Identification of Cobalamin Binding Protein Synthesized *de novo* by Gastric Cancer Cells

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Summary The content of cobalamin-binding protein (Cb1BP) was measured in stomach extracts and various cell lines. Cb1BP was increased in the cancer extract compared with non-cancerous control. The level of Cb1BP was also increased in serum-free culture medium of a gastric cancer cell line, but was almost undetectable in media of control cell lines. This output of Cb1BP in medium was suppressed by cycloheximide. These Cb1BPs were immunologically cross-reactive with anti saliva R binder antibody. The M_r , determined by immunoprecipitation, was 70 kDa and the pI, 2.8–3.2. From these properties, this Cb1BP in cancer cells was considered to be an isoprotein of the cobalamin R binder. Histochemical studies showed that immunoreactive Cb1BP was confined to cytosol of cancer cells and cells in metaplasia. The present results suggest that Cb1BP of the R binder type is synthesized *de novo* in some type of gastric cancer cells and in metaplastic cells.

Key Words: cobalamin binding protein, serum-free culture, immunoprecipitation, immunohistochemistry, R binder

Human cobalamin (B_{12})-binding protein can be divided into three classes [1]: (1) gastric intrinsic factor, which facilitates B_{12} absorption from the small intestine; (2) plasma transcobalamin II, which facilitates cellular uptake of B_{12} by tissue; and (3) R binders, which are found in several body fluids and tissues but whose role in cellular metabolism of cobalamin remains largely a matter of conjecture. Plasma transcobalamin I and -III are considered immunologically equivalent to R binder. The term "R protein" was first used by Graesbeck *et al.* [2] to identify in human

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gastric juice a B₁₂-binding protein without intrinsic factor (IF) activity. A recent report has indicated the absence of R binder in the gastric juice obtained with gastrostomy tubes from patients with complete obstructin to swallowing [3]. Our preliminary studies showed, however, the presence of non-IF-binding protein in the gastric juice and stomach extract obtained from cancer patients [4, 5]. In these regards, a study was undertaken to elucidate derivation and physicochemical characters of CblBP in the stomach of cancer patients.

MATERIALS AND METHODS

Extraction of binding protein. Stomach tissues were obtained from a 56-year-old female with gastric cancer and from a 22-year-old male with a perforated duodenal ulcer. In the former, separate homogenates were prepared from the carcinoma itself and the scrape of mucosa adjacent to the tumor that was not histologically involved in the cancer, though it contained a small metaplastic area. Each specimen was minced by Biomixer (Nissei, Tokyo) for 5 s and homogenized in extraction buffer (6 ml/g specimen) with a Teflon pestle for 15 s at 1,500 rpm on the ice. Extraction buffer consisted of 50 mM Tris-HCl (pH 7.5) with 0.25 mM sucrose, 5 mM EDTA, 5 mM EGTA, 2 mM phenylmethylsulphonyl fluoride, 2 mM pepstatin, and 0.5% Triton X-100. Homogenates were gently stirred for 6 h at 4°C and centrifuged at 20,000×g for 1 h. Supernatants were stored at -80°C. All specimens used were obtained with the patient's prior consent.

Serum-deprived culture. We used gastric cancer cell line KATOIII, which was generously provided by Dr. M. Sekiguchi, Institute of Medical Science, the University of Tokyo [6], HeLa cells (CCL-2; ATCC), and Intestine 407 (CCL-6) as controls. The cells were maintained in suspension in medium consisting of 45% DMEM, 45% RPMI-1640, 10% heat-inactivated FCS, 200 mM/liter glutamine, streptomycin (100 µg/ml) and penicillin G (100 units/ml), and were split at a 1:10 ratio at weekly intervals. After subconfluent growth, cells were harvested, washed three times in Hanks' balanced salt solution, then cultured again at a density of 1×10⁵/ml in supplemented serum-free medium (SSFM) consisting of DMEM containing 5 µg/ml insulin (Sigma, MO), 5 µg/ml transferin (Sigma), 1% non-essential amino acids (Flow Laboratory, VA), and antibiotics [7, 8]. Spent medium was taken after 1 week, and concentrated about a hundred fold using an Amicon membrane (YM-10). There were no striking changes in cell viability after 1 week in SSFM.

Assay of vitamin B₁₂- and cobalamin binding capacity. Unsaturated B₁₂-binding capacity (UBBC) was measured by a modified albumin-coated charcoal method [9]. Saturated binding capacity was calculated from endogenous Cbl determined by radioassay (Immophase, Corning, MA). The results of both assays were expressed as concentration of binder-equivalent cobalamin (pg/ml). In some experiment, we carried out the UBBC assay at various pH's in PBS, and measured endogenous Cbl in peak column-fraction of cancer extract before and after

adsorption of TC-II onto Silica Quso [10], thereby this cobalamin binding protein was discriminated from TC-II.

