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FOLATE RECEPTORS

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ABSTRACT

Glycosyl-phosphatidylinositol-anchored folate receptors (FR) have physiologic and pharmacologic relevance in mediating cellular and transcellular folate/antifolate transport. Three FR isoforms with differing relative affinities for folates and expression patterns in normal and malignant cells/tissues are recognized, but the precise mechanism of cellular entry of folate via FR remains controversial. Although FR expression allows previously FR-deficient cells to survive a reduced folate milieu, an inverse relationship between FR expression and cell proliferation has been established in some cells. The inverse regulation of FR expression by the extracellular folate concentration suggests heterogeneity in underlying mechanisms. Whereas reduced FR expression is yet another mechanism for acquiring antifolate resistance, overexpression of FR does not invariably render cells more sensitive to antifolates. The exploitation of FRs as Trojan horses to deliver folate-tagged liposomes bearing diverse cargo represents a novel therapeutic strategy to target FR-expressing cells. Finally, a critically important role of human placental FR in mediating maternal-to-fetal transplacental transport of folates has been established. Thus, FR appear to have a major impact on several aspects of human physiology and medicine.

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INTRODUCTION

The shutdown of DNA synthesis and one-carbon metabolism arising from folate deficiency perturbs the cell cycle and results in megaloblastosis, which, if uncorrected, leads to premature cell death, with characteristic clinical presentations (5). Acquisition of folate, therefore, is critically important to the viability of proliferating cells (4, 28). In general, folate transport involves translocation of the ligand into cells from the extracellular compartment (i.e. cellular uptake mechanisms) and across cellular barriers from one compartment to another (i.e. transcellular mechanisms). Since the last comprehensive review on the biological chemistry of folate receptors (FR) (4), several studies have provided new insights into their biological function. This review highlights these issues, with an emphasis on nutritional aspects of FR-mediated cellular and transcellular folate transport. [Due to space limitations, the bibliography has been generally restricted (with the exception of molecular biology–related data) to those papers published in the past five years. The reader is referred to previous reviews (4, 28) for important references of historical interest.]

The basis for separate classification of the components and mechanisms for cellular folate transport was outlined previously (4). The reduced-folate carrier (RFC) is a low-affinity, high-capacity system that mediates the uptake of reduced folates into cancer cells, predominantly at pharmacologic (micromolar) extracellular folate concentrations (EFC). A RFC cDNA (22) that restores sensitivity to a methotrexate transport-resistant cell line functionally deficient in RFC encodes a 58-kDa polypeptide and resembles the mammalian glucose transporter (GLUT 1), a member of the 12 transmembrane domain–spanning membrane transporter family. Although RFC systems have not been definitively shown to exist in normal human cells through functional studies, this deficiency in data should be rectified shortly. Cellular folate transport can also be mediated by 38- to 44-kDa membrane-associated folate-binding proteins (FBP) or FR (these terms are used synonymously throughout), which bind physiologic folates with high affinity in the nanomolar range. The third pathway for cellular folate transport via passive diffusion was only documented as a pharmacologic effect (4). However, new data indicate that it is an integral part of transplacental folate transport occurring in concert with FR (29).

Transcellular folate transport systems include transport across the placenta, renal tubular cells, and the blood-brain barrier/blood–cerebrospinal fluid barrier. Although these barriers overexpressed FR (4), their functional role has been unclear until recently.

STRUCTURE OF FOLATE RECEPTORS

Molecular and Biochemical Aspects

Three human FR cDNA isoforms, FR-α (14, 19, 24, 37, 65), FR-ß (53, 58), and FR-γ (69, 70), and two related cDNAs from murine L1210 cells (12) have been cloned. Although the open reading frames and the 3′-untranslated regions of the reported FR-α cDNA are identical, their 5′-untranslated regions are heterogeneous in length and sequence. The genomic organization of human FR genes (14, 57) has been identified in chromosome 11q13.2→q13.5, where four FR-related genes were found within a 140-kilobase (kb) region. The FR-α and FR-ß genes were in sequence (< 23 kb apart), with two additional FR-related genes or pseudogenes located upstream of the FR-α gene. Genomic clones containing FR-ß have been independently isolated by Elwood's and Rothenberg's laboratories (53, 64). The FR-ß gene is ~5 kb long and has five exons and four introns. The promoter lacks TATA and CAAT elements but contains sequences recognized by the *ets* oncogene-encoded transcription factor and SP1, which may regulate expression of FBPs (64). The genomic DNA sequence of the FR-α gene is not yet published, but its structure is more complex than FR-ß. Although the number of exons and introns were different in two preliminary studies (53, 63), a recent report indicates that FR-α contains at least two independent, tissue/cell–specific functional promoters. Interestingly, one promoter is located within an intron and contains three clustered SP1 binding sites and an initiator region, all of which are necessary for basal promoter activity (67).

