Ontogenic and longitudinal activity of Na⁺-nucleoside transporters in the human intestine

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Nucleosides are essential for cell survival because they play a pivotal role in a variety of key cellular processes. Because de novo synthesis of nucleosides is an energy-intensive process, cells fulfill their nucleoside requirement by salvage from both inside and outside the cell. To do so, they use one or more of a family of membrane nucleoside transporters. To date, five Na⁺-dependent concentrative (N1, N2, N3, N4, and csg) transporters and two Na⁺-independent equilibrative (es and ei) transporters have been identified (7). These transporters exhibit differential requirements for a Na⁺ gradient, substrate specificity, and sensitivity to S-(p-nitrobenzyl)-6-thioinosine [nitrobenzylmercapto-
purine riboside (NBMPR)]. Briefly, N1 (hCNT2) and N2 (hCNT1) selectively transport purine and pyrimidine nucleosides, respectively, whereas N3 has broad substrate selectivity, accepting both purine and pyrimidine nucleosides as permeants. N4 has similar substrate specificity to N2 except that it also transports guanosine. Uridine and adenosine are permeants of N1–N4 transporters. The csg transporter is unique in that it is the only Na⁺-dependent transporter that is highly sensitive to inhibition by low concentrations of NBMPR and dipyriddamole (DPA) (5). The permeant selectivity of this transporter, found in isolated human leukemic cells, has not been clearly established. In contrast, both of the equilibrative nucleoside transporters, es (hENT1) and ei (hENT2), exhibit a broad substrate specificity, accepting both purine and pyrimidine nucleosides as permeants. However, es is sensitive to inhibition at low concentrations of NBMPR (IC₅₀ ~ 0.4 nM), whereas ei is unaffected by NBMPR up to 1 μM (IC₅₀ ~ 2.8 μM) (24).

Enterocytes have a limited capacity for de novo nucleoside synthesis. Therefore, they rely heavily on nucleoside transporters to salvage nucleosides from their environment to meet their metabolic demands. We have previously established, using Xenopus oocytes microinjected with mRNA isolated from the human adult intestine, that the human adult intestine expresses two Na⁺-nucleoside transporters, N1 and N2, and two Na⁺-independent transporters, es and ei (3). Although both N1 and N2 Na⁺-nucleoside transporters are found on the apical surface of adult jejunal enterocytes (17), the Na⁺-independent transporters, es and ei, are not present there. In the same study, we reported that the Na⁺-nucleoside transporters have greater activity in the jejunum than in the ileum.

In this study, we have determined whether nucleoside transporters are present in the human fetal intestine, the types of transporters found there, and whether their activity is developmentally regulated. In addition, we have conducted a detailed study to determine the longitudinal distribution of the nucleoside transporters along the entire length of the human adult and fetal intestine. Such information is of fundamental biological and pharmacological interest. With
the rise in the incidence of viral diseases (e.g., AIDS) in pregnant women, there has been an increase in the use of antiviral nucleoside drugs in this population. Anti-AIDS nucleoside drugs such as zidovudine, didanosine, dideoxycytidine, and stavudine readily cross the placenta and accumulate in the amniotic fluid (16, 19, 22, 23). Thus the amniotic fluid serves as a reservoir from which drug can be absorbed from the fetal intestine. Understanding the types and activity of nucleoside transporters found in the intestine during the course of fetal development might shed some light on the extent of exposure of the fetus to nucleoside drugs after maternal administration. In addition, detailed characterization of the types and activity of nucleoside transporters found in the human adult intestine will also provide critical data for optimization of controlled-release formulations of nucleoside drugs.

MATERIALS AND METHODS

Chemicals

[5-3H]uridine (23.6 Ci/mmol), [8-3H]guanosine (14.7 Ci/mmole), and [methyl-3H]thymidine (20 Ci/mmole) were purchased from Moravek Biochemicals, (Brea, CA). Nucleoside analogs valinomycin and phloridzin were obtained from Sigma Chemical (St. Louis, MO). All other chemicals were of the highest analytical grade available.

