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Ribavirin uptake by cultured human choriocarcinoma (BeWo) cells and *Xenopus laevis* oocytes expressing recombinant plasma membrane human nucleoside transporters

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Abstract

We investigated the mechanism of the transport of ribavirin (1-β-D-ribofuranosyl-1,2,4-trizole-3-carboxamide) into placental epithelial cells using human choriocarcinoma (BeWo) cells and *Xenopus* oocytes expressing human nucleoside transporters. In BeWo cells, when a relatively low concentration (123 nM) of ribavirin was used, both Na^+-dependent uptake and -independent uptake of ribavirin were observed. On the other hand, when a higher concentration (100 μM) of ribavirin was used, Na^+-independent uptake was observed, but there was only a slight Na^+-dependent uptake. In *Xenopus* oocytes, influxes of ribavirin mediated by hCNT2 (concentrative nucleoside transporter 2), hCNT3 (concentrative nucleoside transporter 3), hENT1 (equilibrative nucleoside transporter 1) and hENT2 (equilibrative nucleoside transporter 2) were saturable, and apparent K_m values were 18.0 μM, 14.2 μM, 3.46 mM and 3.71 mM, respectively. These data indicate that hCNT2 and hCNT3 have higher affinity for ribavirin than do hENT1 and hENT2. Moreover, analysis by RT-PCR showed that BeWo cells express mRNA of hCNT3, hENT1 and hENT2. These results suggest that ribavirin is taken up by BeWo cells via both the high-affinity Na^+-dependent transporter hCNT3 and the low-affinity Na^+-independent transporters hENT1 and hENT2.

Keywords: Ribavirin; Concentrative nucleoside transporter; Equilibrative nucleoside transporter; BeWo cells; (*Xenopus laevis*) oocytes

1. Introduction

Nucleoside transporter (NT) is membrane proteins that mediate cellular uptake of nucleosides, and these transporters play an important role in the nucleoside salvage pathway for nucleotide synthesis. NT also transports nucleoside analogue drugs used for the treatment of cancers and viral diseases. Since most nucleosides and their analogues are relatively hydrophilic molecules, NT is essential for their transport across the plasma membrane (Cass et al., 1998). In addition, adenosine is an important substance in many physiological processes, including coronary vasodilation, renal vasoconstriction, neurotransmission, platelet aggregation and lipolysis. Therefore, NT also plays a role in regulation of adenosine concentration at cell surface receptors (Bucheimer and Linden, 2004; Boison, 2005; Gessi et al., 2000). NT is classified into ENT (equilibrative nucleoside transporter) and CNT (concentrative nucleoside transporter). The driving force of CNT is Na^+ gradient. In humans, four ENT and three CNT isoforms have been identified (Baldwin et al., 2003; Gray et al., 2004). hENT1 and hENT2 transport purine and pyrimidine nucleosides, and hENT2 also transports nucleobases. These are different in sensitivity to inhibition by nitrobenzylmercaptopurine ribonucleoside (NBMPR) and vasoactive compounds (dipylidamole, dilazep) (Griffiths et al., 1997a,b; Yao et al., 2002). hENT3 transports purine and pyrimidine nucleosides and adenine, and it is localized in the intracellular compartment, whereas hENT1 and hENT2 are localized in the plasma membrane (Baldwin et al., 2005). Although hENT4 exhibits low homology to other ENT members and transports monoamines (serotonin, dopamine and the neuroxin 1-methy-4-phenylpyridinium), it is

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classified as an NT. Since hENT4 is predominantly expressed in the human brain and is widely distributed in the central nervous system, it might supplement the role of other monoamine transporters in the brain (Engel et al., 2004). hCNT1 is pyrimidine nucleoside-selective and hCNT2 is purine nucleoside-selective. hCNT3 recognizes both purine and pyrimidine nucleosides. In addition, all three CNT isoforms transport uridine and adenosine (Ritzel et al., 1997, 1998, 2001a,b; Wang et al., 1997; Toan et al., 2003). Generally, CNT has higher affinities for nucleosides than does ENT. ENT is widely distributed in mammalian tissues, but its relative abundance varies. For example, ENT2 is abundant in the skeletal muscle. On the other hand, expression of CNT has organ specificity. hCNT1 is abundantly expressed in the kidney, liver and small intestine. hCNT2 is expressed in the stomach, small intestine, kidney and colon, and hCNT3 is distributed in the pancreas, trachea, mammary gland, bone narrow, intestine, lung, liver, testis, prostate and placenta (Ritzel et al., 2001b; Pennycooke et al., 2001; Alcorn et al., 2002). Furthermore, in renal epithelial cells, CNT is localized mainly in the apical membrane and ENT is localized mainly in the basolateral membrane, suggesting that CNT and ENT cooperate in the transport of nucleosides and their analogues across epithelial cells (Lai et al., 2002; Mangravite et al., 2003).

