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Folate-mediated delivery of macromolecular anticancer therapeutic agents

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Abstract

The receptor for folic acid constitutes a useful target for tumor-specific drug delivery, primarily because: (1) it is upregulated in many human cancers, including malignancies of the ovary, brain, kidney, breast, myeloid cells and lung, (2) access to the folate receptor in those normal tissues that express it can be severely limited due to its location on the apical (externally-facing) membrane of polarized epithelia, and (3) folate receptor density appears to increase as the stage/grade of the cancer worsens. Thus, cancers that are most difficult to treat by classical methods may be most easily targeted with folate-linked therapeutics. To exploit these peculiarities of folate receptor expression, folic acid has been linked to both low molecular weight drugs and macromolecular complexes as a means of targeting the attached molecules to malignant cells. Conjugation of folic acid to macromolecules has been shown to enhance their delivery to folate receptor-expressing cancer cells in vitro in almost all situations tested. Folate-mediated macromolecular targeting in vivo has, however, yielded only mixed results, largely because of problems with macromolecule penetration of solid tumors. Nevertheless, prominent examples do exist where folate targeting has significantly improved the outcome of a macromolecule-based therapy, leading to complete cures of established tumors in many cases. This review presents a brief mechanistic background of folate-targeted macromolecular therapeutics and then summarizes the successes and failures observed with each major application of the technology.

Keywords: Folate receptor; Macromolecular drug targeting; Liposomal therapeutic agents; Gene therapy vectors; Prodrug-activating enzymes; Immunotherapeutic agents

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1. Introduction

Proteins, gene therapy vectors, liposome-encapsulated drugs, aptamers, antisense oligonucleotides, and drug-derivatized biodegradable polymers all show great promise for the treatment of cancers, largely because of their improved specificity, prolonged delivery, or enhanced potency over more traditional chemotherapeutic agents. However, unlike their low molecular weight counterparts, macromolecular drugs often encounter significant permeability barriers that can limit achievement of the above desirable properties. Thus, while tumor selectivity is often heightened due to a tumor's poorly formed vasculature and the consequent passive accumulation of macromolecules within the malignant mass {known as the enhanced permeability and retention (EPR) effect [1]}, the tumor cell membrane can still constitute a formidable barrier for those macromolecules that must enter their target cells to cause cell death. Increased intratumor pressure can also compromise delivery of macromolecular drugs to sites deep within a malignant mass if the tumor's lymphatic drainage is poorly developed [2]. To overcome such limitations, many workers have employed cancer cell-specific ligands as targeting moieties for the improved delivery and retention of macromolecular therapeutics within the tumor tissue [3]. The purpose of this review is to summarize the progress that has been made in developing the vitamin, folic acid, as a ligand for the selective targeting and delivery of macromolecular drugs into tumor cells.

Folic acid is a vitamin required for one-carbon transfer reactions in several metabolic pathways. Because folic acid is essential for the biosynthesis of nucleotide bases, the vitamin is consumed in elevated quantities by proliferating cells. Normal cells transport physiological folates across the plasma membrane using either of two membrane-associated proteins, the reduced folate carrier or the folate receptor (FR). The former is found in virtually all cells and constitutes the primary pathway responsible for uptake of physiological folates. The latter is found primarily on polarized epithelial cells and activated macrophages [4,5], and preferentially binds and internalizes oxidized folates via receptor-mediated endocytosis [6]. While low concentrations of the reduced folate carrier are probably sufficient to supply the folate requirements of most normal cells, FR is frequently overexpressed on cancer cells, perhaps enabling the malignant cell to compete successfully for the vitamin when supplies are limited [7,8].

While overexpression of FR on many cancer cells obviously identifies the receptor as a potential target for a variety of ligand- and antibody-directed cancer therapeutics [9], FR may be further qualified as a tumor-specific target, since it generally becomes accessible to intravenous drugs only after malignant transformation. That is, because FR is selectively expressed on the apical membrane surface of certain epithelial cells (i.e., the membrane surface facing a body cavity), it is inaccessible to blood born reagents and therefore protected from FR-directed therapeutics delivered in the plasma. However, upon epithelial cell transformation, cell polarity is lost and FR becomes accessible to targeted drugs in circulation. Probably because of this dual mechanism for tumor specificity, the receptor's natural ligand, folic acid, has become a popular molecule for targeting attached drugs to cancer cells. The attractiveness of

