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Cell Proliferation Kinetics of Human Gastric Carcinoma: An Immunohistochemical Study With a Monoclonal Antibody Against DNA Polymerase-α

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Cell proliferation kinetics of human gastric carcinoma were studied immunohistochemically using a monoclonal antibody against DNA polymerase-α (Pol-α). The distribution patterns and percentages of proliferative cells were examined in cases with various histological types of gastric carcinoma and compared with those of normal epithelium of the gastric foveolae. Pol-α-positive epithelial cells were localised at the isthmus of the normal foveola, while Pol-α-positive cancer cells were distributed irregularly in the cancer nests. The percentage of Pol-α-positive cells (%PPC) was significantly higher in the carcinoma [mean (S.D.) 41.6 (12.9)%] than in the normal foveola [24.8 (6.4)%] ($P < 0.01$). Also, the intestinal-type carcinoma showed a relatively higher %PPC [44.9 (12.0)%] than the diffuse type [36.2 (15.1)%] ($P < 0.05$), and the %PPC of signet ring cell carcinoma was extremely low [7.3 (2.2)%] ($P < 0.01$). Pol-α-positive cancer cells were observed most abundantly in the lamina propria of the mucosa. They decreased in number with the depth of cancer infiltration down to the subserosa.
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INTRODUCTION

HUMAN GASTRIC carcinomas show varied proliferation patterns and growth rates. It is important to estimate proliferative activities of the carcinomas to predict the degree of malignancy of each case. For this purpose cell proliferation kinetics of human gastric carcinoma have been studied by detection of S-phase cells with either tritiated thymidine ([^{3}H]TdR) [1–3] or bromodeoxyuridine (BrdU) [4], and some interesting information has been presented. It was reported that the intestinal-type carcinoma showed a higher labelling index than the diffuse-type carcinoma and that the signet ring cell carcinoma had a low labelling index and might be out of cell cycle [1, 2]. The cases with high labelling indices of BrdU were reported to have a poorer prognosis because of a high degree of lymph node metastasis [4]. However, the labelling method using [^{3}H]TdR or BrdU has many restrictions when performed on human tissues, and the findings of these studies were still not sufficient to determine the relation between the proliferation patterns of the carcinoma and the clinicopathological characteristics, such as histological subtype, depth of infiltration, size of carcinoma, age and sex of the patients, and positivity or negativity of lymph-node metastasis.

DNA polymerase-α (Pol-α) is a key enzyme which catalyses ribonucleoside triphosphate-dependent DNA synthesis in cooperation with DNA primase [5, 6], and the cells having Pol-α activities in the nuclei are supposed to be in cell cycle. Recently, monoclonal antibodies against Pol-α were produced which enabled us to detect proliferating cells immunohistochemically on pathological specimens [7–9]. As a result, several studies have been performed on the human cancer tissues indicating

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that Pol-α is a useful marker to estimate the malignancy of the tumour [10–12]. The present study is an attempt to examine proliferative activity of gastric cancer cells and its relation to some pathological features of individual cases, using a monoclonal antibody against Pol-α.

MATERIALS AND METHODS

65 gastric carcinomas resected surgically at Tsukuba University Hospital were used in the present study. The age of the patients ranged from 33 to 80 years, and 43 cases were male and 22 were female. 19 tumours were located at the antrum, while 46 involved the corpus of the stomach. The tumours appeared in various shapes; some cases were elevated, some were flat, and the others were ulcerated. The size of the tumours ranged from 1.0 to 18.5 cm in largest diameter. The tumours were divided into two groups based on their histological type according to Lauren's classification: intestinal and diffuse [13]. 36 cases were of the intestinal-type carcinoma and 29 cases were of the diffuse type. The intestinal-type carcinomas were further subclassified into 27 cases of papillary (pap) and well-differentiated tubular (tub 1) adenocarcinoma, and 9 cases of moderately differentiated tubular (tub 2) adenocarcinoma according to the method used by the Japanese Research Society for Gastric Cancer [14]. The diffuse-type carcinomas were subclassified into 27 cases of poorly differentiated adenocarcinoma (por) and 2 cases of signet ring cell carcinoma (sig). The tumours were also classified into two categories based on their stage of development. There were 12 early-phase carcinomas that were localised in the mucosa and 53 advanced-phase carcinomas that had infiltrated the muscular layer. 41 cases had metastasised to the regional lymph nodes, while 24 cases showed no lymph-node metastasis.

