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Immunohistochemical Analysis of Cell Proliferation in Gliomas using Proliferating Cell Nuclear Antigen

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Abstract Proliferating cell nuclear antigen (PCNA) immunostaining of 46 gliomas was carried out. Their PCNA labeling indices (LIs) of 18 cases with multiform glioblastoma, 14 cases with anaplastic astrocytoma and 14 cases with astrocytoma were 26.2 ± 2.0% (mean ± standard error), 10.7 ± 1.7% and 5.1 ± 0.7%, respectively. The PCNA LIs were related to the histopathological malignancy.

The clinical prognosis was assessed in 42 patients. Those with a PCNA LI less than 10% had a better prognosis than those with a PCNA LI of 10% or more. The proliferative potential of the vascular components in glioma was higher than that of vessels of the normal brain, but lower than that of tumor cells. Therefore, the high proliferative potential of the vascular components in glioma is unlikely to be due to neoplastic transformation, but an angiogenetic factor in the tumor. Immunostaining of PCNA is a simple and reproducible method for estimating the proliferative potential of gliomas and prognosis of patients with them.

Key words:Glioma, PCNA, Histopathological grade, Prognosis.

Introduction

Cell-cycle studies demonstrated the proliferative activity of tumors and their biological behavior. Various methods, such as determination of the mitotic or ^{3}H-thymidine labeling indices[1], have been used to study the cellular kinetics of tumor in the human nervous system. Immunohistochemical detection of bromodeoxyuridine has been used successfully to determine the fraction of cells in the S-phase of the cell cycle originally, which is an indicator of the proliferative activity of various types of human brain tumor[2]. The proliferating cell nuclear antigen (PCNA), so called cyclin, was first identified in 1987[3], in the sera of patients with systemic lupus erythematosus. It is an auxiliary protein of the delta subunit of DNA polymerase[4),5]. It appears in the cell at the G1/S boundary immediately before DNA replication, and its expression level increases during the S phase[6]. Tabuchi et al. demonstrated immunohistochemically the presence of PCNA in human glioma cells in vitro and in situ[7]. This method simplified the assessment of the proliferative potential of glial tumors.

In order to detect PCNA, cryostat sections or specially prepared histopathological materials are usually required. However, PCNA has been stained by applying a new antibody to conventionally fixed and processed histopathological materials[8)-13]. Monoclonal antibodies against genetically engineered rat PCNA were generated using conventional methods. Of the eleven clones with anti-PCNA specificity generated, six were found to react with formalin-fixed histopathological material, and one, designated as

PC10, was chosen for further detailed study, because it showed the highest activity in an ELISA assay. The supernatant from clone PC10 was used at a range of dilutions[9]. Therefore, the author has assessed the potential usefulness of PCNA immunostaining and PCNA labeling index (LI) determination using this antibody to evaluate the proliferative potential of glial tumors.

Unusual vascular proliferation is one of the distinctive features of glial tumors. It is particularly prominent in multiform glioblastoma and has been considered to be a sign of malignancy. Although there are many experimental studies of vascular endothelial cell proliferation information on the cytokinetics of vascular components in tumor is still sparse. In this study, the author evaluated the proliferative activity of the vascular components in relation to that of the surrounding tumor cells in human multiform glioblastoma.

Materials and Methods

Forty-six glial tumors were classified according to the criteria of the WHO classification. Astrocytomas (grade 2), anaplastic astrocytomas (grade 3) and multiform glioblastomas (grade 4) were 14, 14, and 18 cases, respectively.

