Comparison of Bromodeoxyuridine and Proliferating Cell Nuclear Antigen Labeling in Gastric Carcinoma

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The proliferative activity of human gastric carcinoma was measured by means of in vitro incorporation of the thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU), into the newly-synthesized DNA of fresh tumors and immunohistochemical staining of proliferating cell nuclear antigen (PCNA) using avidine-biotin peroxidase method. Eighty-two cases of surgically resected human gastric carcinomas consisting of 18 various histologic types were subjected to study. The mean BrdU labelling index (LI) and PCNA LI were 22.9% and 39.1%, respectively. The correlation between BrdU LI and PCNA LI was statistically significant (correlation coefficient $\mu=0.61334$, $p=0.0001$). We concluded that immunohistochemical staining for PCNA may become a practical method instead of in vitro or in vivo BrdU labeling to assess the proliferation fraction of the gastric cancer patient.

Key Words : Bromodeoxyuridine, Proliferating Cell Nuclear Antigen, Gastric Carcinoma

INTRODUCTION

Evaluation of the proliferative fraction of the neoplastic cell population is one of the most essential parameters of the growth of neoplasms for prognostic and therapeutic purposes (Risio et al., 1986). For the evaluation of the proliferative fraction, mitosis count (Baak, 1990), autoradiography after incubation with tritiated thymidine (Meyer and Conner, 1977), or flow cytometric measurement (Dean et al., 1982) have been mainly used.

Recently it has been reported that monoclonal antibody against bromodeoxyuridine (BrdU) (Gratzer, 1982) and proliferating cell nuclear antigen (PCNA) (Garcia et al., 1989) could be used to identify proliferating cells. BrdU is a non-radioactive thy-

midine analogue that incorporates competitively into the nuclear DNA during the S-phase of the cell cycle. The labeling index of BrdU has been proven to be quite similar to that of tritiated thymidine both in vivo (Langer et al., 1985) and in vitro (Meyer et al., 1989). PCNA is a 36-Kd nuclear protein, the amount of which is increased rapidly from late G1 phase and sustained through the S-phase of the cell cycle. This protein has been described independently by Bravo and Celis (1980) through two dimensional gel electrophoretic studies on proliferating and quiescent cells, and by Miyachi et al (1978) by using human autoantibodies in lupus patients. It has been demonstrated that PCNA is an auxiliary protein of DNA polymerase delta (Bravo et al., 1987) that plays a critical role in the initiation of cell proliferation.

We performed in vitro BrdU labeling and immunohistochemical staining of PCNA in gastric carcinoma and compared the labeling indexes between them. This study was performed to assess the proliferative fraction of gastric carcinoma and to clarify the relationship between in vitro BrdU labeling and PCNA labeling.

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MATERIALS & METHODS

We examined eighty-two cases of surgically resected human gastric carcinomas, including 18 cases of well differentiated adenocarcinoma, 23 cases of moderately differentiated adenocarcinoma, 26 cases of poorly differentiated adenocarcinoma, 4 cases of signet ring cell carcinoma, 5 cases of mucinous carcinoma, 2 cases of small cell carcinoma, and 4 cases of undifferentiated carcinoma. Fresh tumor specimens were cut into 0.5-1.0 mm thick pieces with a blade and put in a 10 ml V-vial (Wheaton) containing RPMI 1640 solution with 100 μ M BrdU (Sigma). The vials were put in an incubator under hyperbaric conditions 3 times above room atmosphere with a mixed gas (95% O₂+5% CO₂). The incubation was performed at 36°C with gentle shaking for 2 hours. After incubation, the slices were fixed in Carnoy's solution (glacial acetic acid 10%, chloroform 30%, and ethanol 60%) for more than 12 hours (up to 80 hours). More detailed procedures were described in a previous paper (Kim et al., 1991).