Ribavirin (1-β-D-ribofuranosyl-1,2,4-trizole-3-carboxamide), a purine analogue, is a broad-spectrum antiviral drug that is active *in vivo* and *in vitro* against both RNA and DNA viruses (Sidwell et al., 1979). It has recently been used in the treatment of patients with hepatitis C virus. Although treatment with ribavirin alone has not been successful, combination therapy with interferon α-2b resulted in higher rates of hepatitis C virus eradication than did IFN alone (Di Bisceglie et al., 1995). In cells, ribavirin is metabolized to monophosphate (RMP), diphosphate (RDP) and triphosphate (RTP). RMP inhibits inosine monophosphatase dehydrogenase (IMPDH) and this leads to depletion of GTP, which is necessary for vial RNA synthesis. RTP inhibits hepatitis C virus RNA replication by hepatitis C virus RNA-dependent RNA polymerase and causes hepatitis C virus RNA mutation of hepatitis C virus RNA polymerase. Furthermore, it has been suggested that ribavirin has the effect of immunomodulation (Feld and Hoofnagle, 2005). As major adverse effects, hemolytic anemia and teratogenicity are known (Sidwell et al., 1979). Ribavirin is distributed in erythrocytes concentration-dependently and is metabolized to mono-, di-, and triphosphates. Since erythrocytes do not express phosphatase, an enzyme that hydrolyzes phospholylated nucleosides, and NT does not transport these metabolites, phospholylated ribavirin accumulates in erythrocytes. Accumulation of the metabolites causes hemolytic anemia (Page and Connor, 1990). As for teratogenicity, it has been observed that ^{14}C-ribavirin passes through the placenta and is distributed in the embryo in the rat. Therefore, it will cause fetal toxicity.

In this study, we investigated the transport mechanism of ribavirin in placental epithelial cells by means of an uptake experiment using human choriocarcinoma (BeWo) cells. The transport of ribavirin via CNT and ENT was also characterized using *Xenopus laevis* oocytes heterologously expressing CNT or ENT.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS) and ribavirin were purchased from ICN Biomedicals (Aurora, OH), and [^{3}H]-ribavirin (8.1 Ci/mmol, 1 mCi/ml) was obtained from Moravek Biochemicals (Brea, CA). BeWo cells (RCB1644) were provided by Riken Cell Bank (Tsukuba, Japan). NBMPR, Nutrient Mixture F12 Ham Kaighn's Modification (F12K) and penicillin–streptomycin were purchased from SIGMA (St. Louis, MO). All other compounds were of reagent grade.

2.2. Cell culture

BeWo cells were cultured in F12K containing sodium bicarbonate (0.25% w/v), 15% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) and were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were removed from the plates by washing once with phosphate-buffered saline (PBS) containing 0.86 mM ethylenediamine-*N,N,N′,N′*-tetraacetic acid (EDTA) and then trypsinized (0.25% trypsin in Hanks' balanced salt solution (HBSS)) and diluted 5-fold with F12K. For transport experiments, 2×10^5 BeWo cells were seeded on 24-well culture plates (Greiner Japan, Tokyo, Japan) and cultured for 4 days until confluence. The medium was changed every day.

2.3. Total RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis of expression of nucleoside transporters in BeWo cells and human placenta

Total RNA was isolated from BeWo cells using TRIzol® Reagent (Invitrogen, Carlsbad, CA). Human placental total RNA was purchased from Cell Applications (San Diego, CA). cDNA was synthesized using ReverTra Ace® (TOYOBO, Osaka, Japan) and oligo-dT primer (18 mer). Reverse transcription conditions were 10 min at 30 °C, 60 min at 42 °C, 5 min at 99 °C and cooling to 4 °C. cDNA was amplified using Blend-Taq® (TOYOBO). The primers used were as follows: hENT1: 5′-cagaatgtgtccttggtcact-3′ and 5′-atgataacagcacaggctgtg-3′; hENT2: 5′-cctccgtctgcttcatcaact-3′ and 5′-ctggaagacagtgaagactga-3′; hCNT1: 5′-tggaaggtctgggacatggagaa-3′ and 5′-atgatgctttgagcaggcaa-3′; hCNT2: 5′-aagaagtagagcctgagggaa-3′ and 5′-aaccaaggagactcctgcaaa-3′; hCNT3: 5′-gagaacgagaacacatcagga-3′ and 5′-ccagaaccaatggctgtttag-3′. PCR conditions were 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C for 35 cycles. Finally, the PCR mixture was heated to 72 °C for 2 min and cooled to 4 °C.

