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Identification of regulatory mechanisms of intestinal folate transport in condition of folate deficiency

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Abstract

Folic acid is an essential micronutrient, deficiency of which can lead to disturbance in various metabolic processes of cell. Folate transport across intestine occurs via the involvement of specialized folate transporters viz. proton coupled folate transporter (PCFT) and reduced folate carrier (RFC), which express at the membrane surfaces. The current study was designed to identify the regulatory mechanisms underlying the effects of folate deficiency (FD) on folate transport in human intestinal cell line as well as in rats and to check the reversibility of such effects. Caco-2 cells were grown for five generations in control and FD medium. Following treatment, one subgroup of cells was shifted on folate sufficient medium and grown for three more generations. Similarly, rats were fed an FD diet for 3 and 5 months, and after 3 months of FD treatment, one group of rats were shifted on normal folate-containing diet. Increase in folate transport and expression of folate transporters were observed on FD treatment. However, when cells and rats were shifted to control conditions after treatment, transport and expression of these genes restored to the control level. FD was found to have no impact on promoter methylation of PCFT and RFC; however, messenger RNA stability of transporters was found to be decreased, suggesting some adaptive response. Overall, increased expression of transporters under FD conditions can be attributed to enhanced rate of transcription of folate transporters and also to the increased binding of specificity protein 1 transcription factor to the RFC promoter only.

Keywords: Folate transport; Caco-2 cells; Intestine; Folate deficiency; Regulatory mechanisms

1. Introduction

Folate being an essential micronutrient is needed for normal human health and well-being. Normal microflora of large intestine can synthesize folate; however, the amount is insufficient to fulfill the metabolic requirements of the body [1]. Therefore, exogenous supply of this vitamin either through diet or through nutritional supplements is important. Folates are water soluble in nature and their extensive absorption takes place within intestine [2]. Since it is a negatively charged molecule, it cannot pass through the cell membranes and therefore requires specific membrane transporters for its transport in and out of the cell. Reduced folate carrier (RFC) and proton coupled folate transporter (PCFT) are the specific folate transporters present on the membrane surfaces of the intestinal cells [3]. Besides these two, folate receptor (FR) is another transporter that is not expressed in the intestine under normal conditions, but its expression is observed in the colon in cancer conditions [4]. PCFT, a 50-kDa protein in human cells, expresses at the apical surfaces of the cells and has acidic pH optima. Since intestinal lumen has an acidic pH, PCFT plays a major role in intestinal folate transport [5]. RFC is an organic anion antiporter and couples the transport of folic acid with organic anions, mostly phosphates [6]. This particular transporter has neutral pH optima and expresses at both brush border and basolateral surface of the cells [7–9]. FR has many isoforms, among which FRα is the most commonly expressed form. It is a glycosylphosphatidyl-inositol-linked protein with high affinity for folic acid and mostly expresses at the apical surfaces of polarized epithelial cells [10]. The expression of this particular transporter increases in cancer conditions, and therefore, its elevated levels are observed in cancerous cell lines as compared to their normal counterparts [11].

Folic acid is known to play important metabolic roles in various biological processes like synthesis of nucleotide precursors, methylation reactions, and so on [1,12]. Dietary folate deficiency is quite common in and around the world, and deficiency of this vitamin is linked to various metabolic disorders [13]. In United States, Canada and in some European countries, mandatory food fortification has been done in order to encounter the effects mediated by folate deficiency [14]; however, in a large proportion of the globe, folate deficiency is quite common because of absence of these fortification strategies [15]. It has been observed from the previous studies conducted either on rats or on cell lines that folate deficiency results in altered expression of folate transporters [9,12,16], although the exact underlying mechanisms are not known till date. Also, whether the effects mediated by folate deficiency are reversible or not has not been studied. So, the current study was designed to examine various

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aspects of regulation of folate transporters in conditions of folate deficiency across intestinal cells.

