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High affinity binding of the transcobalamin II–cobalamin complex and mRNA expression of haptocorrin by human mammary epithelial cells

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Abstract

Little is known about the acquisition of cobalamin by the mammary gland and its secretion into milk. Human milk and plasma contain at least two types of cobalamin binding proteins: transcobalamin II (TC) and haptocorrin (HC). In plasma, TC is responsible for the transport of cobalamin to tissues and cells; however, cobalamin in milk is present exclusively bound to HC. We show that human mammary epithelial cells (HMEC) exhibit high affinity for TC; Scatchard analysis revealed a single class of binding sites for the TC–[^{57}Co]cyanocobalamin complex with a dissociation constant (K_d) of 4.9×10^{-11} M. Uptake of the TC–[^{57}Co]cyanocobalamin complex at 37°C was saturable by 24 h. Binding of free [^{57}Co]cyanocobalamin to HMEC was not saturable and very limited binding of the HC–[^{57}Co]cyanocobalamin complex was observed. Expression of the haptocorrin gene by HMEC was confirmed by Northern blot and PCR analysis. Thus, a specific cell surface receptor for the TC–cobalamin complex exists in the mammary gland and once cobalamin is internalized, it may be transferred to HC and subsequently secreted into milk as a HC–cobalamin complex.

Keywords: Transcobalamin; Transcobalamin II receptor; Haptocorrin; Vitamin B_{12}; Cobalamin; Mammary gland

1. Introduction

Two different binding proteins transport cobalamin (Cbl) in plasma and milk. In human milk, Cbl is bound exclusively to a 60–68 kDa glycoprotein called haptocorrin (HC) [1,2]. In plasma, however, a 43 kDa protein called transcobalamin II (TC) is the primary transport protein that is responsible for the delivery of Cbl to tissues [3]. Studies have demonstrated high affinity, saturable binding of TC–Cbl to plasma membranes from a variety of cell types, such as those of the kidney [4], placenta [5], liver [6], and fibroblasts [7], which is mediated by receptors that recognize the TC–Cbl complex. Because all cells require Cbl as a coenzyme for methylmalonyl-CoA mutase and methionine synthase, it has been hypothesized that receptors that recognize the TC–Cbl complex in plasma are present on the surface of all cells. Recent studies have detected TC–Cbl receptor expression in plasma membranes from various cells [5,8–10]. It is possible that similar receptors also exist on the surface of human mammary epithelial cells (HMEC). On the other hand, receptors that recognize HC–Cbl in circulation may exist on the surface of HMEC since HC–Cbl is the dominant Cbl-binding protein in human milk [2]. Little is known about the mechanisms by which the mammary gland acquires Cbl from the maternal circulation and how it is transported into human milk. This study explored the process by which the mammary gland acquires Cbl from the maternal circulation, either as free Cbl or as a HC–Cbl or TC–Cbl complex, using normal HMEC in culture as a model. We also determined the extent to which HC is expressed by the mammary gland in order to explain the predominant presence of HC–Cbl rather than TC–Cbl in human milk.

2. Material and methods

2.1. Culture of human mammary epithelial cells

Normal HMEC, serum free mammary epithelial growth medium (MEGM) supplemented with epidermal growth factor (10 μg/ml), hydrocortisone (0.5 mg/ml), bovine insulin (5.0 mg/ml), and bovine pituitary extract (13 mg/ml), and custom made MEGM without supplemented Cbl were

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purchased from Clonetics (San Diego, CA). HMEC (passage 9) seeded in 48-well plates were grown to confluence in MEGM. The human mammary epithelial cancer cell line MCF-7 (passage 7, American Type Culture Collection, Manassas, VA) was seeded in 100 cm^3 culture dishes and grown to confluence in minimum essential media with Earle's salts, L-glutamine and non-essential amino acids (Life Technologies-Invitrogen, Carlsbad, CA) supplemented with HEPES buffer (15 mM), fetal bovine serum (10%), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cell culture supplies were purchased from Fisher Scientific (Santa Clara, CA). Both cell lines were incubated in a 37°C, 5% CO_2/95% air humidified incubator. Confluence was determined by microscopic examination.

