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Folic acid transport via high affinity carrier-mediated system in human retinoblastoma cells

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Abstract

The primary objective of this study was to investigate the expression of a specialized carrier-mediated system for folic acid and to delineate its uptake mechanism and intracellular trafficking in a human derived retinoblastoma cell line (Y-79). Uptake of [^{3}H]Folic acid was determined at various concentrations, pH, temperatures, in the absence of sodium and chloride ions and in the presence of structural analogs, methyltetrahydro folate (MTF) and methotrexate (MTX), vitamins, membrane transport and metabolic inhibitors to delineate the mechanism of uptake. Kinetics of uptake was studied in the presence of various intracellular regulatory pathways; protein kinases A and C (PKA and PKC), protein tyrosine kinase (PTK) and calcium–calmodulin modulators. Reverse transcription polymerase chain reaction (RT-PCR) was performed to confirm the molecular identity of folate carrier systems. The uptake was found to be linear up to 30 min. The rate of uptake followed saturation kinetics with apparent K_m of 8.29 ± 0.74 nM, 17.03 ± 1.98 nM and 563.23 ± 115.2 nM and V_{max} of 393.47 ± 9.33, 757.58 ± 26.21 and 653.17 ± 31.7 fmol/(min mg) protein for folic acid, MTF and MTX, respectively. The process was chloride, temperature and energy dependent but sodium and pH independent; inhibited by the structural analogs MTF and MTX but not by structurally unrelated vitamins. Membrane transport inhibitors did not affect the uptake of [^{3}H]Folic acid, however endocytic inhibitor, colchicine, significantly inhibited the [^{3}H]Folic acid uptake indicating the involvement of receptor mediated endocytosis process. PKC, PTK and Ca^{2+}/calmodulin pathways appeared to play important roles in the regulation of folic acid uptake. Molecular evidence of the presence of folate receptor (FR) precursor was identified by RT-PCR analysis. This research work demonstrated, for the first time, the functional and molecular existence of a specialized high affinity carrier-mediated system for folic acid uptake, in human retinoblastoma cells.

Keywords: Folic acid; Human retinoblastoma cell line (Y-79); Uptake mechanism; Cellular translocation; Folate receptor (FR)

1. Introduction

Folic acid, an essential water-soluble vitamin, plays a vital role in growth, differentiation and homeostasis of retinal cells. It acts as a coenzyme in the synthesis of purine and pyrimidine nucleotide precursors of DNA and RNA, interconversion and one-carbon metabolism of several amino acids (Brzezinska et al., 2000). Due to its critical role in visual system development, deficiency of this essential micronutrient result in visual disorders including nutritional amblyopia (Golnik and Schaible, 1994) which leads to reduced central vision, loss of papillomacular-bundle fibers, scotoma, pallor of the optic disk and optic atrophy. Folic acid deficiency in retinal cells may cause retinal edema, dysfunction and damage of photoreceptor cells and may also lead to methanol-induced retinal toxicity by decreasing the metabolism of formic acid, a neurotoxic metabolite of methanol (Seme et al., 1999).

Mammalian cells including retinal cells cannot synthesize folic acid and therefore rely on exogenous source for their metabolic requirements. Despite the fact that retina is a highly vascularized tissue, presence of blood retinal barriers encompassing endothelial cells of the retinal capillary and retinal pigment epithelium (RPE), regulate the exchange of various nutrients between circulation and neural retina (Duvvuri et al., 2003). Therefore, nutrients including folates must be taken up into the retinal cells by specific transport systems (Sirotnak and Tolner, 1999). Two general categories of transport systems have been identified for the entry of folic acid into the mammalian

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cells (Brzezinska et al., 2000) which includes reduced folate carrier (RFC) and folate receptor (FR). RFC is a bidirectional transporter with a twelve transmembrane-spanning domains. RFC exhibits very high affinity for reduced folates and moderate to poor affinity for non-reduced folates. FR is a cell membrane anchored protein with a mass of 38–40 kDa. FR interacts with folic acid much more efficiently with a high affinity than with reduced folates (Brigle et al., 1994; Shen et al., 1995; Spinella et al., 1995).

Recently, Bridges et al. have studied the translocation of folic acid across RPE and suggested that folic acid is transferred from the chorio-capillaries to the adjacent photoreceptor cells by the concerted action of RFC in the apical membrane and FR in the basal membrane (Bridges et al., 2002). However, uptake mechanism and translocation of folic acid into the neural retina are not well understood despite its critical role in the growth and development of retina. Although many studies have focused on the mechanism of folic acid uptake in various tissues such as intestine (Itoh et al., 2001; Kumar et al., 1997), kidney (Birn et al., 2005), placenta (Bisseling et al., 2004), pancreas (Nabokina et al., 2004) and cell lines derived from various species (human and rabbit), the process of intracellular trafficking in the retina has not been clearly delineated. Recent report by Yoo and Park (2004) suggested that folate-receptor-targeted doxorubicin nano-aggregrates could be a potentially useful delivery system for folate-receptor-positive cells. Folic acid conjugated pegylated polyplexes has been shown to generate higher transfection efficiency and targeted gene delivery (van Steenis et al., 2003). Due to the over expression in most tumor cells, folate receptor has been considered an attractive target for tumor-selective anti-cancer drug/prodrugs design, protein and gene delivery systems (Benns et al., 2001; Leamon and Low, 2001; Shinoda et al., 1998).

