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The relationship between folate transport activity at low pH and reduced folate carrier function in human Huh7 hepatoma cells

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Abstract

Transport of folates and antifolates in both hepatocytes and Huh7 human hepatoma cells is characterized by a low-pH optimum. Studies were undertaken to determine the extent to which this transport activity is mediated by the reduced folate carrier (RFC) in Huh7 human hepatoma cells. RFC expression was ablated by chemical mutagenesis and antifolate selective pressure with PT632 resulting in the PT632^R subline in which RFC mRNA could not be detected. Methotrexate (MTX) influx in these cells at pH 7.4 was reduced by 70%, leaving substantial residual RFC-independent influx while influx of MTX and folic acid at pH 5.5 was not significantly decreased. The influx K_t for folic acid and MTX at pH 5.5 in PT632^R cells was 0.36 and 1.5 μM, respectively. The affinity of the low pH transporter in PT632^R cells was highest for pemetrexed (K_i=140 nM), very low for PT632 (K_i=77 μM), and was stereospecific for the natural isomer (6S) of 5-formyltetrahydrofolate. In Huh7 cells transiently transfected with an RFC siRNA, RFC expression was reduced by 60% resulting in a 40% decrease in MTX influx at pH 7.4 but only a very small (5%) reduction in MTX or folic acid influx at pH 5.5. These data indicate that MTX transport in Huh7 cells at neutral pH is mediated largely by RFC while at pH 5.5 the predominant route of transport is independent of RFC.

Keywords: Folate transport; Folic acid; Methotrexate; Pemetrexed; Acidic pH

1. Introduction

Membrane transport of folates and antifolates into cells has been an area of considerable interest because of the important roles that natural folates play in biosynthetic processes, and that antifolates, primarily methotrexate (MTX), have played in cancer chemotherapy. The mechanisms of folate transport were recently reviewed [1]. Best characterized of these transport mechanisms has been the reduced folate carrier, (RFC, SLC19A1), an anion exchanger that has the properties of a classical facilitative carrier. Two folate receptors, FRα and FRβ, high-affinity membrane binding proteins, are endocytotic routes that transport folates unidirectionally into cells [1]. In addition, some members of the organic anion families of transporters (SLC21 and SLC22) transport folates and antifolates [2–4]. Beyond this, it has become clear that there is another folate transport activity (or activities) with a low pH optimum. This low-pH activity represents the most robust of the folate transport routes in small intestine [5], is present in a variety of other primary tissues or cells [6–9] and in the majority of human solid tumor cell lines, where transport at low pH is equal to, or greater than, transport at physiological pH [10].

While it has been suggested that transport at low-pH in small intestinal cells is mediated by RFC [11,12], studies from this laboratory have shown that when RFC function is eliminated due to mutations in the protein in rat small

Abbreviations: 5-CHO-THF, 5-formyltetrahydrofolate; DHFR, dihydrofolate reductase; MTX, methotrexate; RFC, reduced folate carrier; PT523, Nα-(-4-amino-4-deoxypteroyl)-Nδ-hemiphthaloyl-1-ornithine; PT632, 5,8-dideaza analog of PT523

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intestinal cells (IEC-6), there is no change in MTX influx at low pH [13]. Further, when RFC was deleted from the genome, transport of MTX in HeLa cells at low pH was not reduced [10]. This report extends studies on the relationship between RFC function and transport mediated at low pH to hepatic cells where the predominant folate transport activity in freshly isolated hepatocytes and hepatomas cells has a low pH optimum [14,15]. The model chosen was the Huh7 hepatoma cell line which exhibits a very high ratio of low-pH to neutral-pH MTX transport activity [10]. Two approaches were employed to determine the impact of suppression of RFC expression on MTX transport activity at low and physiological pH; chemical mutagenesis with antifolate selective pressure and gene silencing using RFC siRNA.