Small cancer tissues (1 × 1 × 0.5 cm) were removed immediately after the surgical operation and fixed in 4% paraformaldehyde overnight at 4°C. Five samples of the surrounding histologically normal gastaric mucosa, more than 3 cm distant from the carcinoma, were also used. After washing with a series of gradient sucrose-added phosphate-buffered saline (PBS) solutions, the tissues were embedded in OCT-compound (Tissue-Tek), frozen in liquid nitrogen and stored at −80°C until used. Thin sections, 6 μm in thickness, were prepared with a cryostat (Tissue-Tek II, Miles, USA) and treated by the peroxidase–antiperoxidase (PAP) [15] or the avidin–biotin peroxidase complex method (ABC) [16] with a monoclonal antibody against Pol-α.

The monoclonal antibody against Pol-α (CL22-2-42B, Medical & Biological Laboratories, Japan) was a mouse IgG antibody, secreted by a hybridoma clone prepared by the fusion of NS-1 mouse myeloma cells with mouse splenic cells that had been immunised with calf thymic cells. This monoclonal antibody was demonstrated to cross-react selectively with human proliferative cells [8, 9].

After blocking the endogeneous peroxidase activity with 0.3% hydrogen peroxide, the tissue sections of the gastric carcinoma and the normal mucosa were washed with PBS, then treated with normal goat serum. Next, the sections were incubated for 1 h at room temperature with either anti Pol-α antibody or normal mouse serum as a negative control. The sections were then washed with PBS and treated with sheep antimouse IgG antibody and soluble horseradish peroxidase anti–horseradish peroxidase antibody complex (PAP) solution (Proliferative Cell Test Kit, MBL, Japan) successively. The peroxidase reaction was visualised with 3,3′-diaminobenzidine tetrahydrochloride (DAB, 0.03%) and hydrogen peroxide (0.001%) in Tris–HCl buffer, pH 7.4. After incubation with the primary antibody, some of the specimens were treated successively with biotinylated sheep antimouse IgG and avidin–biotinylated horseradish peroxidase complex (ABC) solution (Vectastain Kit, Vector Lab., USA) and were then visualised with DAB and hydrogen peroxide. No differences of immunohistochemical stainabilities were detected between the PAP and ABC methods in the present study. Some of the specimens were counter-stained with haematoxylin or methyl green. For pathological examination, the specimens prepared by routine haematoxylin–eosin staining were used.

Approximately 1000–1500 foveolar epithelial cells of the normal mucosa, and 300–5000 gastric cancer cells were observed using a light microscope. The foveolar epithelium was composed of mucous cells at the surface and immature cells at the isthmus, and was distinguished from the pyloric or fundic glands of the deep layer by microscopic observation. Three to seven fields of the gastric cancer tissue, including the different layers of the gastric wall, were observed. The morphological features of the cancer cells of individual cases were confirmed by the observation of the specimen stained with haematoxylin–eosin. The cells, whose nuclei were stained various shades of brown, were distinguished as Pol-α-positive cells light microscopically. All of the positive cells were counted and the percentage of the Pol-α-positive cells (%PPC) per total normal foveolar cells or gastric cancer cells observed was calculated in each case. Further, the cancer cells were subgrouped according to their location; lamina propria of the mucosa, submucosa, muscularis propria and subserosa. The percentage of the positive cells in each location was also calculated. The data were presented in the form of mean (S.D.), and statistical analysis was performed using Student's *t*-test. Furthermore, the mean %PPC of the gastric cancer cells was comparatively studied between different groups of cases categorised by the histological type, size, sex and age of the patients and the existance of lymph-node metastasis.

