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Expression of Placental Alkaline Phosphatase Isoenzyme in Human Gastrointestinal Carcinomas as Identified Using Monoclonal Antibodies

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Summary. **Mouse monoclonal antibodies (MAbs) against MKN1 human gastric adenosquamous carcinoma cells were produced, and selected to bind to the placental alkaline phosphatase (PLAP) expressed by the cancer cells. One of the MAbs, M1H2, was highly specific for PLAP or PLAP-like isoenzyme and did not cross-react with intestinal or liver alkaline phosphatase enzymes. Another MAb, M1H9, also became bound to PLAP or a PLAP-like isoenzyme with a lower binding activity than M1H2, but also reacted weakly with enzymes other than PLAP or PLAP-like enzymes, indicating that the binding of M1H9 to PLAP or a PLAP-like enzyme was nonspecific. The antigen molecule recognized by MAbs was a *Mr* 65,000 peptide, being equivalent to a PLAP monomer as estimated by Western blot analysis. Immunocytochemical examination showed that these MAbs detected PLAP or a PLAP-like enzyme over the surface of MKN1, SCH, and A431 cells which express the enzyme with a uniform distribution, whereas reactivities to non-PLAP-producing, MKN45 and KATO-III cells were not detected. Immunohistological studies of human gastrointestinal carcinomas using M1H2 demonstrated that 26 of 29 esophageal carcinomas (90%), 12 of 27 gastric carcinomas (44%), and 10 of 18 colorectal carcinomas (56%) were reactive with the MAb. Noncancerous tissues adjacent to cancers did not react with the MAb. These results indicate that PLAP or the PLAP-like enzyme in gastrointestinal cancers may be regarded as cancer-specific antigens, and that M1H2 may be of value as a marker in gastrointestinal cancers, especially esophageal cancers.**

INTRODUCTION

Human placental alkaline phosphatase (PLAP) is an isoenzyme of alkaline phosphatase (AP) which is normally produced in term placental tissue. The Regan isoenzyme, which is identical to the normal PLAP, was first observed in a squamous cell carcinoma of the lung by Fishman et al.[1] Subsequently, Nakayama et al.[2] discoverd a PLAP-like isoenzyme (Nagao isoenzyme) that differed from PLAP by its greater sensitivity to L-Ieucine and EDTA. The PLAP-like isoenzyme is expressed in trace amounts in the testis, uterine cervix, thymus, and lung.[3,4] Enhanced expression of the PLAP-like isoenzyme is found in germ cell tumors such as seminomas,[5] where it serves as a useful marker of these tumors.[6] On the other hand, true PLAP (Regan isoenzyme) is expressed in tumor cells of both trophoblastic and nontrophoblastic origin.[7] The occurrence of PLAP in gastrointestinal carcinomas has been reported to be 30% to 40% by immunohistochemical studies,[8,9] although these studies have used polyclonal antibodies which cannot distinguish PLAP from intestinal AP due to their cross reactivity. Only two investigations of PLAP expression in gastrointestinal carcinomas using monoclonal antibodies (MAbs) have been recently available.[10,11]

In the current study, we have prepared MAbs to PLAP as expressed by a gastric cancer cell line, MKN1,[12] and report here on the characterization of these MAbs and the expression of the enzyme in gastrointestinal cancer tissues in an attempt to assess the clinical potential of PLAP as a marker for gastrointestinal carcinomas.

MATERIALS AND METHODS

Cell lines

The cell line used for immunizing mice was the MKN1 human gastric adenosquamous carcinoma cell line which has been found to express the Regan

isoenzyme monophenotypically.[12] The SCH human gastric choriocarcinoma cell line and the A431 human epidermoid carcinoma cell line (American Type Culture Collection CRL-1555) were used to assess the reactivity of MAbs for the Nagao isoenzyme that was expressed by both cell lines.[12,13] As controls, two gastric cancer cell lines, MKN45 and KATO-III, were used.[14] All the cell lines were grown in RPMI-1640 medium (Nissui Seiyaku, Tokyo) supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY, U. S. A.) in a humidified atmosphere containing 5% CO_2 at 37℃.

