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Biotin uptake and cellular translocation in human derived retinoblastoma cell line (Y-79): A role of hSMVT system

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Abstract

The objective of this research was to investigate the presence of a specialized carrier-mediated system for biotin and delineate uptake mechanism and intracellular trafficking of biotin in the human derived retinoblastoma cell line (Y-79). Human derived retinoblastoma cell line, Y-79, was used for uptake studies. Uptake of [^{3}H]Biotin was determined at various concentrations, pH, temperatures, in the absence of sodium and in the presence of other vitamins and metabolic inhibitors to delineate the mechanism of uptake. Uptake was determined in the presence of various intracellular regulatory pathways (protein kinase A & C, protein tyrosine kinase and calcium-calmodulin) modulators. Reverse transcription polymerase chain reaction (RT-PCR) was performed to confirm the molecular identity of human sodium-dependent multivitamin transporter (hSMVT). Uptake of [^{3}H]Biotin in Y-79 cells were found to be saturable at micromolar concentration range, with apparent K_m of 8.53 μM and V_{max} of 14.12 pmol/min/mg protein, but linear at nanomolar concentration range. Uptake was sodium, pH, temperature and energy-dependent, but chloride independent; inhibited by the structural analogue desthiobiotin, pantothenic acid and lipoic acid at milimolar concentrations and not at nanomolar concentrations. Uptake of [^{3}H]Biotin was *trans*-stimulated by the intracellular biotin. Ca^{2+}/calmodulin pathways appeared to play important roles in the regulation of riboflavin uptake in Y-79 cells via significant reduction in V_{max} (66%) and K_m (28%) of the uptake process. A human sodium-dependant multivitamin transporter, hSMVT, was identified by RT-PCR in Y-79. These studies demonstrated for the first time the existence of a human sodium dependant multivitamin transporter (hSMVT), a specialized carrier-mediated system for biotin uptake, in human derived retinoblastoma cells.

Keywords: Biotin; Human derived retinoblastoma cell line (Y-79); Uptake mechanism; Cellular translocation; Human sodium-dependant multivitamin transporter (hSMVT)

1. Introduction

Retinoblastoma is a relatively uncommon retinal tumor primarily diagnosed in the children (Donaldson, 1993). Systemic chemotherapy has succeeded to certain extent in treating retinoblastoma (Friedman et al., 2000; Shields et al., 2002). The retina is a highly perfused tissue with a complex cellular organization. Drug delivery to the retina is a challenging task, particularly via the systemic route, due to the presence of a blood–retinal barrier (BRB) which regulates permeation of molecules from blood to retina (Duvvuri et al., 2003). Various nutrient transporters, such as peptide, amino acids, glucose, folate, monocarboxylic acid, nucleoside and nucleobase, organic anion and organic cation transporters have been reported to be expressed on the retina and BRB (Lee, 2000). It may be possible to utilize these transporters to enhance the retinal bioavailability of various compounds (Duvvuri et al., 2003).

Biotin (Vitamin H), a water-soluble vitamin, plays a vital role in intermediary metabolism, energy production, cell differentiation and cell proliferation in the retina. It is also known as coenzyme R, which acts as an essential coenzyme for various carboxylases in pathways of gluconeogenesis, fatty acid biosynthesis and catabolism of various odd-chain fatty acids and branched chain amino acids (Manthey et al., 2002; Crisp

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et al., 2004). Recent studies have shown that biotin is involved in the regulation of gene expression of specific membrane transporters and in the regulation of the intracellular cGMP (Said, 1999). Biotin deficiency has been reported to occur in patients with inborn errors of biotin metabolism (Bonjour et al., 1984; Sweetman and Nyhan, 1986; Dakshinamurti and Chauhan, 1988), in patients on long-term therapy with anticonvulsant agents (Krause et al., 1982, 1986) and in patients on long-term parenteral nutrition (Mock et al., 1981; Low et al., 1986). Biotinidase deficiency, a disorder of biotin metabolism, results in optic neuropathy, senosorineural hearing loss, asthma, dermatitis, alopecia and conjunctivitis and treatment with biotin therapy achieved a rapid clinical improvement (Puertas Bordallo et al., 2004). Recent studies have shown that in developing retina, an adequate content of biotin and a precise regulation of retinal cell death are required for the correct morphogenesis of the eye.(Valenciano et al., 2002)

Despite its critical role in developing ocular tissues, the molecular mechanism and regulation of biotin translocation into the retina is not well understood. Although many studies have focused on the mechanism of biotin uptake using various tissues (intestine, liver, kidney, placenta, skin) and cell lines from several species (human, rat, and rabbit), the process of intracellular trafficking of biotin in the retina has not been clearly delineated. Moreover, recent studies by Sinko et al. suggested that the targeting approach utilizing transporters such as human sodium-dependant multivitamin transporter (hSMVT), a biotin transporter, may substantially improve the delivery and anticancer activity of camptothecin through enhancement of cellular permeability and intracellular retention (Sinko et al., 2002). SMVT has been targeted to substantially improve the intestinal permeability and oral bioavailability of retro-inverso Tat nanopeptide (Ramanathan et al., 2001a,2001b). Therefore, biotin transporter targeted drug/prodrugs may be utilized for higher retinal intracellular drug concentration.

The objective of this study was to investigate the presence of specialized carrier system for biotin on the neural retina and to delineate the mechanism, intracellular regulation and possible role of sodium-dependant multivitamin transporter in the uptake of biotin by retinal cells. A human retinoblastoma cell line, Y-79, has been selected as a model for retinoblastoma. The human retinoblastoma (Y-79) is a multipotential neural cell line derived from a tumor of the inner layers of the retina (Yorek et al., 1986). Several studies have provided evidence that this cell line is a primitive neuroctodermal line that contains both neuronal and glial characteristics (Kyritsis et al., 1984) and therefore, is a potentially useful cellular model of the human retina. Various studies provide evidence supporting the neuroblastic potential of the Y-79 cell line (del Cerro et al., 1993; del Pilar Gomez et al., 1993).

