

Cancer Research



Expression of a Hybrid Form of Alkaline Phosphatase Isoenzyme in a Newly Established Cell Line (HuG-1) from a Gastric Cancer Patient

Hiroyasu Imanishi, Toshikazu Hada, Koji Muratani, et al.

Cancer Res 1990;50:3408-3412.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/50/11/3408>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

Expression of a Hybrid Form of Alkaline Phosphatase Isoenzyme in a Newly Established Cell Line (HuG-1) from a Gastric Cancer Patient

Hiroyasu Imanishi, Toshikazu Hada,¹ Koji Muratani, Kazuyuki Hirano, and Kazuya Higashino

Third Department of Internal Medicine, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663 [H. I., T. H., K. M., K. H.], and Gifu Pharmaceutical University, 6-1 Mitahorahigashi 5-chome, Gifu 502 [K. H.], Japan

ABSTRACT

An unusual alkaline phosphatase (AP), named HuG-AP, was found in a newly established cell line (HuG-1) derived from a patient with stomach cancer. The enzyme was purified about 300-fold by affinity chromatography. On polyacrylamide gradient (4–30%) gel electrophoresis, the one band with the enzyme activity was observed. The enzymic properties of HuG-AP did not conform to those of liver, intestinal, placental, and germ cell AP isoenzymes which were recognized as homodimeric structure. Thermostability of the HuG-AP showed an intermediate value between intestinal and placental APs. Immunologically, the HuG-AP reacted with both anti-intestinal and anti-placental AP monoclonal antibodies. Dot blot analysis showed that both intestinal and placental AP mRNAs were expressed in HuG-1 cells concurrently.

Therefore, we concluded that this novel AP had a hybrid form, namely heterodimeric structure, consisting of one subunit each of intestinal and placental APs. However, all of the properties of this hybrid AP did not conform to the intermediate enzymic properties between intestinal and placental APs which would be shown when they both coexist. Because an AP identical to HuG-AP had already been found in the metastatic lesion of the liver of the same patient, the expression of this novel AP seemed to occur in the patient's original cancer cells but did not result from spontaneous transformation of cultured cells.

INTRODUCTION

There are four gene loci for human APs.² The structure of these four AP genes has become clear recently (1–4).

Cancer tissues are known to express unusual APs. For example, placental AP (Regan isoenzyme) (5) and germ cell AP (Nagao isoenzyme) (6), which is normally expressed in trace amounts in testis and thymus except for pregnancy, can be detected in the sera and cancerous tissue of patients with malignant diseases, especially with genitourinary cancers. Kasahara isoenzyme, which is another cancer-associated AP of intestinal type, is used for diagnostic assistance of hepatocellular carcinoma (7, 8). A normal cell usually expresses one type of AP mRNA, which is then translated to one AP protein consisting of the same two subunits, so-called homodimeric structure. However, some cancer cell lines exceptionally express two different types of AP mRNA concomitantly. In such cancer cells, some hybrid form of AP, in other words heterodimeric AP, may exist. Thus far, however, few reports dealing with heterodimeric type of AP expressed in human established cancer cell lines have been encountered except that Wray and Harris showed the heterodimeric AP (placental/intestinal) expressed in Hep2/5 cells, a subline of HeLa, using immunological methods (9).

We (10) recently reported a novel AP (named IP-AP) having unique enzymatic properties different from those of intestinal

and placental APs and reacting with both monoclonal antibodies specific to intestinal and placental APs, suggesting a hybrid form of AP consisting of one subunit of intestinal AP and another subunit of placental AP, found in the metastatic lesion of the liver of a patient with gastric cancer.

In the present study, we report the establishment and characterization of a new gastric cancer cell line, HuG-1, which was derived from cancer cells in the ascitic fluid of the same patient mentioned above, and we describe that this cell line produced an novel AP (HuG-AP) identical to IP-AP expressed in the metastatic lesion of the liver of the same patient.

MATERIALS AND METHODS

Patient Profile

The patient was a 55-year-old Japanese male of the A, Rh+ blood group with advanced gastric cancer (Borrmann 3). Histologically, specimens of the gastric tumor obtained at autopsy were moderately differentiated tubular adenocarcinoma (Fig. 1A). At the time of admission of the patient on October 11, 1986, peritoneal dissemination with ascites and metastasis to the liver was observed with abnormally high serum levels of CEA (225 ng/ml), CA 19-9 (26,400 units/ml), and TPA (5,400 units/liter). The ascites, containing many cancer cells, was aspirated on October 28, 1986. The specimen of the patient's liver containing metastatic cancer tissue was obtained at autopsy.