2.2. Batch purification of haptocorrin from human whey

Human milk HC was batch purified as follows. Separation of whey from whole human milk (day 9) was performed by adjusting the milk to pH 4.3 with 1 M HCl and to a final concentration of 60 mM $CaCl_2$ followed by ultracentrifugation for 1 h at 240 000 × *g* at 4°C. The fat and the casein pellet were removed and the whey was filtered through a 0.22 μm filter (Gelman Sciences, Ann Arbor, MI). The resultant whey fraction (3 ml) was then incubated overnight with 200 μl of radiolabeled Cbl ([^{57}Co]Cbl; approx. 300 μCi/μg cyanocobalamin; Amersham International, Buckinghamshire, UK) at 4°C. The radiolabeled whey samples were repeatedly injected into a Fast Protein Liquid Chromatography system (FPLC, Amersham Pharmacia, Uppsala, Sweden) using a Superose 12 gel filtration column (Amersham Pharmacia) in phosphate buffered saline (pH 7.4) at 0.3 ml/min. Protein and radioactive peaks were monitored continuously at 280 nm and by γ-counting (Beckman, Fullerton, CA), respectively. Radioactive protein fractions (1 ml) recovered from the Superose 12 column were pooled and concentrated on a Mono-Q HR 5/5 anion exchange column (Amersham Pharmacia) in 20 mM ethanolamine (pH 9.5) with a linear NaCl gradient (0.6 M = 100%) at a flow rate of 0.5 ml/min. Radioactive protein peaks were further concentrated using Centriplus-30 (Amicon, Beverly, MA) and purity of the HC–[^{57}Co]Cbl was assessed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). The HC concentration was determined by a charcoal adsorption assay as previously described [11]. The specific activity of HC–[^{57}Co]Cbl was 5.2 cpm/pg.

2.3. Batch purification of transcobalamin II from human serum

Human serum TC was batch purified as follows. Serum was obtained by centrifugation of whole human blood at 2000 × *g* at 4°C for 20 min. The serum was filtered through a 0.22 μm filter and incubated overnight with [^{57}Co]Cbl at 4°C. The radiolabeled serum samples (2 ml) were repeatedly injected into the FPLC using the Mono-Q HR 5/5 anion exchange column in 20 mM ethanolamine (pH 9.5) with a linear NaCl gradient (0.6 M NaCl = 100%) at a flow rate of 0.5 ml/min with monitoring of radioactive protein peaks as described above. The radioactive fractions (1 ml) containing the purified TC–[^{57}Co]Cbl complex were pooled and concentrated as described above, and purity was assessed by SDS–PAGE. The specific activity of TC was 12.6 cpm/pg.

2.4. SDS–PAGE

Molecular weights and purity of the separated proteins were assessed by SDS–PAGE. Samples were diluted 1/1 in sample buffer (1.25 M Tris–HCl (pH 6.8), 10% SDS, 10% sucrose, 2.5 mM β-mercaptoethanol, and 0.05% bromophenol blue) and heated in boiling water prior to application on a 10–12% gradient gel (Bio-Rad, Hercules, CA). Gels were stained with Coomassie brilliant blue (Sigma, St. Louis, MO) and destained with acetic acid:ethanol:water (10:25:65; v/v/v).

2.5. Binding studies

Determination of the binding of Cbl to HMEC was performed by exposing cells to increasing concentrations of free [^{57}Co]Cbl (0–1100 pM), HC–[^{57}Co]Cbl complex (0–80 pM), and TC–[^{57}Co]Cbl complex (0–110 pM) in duplicates and repeated three times. Binding studies were performed at 4°C to minimize internalization of the protein and incubated for 1, 2, 4, and 6 h for free [^{57}Co]Cbl or 1 and 3 h for HC–[^{57}Co]Cbl and TC–[^{57}Co]Cbl complexes. The medium used during the incubation periods in both the binding and internalization studies (described below) was MEGM without Cbl supplementation and bovine pituitary extract to control for exogenous Cbl. At the end of each incubation period, unbound ligand in the medium was removed and the cells were washed three times with ice-cold PBS (pH 7.4). The cells were then solubilized with 0.5% SDS and counted for cell-associated radioactivity. Analysis of the binding data was performed using nonlinear regression analysis (GraphPad Prism Software version 3.02, San Diego, CA). The value of K_d was determined by applying the Scatchard transform to the nonlinear regression curve [12].