2.4. Ribavirin uptake by BeWo cells

All uptake experiments were carried out at room temperature. The transport buffers used in this study were sodium-containing buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose and 25 mM Tris, pH adjusted to 7.4 by HEPES) and sodium-free buffer (in which Na^+ was replaced by *N*-methyl-D-glucamine (NMDG). Since NBMPR has low solubility in the buffer, NBMPR was first dissolved in DMSO and further

diluted in the transport buffer (1% v/v). Only DMSO was added to the buffer without NBMPR (control). BeWo cells were washed with 1 ml transport buffer and incubated in 250 μl transport buffer per well containing 0.25 μCi [^{3}H]-ribavirin. At the end of incubations, the solution was removed and cells were washed twice with ice-cold transport buffer. The cells were dissolved in a solution containing 0.2 M NaOH and 1% sodium dodecyl sulfate (SDS), and radioactivity was measured by a liquid scintillation counter. Before the concentration-dependent uptake experiment, the time course of ribavirin uptake was examined in order to determine how long the uptake followed a rectilinear course.

2.5. Plasmid construction

cDNA fragments containing the whole open reading frames of hCNT1 (GenBank accession no. U62966), hCNT2 (GenBank accession no. AF036109) or hCNT3 (GenBank accession no. AF305210) were obtained by RT-PCR. hCNT1 cDNA was amplified from human kidney total RNA (Cell Applications), and hCNT3 cDNA was obtained from BeWo cell total RNA using AMV Reverse Transcriptase XL (TaKaRa, Tokyo, Japan), oligo-dT primer (18 mer), LA Taq polymerase (TaKaRa) and primers specific to hCNT1 (5′-ggtctgggacatggagaacgacc-3′ and 5′-catgttctgtcctcactgtgcacagat-3′) and hCNT3 (5′-ggtacaaaggacctccagacc-3′ and 5′-tcagagttccactggagaagtg-3′). hCNT2 cDNA was amplified from human placenta total RNA (Cell Applications) using ReverTra Ace® (TOYOBO), oligo-dT primer (18 mer), KOD Dash® (TOYOBO) and primers specific to hCNT2 (5′-aggagccagagggaatcaat-3′ and 5′-gatcaagcagcctaggcacagacggt-3′). Since this first PCR product contained an unspecific product, nested PCR was carried out using KOD Dash® (TOYOBO) and primers specific to hCNT2 (5′-ggagaacaggagatggagaaagcaagt-3′ and 5′-gatcaagcagccttaggcacagacggt-3′). The conditions for reverse transcription were 10 min at 30 °C, 60 min at 42 °C, 5 min at 99 °C and cooling to 4 °C. PCR conditions were 30 s at 94 °C, 30 s at 55 °C (for hCNT1 and hCNT3 amplification) or 2 s at 55 °C (for hCNT2 amplification) and 2.5 min at 72 °C (for hCNT1 and hCNT3 amplification) or 1 min at 72 °C (for hCNT2 amplification) for 35 cycles. Finally, the PCR mixture was heated to 72 °C for 2 min and cooled to 4 °C. PCR products were subcloned in the pGEM-T Easy Vector (Promega, Madison, WI). cDNA clones of hENT1 (GenBank accession no. BC008954) and hENT2 (GenBank accession no. BC011387) were purchased from Invitrogen. However, compared with the other hENT2 sequence (GenBank accession no. NM_001532), purchased hENT2 cDNA lacked a part of the open reading frame and caused a frame shift. Therefore, we adjusted it by replacing the missing part with the RT-PCR product obtained from human placenta total RNA. ReverTra Ace® (TOYOBO), oligo-dT primer (18 mer), Pyrobest® DNA polymerase (TaKaRa) and primers specific to hENT2 (GenBank accession no. NM_001532) (5′-agcacagccaggatcctgagcac-3′ and 5′-cgccaggcacatggtgagggaca-3′) were used for RT-PCR. Reverse transcription conditions were 10 min at 30 °C, 60 min at 42 °C, 5 min at 99 °C and cooling to 4 °C. PCR conditions were 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C for 35 cycles. Finally, the PCR mixture was heated to 72 °C for 2 min and cooled to 4 °C. The PCR product was subcloned into deleted hENT2 clone (GenBank accession no. BC011387) using *Bam*HI and *Dra*III sites. All constructs were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit® (Applied Biosystems, Foster City, CA). Mutations were modified using a QuikChange Site-Directed Mutagenesis Kit® (Stratagene, La Jolla, CA).