Establishment of a New Cell Line (HuG-1) from the Patient

Samples of 15 ml of ascitic fluid were incubated in three 75-cm² flasks (Iwakiglass, Tokyo, Japan) at 37°C in a humidified atmosphere of 5% CO₂ in air for 1 day, then 15 ml of RPMI 1640 containing 10% heat inactivated FBS (Whittaker M.A. Bioproducts Inc.) and antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin) were added to each flask. Subsequently half of the medium was changed every 4–7 days. The cultured cells were observed with a phase-contrast microscope and photographed. At the 55th generation, the cultured cells were cloned twice and a new cell line, HuG-1, was established.

Characterization of HuG-1 Cells

Chromosomal Analysis. Exponentially growing cells in RPMI 1640 containing 10% FBS were incubated with Colcemid (0.2 µg/ml, final) for 7 h. After hypotonic treatment for 10 min at 37°C in 0.075 M KCl, the cells were fixed in methanol:acetic acid (3:1, v/v). Chromosomes were stained by the trypsin-Giemsa banding technique (11). One hundred chromosomes in metaphase were examined.

Heterotransplantation. Female nude mice of BALB/c origin were obtained commercially when 6 weeks old and were used as recipients in heterotransplantation experiments. Suspensions of 1 × 10⁷ cells in 0.5 ml phosphate-buffered saline were inoculated s.c. into the back of the nude mouse, and the animals were observed for 6 months for development of tumors.

Tumor Markers in the Used Medium of HuG-1 Cells and in the Serum of Tumor-bearing Nude Mice. Culture of 1 × 10⁵ cells/ml was incubated for 3 days without medium change. The medium was then collected by centrifugation at 500 × g for 10 min to remove floating cells and stored at –70°C. Sera were obtained from the tumor-bearing nude mice when the s.c. inoculated tumor size reached about 1.0 cm³. Sera of nontreated nude mice were collected as control. These sera were stored at –70°C until use. The used medium was then determined for CEA, CA19-9,

Received 11/20/89; revised 2/22/90.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ To whom requests for reprints should be addressed.

² The abbreviations used are: AP, alkaline phosphatase; IP-AP, intestinal/placental alkaline phosphatase hybrid form; CEA, carcinoembryonic antigen; TPA, tissue polypeptide antigen; CA19-9, carbohydrate antigen 19-9; FBS, fetal bovine serum; EAIA, enzyme-antigen immunoassay.

and TPA with radioimmunoassay kits from Dainabot (Tokyo, Japan), Centocore, Inc. (Malvern, PA), and Daiichi (Tokyo, Japan), respectively. Sera of tumor-bearing nude mice were also measured for CEA, CA19-9, and TPA.

Characterization of AP

Assay of AP. AP activity was determined using phenylphosphate as a substrate by the method of Higashino *et al.* (7). One unit of the enzyme activity was defined as the amount of enzyme causing the release of 1 μmol of phenol/min. Protein was determined by the method of Lowry *et al.* (12). The effects of various inhibitors and thermostability of AP were tested for discrimination of AP isoenzymes according to the method described previously (13). APs from normal intestine, term placenta, and liver and germ cell AP from seminoma tissue were prepared as described previously (7, 10).

Purification of AP. AP was extracted from the established cells (HuG-1) by the method reported previously (7). The *n*-butyl alcohol extract from HuG-1 cells was treated with acetone. The fraction precipitating between 30 and 60% acetone was dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl, 10 μM MgCl₂, 10 μM ZnCl₂, and 0.02% NaN₃ (buffer A). The dialyzed enzyme was applied on a 2HIMS-1 column (Sephacrose 4B coupled with anti-intestinal AP monoclonal antibody) (14, 15) and washed with buffer A. The enzyme was eluted with 0.2 M Na₂CO₃ containing 0.5 M NaCl. The enzymically active fractions were dialyzed against buffer A. The enzyme was then applied on an HPMS-1 column (Sephacrose 4B coupled with anti-placental AP monoclonal antibody) and eluted by use of the same procedure. Thus eluted enzyme was purified about 300-fold from the cell homogenate and its specific activity was 2.6 units/ μg protein. This purified enzyme was used for the following experiments.

