Immunological Characterization and Clinical Implication of Cobalamin Binding Protein in Human Gastric Cancer

Yoshio Wakatsuki, Masami Inada, Hiroyuki Kudo, Gakuji Oshio, Tohru Masuda, Takeo Miyake, and Tohru Kita

ABSTRACT

Cobalamin (vitamin B12) binding protein was purified from gastric cancer extracts and from serum-free culture medium of cancer cell line KATO-III. The molecular weight, determined by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was 70,000 and the pi was 2.8 to 3.2. From biochemical and immunological properties, this cobalamin binding protein was considered to be an isoprotein of cobalamin R binder. Monoclonal antibodies were produced against saliva R and cobalamin binding protein in culture medium to study their antigenic determinants. Monoclonal antibody 55-D reacted to an epitope of peptide in both binders, whereas WK-1 and H-12 reacted to determinants of a carbohydrate moiety, including sialic acid, in cancer cell-derived binder. In addition, we carried out an enzyme-linked immunoenzyme assay and examined plasma levels of immunoreactive R binder in patients with gastric cancer (n = 72), benign gastrointestinal disease (n = 30), and healthy individuals (n = 40). Even in patients without liver metastasis, the level of immunoreactive R binder detected by monoclonal antibody H-12 was elevated in some patients and decreased after excision of the tumor. R binder was also elevated in cancer tissue extract. Immunoreactive binder was histochemically detected in the cytosol of cancer cells and metaplastic cells of the gastric mucosa. The present findings suggest that cobalamin R binder is de novo synthesized in gastric cancer cells and that its plasma level increases in some patients. This binding protein may be a useful diagnostic and therapeutic parameter.

INTRODUCTION

Since an early report by Waxman and Gilbert (1, 2) that

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2 The abbreviations used are: Cbl, cobalamin; UBBC, unsaturated B12 binding capacity; CBBF, cobalamin binding protein; DMEM, Dulbecco's modified Eagle's medium; SSFM, supplemented serum-free medium; IEF, isoelectric focusing; RAR, rabbit anti-R-antibody; MAB, monoclonal antibody; EIA, enzyme immunoassay; RIA, radioimmunoassay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TC, transcobalamin; T-PBS, PBS-0.05% Tween 20; BSA-PBS, PBS-2% BSA.

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polycythemia vera. All samples were stored at −80°C and not thawed until use.

Assay of Vitamin B₁₂ and Cobalamin Binding Capacity. Cobalamin unsaturated binders were measured by a modified albumin-coated charcoal method (14, 20). Saturated binders present in gel-fractionated materials were calculated from the endogenous Cbl determined by radioassay (Immaphase; Cornings, Corning, NY). The results were expressed as the concentration of binder-equivalent cobalamin (pg/ml).

To estimate the level of T-cell contamination in the materials, the UBBC assay was carried out at various pHs in PBS, and endogenous cobalamin was measured after adsorption of T-cell II by silica (fumed; Sigma) (21). Recovery in both assays was evaluated.

Gel Filtration Analysis and Purification of CblBP. Conditioned media of various cell lines and stomach extracts were saturated with appropriate amounts of [³⁰Co]cyanocobalamin (CT.2; Amersham) and applied to a Sephadex G-200 column (13, 22). Immunological cross-reactivity was examined by incubation of the radiolabeled materials with antiserum before column chromatography, antigen-antibody reaction being detected by a shift of the appropriate binder peak toward the void fraction, thus indicating formation of a large complex (14). For further purification of binding protein, Cbl-Sepharose affinity chromatography was carried out (23).

IEF. Polycaryamide rod gel electrophoresis, with a pH gradient of 2.0 to 5.0, was accomplished by modification of the method described by Laas et al. using Pharmalyte (Pharmacia, Uppsala, Sweden) (24). Before application to the gel, samples were labeled with [³⁰Co]Cbl₂ and fractionated on a Sephadex G-200 column. After optimal focusing was obtained, the gel was sectioned into 5-mm slices and assayed for radioactive B₁₂. The pH gradient across each gel slice was determined by eluting with distilled water (1 ml) for 24 h and measuring the pH values of the eluants directly.