2. Materials and methods

2.1. Materials

[^{3}H]Biotin (50 Ci/mmol) was purchased from Parkin-Elmer (Boston, MA). Unlabeled biotin, pantothenic acid, desthiobiotin, lipoic acid, biocytin, valeric acid, folic acid, nicotinic acid, ascorbic acid, riboflavin, pyridoxine, sodium azide, ouabain, 2,4-dinitrophenol, protein tyrosine kinase (PTK) modulators (genistin and genistein), protein kinase (PKC and PKA) pathways modulators (bisindolylymaleimide I, phorbol-12-myristate-13-acetate, forskolin, and 3-isobutyl-1-methylxanthine), calcium-calmodulin pathway modulators (calmidazolium, KN-62 and trifluoperazine), choline chloride, Triton X-100, HEPES, D-glucose and all other chemicals were purchased from Sigma Chemical Co (St. Louis, MO). All chemicals were products of special reagent grade and used without further purification.

2.2. Cell culture

Y-79 cells, obtained from American Type Culture Collections (ATCC). Cells were incubated in 75 cm^2 tissue culture flasks as a suspension in RPMI 1640 medium supplemented with 15% non-heat-inactivated fetal bovine serum, 1 mM glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml) and were maintained at 37 °C, in a humidified atmosphere of 5% CO_2 and 90% relative humidity.

2.3. Uptake experiments

Cells were collected following centrifugation and then washed three times with a Dulbecco's phosphate-buffered saline (DPBS), pH 7.4, containing 130 mM NaCl, 0.03 mM KCl, 7.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM Mg SO_4, and 5 mM glucose. Aliquots of approximately 5×10^6 cells were then preincubated in 1 ml DPBS for 10 min at 37 °C. Biotin uptake was initiated by the addition of a fixed amount of [^{3}H] Biotin. Cells were incubated for a suitable time period at 37 °C. At the end of the each experiment tubes were immediately centrifuged, the solution was aspirated off and cells were washed with 3 × 1 ml of ice-cold stop solution (210 mM KCl, 2 mM HEPES), pH 7.4, to arrest the reaction. The cells were then solubilized in 1 ml of 0.1% Triton-X solution in 1% NaOH and an aliquot was then transferred to scintillation vials containing 5 ml of scintillation cocktail. Radioactivity associated with the cells was quantified using a scintillation counter (Beckman Instruments Inc., Model LS-9000, Fullerton, CA) and the protein content of each sample measured by the methods of Bradford (Bradford, 1976) using bovine serum albumin as the standard (Bio-Rad protein estimation kit, Hercules, CA, USA). Cell viability under all treatment regimens was monitored by the trypan blue exclusion test and was routinely observed to be between 90 and 95%.

2.3.1. Time and concentration dependence

Uptake of [^{3}H] Biotin was carried out for various time period (3, 5, 10, 15, 30 and 45 min.) to determine optimum time period for uptake studies. To determine the contribution of hSMVT and high affinity biotin specific transporter various concentrations of unlabeled biotin ranging from micromolar (0.1–100 μM) and nanomolar (0.1–100 nM) solutions were prepared in DPBS, pH 7.4, and then spiked with fixed amount of [^{3}H]Biotin for con-

centration dependence studies. Uptake experiments were carried out as mentioned earlier.

2.3.2. Ion and energy dependence

The effect of sodium was examined by adding equimolar quantities of potassium, ammonium and choline chloride to substitute sodium chloride (NaCl) and sodium phosphate monobasic (Na_2HPO_4), in DPBS, pH 7.4. Effect of chloride ion was studied by using equimolar quantities of sodium phosphate dibasic (NaH_2PO_4), potassium phosphate (KH_2PO_4), and calcium acetate to substitute NaCl, potassium chloride (KCl) and calcium chloride ($CaCl_2$), respectively. To delineate the energy dependency, cells were preincubated with metabolic inhibitors (1 mM), like ouabain (an inhibitor of Na^+/K^+-ATPase), 2,4-dinitrophenol (intracellular ATP reducer) and sodium azide (an inhibitors of oxidative phosphorylation), for 1 h. Uptake was then carried out as described earlier with pH 7.4 buffer solution containing [^{3}H]Biotin (10 nM).

2.3.3. pH and temperature dependence

The pH of the buffer was adjusted to 5, 6, 7.4, and 8 for pH dependence studies. When effect of temperature on uptake of [^{3}H]Biotin was studied, buffer was adjusted to different temperatures (4 °C, room temperature (20 °C), and 37 °C). Uptake of [^{3}H]Biotin (10 nM) was then carried out at various pH levels and temperature settings.

2.3.4. Substrate specificity

To delineate the structural requirements for interactions with biotin carrier system, uptake experiments were carried out with competitive inhibitors (various vitamins and structural analogs). The unlabeled competitor was simultaneously incubated with the respective radiolabeled biotin (10 nM) and uptake was carried out as described previously. The unlabeled vitamins (biotin, pantothenic acid, desthiobitoin, lipoic acid, folic acid, riboflavin, nicotinic acid, pyridoxine and thiamine) at a concentration of 50 and 100 μM and structural analogs (biocytin, biotin methyl ester, valeric acid) at a concentration of 100 μM were used in these studies. Effect of the anion transport inhibitors 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) on biotin uptake was also examined at a concentration of 1 mM. To examine the presence of biotin specific transporter system, uptake of [^{3}H]Biotin (2 nM) was carried out in the presence of unlabeled vitamins (biotin, pantothenic acid, desthiobitoin, lipoic acid) at concentrations of 50 and 100 nM.

2.3.5. trans-stimulation study

In these studies, cells were preincubated with 1 ml of DPBS buffer (control) or 1 ml of DPBS buffer plus unlabeled Biotin, ranging from 10–500 μM, at 37 °C for 30 min. Cells were then rinsed with ice-cold buffer before the uptake studies were performed as described previously.