2.6. Internalization studies

Internalization studies were performed using 20 pM TC–[^{57}Co]Cbl over a progressive time period (0–36 h) in triplicates and repeated three times. At the end of each incubation period, the medium was removed and the cells washed, then trypsinized using 0.1% trypsin/EDTA (Sigma) at 37°C for 10 min. Cells were gently pipetted out of the wells, centrifuged at 1000 × *g* for 5 min, and cell-asso-

ciated radioactivity was determined. Protein content of the cell pellet was determined by the modified Lowry method [13].

2.7. Northern blot analysis

Northern blot analysis was performed to examine the expression of HC in HMEC and MCF-7. HC mRNA expression was examined in MCF-7 to determine whether or not this cell line would be an appropriate model for further studies of Cbl metabolism in the mammary gland. Messenger RNA from confluent HMEC and MCF-7 was extracted using the Micro-Fast Track 2.0 kit as per the manufacturer's instructions (Invitrogen, Carlsbad, CA) and electrophoresed through a formaldehyde/agarose gel and transferred to a nylon membrane (Roche Molecular Biochemicals, Indianapolis, IN) according to standard procedures [14]. Hybridization was performed overnight at 60°C using a 1.5 kb HC cDNA probe [15] labeled with [α-^{32}P]dCTP (Amersham Pharmacia, Piscataway, NJ) by random priming (DNA Labeling Kit, Roche Molecular Biochemicals). The blot was then washed three times for 15 min at 60°C with medium stringency (3% SDS, 40 mM Na_2HPO_4 (pH 7.2), 1 mM EDTA, pH 8.0) and exposed to film at −80°C using an intensifying screen. RNA integrity was assessed by hybridizing the blot using a 2.0 kb β-actin cDNA probe.

2.8. Polymerase chain reaction (PCR)

PCR was performed on a human mammary gland cDNA gene pool (Invitrogen) using oligonucleotide primers based on the sequence of human asialoglycoprotein receptor 2 (ASGPR; GenBank accession No. NM_001181) and HC (GenBank accession No. J05086). Primers were designed using Primer3 software [16]. The ASGPR primers used were 5′-AGCTGGAGAAACAG-CAGCAGGA-3′ (sense) and 5′-TCCCCATACCCC-TGTCTCAGTG-3′ (antisense) and the HC primers used were 5′-AGGGGAACCAGCGCTGTCAA-3′ (sense) and 5′-GCCTGCATGGCTTCTCCTGT-3′ (antisense). PCR was performed in a reaction mixture containing 1 μl of cDNA, 0.25 μM of both sense and antisense primers, 40 mM Tris–KOH (pH 9.2), 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 3.75 μg/ml bovine serum albumin, 0.2 mM of each dNTP, and cDNAdvantage polymerase mix which includes KlenTaq-1 DNA polymerase and TaqStart Antibody (Clontech, Palo Alto, CA). In a Perkin Elmer 9700 automated thermal cycler (Foster City, CA), both the ASGPR and HC PCR reaction mixture were subjected to an initial denaturation at 94°C for 1 min, followed by 30 cycles of amplification and denaturation at 94°C for 30 s, primer annealing at 65°C for 1 min, and elongation at 72°C for 2 min and a final extension at 72°C for 7 min. The PCR product was visualized after electrophoresis with ethidium bromide using a 0.7% agarose gel.

