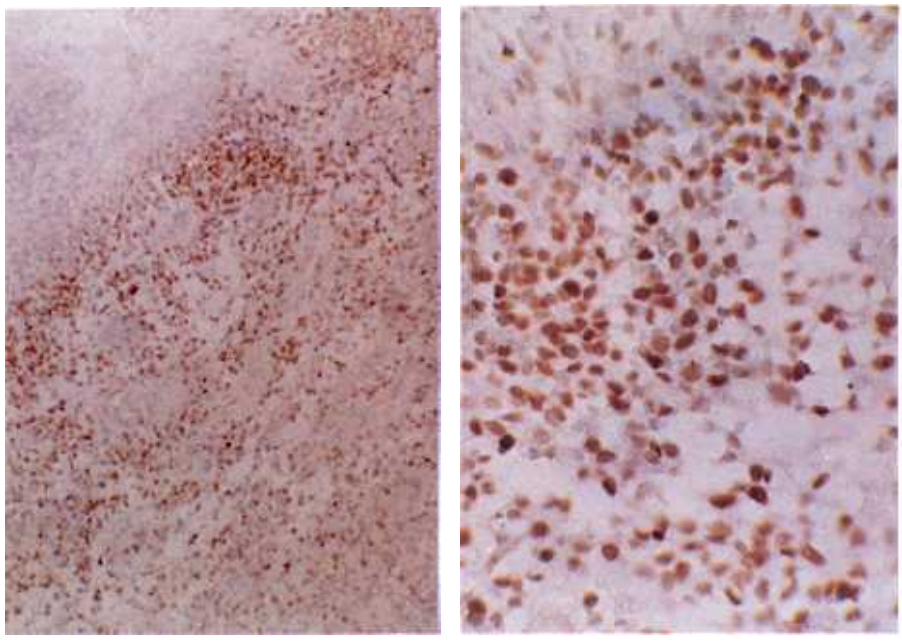

Fig. 1. Photomicrographs of BrdU stain of glioblastoma multiforme case.
A: Immunoperoxidase stain for bromodeoxyuridine showing numerous labeled nuclei. Diaminobenzidine (DAB) was used as a chromogen and counterstained with Harris hematoxylin (× 100).
B: Magnified view (× 40) of the same specimen showing a BrdU labeling index over 30%.

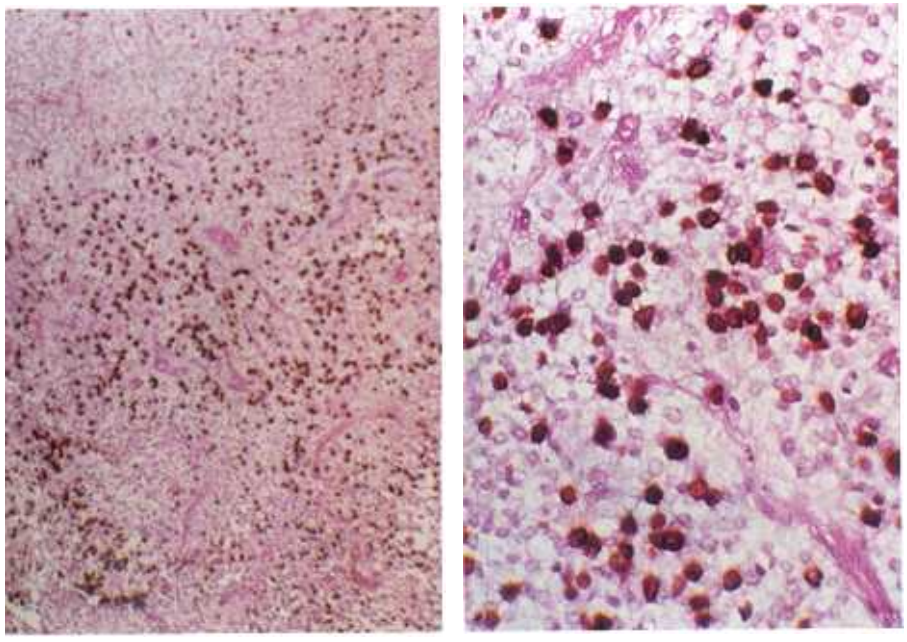

Fig. 2. Photomicrographs of PCNA stain of the same case as Fig. 1., but different field.
A: Immunoperoxidase stain for PCNA at low power (× 100). Aminoethyl carbamazole (AEC) was used as a chromogen and counterstained with Mayer hematoxylin.
B: Magnified view (× 400) of the same specimen showing a PCNA labeling index of 14.5%.

hematoxylin.