2.3.6. Intracellular regulation

Since intracellular uptake and trafficking of biotin is poorly understood, different signal transduction pathways modulators (PTK, PKC, PKA and Ca^{+2}/calmodulin pathways) were employed to explore the involvement of biotin in various cell signaling pathways. Various modulating agents (genistin, genistein, bisindolylmaleimide-I, phorbol-12-myristate-13-acetate, forskolin, *N*-[2-bromocinnamyl(amino)ethyl]-5-isoquinolinesulfonamide(H-89), 3-isobutyl-1-methylxanthine (IBMX), calmidazolium, KN-62 and trifluoperazine) were prepared in DMSO or absolute ethanol (final concentration of the organic solvent was less than 1% v/v). Cells were incubated for 1 h and uptake was initiated by adding [^{3}H]Biotin (10 nM) to examine the effects of various signaling pathways modulators. An identical amount of drug-dissolving vehicle (DMSO or ethanol) was incorporated in the bathing medium for control experiments to determine the effect of these solvents on untreated cells.

2.4. RT-PCR analysis

Total RNA was isolated from Y-79 cells with Trizol® reagent (Invitrogen) following manufacturer's protocol. Reverse transcription (RT) was performed according to standard protocol with 1 μg total RNA to obtain first strand cDNA. One micro liter of cDNA was then introduced into PCR and the product was subjected to gel electrophoresis with 3% agarose gel. Primers used for the amplification of human SMVT were: forward 5′-CGATTCAATAAAACTGTGCGAGT-3′ and reverse 5′-GGACAGCCA CAGATCAAAGC-3′. These primers were adopted from a published human SMVT cDNA sequences (Balamurugan et al., 2005). The PCR conditions were as follows: denaturation (94 °C, 45 s), annealing (58 °C, 1 min), and extension (72 °C, 45 s) for 35 amplification cycles, followed by a final extension of 72 °C for 10 min. The resultant product was sequenced from both directions by SeqWright using an automated Perkin-Elmer Applied Biosystems 3730 × 1 Prism TM DNA sequencer to establish its molecular identity.

2.5. Computer analysis

Nucleotide sequence homology matching was performed with a basic local aligment tool (BLAST) via on-line connection to the National Center of Biotechnology Information (NCBI). Multiple nucleotide sequence comparisons were done using CLUSTAL W (1.81) multiple sequence alignment tool from Swiss-Prot.

2.6. Data analysis

Uptake data was fitted to a modified Michaelis–Menten equation. This equation takes into account the carrier-mediated process (as describe by the classical Michaelis–Menten equation) and a linear non-saturable diffusional process.

$$V = \frac{V_{\max}[C]}{K_m + [C]} + K_d[C] \quad (1)$$

where V is the total rate of uptake, $V_{\max}$ the maximum uptake rate for the carrier-mediated process, K_m (Michaelis–Menten constant) the concentration at half-saturation, C the substrate

concentration, K_d the rate constant for the non-saturable diffusion component and K_d [C] represents the nonsaturable component, whereas the saturable component of total uptake is represented by $(V_{max}\ [C])/(K_m + [C])$. Data were fitted to above equation with a nonlinear least square regression analysis program (Kaleida Graph Version 3.09, Synergy Software, PA). The kinetic parameters, calculated using Kaleida Graph, were substituted into the equation to determine the contribution of the saturable and nonsaturable components. The quality of the fit was determined by evaluating the coefficient of determination (R^2), the standard error of parameter estimates, and by visual inspection of the residuals.

2.7. Statistical analysis

All experiments were conducted at least in quadruplicate and results are expressed as mean ± S.D. Michaelis–Menten parameters K_m and V_{max} are expressed as mean values. Unpaired Student's *t*-test was applied to calculate statistical significance. A difference between mean values was considered significant if $p < 0.05$.

3. Results

3.1. Uptake studies

3.1.1. Time and concentration dependence

Time-dependent uptake of [^{3}H]Biotin (2 and 10 nM) was carried out with Y-79 cells. As shown in Fig. 1(A) and (B), uptake increased linearly up to 20 min ($r^2 = 0.99$) and 45 min ($r^2 = 0.98$) for 10 and 2 nM [^{3}H]Biotin, respectively. Based on these results, 10 and 30 min incubation periods were selected as the standard incubation time for 10 and 2 nM [^{3}H]Biotin respectively for all the experiments (unless otherwise specified).

The presence of a biotin transport system in the Y-79 cell line was determined by assessing the kinetics of biotin uptake. Analysis of total [^{3}H]Biotin (10 nM) uptake data reveals an uptake mechanism consisted of two pathways: a saturable pathway at low concentrations and an apparently nonsaturable (passive) pathway that dominated at concentrations above 40 μM (Fig. 1(A)). After fitting the Biotin data to modified Michaelis–Menten equation, an uptake system with an apparent K_m of 8.53 μM and a V_{max} of 14.12 pmol/min/mg protein was identified. The dotted line represents the uptake for the saturable component and the dashed line represents the uptake for nonsaturable component of biotin uptake. Saturable and nonsaturable components were determined by substituting the values of the kinetic constants into modified Michaelis–Menten equation. As shown in Fig. 1(B) rate of [^{3}H]Biotin (2 nM) uptake was found to be linear as a function of concentration in the nanomolar range (0.1–50 nM).