2.6. Expression and transport assay in X. laevis oocytes

cRNA was synthesized from linearized plasmids with T7 RNA polymerase or SP6 RNA polymerase and a poly(A) tail was added by using a mMESSAGE mMACHINE and Poly(A) Tailing Kit (Ambion, Austin, TX). Mature oocytes from *X. laevis* were isolated by treatment with 2 mg/ml of collagenase (Wako, Okasa, Japan), manually defolliculated, and maintained at 16 °C in modified Barth's medium supplemented with 50 mg/l of gentamicin. On the following day, oocytes were microinjected with either 50 nl of water containing 50 ng cRNA or 50 nl water alone. Uptake of ribavirin was measured 3 days after microinjection. Uptake experiments were performed at room temperature. The transport buffers used in this study were sodium-containing buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH adjusted to 7.4 by Tris) and sodium-free buffer (in which Na^+ was replaced by NMDG). Oocytes were incubated in 100 μl of transport buffer containing 0.4 μCi [^{3}H]-ribavirin (494 nM) for hCNT2 and 0.2 μCi [^{3}H]-ribavirin (247 nM) for hCNT3 and 1 μCi [^{3}H]-ribavirin (1.23 μM) for hENT1 and hENT2 uptake assay. At the end of incubation, oocytes were washed five times with ice-cold transport buffer. Then oocytes were dissolved in 10% SDS solution and radioactivity was measured by a liquid scintillation counter. Before the concentration-dependent uptake experiment, the time course of ribavirin uptake was examined in order to determine how long the uptake was rectilinear with time.

3. Results

3.1. Expression of nucleoside transporters in BeWo cells and human placenta

Analysis of nucleoside transporters showed that the mRNA of hCNT3, hENT1 and hENT2 was expressed in BeWo cells

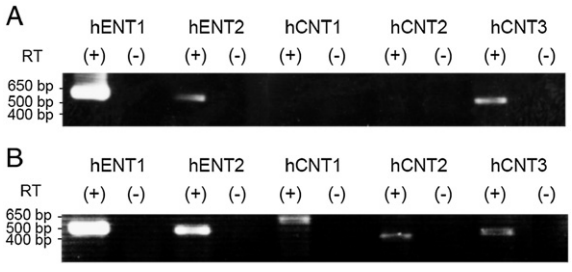

Fig. 1. Qualitative RT-PCR analysis of hCNT1, hCNT2, hCNT3, hENT1 and hENT2 expression in BeWo cells (A) and human placenta (B). The expected sizes of the PCR products were 0.51 kb for hENT1, 0.47 kb for hENT2, 0.61 kb for hCNT1, 0.39 kb for hCNT2 and 0.44 kb for hCNT3.

(Fig. 1A). Although this experiment was not designed as a quantitative approach, it seemed that hENT1 is the most abundant subtype of nucleoside transporters. It has been reported that BeWo cells express hENT1 and hENT2 and that NBMPR-sensitive transport activity level is very high compared with that in other cells, such as HeLa, S49 and CHO cells (Boumah et al., 1992; Mani et al., 1998). On the other hand, analysis of human placental total RNA showed that mRNA of hCNT1, hCNT2, hCNT3, hENT1 and hENT2 was expressed (Fig. 1B). As in BeWo cells, it is likely that hENT1 was expressed abundantly. It has been reported that the results of real-time PCR suggested mRNA of hENT1 is expressed at a higher level than that of other nucleoside transporters in placenta (Alcorn et al., 2002). In this study, although BeWo cells were derived from placenta, mRNA of hCNT1 and hCNT2 was not detected.

3.2. Effects of Na^+ and NBMPR on ribavirin uptake by BeWo cells

The effects of Na^+ and NBMPR (an ENT-specific inhibitor) on the uptake of ribavirin by BeWo cells were examined. When a relatively low concentration (123 nM, labeled compound only) of ribavirin was used, replacement of Na^+ by NMDG resulted in a decrease in the uptake of ribavirin (Fig. 2A). Furthermore, in the absence of Na^+, the uptake of ribavirin was decreased by the addition of 100 μM NBMPR (a concentration at which hENT1 and hENT2 were inhibited completely (Ward et al., 2000)). These findings suggest that ribavirin is transported via both Na^+-dependent and Na^+-independent transporters. Fig. 2B shows the effects of Na^+ and NBMPR on the uptake of ribavirin when the concentration of ribavirin was increased up to 100 μM. When the concentration of ribavirin was 100 μM, only a slight Na^+-dependent uptake was observed by replacement of Na^+ by NMDG (No significant difference was observed.). On the other hand, in the absence of Na^+, the uptake of ribavirin was decreased by the addition of 100 μM NBMPR. Furthermore, when the concentration of ribavirin was increased up to 100 mM, neither Na^+-dependent uptake nor NBMPR-sensitive uptake was observed.