Procurement of Human Intestines

This study was approved by the institutional review board of the University of Washington.

Fetal intestines. Human fetal small intestines of various gestational ages (9–18 wk old) were collected from normal abortuses of both sexes, immediately snap frozen in liquid nitrogen, and then stored at −70°C until use. Five to twenty-one fetal intestines of similar gestational age (within an age range of 14 days) were pooled for each transport experiment. Except for longitudinal activity studies, in which fetal intestines (from duodenum to cecum) were divided into two equal segments, proximal and distal, brush-border membrane vesicles (BBMV) were isolated from whole fetal intestines.

Adult intestines. Human intestines (from the ligament of Treitz to the cecum) were obtained from breathing adult organ donors (victims of vehicular or cerebrovascular accidents in otherwise good health) of both sexes. After the duodenum (the first foot) was separated, the small intestines were divided into two equal segments, with the proximal half representing the jejunum and the distal half the ileum. The jejunal and ileal segments were subdivided into 1-foot segments, each of which was rinsed with ice-cold saline solution to remove luminal debris. The last 2–3 feet of the intestine was defined as the distal ileum, and the last foot was defined as the terminal ileum. The tissues were stored at −70°C until use. For longitudinal activity studies, BBMV were prepared separately from the duodenum (1st foot), proximal jejunum (3rd foot), midpoint of the small intestine (usually 7th–9th foot), and distal ileum (last 2–3 feet) of each subject (total of 5 subjects) on the same day.

Preparation of BBMV

Fetal and adult intestines were thawed on ice, and BBMV were purified by the Mg2+- precipitation method as described previously (17). Membrane potential was clamped using the K+ ionophore valinomycin (3 μM). The purity of these preparations was routinely determined by assaying enzyme marker activities for the apical (alkaline phosphatase) and basolateral (ouabain-sensitive K+-phosphatase) membranes in both the starting homogenates and the vesicle suspensions.

Transport Studies

Uptake studies were performed under zero-trans conditions by the rapid filtration technique as described previously (17). The exact composition of the resuspension buffers and the incubation media are given in the legends to the figures. To determine whether Na+-dependent nucleoside transporters were present in the fetal BBMV, the uptake of [3H]uridine (2 μM) by fetal BBMV was measured in the presence and absence of a Na+ gradient (150 mM, out > in) as a function of time. In the latter case, an equivalent amount of KCl was used instead of NaCl. To distinguish the types of nucleoside transporters present along the adult and fetal intestine, we measured the uptake of [3H]guanosine (1 μM) or [3H]thymidine (1 μM) as prototypical purine and pyrimidine nucleoside substrates, respectively, by the intestinal BBMV in the presence and absence of inhibitors, including inosine (100 μM), cytidine (100 μM), NBMPR (1 μM), and DPA (10 μM) in Na+-containing or Na+-free media. Where applicable, the net Na+-dependent uptake of the labeled nucleosides was calculated as the difference in uptake in the presence and absence of Na+ . Because NBMPR and DPA, inhibitors of es and ei, respectively, were dissolved in N,N-dimethylformamide (DMF, final concentration ≤ 0.25% vol/vol), appropriate controls (solvent only) were conducted for every experiment. DMF (≤0.25% vol/vol) did not significantly affect the uptake rate of permeants (P > 0.05). Unless otherwise indicated, 10-μl aliquots of BBMV were incubated at room temperature with 40 μl of incubation medium (pH 7.4) for 10 s for adult BBMV and 20 s for fetal BBMV. Reactions were terminated by the addition of 3 ml ice-cold STOP solution (containing, in mM: 225 NaCl, 50 HEPES-Tris buffer, 0.1 MgSO4, and 1 phloridzin), followed by three washes each with 3 ml ice-cold STOP solution.