3.1.2. Ion and energy dependence

In order to investigate sodium dependency, Na^+ ions in the bathing media was replaced with equimolar quantity of K^+, NH_4^+ and choline chloride. As shown in Fig. 2, significant inhibition (>80%) was observed in the uptake of [^{3}H]Biotin (10 nM)

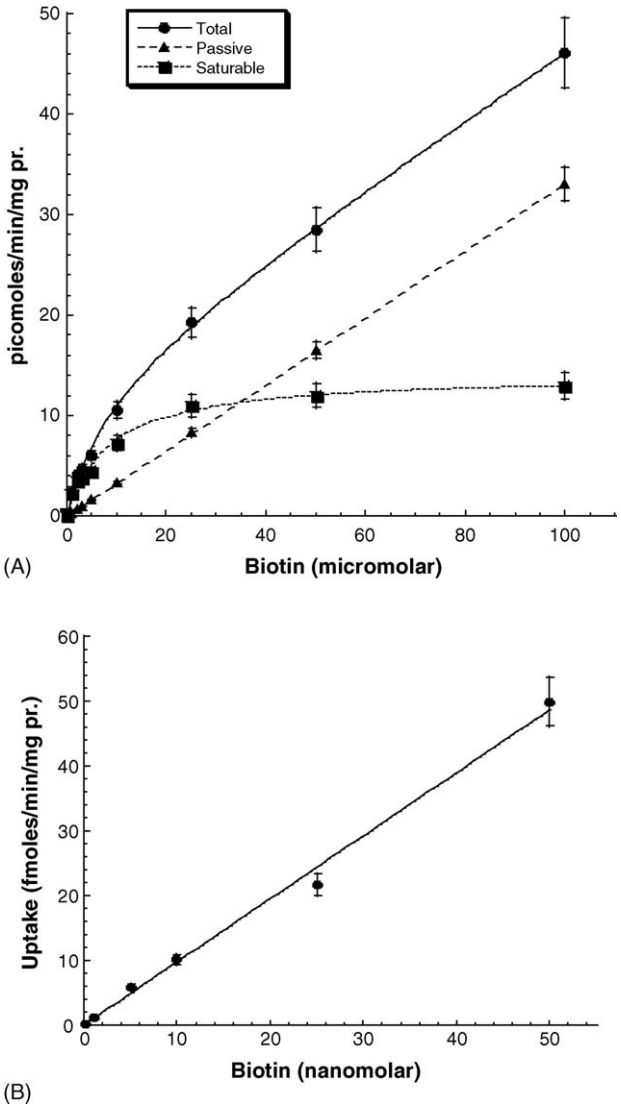

Fig. 1. Uptake of [^{3}H]Biotin by Y-79 cells as a function of micromolar (A) and nanomolar (B) substrate concentration at 37 °C, pH 7.4. (●) total uptake, (▲) linear, non-saturable component, (■) Michaelis–Menton component. Solid line represents the calculated fit of the data to modified Michaelis–Menton equation as described under Section 2. The estimated active component to the total biotin uptake is simulated with a dotted line; the estimated passive component is indicated with a dashed line. Each data point represents the mean ± S.D. of 4–5 separate uptake determinations.

indicating that uptake process is highly sodium-dependent. To test the hypothesis that biotin uptake depends on selective cotransport of Cl^-, experiments were conducted in media containing salts of alternative organic and inorganic monovalent anions (phosphate and acetate). No significant difference was observed in the uptake of [^{3}H]Biotin (10 nM) in Y-79 cells when chloride ion was substituted with phosphate and acetate.

We further studied the effect of metabolic inhibitors on the uptake of [^{3}H]Biotin (10 nM). A Na^+/K^+-ATPase inhibitor (ouabain), intracellular ATP reducer ((2,4-dinitrophenol (DNP)) and oxidative phosphorylation inhibitor (sodium azide) were added as metabolic inhibitors. Uptake was reduced significantly in the presence of sodium azide, ouabain and DNP. The rates of uptake were 16.38 ± 0.97, 7.96 ± 0.39, 6.02 ± 0.22 and

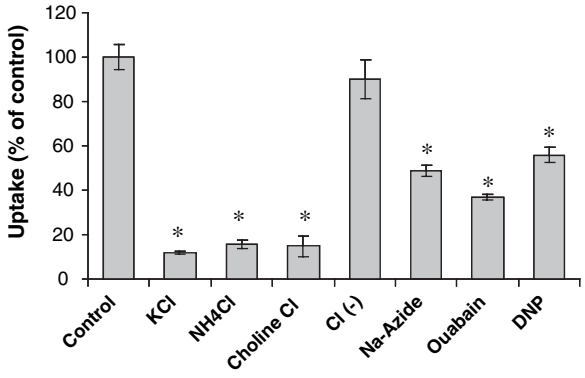

Fig. 2. Uptake of [^{3}H]Biotin by Y-79 cells in presence and absence of sodium and chloride (replaced with equimolar concentration of other monovalent cations) in DPBS buffer (pH 7.4) and in presence of metabolic inhibitors ouabain (1 mM), sodium azide (1 mM) and 2,4-dinitrophenol. Here, uptake is expressed as a percentage of control. Each data point represents the mean ± S.D. of 4–5 separate uptake determinations. Asterisk (*) represents significant difference from control ($p < 0.05$).

9.14 ± 0.57 fmol/min/mg pr. for control and in the presence of sodium azide, ouabain, and DNP, respectively (Fig. 2).

3.1.3. pH and temperature dependence

To test the potential involvement of a hydrogen-coupled uptake pathway, incubation media with pH over a range of 5–8 were prepared by adjusting the pH of DPBS. Biotin uptake was found to be decreased as a function of decreasing (or increasing) buffer pH from 7.4. Therefore, all experiments were carried out at pH 7.4. The biotin uptake pathway in Y-79 cells was further characterized using additional criteria for active membrane transport, namely temperature dependence. To determine temperature dependence of the transport pathway, uptake of [^{3}H]Biotin (10 nM) was performed at different temperatures. The rates of uptake were 20.77 ± 1.01, 13.38 ± 1.62, and 4.78 ± 1.34 fmol/min/mg pr. at 37°, 20°, and 4 °C, respectively, indicating that biotin uptake was substantially reduced with the decrease of temperature.