Samples

Human placental tissues were obtained immediately at full-term delivery. Liver tissues and small intestinal mucosae were obtained at autopsy and during surgery, respectively. Gastrointestinal cancer tissues were obtained from surgically resected specimens.

Extraction of AP from cultured cells and tissues

Cultured cells and normal human tissues (placenta, small intestine, liver) were homogenized in 10 mM Tris-HCl buffer, pH 8.0, containing 0.9% NaCl, and suspended in 40% (v/v) n-butanol. Each mixture was ultrasonicated at 200W for 5 min, then centrifuged, and the lower aqueous layer was dialyzed against 10 mM Tris-HCl buffer, pH 8.0, containing 0.9% NaCl and 1 mM $MgCl_2$ at 4℃ for 12 h, and the extracts stored at −70℃ until use.

AP activities were determined using the King-King modification of the King-Armstrong method. The protein concentration was measured using Lowry's method. The MKN1 cell extracts contained 3.05 IU/mg protein of AP.

Production of monoclonal antibodies to PLAP

Antibody-secreting hybridomas were prepared according to the method by Köhler and Milstein.[15] BALB/c mice (Charles River Japan, Atsugi) were immunized with three intraperitoneal injections of MKN1 cells. Four days after the last immunization, spleen cells were fused with SP2/0-Ag14 cells[16] using 50% (w/v) polyethylene glycol 4000 (Merck, Darmstadt, Germany). Antibody-secreting clones were selected using an enzyme-linked immunosorbent assay (ELISA) as described below, and subcloned twice by limiting dilution. Subcloned cells were intraperitoneally injected into pristane-primed BALB/c mice to produce ascites. Antibodies were purified from the ascites fluids by precipitation in 60% ammonium sulfate, followed by gel filtration using Sephacryl S-200 (Pharmacia, Uppsala, Sweden).

Enzyme-linked immunosorbent assay (ELISA)

For the micro-ELISA testing, 96-well microtiter plates (Corning, Corning, NY, U.S.A.) were coated with the AP extracts from MKN1 cells or normal human tissue (10 μg/ml), and incubated for 12 h at 4℃. The following incubation steps were done at room temperature. After blocking nonspecific binding sites with 100 μl of 1% bovine serum albumin (BSA, Sigma, St. Louis, MO, U.S.A.) in phosphate-buffered saline (PBS) for 2 h, 100 μl of the supernatant of growing hybridomas was added to the wells and incubated for 2 h. After three washings with 0.05% Tween 20 in PBS (pH 7.4), 50 μl of peroxidase-conjugated rabbit antimouse immunoglobulin (Dakopatts, Copenhagen, Denmark), diluted to 1 : 500 in 0.05% BSA in PBS (pH 7.4), were allowed to react for 1 h. After three washings with 0.05% Tween 20 in PBS, the plates were incubated with the substrate solution (0.5 mg/ml o-phenylene diamine in 0.15 M citrate-phosphate buffer, pH 5.0, containing 0.03% H_2O_2) for 30 min. The reaction was stopped with 4 N H_2SO_4. Absorbance was measured at 492 nm on a microplate reader (Model 450, Bio-Rad Japan, Tokyo).