The specimens were embedded in paraffin and sections were cut in 4 μm thick, mounted on poly-L-lysine coated glass slides and deparaffinized with a series of graded alcohol. The sections were incubated in 0.3%v/v H_2O_2 in 99.5%v/v methanol for 20 min. at room temperature to eliminate any endogenous peroxidase activity, rinsed three times in phosphate-buffered saline solution (PBS) and immunostained by the avidin biotin peroxidase complex (ABC) method, as follows. Anti-PCNA-monoclonal antibody (PC 10, DAKO, Denmark) diluted 1:50 in PBS, which contained 2%v/v horse serum albumin, was overlaid on the tissue sections for 1 hour at room temperature. Then, they were washed three times, for 5 min. each, with cold PBS. Biotinylated anti-mouse IgG (diluted 1:200 with PBS) (Vector Laboratories, Burlingame, CA, USA) was applied and incubated for 1 hour at room temperature. Then, the sections were washed three times, for 5 min. each, with cold PBS, incubated with streptavidin-horseradish peroxidase (diluted 1:25 with PBS) for 30 min. at room temperature. The peroxidase activity was demonstrated with 0.02%v/v 3-3' diaminobenzidine (DAB)-0.005%v/v H_2O_2 in Tris-buffered saline, pH 7.6, as the substrate, which was applied to the sections and allowed to react for 3 min. at room temperature. The reaction was stopped by rinsing the slides with distilled water, each section was counterstained with hematoxylin, dehydrated with methanol, cleaned with xylene and mounted under a glass coverslide. The author carried out PCNA staining of several sections from the same specimen, and the PCNA LIs were virtually identical.

In control studies, in which cells were incubated with normal mouse serum as the first antibody, neither cytoplasmic nor nuclear staining was observed.

The PCNA LI was calculated as the percentage of PCNA-labeled to the total number of nuclei at 5 sites in every specimen, which was scanned at low power to determine the areas that were most evenly and heavily labeled. One thousand to 2000 tumor cells per specimen were counted at a magnification of $\times 400$.

The tissue samples were scored by two observers who has no knowledge of the diagnosis.

The results were expressed as means $\pm$ standard errors of the mean. Differences between the means of the groups were analyzed using the Wilcoxon signed-rank test and those at $p<0.05$ were considered to be significant.

Results

PCNA LI for each histopathological malignancy grade

The mean PCNA LIs of the multiform glioblastomas, anaplastic astrocytomas and astrocytomas were 26.2 $\pm$ 2.0% (range, 14.1 to 41.0%), 10.7 $\pm$ 1.7% (range, 7.2 to 24.2%) and 5.1 $\pm$ 0.7% (range, 2.1 to 9.9%) respectively. The mean PCNA LI of the multiform glioblastomas was significantly higher than those of the orher grade of glioma ($p=0.001$). The PCNA LI was related to the grade of histopathological malignancy and the means of all three groups differed significantly from each other (Fig. 2). Inter-observer variations were present, but were slight, 1.5-3.6% for multiform glioblastomas, 0.6-2.1% for anaplastic astrocytomas and few % for astrocytomas.

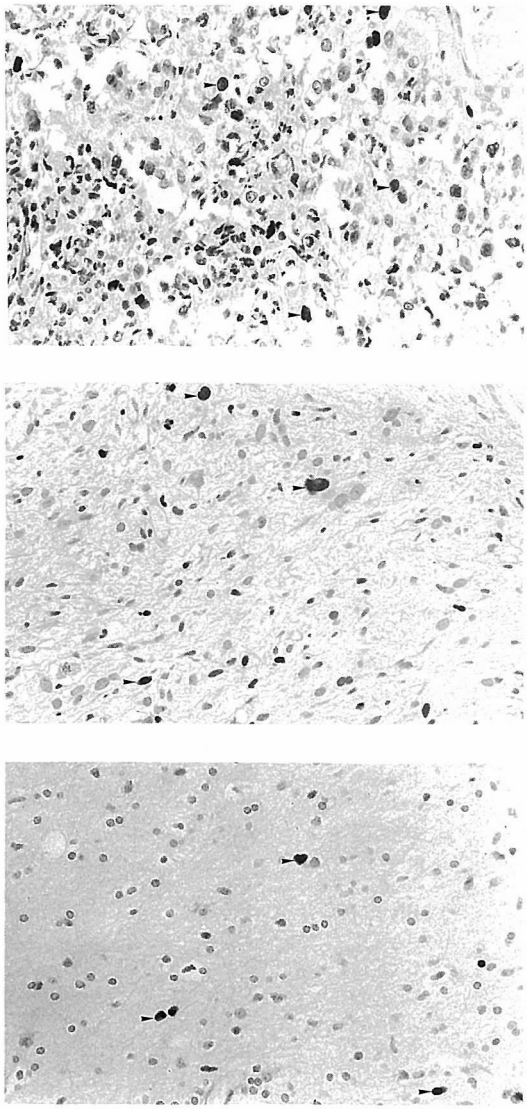

Fig. 1. Photomicrographs of tumor specimens. Proliferating cell nuclear antigen immunochemical stain, magnification, × 200. A: Multiform Glioblastomas (Case 8), Proliferating cell nuclear antigen labeling index (PCNA LI) is 36.9%. B: Anaplastic astrocytoma (Case 27). PCNA LI is 23%. C: Astrocytoma (Case 35). PCNA LI is 6.2%. Typical positive cells were indicated by arrows.