3.1.4. Substrate specificity

To investigate the substrate specificity of the saturable uptake process, we studied the effects of various structural analogs on [^{3}H]Biotin (10 nM) uptake in Y-79 cells. As shown in Fig. 3(A), 45.2% and 73.6% of [^{3}H]Biotin uptake was blocked in the presence of 50 and 100 μM unlabeled biotin, respectively. Interestingly, significant inhibition in the uptake of [^{3}H]Biotin was observed with pantothenic acid (39.2% and 65.6%) and lipoate (53.9% and 74.1%) at a concentration of 50 and 100 μM. Of the various biotin analogs tested, only desthiobiotin strongly reduced uptake but no significant inhibition was observed with biocytin and biotin methyl ester. Valeric acid caused a substantial inhibition of [^{3}H]Biotin uptake (38.6%). As shown in Table 1, none of the unlabeled vitamins (folic acid, riboflavin, nicotinic acid, pyridoxine and thiamine) showed any effect in the uptake of [^{3}H]Biotin in Y-79 cells. No significant inhibition in [^{3}H]Biotin uptake was observed in the presence of DIDS (1 mM).

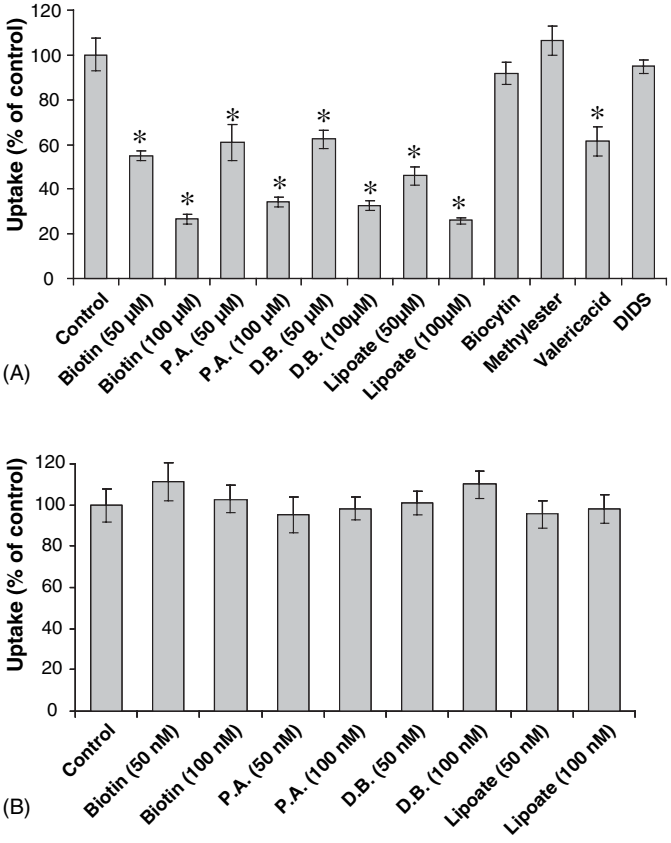

Fig. 3. (A) Uptake of [^{3}H]Biotin (10 nM) by Y-79 cells in presence of micromolar concentration of biotin structural analogs, pantothenic acid, lipoic acid, valeric acid and DIDS. Here, uptake is expressed as a percentage of control. Each data point represents the mean ± S.D. of 4–5 separate uptake determinations. Asterisk (*) represents significant difference from control ($p < 0.05$); (B). Uptake of [^{3}H]Biotin (2 nM) by Y-79 cells in presence of nanomolar concentration of biotin, desthiobiotin, pantothenic acid and lipoic acid. Here, uptake is expressed as a percentage of control. Each data point represents the mean ± S.D. of 4–5 separate uptake determinations.

To confirm the involvement of a carrier-mediated system for biotin uptake that saturates at the micromolar range, the effect of nanomolar concentrations of the bitotin structural analogs on the rate of carrier-mediated [^{3}H]Biotin (2 nM) uptake was also examined. As shown in Fig. 3(B), no significant difference in the [^{3}H]Biotin uptake was observed in the presence of 50 and 100 nM of unlabeled biotin, pantothenic acid, desthiobiotin and lipoic acid.

Table 1
Effect of structurally unrelated vitamins on uptake of [^{3}H]Biotin in Y-79 cells

Compound (100 μM)	Uptake (fmoles/min/mg pr.)
Control	20.80 ± 0.66
Folic acid	17.27 ± 1.65
Riboflavin	17.45 ± 1.64
Nicotinic acid	19.58 ± 1.99
Pyridoxine	18.01 ± 1.71
Thiamine	17.53 ± 1.01

Values are means ± S.E. of four separate uptake determinations.

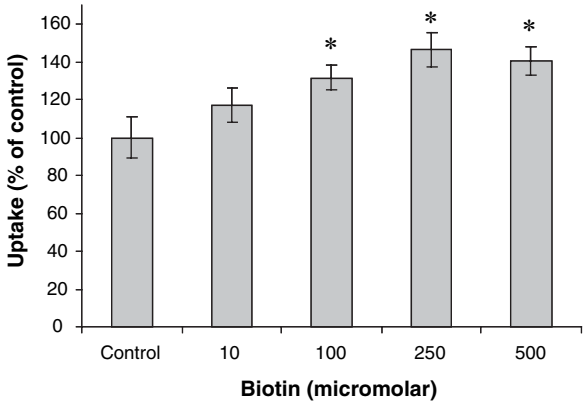

Fig. 4. *trans*-Stimulation study of [^{3}H]Biotin uptake by Y-79 cells. Uptake is expressed as a percentage of control. Each data point represents the mean ± S.D. of 4–5 separate uptake determinations. Asterisk (*) represents significant difference from control ($p < 0.05$).