Class analysis of the MAbs was performed using a Mouse Typer Sub-Isotyping Kit (Bio-Rad Japan).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Whole MKN1 cell extracts were prepared by directly lysing cells with Laemmli sample buffer for SDS-PAGE.[17] SDS-PAGE of antigens (MKN1 cell-lysates and AP extracts from placenta),[17] followed by transfer to nitrocellulose sheets (0.45 μm pore size, Bio-Rad, Richmond, CA, U.S.A.) and immunostaining with MAbs, was done according to the method of Towbin et al.[18]

Enzyme immunoassay (EIA) of alkaline phosphatase with MAbs

For solid-phase EIA of AP with MAbs, samples of AP extracted from normal human tissues, MKN1 and SCH cells as described above were used. The MAbs or normal mouse IgM as an irrelevant antibody were attached to 96-well microplates (Corning), and incubated for 12 h at 4℃. The plates were next blocked for the nonspecific adsorption of protein for

1 h with 100 μl of 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), followed by incubation with 100 μl samples for 4 h at room temperature. After three washings with 0.05% Tween 20 in PBS, the plates were incubated with 100 μl of substrate solution (1 mg/ml p-nitrophenylphosphate in diethanolamine buffer, pH 10.0) for 20 min at 37℃. The reaction was stopped with 100 μl of 2 N NaOH. The OD_{405} of each well was determined using a microplate reader (Bio-Rad Japan).

Immunoperoxidase staining

The cultured cell lines and gastrointestinal carcinoma tissues from surgical specimens were used to examine the immunochemical localization of the reactive antigens by indirect immunoperoxidase staining. Pellets of cultured cells and human cancer tissues were fixed in Bouin's fixative or 10% formaldehyde, respectively, and embedded in paraffin. As the first antibody, M1H2 diluted to 1 : 60 or normal mouse immunoglobulin (Ig) M (Dakopatts) as a negative control was added and incubated for 12 h at 4℃. The sections were counterstained with methylgreen. The immunoreactivity in the section was determined as follows: a section with more than two thirds of tumor cells showing clear staining was defined as +++, one third to two thirds as ++, less than one third as +, and negative as −. Histologic classification of the esophageal, gastric, and colorectal cancers was performed according to the criteria of the Japanese Research Society for Esophageal Diseases, Gastric Cancer, and Cancer of the Colon and Rectum.

RESULTS

Production of MAbs

Antibody reactivity in the hybridoma culture supernatant was tested against n-butanol-extracted MKN1 enzyme by ELISA. Two positive clones stably secreting MAbs (M1H2 and M1H9) against PLAP extract from MKN1 cells were established. Both MAbs belonged to the IgM κ isotype.

Characterization of MAbs

The binding activities of MAbs to various types of AP were determined using solid-phase EIA. As shown in Table 1, PLAP expressed by MKN1 cells and term placenta, and a PLAP-like enzyme expressed by SCH cells were both detected with M1H2 and M1H9. However, the binding activity to PLAP of MAbs was higher than that to the PLAP-like enzyme. APs of the small intestine and liver were not detected with M1H2. M1H9, however, showed a predominant binding activity to PLAP or the PLAP-like enzyme; it was also reactive with APs of small intestine and liver. These results suggest that M1H2 is specific for PLAP or the PLAP-like enzyme, although it is difficult to state that the binding of M1H9 to PLAP or the PLAP-like enzyme was specific. Western blot analysis of lysates from MKN1 cells and PLAP extracted from term placenta with MAbs revealed the same single staining of the band (Fig. 1). The molecular weight of this protein was approximately 65,000, this being identical with that of a PLAP monomer.

Immunoperoxidase staining with the MAbs

Preliminary investigation on the staining pattern and positivity of PLAP in paraffin and frozen sections in 5 cases of esophageal cancer revealed that there was no difference in PLAP immunoreactivity between paraffin and frozen sections.

Both M1H2 and M1H9 showed strong, clear staining of the syncytiotrophoblast plasma membrane of term placenta, and did not bind to the liver or small intestinal mucosae (data not shown).

Immunocytochemically, both MAbs became bound to PLAP or the PLAP-like enzyme expressed by

Table 1. Binding activity of MAbs to various APs as estimated by solid-phase EIA

MAb	Binding activity[a] to various APs[b]				
	MKN1-PLAP	SCH-PLAP	Term-PLAP	Small intestinal-AP	Liver-AP
M1H2	0.315±0.015	0.093±0.012	0.227±0.015	0.012±0.006	0.012±0.002
M1H9	0.148±0.053	0.098±0.037	0.226±0.015	0.107±0.022	0.028±0.02

[a] Absorbance was done at 405 nm. Each value represents the mean±SD of quadruplicate readings. Normal mouse IgM was used as an irrelevant antibody whose reading was less than 0.01.