With possible exceptions, FR are glycosyl-phosphatidylinositol (GPI) anchored (43, 73, 77). [Although FR-γ was originally believed to be GPI anchored (69), further analysis (70) revealed it was a secretory form predominantly expressed in hematopoietic cells.] The ω locus is the amino acid to which a preformed GPI anchor is added following posttranslational cleavage of a carboxyl-terminal domain of the nascent polypeptide by a GPI transamidase (8). Although data from the cDNA and amino acid analysis of a GPI-anchored protein can predict the ω locus with ~100% accuracy (8), this locus has not been ascertained directly from mature FR. Now, recent molecular studies on FR-α and FR-ß suggest Ser-234 and Asn-230, respectively, as the preferred sites of GPI-anchor attachment (85).

Ligand Binding by FR

Analysis by circular dichroism has identified conformational changes following folate binding to FBP (35), and the increase in folding stability induced by ligand binding is derived from ligand-induced aggregation of these proteins.

There are diverse intracellular folate-dependent enzymes with little homol-

ogy in their primary structure to FR/FBP that interact with folates (5). Although human saliva contained species with low epitope relatedness to FBP, only a minor fraction of these FBP specifically bound folate with high affinity. The major fraction did not contain bound endogenous folate and did not bind radiolabeled folates (i.e. nonfunctional FBP) (76). Other mutant FBPs have subsequently been described (51). In general, however, nonfunctional FBPs can be mimicked in numerous trivial ways: These relate to prior occupancy of the ligand-binding site by folate (21), the presence of potential inhibitors of folate binding (72), destruction of the ligand-binding site of FBPs shortly after synthesis by potential proteases, cross-reacting species with a different preferred ligand than folates (86), or aglycosylated forms not yet posttranslationally processed to acquire functionally active ligand-binding sites (46). Thus, it is imperative to subject putative nonfunctional proteins to rigorous biochemical tests (ideally, after their isolation) before a FBP can confidently be assigned nonfunctional status. For example, in the instance that a nonfunctional FBP binds endogenous folate with a higher affinity than normal folate, there is the possibility that bound endogenous folate may not be dissociated by low pH (the method conventionally used). Naturally, this FBP will not bind exogenous radiolabeled folate, leading to the erroneous conclusion that the FBP is a nonfunctional species. Denaturation of FBPs will release endogenous-bound folates, with restoration of ligand-binding capacity on subsequent renaturation (76). Therefore, at a minimum, any putative nonfunctional species must withstand this degree of experimental scrutiny to eliminate trivial explanations. No less unusual are dominant negative phenotypes of mutant FR (52), but here additional controls are necessary. Clearly, demonstration that isolated mutant FR also confer a dominant negative phenotype to endogenous FR and assurance that transfected plasmids did not alter the regulation of endogenous FR, or sequester them into subcellular compartment(s) inaccessible to radiolabeled folate applied to the cell surface, would reduce ambiguity. Such studies could also provide an intellectual framework for understanding the functional basis of these mutant phenotypes. This concept (52) is nevertheless intriguing and again highlights a relationship between aggregation of FR and changes in affinity for folate. Finally, it would be of exceptional interest if dominant negative FR were actually identified in vivo.

Ratnam's laboratory has determined that FR-α are predominantly expressed in most normal and malignant epithelial tissues (with the exception of sarcomas) (60). This is significant because FR-α and FR-ß exhibit differences in relative affinities (compared with folic acid) for the (6*S*) (physiologic) and (6*R*) (unphysiologic) diastereoisomers of various folates (80). For example, (6*S*)5-methyltetrahydrofolate, (6*S*)5-formyltetrahydrofolate, and methotrexate bound FR-α with ~50-, ~100-, and ~20-fold more affinity, respectively. However, (6*R*) forms bound FR-α only ~2- and 4-fold more avidly. In contrast,

FR-ß bound (6*R*) forms ~7 and 12 times more tightly than (6*S*) forms. Such data have been confirmed with murine FR (11). The newer antifolate, (6*S*)5,10-dideazatetrahydrofolate, which is preferentially transported at nanomolar concentrations via FR (54), showed ~10-fold greater affinity for FR-α than for FR-ß; these (6*S*) forms also had ~3-fold higher affinity for FR-α than (6*R*) forms had. Conversely, (6*S*) and (6*R*) forms had comparable affinity for FR-ß. Thus, pending direct experiments, a reasonable assumption is that the net efficiency of transport of various folates into cells would depend on the relative expression of one FR isoform over the other. Such data may eventually be important in optimizing efficient delivery of antifolates to effect maximal cytotoxicity of target cells while protecting normal cells.

Although limited site-specific mutagenesis has not yet characterized the ligand-binding site of FR (16), some unusual issues related to this domain in salivary FBPs (76), in different FR isoforms (11, 80), and in nonfunctional/mutant FBPs may only be resolved by analysis of the crystal structure of these proteins, which will shortly be reported on.