3.1.5. *trans*-stimulation studies

In these studies, cells were preincubated with 1 ml of DPBS buffer (control) or 1 ml of DPBS buffer plus unlabeled biotin (10–100 μM) and the uptake of [^{3}H]Biotin (10 nM) was measured. The efflux rate of [^{3}H]Biotin would be expected to be higher in the control compared with cells preincubated with unlabeled biotin, if the uptake is carrier mediated. As shown in Fig. 4, [^{3}H]Biotin uptake was significantly enhanced (>30%) in cells preincubated with biotin compared with cells preincubated with only buffer (control). Varying the concentration of unlabeled biotin in the incubation medium, a concentration dependant *trans*-stimulation of uptake was observed. There was only a small difference in the stimulatory effect between 250 and 500 μM unlabeled biotin, demonstrating saturation of uptake.

3.1.6. Role of intracellular regulatory pathways

Involvement of intracellular regulatory pathways such as protein kinase C (PKC), protein kinase A (PKA), protein tyrosine kinase (PTK), and Ca^{2+}/calmodulin-mediated pathways in the regulation of [^{3}H]Biotin (10 nM) uptake into Y-79 cells was investigated. The possible role of PKC, PTK and PKA in the regulation of biotin (10 nM) uptake was studied by examining the effect of pretreating Y-79 cells for 1 h with either the PKC activator ((phorbol 12 myristate 13-acetate (PMA)), or with the PKC inhibitors (bisindolylmaleimide I), PTK pathway modulators (genistein and genistin) and PKA pathway modulators (activators, IBMX and forskolin and specific inhibitor, H-89). The results show that none of these compounds had significant effect on the biotin uptake.

The role of Ca^{+2}/calmodulin-mediated pathways in the regulation of [^{3}H]Biotin (10 nM) uptake in Y-79 cells was examined by pretreating these cells for 1 h with the calmodulin inhibitors (calmidazolium and tri-fluoperazine) and with the inhibitor of Ca^{+2}/calmodulin-dependent protein kinase II (KN-62). These compounds caused a significant and concentration-dependent inhibition in biotin uptake (Table 2). We also examined the effect of calmidazolium on the kinetic parameters of biotin uptake by Y-79 cells. This was done by examining the effect of calmidazolium on the uptake of biotin as a function of concentration. The results showed biotin uptake to be saturable both in the absence and presence of calmidazolium; however, uptake in the presence of calmidazolium was lower than that of control. There was a decrease (66%) in the V_{max} of biotin uptake in calmidazolium-pretreated cells compared with control cells (4.77 versus 14.12 pmol/min/mg protein, respectively) and a decrease (28%) in the apparent K_m (6.22 nM versus 8.53 nM, respectively) (Fig. 5).

Table 2
Effect of Ca^{2+}/calmodulin-mediated pathways modulators on uptake of [^{3}H]Biotin in Y-79 cells

Modulators (μM)	Uptake (fmoles/min/mg pr.)
Control	18.62 ± 1.98
TFP	
10	18.10 ± 0.72
100	13.44 ± 1.84*
250	11.02 ± 0.47*
KN-62	
0.1	17.35 ± 1.39
1	12.22 ± 2.71*
10	9.53 ± 0.81*
Calmidazolium	
Control	49.69 ± 2.20
10	29.26 ± 1.70*
50	21.03 ± 1.75*
100	14.48 ± 0.41*
250	6.47 ± 0.43*

In these studies cells were preincubated with 1 ml of Ca^{2+}/calmodulin-mediated pathway modulators (TFP, KN-62 and calmidazolium) and the uptake of [^{3}H]Biotin (10 nM) was measured. Values are means ± S.E. of four separate uptake determinations. Asterisk (*) represents significant difference from control ($p < 0.05$).

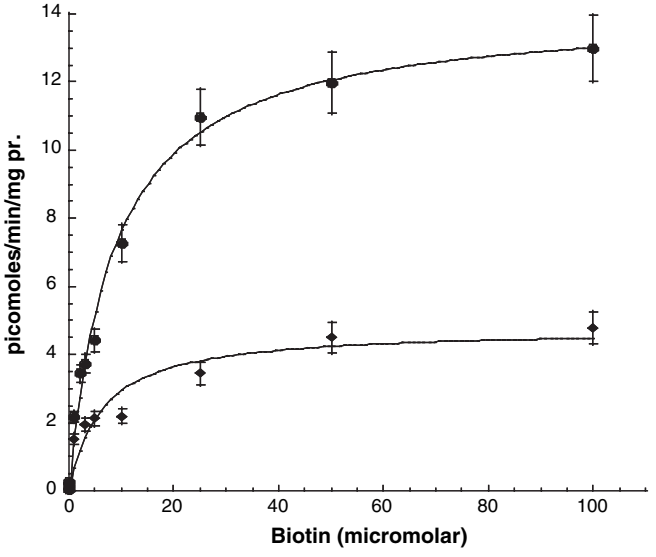

Fig. 5. Concentration-dependant uptake of [^{3}H]Biotin on calmidazolium-treated cells. (●) Control; (♦) Calmidazolium treated cells. The passive diffusion component is obtained using Michaelis–Menten equation as described in Section 2 and subtracted from total uptake values to represents only the saturable uptake process. Each data point represents the mean ± S.D. of 4–5 separate uptake determinations.

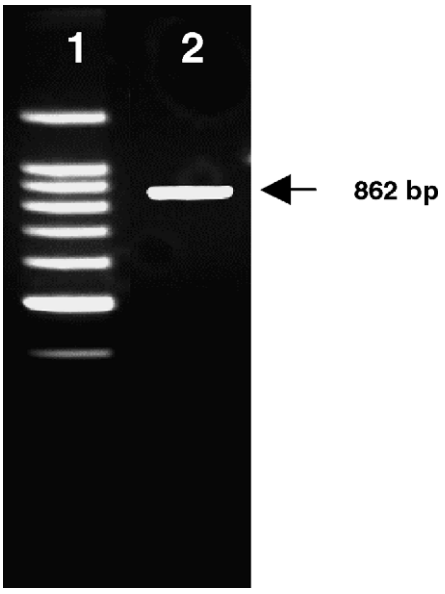

Fig. 6. Molecular identity of the hSMVT in Y-79 cells at the mRNA level. PCR product obtained using hSMVT specific primers and total RNA isolated from Y-79 cells. Aliquots of PCR products were analyzed by gel electrophoresis on 3% agarose gel. Ethidium bromide staining of the gel showed a major ~862 bp (lane 2) band corresponding to the amplified human SMVT cDNA. Lane 1 represents 1-kbp DNA ladder.