[b] Samples were prepared as outlined in "MATERIALS AND METHODS".

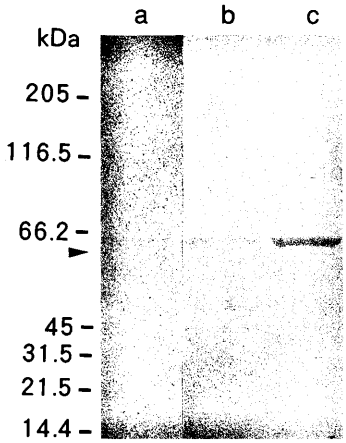

Fig. 1. Western blot. SDS-PAGE of MKN1 cell-lysates (lanes **a** and **b**) and AP extract from term placenta (lane **c**) was performed, followed by transfer to nitrocellulose sheets and immunostaining with the MAbs indicated. **a**, M1H9; **b** and **c**, M1H2. Positions of molecular weight markers are indicated. $\times 10^3$

MKN1, SCH, or A431 cells with a uniform distribution (Fig. 2, A-F). Two other non-PLAP-producing cancer cell lines, MKN45 and KATO-III, were not stained with either MAb.

Next, 74 various gastrointestinal carcinoma tissues from surgical specimens were examined for reactivity with both MAbs. The results are summarized in Table 2. Twelve of 27 gastric cancers (44%) were positive for M1H2 and M1H9. The tumors demonstrated a typical plasma membrane reactivity and some cytoplasmic staining (Fig. 3A). On the other hand, a high frequency of positive staining of tumor tissues by both MAbs (90% for M1H2, and 79% for M1H9)was observed in esophageal cancers without regard to differentiating grades of the cancer cells. Moreover, the localization of staining was observed mainly in the cytoplasm of cancer cells (Fig. 3B). Colorectal cancers also reacted with the MAbs, its incidence being 56% (10 of 18) for M1H2 and 39% (7 of 18) for M1H9. The staining pattern was almost identical with that of gastric cancers (Fig. 3C). None of the noncancerous tissues adjacent to cancers was stained in any tumor. Throughout the immunohistochemical study, staining with M1H9 showed a speckl-

Table 2. Immunohistochemical staining of gastrointestinal cancer tissues with MAbs

Site	Histologic classification[b]	Immunoreactivity[a]									
		M1H2					M1H9				
		+++	++	+	−	Incidence	+++	++	+	−	Incidence
Stomach	tub1	0	0	3	7	3/10(30%)	1	0	2	7	3/10(30%)
	tub2	2	1	1	1	4/5 (80%)	1	2	1	1	4/5 (80%)
	por and sig	1	0	3	6	4/10(40%)	1	0	3	6	4/10(40%)
	ECC	0	1	0	1	1/2 (50%)	0	1	0	1	1/2 (50%)
	total	3	2	7	15	12/27(44%)	3	3	6	15	12/27(44%)
Esophagus	SCC	10	8	5	3	23/26(88%)	3	9	9	5	21/26(81%)
	adenocarcinoma	1	0	0	0	1/1 (100%)	1	0	0	0	1/1 (100%)
	muc	1	1	0	0	2/2 (100%)	1	0	0	1	1/2 (50%)
	total	12	9	5	3	26/29(90%)	5	9	9	6	23/29(79%)
Colon and Rectum	well	0	2	5	6	7/13(54%)	0	0	5	8	5/8 (63%)
	mod	1	1	0	1	2/3 (67%)	1	1	0	1	2/3 (67%)
	por	0	0	1	1	1/2 (50%)	0	0	0	2	0/2 (0%)
	total	1	3	6	8	10/18(56%)	1	1	5	11	7/18(39%)

[a] Immunoreactivity is defined as follows: −, negative; +, less than 1/3; ++, 1/3 to 2/3; +++, more than 2/3 of tumor cells were stained.