Therefore, the objective of this study was to investigate the presence of a carrier-mediated system for folic acid on the neural retina and to delineate mechanism, intracellular regulation and possible role of high affinity FR in the uptake of folic acid by retinal cells. A human retinoblastoma cell line, Y-79, has been selected as a model for retinoblastoma. Y-79 is a multipotential neural cell line derived from a tumor of the inner retinal 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 and therefore, is a potentially useful cellular model of the human retina (Kyritsis et al., 1984). Kansara et al. (2005, 2006) have characterized vitamin transporters using Y-79 cells, as an in vitro model for human retinal cells.

2. Materials and methods

2.1. Materials

[^{3}H]Folic acid (50 Ci/mmol) was purchased from Perkin-Elmer (Boston, MA). Unlabelled folic acid, methyltetrahydrofolate (MTF), methotrexate (MTX), biotin, ascorbic acid, riboflavin, pantothenic acid, sodium azide, ouabain, 2,4-dinitrophenol, protein tyrosine kinase (PTK) modulators (genistin and genistein), protein kinase (PKC and PKA) pathway modulators (bisindolylymaleimide I, phorbol-12-myristate-13-acetate, forskolin, and 3-isobutyl-1-methylxanthine (IBMX)), calcium–calmodulin pathway modulators (calmidazolium, KN-62 and trifluoperazine), probenecid, 4,4′-di-isothiocyanatostilbene-2,2′-disulphonic acid (DIDS), 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITC), colchicine, choline chloride, Triton X-100, HEPES, D-glucose and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals were of special reagent grade and used without further purification.

2.2. Cell culture

Y-79 cells were 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 studies

Uptake studies were performed according to previously published methods (Kansara et al., 2006) with minor modifications. Following centrifugation cells were acid washed three times with a Dulbecco's phosphate-buffered saline (DPBS), pH 3.5, 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 to remove endogenous folates bound to FR at the cell surface. Cells were then washed with DPBS, pH 7.4, at 0 °C and resuspended in uptake buffer, DPBS, pH 7.4. Aliquots of approximately 5×10^6 cells were then preincubated in 1 ml DPBS for 10 min at 37 °C. Folic acid uptake was initiated by the addition of a fixed amount of [^{3}H]Folic acid. Cells were incubated for a suitable time period at 37 °C. At the end of 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. 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 using bovine serum albumin as the standard (Bio-Rad Protein Estimation Kit, Hercules, CA, USA). Cell viability under all treatment conditions was monitored by the trypan blue exclusion test and was routinely observed to be between 90 and 95%.

2.3.1. Time and concentration dependency

Uptake of [^{3}H]Folic acid was carried out over various time period (1, 2, 5, 10, 20 and 30 min) to determine optimum time necessary for influx (uptake) studies. To assess the contribution of a carrier-mediated system of folic acid, various concentra-

tions of unlabeled folic acid and MTF (0.1–100 nM) and MTX (0.01–10 μM) solutions were prepared in DPBS, pH 7.4, and then spiked with fixed amount of [^{3}H]Folic acid. Uptake experiments were carried out as described previously.

2.3.2. Effect of pH and temperature

Buffer pH was adjusted to 5, 6, 7.4, and 8 for pH dependence studies. In order to study the effect of temperature on uptake of [^{3}H]Folic acid, buffer temperatures were adjusted to 4 °C, room temperature (20 °C), and 37 °C. Uptake of [^{3}H]Folic acid (10 nM) was then carried out under various pH and temperature conditions.

2.3.3. Role of ions, energy and membrane transport inhibitors

Effect of sodium ion 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 incorporating equimolar quantities of sodium phosphate dibasic (NaH_2PO_4), potassium phosphate (KH_2PO_4), and calcium acetate as substitute for NaCl, potassium chloride (KCl) and calcium chloride ($CaCl_2$), respectively. To delineate the energy dependence, 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 inhibitor of oxidative phosphorylation), for 1 h. To study the effect of the anion transport inhibitors on folic acid uptake, cells were preincubated with probenecid, DIDS, SITC at 0.5 mM concentration. To delineate the uptake mechanism, cells were incubated with an endocytic inhibitor, colchicine (100 μM). Uptake was then carried out as described earlier with buffer solutions containing [^{3}H]Folic acid (10 nM).

2.3.4. Substrate specificity

To delineate the structural requirements for interactions with folate carrier system, uptake studies were carried out in the presence of vitamins and structural analogs. The unlabeled competitor was simultaneously incubated with the respective radiolabeled folic acid (10 nM). Unlabeled vitamins (ascorbic acid, biotin, pantothenic acid, riboflavin and niacin) were used at a concentration of 10 μM. Unlabeled folic acid and structural analogs (MTF and MTX) at a concentration of 0.1 and 1 μM were employed in these studies.

2.3.5. trans-Stimulation study

Cells were preincubated with 1 ml DPBS buffer (control) or 1 ml DPBS buffer containing unlabeled folic acid, ranging from 1 to 100 μM, at 37 °C for 30 min. Cells were then rinsed with ice-cold buffer before the uptake of [^{3}H]Folic acid were performed as described previously.

2.3.6. Intracellular regulation

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]Folic acid uptake into Y-79 cells was investigated. Cells were preincubated for 1 h separately with PKC pathway activator (phorbol 12 myristate 13-acetate), or with the PKC pathway inhibitor (bisindolylmaleimide I), PTK pathway modulators (genistein and genistin), PKA pathway modulators activators (IBMX and forskolin and specific inhibitor, H-89), calmodulin inhibitors (calmidazolium and tri-fluoperazine) and Ca^{+2}/calmodulin dependent protein kinase II inhibitor (KN-62). Solutions of these modulating agents 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]Folic acid (10 nM). An identical amount of drug-dissolving vehicle (DMSO or ethanol) was incorporated in the bathing medium for control studies to determine the effect of these solvents on untreated cells.