3. Results

3.1. Binding of TC–[^{57}Co]Cbl, HC–[^{57}Co]Cbl, and free [^{57}Co]Cbl to HMEC

Binding studies were performed on HMEC using partially purified HC from human milk and TC from human serum (Fig. 1). Significant binding of TC–[^{57}Co]Cbl to HMEC was observed when cells were incubated with increasing concentrations of the ligand. Fig. 2A shows a linear dose response for the binding of TC–[^{57}Co]Cbl to HMEC (0.02–1.6 fmol/mg protein) at 1 h. The amount of TC–[^{57}Co]Cbl complex bound to HMEC at 3 h was similar to that at 1 h. In contrast, low binding of HC–[^{57}Co]Cbl complex to HMEC (approx. 0.1 fmol/mg protein) was obtained at all concentrations at 1 h. A similar binding capacity was also observed at 3 h.

The Scatchard transform to the nonlinear regression binding curve of the TC–[^{57}Co]Cbl complex revealed the presence of a single class of high affinity binding sites with a K_d of 4.9×10^{-11} M (Fig. 2A inset).

In the circulation, the majority of Cbl is protein-bound to either HC or TC; thus from a quantitative point of view, the amount of free Cbl is small. Even though the mammary gland would come into contact with very little free Cbl, we could not exclude the possibility that the free form may be taken up by the mammary gland. A linear increase in binding of free [^{57}Co]Cbl was observed (approx. 2–10 fmol/mg protein) with increasing [^{57}Co]Cbl

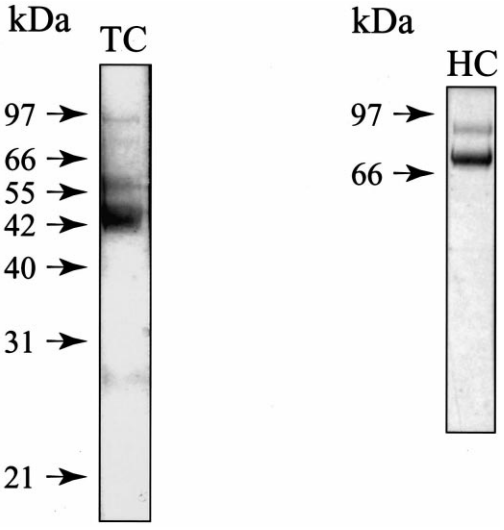

Fig. 1. Purification of HC and TC. HC and TC were batch purified from the whey portion of human milk and human serum, respectively. Both whey and serum were incubated with [^{57}Co]Cbl overnight at 4°C. Whey was repeatedly injected into the FPLC using a Superose 12 gel filtration column. Radioactive protein fractions were pooled then concentrated on a Mono-Q HR 5/5 anion exchange column. Serum was repeatedly injected into the FPLC using a Mono-Q HR 5/5 column. Radioactive protein peaks from multiple runs were concentrated and purity was assessed by SDS–PAGE under reducing conditions.

concentration, but saturation was not attained even at the highest concentration (Fig. 2B).

3.2. Internalization of TC–[^{57}Co]Cbl by HMEC

Since the TC–[^{57}Co]Cbl complex displayed saturable binding, we next examined the extent of Cbl internalization (Fig. 3). When cells were incubated with 20 pM of the TC–[^{57}Co]Cbl complex, we observed an increase in the binding and internalization of Cbl over time which approached a plateau by 24 h (0.6–1.6 fmol/mg protein). This result also strongly supports the possible existence of a receptor that recognizes the TC–[^{57}Co]Cbl complex.