[b] tub 1, well differentiated tubular adenocarcinoma; tub 2, moderately differentiated tubular adenocarcinoma; por, poorly differentiated adenocarcinoma; sig, signet-ring cell carcinoma; ECC, endocrine cell carcinoma; SCC, squamous cell carcinoma; muc, mucoepidermoid carcinoma; well, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma.

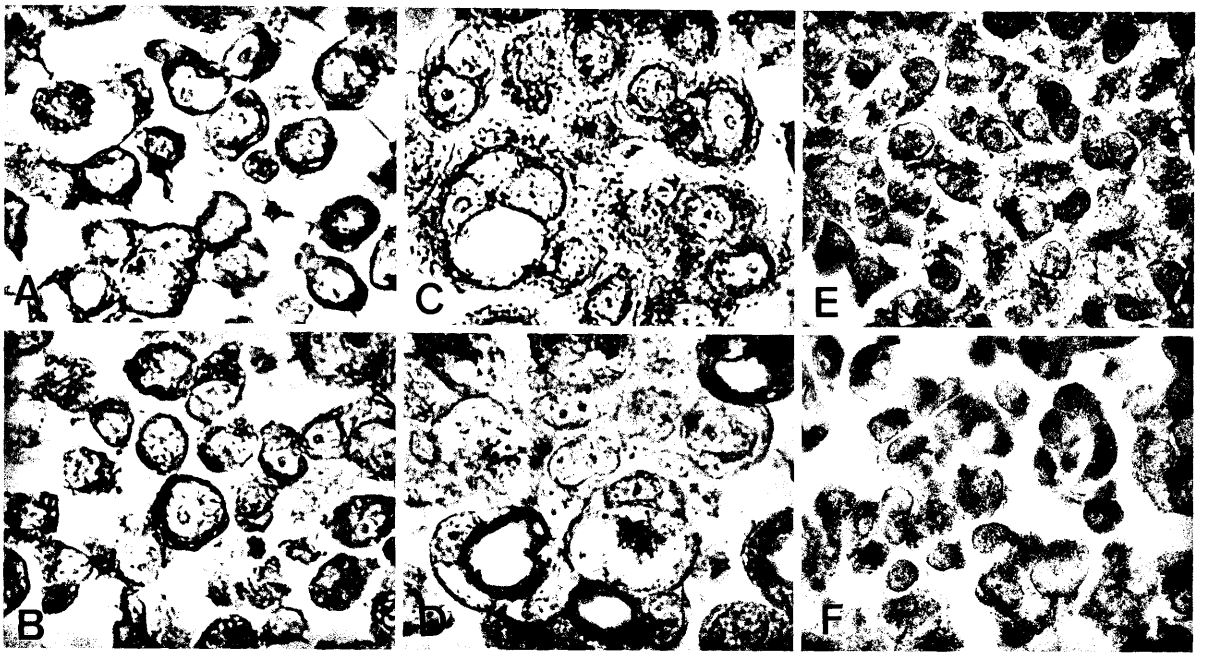

Fig. 2. Immunoperoxidase staining of cultured cancer cells with MAbs, M1H2 and M1H9. Bouin's solution-fixed and paraffin-embedded specimens of cell pellets have been stained with the MAbs indicated. **A** and **B**, MKN1 cells; **C** and **D**, SCH cells; **E** and **F**, A431 cells. **A**, **C** and **E** stained with antibody M1H2; **B**, **D** and **F**, stained with antibody M1H9. ×680

ed distribution in all cases as compared to M1H2, reflecting a lower binding activity of M1H9 to PLAP or the PLAP-like enzyme.