2.4. Molecular evidence: RT-PCR analysis

Total RNA was isolated from Y-79 cells using Trizol® reagent (Invitrogen) by a standard protocol. Briefly, cells were lysed by adding 800 μl of Trizol reagent. The lysate was then transferred to Eppendorf tubes. RNA was extracted by the phenol–$CHCl_3$–isopropranolol method, purified, and dissolved in 50 μl of RNase–DNase-free water. For single strand cDNA synthesis, 5 μg total RNA was reverse transcribed according to a standard protocol using MMLV-Reverse transcriptase (Promega, Madison, WI). The conditions for reverse transcription were denaturation of template RNA for 2 min at 94 °C and reverse transcription for 60 min at 72 °C. Amplification was performed using 1 μg cDNA and primers used for the amplification were: forward 5′-GTG TCG ACC CTG GAG GAA GA-3′ and reverse 5′-GCA CGT TCA GTA CCC GCT CCT CT-3′. PCR conditions were as follows: denaturation (94 °C, 45 s), annealing (55 °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 product was separated by gel electrophoresis using 3% agarose gel and visualized by chemiluminescence.

2.5. Computer analysis

Nucleotide sequence homology matching was performed with a basic local alignment search 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 (Eq. (1)). This equation takes into account the carrier-mediated process (as describe by the 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} is the maximal uptake rate for the carrier-mediated process, K_m (Michaelis–Menten constant) is the concentration at half-saturation, C is the substrate concentration, K_d represents rate constant for the non-saturable diffusion component and $K_d[C]$ represents the nonsaturable component, whereas the saturable component of total uptake is given 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 with Kaleida Graph, were substituted into Eq. (1) 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. unless otherwise specified. Michaelis–Menten parameters K_m and V_{max} are expressed as mean ± S.E. 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. Identification and functional characterization of folate carrier system

3.1.1. Time and concentration dependent uptake

Time dependent uptake of [^{3}H]Folic acid was carried out with Y-79 cells. Uptake was found to be linear up to 30 min ($r^2 = 0.99$) and occurred at a rate of 27.45 ± 2.36 fmol/(min mg) protein. Based on these results, 10 min incubation time was selected as the standard uptake period for all the experiments (unless otherwise specified).

The presence of a carrier-mediated folic acid uptake system in the Y-79 cell line was determined by assessing the uptake kinetics of folic acid in the presence of unlabeled folic acid, MTF and MTX. Analysis of total [^{3}H]Folic acid uptake data reveals that the uptake mechanism consisted of two pathways: a saturable pathway at low concentrations and an apparently nonsaturable (passive) pathway that dominated at higher concentrations above 0.1 μM for folate and MTF and 10 μM for MTX (Fig. 1). Saturable and nonsaturable components were determined by substituting the values of the kinetic constants into Michaelis–Menten equation. Uptake by the saturable components was determined by subtracting the diffusional component from the total uptake at each concentration. After fitting the data to modified Michaelis–Menten equation, an uptake process with apparent K_m of 8.29 ± 0.74 nM, 17.03 ± 1.98 nM and 563.23 ± 115.2 nM and V_{max} of 393.47 ± 9.33, 757.58 ± 26.21 and 653.17 ± 31.7 fmol/(min mg) protein for folic acid, MTF and MTX, respectively.

3.1.2. pH and temperature dependence

To examine 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. No statistically significant difference was observed in the uptake of folic acid as a function of pH (Table 1). Therefore all experiments were carried out at pH 7.4. To determine temperature dependence of the transport mechanism, uptake of [^{3}H]Folic acid was performed at different temperatures. The rates of uptake were reduced by 26.6 and 53.4% at 20 and 4 °C, respectively, relative to 37 °C indicating that folic acid uptake is temperature dependant and the process works optimally at physiological temperature, 37 °C (Table 1).

3.1.3. Role of ions, energy and membrane transport inhibitors

In order to investigate ion dependency, Na^+ ions in the bathing media was replaced with equimolar quantity of K^+, NH_4^+ and choline chloride and Cl^- ions with salts of alternative organic and inorganic monovalent anions (phosphate and acetate). Significant inhibition (46.62%) was observed in the uptake of [^{3}H]Folic acid when chloride ion was substituted

Table 1
Effects of pH and temperature and role of ions in the uptake of [^{3}H]Folic acid in Y-79 cells

Characteristics	Conditions	No. of replicates	Uptake (fmol/(min mg) protein)
pH	5.0	5	28.61 ± 4.98
	6.0	5	22.71 ± 1.71
	7.4	5	25.11 ± 3.18
	8.0	5	24.13 ± 2.76
Temperature (°C)	37	4	27.51 ± 3.07
	22	4	20.19 ± 1.99
	4	4	12.82 ± 1.81
Sodium ions	Control (Na^+)	5	27.52 ± 3.51
	$Na^+ \rightarrow K^+$	5	21.98 ± 3.45
	$Na^+ \rightarrow$ choline	5	28.06 ± 2.51
	$Na^+ \rightarrow NH_4^+$	5	24.90 ± 3.08
Chloride ions	Control (Cl^-)	5	24.77 ± 3.67
	$Cl^- \rightarrow PO_4^{3-}$ and CH_3COO^-	5	13.22 ± 0.95

Values are expressed as means ± S.D.