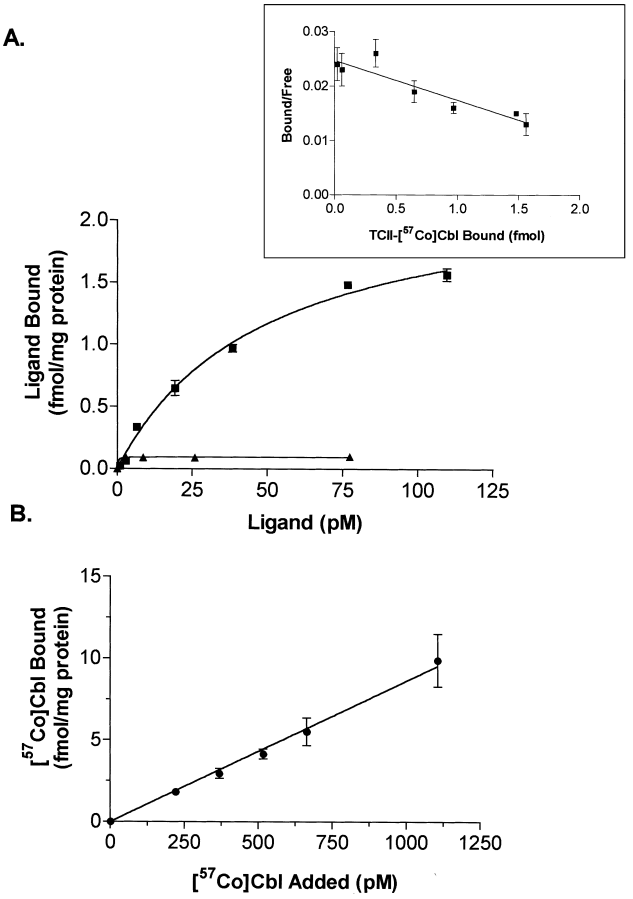

Fig. 2. (A) Binding of HC–[^{57}Co]Cbl (▲) and TC–[^{57}Co]Cbl (■) to HMEC. Confluent cells were exposed to HC–[^{57}Co]Cbl complex (0–80 pM) and TC–[^{57}Co]Cbl complex (0–110 pM) at 4°C for 1 h. The medium used was MEGM without Cbl supplementation and bovine pituitary extract to control for exogenous Cbl. At the end of the incubation period, unbound ligand in the medium was removed and the cells were washed with ice-cold PBS. The cells were then solubilized with 0.5% SDS and counted for cell-associated radioactivity. The experiment was repeated at 3 h with similar results. (Inset) Analysis of TC–[^{57}Co]Cbl complex binding to HMEC. Binding data were analyzed using nonlinear regression analysis. The value of K_d was determined by applying the Scatchard transform to the nonlinear regression curve. *x*-Axis: TC–[^{57}Co]Cbl complex bound (fmol) and *y*-axis: bound/free ($\times 10^6$). (B) Binding of [^{57}Co]Cbl to HMEC. Confluent cells were exposed to free [^{57}Co]Cbl (0–1100 pM) at 4°C for 1 h. The medium used was MEGM without Cbl supplementation and bovine pituitary extract to control for exogenous Cbl. At the end of the incubation period, unbound ligand in the medium was removed and the cells were washed with ice-cold PBS. The cells were then solubilized with 0.5% SDS and counted for cell-associated radioactivity. The experiment was repeated at 2, 4, and 6 h with similar results.

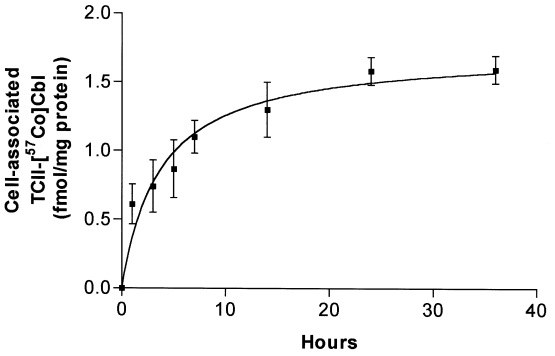

Fig. 3. Internalization of TC–[^{57}Co]Cbl by HMEC. Confluent cells were exposed to 20 pM TC–[^{57}Co]Cbl complex at 37°C over time (0–36 h). The internalization study was performed using MEGM without Cbl supplementation and bovine pituitary extract to control for exogenous Cbl. Medium was removed at subsequent time points and the cells were washed with ice-cold PBS. Cells were then trypsinized, using 0.1% trypsin/EDTA, for 15 min at 37°C, centrifuged, and cell-associated radioactivity was determined.