Gel filtration and purification of CblBP. One milliliter of samples (2.0 mg protein/ml) was incubated with 20 μ l of [^{57}Co]cyanocobalamin (210.0 $\mu\text{Ci}/\mu\text{g}$, 36.2 pmol/ml, Amersham, UK) for 30 min at room temperature, applied to a Sephadex G-200 (SG-200) column, and eluted as described elsewhere [9, 11]. Immunological crossreactivity was examined by the same column chromatography; radiolabeled materials mentioned above were incubated again with appropriate amount of antisera for 30 min at room temperature before application to the column, thus antigen-antibody reaction was detected by a shift of the appropriate binder peak toward the void fraction [9]. For purification of binding protein, Cbl-Sepharose affinity chromatography was carried out according to the method described by Allen and Majerus [12].

Isoelectric focusing. Radioactive peaks, except free cobalamin, in SG-200 gel filtration were analyzed by polyacrylamide rod gel electrophoresis with a pH gradient of 2.5–5.0, which was made by using Pharmalyte (Pharmacia, Uppsala) [13]. After optimal focusing was obtained, the gel was sectioned into 5-mm slices and assayed for radioactive B_{12} . The pH gradient across each gel was determined by elution of each slice with distilled water (1 ml) for 24 h and direct measurement of the pH value of the eluants.

Preparation of antibodies. Anti-intrinsic factor antibody was prepared from serum of a patient with pernicious anemia. Rabbit anti-R antibody (RAR) was raised against affinity-purified saliva R binder. Antisera were fractionated by anion-exchange chromatography (Whatman; DE32).

Immunoprecipitation. Saliva and plasma were obtained from healthy volunteers. Saliva, conditioned SSFM of KATOIII (K-SSFM), gastric cancer extract, and extracts of non-cancerous mucosa both with and without metaplasia were radioiodinated by the lactoperoxidase method [14]. Radio-labeled materials were then immunoprecipitated with RAR and Protein A Sepharose overnight at 4°C according to a previously described procedure [15]. Final precipitates were analyzed by SDS-PAGE and autoradiography.

Immunostaining. Sections of formalin-fixed and paraffin-mounted cancer tissue, normal stomach tissue, and KATOIII smears were stained by the indirect peroxidase technique. Deparaffinized sections and cell smears were treated with 0.3% hydrogen peroxide in absolute methanol for 20 min for blocking of endogenous peroxidase activity. After blocking the non-specific binding of immunoglobulin by incubation in 10% normal goat serum for 1 h, we further incubated sections and smears with RAR at a dilution of 1 : 500 overnight at 4°C, followed by treatment with peroxidase-conjugated goat anti-rabbit IgG antiserum (Cappel, CA) at a dilution of 1 : 100, at room temperature for 30 min. The histochemical determination of peroxidase was carried out according to the modified method of Graham and Karnovsky [16].

RESULTS

Gel filtration

In either cancer or control extracts, UBBC was less than 10% of the total binding capacity. A large peak with estimated molecular weight of 120 kDa was observed in both cancer extract and K-SSFM (Fig. 1A and C). In the control extract, binders caused a broad-based peak at 80–90 kDa with a small shoulder at 120 kDa (Fig. 1B). Free Cbl was eluted at the end of the column run. In condi-

Fig. 1. Elution profiles of $[^{57}\text{Co}]\text{B}_{12}$ -labeled samples. Labeled samples of 1 ml were applied to a column (1.4×28 cm) of Sephadex G-200 equilibrated and eluted with 0.05 M potassium phosphate (pH 7.4) containing 0.5 M NaCl. Concentration of endogenous cobalamin ($\bullet$ — $\bullet$) and UBBC ($\circ$ — $\circ$) were determined for each fraction (2.5 ml); cobalamin due to $[^{57}\text{Co}]\text{B}_{12}$ -labeling was subtracted in endogenous cobalamin assay. A, Cancer extract; B, extract from non-cancerous mucosa adjacent to tumor; C, SSFM of KATOIII; D, SSFM of Intestine 407; E, saliva; F, SSFM of HeLa cell; in C, cycloheximide was added ($5 \mu\text{g}/\text{ml}$) ($\triangle$ — $\triangle$); V_o , void volume; Apparent molecular weight was estimated from calibration kit markers.