3.3. Na^+-dependent and -independent nucleoside transporter-mediated ribavirin uptake by BeWo cells

The concentration dependence of ribavirin uptake via Na^+-dependent and -independent transporters by BeWo cells was examined. At the first, we tried to calculate the Na^+-dependent uptake by subtracting the uptake rate measured in the absence of Na^+ from that measured in the presence of Na^+. However, at a relatively high concentration, Na^+-dependent uptake was hardly observed (Fig. 2B). On the other hand, in the buffer with 100 μM NBMPR, Na^+-dependent uptake was observed even at

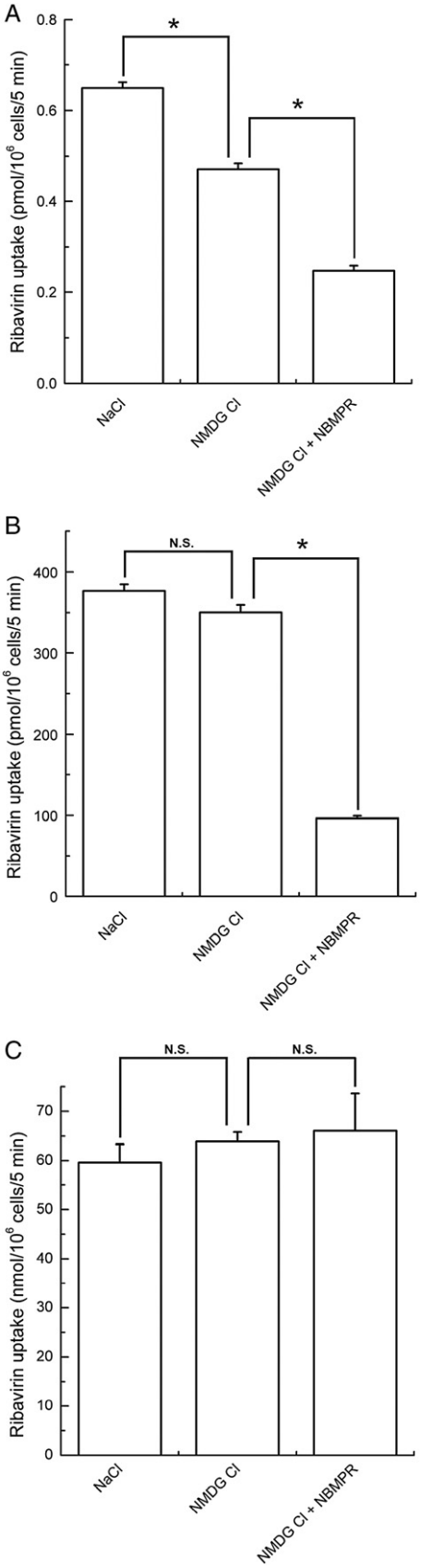

Fig. 2. Effects of Na^+ and NBMPR on ribavirin uptake in BeWo cells. Cells were incubated for 5 min with 123 nM (A) or 100 μM (B) or 100 mM (C) ribavirin in a buffer of 140 mM NaCl and *N*-methyl-D-glucamine chloride with or without 100 μM NBMPR. Each value of uptake is the mean±S.E.M. of three determinations (*$P<0.001$ significantly different).