Data Analysis

Kinetic parameters of transport were estimated by applying least-square nonlinear regression analysis (WinNONLIN) to tracer displacement curves according to the following equation (4)

\[ \frac{v^*}{K_m + S_{\text{cold}} + T} + k_D \cdot T \]

where \( v^* \) is the initial rate of tracer uptake, T and Scold are the tracer and unlabeled substrate concentrations, respectively, \( K_m \) is the Michaelis-Menten constant, \( V_{\text{max}} \) is the maximum velocity, and \( k_D \) is the rate constant of passive diffusion.

Statistical Analysis

The data were analyzed using one-way ANOVA with an alpha set at 0.05. When a significant F-ratio was detected, a Student’s t-test with Bonferroni correction (for multiple comparisons) was performed to detect which treatments were significantly different from the corresponding control (tracer only).

RESULTS

Purity of Human Intestinal Brush-Border Membranes

Protein content was 6.0–14.4 mg protein/ml for adult BBMV and 4.4–16.9 mg protein/ml for fetal
BBMV. The activity of brush-border marker enzyme alkaline phosphatase was enriched 12- to 34-fold and 12- to 19-fold for adult and fetal BBMV, respectively.

**Time Course of Uridine Uptake by Fetal BBMV**

Na⁺-dependent transport of nucleosides by fetal BBMV was evident as early as 11–12 wk (82 ± 1 days; n = 9) of gestation. In the presence of a Na⁺ gradient (150 mM, out > in), [3H]uridine (2 μM) was transiently taken up by fetal BBMV against its own concentration gradient and peaked at 120 s (Fig. 1). When Na⁺ was replaced by K⁺, the overshoot phenomenon was abolished. The close agreement between the uptake rates at equilibrium (~30 min) in the presence and absence of a Na⁺ gradient suggested that there was no loss in the integrity of the BBMV. Because nucleoside uptake was found to be linear up to at least 20 s, and this time period allowed a greater differentiation from passive diffusion when compared with uptake at 10 s, this time was used in all experiments conducted with fetal intestinal BBMV.

**Identity of Nucleoside Transporters Along the Human Adult and Fetal Small Intestine**

The profiles of inhibition of the uptake of [3H]thymidine (2 μM) or [3H]guanosine (2 μM) by the fetal BBMV were similar at both the 12th (81 ± 2 days; n = 21) and 18th (122 ± 5 days; n = 5) week of gestation (the findings for the latter age group are depicted in Fig. 2). Briefly, the net Na⁺-dependent uptake of [3H]guanosine (2 μM) by fetal BBMV was completely inhibited in the presence of inosine (100 μM) but was not significantly inhibited by cytidine (100 μM). Conversely, the net Na⁺-dependent uptake of [3H]thymidine (2 μM) was significantly inhibited by cytidine (100 μM; ≥95% inhibition) but not significantly inhibited by inosine (100 μM). These data suggest that two distinct Na⁺-dependent N1 and N2 transporters are present on BBMV. The activity of brush-border marker enzyme alkaline phosphatase was enriched 12- to 34-fold and 12- to 19-fold for adult and fetal BBMV, respectively.
the brush-border membrane of the fetal small intestine. The lack of partial inhibition of Na\(^+\)-dependent \(^{[3}H\)guanosine uptake by cytidine (100 µM) ruled out the possibility of the existence of N3 and N4. In addition, NBMPR (1 µM) and DPA (10 µM) did not have an inhibitory effect on the uptake of \(^{[3}H\)guanosine and \(^{[3}H\)thymidine in both Na\(^+\)-containing and Na\(^+\)-free media. These data suggest that Na\(^+\)-dependent csg and Na\(^+\)-independent es and ei transporters are not present on the brush-border membrane of fetal enterocytes.