Electrophoresis. Polyacrylamide gradient (4–30%) gel electrophoresis was performed according to the method of Higashino *et al.* (13). After electrophoresis, the bands made visible by staining the enzyme activity with 1-naphthyl phosphate and Fast Blue BB salt (Sigma) as described previously (7).

Immunological Analysis. EAIA was performed using three kinds of monoclonal antibodies (HPMS-1, 2HIMS-1, and HLMS-1) each of which is specific for human placental, intestinal, or liver AP isoenzyme (15). Briefly, each well of a 96-well plate was incubated with 100 μl of goat anti-mouse IgG antibody (Kirkegaard and Perry Lab. Inc.; 20 $\mu\text{g}/\text{ml}$). After the plate had been washed and blocked with 1% bovine serum albumin, 100 μl of the monoclonal antibody solution (5 $\mu\text{g}/\text{ml}$) were added to each well, followed by incubation. After the plate had been washed, 100 μl of AP (10 milliunits) were added and incubated at 4°C for 12 h. The plate was washed, and then 200 μl of substrate solution (2.7 mM *p*-nitrophenyl phosphate) were added to each well, followed by incubation at 37°C for 30 min. The absorbance of the resulting solution in each well was measured against substrate blank at 405 nm.

Dot Blot Analysis of mRNA. Two kinds of probe which are specific for intestinal AP mRNA or placental AP mRNA were prepared as described previously (8). Total cellular RNA was isolated from HuG-1 cells by the guanidine isothiocyanate/CsCl method (16). The polyadenylated RNA fraction was separated by chromatography on an oligodeoxythymidylate cellulose column as described previously (17). Pre-hybridization and hybridization were performed as described previously (8).

Analysis of Data. Values reported are the mean \pm SEM for three determinations. Differences between groups were evaluated using Student's *t* test, with significant difference defined as those associated with $P < 0.01$. The results of EAIA, inhibition assay, and thermostability of APs represent the average of two determinations (the difference of two determinations; within 5%).

RESULTS

Characterization of the HuG-1 Cell Line

The ascitic fluid containing floating cells in the flask was gradually replaced by RPMI 1640 containing 10% FBS. Finally,

after about 1 month of primary culture, the cells could be cultivated in RPMI 1640 containing FBS. The floating cancer cells showed good viability and grew faster than those attached to the flask; therefore we cultured only these floating cells. The floating cells have been cultivated for 220 generations over a period of 31 months (Fig. 1B). The cells were split 1:2 every 3 to 4 days and could be expanded. At the 36th generation some cells were transferred to serum-free medium and were found to grow as well in the medium containing 10% FBS. Using limiting dilution, we obtained cloned cells at the 55th generation (HuG-1). The doubling time (exponential phase) of HuG-1 cells was