RESULTS

Normal gastric mucosa

The normal gastric mucosa was composed of the foveolae at the superficial layer and the pyloric or fundic glands at the deeper layer. The foveolae were further composed of tall columnar mucous cells at the surface and immature columnar or cuboidal cells at the isthmus, just above the pyloric gland at the antrum and the fundic gland at the corpus. Pol-α-positive epithelial cells appeared to be localised at the isthmus of the normal foveolae, and virtually no positive cells were observed at the surface of the foveolae or the pyloric and fundic glands (Fig. 1). Pol-α was found in the nuclei of the positive cells which had been stained various shades of brown. The %PPC of the normal foveolar epithelium cells was calculated, based on the ratio of positive cells found at the isthmus, to the total no. of both positive and negative foveolar cells (the negative cells having been found at the surface), and this ranged from 16.1% to 30.0% [mean (S.D.) 24.8 (6.4)%] (Table 1). Immature lymphocytes were also positively stained and were observed in a group at the germinal centre of the lymph follicle or sparsely distributed in the stroma.

Gastric cancer tissue

Pol-α-positive cancer cells were distributed irregularly in the cancer nests of the varied histological types found in the various layers of the gastric wall (Figs 2 and 3). The %PPC of each carcinoma case calculated in several different fields of the cancer nests showed values ranging from 5.8% to 71.8%. However, the

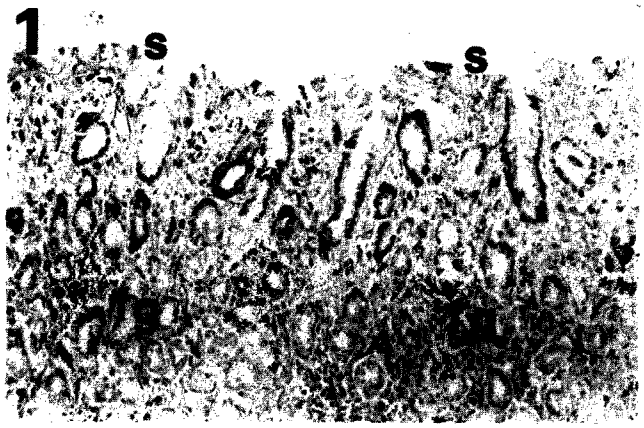

Fig. 1. Photograph of normal gastric mucosa, stained with anti-Pol-α antibody. The positive cells at the isthmus of the foveolae have darkly stained nuclei. Almost all of the surface epithelium (s) and fundic glands (fg) in the lower portion of the photograph have no positive nuclei. Several immature lymphocytes in the stroma are also positively stained. Immunoperoxidase staining without counter-stain. × 100.

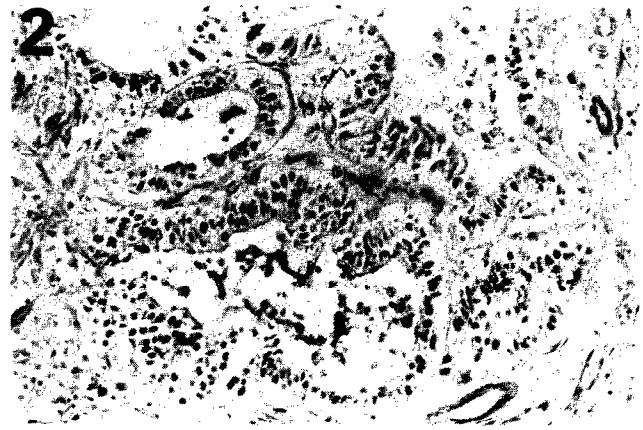

Fig. 2. Photograph of a intestinal-type carcinoma, stained with anti-Pol-α antibody. Approximately 60% of the cancer cells have positively stained nuclei. Immunoperoxidase staining without counter-stain. × 160.

mean value of all the carcinoma cases was significantly higher [41.6 (12.9)%] than that of the normal foveolar epithelium ($P < 0.01$; Table 1).