TRANSCELLULAR FOLATE TRANSPORT VIA FOLATE RECEPTORS

Maternal-to-Fetal Transplacental Folate Transport

Pregnancy is the most common cause of megaloblastic anemia in adults worldwide; and with lactation, folate requirements increase 5- to 10-fold more than in nonpregnant women (to 300 – 400 g/day), for growth of the fetus, placenta, and maternal tissues (5). This demand for folate, which is further aggravated by increased folate catabolism during pregnancy (50), must be met by adequate dietary intake. Since folate deficiency during pregnancy leads to decreased placental weight and premature, low-birth-weight infants, administration of folate supplements from the outset of pregnancy diagnosis has been routine (5, 6). However, the landmark study from Hungary indicates the necessity of periconceptional folate supplementation for healthy women to reduce the incidence of neural-tube defects (spina bifida, meningocoele, anencephaly) in their babies (20). In addition to demonstrating that periconceptional folic acid in higher doses (4 mg/day) protects ~75% of fetuses of women at risk (50a), such data highlight the importance of folates in neurologic development. But how do folates traverse the placenta from mother to fetus?

The clinical observations (5) that folate-deficient mothers deliver babies with normal folate stores invariably recall the metaphor of the fetus as a parasite. But this fact is also a vivid, physiologically relevant "experiment of nature" (and thank God for such reproducible and successful experiments!) that proves there exists a clearly defined mechanism to protect the fetus from

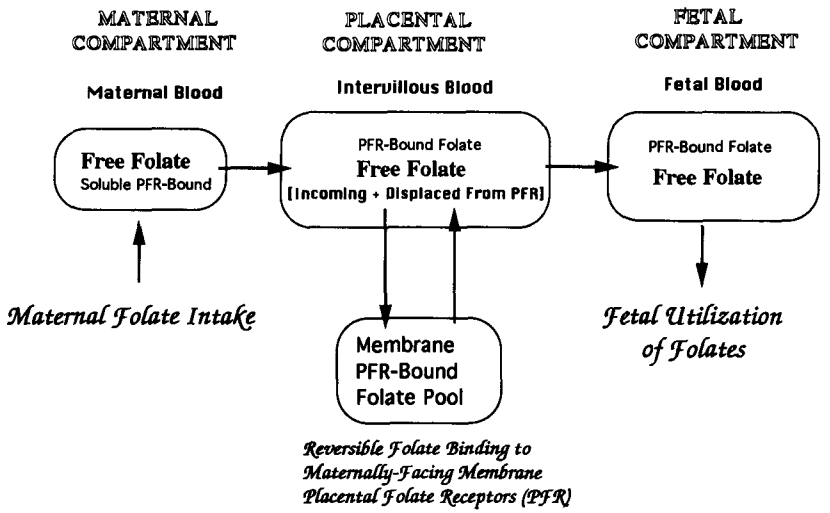

Figure 1 Diagrammatic representation of maternal-to-fetal transplacental folate transport involving folate receptors. (Adapted from Reference 29.)

the consequences of folate deficiency during critical stages in its growth and development. Curiously, although this system is concentrative and resistant to decreased maternal folate, it is sensitive to maternal folate loads such that, when presented with higher-than-normal folate levels, transport to the fetus is enhanced. So why has such a remarkable homeostatic system that favors fetal folate acquisition not been mechanistically defined? A major stumbling block has been the paucity of good models. Accordingly, with the collective experience of GI Henderson and S Schenker, acknowledged leaders in this field, an ex vivo placental cotyledon perfusion model was used to test the hypothesis that under physiologic conditions, placental FRs played a major functional role in transplacental folate transport. This model had a decided advantage in allowing individual perfusion and sampling of maternal and fetal compartments in studies that lasted ~4 h per freshly delivered placenta.

The results of these collaborative studies (29) indicate that net maternal-to-fetal folate transfer is a process consisting of two steps. The first step is the concentrative component in which circulating 5-methyltetrahydrofolate is bound to (captured by) placental FR on the maternally facing chorionic surface. Although kinetics favor binding, a dynamic state exists wherein a gradual release of 5-methyltetrahydrofolate from this pool adds to incoming circulating folates to generate an intervillous blood level approximately three times that in the maternal blood. In the second step, folates are transferred to the fetal

circulation along a downhill concentration gradient. Thus, it turns out that the prodigious, reversible, high-affinity binding of maternal folates by placental FR (77) is the key to mediation of transplacental folate transport. In fact, once captured, placental FR-bound folates are predestined for transplacental folate transport, since incoming (dietary) folates displace placental FR-bound folates that then passively diffuse down a concentration gradient to the fetus. And so this elegant cycle goes on, assuring continued unidirectional transplacental folate transport (Figure 1). As only FR-α was identified in normal trophoblasts (56), this form likely mediates this process. [FR-ß may have derived (4, 58) from sheared maternal decidua during delivery.]