DISCUSSION

The aim of this study was to produce MAbs to cancer-associated PLAP and to evaluate the occurrence of enzyme expression in gastrointestinal cancers.

A modest number of MAbs to PLAP has been established by other investigators,[11,19–25] although for the most part these MAbs have been raised against the PLAP protein isolated from term placenta, not against cancer-associated PLAP as expressed by neoplastic cells. We have produced MAbs to cancer-associated PLAP by immunizing mice with MKN1 cells in which PLAP is ectopically expressed; however, the resultant MAbs (M1H2 and M1H9) reacted both with normal term PLAP and PLAP of MKN1 cells. Moreover, both MAbs also reacted with the PLAP-like enzyme as expressed by the SCH and A431 cell lines, although the binding activity estimated by EIA was less than that to PLAP. These results indicate that PLAP expressed in cancer is probably identical to the normal term PLAP, and the present MAbs cannot recognize structural differences between PLAP and the PLAP-like enzyme arising in cancer cells. Structural differences and distinct genes in PLAP and PLAP-like enzymes have been resolved by studies using electrophoresis on starch gels,[26] allotype-specific monoclonal antibodies,[27] and cDNA library;[28] however, in terms of MAb recognition, these two closely related enzymes have not as yet been distinguishable.

PLAP or the PLAP-like enzyme has been regarded as a tumor marker for germ cell tumors[6,29] and uterine and ovarian cancers.[30–32] However, the occurrence of PLAP or the PLAP-like enzyme is unlikely to be restricted to germ cell tumors or ovarian cancers, being often expressed by gastrointestinal cancers.[10] We have also examined immunohistochemically the expression of the enzymes in gastrointestinal cancers using M1H2 and M1H9. In contrast to earlier immunohistochemical studies using polyclonal or monoclonal antibodies,[8–11] the current study using M1H2 and M1H9 has shown that a high proportion of the tumors is positive for PLAP or the PLAP-like enzyme. The most significant numbers of the enzyme-positive cases were observed in esophageal cancers. Hamilton-Dutoit et al. also reported that proximal