PCNA Immunohistochemistry

The tissue slides were incubated for 30 minutes in methanol with 0.3% hydrogen peroxide to avoid nonspecific tissue reaction of peroxidase. After a ten-minute incubation with blocking serum (Goat limmunoglobulins), a 1 : 20 dilution of purified anti-PCNA monoclonal antibody (PC10, DAKO Corp., Santa Barbara, California) was used as the primary antibody and applied to cover the tissue section for 30 minutes at room temperature. Immuno-peroxidase staining was done with a labeled streptavidin-biotin (LSAB, DAKO Corp., Santa Barbara, California) kit with aminoethyl carbamazole (AEC) as a chromogen and counterstaining with Mayer hematoxylin.

Scoring of the Immunohistochemical Staining

Regardless of the intensity of the nuclear immunostaining, all stained nuclei were scored as positive. Labeling indices of the BrdU and of the PCNA were scored as a percentage of positive immunostained nuclei out of the total number of nuceli in the same field. Five to ten high-power fields were examined in all cases. Areas of dense staining were evaluated when immunostaining showed marked heterogeneity, since they represent the proliferative potential of the tumor mass. Random sampling of the fields was avoided because the mean value of such labeling indices dose not reflect the actual growth potential of each tumor.

Comparison of the BrdU and PCNA Labeling Indices

Histological diagnosis and the grade of malignancy were compared with the labeling indices of BrdU and PCNA. A linear regression analysis was done to fine the relation formula and the correlation coefficient between the BrdU LI and PCNA LI.

RESULTS

The immunohistochemically labeled cells were easily discriminated from the unlabeled nuclei in the same microscopic fields with both BrdU (Fig. 1) and PCNA (Fig. 2) monoclonal antibodies. Both of the labelings were confined to the nuclei. Labeling patterns showed topographic variation, but identical or similar areas of the tumors were stained by both antibodies.

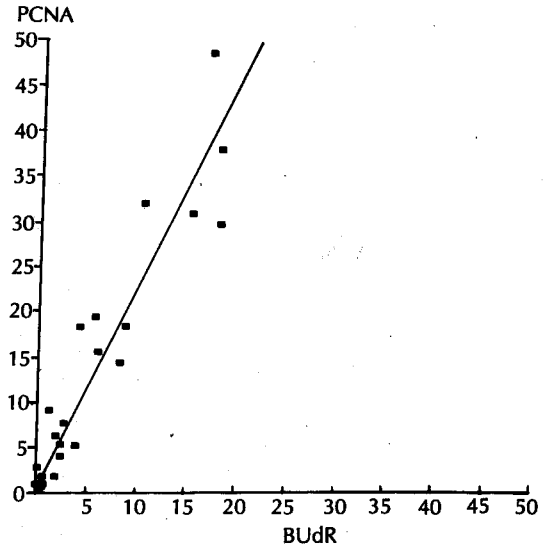

Fig. 3. *Scattergram of the BrdU LI(%) and PCNA LI(%) of 31 cases of brain tumor. A regression line of Y=2.2X+0.8 (r^2=0.86) shows close reltationship between the two LIs. Y=PCNA LI, X=BrdU LI, and r^2=coefficient of determination.*

Labeling indices of BrdU and PCNA varied greatly from case to case, but they varied accordingly with the histopathological diagnosis. Results of the examined cases and the labeling indices of BrdU and PCNA are summarized on Table 1. Mean value of LIs paralleled the degree of histopathological malignancy, although some overlap was noted between the various grades of tumor. The BrdU LI ranged from 0% to 18%, with an average value of 4.3%, and PCNA LI ranged from 0.1% to 48.7%, with an average of 10.5%. Tumors with a higher BrdU LI also showed a higher PCNA LI, and the mean value of PCNA LI paralleled but was higher than that of BrdU LI. A regression line was obtained by the least square method as follows:

$Y=2.2X+0.8$ ($r^2=0.86$)

where Y=PCNA LI, X=BrdU LI, and r^2=coefficient of determination (Fig. 3).

DISCUSSION

The histopathological diagnosis and grading of

gliomas or other brain tumors are sometimes ambiguous because bilogical behavior has been judged by morphology (Russell and Rubinstein 1989). Generally, tumor malignancy is graded on the basis of the appearance of tumor cells such as cellularity, nuclear and cytoplasmic pleomorphism, invasion of adjacent structures, and accompanying histological alterations as vascular changes and necrosis.

Mitotic figures do not necessarily correlate with the biological malignancy of the glial tumor, because more slowly growing oligodendrogliomas show more frequent mitoses than more malignant glioblastomas (Burger and Vollmer 1980; Rusell and Rubinstein 1989). Although the pathology of brain tumors is reasonably wel defined, the predicted prognosis of each patient based on a histopathological study does not often match the outcome. Quantitiative measuring of the proliferative activity is needed not only to predict the prognosis but also to evaluate the responses to treatments of individual patients (Hoshino *et al.* 1972; Murovic *et al.* 1986).

