The Mechanism of Carrier-Mediated Transport of Folates in BeWo Cells: The Involvement of Heme Carrier Protein 1 in Placental Folate Transport

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The aim of this study was to elucidate the mechanism of folate transport in the placenta. A study of folate was carried out to determine which carriers transport folates in the human choriocarcinoma cell line BeWo, a model cell line for the placenta. We investigated the effects of buffer pH and various compounds on folate uptake. In the first part of the study, the expression levels of the mRNA of the folate receptor/C11 (FR/C11), the reduced folate carrier (RFC), and heme carrier protein 1 (HCP1) were determined in BeWo cells by RT-PCR analysis. Folate uptake into BeWo cells was greater under an acidic buffer condition than under a neutral one. Structure analogs of folates inhibited folate uptake under all buffer pH conditions, but anion drugs (e.g., pravastatin) inhibited folate uptake only under an acidic buffer condition. Although thiamine pyrophosphate (TPP), a substrate of RFC, had no effect on folate uptake, hemin (a weak inhibitor of folate uptake via HCP1) decreased folate uptake to about 80% of the control level under an acidic buffer condition. Furthermore, kinetic analysis showed that hemin inhibited the low-affinity phase of folate uptake under an acidic buffer condition. We conclude that pH-dependent folate uptake in BeWo cells is mediated by at least two carriers. RFC is not involved in folate uptake, but FRα (high affinity phase) and HCP1 (low affinity phase) transport folate in BeWo cells.

Key words: folate; BeWo; FRα; RFC; HCP1

Folates are essential nutrients required for the provision of one-carbon moieties in biosynthetic processes.1) It is known that folates are essential for cell division and growth and that deficiencies in folates impair fetal development.2,3) It has been reported that folate deficiency during pregnancy increases the risk of neural-tube defects developing in an infant.4,5) Although folate is an important vitamin, mammals cannot synthesize folates by themselves. Therefore intake of folates from dietary sources is essential, and systems for the absorption of folates from the intestine play an important role in the process of folate intake. These systems have been elucidated, and mechanisms of carrier-mediated absorption of folates have been reported.6,7) Folate receptor α (FRα), a glycosylphosphatidylinositol-linked glycoprotein, and reduced folate carrier (RFC/SLC19A1) have been found to be folate carriers.8,9) It has been found that both of these carriers are pH-sensitive and that they are expressed not only in the intestine but also in other organs, including the placenta.10–12)

The placenta is viewed as a protective barrier and as a site for nutrient and waste exchange between mother and fetus. Since folate uptake is pH-sensitive and is inhibited by anion exchanger inhibitors in placental cell lines,13) it appears that FRα and RFC also contribute to folate transfer through the placenta. Moreover, folates are also substrates for ATP-binding cassette transporters, MRPs (multidrug resistance-associated proteins), and BCRP (breast cancer resistance protein).14–16) Hence it is thought that these transporters contribute greatly to folate kinetics. Although there have been reports on folate transport in the placenta,17,18) the mechanism of folate transport in the placenta has not been elucidated in detail.

Recently, it was suggested that heme carrier protein 1 (HCP1) is a proton-coupled folate carrier in the intestine, and it has been shown that HCP1 is expressed in the placenta.19) Since folate uptake in placental cell lines has been found to be pH-sensitive, it is conceivable that HCP1 also contributes to folate transfer from mother to fetus, the function of HCP1 in the placenta has not yet been investigated.

The aim of this study was to determine the mechanism of folate uptake in the placenta. We investigated the effects of various substrates and inhibitors of folate carriers to identify the carriers involved in folate uptake in the human choriocarcinoma cell line BeWo, a line
that has been widely used in studies of the trophoblast transport mechanism.\textsuperscript{20,21}

**Materials and Methods**

*Chemicals.* \[3',5',7,9-^3\text{H}]Folic acid (23.0 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ). All other reagents were of the highest grade available and were used without further purification.

*Cell culture.* BeWo cells were obtained from the Riken Cell Bank (Saitama, Japan). BeWo cells were cultivated in the nutrient mixture F-12 Ham Kainh’s modification (Sigma-Aldrich, Tokyo) supplemented with 15% fetal bovine serum (MP Biomedicals, Solon, OH) and 1% penicillin-streptomycin at 37 °C under 95% air-5% CO\textsubscript{2}. The cells were grown for 4–5 d, and after reaching confluency were washed with PBS and harvested by exposure to a trypsin-EDTA solution, and then passed into new flasks. For the uptake study, BeWo cells were seeded at a density of \(1 \times 10^5\) cells/ml on 24-well plastic plates (Corning Coster, Cambridge, MA). The cell monolayers were fed a fresh growth medium every 2 d, and were then used at 5 d in uptake experiments.