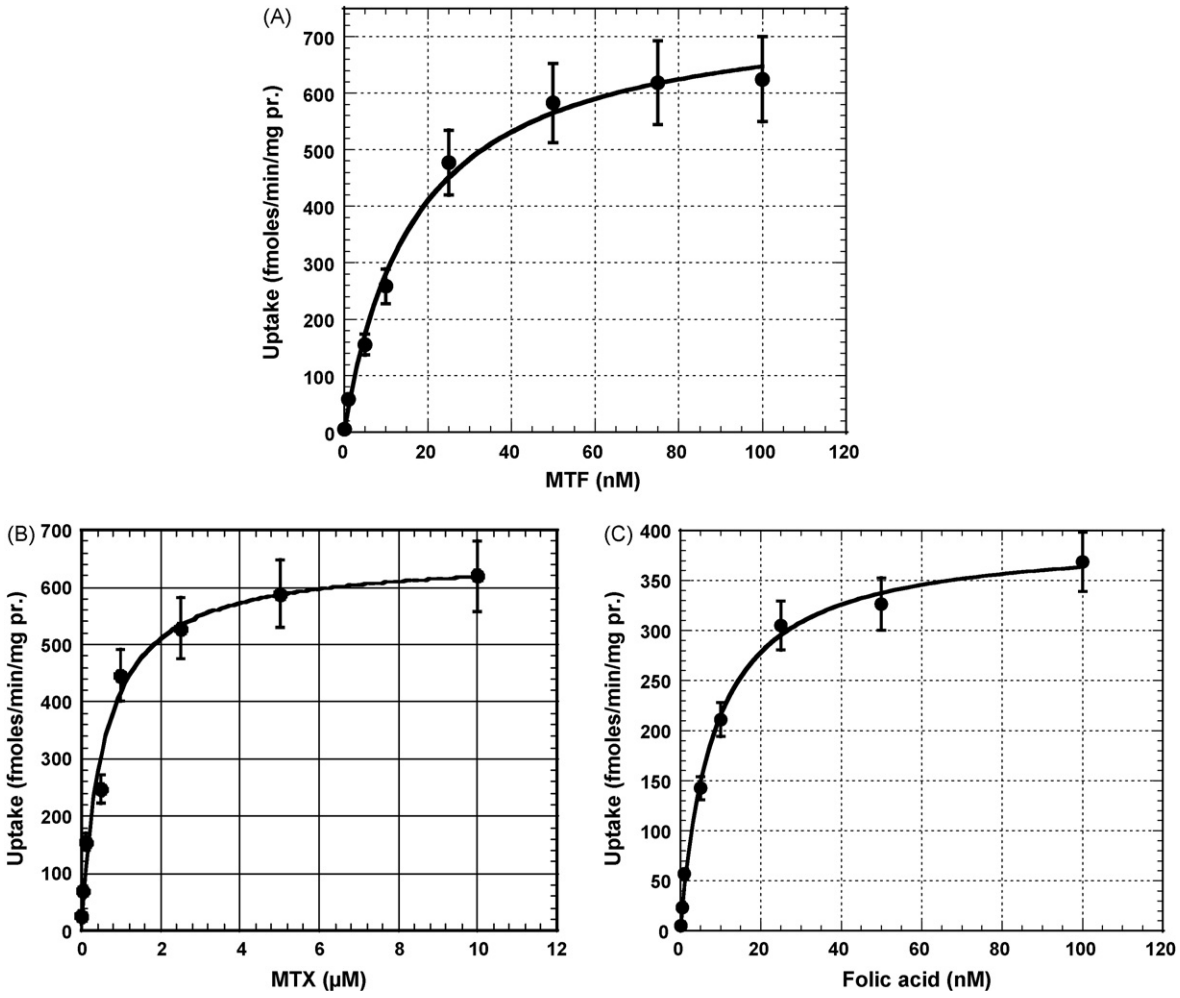

Fig. 1. Uptake of [^{3}H]Folic acid by Y-79 cells as a function of substrate concentration at 37 °C, pH 7.4. (A) MTF; (B) MTX; (C) folic acid. Solid line represents the calculated fit of the data to modified Michaelis–Menton equation as described in Section 2. The estimated active component to the total folic acid uptake is simulated with a solid line; the estimated passive component is deducted from the total uptake. Each data point represents the mean ± S.D. of four to five separate uptake determinations.

with phosphate and acetate, indicating that the uptake process is highly depend on the presence of Cl^- ions. However, no significant difference was observed in the uptake of [^{3}H]Folic acid in the absence of Na^+ ions (Table 1).

We further studied the effect of metabolic inhibitors on the uptake of [^{3}H]Folic acid. A Na^+/K^+-ATPase inhibitor (ouabain), intracellular ATP reducer (2,4-dinitrophenol; DNP) and oxidative phosphorylation inhibitor (sodium azide) were employed as metabolic inhibitors. The uptake was significantly inhibited by 44.1 and 53.7% as compared to control in the presence of sodium azide and DNP, respectively (Table 2). However, no significant difference was observed in the [^{3}H]Folic acid uptake

Table 2
Effects of energy inhibition by metabolic inhibitors (ouabain, DNP and sodium azide), membrane anion exchanger inhibitors (probenacid, DIDS and SITC) and endocytic inhibitor (colchicine) on the uptake of [^{3}H]Folic acid in Y-79 cells

Characteristics	Conditions	No. of replicates (n)	Uptake (fmol/(min mg) protein)
Control	–	6	28.51 ± 2.91
Metabolic inhibitors	Ouabain (1 mM)	6	21.32 ± 0.98
	DNP (1 mM)	6	15.94 ± 2.77
	Na-azide (1 mM)	6	13.20 ± 3.55
Membrane transport inhibitors	Probenacid (0.5 mM)	4	27.32 ± 2.71
	DIDS (0.5 mM)	4	29.02 ± 2.41
	SITC (0.5 mM)	4	22.01 ± 1.33
Endocytic inhibitor	Colchicine (100 μM)	5	5.21 ± 1.01

Values are expressed as means ± S.D. DNP, 2,4-di-nitrophenol; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; SITC, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid.

in the presence of ouabain (1 mM). Role of membrane transporter inhibitors was investigated by incubating the cells with probenecid, DIDS and SITC at 0.5 mM. No significant change in the uptake of [^{3}H]Folic acid was observed indicating that anion exchanger may not be involved in the uptake of folic acid. Moreover, significant inhibition (80.21%) of [^{3}H]Folic acid uptake in the presence of 100 μM colchicine suggests the involvement of receptor mediated endocytic process.