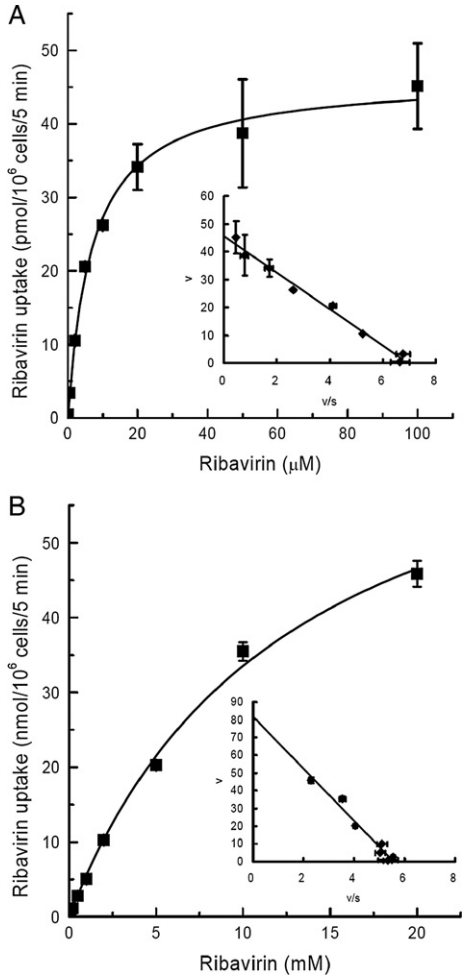

Fig. 3. Concentration dependence of Na^+-dependent (A) and Na^+-independent (B) uptake of ribavirin into BeWo cells. Na^+-dependent uptake of ribavirin for 5 min was analyzed in 100 μM NBMPR buffer with or without 140 mM Na^+ over a ribavirin concentration range of 123 nM–100 μM and calculated by subtracting rates measured in the absence of Na^+ from rates measured in the presence of Na^+. Na^+-independent uptake of ribavirin for 5 min was analyzed in a Na^+-free buffer with or without 100 μM NBMPR over a ribavirin concentration range of 0.1–20 mM and calculated by subtracting rates measured in the presence of 100 μM NBMPR from rates measured in the absence of NBMPR. Each value is the mean±S.E.M. of three determinations.

a relatively high concentration. Therefore, ribavirin uptake via an Na^+-dependent transporter by BeWo cells was calculated by subtracting the uptake rate measured in the absence of Na^+ and presence of 100 μM NBMPR (in which no NT is active) from that measured in the presence of Na^+ and 100 μM NBMPR. Similarly, the uptake via an Na^+-independent transporter was calculated by subtracting the uptake rate measured in the absence of Na^+ and presence of 100 μM NBMPR (in which no NT is active) from that measured in the absence of Na^+ and 100 μM NBMPR. Before examining the concentration-dependent uptake, we examined the time course of 123 nM ribavirin uptake in BeWo cells. We found that the uptake of ribavirin increased linearly for at least 1 h (data not shown) in BeWo cells. However, after ribavirin has been transported into cells, it should be metabolized. This metabolism might significantly decrease the amount of non-metabolized ribavirin in the cells and affect the transport of ribavirin. Therefore, to avoid the effect of metabolism, we shortened the incubation time as much as possible (5 min) to calculate K_m and Vmax. As shown in Fig. 3, both Na^+-dependent and -independent transporter-mediated uptakes were saturable. K_m and Vmax values were 7.0±0.67 μM and 46.3±1.21 pmol/10^6 cells/5 min for Na^+-dependent transport (Fig. 3A) and 12.7±1.48 mM and 76.2±4.57 nmol/10^6 cells/5 min for Na^+-independent transport, respectively (Fig. 3B).

3.4. Functional expression of hCNT1, hCNT2, hCNT3, hENT1 and hENT2 in X. laevis oocytes

To characterize the transport of ribavirin via a nucleoside transporter and also compare the values of kinetic parameters with those obtained from the uptake study using BeWo cells, we measured the uptake of ribavirin by *X. laevis* oocytes that heterologously expressed nucleoside transporters. At first, we examined if these NT recognize ribavirin as a substrate. We found that hCNT2, hCNT3, hENT1 and hENT2 recognized ribavirin but that hCNT1 did not (data not shown). Therefore, we examined the uptake of ribavirin via hCNT2, hCNT3, hENT1 and hENT2 in detail. Before examining concentration-dependent uptake, we examined the time course of 123 nM ribavirin uptake in *X. laevis* oocytes expressing hCNT2, hCNT3, hENT1 and hENT2. We found that the uptake of ribavirin increased linearly in the oocytes expressing hCNT3 for at least 30 min and in the oocytes expressing hCNT2, hENT1 and hENT2 for 1 h (data not shown). However, for the same reason as that for BeWo cells, we shortened the incubation time by 10 min. Fig. 4 shows the saturation kinetics of the uptake of ribavirin. Fig. 4A and B shows the uptake of ribavirin via hCNT2 and hCNT3, respectively. The hCNT2- and hCNT3-specific transport of ribavirin was saturable with K_m and Vmax values for the transport process of 18.0±3.69 μM and 22.5±1.58 pmol/oocyte/10 min and 14.2±0.83 μM and 78.0±1.47 pmol/oocyte/10 min, respectively (Fig. 4A, B). The hENT1- and hENT2-specific transport of ribavirin was also saturable with K_m and Vmax values of 3.46±0.19 mM and 1.12±0.02 nmol/oocyte/10 min and 3.71±0.35 mM and 1.23±0.04 nmol/oocyte/10 min, respectively (Fig. 4C, D).