Renal Folate Conservation

Selhub and colleagues have accumulated data that strongly support the conclusion that folate transport across proximal renal tubular cells involves FR-mediated endocytosis (briefly reviewed in 68). Significantly, electron microscopy and autoradiography following infusion of radiolabeled folate revealed movement of the label as a function of time from proximal tubule brush borders to endocytotic vesicles and lysosomes (30). Thus, a likely scenario is that after glomerular filtration, the luminal folate binds FR in the brush-border membranes of proximal renal tubular cells and is internalized rapidly via FR-mediated endocytosis; in the low pH of endocytotic vesicles, there is dissociation of the folate and slow transport across the basolateral membranes into the blood with recycling of apo-FR back to the luminal brush-border membrane (68).

CELLULAR TRANSPORT OF FOLATES VIA FOLATE RECEPTORS

As highlighted earlier (4), the pathway(s) for entry of folates and antifolates is likely to be distinct in different cells, depending on the relative efficiency of FR- and RFC-mediated mechanisms, as well as on the intra- and extracellular concentration of folates and antifolates.

Is there anatomic or functional coupling between the FR and RFC? Earlier studies had established that FR-mediated uptake was quite independent of, and did not require the participation of, the RFC (4). Independent data from a variety of models, including transplacental folate transport (29), cloning of RFC cDNA (22), and transfection of RFC-defective cells with FR cDNA (23, 72), have come to similar conclusions. However, this ignores earlier data from Kamen and Anderson's group, which spawned the concept of linkage of FR and the RFC/(anion channels) in potocytosis, the paradigm for receptor-mediated uptake of small molecules via caveolae (caveolae are invaginations in the

plasma membrane and are distinct from classic clathrin-coated pits, which are involved in receptor-mediated endocytosis) (2, 3, 61). In this hypothetical model, folates bind to GPI-anchored FR, which then moves into caveolae. The mouths of caveolae then transiently seal, and the enclosed FR-bound folate is dissociated by acidification followed by transport of the released folate into the cytoplasm via anion channels, while apo-FRs are recycled to bind more folate.

The finding that probenecid-inhibited uptake of folate suggested that anion channels were involved in *trans*-caveolar translocation of the ligand to the cytosol (36). Moreover, while activators of protein kinase C did not inhibit endocytosis via clathrin-coated pits, they inhibited the internalization of folate coincident with demonstration of a reduction in the number of invaginations of caveolae (71). Furthermore, lowering the cholesterol content of cells inhibited FR-mediated folate transport coincident with disruption of the clustered organization of FR and the integrity of caveolae (15). However, Goldman's laboratory recently reported (72) that probenecid inhibits folate interaction with FR in a dose-dependent manner. These data have indirectly raised questions about the validity of earlier conclusions from studies with probenecid (36) and have also served to highlight the importance of the electron microscope demonstration of movement of directly labeled folate or FR into caveolae.

Now a new revelation (49) in the form of additional control experiments formally questions the validity of the entire concept of potocytosis and the role of caveolae in FR-mediated folate uptake (2, 3). This controversy is dramatized by the fact that the protagonists involve the lead and senior author of the original report describing the potocytosis model (62). At issue is the surprising identification (49) that sequestration of FR (and other GPI-anchored proteins) into discrete clusters is dependent on cross-linking by a second antibody. Thus, when the primary anti-FR antibody was directly labeled, there was no clustering in the absence or presence of folate (49). These authors concluded that their data "[argue] strongly against the potocytosis model of folate uptake." Moreover, the earlier data that "GPI-anchored proteins are excluded from coated pits may be explained by the ability of GPI-anchored proteins to redistribute to caveolae artifactually during the process of immuno-localization, thereby apparently depleting coated pits of GPI-anchored proteins" (49). Anderson's group have countered that the primary antibody to FR per se may have prevented clustering (59). They generated additional data pointing to a differential regulatory role of wild-type (GPI-anchored) FR versus chimeric FR targeted to clathrin-coated pits; this provides some more support for a role of caveolae in regulating FR-mediated folate uptake (59). However, since net uptake of folate was equally good when folate was internalized by wild-type and chimeric FR, it is still a matter of debate whether or not the differential

behavior observed "formally establishes that coated pits are not normally used for the endocytosis of folate" (59). Thus, although there has been substantial (albeit mostly indirect) support for involvement of caveolae in GPI-anchored, FR-mediated folate transport via potocytosis, direct demonstration of FR within these organelles has remained elusive. Notwithstanding, there is independent support for a probenecid-sensitive folate transporter in choriocarcinoma cells that is driven by a transmembrane H+ gradient generated by a membrane-associated vacuolar type H(+)-pump (55). Although studies on transcellular folate transport have ruled out a role for anion channels, such channels could be uniquely required for cellular folate transport into malignant/immortalized cells. Thus, it is entirely plausible that FR may interact with this (non-RFC) pump in some cells to explain the earlier data (36). However, as of now, we are still some way from resolving this charged controversy.