Comparison between the two main histological types of gastric carcinoma revealed that the 36 intestinal-type carcinoma cases showed a relatively higher %PPC [44.9 (12.0)%] than did the 29 diffuse type carcinoma cases [36.2 (15.1)%; $P < 0.05$]. Comparison between histological subtypes also revealed that pap and tub 1 adenocarcinoma cases showed relatively higher %PPC values than poorly differentiated adenocarcinoma cases (por). Histological discrimination of pap from tub 1 was rather difficult by the observation of a single piece of tissue, so the total value of %PPC of pap and tub 1 was calculated (pap+tub 1). tub 2 adenocarcinomas showed an intermediate %PPC value between pap+tub 1 and por. The %PPC of sig, a subtype of the diffuse-type carcinoma, was 7.3 (2.2)%; significantly lower than those of other histological subtypes ($P < 0.01$; Table 1).

It was observed that the Pol-α-positive cancer cells were abundant in the lamina propria of the mucosa in every case, and that they decreased in number with the depth of cancer infiltration down to the submucosa, muscularis propria and

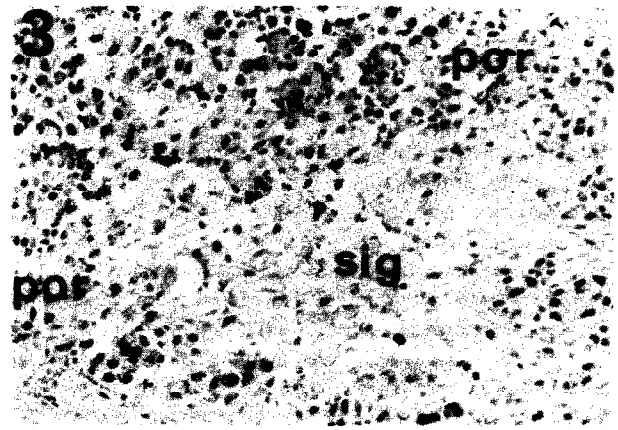

Fig. 3. Photograph of a diffuse-type carcinoma, stained with anti-Pol-α antibody. The carcinoma consists of two subtypes of cancer cells; non-mucus-containing poorly differentiated cells (por) and mucus containing signet ring cells (sig). Most of the por at the upper, lower and right portions have positively stained nuclei; however, the sig in the middle portion have unstained nuclei. Immunoperoxidase staining without counter-stain. × 280.

Table 1. The mean percentage of P-α *positive cells (%PPC) in the normal foveolar epithelium and various types of gastric carcinoma*

Histological type	No. of cases	Range of %PPC	Mean value of %PPC (S.D.)	
Normal foveolar epithelium	5	16.0–30.1	24.8 (6.4)	$P < 0.01$
Cancer tissue, total cases	65	5.8–71.8	41.6 (12.9)	
Intestinal type	36	13.1–71.8	44.9 (12.0)	
pap+tub 1	27	13.1–71.8	46.3 (12.7)	$P < 0.05$
tub 2	9	31.5–58.0	40.5 (8.1)	
Diffuse type	29	5.8–60.9	36.2 (15.1)	
por	27	15.3–60.9	38.4 (13.3)	$P < 0.01$
sig	9	5.8– 8.8	7.3 (2.2)	

pap = papillary adenocarcinoma; tub 1 = well-differentiated tubular adenocarcinoma; tub 2 = moderately differentiated tubular adenocarcinoma; por = poorly differentiated adenocarcinoma; sig = signet ring cell carcinoma.

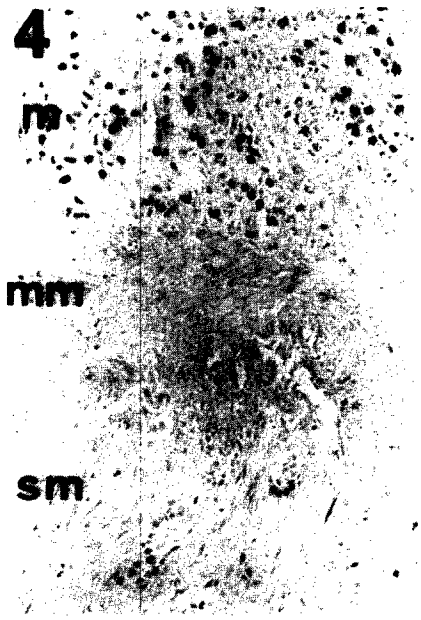

Fig. 4. Photograph of a diffuse-type carcinoma, stained with anti-Poly-α antibody. The cancer cells proliferate in the lamina propria of the mucuosa (m) and the submucosa (sm). The muscularis mucosae (mm) is observed at the middle layer. Many cancer cells in the lamina propria are positively stained, while only a few cancer cells in the submucosa are positive. Immunoperoxidase staining without counter-stain. × 200.

subserosa, successively (Fig. 4). The mean values of %PPC in each location of the gastric wall decreased in order from the lamina propria to the subserosa (Table 2).