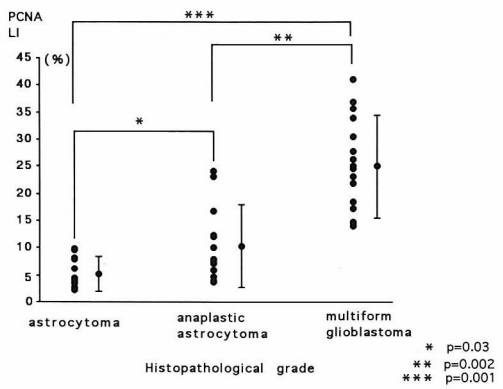

Fig. 2. Distribution of PCNA LI and histological grading. The PCNA LI is related to histological diagnosis. The PCNA LIs are significantly different among the three groups (Wilcoxon signed-rank test).

PCNA LI of tumor and endothelial cells

The PCNA LIs of the vascular components in the 8 multiform glioblastomas ranged from 6.5% to 20.7%, and were significantly higher than those of vessels in the normal brain, but significantly lower than those of the tumor cells (Table 1). The PCNA LIs of the anaplastic astrocytomas, astrocytomas and vascular components in the normal brain tissue could not be determined, as virtually no PCNA immunostaining was observed.

Relationship between PCNA LI and clinical prognosis

The clinical prognosis was assessed in 42 of the 46 patients, while the other 4 with complications were excluded for this part of the study (Table 1). Forty two patients could be followed up for as long as possible up to 12 years.

Twenty three patients had died and 19 were still alive. Only 5 of the 20 patients with PCNA LIs of less than 10% died, whereas 18 of the 22 patients with PCNA LIs of 10% or more died. The mean survival time for the latter patients was 10 months. The survival rates of these 42 patients were anal-

Table 1 Three cases, 19, 21, and 24, died of postoperative complications and the case 9 was a recurrent of the case 8. These four were excluded from the analysis of the relationship between PCNA LI and prognosis.

Case	PCNA LI (%)	PCNA LI (%) of vascular component	Prognosis (months)	Radiation or Chemothrapy	Location
Multiform glioblastoma					
1	25.0	6.5	dead,11	R,C	P
2	30.6	20.7	dead,11	R,C	F
3	24.6	12.6	dead,20	R,C	F
4	14.8		dead,12	R,C	F
5	22.0		dead,16	R,C	T
6	34.0		alive,74	R,C	O
7	23.0	12.8	dead,4	R,C	F
8	36.9	12.1	dead,17	R,C	T
9	41.0	15.4	dead,17	R,C	T
10	27.9		dead,9	R,C	F
11	17.2		dead,14	R,C	T
12	35.6		alive,48	R,C	T
13	18.5		dead,11	R,C	F
14	14.1		alive,27	R,C	F
15	37.0	12.7	dead,11	R,C	F
16	14.6	6.6	alive,48	R,C	F
17	27.7		dead,50	R,C	F
18	26.4		dead,10	R,C	F
Anaplastic astrocytoma					
19	7.7		dead,3		F
20	5.8		alive,84	R,C	F
21	7.9		dead,16	R,C	T
22	12.0		dead,4	R,C	T
23	12.3		dead,12	R,C	F
24	10.1		dead,8	R	F
25	3.9		dead,3	R,C	F
26	24.2		alive,144	R,C	F
27	23.0		alive,12	R,C	F
28	7.2		alive,144	R,C	F
29	7.2		alive,49	R,C	F
30	5.4		alive,56	R,C	T
31	16.7		dead,24	R,C	F
32	6.0		dead,60	R,C	P
Astrocytoma					
33	2.3		alive,144	R	F
34	4.3		alive,144	R,C	F
35	6.2		alive,108	R,C	T
36	2.7		alive,48	R,C	F
37	3.9		alive,25	R,C	F
38	9.5		dead,77	R,C	T
39	9.9		dead,41	R,C	T
40	7.9		alive,84		P
41	8.1		alive,144	R,C	F
42	2.1		alive,144		F
43	4.5		alive,96	R,C	F
44	4.1		dead,134	R,C	F
45	2.3		dead,48	R,C	T
46	3.4		alive,122	R,C	F