Is there an interrelationship between FR and the RFC? Both methotrexate and 5,10-dideazatetrahydrofolate utilize both RFC and FR for transport into cells (4, 54). However, the major determinants of net antifolate transport are the prevailing EFC, the concentration of antifolate, and the extent of RFC and FR expression (4). Cell lines have been isolated for primary resistance to methotrexate through defective RFC, but which also overexpress FR (10). Because this mutation in overexpression of FR in these cells allows for continued growth in folate-depleted media, the expression of FR clearly had a salutary effect for these neoplastic cells in that it compensated for defective RFC expression.

Do the RFC- and FR-mediated folate-transport systems communicate with one another? Apparently not. Thus, beginning with wild-type breast cancer cells, which only exhibited RFC-mediated folate transport, and a methotrexate-resistant mutant cell line, Cowan's laboratory studied the potential interaction of the RFC and FR after transfection of FR-α cDNA (23). After transfection, both wild-type and methotrexate transport-resistant cells that previously required >100 nM of folic acid for growth were able to grow in 1 nM folic acid. Furthermore, methotrexate transport-resistant cells, which were unable to grow in 1 μM 5-formyltetrahydrofolate were now also able to grow in 1 nM 5-formyltetrahydrofolate. Since FR expression allowed both cell types to accumulate similar (albeit increased) amounts of folic acid, 5-formyltetrahydrofolate, and methotrexate, these data allowed for the conclusion that the FR functioned independently of RFC expression, and vice versa (i.e. alteration of RFC function failed to affect FR function). Parenthetically, in contrast to data from Elwood's laboratory (17), these cells did not exhibit an increased sensitivity to methotrexate. Moreover, their apparently paradoxical resistance to the lipophilic antifolate, trimetrexate, suggests that these cells were rescued by FR-mediated uptake of 5-formyltetrahydrofolate. These contradictory results (17, 23) from different breast cancer cell lines are examples where additional

variables likely play a role. Thus, the sensitivity of cells to antifolates may not be invariably enhanced by transfection or transduction of FR genes, and specific studies with several malignant and normal cells will be required to comprehensively test this hypotheses in vitro and in vivo. Recently, Schornagel and Jansen's group have also independently arrived at a similar conclusion (84).

How efficient are the two systems in transport of methotrexate? Goldman's laboratory compared the transport of methotrexate in L1210 cells, which expressed only RFC, and a mutant L1210 cell line with a defective RFC that was transfected with FR cDNA. The conclusion of this study was significant in that the RFC and FR functioned essentially independently of one another (72). More importantly, however, when enough FR were expressed, these proteins mediated the uptake of methotrexate and 5-methyltetrahydrofolate with comparable rates to cells expressing only the RFC. Thus, FR have both physiologic and pharmacologic importance, directly confirming several earlier studies (4). Once again, this may not be the case with all malignant human cells; for example, despite high FR expression, the RFC was recently found to be the preferential carrier of antifolates in some cells (84).

Is there a role for FR in antifolate resistance? Yes. Methotrexate resistance can be due to alterations in dihydrofolate reductase (gene amplification or diminished affinity), reduced intracellular polyglutamation, increased efflux, or reduced transport via the RFC. Recent studies in cells that primarily depend on FR for folate uptake now also implicate a reduction in expression of FR (66). Thus, if similar mechanisms operate in vivo, it could likewise adversely influence methotrexate and 5,10-dideazatetrahydrofolate uptake (23, 27) into tumors that mainly depend on FR-mediated transport, and negatively impact therapy.

EXPRESSION AND REGULATION OF FOLATE RECEPTORS

Expression of FR

Polyclonal and monoclonal antibodies used against FR from a variety of sources demonstrated cross-reacting proteins in several normal and malignant human cells/tissues (Table 1). However, without confirmatory data with molecular and other biochemical methods (60), it may be premature to make definitive conclusions on the extent of expression of FR, especially in cells/tissues that apparently do not have cross-reactive FR-like species on cell surfaces. This relates to the fact that FR-negative tissues can be generated in vitro even from tissues that overexpress FR. A good example is human placenta, where a time-dependent release of hydrophilic FR from placental membranes medi-