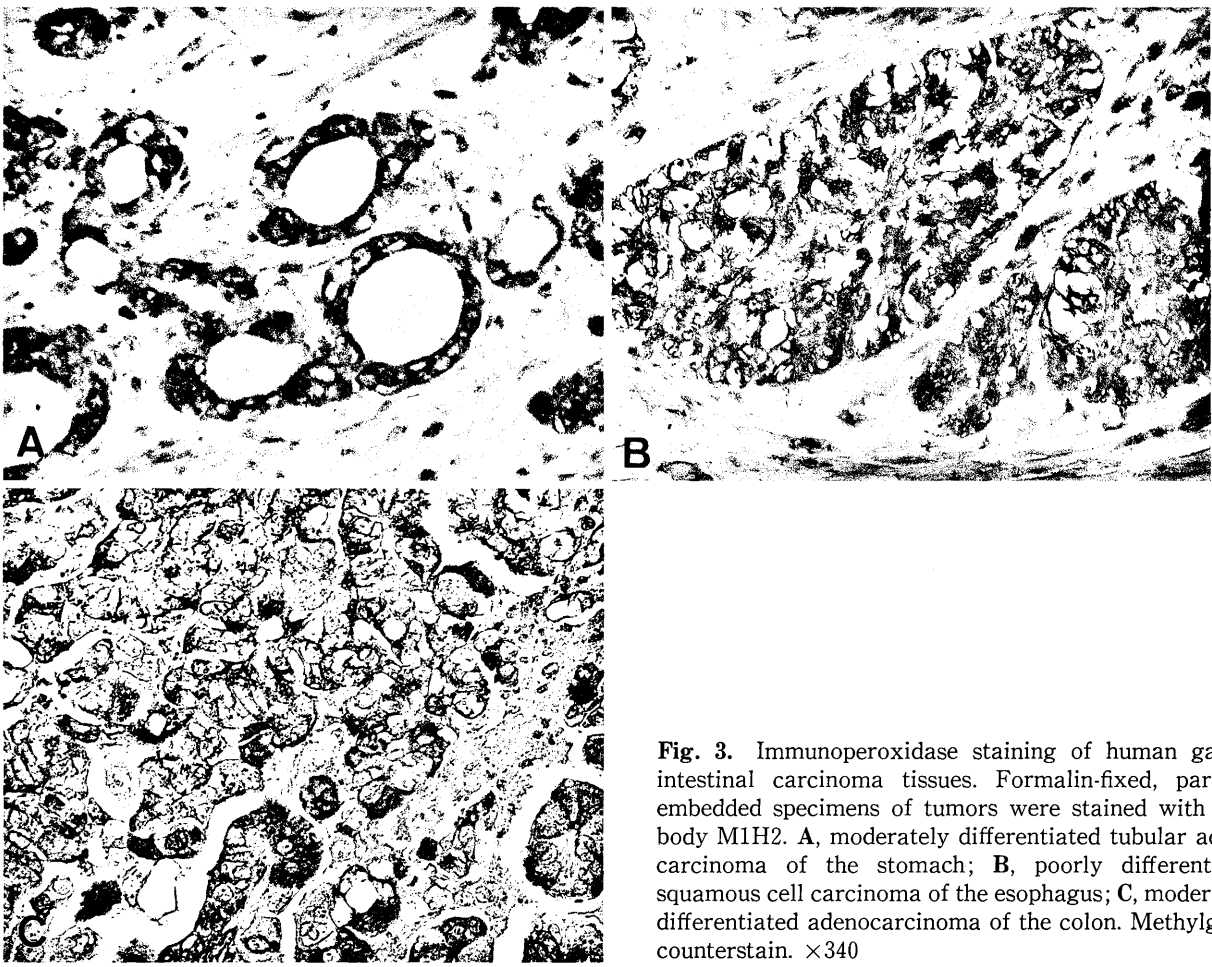

Fig. 3. Immunoperoxidase staining of human gastrointestinal carcinoma tissues. Formalin-fixed, paraffin-embedded specimens of tumors were stained with antibody M1H2. **A**, moderately differentiated tubular adenocarcinoma of the stomach; **B**, poorly differentiated squamous cell carcinoma of the esophagus; **C**, moderately differentiated adenocarcinoma of the colon. Methylgreen counterstain. ×340

gastrointestinal tract tumors, particularly esophageal cancers, had a high incidence of PLAP expression (67%).[10] Their cases were exclusively esophageal adenocarcinomas; however, the present cases showed squamous cell carcinomas for the most part (26 squamous cell carcinomas out of 29 cases). No report demonstrating a high proportion of the expression of PLAP or the PLAP-like enzyme in esophageal squamous cell carcinomas has appeared in the literature. Although the reason why esophageal cancers demonstrate a high incidence of the enzyme expression is unclear at present, PLAP or the PLAP-like enzyme is expected to be a tumor marker for esophageal squamous cell carcinomas. To confirm the usefulness of the enzyme as a marker in esophageal carcinomas, larger surveys including the measurement of enzyme levels using sera from patients and tumor tissue extracts assays using MAbs would be needed.

In summary, two MAbs have been produced to PLAP as expressed by MKN1 gastric adenosquamous carcinoma cells. These MAbs, particularly M1H2, were highly specific for PLAP or the PLAP-like enzyme. By immunohistochemistry, PLAP or the PLAP-like enzyme recognized by M1H2 was able to be detected in many gastrointestinal tract cancers, especially esophageal squamous cell carcinomas. Therefore, M1H2 may be of value as a marker in patients with esophageal carcinomas. Moreover, since the PLAP or PLAP-like enzyme is a tightly membrane-bound protein, M1H2 should therefore be useful as a tool for experiments in immunotargeting[33,34] and immunotoxin therapy.[35] Subsequent studies regarding these aspects using M1H2 are currently underway.

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