3.3. Expression of HC by HMEC

To explain the high concentration of HC present in breast milk as compared to TC, we examined whether or not HC was expressed by the epithelial cells of the mammary gland (Fig. 4). A 1.5 kb HC message was clearly detected from the HMEC, suggesting the HC gene is expressed by the mammary gland. On the other hand, no message was detected in the MCF-7 cell line. The presence of a single 2.0 kb β-actin band confirmed the integrity of the RNA in both cell lines. PCR performed using human mammary gland cDNA and HC primers resulted in a single product in the expected region of approx. 600 bp (figure not shown).

3.4. Identification of ASGPR transcript in the human mammary gland

Desialylated, galactose-terminal HC is cleared from the circulation by ASGPR present in hepatocytes. Thus, we determined whether or not ASGPR is also present in the mammary gland for HC–Cbl binding/uptake. PCR of the human mammary gland cDNA using ASGPR primers did not result in a product in the expected region of 650 bp (figure not shown).

4. Discussion

This study demonstrates that the surface of normal human mammary epithelial cells in culture has a high affinity for TC–Cbl. Scatchard plot analysis revealed the presence

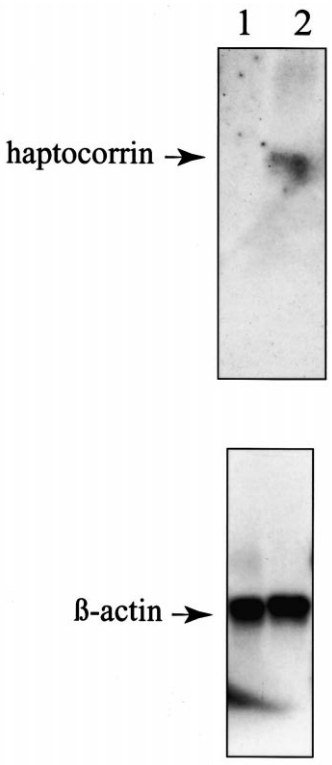

Fig. 4. Expression of HC by (1) MCF-7 and (2) HMEC. Messenger RNA was extracted from confluent cells and electrophoresed through a 1% formaldehyde/agarose gel and hybridized with [α-^{32}P]dCTP-labeled HC cDNA probe. Integrity of the mRNA was assessed by hybridizing with [α-^{32}P]dCTP-labeled β-actin cDNA probe.

of only one high affinity binding site for TC–Cbl. Previous studies have identified the presence of TC–Cbl receptors on human intestinal cells [8,9], leukemia cells [17], placental cell membranes [5,8,10], kidney, and liver [8]. We report that the TC–Cbl binding sites on the surface of the mammary gland may be responsible for the internalization of Cbl. Although the dissociation constant was of similar magnitude to that of previous studies on other cell types [5–7,10,18,19], the amount of TC–Cbl bound per mg of protein was less than what has been reported – previous studies have used membrane preparations, which would have a higher number of receptors in comparison to cells [4,6,18,20,21]. It is also possible that an increase in the number of receptors may be found during lactation since the mammary gland may have a higher demand for Cbl not only for its use in intracellular Cbl metabolism, but also for secretion into milk. This increase in requirement for the vitamin may result in the upregulation of TC–Cbl receptor expression on the surface of the mammary gland to enhance Cbl uptake from the maternal circulation. An increase in TC–Cbl receptor expression may also be possible during periods of low maternal Cbl consumption during lactation. Sneed et al. [22] showed that dietary Cbl supplementation to lactating mothers with low Cbl intake increased milk Cbl concentrations, but supplementation did not have the same effect if the mother's intake was sufficient [23,24]. The possibility of hormonal influence on TC–Cbl receptor expression during lactation needs further investigation.

In circulation, Cbl is virtually all protein-bound to HC or TC [2]. Nevertheless, we determined whether or not free Cbl would bind to HMECs. Unlike the TC–[^{57}Co]Cbl complex for which saturation was reached with increasing concentrations of the ligand, increasing the concentration of [^{57}Co]Cbl to levels considered pharmacological (>800 pM) was not saturable, but instead exhibited an effect of mass action diffusion. Similar binding effects using free Cbl have also been observed in rat kidney tubule cells [4], rabbit liver and human placental membranes [6]. Under physiological conditions, free Cbl uptake is thus highly unlikely to quantitatively contribute to mammary gland Cbl uptake.