Preparation of Polyclonal and Monoclonal Antibodies. Serum of patients with pernicious anemia was used as anti-intrinsic factor antibody. RAR was raised against affinity-purified saliva R binder (25). MAbs were prepared by immunizing BALB/c mice, separately, with affinity-purified saliva R and CblBP purified from conditioned SSFM of KATO-III (K-SSFM). Hybridization was carried out according to the modified method of Herzenberg et al. (26), using P3-NS1-1Ag4-1 as a fusion partner and polyethylene glycol (M, 1500; BDH Chemicals, Poole, England). Hybridomas were cloned more than twice by limiting dilution. The screening assay was performed by ELISA, as described below. These cloned hybridomas were then injected into pristane-primed BALB/c nude mice for ascites production, and the resulting antibodies were fractionated by anion exchange chromatography (Whatman; DE32).

ELISA. For screening hybridomas, ELISA was prepared as follows. Poly-styrene microassay plates (Falcon No. 3912) were coated with 50 µl per well of RAR (20 µg/ml in PBS) and left overnight at 4°C. After being washed 3 times with T-PBS, the plates were blocked by using BSA-PBS for 1 h at room temperature and washed again. Fifty µl of partially purified saliva R or K-SSFM (50 µg of protein/well) were incubated for 2 h and washed with T-PBS. Fifty µl of culture supernatant of each hybridoma were then incubated for 2 h at room temperature and washed again in T-PBS. Bound antibody was determined using 50 µl of affinity-purified goat F(ab')₂ anti-mouse IgG (TAGO 4550) at a 1:1500 dilution in BSA-PBS (27, 28). Color was developed with 0.04% o-phenylenediamine in 0.1 M citrate buffer (pH 5.0) containing 0.006% H₂O₂. Hybridomas from mice immunized with CblBP from K-SSFM were screened by dual antigens in each cloning procedure, first against saliva R, and then against saliva K, to exclude clones that produced antibodies with various antibodies and immunoprecipitated similarly. After several washings, the radioactivity in the final precipitates was counted with a well-type gamma counter.

Chemical and Enzymatic Treatment of Antigens. KATO-III in PBS was disrupted by sonication and then centrifuged at 100,000 × g for 30 min with the resulting supernatant being used for antigen preparation. Digestion by Pronase (EC 3.4.24.4; Pronase E from Streptomyces; Kaken Kagaku, Tokyo, Japan) was carried out in 0.05 M Veronal buffer (pH 7.7) at 37°C for 46 to 48 h at an enzyme-substrate ratio of 1:40 (31). For destruction of carbohydrate structure, antigen preparations were treated with neuraminidase (EC 3.2.1.18; Vibrio cholerae neuraminidase; Behringwerke AG, Marburg, West Germany) and then with periodate according to the method of Colligan and Todd (32). Neuraminidase (1 unit/ml) was added in a ratio of 1 ml per 100 µg of each antigen. Periodate oxidation was performed using sodium metaperiodate (Nakarai Chemicals, Kyoto, Japan), in a final concentration of 5.33 mmol, at room temperature and in the dark for 40 h. The reaction mixture was then intensively dialyzed against PBS. Reactivities of treated antigens to MAbs 55-D, WK-1, and H-12 were investigated using solid-phase RIA in which assay plates were precoated with 100 µl of each MAb (30 µg/ml) and blocked by BSA-PBS. After incubation with treated cell lysate of KATO-III, bound antigen was detected by radiiodinated RAR (approximately 3 × 10⁵ cpm). Using the same precoated assay plates, competitive inhibition was also studied. Briefly, to a constant amount of radioiodinated cell lysate of KATO-III, we added increasing amounts of cold inhibitors, which were treated lysates of the same cell, untreated lysates of granulocytes (5.0 mg of protein/ml), and purified plasma TC I and TC III. After aspiration of the radiolabeled materials, the plates were washed extensively, and the radioactivity of each separated well was determined by a gamma counter.

Assay for Patient Plasma. For the CblBP assay in the patient plasma, a sandwich-type ELISA was developed. Briefly, assay plates were coated with 50 µl of MAb 55-D or H-12 (20 µg/ml) and blocked with BSA-PBS. After washing with T-PBS, 50 µl of plasma, diluted 1:2 with PBS, were incubated. Fifty µl of gastric cancer extracts in a dilution series from ×10 to ×10² were used as an assay standard. Bound antigen was detected by adding 50 µl of RAR (Fab') which was labeled with horseradish peroxidase by the maleimide method (33). Immunoreactive binder contained in the extract at 5 × 10⁵ dilution was tentatively assumed to have a concentration of 1 unit/ml; this value was approximately equivalent to 0.13 pg of cobalamin/ml. Determination of endogenous Cbl was accomplished by modification of the method of Graham and Karnofsky (34). Immunoreactive binder in the extract at 5 × 10⁵ dilution was tentatively assumed to have a concentration of 1 unit/ml; this value was approximately equivalent to 0.13 pg of cobalamin/ml. Separation of the total binding capacity was carried out by column chromatography and the binder assay described earlier. Unknown samples were quantified from the calibration curves. For statistical analysis, Student's t test was used.