*RT-PCR analysis.* Total RNA was prepared from BeWo cells using Isogen (Nippon Gene, Tokyo). Single-strand cDNA was made from 2 μg of total RNA by reverse transcription (RT) using an Omniscript RT Kit (Qiagen, Tokyo). PCR was performed with Hot Star Taq PCR (Qiagen) with FR\textalpha, RFC, and HCP1-specific primers through 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. The primers specific to FR\textalpha, RFC and HCP1 were designed on the basis of sequence data in the GenBankTM database (accession nos., BT007158, NM_194255 and NM_080669 respectively).

The sequences of the specific primers were as follows: 5′-GCA TTT CAT CCA GGA CAC ct-3′ (sense) and 5′-GAC AAT CTT CCC ACC ATT GC-3′ (antisense) for FR\textalpha, 5′-CAG CAT CTT GCT GTG CTA TG-3′ (sense) and 5′-TGA TGG TCT TGA CGA TGG TG-3′ (antisense) for RFC, and 5′-TGA TGG TCT TGA CGA TGG TG-3′ (antisense) for HCP1. The PCR products were subjected to electrophoresis on a 2% agarose gel and then visualized by ethidium bromide staining.

*Uptake experiments.* Uptake experiments using BeWo cells were carried out as described previously.\textsuperscript{21} The cells were washed twice with Hanks’ balanced salt solution (HBSS), containing 137 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl\textsubscript{2}, 1.0 mM MgSO\textsubscript{4}, 0.3 mM KH\textsubscript{2}PO\textsubscript{4}, 0.4 mM Na\textsubscript{2}HPO\textsubscript{4}, 4.2 mM NaHCO\textsubscript{3}, 25 mM d-glucose, and 10 mM HEPES, and then equilibrated in HBSS at 37 °C for approximately 10 min. [3',5',7,9-^3\text{H}]Folic acid (0.5 μCi/ml) premixed with or without inhibitors in warm HBSS was added to the cells in a shaking hot box (37 °C). The dosing solutions were then aspirated, and the cells were washed twice with ice-cold HBSS, followed by the addition of 0.5 ml of lysing solution (1% SDS in 0.2 N NaOH), and shaking of the plate in the hot box was continued. Samples of the lysate were then collected for scintillation spectrometry (1600TR, Packard Instruments, Meriden, CT) and protein assay by Lowry’s method with bovine serum albumin as the standard.

*Analytical procedures.* Student’s t-test was used to determine the significance of differences between two group means. For kinetic studies, the Michaelis-Menten constant was fitted to the following equation:

\[
V = \frac{V_{max} \times S}{K_m + S}
\]

(1)

**Results**

*Expression of mRNA of folate carriers in BeWo cells.* In the first part of the study, we investigated the expression levels of the mRNA of folate carriers by RT-PCR analysis using total RNA isolated from BeWo cells and specific primers of FR\textalpha, RFC, and HCP1. Each mRNA was clearly detected at the appropriate size clearly (146 bp, 161 bp, and 182 bp respectively) (Fig. 1).

*Time- and pH-dependent uptake of folate into BeWo cells.* We investigated the manner of folate uptake into BeWo cells. This was linear over a period of 10 min, as reported by Takahashi et al. (Fig. 2A).\textsuperscript{13} Hence, in subsequent experiments, we investigated the manner of folate uptake at 10 min. Folate uptake increased with decreases in buffer pH from neutral to acidic. The amount of folate uptake under a pH 5.5 buffer condition was about 10-fold greater than that under a pH 7.4 buffer condition (Fig. 2A, B), suggesting that folate carriers in BeWo cells are pH-sensitive.

*Inhibitory effect of various compounds on folate uptake.* To determine the folate uptake mechanism in BeWo...
cells, we investigated the inhibitory effects of various compounds on folate uptake. Non-labeled folate significantly inhibited folate uptake under both pH 5.5 and pH 7.4 conditions. Leucovolin (50 μM) and methotrexate (50 μM), structural analogs of folate, also decreased folate uptake, to about 50–70% of the control level under both acidic and neutral buffer conditions. On the other hand, although inhibitory effects of pravastatin, probenecid, indomethacin, nicotic acid, and p-aminobenzamide (50 μM) were observed under a pH 5.5 buffer condition, these anions had no effect under a pH 7.4 condition even at concentrations of 1 mM. Pravastatin, probenecid, indomethacin, nicotic acid, and p-aminohippurate; DIDS, 4,4'-Diisothiocyanatostilbene-2,2'-disulfonate.