The HC–Cbl complex exhibited minimal affinity towards HMEC as compared with the TC–Cbl complex, suggesting that HC is not likely to be responsible for the delivery of Cbl to the mammary gland. A previous study obtained similar results using kidney tubule cells [4]. Alternatively, HC–Cbl delivers Cbl to the liver via the asialoglycoprotein pathway [25]. Hepatocytes express ASGPR and are responsible for the clearance of desialylated, galactose-terminal HC in the circulation. We did not identify an ASGPR transcript in the human mammary gland suggesting that the mammary gland does not acquire Cbl bound to circulating HC.

HMECs were shown to take up TC–Cbl; however, HC–Cbl is the dominant form of Cbl in breast milk [1,2,26]. Thus, two different ligands are involved in the uptake and transfer of Cbl into milk. Northern blot as well as PCR analysis showed that HC is expressed by HMEC, strongly indicating that the HC–Cbl present in breast milk does not originate from maternal circulation. The lack of a HC transcript in MCF-7 suggests altered Cbl metabolism; therefore, careful consideration should be made when using this cell line as a model to study Cbl metabolism in the normal mammary gland. This study did not investigate the fate of TC–Cbl after the uptake by HMEC; however, it has been proposed for other tissues that once the complex is internalized, the TC is degraded by lysosomal enzymes, thereby releasing Cbl [9]. If a similar mechanism is in place in mammary epithelial cells, an intracellular transfer of Cbl from TC to HC would explain the predominance of HC–Cbl in human milk. Further studies are needed to investigate the fate of Cbl in mammary epithelial cells.

The mechanisms by which the mammary gland acquires Cbl from the maternal circulation and how the vitamin is transferred to breast milk have not been investigated previously. This study provides the first evidence that Cbl is taken up by HMEC from TC–Cbl, and not from HC–Cbl or as free Cbl, through a receptor-mediated process. Further studies are needed to understand the intracellular mechanisms of transfer of Cbl from TC to HC in HMEC.