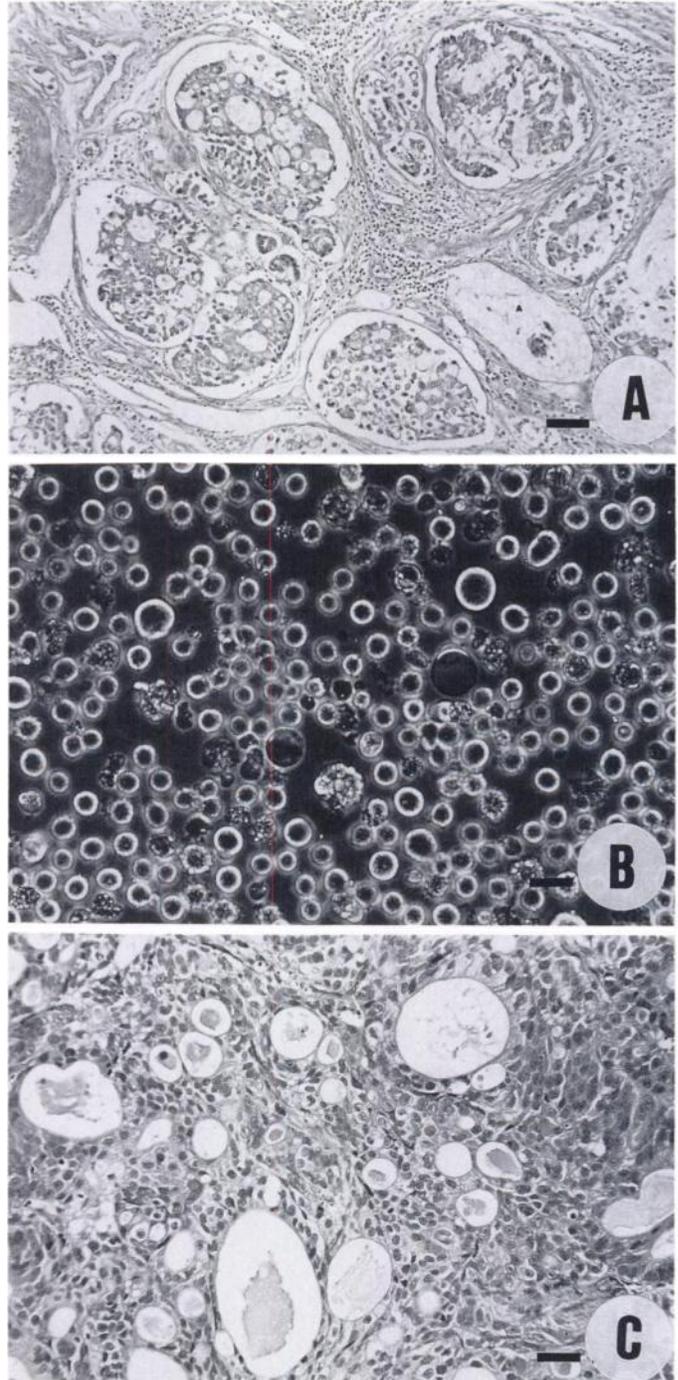


Fig. 1. A, light micrograph of moderately differentiated tubular adenocarcinoma of the original tumor tissue. H&E; bar, 60 μm . B, phase-contrast micrograph of HuG-1 cells in medium containing 10% FBS. Some signet ring cells are seen. Bar, 30 μm . C, light micrograph of transplanted HuG-1 cells in the nude mouse shows moderately differentiated tubular adenocarcinoma. H&E; bar, 30 μm .

about 38 h at the 60th and 160th passages. The chromosome number of the cells at the 72nd generation was between 55 and 63 with a hypotriploid modal number of 60. Giemsa banding carried out at the 72nd generation on the cells with the modal chromosome number showed trisomy of chromosomes 2, 6, and 12; monosomy of chromosomes 4, 5, and 21; and nullisomy of the Y chromosome. In addition, 9 marker chromosomes, 1q-, 3p+, 7p+, 8p+, 9p-, 11p+, 11p+, 14p+, and 17p+, were identified in all karyotypes studied. Three structurally abnormal chromosomes, i(10q), 15q+, and 16q+, were present in 7 of 8 karyotypes.

Inoculation of nude mice with 1×10^7 HuG-1 cells resulted in the development of tumors. Histologically, the tumor revealed moderately differentiated adenocarcinoma (Fig. 1C) and resembled a part of original patient's cancerous tissue.

The available tumor markers in the used medium were assayed. Table 1 shows that HuG-1 cells at a saturation density released CEA, CA19-9, and TPA. The ability of HuG-1 cells to produce CEA, CA19-9, and TPA has continued from the primary culture up to the present time (220th generation). The HuG-1 cells released CEA, CA19-9, and TPA even in serum-free medium, but in smaller amounts than in medium containing 10% FBS (Table 1). Serum levels of CEA (6.9 ± 2.2 ng/ml), CA19-9 (170 ± 32 units/ml) and TPA (4200 ± 1600 units/liter) in the tumor-bearing nude mice (about 1.0 cm^3 in tumor volume) were high compared with those of CEA (below 0.5 ng/ml), CA19-9 (below 6 units/ml), and TPA (180 ± 50 units/liter) in nontreated nude mice ($P < 0.01$).

Characterization of AP of HuG-1 Cells

Electrophoretic Mobility. As shown in Fig. 2, HuG-AP extracted and purified from HuG-1 cells electrophoresed in the intermediate position between placental and intestinal APs on polyacrylamide gradient (4–30%) gel electrophoresis, just as the IP-AP obtained directly from patient's cancer tissue did.