Fixed tissues were processed through successive treatment of 100% ethanol (3 changes), xylene (2 changes), and paraffin (2 changes). The paraffin blocks were cut at 4 μ m and mounted on poly-L-lysine coated slides. We obtained two tissue sections in each paraffin block. One was used for immunohistochemical staining of BrdU and the other for PCNA. After deparaffinization and rehydration through xylene and alcohol in series, the slides were incubated in 1 N hydrochloric acid at 60°C for 10 minutes to denature double-stranded DNA. The denaturation was stopped by immersion in 0.1 M Borax solution. The slides were blocked with normal goat serum. After 20 minutes, the goat serum was replaced by 1 : 250 monoclonal anti-BrdU antibody (Synbio). Negative control slides were run by omitting the anti-BrdU procedure. The slides were placed in a humid chamber for 30 minutes at room temperature. The following procedures included rinsing the slides with Tris buffer, applying biotinylated anti-mouse IgG (Vector ABC Elite kit), rinsing the slide again, and applying avidin-biotin-peroxidase complex. We used 0.01% diaminobenzidine (DAB) with 0.02% hydrogen peroxide as chromogen. After hematoxylin staining, the slides were mounted with balsam after dehydration. The BrdU labeling index (BLI) was calculated as the percentage of labeled nuclei among 100 tumor cell nuclei.

Immunohistochemical staining of PCNA using avidin-biotin peroxidase method was done using the same procedures. The labeling indexes for PCNA were determined with the same method as the BLI. We compare the labeling indexes of BrdU and those of PCNA in the adjacent sections.

RESULTS

BrdU and PCNA were detected in the nuclei with dark brown color showing a granular or uniform pattern (Fig. 1, 2). Nuclear immunostaining for BrdU was mostly seen in the superficial layers of the tissue slices but for PCNA positive cells were encountered in also the deeper portion.

Fig. 1. In vitro BrdU labeling and immunohistochemical staining using anti-BrdU on well differentiated adenocarcinoma. ($\times 400$, ABC method, hematoxylin counter staining)

Fig. 2. Immunohistochemical staining of PCNA on well differentiated adenocarcinoma. ($\times 400$, ABC method, hematoxylin counter staining)

Table 1. Correlation of BrdU labeling index and PCNA labeling index in various histologic types of gastric carcinoma

Histology	Mean BrdU LI	Mean PCNA LI	Correlation coefficient	p value	No. of Cases
WD	25.7	41.0	0.0736	(p=0.0005)	18
MD	23.6	40.3	0.4679	(p=0.0243)	23
PD	21.3	36.0	0.6048	(p=0.0013)	26
SR	16.5	30.5	-0.2558	(p=0.7442)	4
MU	21.8	40.4	0.8324	(p=0.0802)	5
Total	22.8	39.1	0.6156	(p=0.0001)	82

WD : well differentiated adenocarcinoma

MD : moderately differentiated adenocarcinoma

PD : poorly differentiated adenocarcinoma

SR : signet ring cell carcinoma

MU : mucinous carcinoma

LI : labeling index

Table 2. Regression analysis of BrdU labeling index (BLI) and PCNA labeling index (PLI) in various histologic types of gastric carcinoma

WD	BLI = $3.7 + 0.5357 \times \text{PLI}$		(p=0.0005)
MD	13.0	0.2646	(p=0.243)
PD	13.0	1.0827	(p=0.0011)
SR	40.7	-0.6190	(p=0.7442)
MU	-17.8	0.9808	(p=0.0802)
Total	8.4	0.3703	(p=0.0000)

Fig. 3. In vitro BrdU labeling and immunohistochemical staining using anti-BrdU on signet ring cell carcinoma. (X 400, ABC method, hematoxylin counter staining) **Fig. 4.** Immunohistochemical staining of PCNA on signet ring cell carcinoma. (X 400, ABC method, hematoxylin counter staining).