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]Folic acid uptake in Y-79 cells. The uptake rate was found to be 24.48 ± 2.41, 3.06 ± 0.61, 1.32 ± 0.44, 4.27 ± 0.67, 2.69 ± 0.56, 8.60 ± 0.95, 5.57 ± 1.56, 25.96 ± 4.18, 22.81 ± 2.30, 23.79 ± 0.46, 25.53 ± 2.52 and 21.76 ± 3.83 fmol/(min mg) protein for control and in the presence of folic acid (0.1 μM), folic acid (1 μM), MTF (0.1 μM), MTF (1 μM), MTX (0.1 μM), MTX (1 μM), biotin (10 μM), pantothenic acid (10 μM), riboflavin (10 μM), niacin (10 μM) and ascorbic acid (10 μM), respectively. As shown in Fig. 2, 87.5 and 94.6% of [^{3}H]Folic acid uptake was blocked in the presence of 0.1 and 1 μM of unlabeled folic acid, respectively. Significant inhibition in the uptake of [^{3}H]Folic acid was observed with MTF (82.6 and 89.1%) and MTX (64.8 and 77.2%) at a concentration of 0.1 and 1 μM. None of the unlabeled vitamins (biotin, pantothenic acid, riboflavin, niacin and ascorbic acid) showed any effect on the uptake of [^{3}H]Folic acid.

3.1.5. trans-Stimulation studies

Cells were preincubated with 1 ml of DPBS buffer (control) or 1 ml of DPBS buffer plus unlabeled folic acid (1–100 μM) and the uptake of [^{3}H]Folic acid was measured. If the uptake is a bidirectional transport system, the efflux rate of [^{3}H]Folic acid would be expected to be higher in the control compared with cells preincubated with unlabeled folic acid. As shown in Fig. 3, no significant difference was observed in the [^{3}H]Folic acid uptake between control and cells preincubated with folic acid (1–100 μM).

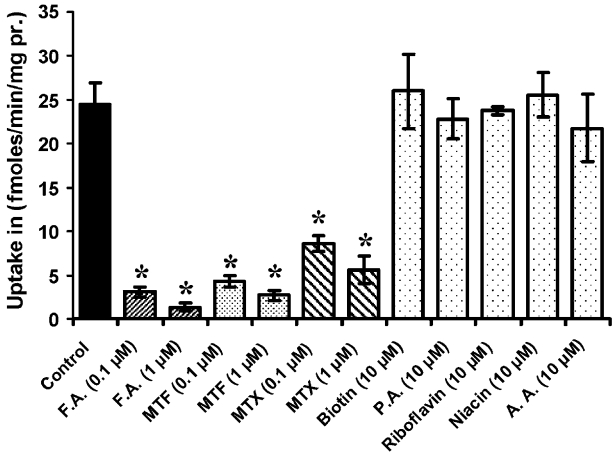

Fig. 2. Substrate specificity of uptake of [^{3}H]Folic acid (10 nM) by Y-79 cells in presence of structurally related and unrelated anagoges. Each data point represents the mean ± S.D. of four to five separate uptake determinations. Asterisk (*) represents significant difference from control ($p < 0.05$).

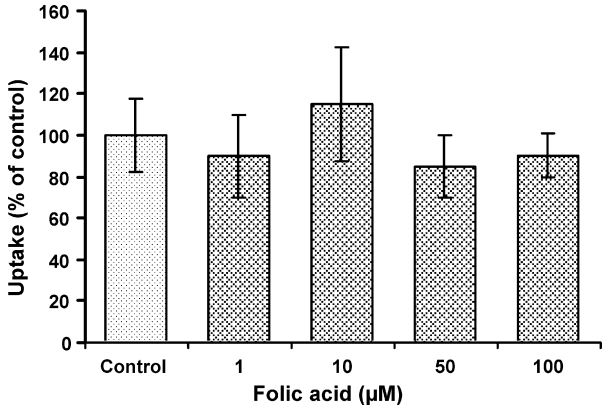

Fig. 3. *trans*-Stimulation study of [^{3}H]Folic acid uptake by Y-79 cells. Uptake is expressed as a percentage of control. Each data point represents the mean ± S.D. of four to five separate uptake determinations.