The mean %PPC of the 22 cases of small-sized carcinomas smaller than 5 cm in largest diameter [44.3 (15.2)%] was slightly higher than that of the 15 cases of large-sized carcinomas larger than 10 cm in largest diameter [37.9 (11.0)]. The mean %PPC of the 19 cases of patients over 70 years old [46.8 (13.7)%] was also slightly higher than that of the 13 cases of patients under 50 years old [39.3 (14.5)%]. However, no significant differences were noted statistically between the smaller and larger carcinoma groups, or between the younger and older patient groups. The mean %PPC of the 43 male cases [42.0 (12.1)%] was similar in value to that of the 22 female cases [40.0 (14.4)%]. The %PPC of the 41 cases with lymph-node metastasis [40.1 (12.5)%] was also similar in value to that of 24 cases without metastasis [44.7 (13.3)%]. Neither the sex of the patients nor the positivity or negativity of lymph-node metastasis had any effect on the %PPC.

DISCUSSION

Pol-α-positive cells were found to be localised at the isthmus of the normal foveolae, just above the pyloric gland at the antrum and the fundic gland at the corpus. This finding was consistent with those of the previous studies with [^{3}H]TdR and BrdU on human tissues [1–3] and experimental animals [17, 18]. Immature lymphocytes in the germinal centre of the lymph follicles and in the stroma were also found to be positive. The epithelial cells at the isthmus of the foveolae and the immature lymphocytes at the germinal centre of the lymph follicle are supposed to be proliferating cells, and the results of the present study suggest that the anti-Pol-α antibody used was able to detect selectively the proliferating cells in the gastric mucosa. The %PPC values of the normal foveolar epithelium and the carcinoma were higher than the labelling indices of the studies

Table 2. The mean percentage of Pol-α-positive cells (%PPC) in the gastric carcinoma at various depths of infiltration in the gastric wall

Site	No. of cases	Range of %PPC	Mean value of %PPC (S.D.)	
Lamina propria	56	5.8–62.6	46.9 (12.7)	$P < 0.01$
Submucosa	43	12.6–56.2	38.0 (12.4)	
Muscularis propria	21	7.9–34.9	27.6 (16.3)	$P < 0.01$
Subserosa	10	8.8–39.5	25.6 (15.4)	

using [^{3}H]TdR and BrdU. [^{3}H]TdR and BrdU are known to be incorporated by cells in S-phase. On the other hand, cells in the entire cell cycle, not only those in S-phase but also those in G1, G2 and M-phases, are supposed to have Pol-α activity in the nuclei or cytoplasms [8, 9]. Higher positivity in the present study is attributable to the different targets between the two different cell cycle markers, [^{3}H]TdR or BrdU and the anti-Pol-α antibody [10, 11].

The mean %PPC of the gastric carcinomas was significantly higher than that of the normal foveolar epithelium, and many cancer cells were found to be in the cell cycle. We counted the %PPC of the normal foveolar epithelium to compare its proliferative activity with that of the gastric carcinoma. This is because the foveolar epithelium and carcinoma are rapid-renewing cells, whereas the pyloric and fundic glandular cells are slow-renewing cells [17, 18]. Therefore it is more appropriate to compare the proliferative activity of the cancer cells with that of the foveolar epithelium than that of the pyloric or the fundic glandular cells. Although the mean %PPC of the gastric carcinoma was high, individual gastric carcinoma cases showed widely varying values of %PPC. These differences may be due to various degrees of proliferative activity; however, analysis of the clinical data suggesting how rapidly the carcinoma grew and a long-term follow-up study to examine the survival curve for the present cases would be necessary to test whether the %PPC is correlated to proliferative activity of the carcinoma. The prognosis of the cases examined using BrdU that showed higher labelling indices was reported to be poorer because of a higher frequency of lymph-node metastasis [4]. However, lymph-node metastasis made no difference to the %PPC of the gastric carcinoma in the present study.