Immunostaining. Sections of formalin-fixed and paraffin-mounted cancer tissue, normal stomach tissue, and KATO-III smears were stained with MAb 55-D and H-12 as follows. Sections with paraffin removed and cell smears were treated with 0.3% hydrogen peroxide in absolute methanol for 20 min in order to block endogenous peroxidase activity. After blocking the nonspecific binding of immunoglobulin by incubation in 30% normal horse serum for 1 h, the sections and cell smears were further incubated with MAb 55-D and H-12 at a 1:2000 dilution overnight at 4°C. Samples were washed with PBS and incubated for 2 h at 4°C, with biotinylated anti-mouse immunoglobulins (Vector, Burlingame, CA) at a 1:300 dilution in PBS-PBS containing 2% normal human serum. Following washing with PBS, the sections and smears were incubated with horseradish-avidin D (Vector) for 1 h at 4°C. The histochemical determination of peroxidase was carried out according to the modified method of Graham and Karnofsky (34). Nonimmunized mouse serum was used as the negative control.

RESULTS

Gel Filtration Analysis and Binding Study. In extracts from either cancerous or control tissues, UBBC was less than 10% of the total binding capacity. A large peak representing a M₁₂,500 binder was observed in both cancer extract and K-SSFM (Fig. 1, D and G). On the other hand, binders caused a

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Fig. 1. Elution profiles of [15Co]B12-labeled with and without antisera. Labeled samples (1 ml were applied to a column (1.4 x 28 cm) of Sephadex G-200, allowed to equilibrate, and then eluted with 0.05 m potassium phosphate (pH 7.4) containing 0.5 m NaCl. Fractions of 2.5 ml were collected, and UBBC and endogenous cobalamin were assayed. Apparent molecular weights were estimated from a calibration kit. A, control extract; B, control extract with anti-R antibody; C, control extract with anti-IF antibody; D, cancer extract; E, cancer extract with anti-R antibody; F, cancer extract with anti-IF antibody; G, conditioned SSFM of KATOH-III (K-SSFM); H, K-SSFM with anti-R antibody; l, K-SSFM with anti-IF antibody. O, UBBC; , endogenous cobalamin including [57Co]B12 added; A, UBBC when cyano-400

$M$, 80,000 to 90,000 broad-based peak with a small $M$, 125,000 shoulder in the control extract (Fig. 1A). Free Cbl was eluted at the end of the column. UBBC was markedly elevated in K-SSFM (Fig. 1G), but was almost at background level (10 to 20 pg/ml) in the case of Hela cells and Intestine 407 (data not shown). UBBC in K-SSFM became undetectable when cycloheximide (5 pg/ml) was added in the culture medium.

Fig. 2. Isoelectric focusing of [15Co]B12-labeled, gel-fractionated samples. Electrophoresis was started with an initial voltage of 200 V for 1 h, then 300 V for 5 h, and finally 400 V for 1 h. A, cancer extract; B, K-SSFM; C, saliva R; D, extract of control mucosa with intestinal metaplasia. , pH of the eluant of sliced gel; O, radioactivity of sliced gel.

IEF. Isoelectric points (pI) of the binder in cancer extract were distributed between 2.8 and 3.8. The pIs determined were: 3.2 in K-SSFM; 3.6 in the control extracts (from noncancerous mucosa with metaplasia); 5.0 in the extract from a young patient without metaplasia (data not shown); and, for reference, 3.8 to 4.3 in saliva R (Fig. 2).

Immunoprecipitation. Despite different origins, an essentially identical $M$, 70,000 group was immunoprecipitated in all samples, except for the extract obtained from one young patient (Fig. 3).