3.1.6. Role of intracellular regulatory pathways

The role of Ca^{+2}/calmodulin-mediated pathways in the regulation of [^{3}H]Folic acid uptake 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 ($p < 0.05$) and concentration dependent inhibition in folic acid uptake (Table 3). Involvement of a PKA-mediated pathway in the regulation of folic acid uptake was studied by examining the effect of pretreating Y-79 cells for 1 h with compounds that are known to increase intracellular cAMP levels (3-isobutyl-1-methylxanthine and forskolin) thus activating PKA. Results presented here suggest that 3-isobutyl-1-methylxanthine and forskolin significantly ($p < 0.05$) inhibit

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

Modulators (μM)	Uptake (fmol/(min mg) protein)
Control	28.61 ± 4.98
Calmidazolium	
10	22.19 ± 3.01*
50	16.87 ± 2.11*
100	11.97 ± 1.59*
250	7.38 ± 0.91*
TFP	
10	22.03 ± 0.92
100	19.87 ± 1.29*
250	12.06 ± 1.13*
KN-62	
0.1	27.11 ± 3.04
1	18.91 ± 2.90*
10	9.09 ± 1.52*

In these studies cells were preincubated with 1 ml of Ca^{2+}/calmodulin-mediated pathway modulators (calmidazolium, TFP and KN-62) and the uptake of [^{3}H]Folic acid was measured. Values are means ± S.E. of five separate uptake determinations.

* Significant difference from control ($p < 0.05$).

Table 4
Effect of PKA pathway modulators on the uptake of [^{3}H]Folic acid in Y-79 cells

Modulators (μM)	Uptake (fmol/(min mg) protein)
Control	23.11 ± 3.19
IBMX	
0.1	21.44 ± 1.30
1	18.09 ± 0.97*
2.5	15.07 ± 2.48*
5	10.83 ± 1.01*
IBMX (2.5 μM) + H-89 (100 μM)	25.18 ± 1.91
Forskolin	
1	24.08 ± 3.11
10	19.71 ± 2.09
50	13.29 ± 0.97*
100	10.01 ± 2.17*
Forskolin (50 μM) + H-89 (100 μM)	22.17 ± 3.15

In these studies cells were preincubated with 1 ml of PKA pathway modulators (IBMX, H-89 and Forskolin) and the uptake of [^{3}H]Folic acid was measured. Values are means ± S.E. of five separate uptake determinations.
* Significant difference from control ($p < 0.05$).

uptake of [^{3}H]Folic acid in a concentration dependent manner. The effect of the specific PKA inhibitor H-89 on the folic acid uptake was also examined. Forskolin and IBMX induced PKA activity was abolished by H-89 (Table 4). Reduction of folic acid uptake by cAMP modulators suggests involvement of cAMP-dependent protein kinase A (PKA) pathways in the regulation of membrane folic acid transport. In another study, we examined the involvement of PTK pathway in the regulation of folic acid uptake in Y-79 cells which were pretreated for 1 h with the PTK inhibitors, genistein, before initiating uptake of [^{3}H]Folic acid. Genistin is considered as a negative control for this inhibitor. No significant difference was observed in the uptake of [^{3}H]Folic acid in the presence of genistein (10–100 μM) or genistin. To delineate the possible role of PKC, uptake was studied by examining the effect of pretreating Y-79 cells for 1 h with PKC activator, phorbol-12-myristate-13-acetate (PMA) alone or with the PKC inhibitors, bisindolylmaleimide I. The PKC activator, PMA (1–100 μM), significantly ($p < 0.05$) inhibit the folic acid uptake. The inhibitory effect of PMA was abolished by bisindolylmaleimide I at a concentration of ≥50 μM (Table 5).

Table 5
Effect of PKC pathway modulators on the uptake of [^{3}H]Folic acid in Y-79 cells

Modulators (μM)	Uptake (fmol/(min mg) protein)
Control	25.17 ± 2.16
PMA	
10	20.01 ± 1.21*
50	14.29 ± 2.64*
100	10.72 ± 1.43*
PMA (100 μM) + bisindolylmaleimide I	
10	27.03 ± 3.11
50	21.92 ± 2.92
100	23.02 ± 1.56

In these studies cells were preincubated with 1 ml of PKC pathway activator (phorbol-12-myristate-13-acetate) and PKC pathway inhibitor (bisindolylmaleimide I) and the uptake of [^{3}H]Folic acid was measured. Values are means ± S.E. of five separate uptake determinations.
* Significant difference from control ($p < 0.05$).

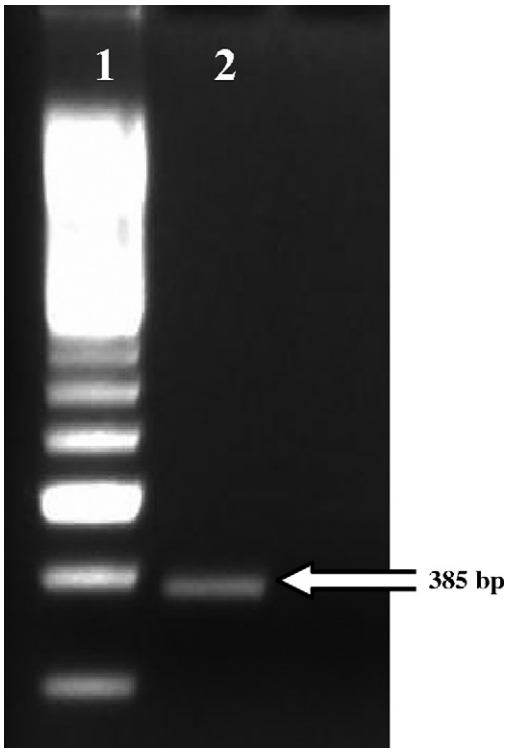

Fig. 4. Molecular identity of folate carrier systems in Y-79 cells at the mRNA level. PCR product obtained using RFC and FR alpha-specific primers and total RNA isolated from Y-79 cells. Aliquots of PCR products were analyzed by gel electrophoresis on 3% agarose gel. Ethidium bromide staining of the gel showed a major ~385 bp (lane 2) band corresponding to the amplified folate receptor precursor mRNA. Lane 1 represents 1-kbp DNA ladder.