In the human adult small intestine, the profiles of inhibition of uptake of \(^{[3}H\)guanosine (1 µM) or \(^{[3}H\)guanosine (1 µM) by BBMV were similar to those observed with the fetal intestinal BBMV. For the sake of brevity, data obtained from the duodenum (1st foot) or the distal ileum (last 2–3 feet) of a human adult small intestine are illustrated in Fig. 3. Net Na\(^+\)-dependent uptake of \(^{[3}H\)guanosine was almost completely inhibited (≥90% inhibition) in the presence of inosine (100 µM), and that of \(^{[3}H\)thymidine was significantly inhibited (≥72% inhibition) by cytidine (100 µM). Neither NBMPR (1 µM) nor DPA (10 µM) inhibited Na\(^+\)-dependent (NBMPR only) or Na\(^+\)-independent uptake of \(^{[3}H\)guanosine or \(^{[3}H\)thymidine by adult BBMV. These profiles were qualitatively replicated when BBMV isolated from the proximal jejunum (3rd foot) and the midpoint (7th foot) of the intestines of the same individual were examined or when uptake into BBMV isolated from these four regions was examined in two additional individuals. Collectively, N1 and N2 transporters are found on the brush-border membrane along the entire length of the adult small intestine. However, none of the other nucleoside transporters, namely N3, N4, csg, es, and ei is present there.

Kinetics of Nucleoside Transport

To characterize the nucleoside transporters found in the fetal intestine at different gestational ages, uptake of a \(^{[3}H\)nucleoside (1 µM) into BBMV (2 separate batches per gestational age) isolated from 11- to 12- (76 ± 3 days; n = 11 and 15) and 14- to 15- (100 ± 3 days; n = 9 and 10) wk-old fetuses was measured in the presence of increasing concentrations of the corresponding unlabeled nucleoside and a constant Na\(^+\) gradient (150 mM, out > in). The uptake of both \(^{[3}H\)guanosine and \(^{[3}H\)thymidine obeyed the Michaelis-Menten relationship, suggesting that transport kinetics of N1 and N2 are saturable. In addition, the
Table 1. Summary of kinetic parameters of human intestinal N1 and N2 transporters

<table>
<thead>
<tr>
<th></th>
<th>Fetal 11–12 wk</th>
<th>Fetal 14–15 wk</th>
<th>Adult</th>
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</thead>
<tbody>
<tr>
<td>Guanosine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$</td>
<td>10.7, 14.1</td>
<td>14.5, 6.09</td>
<td>12.0 ± 1.34</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>9.8, 2.8</td>
<td>5.7, 5.7</td>
<td>63.9 ± 10.9</td>
</tr>
<tr>
<td>$K_{Na}$</td>
<td>9.38</td>
<td>13.0 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Hill coefficient</td>
<td>1.08</td>
<td>1.20 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>Thymidine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$</td>
<td>2.06, 3.22</td>
<td>5.56, 3.20</td>
<td>2.74 ± 0.54</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>5.8, 6.4</td>
<td>3.8, 3.8</td>
<td>16.1 ± 3.64</td>
</tr>
<tr>
<td>$K_{Na}$</td>
<td>49.6, 40.5</td>
<td>26.7 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>Hill coefficient</td>
<td>0.98, 0.94</td>
<td>0.95 ± 0.08</td>
<td></td>
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</table>

Values for fetal parameters are best fit means from least square nonlinear regression analyses of the data obtained from 2 different batches of pooled vesicles. Values for adult parameters are means ± SD of 3–4 individuals (17). $K_m$, Michaelis-Menten constant ($\mu$M); $V_{max}$, maximal transport activity for nucleoside uptake (pmol/mg protein/10 s); $K_{Na}$, $Na^+$ concentration required to achieve half of the maximal uptake velocity in a Na$^+$-activation study (mM).

close agreement of the $K_m$ values obtained using the fetal and the adult BBMV (Table 1) suggested that the fetal N1 and N2 transporters had similar affinities for their permeants compared with the adult N1 and N2 transporters, respectively. The lower $V_{max}$ values may have been due to a lower yield of BBMV from the fetal tissue per milligram of protein or may reflect a lower maximal activity of these transporters in fetal intestines.

The stoichiometric relationship of $Na^+$ to nucleoside uptake by BBMV obtained from 14- to 15-wk-old fetal intestines was estimated using the Na$^+$-activation method. Increasing the concentration of NaCl in the extravesicular medium (isosmolarity maintained with choline chloride) produced a hyperbolic stimulation in the uptake rate of $[^3H]$guanosine and $[^3H]$thymidine. To estimate the Hill coefficient, the Hill equation (21) was fitted to the data. The Na$^+$-nucleoside stoichiometry was found to be 1:1 (Table 1), which suggests transport of a single nucleoside molecule with a single Na$^+$ ion.