4. Discussion

In this study, we investigated the uptake of ribavirin by BeWo cells. When a relatively low concentration (123 nM) of ribavirin was used, the replacement of Na^+ by NMDG resulted in a decrease in the uptake of ribavirin. Furthermore, in the buffer without Na^+, the uptake of ribavirin was decreased by the addition of NBMPR. These results suggest that ribavirin was transported via Na^+-dependent and -independent nucleoside transporters. Since BeWo cells express the mRNA of hCNT3 and hENT1 and hENT2 (Fig. 1A), these nucleoside transporters would be involved in the uptake of ribavirin in BeWo cells. Though human placenta expressed the mRNA of hCNT1 and hCNT2, BeWo cells that were derived from human placenta did not. These transporters might be lost in transformed cells

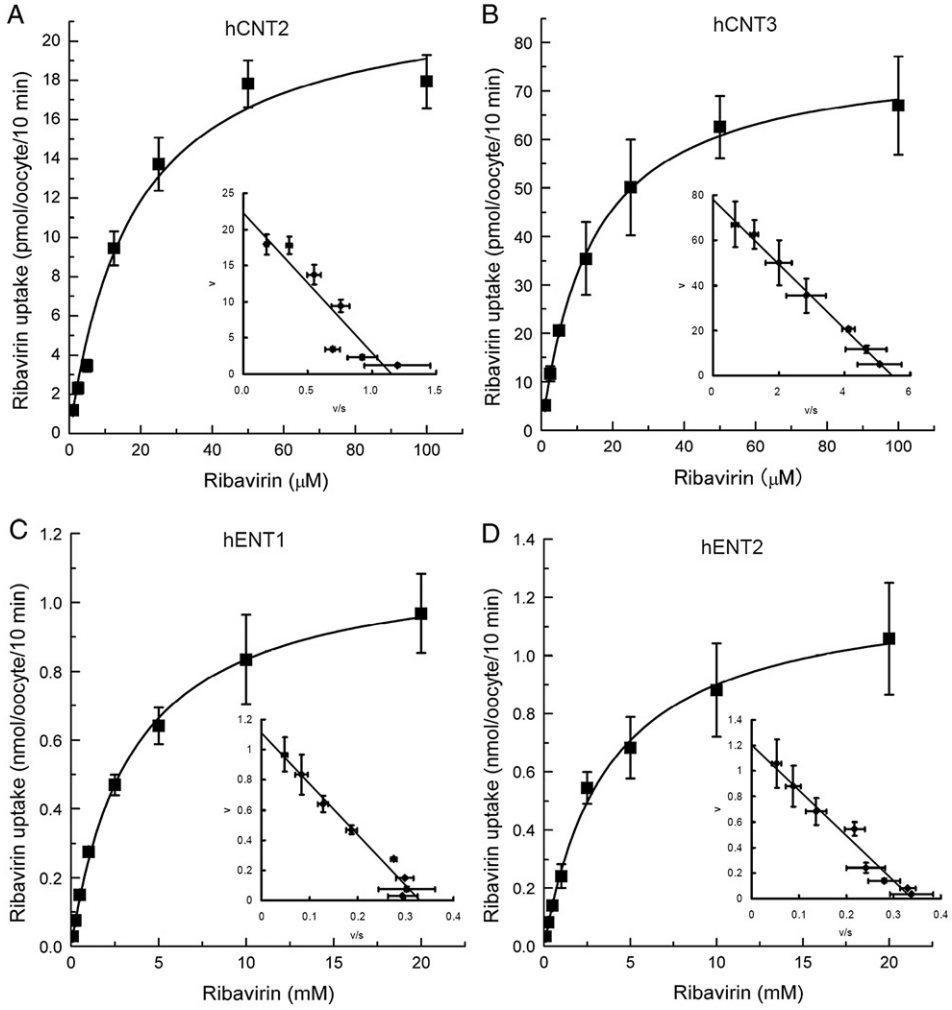

Fig. 4. Concentration dependence of hCNT2-mediated (A), hCNT3-mediated (B), hENT1-mediated (C) and hENT2-mediated (D) ribavirin uptake into *Xenopus* oocytes. Ribavirin uptake (10 min) was determined by analysis of permeant influx in RNA-injected oocytes minus that determined in water-injected oocytes over the ribavirin concentration range of 1 μM–100 μM (hCNT2, hCNT3) or 0.1–20 mM (hENT1, hENT2). Each value is the mean±S.E.M. of 5–7 determinations.