Immunological Analysis. Specificities of monoclonal antibodies are shown in Table 2. HuG-AP extracted with *n*-butyl alcohol from HuG-1 cells reacted with both anti-placental and anti-intestinal AP specific monoclonal antibodies, just as the IP-AP did (10). This suggests that HuG-AP consists of two different subunits, one from intestinal AP subunit and the other from placental AP.

Thermostability. The activity of HuG-AP remaining after incubation at 65°C is shown in Fig. 3. HuG-AP was more heat-labile than placental AP but more heat-stable than intestinal AP. This was consistent with data for IP-AP (10).

Effect of Amino Acids on Enzyme Activity. HuG-AP was sensitive to L-leucine and L-phenylalanine but resistant to L-homoarginine. However, the degree of the sensitivity of HuG-AP to these amino acids was not always compatible with that of placental or intestinal AP (Table 3). These properties were the same as those of the IP-AP reported previously (10).

Table 1 Assays of tumor markers in the used medium of HuG-1 cells

	Culture supernatant ^a		10% FBS ^b (control)	Patient's serum
	10% FBS	Serum-free		
CEA (ng/ml)	6.3 ± 1.3^c	1.0 ± 0.1	0.3 ± 0.1	225
CA19-9 (units/ml)	888 ± 259^c	64 ± 8.3	5 ± 2	26,400
TPA (units/liter)	$6,129 \pm 1,600^c$	$1,822 \pm 611$	13.4 ± 4	5,400

^a Tumor marker levels were shown for 10^5 cells/ml/72 h incubation under confluent conditions.

^b RPMI 1640 containing 10% FBS was assayed as negative control. Each value is the mean \pm SEM of triplicate measurements.

^c $P < 0.01$ by Student's *t* test when compared with samples from used serum-free medium.

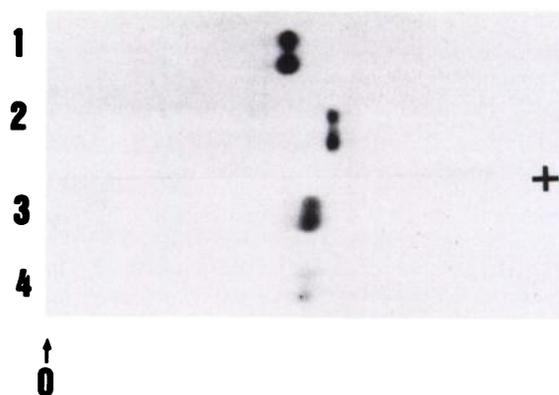


Fig. 2. Electrophoretogram of APs on polyacrylamide gradient (4–30%) gel. 1, intestinal AP; 2, placental AP; 3, IP-AP; 4, HuG-AP. O, origin. Anodal direction is from the origin (O) to the right (+) of the figure.

Table 2 Immunological study of alkaline phosphatases

Enzyme activity of each specimen used was 4 milliunits. Values are the actual absorbance at 405 nm of *p*-nitrophenol produced in each well and represent the average of two determinations.

Alkaline phosphatase	Monoclonal antibodies		
	Anti-intestinal AP	Anti-placental AP	Anti-liver AP
Intestinal	0.80	0	0
Placental	0	0.65	0
Liver	0	0	0.91
IP-AP	0.46	0.50	0
HuG-AP	0.44	0.54	0

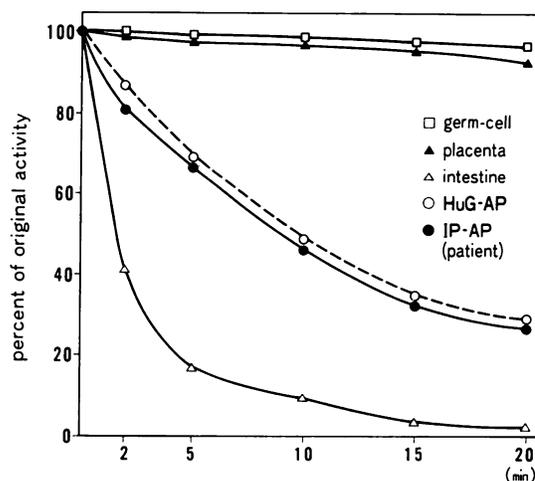


Fig. 3. Thermostability of AP. Thermostability was examined by incubating the enzyme at 65°C in 20 mM Tris-HCl, pH 7.4, containing 2 mM MgCl₂ and 2 mM ZnCl₂ for the time indicated.