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References

[1] R.L. Burger, R.H. Allen, Characterization of vitamin B_{12}-binding proteins isolated from human milk and saliva by affinity chromatography, J. Biol. Chem. 249 (1974) 7220–7227.
[2] D.P. Sandberg, J.A. Begley, C.A. Hall, The content, binding, and forms of vitamin B_{12} in milk, Am. J. Clin. Nutr. 34 (1981) 1717–1724.
[3] R.H. Allen, P.W. Majerus, Isolation of vitamin B_{12}-binding proteins using affinity chromatography. III. Purification and properties of a human plasma transcobalamin II, J. Biol. Chem. 247 (1972) 7709–7717.
[4] J. Lindemans, J.V. Kappel, J. Abels, Uptake of transcobalamin II-bound cobalamin by isolated rat kidney tubule cells, Scand. J. Clin. Lab. Invest. 46 (1986) 223–232.
[5] P.A. Seligman, R.H. Allen, Characterization of the receptor for transcobalamin II isolated from human placenta, J. Biol. Chem. 253 (1978) 1766–1772.
[6] E. Nexø, M.D. Hollenberg, Characterization of the particulate and soluble acceptor for transcobalamin II from human placenta and rabbit liver, Biochim. Biophys. Acta 628 (1980) 190–200.
[7] P. Youngdahl-Turner, L.E. Rosenberg, Binding and uptake of transcobalamin II by human fibroblasts, J. Clin. Invest. 61 (1978) 133–141.
[8] S. Bose, S. Seetharam, B. Seetharam, Membrane expression and interactions of human transcobalamin II receptor, J. Biol. Chem. 270 (1995) 8152–8157.
[9] S. Bose, S. Seetharam, N.M. Dahms, B. Seetharam, Bipolar functional expression of transcobalamin II receptor in human intestinal epithelial Caco-2 cells, J. Biol. Chem. 272 (1997) 3538–3543.
[10] E.V. Quadros, P. Sai, S.P. Rothenberg, Characterization of the human placental membrane receptor for transcobalamin II-cobalamin, Arch. Biochem. Biophys. 308 (1994) 192–199.
[11] C. Gottlieb, K.-S. Lau, L.R. Wasserman, V. Herbert, Rapid charcoal assay for intrinsic (IF), gastric juice unsaturated B_{12} binding capacity, antibody to IF, and serum unsaturated B_{12} binding capacity, Blood 25 (1965) 875–884.
[12] G. Scatchard, The attractions of proteins for small molecules and ions, Ann. NY Acad. Sci. 51 (1949) 660–672.
[13] J.P. Bennet, in: T.R. Hesketh, H.L. Kornberg, J.C. Metcalfe (Eds.), Solubilization of Membrane-bound Enzymes and Analysis of Membrane Protein Concentration, Techniques in Lipid and Membrane Biochemistry, Elsevier Biomedical, New York, 1982, pp. 1–22.
[14] J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Vol. 2, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989 pp. 7.39–37.50.
[15] J. Johnston, J. Bollekens, R.H. Allen, N. Berliner, Structure of the cDNA encoding transcobalamin I, a neutrophil granule protein, J. Biol. Chem. 264 (1989) 15754–15757.
[16] S. Rozen, H.J. Skaletsky, Available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html ed., Center for Genome Research at the Whitehead Institute for Biomedical Research, Cambridge, MA, 1998.
[17] T. Amagasaki, R. Green, D.W. Jacobsen, Expression of transcobalamin II receptors by human leukemia K562 and HL-60 cells, Blood 76 (1990) 1380–1386.
[18] C. Fiedler-Nagy, G.R. Rowley, J.W. Coffey, O.N. Miller, Binding of vitamin B_{12}-rat transcobalamin II and free vitamin B_{12} to plasma membranes isolated from rat liver, Br. J. Haematol. 31 (1975) 311–321.
[19] E. Boukhzer, A. Ennya, F. Felden, A. Gérard, E. Nexø, J.P. Nicolas, H. Gérard, J.L. Guéant, Transcobalamin II-cobalamin binding sites are present on rabbit germ cells, Biochim. Biophys. Acta 1175 (1992) 128–131.
[20] P.A. Friedman, M.A. Shia, J.K. Wallace, A saturable high affinity binding site for transcobalamin II-vitamin B_{12} complexes in human placental membrane preparations, J. Clin. Invest. 59 (1977) 51–58.
[21] V.I. Mathan, B.M. Babior, R.M. Donaldson Jr., Kinetics of the attachment of intrinsic factor-bound cobamides to ileal receptors, J. Clin. Invest. 54 (1974) 598–608.
[22] S.M. Sneed, C. Zane, M.R. Thomas, The effects of ascorbic acid, vitamin B_6, vitamin B_{12}, and folic acid supplementation on the breast milk and maternal nutritional status of low socioeconomic lactating women, Am. J. Clin. Nutr. 34 (1981) 1338–1346.
[23] R. Thomas, S. Sneed, C. Wei, P. Nail, M. Wilson, E.E. Sprinkle, The effects of vitamin C, vitamin B_6, vitamin B_{12}, folic acid, riboflavin, and thiamin on the breast milk and maternal status of well-nourished women at 6 months postpartum, Am. J. Clin. Nutr. 33 (1980) 2151–2156.
[24] C.M. Donangelo, N.M.F. Trugo, J.C. Koury, M.I. Barreto-Silva, L.A. Freitas, W. Feldhelm, C. Barth, Iron, zinc, folate and vitamin B_{12} nutritional status and milk composition of low-income Brazilian mothers, Eur. J. Clin. Nutr. 43 (1989) 253–266.
[25] G. Ashwell, A.G. Morrell, The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins, Adv. Enzymol. 41 (1974) 99–128.
[26] E. Nexø, H. Olesen, D. Bucher, J. Thomsen, Purification and characterization of rabbit transcobalamin II, Biochim. Biophys. Acta 494 (1977) 395–402.