3.2. RT-PCR analysis

To determine molecular expression of the biotin transporter in Y-79 cells at the mRNA level, total RNA from the Y-79 cells were isolated. The PCR product was sequenced in both directions. The sequence obtained showed maximum homology to hSMVT (GenBank accession no. AF116241) using the BLAST search program (NCBI). A major band (862 bp), corresponding to the amplified human SMVT cDNA, with total product length of 15012 bp, was observed (Fig. 6).

4. Discussion

Human retina is a delicate organization of neurons, glia and nourishing blood vessels. Biotin, an essential water soluble vitamin, appears to play a critical role in cellular homeostasis and pathological conditions of retina including retinoblastoma. Treatment strategies for retinoblastoma have gradually changed over the past few decades. Systemic chemotherapy (chemoreduction) in the treatment of intraocular tumors has succeeded to certain extent in conjunction with cryotherapy or photocoagulation (Friedman et al., 2000; Shields et al., 2002). Various nutrient transporters (peptide, amino acids, glucose, folate, monocarboxylic acid, nucleoside and nucleobase, organic anion and organic cation transporters), present on the retina, have been known to play an important role in tissue nutrition and regulation of endogenous and exogenous substances. Biotin transporter expressed on the retina, may be targeted following systemic and intravitreal administration to generate enhanced drug availability in the retina.

A carrier mediated transport system for biotin is well studied in the various other tissues including placenta (Prasad et al., 1997), intestine (Said, 1991; Prasad et al., 1999; Balamurugan et al., 2003), kidney (Kumar et al., 1998; Balamurugan et al., 2005), liver (Said et al., 1992; Balamurugan et al., 2003) and skin (Grafe et al., 2003), but little is known about the uptake and intracellular regulation of biotin in the retina. The major aim of the present study was to identify the presence of biotin carrier system on human derived retinoblastoma cell line (Y-79), a model cell line for human retinoblastoma, and to investigate the mechanism and intracellular regulation of biotin uptake. In the present study, we have also explored the possible role of the hSMVT as a biotin carrier system on Y-79 cells and have attempted to establish its functional and molecular expression. Y-79 cell line was selected because it is a multipotential human cell line derived from a tumor of the inner plexiform layer of the retina, and it retains many neural characteristics. The Y-79 has been used as an in vitro model of retinoblastoma to study normal differentiation and maintenance of function of the neural retina and to facilitate the evaluation of antitumoral treatments (McFall et al., 1977; Olianas et al., 1992). Y-79 cell line has been selected as a model system to investigate metabolic characteristics and membrane properties of the retina (Tombran-Tink et al., 1992).

[^{3}H]Biotin (10 nM) uptake was found to be saturable as a function of concentration with an apparent K_m and V_{max} of 8.53 μM and 14.12 fmol/min/mg protein, respectively. Involvement of this carrier-mediated system in the retinal uptake of biotin is consistent with various other cell systems such as colonic, intestinal, placental, renal and liver hepatocytes cells. The previously reported half maximal transport constants (K_m) values range from 2.1 μM in placental choriocarcinoma (BeWo) (Prasad et al., 1997), to 22 μM in human keratinocytes, (HaCaT cells) (Grafe et al., 2003) and V_{max} values ranges from ~32 to 250 pmol/mg protein/min. More recently, a second human high-affinity sodium-dependant bitotin uptake system, with an apparent K_m in the nanomolar range has been described in peripheral blood mononuclear cells (PBMCs) (Zempleni and Mock, 1998, 1999) and in human keratinocytes (Grafe et al., 2003). Concentration dependence uptake of biotin (2 nM) showed no saturation in nanomolar range (0.1–50 nM) indicating that recently described human high-affinity biotin uptake system may not be functional in Y-79 cells. These variations could be due to the existence of different biotin uptake mechanisms at varying levels of expression depending on cell lines and/or inherent nature of cancer cell lines. Despite these differences, a common trend is observed among various studies showing that biotin uptake mechanism is specific and saturable in the range of micromolar concentration suggesting the involvement of hSMVT in the uptake of biotin in Y-79 cells.

Uptake process of biotin was found to be pH-dependent as significant difference was observed within a pH range of 5–8. Temperature-dependent regulation of vitamin uptake defines an active uptake mechanism (Foraker et al., 2003). Uptake of biotin was found to be temperature-dependent. Presence of sodium-free buffer caused significant inhibition of [^{3}H]Biotin (10 nM) uptake. These results suggest that sodium ions play a significant role in biotin translocation. Additional support for Na^+ dependence has been shown through uptake studies performed in the presence of ouabain, a well-known Na^+/K^+-

ATPase inhibitor. Significant inhibition was observed in the presence of ouabain, suggesting that this carrier-mediated transport process is sodium-dependent. In order to determine whether the uptake of biotin is dependent on a motive energy force, known metabolic inhibitors (sodium azide and 2,4-dinitrophenol) have been employed. Significant inhibition of [^{3}H]biotin uptake was observed when cells were treated with sodium azide and 2, 4-dinitrophenol, which is known to reduce intracellular ATP. Thus, process of biotin uptake by Y-79 cells was found to be energy dependant and appears to be directly coupled to ATP energy sources. These results indicate the involvement of a specialized, sodium-dependant high-affinity carrier-mediated system in the retinal biotin uptake which saturates at micromolar range.