Table 1 Folate receptor (FR) expression in normal and malignant human tissues

Cells/tissues	Determinants	Expression
Normal		
	Genitourinary system	Placenta, chorionic villus, trophoblastic cells (29, 56, 75, 77, 83); fallopian tube, uterus, ovary (83); vas deferens, epididymis, semen (83); kidney (proximal tubules) (83)
	Central nervous system	Choroid plexus epithelial cells, CSF (31, 83)
	Hematopoietic system	Hematopoietic progenitor cells [CFU-GEMM, CFU-GM, BFU-E, CFU-E] (7)
	Gastrointestinal system	Salivary (submandibular) (76, 83); colon (32)
	Respiratory system	Bronchial glands and alveolar lining (type-I and-II pneumocytes) (83).
	Endocrine systems	Breast (acinar cells) (83) and human milk (4); thyroid, pancreas (83)
	Miscellaneous	Fibroblasts (4)
Malignant		
	Consistently high and uniform expression	Nasopharyngeal carcinoma (4); cervical carcinoma (73); ovarian carcinoma (19, 26, 74); choriocarcinoma (56)
	Relatively lower, inconsistent expression	Endometrial carcinoma (26); breast carcinoma (4); primary brain tumors (81, 82); colorectal carcinoma (26, 37); sarcomas (60); renal cell carcinoma (26)

ated by an EDTA-sensitive endogenous metalloprotease was demonstrated (77). Similar activation of the metalloprotease may also occur with apparently innocuous agents/buffers used in cell culture (25, 75). Hydrophobic FR can also be cleaved off the cell surface by GPI-specific phospholipases present in media (77) and trypsin (4). Furthermore, there may be FR isoform-specific differences in affinity for various antibodies (an unknown variable) or other intrinsic nuances in detecting FR expression (e.g. use of monoclonal versus polyclonal antibodies, or cells in suspension versus frozen/fixed tissues, etc). Thus, it is quite possible that additional tissues (not listed in Table 1) may be found to express FR.

Regulators of FR Expression

Conversion of hydrophobic GPI-anchored FR to hydrophilic FR as mediated by a metalloprotease (25, 75) or by GPI-specific phospholipases C or D (43,

77) can potentially be an important mechanism for posttranslational regulation of the expression of FR. However, no probes are available to directly study these hydrophobic FR-directed enzymes. We have recently isolated a FR-directed hydrophobic metalloprotease (85a) and should eventually be able to determine the potential significance of this protein in the regulation of FR expression and net folate transport. Although a major role for the metalloprotease was not identified in the placenta at term (29), its activity could easily determine the extent of acquisition of folates by FR on trophoblasts, thereby influencing placental growth and development. The extent of contribution of hematopoietic cell FR-γ (70), and the relative role of GPI-phospholipases versus metalloprotease in generating hydrophilic FBPs in serum, also awaits clarification.

Regulation by the EFC

The EFC inversely regulates FR expression (4, 28). In human nasopharyngeal carcinoma (KB) cells, when FR was increased in response to low EFC, there was an increase in FR mRNA (65) as a result of increased mRNA stability (33). Conversely, with excess EFC of folates and antifolates, FRs were down-regulated with a reduction in FR mRNA (34). Although low-EFC-adapted L1210 cells exhibited a rearrangement in the locus upstream from the start codon of the FR gene (involving insertion of an intracisternal A particle) (13), this retrovirus-like sequence was unresponsive to the folate status of the cell. Thus, the reversible, physiologic mechanism(s) for transcriptional FR regulation by the EFC remains undiscovered.

In general, steady-state up-regulation of FR accompanied by an increase in FR mRNA can result from increased rates of transcription of FR genes and/or increased stability of FR mRNA. In addition, FR up-regulation may involve independent changes in the translational or posttranslational pathways involving FR metabolism (i.e. increased rate of FR synthesis, decreased rate of degradation of FR, or reduced activity of potential regulators of cell surface FR membrane association). Conversely, down-regulation of FR could be a function of independent or combined alterations in these parameters in the opposite sense, all of which constitute cellular FR metabolism. Thus far, however, studies on up- or down-regulation of FR have not comprehensively investigated each of these parameters of FR metabolism in response to low and high EFC. And whether one or more of the steps in the biosynthesis and/or degradation of FR is a dominant responsive parameter to changes in the EFC also remains to be determined. We have recently determined that in cervical carcinoma cells, regulation of FR expression by the EFC is primarily controlled at the translational level through dominant alterations in FR synthetic rates (45a). In addition, we have identified an 18-base *cis*-element in the 5′ untrans-

lated region of FR-α mRNA that specifically binds a 46-kDa cytosolic (*trans*-factor) protein (72a). Therefore, our immediate challenge is to determine the functional basis of this interaction in the regulation of FR, and specifically in response to changes in the EFC.

FOLATE RECEPTORS AND CONTROL OF CELL PROLIFERATION

Interaction of anti-FR immunoglobulin (Ig) G with FR on hematopoietic progenitors earlier led to increased cell proliferation that was independent of induction of megaloblastosis and intracellular folate deficiency (7). However, it was unclear whether anti-FR IgG perturbed a normally inhibitory role of FR or accentuated a stimulatory function of FR in cell proliferation (4). Furthermore, was there a similar constitutive role for FR in control of cell proliferation in malignant cells?