Longitudinal Distribution of Nucleoside Transporters Along the Human Adult and Fetal Small Intestines

The proximal-distal distribution of the N1 and the N2 Na$^+$-nucleoside transporters along the fetal small intestines (16–18 wk old or 116 ± 5 days; $n$ = 6) demonstrated a modest gradient; transporter activity was higher in the proximal half compared with the distal half (Fig. 4). In the adult intestine, the proximal-distal distribution of the N1 and the N2 Na$^+$-nucleoside transporters, as illustrated by $[^3H]$guanosine and $[^3H]$thymidine uptake, respectively, demonstrated considerable intersubject variability in magnitude and pattern (Fig. 5). In general, the N1 transporter activity increased (by 1.7-fold in most cases) from the duodenum (1st foot) to the proximal jejunum (3rd foot) and then declined toward the distal ileum. In some individuals the proximal-distal distribution of the N2 transporter activity paralleled that of the N1 transporter, whereas in other individuals the N2 transporter activity increased along the length of the intestine and reached a peak in the distal ileum.

DISCUSSION

This is the first study to examine the ontogenic profile of nucleoside transporters in the human fetal small intestine and to compare the type, functional characteristics, and regional activity of these transporters to those found in the adult small intestine. We have used 11- to 18-gestational-wk-old fetal intestines because this represents a period of the most rapid morphogenetic development. Previous studies have shown that during the first few weeks of gestation, the fetal intestinal epithelium consists of proliferative, undifferentiated cells 2–3 cell layers thick (9, 15). There is no evidence of villi in the fetus at ~7 wk old (6). Villus formation begins at 8–10 wk of gestation and progresses from the duodenum toward the colon. Similarly, cellular differentiation of the intestinal epithelium to form various cell types including enterocytes begins as early as 8–10 wk of gestation and progresses in a proximal-to-distal direction. At 20 wk of gestation, the colonic villi start to disappear and the structure of

Fig. 4. $[^3H]$guanosine (A) and $[^3H]$thymidine (B) uptake by BBMV isolated from the proximal and distal parts of 16- to 18-wk-old human fetal small intestines (116 ± 5 days old; pooled from 6 intestines). The BBMV were resuspended in 50 mM HEPES-Tris buffer (pH 7.4) containing 0.1 mM MgSO$_4$, 225 mM KCl, and valinomycin (3 μM). The final concentrations in the incubation media were 50 mM HEPES-Tris buffer (pH 7.4) containing 0.1 mM MgSO$_4$, 1 μM $^3$H substrate, and either 150 mM NaCl with 75 mM KCl or 225 mM KCl. An aliquot (10 μl) of BBMV was incubated with 40 μl of incubation medium at room temperature for 20 s. Values are means ± SD of triplicate determinations of net Na$^+$-dependent uptake.
enterocytes in the fetal jejunum resembles those found during adulthood (14). We initially attempted to isolate BBMV from fetal small intestine of younger age (8 wk old) by the Mg\(^{2+}\)-precipitation method. However, because of extremely low yield of BBMV and the limited supply of 8- to 10-wk-old intestinal tissues, functional assay for nucleoside uptake could not be performed. Therefore, data obtained from an older gestational age (11–18 wk old) only are reported. For ethical reasons, fresh fetal intestinal tissue of gestational age greater than 18–20 wk cannot be obtained.