tioned SSFM, UBBC was markedly elevated in KATO-III (Fig. 1C), but was at almost background level in the case of Intestine 407 (Fig. 1D) and HeLa cell (Fig. 1F). Cycloheximide, at a concentration as small as 5 $\mu\text{g/ml}$ in culture medium, suppressed the UBBC in K-SSFM. In the cancer extract and K-SSFM, the main radioactive peak was eluted at the void volume only when samples had been incubated with RAR (Fig. 2A and E). In the control extract, however, the major peak binder reacted with anti-intrinsic factor antibody and the small peak binder reacted with RAR (Fig. 2C and D). Unsaturated binding capacity in the cancer extract and in K-SSFM were relatively stable in the pH range of 3.0–11.0; retention of binding capacity was 88% at pH 3.0 and 85% at pH 11.0 when 100% capacity was defined as the level at pH 7.4. The level of endogenous cobalamin in peak fraction of cancer extract before and after silica-adsorption were $1,980 \pm 108.5$ (pg/ml) and $1,834.7 \pm 125.4$ (pg/ml), respectively, in quadruplicate assays.

Fig. 2. Elution profiles of radiolabeled and antiserum-incubated samples. (●—●), Endogenous cobalamin; (○—○), UBBC; A and B, Cancer extract; C and D, control extract; E and F, K-SSFM; for experimental details, see "Materials and Methods" section.

Fig. 3. Isoelectric focusing of SG-200 gel-fractionated samples. Electrophoresis was started with an initial voltage of 200 V for 1 h and 300 V for 5 h and finally 400 V for 1 h. A, Cancer extract (fraction no. 16 in SG-200 filtration); B, K-SSFM (no. 16); C, saliva (no. 16); D, control extract (no. 20). —●—, pH; —○—, CPM.

Isoelectric focusing

Isoelectric points (pI) of the binder in the cancer extract were distributed between 2.8 and 3.8. Other pIs determined were as follows: 3.2 in K-SSFM, 3.6 and 5.0 in the control extract (with and without metaplasia, respectively), and 3.8–4.3 in saliva (Fig. 3).

Immunoprecipitation

RAR immunoprecipitated an essentially identical 70 kDa molecule in all samples studied except in the extract obtained from the young patient with duodenal ulcer (Fig. 4).

Immunostaining

In stomach tissue, positive staining was confined to the cytosol of cancer cells, and to goblet and columnar epithelial cells in the area showing intestinal metaplasia, but no significant staining was observed in normal stomach mucosa (Fig. 5). In cell smears from KATOIII, positive staining was also observed in the cytosol.

DISCUSSION

We observed an increased level of Cb1BP in the gastric cancer extract. Binding capacity of this Cb1BP was acid resistant and not adsorbed onto Silica Quso,

Fig. 4. Immunoprecipitation with anti-R antibody. 1, Saliva; 2, K-SSFM; 3, extract of gastric cancer; 4, extract of control mucosa with intestinal metaplasia; 5, plasma; 6, extract of control mucosa (without metaplasia) from patient with ulcer. Molecular weight values were determined by a calibration kit.

Fig. 5. Immunoperoxidase staining for R binder in the gastric mucosa. (a) Papillary adenocarcinoma, and (b) intestinal metaplasia. Counterstained with methyl green.

which discriminated this Cb1BP from TC-II.

To exclude plasma-derived factors, granulocytes and saliva, we have included studies on cell lines cultured in serum-free medium. An increase in holo- and apo-binding capacities was observed in the medium from a gastric cancer cell line but not in that from controls. These binding capacities became undetectable in the presence of cycloheximide in the culture medium.

Binding capacities in the cancer extract and K-SSFM were acid resistant and not adsorbed to silica, which was discriminative from that of TC-II.

Chromatographic and immunological properties of these Cb1BP in the cancer extract and K-SSFM were very similar to those of R binder, although the pIs of the former were more acidic than the pI of saliva R. The M_r determined by immunoprecipitation and SDS-PAGE were the same as that of saliva R.

In histochemical studies, immunoreactive R binder was detected in the cytosol of cancer cells and in cells showing intestinal metaplasia.

All these lines of evidences suggest that gastric cancer cells *de novo* synthesize a Cb1BP that has the properties of an isoprotein of R binder.

In the present study, a 70 kDa Cb1BP was immunoprecipitated in the extracts of carcinoma and mucosa with intestinal metaplasia, but not in the extract of mucosa without intestinal metaplasia. Our recent work demonstrated that immunoreactive R binder was not detected in normal stomach mucosa but was found in goblet cells of the colon and in surface epithelium of the small intestine [17]. Therefore, this binder-production by gastric mucosa may suggest a common histogenetic background for intestinal metaplasia and some type of gastric cancer [18].

R binders from various sources are known to have the same protein backbone and microheterogeneous carbohydrate moieties [19, 20], and cancer-associated aberrant glycosylation of glycolipids and glycoproteins have been recently reported [21, 22]. In these regards, studies are now in progress to elucidate the structural alterations of carbohydrate residues that are probably responsible for the lower pI values of the cancer derived R binder.

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