Effect of P_i, Sodium Dodecyl Sulfate, and EDTA on Enzyme Activity. The effect of EDTA on the activity of HuG-AP was similar to that on intestinal AP, whereas HuG-AP showed properties intermediate between those of placental and intestinal APs against inorganic phosphate and SDS.

Dot Blot Analysis. Biochemically and immunologically, HuG-AP was identical to IP-AP. Therefore we used HuG-1 cells as a model system to investigate the expression of novel AP mRNA in the gastric cancer cells of the patient. The specificity of probes for intestinal AP mRNA (D-I) and placental AP mRNA (D-P) was described previously (8). Fig. 4 shows that HuG-1 cells derived from one cloned cell expressed both intestinal and placental AP mRNA concurrently.

Table 3 Enzymic properties of alkaline phosphatases^a

Inhibitors ^a	% of inhibition ^b				
	Intestinal AP	Placental AP	Germ-cell AP	IP-AP	HuG-AP
L-Phenylalanine					
5 mM	59.0	73.8	86.9	87.8	85.7
10 mM	68.6	85.1	91.1	92.4	92.3
L-Homoarginine ^a					
5 mM	30.1	5.2	28.8	22.5	19.2
10 mM	38.7	14.0	38.8	31.9	34.8
L-Leucine					
5 mM	53.9	35.8	85.2	80.2	76.7
10 mM	67.1	57.9	90.9	89.8	85.8
K ₂ HPO ₄					
5 mM	7.5	62.6	48.8	25.1	24.9
10 mM	17.3	76.4	64.9	39.2	37.0
Sodium dodecyl sulfate					
0.5%	0	97.7	97.9	61.6	65.4
5%	1.2	99.1	99.4	84.4	89.1
EDTA					
0.1 mM	56.2	14.7	49.5	56.2	51.5

^a Each inhibitor was incorporated in the standard assay mixture so as to give the final concentration indicated. The effect of EDTA or sodium dodecyl sulfate on the enzyme activity was studied as described previously (13).

^b The values represent the average of two determinations.

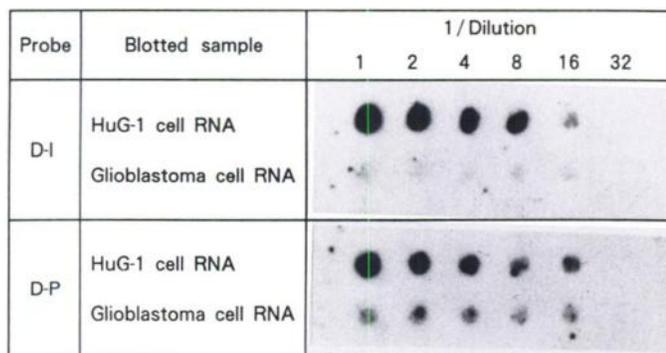


Fig. 4. Expression of intestinal AP mRNA and placental AP mRNA. Initial dose of dotted polyadenylated RNA was 10 μ g. D-I, probe specific for intestinal AP mRNA, is a 439-base pair *MspI-MspI* fragment of 3'-untranslated region of intestinal AP complementary DNA. D-P, probe specific for placental AP mRNA, is a 652-base pair *MspI-SacI* fragment of 3'-untranslated region of placental AP complementary DNA. Both probes were prepared as described previously (8). Glioblastoma cells in which AP was not detected were used as negative control.

DISCUSSION

In the present study, we newly established a gastric cancer cell line (HuG-1) from our patient's ascites. Some human cancer cell lines have been reported to produce CEA and CA 19-9 (18-20). In our patient, his serum levels of CEA, CA 19-9, and TPA were high and the production of these tumor markers has continued in both HuG-1 and transplanted tumors of HuG-1 cells in nude mice.

Recently, we (10) first proved that IP-AP was expressed in the cancer tissue in humans. The production of this hybrid form of AP has also continued in our established cell line (HuG-1). Furthermore, AP extracted from transplanted tumors of HuG-1 cells in nude mice showed the same properties as did IP-AP and HuG-AP electrophoretically, immunologically, and enzymically (not shown). Although this hybrid form of AP has been suggested to exist in a few cancer cell lines *in vitro* (21), this type of AP has not been confirmed hitherto in any human cancer tissues or in sera of patients with malignant diseases. Our finding of a hybrid form of AP in human native materials suggests that expression of a hybrid form of AP in some cancer

cell lines did not result from spontaneous transformation of cultured cell lines during long term culture *in vitro* but it had already occurred in original cancer tissue.