R: radiation, C: chemotheraphy, F: frontal lobe, T: temporal lobe, P: parietal lobe, O: occipital lobe

yzed by the Kaplan-Meier method (Fig. 3). The log-rank test demonstrated a significant difference (chi=9.907, $p<0.01$) between the survival rates of patients with PCNA LIs of 10% and above (n=22) and less than 10% (n=20). Tumor specimens were assessed from 23 patients who died. The mean PCNA LI was higher and their prognosis was likely to be worse (r=0.538 $p<0.01$). Therefore, the PCNA LI of the tumor was associated closely with the clinical prognosis.

Relationships between location and prognosis, and between mode of therapy (radiation and/or chemotherapy) and prognosis were not significant.

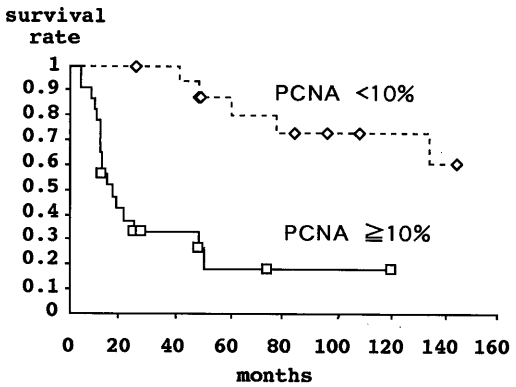

Fig. 3. Survival curves for patients with gliomas. There is a statistically significant difference between the groups with PCNA LI values less than 10% (dotted line) and that with a higher LI (solid line). Log-rank test is chi=9.907, $p<0.01$.

Discussion

Several authors have described immunohistochemical cellular kinetic studies by PCNA[3-14]. This method was applied to gliomas in order to determine their malignancy grades. The PCNA LI was related to the histopathological grade of malignancy and it could be used to estimate the clinical prognosis. The scores for tumors of the same histopathological grade were scattered, so that the tumor cells exhibited varying degrees of biological aggressiveness. Therefore, this method would appear to be useful for estimating the biological aggressiveness of individual tumors. Tumor specimens were taken from 23 patients who had died. The mean PCNA LI was higher, their prognosis was likely to be worse (r=0.538 $p<0.01$). The PCNA LI indicates the proliferative potential of individual glial tumors more accurately than the histopathological diagnosis. The proliferative potential of individual tumors can be estimated by formalin-fixed, paraffin-embedded specimens and may be used as routine assessment of tumor proliferative potential.

Nagashima et al. reported that the proliferative potential of the vascular components of multiform glioblastoma was higher than that of vessels in normal brain, but lower than and independent of that of tumor cells[15]. Denekamp et al. reported that the ^{3}H-thymidine LI of endothelial cells was independent of the LI of tumor cells, and proved that the LI of the endothelial cells did not correlate closely with the actual rate of tumor growth measured by the potential doubling time or tumor doubling time. It was suggested that this difference between the endothelial LI and tumor growth rate reflected differences in the ability of each tumor to produce an angiogenetic factor[16]. This study supports this hypothesis.

There are two populations of PCNA[17]. The one is associated with DNA replication sites and the other with unscheduled DNA synthesis. Both forms are conserved in formalin-fixed tissues, but only the one is present in methacarn-fixed tissues and the PCNA LIs differ in the different fixatives. In addition, differences among the PCNA LIs determined using different monoclonal antibodies have been reported[18].

In conclusion, the PCNA immunostaining method can be used to assess the proliferative potential of conventionally fixed and processed glial specimens. This method is valuable, because it is quick and simple to perform and the results are reproducible. Furthermore, retrospective studies can be performed by paraffinembedded specimens and the long-term prognoses of patients with gliomas can be assessed.

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