Recent studies have documented that transfection of FR cDNA into various cells that do not constitutively express FR (9, 17, 47, 48) led to greater proliferation and survival when compared with controls cultured in low EFC. Thus, FR clearly have an overall growth-promoting function. However, it is unclear whether these findings were due to independent effects of (*a*) a greater concentration of intracellular folates accumulated by FR cDNA-transfected cells (which prevented folate-deficient cell death at low EFC), (*b*) a proliferative signal generated at the level of FR per se, or (*c*) a combination of both. To sort out these possibilities, we encapsidated FR cDNA in the sense/antisense orientation into infectious adeno-associated virions, transduced cervical carcinoma cells, and determined the functional consequences of over- and under-expression of FR on cell proliferation at high (micromolar) EFC (73). This latter issue was crucial to eliminate the variable of intracellular folates on cell proliferation among various cohorts studied, since micromolar EFC led to passive diffusion of folic acid into cells (4). When compared with antisense FR cDNA-transduced and untransduced cells, sense FR cDNA-transduced cells exhibited statistically significant increases in total FR, smaller colonies, lowered cell proliferation in vitro, and less tumor volumes with dramatic prolongation of tumor doubling times (225 versus 96 h) after transplantation into nude mice. In addition, with single cell–derived transduced clones, an inverse relationship between cell proliferation and FR expression was formally established (Figure 2) (73).

Can these apparently opposing [e.g. growth-promoting (9, 17, 47, 48) and growth-inhibiting (73)] functions of FR be unified into a hypothesis linking folate deficiency, FR expression, and cell proliferation? That malignant cells, which constitutively overexpress FR, can inversely regulate FR in response to EFC (4) is well documented. However, in some (but not all) cell lines, up-

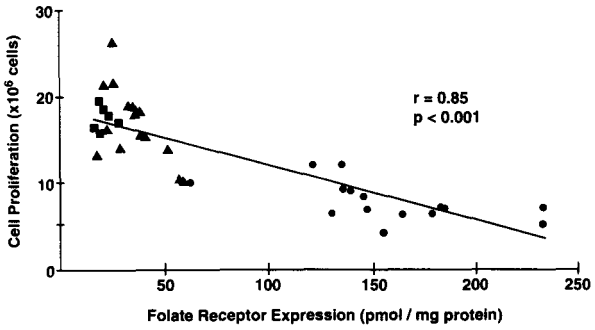

Figure 2 Relationship between cell proliferation and folate receptor expression. A total of 36 randomly isolated, single cell clone–derived cell lines, 15 from sense FR cDNA-transduced cells (*circles*), 15 from antisense FR cDNA-transduced cells (*triangles*), and 6 from untransduced control cells (*squares*), were analyzed for cell proliferation and FR expression. (From Reference 73.)

regulation of FR is also accompanied with a prolongation in doubling time, and vice versa. Our recent data (73) therefore allow us to propose a simple hypothesis wherein the up-regulated FR in response to low EFC could serve to check proliferation of cells by moving them into the resting phase of the cell cycle—a beneficial way to reduce folate requirements under the stress of nutrient deficiency. Conversely, with nutrient excess, the ensuing down-regulation of FR could concomitantly serve to release the normal inhibitory influence of FR on cell proliferation, thereby leading to a permissive state for cell proliferation. If there is a similar inverse relationship between FR expression and hematopoietic progenitor cell proliferation, this could have other clinical significance. For example, clonally derived hematopoietic cells from patients with paroxysmal nocturnal hemoglobinuria, and which lack GPI-anchored proteins (including FR), have a selective proliferative advantage over normal cells—this results in excess hemolysis and impacts on the severity of clinical presentation. So it is not entirely unreasonable to ask if the lack of FR in affected cells has any role in determining their proliferative advantage over normal cells.

EXPLOITATION OF FOLATE RECEPTORS AS TROJAN HORSES

Colnaghi's group have used chimeric murine-human anti-FR antibodies to mediate cell kill of ovarian carcinoma cells in vitro (18); demonstration of similar data in vivo would indeed be an important and welcome addition to the oncologist's meager therapeutic armamentarium.

By covalently attaching folic acid via one of its free carboxyls to toxic proteins or liposomes containing biologically active agents, Low's laboratory exploited FR as Trojan horses to mediate the cellular uptake of these folate conjugates for therapeutic and diagnostic use. Of significance, the rate and the extent of endocytosis of internalized folate-conjugates was independent of the conjugated moiety but was dependent on the expression of apo-FRs (38, 40). Equally important, folate-conjugated proteins also retained their function once internalized via FRs (39). Thus, although the ribosome-inactivating protein, momordin, was nontoxic at micromolar concentrations, conjugation with folate rendered it cytotoxic at nanomolar concentrations (through high affinity interaction with FRs). Actual translocation into the cytoplasm was also shown by using a translocation-defective (from endosomes to cytoplasm) *Pseudomonas* exotoxin coupled to folate that was internalized via FR into the cytoplasm, where it was lethal (42). Furthermore, since internalization was dependent on expression of FR, malignant cells that overexpressed FR were selectively targeted (41).