Our newly discovered hybrid form of AP bound to both anti-placental AP and anti-intestinal AP monoclonal antibodies and had more heat stability than adult intestinal AP but less heat stability than term placental AP. However, the sensitivity of our hybrid form of AP to various inhibitors did not show the intermediate value between intestinal and placental APs, suggesting that these properties may be based on the characteristics of the heterodimeric structure of our enzyme and may not be due to the simple mixture of the two enzymes, intestinal and placental AP.

Although the HuG-1 cell line was not established from the primary gastric cancer tissue, we concluded that it originated from the gastric cancer because the HuG-1 tumor that developed in nude mice after inoculation of HuG-1 cells histologically resembled the primary gastric cancer tissue of the patient and because HuG-1 cells produced CEA, CA19-9, and TPA which could be stained in our patient's gastric cancer tissue histochemically (not shown) and were observed as abnormally high level in our patient's serum. HuG-1 cells also produced the abnormal AP, which has the same enzymic properties as the one found and named IP-AP in the cancer tissue of the same patient. HuG-1 cells were cloned twice consecutively because it remained possible that there were two cloned cells which expressed intestinal and placental APs, respectively. As a result of cloning of the cells, we concluded that a HuG-1 cell, a single cloned cell, expressed CEA, CA19-9, TPA, and an AP identical to IP-AP, a newly discovered AP, concomitantly.

HuG-1 cells expressed both intestinal AP mRNA and placental AP mRNA. Dot blot analysis rendered to confirm the result of immunological analysis using monoclonal antibodies. On the basis of immunological, enzymic, and dot blot analyses, we concluded that our newly discovered AP was a hybrid form of AP consisting of one subunit each of intestinal and placental APs. However, another possibility remains to be excluded, that this hybrid form of AP consists of intestinal and germ cell AP subunits, because anti-placental AP monoclonal antibody which we used in the present study also reacted with germ cell AP. Because the nucleotide sequence of our probe to placental AP mRNA is highly specific for placental AP, thus not reacting to germ cell AP mRNA, one subunit of our hybrid form of AP seems to come from placental AP, but not from germ cell AP.

As for APs as tumor marker, Regan isoenzyme (term placental AP), Nagao isoenzyme (germ cell AP), and Kasahara isoenzyme (modified intestinal AP) have been described. Our hybrid form of AP (intestinal/placental) may also exist in some cancer tissues and it is possible that we have taken the hybrid form of AP for Regan isoenzyme or Nagao isoenzyme because of its heat stability, L-leucine sensitivity, and reactivity to anti-placental AP antibody. Our present study indicates that we must reinvestigate carefully if a hybrid form of AP has been expressed in human cancerous tissues or in sera of patients with malignant disease.

The HuG-1 cell line could be useful for studies on expression of AP, conventional tumor markers of cancer cells, and the autocrine growth in serum-free medium.

REFERENCES

- Weiss, M. J., Ray, K., Henthorn, P. S., Lamb, B., Kadesch, T., and Harris, H. Structure of the human liver/bone/kidney alkaline phosphatase gene. *J. Biol. Chem.*, 263: 12002-12010, 1988.
- Henthorn, P. S., Ruducha, M., Kadesch, T., Weiss, M. J., and Harris, H.