2. Materials and methods

Folic acid [3,5,7,9-^{3}H]-sodium salt acid with a specific activity of 40 Ci/mmol (>99% purity) was purchased from American Radiolabeled Chemicals, Inc., MO, USA. Cell culture reagents were all of cell culture grade and were obtained either from Sigma-Aldrich Co. or from Invitrogen, Life Technologies. TRIzol Reagent for RNA isolation was purchased from Ambion, Life Technologies Corporation, CA, USA, and complementary DNA (cDNA) synthesis was done using RevertAidTM M-MuLV-RT kit (MBI Fermentas, Life Sciences, USA). DyNAmo Flash SYBR Green qPCR Kit was obtained from Thermo Fisher Scientific Inc. Primary polyclonal antibodies against human RFC, PCFT, FR and rat RFC and PCFT were raised in rabbits using antigen specific to these transporters. Antigens specific to the transporters are described below under Western blot analysis. Other antibodies like β-actin, chromatin immunoprecipitation assay (ChIP)-grade KLF, HNF antibody and HRP-labeled goat–antirabbit immunoglobulin G (IgG) and antimouse IgG were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. ChIP-grade specificity protein 1 (SP1) primary antibody was purchased from Cell Signaling Technology, Inc. Enhanced chemiluminescence detection kit was purchased from Biological Industries Ltd. Kibbutz beit Haemek, Israel. EZ-DNA methylation gold kit was purchased from Zymo Research, Irvine, CA, USA, whereas M.SssI enzyme was purchased from New England Biolabs, Beverly, MA, USA. Protein A/G polyacrylamide beads (UltraLink Resin) were purchased from Thermo Scientific, Rockford, IL, USA. Dynabeads M-280 streptavidin was obtained from Invitrogen, Life Technologies, AS, Norway. Biotin-16-UTP was purchased from Roche Diagnostics GmbH, Mannheim, Germany. All other routine biochemicals were of molecular grade and were purchased from Sigma Chemical Co.

2.1. Cell culture

Caco-2 is a colon adenocarcinoma cell line that was obtained from NCCS, Pune. Caco-2 cells were routinely cultured on Eagle's modified minimum essential medium supplemented with 20% fetal bovine serum (FBS), sodium bicarbonate, penicillin (100,000 U/l) and streptomycin (10 mg/l). Cells were maintained in a humified incubator (95% humidity) with 5% CO_2 at 37°C. Medium of the cells was changed twice a week, and subculturing was done routinely once after 10 days. This cell line was chosen as it has the potential of spontaneous differentiation to small intestine columnar epithelial like absorptive cells in culture conditions. Besides this, these epithelial cells show polarity when grown on inserts and thus are an excellent model for transport studies.

For folate deficiency treatment, cells were grown on a commercially available folate-deficient medium supplemented with 20% dialyzed FBS for five generations. Five generations corresponded to around 13 days in control Caco-2 cells with a doubling time of 62 h. Dialyzed FBS has very low amounts of folic acid, that is, 0.6 nM [17]. This amount of folic acid is sufficient to sustain normal growth of cells up to a certain time period; thereafter, due to severe folate deprivation, cells may undergo apoptosis as severe folate deficiency is cytotoxic to cells. Culturing cells for five generations [16] on folate-deficient medium resulted in minimum loss of viability. Control cells were maintained under similar conditions in folate-containing medium (control for folate deficiency and control for folate repletion). After folate deficiency treatment, one group of cells was shifted on control medium with normal levels of folate (folate repletion) and cultured for three more generations (8 days) to see whether the effects mediate by folate deficiency are reversible or not.

After folate deficiency treatment, cellular viability determination was done using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT). For this assay, cellular medium was removed and replaced by medium containing MTT (0.5 mg/ml) followed by incubation at 37°C for 4 h. After that, cellular medium was replaced by dimethyl sulfoxide in order to dissolve formazan crystals. Absorbance was taken at 570 nm in a microplate reader, and viability of control cells was considered to be 100%.

2.2. Animals

Young adult male Wistar rats (120–150 g) were procured from central animal house facility available in the institute. Rats were maintained under proper hygienic conditions in polypropylene cages placed in the rooms of the animal house facility with controlled humidity (44%–55%), temperature (23±1°C) and 12-h dark–light cycle. Rats were randomized into five groups, with each group having six rats. Rats of group 1 (3-month control) and group 4 (5-month control) were fed on semisynthetic diet containing 2-mg folic acid/kg diet for 3 and 5 months, respectively. Group 2 (3-month folate-deficient) and group 5 (5-month folate deficient) rats were fed on folate-deficient diet for 3 and 5 months, respectively, whereas group 3 rats (3-month folate-deficient with 2-month folate supplementation) were fed for 3 months on folate-deficient diet and thereafter for 2 months on folate-containing diet (2-mg folic acid/kg diet). After treatment, animals from each group were sacrificed using sodium pentothal, and the small intestine was removed by cutting at the ligament of Trietz. Upper two-third of the small intestine was removed and flushed with saline 2–3 times using the blunt needle and syringe until the intestine was cleared.

The study protocol was approved by Institutional Animal Ethical Committee and Institutional Biosafety Committee.

2.3. Microbiological assay for folate level determination

Lactobacillus casei assay was performed as previously described in order to determine folate levels in rat serum, intestinal cells and Caco-2 cells [18,19]. For determination of intracellular total folate levels, folate extraction buffer (0.1 M potassium phosphate buffer, pH 7.0; 50 mM ascorbic acid; 10 mM 2-mercaptoethanol; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; and 1% Triton X100) was used to lyse Caco-2 and rat intestinal cells followed by centrifugation, and the supernatant was thus separated and treated with rat plasma conjugase. This enzyme hydrolyzed the polyglutamated folate to monoglutamated folate form which can now be utilized by the *L. casei* for its growth. Growth of this bacterium is directly proportional to folate levels. In order to calculate folate levels, standards of folic acid with known concentration were run along with the samples.