The capacity to conjugate liposomes to folate by using polyethylene glycol (PEG, as a spacer) has significantly expanded the potential repertoire of this technology (44). When compared with conventional drug delivery, encapsulation of substances into liposomes (drugs, toxins, oligonucleotides) significantly protects this cargo from premature plasma degradation and retards their renal disposition. The generation of several-hundred folate-PEG tethers built into each liposome led to multiple simultaneous attachments to cell surface FRs. This fortuitously led to an increase in the binding affinity to FR by six orders of magnitude. Selective targeting to FR-expressing cells has also been demonstrated with liposome-entrapped Doxorubicin (45) by using co-cultures of FR-overexpressing cancer cells and low-FR expressing normal cells. Interestingly, whereas free Doxorubicin entered both normal and cancer cells equally nonselectively, the folate-PEG-liposomes containing Doxorubicin exclusively entered, and thereby selectively killed, only cancer cells. FR-targeted liposomes have also been successfully employed for targeted cytoplasmic delivery of antisense oligodeoxynucleotides against the human epidermal growth factor receptor in KB cells (78). This technology is therefore ripe for clinical exploitation because agents of various molecular sizes, composition, and metabolism can be targeted for delivery to cells that express FR.

Folate conjugated to ^{67}Ga-Deferoxamine has also shown promise in nuclear medicine and could replace ^{67}Ga-citrate, which is clinically employed as a tumor-specific imaging agent. Normally, tumor selectivity (usually >5:1 tumor:blood ratio) arises from transfer of ^{67}Ga-citrate to transferrin with subsequent binding of ^{67}Ga-transferrin to cancer cells that express transferrin receptors. With ^{67}Ga-Deferoxamine-folate (79) used to image FR-expressing tumors in nude mice (when compared with ^{67}Ga-Deferoxamine or ^{67}Ga-cit-

rate), by 4 and 45 h postintravenous injection, the tumor:blood ratio of the ^{67}Ga-Deferoxamine-folate had exceeded 400:1 and 1400:1, respectively (P Low, personal communication). Aside from the tumor, significant uptake was seen only in the liver and kidney through non-FR-mediated and FR-mediated mechanisms, respectively. Preclinical studies are underway and it may only be a matter of time before clinical trials are initiated.

FUTURE DIRECTIONS

Recent research on FRs has widened their scope and interest in several branches of biology and medicine. Because cellular folate/antifolate transport is a major determinant of sensitivity and/or resistance to cell proliferation, studies on transport of these ligands via FR into normal and malignant cells are of continued importance to hematology and oncology. Extension of studies on transduction of FR cDNA to modulate cell proliferation and their sensitivity to antifolates, combined with the exploitation of FR as Trojan horses, could have a major diagnostic and therapeutic impact in nuclear medicine, pharmacology, and therapeutics. Discovery of the mechanism of placental FR in maternal-to-fetal folate transfer impacts on reproductive physiology and human development within the context of nutrition, as well as on obstetrics and perinatology. This mechanism may be a paradigm for the transplacental transport of other small M_r ligands that bind cognate placental receptors with high affinity. Together with data implicating FR in renal conservation of folates, these studies will be a beacon for future investigations on folate transport into the central nervous system. The studies with GPI-anchored FR-mediated folate uptake system as a paradigm for potocytosis (fueled by the recent controversy) will undoubtedly continue and, it is hoped, will resolve into a clearer understanding of the underlying mechanism(s). The independent role of FR in constitutive control of cell proliferation has now evolved into a testable hypothesis that potentially places FR within the circuitry of the cell cycle. However, the domain of the FR molecule responsible for the generation, as well as the type, path, and target, of the signal(s) transduced remains undiscovered. The molecular basis of regulation of FR by the EFC and selective tissue expression is still unclear, but the recent isolation of promoters for FR-α and FR-ß genes and identification of *cis*-elements in FR mRNA and the 46-kDa *trans*-factors promise significant discoveries ahead. Such investigations will likely provide comprehensive information on the molecular mechanisms controlling regulation of FR at the transcriptional and posttranscriptional level. Moreover, having isolated the metalloprotease, we are also in a position to identify its regulatory role in FR expression. In summary, based on the sustained interest generated in the recent past along multiple fronts, the next few years will likely prove a period of intense activity and excitement arising from

new discoveries related to FR and FR metabolism. This would likely stem from advances developing at the molecular, biochemical, and cell biological realm, with subsequent translation to the area of diagnostics and experimental therapy encompassing various areas of clinical medicine.

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This review on the state of the art is dedicated to all the investigators referenced below. It has been my honor and pleasure to analyze your work within the context of The Big Picture. However, the words from Paul's interpretation of (agape) love (naturally taken completely out of context) is worth recalling: "But when that which is perfect is come, then that which is in part shall be done away" (1 Cor. 13:10, King James Version). So I hope that future reviews will be able to highlight how little we really knew in 1995!

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