Reactivity with Monoclonal Antibodies. Of 50 clones from the 700 positive wells resulting from 5 consecutive hybridizations in mice immunized with K-SSFM, 12 clones were retained after the cloning procedure. Among these clones, WK-1 (IgG3) and H-12 (IgG3) were used for further study. Clones 55-D (IgGl) and 42-C (IgG3) were derived from mice immunized with saliva R. All these MAb's immunoprecipitated $M$, 70,000 antigen in cell lysate of KATOH-III (Fig. 4A); however, MAbs WK-1 and H-12 did not react with saliva R (Fig. 4B). The findings were further confirmed by an additional radioimmunoprecipitation assay using [15Co]B12 (Table 1). Binding of MAbs to this $M$, 70,000 antigen was reduced by Pronase treatment in 55-D and by periodate and neuraminidase treatment in 55-D and H-12 did not react with saliva R (Fig. 4B). The findings were further confirmed by an additional radioimmunoprecipitation assay using [15Co]B12 (Table 1). Binding of MAbs to this $M$, 70,000 antigen was reduced by Pronase treatment in 55-D and by periodate and neuraminidase treatment in 55-D and H-12 did not react with saliva R (Fig. 4B). The findings were further confirmed by an additional radioimmunoprecipitation assay using [15Co]B12 (Table 1). Binding of MAbs to this $M$, 70,000 antigen was reduced by Pronase treatment in 55-D and by periodate and neuraminidase treatment in 55-D and H-12 did not react with saliva R (Fig. 4B). 

In summary, the epitope of 55-D appeared to be a peptide, while those of H-12 and WK-1 appeared to be carbohydrate moieties (containing terminal sialic acid) of cancer-derived binders.
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1 2 3 4 5 6
cytosol by 55-D, but not by H-12 (data not shown).

Plasma Assay. Fig. 8A shows the calibration curve of the EIA, of which sensitivity was estimated to be $10^{-15}$ M, about 100 times higher than that of gel filtration combined with endogenous cobalamin assay. Since the inhibition study indicated cross-reactivity of 55-D to TC I and TC III (Fig. 8B), we used H-12 in the plasma assay. Cancer patients, even those without liver metastasis, showed significant levels of immuno-reactive binder ($5.5 \pm 0.96$ units/ml; mean $\pm$ SE) compared to healthy controls ($2.5 \pm 0.07$ units/ml) (Fig. 9). The differences of WBC count between these two groups were not significant (data not shown). In 4 cases without metastasis, originally elevated levels of R binder were decreased subsequent to tumor excision, whereas, in 2 patients who underwent gastrojejunal anastomosis necessitated by liver and peritoneal metastasis, binder levels remained high (Fig. 10). Levels of R binder were not affected by surgery in 3 cases of nonmalignant disease.

**DISCUSSION**

Since granulocytes at some differentiation stage produce CblBP of the R binder class (TC I and TC III) (35, 36), increased concentrations of transcobalamin have been noted in some myeloproliferative disorders (36-41). In addition to the total-body granulocyte pool and granulocyte turnover (42), several

<table>
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<th>Antibodies</th>
<th>Saliva R</th>
<th>K-SSFM</th>
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<tr>
<td>NRS$^a$</td>
<td>529 $\pm$ 63$^b$, 450 $\pm$ 55</td>
<td></td>
</tr>
<tr>
<td>RAR</td>
<td>10,209 $\pm$ 553, 9,503 $\pm$ 680</td>
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</tr>
<tr>
<td>55-D</td>
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<td></td>
</tr>
<tr>
<td>WK-1</td>
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<td></td>
</tr>
<tr>
<td>H-12</td>
<td>613 $\pm$ 84, 8,514 $\pm$ 276</td>
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</table>

$^a$ NRS, normal rabbit serum; NMS, normal mouse serum.

$^b$ Mean $\pm$ SD (cpm) of triplicate assays.

**Immunostaining.** In stomach tissue, positive staining was confined to the cytosol of cancer cells, goblet, and columnar epithelial cells in the intestinal metaplasia, but no significant staining was observed in normal stomach mucosa (Fig. 7). In cell smears from KATOH-III, positive staining of granular components in the cytosol and parts of the membrane was noted, while smears from granulocytes showed staining of the cytosol by 55-D, but not by H-12 (data not shown).

**Fig. 3.** Immunoprecipitation using polyclonal anti-R antibody. Molecular weight values were determined by a calibration kit (Pharmacia). Lane 1, saliva; Lane 2, K-SSFM; Lane 3, extract of gastric cancer; Lane 4, extract of control mucosa with intestinal metaplasia; Lane 5, plasma; Lane 6, extract of control mucosa without metaplasia.