3.2. Molecular evidence: RT-PCR analysis

The expression of the folate carrier systems in Y-79 cells at the mRNA level was determined by RT-PCR analysis. The PCR product was sequenced in both directions. A major band (385 bp) corresponding to the amplified folate receptor precursor mRNA (GenBank accession no. NT_033927.7) was observed (Fig. 4) by gel electrophoresis. The BLAST analysis showed that the primers used in this study can result in a PCR product size of 385 bp by binding to the folate receptor precursor mRNA.

4. Discussion

Human retina is a delicate organization of neurons, glia and nourishing blood vessels. Folic acid, a fundamental water-soluble vitamin, is essential for the survival of retinal cells since it is required for the synthesis of purine and pyrimidine based sysnthesis of nucleic acids, DNA and RNA (Prasad et al., 1995). Various reports have examined the critical role of folates 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 treat-

ment of intraocular tumors has succeeded to a certain extent in conjunction with cryotherapy or photocoagulation (Friedman et al., 2000; Shields et al., 2002). Various nutrient transporters (peptide, amino acids, glucose, 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 (Duvvuri et al., 2003). A specific carrier-mediated system for folic acid, if expressed on the retina, may be targeted following systemic and intravitreal administration to generate enhanced drug concentration in the retina.

A carrier-mediated transport system for folic acid has been well studied in various tissues including placenta, intestine, kidney, lungs, choroid plexus, liver and thyroid (Sabharanjak and Mayor, 2004). Presence of folate receptor has been investigated in the buccal carcinoma cell line (KB cells) (Ross et al., 1994), ovarian cancer (Miotti et al., 1995) and brain tumors (Weitman et al., 1992). Recently, Smith et al. (1999b) have analyzed the function, expression and distribution of two folate transport proteins in RPE. These studies have also demonstrated the polarized distribution of these two proteins in intact mammalian mouse retina and in cultured human RPE cells (ARPE-19). Various studies suggested that FRα is anchored to the basolateral side of the RPE whereas RFT-1 is distributed on the apical surface of the RPE (Smith et al., 1999a). Based on cellular distribution on the RPE, Smith et al. have hypothesized that FRα and RFT-1 function in a coordinated manner to perform the vectorial transfer of folate across the RPE cells. To date, however, only the transfer of folate on the RPE has been investigated in retinal tissue, but exact mechanism of uptake and intracellular regulation of folic acid translocation from RPE to the neural retina has not been studied.

Various studies suggested over expression of FR in the malignant cells and relatively low expression in most normal tissues. These differential expressions offer the development of diagnostic agents and folate receptor-mediated tumor targeted therapy in the treatment of various cancers (Theti and Jackman, 2004). Disruption of folate metabolism has long been known to inhibit cell growth and has been established as a target in chemotherapy with the use of antifolates (Brzezinska et al., 2000). Several antifolate drugs have high affinity for the FRs, similar to that of the folic acid and reduced folate cofactors (Wang et al., 1992). However, the role of FR, if present, in the activity of the antifolates for the possible treatment of retinoblastoma has never been evaluated.

Therefore, the major aim of the present study was to identify the presence of folic acid carrier system on human derived retinoblastoma cell line (Y-79) and to investigate the mechanism and intracellular regulation of folic acid uptake. In the present study, we have also explored the possible role of FR in the uptake of anticancer agent, MTX. 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 the neural retina and to facilitate the efficacy of antitumor regimens (McFall et al., 1977). Y-79 cell line has also been utilized as a model in vitro system to investigate metabolic characteristics and membrane properties of the retina (Tombran-Tink et al., 1992).

The use of [^{3}H]Folic acid to trace its structural analogs (MTF and MTX) has been reported earlier (Spinella et al., 1995; Verwei et al., 2005). In our study, [^{3}H]Folic acid (10 nM) uptake was found to be saturable as a function of concentration with an apparent K_m of 8.29 ± 0.74 nM, 17.03 ± 1.98 nM and 563.23 ± 115.2 nM and V_{max} of 393.47 ± 9.33, 757.58 ± 26.21 and 653.17 ± 31.7 fmol/(min mg) protein for folic acid, MTF and MTX, respectively. More recently, RFC, a high capacity–low affinity folic acid uptake system, with an apparent K_m in the micromolar range has been described in tissues such as the intestine and the liver (Horne et al., 1992; Said, 2004). However, in our study, concentration dependence uptake of folic acid (10 nM) showed saturation in nanomolar range (0.1–50 nM) and was linear at higher concentration in micromolar range indicating that high capacity–low affinity uptake system, RFC, may not be functional in Y-79 cells. These could be due to the fact that RFC has a lower affinity for folic acid (100–200 μM) than for MTX and reduced folates (∼1–5 μM) (Brzezinska et al., 2000). Existence of different folic acid uptake mechanisms at varying levels of expression depends on cell lines and/or inherent genetics of cancer cell lines. Despite these differences, a common trend is observed among various studies that folic acid uptake mechanism is specific and saturable in the range of nanomolar concentration within physiological levels of folic acid (10–50 nM) suggesting the involvement of FR in the uptake of folic acid in Y-79 cells.