**Table 1. Effects of Various Compounds on Folate Uptake**

<table>
<thead>
<tr>
<th>Compound</th>
<th>% of control pH 5.5</th>
<th>% of control pH 7.4</th>
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<tbody>
<tr>
<td>No addition</td>
<td>100 ± 8</td>
<td>100 ± 16</td>
</tr>
<tr>
<td>Folate (5 μM)</td>
<td>13 ± 3*</td>
<td>52 ± 10*</td>
</tr>
<tr>
<td>Leucovolin (50 μM)</td>
<td>34 ± 3*</td>
<td>48 ± 3*</td>
</tr>
<tr>
<td>Methotrexate (50 μM)</td>
<td>26 ± 1*</td>
<td>41 ± 2*</td>
</tr>
<tr>
<td>Pravastatin (1 mM)</td>
<td>73 ± 9*</td>
<td>104 ± 13</td>
</tr>
<tr>
<td>Probenecid (1 mM)</td>
<td>56 ± 9*</td>
<td>113 ± 4</td>
</tr>
<tr>
<td>Indomethacin (1 mM)</td>
<td>35 ± 5*</td>
<td>119 ± 15</td>
</tr>
<tr>
<td>Nicotic acid (1 mM)</td>
<td>58 ± 2*</td>
<td>106 ± 22</td>
</tr>
<tr>
<td>PAH (1 mM)</td>
<td>57 ± 3*</td>
<td>95 ± 24</td>
</tr>
<tr>
<td>Furosemide (3 mM)</td>
<td>45 ± 5</td>
<td>80 ± 15</td>
</tr>
<tr>
<td>DIDS (1 mM)</td>
<td>29 ± 3*</td>
<td>69 ± 16</td>
</tr>
</tbody>
</table>

Effects of various compounds on the folate uptake: [3H]-folate (22 nM) uptake was measured with and without each inhibitor in a buffer with a pH of 5.5 or pH 7.4. Each value is the means ± S.D. for four different samples.

*P < 0.05 as compared to no addition, by Student’s unpaired t-test. PAH, p-aminobenzamide; DIDS, 4,4'-Diisothiocyanatostilbene-2,2'-disulfonate.

Folate uptake was not affected at all under either acidic or neutral buffer conditions (Fig. 3).

It has been reported that hemin is a weak inhibitor of folate transport via HCP1. We investigated the effects of hemin and hematoporphyrin on folate uptake in BeWo cells. Folate uptake was decreased to about 80% of the control level by both hemin and hematoporphyrin under an acidic buffer condition, but no inhibitory effect was observed under a neutral buffer condition (Fig. 4). These results suggest that HCP1 is a candidate for a pH-sensitive carrier involved in the folate uptake mechanism in BeWo cells.

**Inhibitory effect of hemin on folate uptake in BeWo cells**

We also investigated in detail the inhibitory effect of hemin on folate uptake under an acidic buffer condition. An inhibitory effect of hemin on folate uptake was observed at folate concentrations of 22–250 nM, folate uptake being decreased to 80–90% of the control level. It was also found that the inhibitory effect of hemin on folate uptake was stronger at folate concentrations above 250 nM than at lower concentrations (22 nM–250 nM). Folate uptake decreased to 55–70% of the control level at a high concentration of folate (Fig. 5A). Two-phase uptake of folate was evident in an Eadie-Hofstee plot.
and apparent Km values were calculated to be 0.06 μM for the high-affinity phase and 0.45 μM for the low-affinity phase. Folate uptake changed to one-phase uptake in the presence of hemin, and the Km value was calculated to be 0.07 μM (Fig. 5B).

Discussion

Folates are essential nutrients for the fetus to protect it against various risks. There have been several studies on folate transport mechanisms using placental cell lines or mammalian placenta.24,25) FR/C11, which transports folates by endocytosis, and RFC, which mediates bidirectional folate transport, are thought to be involved in the folate transport mechanisms in the placenta, but Keating et al. have suggested that other carriers, which differ from both FR/C11 and RFC, are also involved in folate transport under an acidic condition in BeWo cells. Hence it has been suggested that there are more than two transport mechanisms in folate transport in the placenta, and further investigation is necessary to clarify these complex mechanisms.

Recently, HCP1 was reported to be a novel candidate for folate transport.19) It has been characterized as a proton-coupled and pH-sensitive folate carrier and it has been founded that it is also expressed in the placenta. Hence we hypothesized that HCP1 also mediates folate uptake in BeWo cells.