Cytokinetic studies have been started by autoradiographic analysis (Kury and Carter 1965, Tym 1969) of tissue exposed to tritiated thymidine.

Development of flow cytometry made it possible to measure S phase fraction or modal DNA population (Van Dilla *et al.* 1969; Braylan *et al.* 1980; Dolbeare *et al.* 1983; Hedley *et al.* 1985). However, inclusion of non-neoplastic cells could be as much as 20% (Nagashima *et al.* 1985), and heterogenic labeling pattern or spatial orientation could not be appreciated with this technique. Modal DNA population numbers could give some clues of biological behavior, but do not necessarily correlate with tumor growth rate or prognosis in individual patients (Hoshino 1986).

Introduction of the BrdU labeling study was a breakthrough in cytokinetic studies, because of its relatively simple method of in situ characterization of growth potential (Gratzner 1982; Dean *et al.* 1984; Hoshino *et al.* 1989).

Recently, another monoclonal antibody Ki-67 was developed that could discriminate human tumor cells in the cycling pool (Gerdes *et al.* 1983; 1984; Burger *et al.* 1986; Giangaspero *et al.* 1987). But both of the above methods have limitations that preclude routine application of these techniques for estimation of proliferative potentials. Although many authors have reported the successful application of the BrdU labeling study for measurement of S phase fraction in tumor specimen (Dean *et al.* 1984; Nagashima *et al.* 1985; Hoshino *et al.* 1986b), the following may be the limiting factors for its routine clinical use. BrdU should be given before surgery for cytokinetic studies, and it could not be used in unprepared cases or for retrospective analysis. As the uptake of BrdU depends on the regional blood flow, nonuniform perfusion in solid tumors may cause considerable variation in labeling. It has been documented that BrdU LI decreases as a function of the distance from blood vessels (Pavelic *et al.* 1981). Nonspecific uptake of BrdU by marcrophage and endothelial labeling should also be considered when calculating the LI (Lee *et al.* 1990). Some investigators are reluctant to give the patient a possible teratogen for purely diagnostic purpose. It has been well accepted that the monoclonal antibody, Ki-67, reacts with human nuclear antigen, which is expressed only in cycling cells (Sasaki *et al.* 1982; Zuber *et al.* 1988).

However, the nuclear antigen which reacts with Ki-67 has neither been isolated nor identified, and it could be detected only in frozen sections because of the unstable nature of its antigenicity. Furthermore, the tumor specimen examined by frozen sections may not represent the entire tumor cell population because of its heterogeneity. The technical difficulties of obtaining satisfactory staining, and the sampling error inherent in small frozen section specimen are the major drawbacks of the Ki-67 method. Another theoretical issue is that we remain uncertain as to exactly what is being measured with Ki-67 immunostaining (Hall and Levison, 1990).

Ahnen *et al.* (1987) have shown that PCNA immunostaining provides similar information about proliferation in rat colon as that obtained by tritiated thymidine autoradiography, ornithine decarboxylase activity, and flow cytometry. PCNA immunostaining gave a reproducibly higher LI compared with autoradiography. This coincides with the results of our analysis in that PCNA LI outnumbers the LI of thymidine labeling.

Our results appear to be sensitive enough to correlate the growth potential measured with PCNA LI and the S phase fraction determined with BrdU LI, and with the histopathological appearance of biological malignancy of individual tumors. A specimen with a high PCNA LI also showed a high BrdU LI, and vice versa. A close relationship between these two parameters was observed, and a regression line, $Y=2.2X+0.8$, was obtained. This is as expected since PCNA is expressed and detectable in late G1, S, and early G2 phase while a short pulse of BrdU labels a fraction of cells in S phase. PCNA

method gives reproducible results, comparable to BrdU immunostaining. PCNA immunostaining is much simpler because it can be done without any preparation before the surgery and in routinely processed formalin fixed paraffin embedded tissue. PCNA labeling is much safer to the parients than the BrdU method, which depends on the replacement of the methyl group with bromine at the 5-position of the uridine molecule in DNA.

In conclusion, the results of our study demonstrated PCNA labeling study is a suitable marker for assessing cell proliferation comparable to BrdU method.

PCNA study is a simple and safe method which does not require any preparation of the patiient, and could be done in routinely prepared tissue. Further investigation of PCNA as a marker of cellular proliferation and as a possible indicator of prognosis for brain tumors or other tumors will be necessary to define the clinical utility of this method.

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