Gastric carcinomas were classified into two groups by histological types; intestinal and diffuse [13]. The mean %PPC of the intestinal-type carcinoma cases was higher than that of the diffuse-type carcinoma cases. This is consistent with the results obtained using [^{3}H]TdR and BrdU [1–4]. The intestinal-type carcinoma was further subclassified into pap, tub 1 and tub 2, based on the degree of glandular formation. The diffuse-type carcinoma was subclassified into por and sig according to the volume of intracytoplasmic mucus [14]. The pap and tub 1 cases showed a relatively higher %PPC than the por cases in this study. The por cases frequently contained some signet ring cells. Therefore the %PPC of por was calculated without counting the signet ring cells. We examined two cases that consisted exclusively of signet ring cells to calculate %PPC of the sig. The present study revealed that the %PPC was significantly lower in sig than in other subtypes. This result was consistent with that of the previous report by Fujita using [^{3}H]TdR [1]. However, in spite of the lower proliferative activity suggested in these studies, it is known that the biological behaviour and prognosis of sig are rather worse than those of

other subtypes [1, 19, 20]. The life span of cancer cells might be another factor, besides the %PPC, which may determine the growth rate of carcinomas [2, 21, 22].

It was interesting that the %PPC decreased with the depth of infiltration of the cancer cells in the gastric wall. This result suggests that the best environment for cancer cell proliferation is the lamina propria of the mucosa, and that the proliferative activity is hampered as cancer cells infiltrate deeper into the muscularis propria. However, clinically, the growth rate of the intramucosal early-phase carcinoma is low, and that of the deeply infiltrating advanced phase carcinoma is high. This slow growth of the early-phase carcinoma may be due to the loss of many more cells in the mucosal surface, where the cancer cells are injured by food, heat and gastric juice.

The present study showed that the %PPC of smaller-sized carcinomas was slightly higher than that of larger-sized carcinomas, and that the %PPC of carcinomas was slightly higher in older patients (over 70 years) than in younger patients (under 50 years). These results have no significant meaning, because many cases of smaller-sized carcinomas were in early phase and were located in the lamina propria and submucosa where the %PPC was higher. Many of the carcinoma cases of older patients were of the intestinal type, which showed a relatively higher %PPC than the diffuse type.

This study demonstrated that proliferative cancer cells could be detected more easily by using the monoclonal antibody against Pol-α than by using [^{3}H]TdR or BrdU. The distribution of the positive cancer cells was clearly shown in larger tissue blocks by our method. The %PPC of the gastric carcinomas could be compared between various histological subtypes, different depths in the gastric wall, and other clinco-pathological characteristics. As a possible next step, a biochemical technique such as DNA polymerase assay (PDP-LI) would be effective for a quantitative study of Pol-α in the individual cancer tissue [23].

The anti-Pol-α antibody seemed to detect the cells similar to those detected by the anti-Ki-67 antibody [24, 25] and the anti-PCNA/cyclin antibody [26, 27]. The Ki-67 nuclear antigen was reported to appear in the proliferating cells and disappear when the cells were induced to differentiate into resting cells. The PCNA/cyclin was recently reported to be the auxiliary protein of DNA polymerase δ in the nuclei of proliferating cells [28]. All of the cells in cell cycle G1, S, G2 and M phases were supposed to be positive for the anti-Ki-67 and anti-PCNA/cyclin antibodies. However, the positive ratio of Ki-67 on the human cancer cells reported was lower than the %PPC in the present study [25]. It was also reported that the more abundant tumour cells were positively stained with the anti-PCNA/cyclin antibody [27]. It might be interesting to make a comparative analysis of the different targets of these three antibodies to investigate the differences in proliferative activities of the varied cancer cells.

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