In the first phase of this study, we investigated the expression of several folate carriers in BeWo cells. RT-PCR analysis showed that FR/C11, RFC, and HCP1 mRNA were all expressed in BeWo cells, suggesting that these carriers are candidates for folate transport (Fig. 1).

Folate uptake into BeWo cells was linear over a period of 10 min, suggesting that efflux transporters such as MRPs and BCRP were not involved in folate transport in this study (Fig. 2). Furthermore, since folate uptake increased with decreases in buffer pH, and non-labeled folate and its structural analogs had inhibitory effect on folate uptake under both acidic and neutral conditions, these results suggest that folate uptake into BeWo cells is carrier-mediated. However, pravastatin, probenecid, indomethacin, nicotic acid and p-aminohippurate, which have been reported to be substrates or inhibitors of various anion transport mechanisms,27–30) had inhibitory effects only under an acidic buffer condition. These results suggest that BeWo cells also have a folate carrier of which the function differed depending on buffer pH. Hence it is thought that there are more than two carriers that mediate folate uptake into BeWo cells. Furthermore, since the inhibitory effects of DIDS and furosemide were stronger under an acidic condition than under a neutral one, the carrier that transports folates under an acidic condition might be similar to an anion exchanger (Table 1). It has been reported that RFC transports folates as a anion exchanger.31,32) Hence it has been hypothesized that RFC is one of the carriers of folate under acidic conditions,

![Fig. 4.](image1)

**Fig. 4.** Effects of Hemin and Hematoporphyrin on Folate Uptake. 
[^3H]-folate (22 nM) uptake was measured with and without each inhibitor in a buffer with a pH of 5.5 or 7.4. Bars are means ± S.D. for four different determinations. *P < 0.05 compared to no addition, by Student’s unpaired t-test.

![Fig. 5.](image2)

**Fig. 5.** Inhibitory Effect of Hemin on Folate Uptake. 
[^3H]-folate uptake by BeWo cells at various concentrations of[^3H]-folate from 22 nM to 1,000 nM was measured for 10 min with or without 25 μM of hemin (A). B, Eadie-Hofstee plot analysis of the effects of hemin on the folate uptake. Each point represents the mean ± S.D. of four determinations.
but TPP, a substrate of RFC, had no inhibitory effect on folate uptake even at a high concentration (5 mM) (Fig. 3). Keating et al. reported that folates are transported by RFC at both pH 5.5 and pH 7.4 into BeWo cells,26 but Chancy et al. suggested that RFC is expressed on the basolateral side of BeWo cells.33 Taking these facts into consideration, it is probable RFC does not transport folate from the apical side of BeWo cells under acidic buffer conditions.

We investigated the inhibitory effects of hemin, a weak inhibitor of HCP1, and its analog hematoporphyrin, on folate uptake. Although they did not inhibit folate uptake under a neutral buffer condition, about 20% of folate uptake was inhibited under an acidic buffer condition, suggesting that HCP1 is involved in folate uptake into BeWo cells under acidic buffer conditions (Fig. 4).

Furthermore, the inhibitory effect of hemin on folate uptake was stronger under a high concentration of folate (500–1,000 nM) than under a lower concentration (Fig. 5A). Eadie-Hofstee plot analysis showed that folate uptake was two-phase uptake under an acidic buffer condition, and that folate uptake changed to one-phase uptake in the presence of hemin, suggesting that two carriers transport folates under an acidic condition in BeWo cells (Fig. 5B). These facts suggest that hemin has very low affinity for high-affinity folate carriers, and that it inhibits folate uptake mediated by low-affinity folate carriers. Kinetic analysis indicated that the Km values of the carriers were 0.06 μM and 0.45 μM respectively. It has been reported that FRα is a very high-affinity carrier of folate (Km value of nM order),34 suggesting that the high-affinity phase of folate uptake, which has low affinity for hemin, is mediated by FRα in BeWo cells. Furthermore, the Km value of HCP1 at pH 5.5 has been reported to be 1.3 μM.19 Since the Km value of the low-affinity phase of folate uptake, which was sensitive to hemin, is similar to that of HCP1, it is probable that this phase was mediated by HCP1. These facts suggest that folate uptake under the acidic condition was mediated by two carriers, FRα and HCP1.

In summary, more than two pH-sensitive carriers were involved in folate uptake in BeWo cells, but it appears that RFC does not play a role in the folate uptake mechanism on the apical side of BeWo cells. Since the Km values and sensitivity to hemin of these carriers were different, it is shown that FRα (a high-affinity folate carrier) and HCP1 (a low-affinity folate carrier) are involved in folate uptake in BeWo cells.

References