\(\text{Na}^+\)-dependent uptake of nucleosides across the brush-border membrane of the fetal small intestine was clearly evident at an early stage of morphogenesis (11–12 wk of gestation; Fig. 1). Furthermore, we identified the existence of two functionally distinct subtypes of nucleoside transporters that behaved identically to the purine-specific (N1) and pyrimidine-specific (N2) \(\text{Na}^+\)-dependent nucleoside transporters found in the adult intestine (Figs. 2 and 3). In addition, similar to the adult small intestine, functional activities of other \(\text{Na}^+\)-dependent nucleoside transporters (N3, N4, and \(\text{csg}\)) and equilibrative transporters (es and ei) were not detected on the brush-border membrane of early differentiating (12 wk old) and maturing (18 wk old) fetal intestines. These findings suggest that the nucleoside transporters and other apical membrane proteins such as glucose transporters (10, 13), amino acid transporters (12), alkaline phosphatases (11), and dipeptidases (6) are expressed during early stages of intestinal morphogenesis. However, unlike alkaline phosphatase (2) and sucrase-isomaltase (1), for which complex differences exist between the fetal and the adult isoforms, there are no fetal-specific isoforms of the \(\text{Na}^+\)-dependent transporters. The fetal N1 and N2 transporters exhibited identical substrate selectivity and high affinity for their respective prototypical substrates compared with their respective adult counterparts (Table 1). The \(K_m\) values obtained from both early differentiating (11–12 wk old) and maturing (14–15 wk old) fetal intestines did not differ from those obtained from the adult intestines (Table 1). In addition, the close-to-unity values of the Hill coefficient suggest that, like the adult N1 and N2 transporters, the N1 and N2 transporters present in the fetal small intestine have a \(\text{Na}^+\)-nucleoside stoichiometry of 1:1. In addition, these nucleoside transporters had a similar affinity for \(\text{Na}^+\) to their adult counterparts (Table 1). Hence, the N1 and N2 transporters present in the fetal small intestines share the same kinetic characteristics as the respective transporters found in adult intestines. Moreover, the lack of difference between the kinetics of nucleoside transport across BBMV isolated from early differentiating (11–12 wk old) and maturing (14–15 wk old) fetal intestines suggests that there is no age-specific regulation of the types and characteristics of the \(\text{Na}^+\)-nucleoside transporters in the human fetal intestine during this period.

The distribution of the N1 and N2 transporters in the fetal intestine showed a modest proximal-distal gradient; transport activities in the proximal region were slightly higher than those in the distal small intestine. Limited availability of tissue precluded us from examining this gradient in detail. A similar proximal-distal gradient was also demonstrated for the adult N1 and N2 transporters. The indicated transporter activity pattern was found at subsaturating and saturating concentrations (when \(V_{\text{max}}\) is observed) in both the adult and the fetal intestine (data not shown). Since \(V_{\text{max}}\) is independent of the affinity of the transporter for its substrate but is dependent on the density of the transporters, we speculate that it is the density of the transporters that changes along the length of the intestine. The availability of specific antibodies to these transporters will help confirm this hypothesis. Such a proximal-distal gradient in transporter activity in the human intestine has been observed by others for glucose (8) and biotin (20). Similar to the N1 and N2 \(\text{Na}^+\)-nucleoside transporters, glucose transport activity was found to be highest in the distal jejunum and...
lowest in the distal ileum (8). This gradient in glucose transport activity was a result of a decreased density of transporter expression as measured by V_{\text{max}}. In contrast, transport activity of biotin was found to decrease in the order duodenum > jejunum > ileum. Thus the activity of distinct transporters in the adult human intestine demonstrates different patterns of distribution.

This is the first report demonstrating that the N1 and N2 Na⁺-nucleoside transporters are present in the human fetal intestinal brush-border membrane. Moreover, the types of transporters present in 100- to 120-day-old fetal intestine were uniform, with a modest gradation in activity along the length of the intestine. Similarly, the human adult intestine demonstrated only a modest proximal-distal gradient in activity, with the highest activity residing in the jejunum. These results should be useful in developing site-directed delivery of nucleoside drugs, which are absorbed in the human intestine via transport by the nucleoside transporters [e.g., ribavirin (18)]. In addition, the presence of nucleoside transporters in the fetal intestine may have implications for the absorption of drugs from the amniotic fluid after maternal administration of nucleoside drugs.

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REFERENCES