(Dragan et al., 2000). On the other hand, when a higher concentration (100 μM) of ribavirin was used, Na^+-dependent uptake was not clearly observed (A significant difference was not observed.). It is likely that this result was due to the affinity to ribavirin and expression levels of CNT and ENT in BeWo cells. Generally, CNT has higher affinity for a substrate than does ENT. Therefore, when the concentration of ribavirin was 100 μM, hCNT3 would be saturated but hENT would not. Actually, in BeWo cells, the Na^+-dependent component was saturated at 100 μM ribavirin, but the Na^+-independent component was not saturated at that concentration (Fig. 3). Furthermore, it has been reported that BeWo cells expressed a high level of hENT1 (Boumah et al., 1992; Mani et al., 1998). Actually, from the results of uptake of ribavirin, the Vmax value of the Na^+-independent component was much higher than that of the Na^+-dependent component (Fig. 3). Therefore, the uptake via hCNT3 (Na^+-dependent transporter) was observed at only a relatively low concentration of ribavirin. However, when the concentration was increased, hCNT3 was saturated and hENT, which has low affinity for ribavirin, was not saturated. Moreover, since hENT was expressed at a high level in BeWo cells, the uptake via hCNT3 would be masked by the uptake of hENT and would not be observed. Because of this phenomenon, NBMPR would be needed to observe Na^+-dependent uptake in BeWo cells. When excess non-radioactive ribavirin (100 mM) was added to the transport buffer, neither Na^+-dependent nor NBMPR-sensitive uptake was observed. Therefore, this uptake would be passive diffusion. Though ribavirin is a hydrophilic molecule, when ribavirin is present at a high concentration, some of it would permeate the plasma membrane.

Our results demonstrated that ribavirin was transported via hCNT2, hCNT3, hENT1 and hENT2 and was not transported via hCNT1. Since ribavirin is a purine derivative nucleoside, hCNT1, which is pyrimidine nucleoside-selective, would not recognize ribavirin. K_m values of hCNT2 and hCNT3 in *Xenopus* oocytes were 18.0±3.69 μM and 14.2±0.83 μM, respectively. Although these values are slightly larger than the K_m values of physiologically occurring nucleosides (Wang et al., 1997; Ritzel et al., 1998; Toan et al., 2003), CNT had higher affinity for ribavirin than did ENT. This result supports that Na^+-dependent uptake in BeWo cells was saturated and was not observed when ribavirin concentration was increased from

123 nM to 100 μM. On the other hand, in *Xenopus* oocytes, K_m values of hENT1 and hENT2 were 3.46±0.19 mM and 3.71± 0.35 mM, respectively. Although these values are larger than those of physiologic nucleosides (Ward et al., 2000), they are similar to those in BeWo cells. However, the K_m value of ribavirin uptake by human erythrocytes (in which the transport of ribavirin is mediated by the NBMPR-sensitive nucleoside transporter (hENT1)) is much smaller and reported to be 420 μM (Jarvis et al., 1998). Recently, it has been reported that some transporters are involved in cell entry and export of nucleoside analogues (Pastor-Anglada et al., 2005). Erythrocytes might express some kinds of transporters other than the tested nucleoside transporters.

Ribavirin has teratogenicity, and it has been suggested that ribavirin and its metabolite permeate through the placenta in the rat. Both CNT and ENT are thought to be responsible for the placental transport. hCNT2 and hCNT3 had higher affinity for ribavirin than did hENT1 and hENT2. The concentration of ribavirin in plasma is reported to be about 10 μM (Jen et al., 2000). Therefore, it is possible that ribavirin is taken up by cells via hCNT2 and hCNT3 rather than via hENT1 and hENT2. However, from the results of ribavirin uptake in BeWo cells, the ratio of Vmax to K_m of ENT is greater than that of CNT. The relative contribution of a transporter depends not only on the K_m value but also on the level of expression. Further study is needed to determine which NT plays an important role in ribavirin uptake in the placenta. Further investigation of the role of hCNT3 in ribavirin absorption is also needed. In conclusion, the results of this study suggest that ribavirin is taken up by BeWo cells via both a high-affinity Na^+-dependent transporter (hCNT3) and low-affinity Na^+-independent transporters (hENT1 and hENT2). RT-PCR analysis showed that human placenta expresses hCNT2. Therefore, hCNT2 might also be responsible for placental transport of ribavirin together with hCNT3, hENT1 and hENT2.

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