- Sequence and characterization of the human intestinal alkaline phosphatase gene. *J. Biol. Chem.*, 263: 12011–12019, 1988.
3. Knoll, B. J., Rothblum, K. N., and Longley, M. Nucleotide sequence of the human placental alkaline phosphatase gene. *J. Biol. Chem.*, 263: 12020–12027, 1988.
 4. Millán, J. L., and Manes, T. Seminoma-derived Nagao isozyme is encoded by a germ-cell alkaline phosphatase gene. *Proc. Natl. Acad. Sci. USA*, 85: 3024–3028, 1988.
 5. Fishman, W. H., Inglis, N. R., Green, S., Anstiss, C. L., Ghosh, N. K., Reif, A. E., Rustigan, R., Krant, M. J., and Stolbach, L. L. Immunology and biochemistry of Regan isoenzyme of alkaline phosphatase in human cancer. *Nature (Lond.)*, 219: 697–699, 1968.
 6. Nakayama, T., Yoshida, M., and Kitamura, M. L-Leucine sensitive, heat-stable alkaline phosphatase isoenzyme detected in a patient with pleuritis carcinomatosa. *Clin. Chim. Acta*, 30: 546–548, 1970.
 7. Higashino, K., Hashinotsume, M., Kang, K.-Y., and Yamamura, Y. Studies on a variant alkaline phosphatase in sera of patients with hepatocellular carcinoma. *Clin. Chim. Acta*, 40: 67–81, 1972.
 8. Higashino, K., Muratani, K., Hada, T., Imanishi, H., Amuro, Y., Yamamoto, Y., Furuyama, J., Hirano, K., Hong, Y.-M., Shimokura, M., Hirano, T., and Kishimoto, T. Purification and some properties of the fast migrating alkaline phosphatase in FL-amnion cells (the Kasahara isoenzyme) and its cDNA cloning. *Clin. Chim. Acta*, 186: 151–164, 1989.
 9. Wray, L., and Harris, H. Demonstration using monoclonal antibodies of inter-locus heterodimeric isozymes of human alkaline phosphatase. *J. Immunol. Methods*, 55: 13–18, 1982.
 10. Imanishi, H., Hada, T., Muratani, K., Hirano, K., and Higashino, K. Expression of a novel alkaline phosphatase reacting with both monoclonal antibodies to intestinal and placental alkaline phosphatases. *Clin. Chim. Acta*, 186: 309–314, 1989.
 11. Seabright, M. A rapid banding technique for human chromosomes. *Lancet*, 2: 971–972, 1971.
 12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193: 265–275, 1951.
 13. Higashino, K., Otani, R., Kudo, S., and Yamamura, Y. A fetal intestinal-type alkaline phosphatase in hepatocellular carcinoma tissue. *Clin. Chem.*, 23: 1615–1623, 1977.
 14. Hirano, K., Iizumi, Y., Hayashi, Y., Tanaka, T., Sugiura, M., Hayashi, K., Lu, Z.-D., and Iino, S. A highly sensitive assay method for human alkaline phosphatase involving a monoclonal antibody bound to paper disk. *Anal. Biochem.*, 154: 624–631, 1986.
 15. Hirano, K., Matsumoto, H., Tanaka, T., Hayashi, Y., Iino, S., Domar, U., and Stigbrand, T. Specific assays for human alkaline phosphatase isozymes. *Clin. Chim. Acta*, 166: 265–273, 1987.
 16. Chirgwin, J. M., Przybyla, A. E., Macdonald, R. J., and Rutter, W. J. Isolation of biologically active ribonucleic acid from source enriched in ribonuclease. *Biochemistry*, 18: 5249–5299, 1979.
 17. Aviv, H., and Leder, P. Purification of biologically active globulin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA*, 69: 1408–1412, 1972.
 18. Klug, T. L., Salzman, S., Quinn, A., Melincoff, G. A., Sedmak, D. D., Tubbs, R. R., and Zurawski, V. R., Jr. Tumorigenicity in athymic mice of the human colon carcinoma cell line SW1116 expressing the tumor-associated antigenic determinant CA 19-9. *Cancer Res.*, 44: 5212–5218, 1984.
 19. Yamaguchi, N., Morioka, H., Ohkura, H., Hirohashi, S., and Kawai, K. Establishment and characterization of the human cholangiocarcinoma cell line HChol-Y1 in a serum-free, chemically defined medium. *J. Natl. Cancer Inst.*, 75: 29–35, 1985.
 20. Iwamura, T., Katsuki, T., and Ide, K. Establishment and characterization of a human pancreatic cancer cell line (SUIT-2) producing carcinoembryonic antigen and carbohydrate antigen 19-9. *Jpn. J. Cancer Res.*, 78: 54–62, 1987.
 21. Behrens, C. M., Enns, C. A., and Sussman, H. H. Characterization of human foetal intestinal alkaline phosphatase. Comparison with the isoenzymes from the adult intestine and human cell lines. *Biochem. J.*, 211: 553–558, 1983.