Uptake process of folic acid was found to be pH independent as no significant difference was observed within a pH range of 5–8. However, it was found to be temperature dependent with optimal uptake at a physiological temperature of 37 °C. These observations were consistent with the previously published results using L1210, leukemia cells (Spinella et al., 1995). Presence of chloride-free buffer caused significant inhibition of [^{3}H]Folic acid uptake. These results suggest that chloride ions play a significant role in folic acid translocation. No significant difference in the rate of [^{3}H]Folic acid uptake was observed with sodium free buffer. Additional support for Na^+-independence has been shown through uptake studies performed in the presence of ouabain, a well-known Na^+/K^+-ATPase inhibitor. Presence of ouabain (1 mM) in the uptake buffer did not show any effect on the folic acid uptake, suggesting that this carrier-mediated transport is sodium independent process. In order to determine whether the uptake of folic acid is dependent on a motive energy force, known metabolic inhibitors (sodium azide and 2,4-dinitrophenol) have been employed. Significant inhibition of [^{3}H]Folic acid uptake was observed when cells were treated with sodium azide (1 mM) and 2,4-dinitrophenol (1 mM), which is known to reduce intracellular ATP. Thus, process of folic acid uptake was found to be energy dependant and appears to be directly coupled to ATP energy sources. These results clearly indicate the involvement of a specialized, energy and chloride dependant, high-affinity carrier-mediated system in the folic acid uptake which saturates at nanomolar range.

Possible involvement of a high affinity FR in the folic acid uptake was further supported by the finding of a significant inhibition of [^{3}H]Folic acid in the presence of unlabelled folic acid, MTF and MTX at concentrations $\leq$1 μM. Previous studies by Sierra et al. suggested that FR expressed at sufficient levels can be a significant transport route for folic and MTX at low blood levels (100–500 nM) and hence has pharmacological and physiological importance, specially in cells with impaired RFC function (Sierra et al., 1995). No significant inhibition in folic acid uptake was observed in the presence of various unlabelled vitamins (biotin, pantothenic acid, riboflavin, niacin and ascorbic acid). Taken together, these results provide additional support for the presence of a carrier system that specifically mediates the uptake of folic acid, MTF and MTX into Y-79 cells at nanomolar concentrations. Involvement of anion-exchange transport mechanism for MTX influx was observed in L1210 leukemia cells (Spinella et al., 1995). To delineate the involvement of anionic exchange transporter in the Y-79 cells, uptake of [^{3}H]Folic acid was performed in the presence of anion transport inhibitors i.e. probenecid, DIDS and SITC. No significant inhibition by any of this anion exchange inhibitors rules out the possibility of the involvement of an anion-exchange transport mechanism for folic acid uptake in the Y-79 cells. However, colchicines (100 μM) causes significant inhibition suggests that folic acid may be taken up via receptor mediated endocytosis process. However, further studies are required to confirm this conclusion.

A *trans*-stimulation phenomenon is a characteristic feature of an exchange transporter. Therefore, this study was performed to distinguish the role of FR and RFC in the uptake of folic acid in Y-79 cells, by evaluating whether folic acid is taken up by a unidirectional high affinity FR or by a bidirectional RFC mediated system. The presence of the unlabeled folic acid on the opposite side (*trans*) of the membrane had no effect on the flux of the radiolabeled folic acid suggests that the intracellularly accumulated unlabeled folic acid did not inhibit the efflux of the [^{3}H]Folic acid. This finding further supports the involvement of a high affinity carrier-mediated system, FR, in the uptake of folic acid on the Y-79 cells.

RT-PCR analysis provides the evidence of the molecular expression of folate receptor precursor mRNA and therefore further support the presence of a specific transport system for folic acid in Y-79 cells. This result was not consistent with the expected PCR product size (229 bp) of the seven known folate receptor transcription variants (variants 8, 2, 3, 4, 1 and 7) from BLAST analysis. However, post-transcription processing of this precursor mRNA may give rise to aforementioned transcription variants. RT-PCR analysis thus indicate that Y-79 cells may express folate receptor precursor mRNA.

Various 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 (Feschenko et al., 2000). We also investigated possible regulation of the folic acid uptake process by intracellular regulatory pathways. Concentration dependent inhibition by TFP, KN-62, and calmidazolium suggest the involvement of Ca^{2+}/calmodulin mediated pathways in the regulation of folic acid uptake. PKC pathway modulators (PMA and bisindolylmaleimide I) and PKA pathway modulators (IBMX, forskolin and H-89) also seem to play important role in the regulation of folic acid uptake. No significant effect was observed in the presence of PTK pathway modulators (genisetin and genistin) ruled out the involvement of PTK pathway on folate uptake. Summarizing, our results indicate the involvement of a Ca^{2+}/calmodulin, PKC and PKA pathway but not PTK pathways in the regulation of folic acid uptake. The physiological mechanism(s) through which calmidazolium and protein kinase pathways exert its regulatory effect on folic acid uptake is not very clear. Most signal transduction pathways are involved in diverse and critical cellular functions. Currently, physiological significance behind the multiple-signal regulating pathways involved in folic acid uptake in retina is poorly understood. Extensive cross-signaling between cAMP and calmodulin-mediated signal transduction pathways exists at several levels of cellular control. Therefore calmodulin-mediated reduction of folic acid 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 FR, a specialized high affinity carrier-mediated system for folic acid, on Y-79 cells. This carrier-mediated active transporter system is temperature, energy and chloride ion dependant. The process appears to be regulated by PKA, PKC and Ca^{2+}/calmodulin-mediated pathways. The study also provides useful information on the substrate specificity of this carrier system. The study suggests that Y-79 cells may serve as a useful in vitro experimental model of human retinoblastoma for delineating retinal uptake mechanism and intracellular translocation of various antifolate agents. Moreover, folate carrier-mediated system can be targeted through design of folate-conjugated prodrugs to achieve enhanced permeability which can result in significant improvement in therapeutic outcome of retinoblastoma.

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