2. Materials and methods

2.1. Chemicals

[3′,5′7-^{3}H] MTX and [3′,5′,7′,9-^{3}H] folic acid were purchased from Moravek Biochemicals (Brea, CA). Non-labeled folic acid and 5-CH_3-THF were obtained from Sigma while 6S- and 6R-5-CHO-THF were purchased from Schircks Laboratories (Jona, Switzerland). PT 523 and PT 632 were gifts from Dr. Andre Rosowsky (Dana-Farber Cancer Institute, Boston). Pemetrexed was provided by Eli Lilly Co. (Indianapolis, IN) while trimetrexate was obtained from Warner-Lambert (Ann Arbor, MI). All other reagents were obtained in the highest purity available from commercial sources. Radiochemicals and nonlabeled folates and antifolates were purified by liquid chromatography [16].

2.2. Cell culture and selection of RFC-null sublines

Huh7 cells were obtained from the liver center of Albert Einstein College of Medicine and maintained in RPMI 1640 medium containing 2.0 μM folic acid, supplemented with 10% fetal calf serum (Hyclone), 2 mM glutamine, 20 μM 2-mercaptoethanol, penicillin (100 U/ml), and streptomycin (100 μg/ml). For selection of RFC-null Huh7 sublines, Huh7 cells were treated with 2.4 mM ethylmethanesulfonate for 20 h to achieve about 90% cell kill. Cells were allowed to recover for 2 days after the mutagen was washed away and were then reseeded in 100 mm dishes in the presence of 5 or 15 nM PT 632. After 2 weeks, one surviving clone was detected in a plate containing 5 nM PT632 and another clone was visible in a plate containing 15 nM PT632. Both clones were expanded and maintained in medium containing the respective amount of PT632. Preliminary results indicated that both clones had similar resistance to antifolates, lack of RFC mRNA expression, and impaired influx of MTX and folic acid (see Results). The clone selected with 5 nM PT632 was chosen for detailed studies and named PT632^R. Cell cultures were monitored regularly with a mycoplasma detection kit (ATCC) and were shown to be free of this microorganism.

2.3. Cell growth inhibition

Cells were trypsinized, transferred to 96-well plates (1000 cells/well) and exposed continuously to a spectrum of antifolate concentrations for 6 days. Cell growth rate was quantified by sulforhodamine B staining [17].

2.4. Transport studies

Cells ($4-5\times10^5$) were seeded in 20-ml Low Background glass vials (Research International Corp., Prospect, IL) and grown for 3 days to reach confluency. For RFC-null cells, PT632 was present in the medium to maintain clonal stability but was not added to vials seeded for transport studies. Transport measurements were made in cells in monolayer cultures adherent to the glass vials as previously described [18]. Briefly, cells were washed twice in ice cold transport buffer following which 1 ml of the buffer at 37° was added and cells equilibrated at this temperature for 20 min. The buffer was then aspirated and uptake initiated by the addition of 0.5 ml of fresh buffer containing the radioactive substrate and other reagents. Uptake was terminated by the addition of 5 ml of ice cold HBS (see below) following which cells were washed three times with the same buffer and lysed in 0.5 ml of 0.2 N NaOH at 65 °C for 30 min. A 0.4-ml portion of cell lysate was assayed for radioactivity after addition of scintillation fluor (8 ml). Another 10 or 20 μl of lysate was processed for protein determination (BCA Protein Assay, Pierce, Rockford, IL). Cell antifolate is expressed as pmoles per milligram of protein.

The following buffers were used in these studies. HBS (HEPES-buffered saline) at pH 7.0, 7.4, and 8.0 adjusted with NaOH (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose). MBS (4-morpholinepropanesulfonic acid-buffered saline) at pH 5.0, 5.5, 6.0 and 6.5 adjusted with NaOH (20 mM 4-morpholinepropanesulfonic acid, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose). Na^+-free HBS at pH 7.4 or Na^+-free MBS at pH 5.5 were prepared by replacing 140 mM NaCl in HBS or MBS with equimolar choline chloride. Some HBS at pH 7.4 or MBS at pH 5.5 was made glucose-free. Sucrose buffer at pH 7.4 consisted of 20 mM HEPES, 225 mM sucrose, adjusted to pH 7.4 with $Mg(OH)_2$. Sucrose buffer at pH 5.5 consisted of 20 mM 4-morpholinepropanesulfonic acid, 225 mM sucrose, pH adjusted with $Mg(OH)_2$.

2.5. RNA isolation and Northern Blots

Total RNA was isolated from cells with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA (30 μg) was resolved by