2.4. Isolation of rat intestinal epithelial cells and preparation of brush border and basolateral membrane vesicles

Epithelial cells were isolated from rat intestine using the protocol of Weiser [20] with modifications as described earlier [21]. Thereafter, brush border membrane vesicles (BBMVs) were prepared from these intestinal cells using the protocol as described by Kessler et al. [22] with some modifications [23]. The basolateral membrane vesicles (BLMVs) were prepared as described earlier [8] by using the self-generating Percoll gradient method [24]. The vesicles were thus isolated and used further for transport and protein expression studies.

2.5. Transmembrane transfer of [^{3}H]-folic acid in Caco-2 cells and rat BBMVs and BLMVs

For transmembrane transfer experiments, cells were cultured on 12-well inserts (1.12 cm^2, 0.4-μm pore size; Corning, NY, USA). After a week of seeding, folate-deficient treatment (as mentioned above) was given to the cells, whereas control cells were grown as such. Culture medium was changed every second day. Before performing the transmembrane transfer experiments, monolayer integrity was checked by transepithelial electrical resistance (TEER) measurement using Millicell ERS epithelial voltohmeter. The TEER values were found to be greater than 400 Ω cm^2 and therefore considered to be nonleaky [25]. Transmembrane transfer experiments were carried out in Kreb's Ringer (KR) buffer containing [^{3}H]-folic acid. In order to study the membrane transfer of folic acid across apical surfaces where transport is mainly governed by PCFT, KR buffer of pH 5.5 was used as PCFT has acidic pH optima and transport efficiency of this transporter is maximum at this pH. On the other hand, for assessing transmembrane movement of [^{3}H]-folic acid across the basolateral side, KR buffer of pH 7.4 was used because RFC is the main folate transporter at this surface and works maximally at the neutral pH optima. Culture medium was removed from both the compartments, and inserts were washed with prewarmed blank KR buffer (without radiolabeled folic acid) followed by the addition of 0.5 ml of KR buffer containing [^{3}H]-folic acid in the apical compartment and 1.5 ml of blank KR buffer in the basolateral compartment. The movement of folic acid from apical (AL) to basolateral (BL) compartment was measured by taking out 100 μl of the sample from basolateral compartment after 60, 120, 180 and 120 min of incubation. Similarly, for studying transmembrane transfer from basolateral (BL) to apical compartment (AL), [^{3}H]-folic acid was added on the basolateral side and samples were collected from the apical side after particular time periods. To the collected samples, 10 ml of scintillation fluid was added and read on a liquid scintillation counter (Beckman Coulter LS 6500).

The apparent permeability coefficients (P_{app}) were calculated using the following formula [25]: $P_{app}=(dQ/dt)/A^*C_0$ (cm/s), where dQ/dt=permeability rate (mol/s), A=cross-sectional surface area of the filter insert (1.12 cm^2) and C_0=initial concentration in the donor compartment (mol/ml).

In order to study effect of folate deficiency on cellular accumulation of folic acid, inserts were removed from 12-well plate after transport studies and monolayers were washed twice with phosphate-buffered saline (PBS) followed by the addition of 0.5 ml of 1.5 M KOH solution in ethanol. Inserts were incubated overnight at 37°C, and next day, the cell lysates were added into 10 ml scintillation liquid and radioactivity was read in a scintillation counter [25].

To assess the specificity of transmembrane transfer, structural analogs and inhibitors of transporters were used. Among the structural analogs, unlabeled folic acid (5 μM) and methotrexate (5 μM) were used and added in the [^{3}H]-folic acid containing KR buffer. Among the inhibitors, hemin (weak inhibitor of PCFT) and thiamine pyrophosphate (TPP) (strong inhibitor of RFC) were used at the concentration of 100 μM.

Uptake of [^{3}H]-folic acid across rat BBMV and BLMV was studied as previously described [8,26,27] using incubation buffer (100 mM NaCl, 80 mM mannitol, 10 mM HEPES, 10 mM 2-morpholinoethanesulfonic acid), containing 0.5 μM of [^{3}H]-folic acid. Uptake of [^{3}H]-folic acid across BBMV and BLMV was studied as a function of time, pH, substrate concentration and structural analogs. Kinetic parameters, K_m and V_{max}, were determined among all the groups.

2.6. Quantitative reverse transcriptase polymerase chain reaction analysis

RNA was isolated from Caco-2 cells and rat intestine using TRIzol reagent (Ambion, Life Technologies Corporation) according to the manufacturer' instructions. After