The mean BrdU labeling index (LI) and PCNA LI were 22.9% and 39.1% in total cases, respectively. The correlation between BrdU LI and PCNA LI was statistically significant (correlation coefficient $r = 0.61334$, $p = 0.0001$) (Table 1). BrdU LI positively correlated with PCNA LI. Among the various histolo-

gic types, small cell carcinoma had the highest LI (BrdU LI : 24%, PCNA LI : 53%), in contrast to the low LI (BrdU LI : 16.5%, PCNA LI 30.5%) in signet ring cell carcinoma (Fig 3, 4). Regression analysis shows that the BrdU LI can be expressed as $8.4 + (0.3703) \times \text{PCNA LI}$. The correlation of the signet

ring cell carcinoma is different from the other types in the aspect of negative correlation between BrdU LI and PCNA LI and lack of statistical significance.

DISCUSSION

Studies on cell kinetics and nucleotide incorporation into the DNA of various tissue are important for the understanding of the proliferative fraction of malignant tumors.

Radiolabeling with tritiated thymidine has numerous drawbacks including the requirement of fresh, viable tissue for thymidine incorporation, difficulty in uniform uptake and potential alteration of cell kinetics in the tumor samples incubated in vitro, and the expense and dangers in handling radioactive substances. (Meyer and Conner, 1977)

Although flow cytometrically determined kinetic analysis has many advantages including the ability to use fresh as well as archival, paraffin embedded material, flow cytometric DNA measurement of S phase fraction has been proven to be not well correlated with the result of tritiated thymidine labeling in solid tumors especially of non-diploid DNA pattern. (Dean et al., 1982)

In vitro BrdU labeling method is a reliable and specific method evaluating the proliferative fraction of the tumor, but it still requires fresh, viable tissue for incubation and it is impossible to do a retrospective study. (Kim et al., 1991)

Proliferating cell nuclear antigen has been proven to be an auxiliary protein of DNA polymerase delta, and it is synthesized in correlation with the proliferative state of the cell. Bravo and Macdonald-Bravo (1987) have demonstrated that PCNA/cyclin is such a kind of stable protein, that it can be detected in quiescent cells until at least 24 to 48 hours after cells have stopped dividing. By immunofluorescence studies the existence of two patterns of PCNA/cyclin positivity during the S-phase was found: one is nucleoplasmic positive usually in quiescent cells and in the other it is associated with specific nuclear structures. By immunoblotting analysis it is confirmed that the amount of PCNA/cyclin in non-proliferating tissue is negligible. Based on these observations, it is thought that PCNA/cyclin exists throughout the cell cycle except for the G0 state and could be a reliable marker of the proliferative fraction. The immunohistochemical method has several intrinsic advantages over destructive techniques such as flow cytometry,

the most valuable one of which is the good preservation of tissue architecture. Although several authors have analyzed the proliferative fraction of gastric carcinoma using BrdU or PCNA, we cannot find any comment about the correlation of the two methods.

Ohyama et al (1990) reported that the mean value of the S phase fraction of gastric carcinoma was 12.3% using in vivo BrdU labeling according to an analysis of 117 cases, which is markedly lower than our result. They administered the BrdU intravenously in vivo 20 minutes before the laparotomy. It is known that intravenously administered BrdU rapidly disappears in venous blood within 45 minutes. In our study, in vitro incubation was done for 2 hours. The difference of exposure time can explain the difference of S-phase fraction between the two results.

In this study, the labeling indexes of gastric cancer are higher in well differentiated types than in poorly differentiated or signet ring cell types. This result is an unexpected one because less differentiated tumors usually grow faster than the more differentiated ones. It might be related to the fact that the degree of differentiation is not a prognostic factor in gastric carcinoma.

As our results have shown a statistically significant correlation between the LI of BrdU and PCNA, immunohistochemical staining for PCNA may become a practical method instead of in vitro or in vivo BrdU labeling to assess the proliferation fraction of the gastric cancer patient.

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