**Fig. 4.** Left, immunoprecipitation of KATOH-III cell lysate by different MAbs. Lane 1, 55-D; Lane 2, WK-1; Lane 3, H-12; Lane 4, 42-C. Right, immunoprecipitation of saliva (Lanes 1 and 2) and cell lysate of KATOH-III (Lanes 3 and 4) with MAbs WK-1 (Lanes 3 and 4) and H-12 (Lanes 2 and 4).

**Table 1** Immunoprecipitation using $^{125}$I-labeled materials

<table>
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<th>Antibodies</th>
<th>Final precipitates $\times 10^4$ (cpm)</th>
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<tbody>
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$^b$ Mean $\pm$ SD (cpm) of triplicate assays.
reports have indicated other factors which affect the concentrations of plasma R binder (6, 43–45), particularly in the neutropenic state (43) or in some malignancies without bone marrow abnormalities (3–8). Except in adolescent-type hepatoma (4), direct evidence for the production of CblBP by cancer cells has been insufficient to date. Using a serum-free, in vitro system, we observed an increase of CblBP in the culture supernatant of a gastric cancer cell line. This increase was inhibited by the presence of cycloheximide. In extracts from cancerous tissue, the level of a M, 125,000 binding protein was elevated. Histochemical studies showed this binding protein was localized in cancer cells. The level of immunoreactive CblBP in plasma was significantly elevated in cancer patients, even if neither liver
nor bone marrow metastasis was present. A subsequent decrease in concentration after tumor excision was also observed. Together, all these lines of evidence lead us to conclude that the binder was produced by the de novo synthesis of gastric cancer cells and was not the result of the release of preexisting tissue stores of CblBP secondary to tumor invasion.

Although some heterogeneity in charge was observed, the biochemical properties and immunological cross-reactivity of these CblBPs were very similar to those of R binders. The discrepancy in molecular weight between SDS-PAGE and gel filtration has also been documented (25, 46). Conventionally, characterization of R binders from various sources has been performed by anion exchange chromatography (14) and isoelectric focusing (47); however, these procedures are impractical for routine examination. Since microheterogeneity of R binders from various sources has been ascribed to differences in carbohydrate moieties (25, 48, 49), we speculated that such minute differences in carbohydrate residue might lead to the definition of a new group of epitopes. Using pooled saliva R as the standard antigen for normal "glandular"-type R binder (47) and eliminating clones reactive to shared epitopes, we were able to obtain hybridomas producing antibodies reactive to determinants of binders from cancer cells. Our findings suggested a higher sialic acid content in CblBP of K-SSF than in saliva R.

Our preliminary study showed that no significant difference was observed in plasma B₁₂ and UBBC levels between the patients and control groups by radioassay, apparently because of the varying amounts of free cobalamin and TC II present. Neither the UBBC nor B₁₂ level was, therefore, informative for determining total (holo- and apo-) R binder levels. Immunoreactivity using 55-D, which measures total immunoreactive R binder, disclosed markedly increased levels of this binding protein in two cases of polycythemia vera (395 units/ml and 330 units/ml) and a significant increase in cancer patients with liver metastasis. However, the difference of mean values between gastric cancer patients without liver metastasis and patients with benign disease was not significant. Using the H-12 assay, which measures a carbohydrate epitope that is probably more associated with cancer cells, this difference has become significant, and the values obtained in polycythemia vera were both less than 20 units/ml. Since we observed a considerable increase of immunoreactive R binder in one patient with cholecytitis and obstructive jaundice, we think that delayed hepatic clearance of R binder (50, 51) might have contributed to the increase in this case.

It is of considerable interest that positive staining was confined to cancer cells and cells involved in intestinal metaplasia. Using polyclonal antibodies, we recently demonstrated that immunoreactive R binder is not present in normal stomach mucosa but is found in goblet cells of the colon and surface epithelial cells of the small intestine (52). In the present study, R binder with a molecular weight of 70,000 was immunoprecipitated in the extracts from carcinoma and mucosa with intestinal metaplasia, but not in the extract without metaplasia. Therefore, binder production by the gastric mucosa suggests some histogenetic background common to intestinal metaplasia and some types of gastric cancer (53).

Although a high level of R binder in plasma is not specific to gastric cancer, its decrease after therapeutic intervention may be useful in clinically monitoring the course of the disease.

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REFERENCES

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