Possible involvement of hSMVT is further supported by the finding of a significant inhibition of [^{3}H]Biotin uptake by unlabeled biotin and by its structural analogs at micromolar but not at nanomolar concentration range. Effect of structural analogs on biotin uptake also provided useful information about the structural–functional relationship between biotin and its uptake process. Desthiobiotin significantly inhibited the uptake of biotin. It appears that the thiophane portion in the biotin molecule plays an important role for binding to the biotin transporter. These results also suggest that the keto group at the second position of the imidazole ring in biotin is essential for the substrate to interact with the transporter. The tetrahydrothiophene ring which is absent in desthiobiotin does not appear to be essential because this analog is a potent inhibitor. However, lipoate which lacks the ring structure and the keto group is also a potent inhibitor of the biotin uptake. Similar results were obtained in biotin transfer studies using the placental brush-border membrane vesicles. In addition to the involvement of the keto group, it was shown that the terminal carboxyl groups of the valeric acid side chain as well as the length of the side chain are key factors determining the interaction of biotin with the transporter. Biocytin and biotin methyl ester, where biotin was modified at the carboxyl group had no effect on the biotin uptake. No significant inhibition in biotin uptake was observed in the presence of various unlabeled vitamins (nicotinic acid, folic acid, ascorbic acid, riboflavin, and pyridoxine). Significant and concentration dependant inhibition was observed on the uptake of [^{3}H]Biotin in the presence of unlabeled biotin (0.1–100 μM). Taken together, these results provide additional support for the presence of a carrier system that specifically mediates the uptake of biotin, pantothenic acid, desthiobiotin and lipoic acid into Y-79 cells and is saturable at a micromolar range. RT-PCR analysis provides the evidence of the molecular expression of hSMVT and further support the presence of hSMVT in Y-79 cells.

Involvement of anion-exchange transport mechanism for biotin transport across basolateral membrane was observed by Said et al. (Said, 1991). To delineate the involvement of anionic exchange transporter in the Y-79 cells, uptake of [^{3}H]Biotin (10 nM) was performed in the presence of anion transport inhibitor, i.e., DIDS. No significant inhibition of biotin uptake by DIDS rule out the possibility of the involvement of an anion-exchange transport mechanism for biotin uptake in the Y-79 cells.

A *trans*-stimulation study was used to further evaluate whether biotin is translocated by a carrier-mediated system, a characteristic feature of an exchange transporter. The presence of the unlabeled biotin on the opposite side (*trans*) of the membrane resulted in an enhanced flux of the radiolabeled biotin. Results show that uptake of [^{3}H]biotin was *trans*-stimulated by the intracellular biotin. One possible mechanism is that the intracellularly accumulated unlabeled biotin inhibits the efflux of the [^{3}H]Biotin resulting in an enhanced uptake. This finding further supports the presence of carrier-mediated system involved in the uptake of biotin on the Y-79 cells.

Several studies have demonstrated that the activity of membrane transporter systems are rapidly regulated by the major signaling pathways, namely protein tyrosine kinase, protein kinase A-,C-, and Ca^{2+}/calmodulin-mediate pathways (Said et al., 1998; Said, 1999; Feschenko et al., 2000). We also investigated possible regulation of the biotin uptake process by intracellular regulatory pathways. Concentration-dependent decrease in biotin uptake by TFP, KN-62, and calmidazolium suggest the involvement of Ca^{2+}/calmodulin mediated pathways in the regulation of biotin uptake. The effect of calmidazolium appears to be mediated via significant inhibition (39%) in the V_{max} and a significant increase (112%) in apparent K_m of the biotin uptake by Y-79 cells. These results suggest that the effect is mediated via decrease in both the capacity of the biotin uptake system and its affinity. PTK pathway modulators (genistin and genistein), PKA pathway modulators (IBMX, forskolin and H-89) and PKC pathway modulators (PMA and bisindolylmaleimide I) had no effect on biotin uptake. Therefore, our results indicate involvement of a Ca^{2+}/calmodulin pathway modulators but not PTK, PKA and PKC-mediated pathways in the regulation of biotin uptake. Balamurugan et al. showed the activity of PKC and Ca^{+}/calmodulin pathways for the mechanism of Biotin uptake in HK-2 cells. We have seen PKC independent Ca^{+}/calmodulin dependant pathway for the uptake of Biotin in Y-79 cells. The physiological mechanism(s) through which calmidazolium and protein kinase pathways exerts its regulatory effect on biotin uptake is not very clear. However, different mechanisms associated with this pathway have been proposed, including activation of specific protein kinases and possibly direct effect on the uptake system involved. Most signal transduction pathways are involved in diverse and critical cellular functions. Currently, physiological significance behind the multiple-signaling regulating pathways involved in biotin uptake in retina is poorly understood. Extensive cross-signaling between cAMP and calmodulin-mediated signal transduction pathways exists at several levels of cellular control mechanisms (Alberts, 1994). Therefore, CaM-mediated reduction of biotin uptake in Y-79 cells might be a manifestation of intertwined regulation of these processes.

In conclusion, this is the first report indicating the functional and molecular expression of a specialized human sodium-dependant multivitamin transporter (hSMVT) system on Y-79 cell line. This carrier-mediated active transporter system is temperature, energy and pH-dependant in nature. Uptake of [^{3}H]Biotin was *trans*-stimulated by the intracellular biotin. No involvement of PTK, PKA and PKC-mediated pathways were

found in regulating biotin uptake. This system appears to be regulated by Ca^{2+}/calmodulin-mediated pathways. The study also provides useful information on the substrate specificity of this carrier system. This study suggests that Y-79 cells express the hSMVT system and may serve as a useful in vitro experimental model of human retinoblastoma for studying retinal uptake and intracellular translocation of biotin. Moreover, hSMVT system can be targeted for the design of prodrugs to achieve enhanced permeability for highly potent, but poorly bioavailable anticancer agents where a small increase in the retinal availability could result in a significant improvement in therapeutic response in the treatment of retinoblastoma.

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