folate has been further enhanced by its high binding affinity ($K_d \sim 10^{-10}$ M), low immunogenicity, ease of modification, small size (M_r 441.4), stability during storage, compatibility with a variety of organic and aqueous solvents, low cost, and ready availability [10]. To date, many chemical and biological therapeutic agents have been successfully conjugated to folic acid, most of which have demonstrated significantly enhanced delivery to FR-positive tumor cells both in vitro and in vivo [10,11]. In the following summary of folate-targeted macromolecular anticancer agents, we not only identify areas of significant success in macromolecular targeting, but we also point out obstacles that must still be surmounted before the various targeting applications of folic acid can achieve their full potential.

2. Basic aspects

2.1. Structure and function of the folate receptor

The family of human FR ($M_r \sim 38$ kDa) consists of three well-characterized isoforms (FR-α, -β, and -γ/γ′) that are ~70–80% identical in amino acid sequence, but distinct in their expression patterns [12]. FR-α and FR-β are both membrane-associated proteins as a consequence of their attachment to a glycosylphosphatidylinositol (GPI) membrane anchor [12]. FR-α, however, can be distinguished from FR-β by its higher affinity for the circulating folate coenzyme, (6*S*)-5-methyltetrahydrofolate (5-CH_3H_4folate), and by its opposite stereospecificity for reduced folate coenzymes [13]. FR-α also binds folic acid and physiologic folates with slightly higher affinity ($K_D \sim 0.1$ nM) [14] than FR-β ($K_D \sim 1$ nM) [15]. FR-γ and a truncated form of the protein, FR-γ′, lack the GPI anchor and are constitutively secreted in barely detectable amounts as soluble forms of the human FR [12]. The binding affinity of the secreted FR-γ for folic acid is reportedly to be ~0.4 nM [16]. Recently, a gene encoding a possible fourth isoform of the receptor (FR-δ) was identified in an uncharacterized region of the human genome [17]. Analysis of FR-δ expression, however, did not reveal detectable levels of the protein in tissues from either adult or embryonic sources, suggesting a possible transient expression pattern, a splice variant, or an FR pseudogene.

2.2. Expression of folate receptor in normal and malignant tissues

Expression of the various FR isoforms (α, β, γ/γ′) is both tissue-specific and differentiation dependent [12,18]. With the exception of a few normal tissues (kidney, placenta, and choroid plexus), FR-α is present at only low levels on normal epithelia, but often elevated in malignant tissues of epithelial origin, particularly the ovary [8], uterus [18], endometrium [19], brain [20], kidney [18], head and neck [18], and mesothelium [21]. As measured by ^{3}H-folic acid binding to crude plasma membrane preparations (Fig. 1), the difference in FR-α expression between normal and malignant tissues of the same origin can often be quite striking, showing levels of upregulation approaching two orders of magnitude [18]. In patients diagnosed with epithelial ovarian cancer, the degree of FR-α overexpression is further correlated with a higher histologic grade and more advanced stage of the cancer [8], suggesting a possible need for elevated folates in more rapidly growing tumors (Fig. 2). An additional correlation has also been reported between the degree of FR expression and resistance to standard chemotherapy [22]. That is, tumors that survive standard chemotherapy commonly have higher levels of FR. Taken

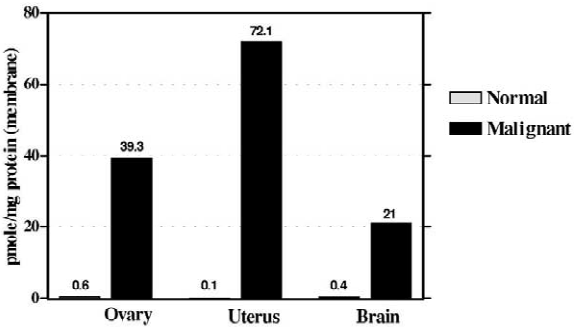

Fig. 1. Comparison of the levels of folate receptor expression between normal and malignant human tissues. All malignant tissues were classified as medium to high grade tumors. For each pair of normal and malignant tissues, 100 μg total protein from crude membrane preparations was isolated and assayed for specific binding of ^{3}H-folic acid (data replotted from Ref. [18]).

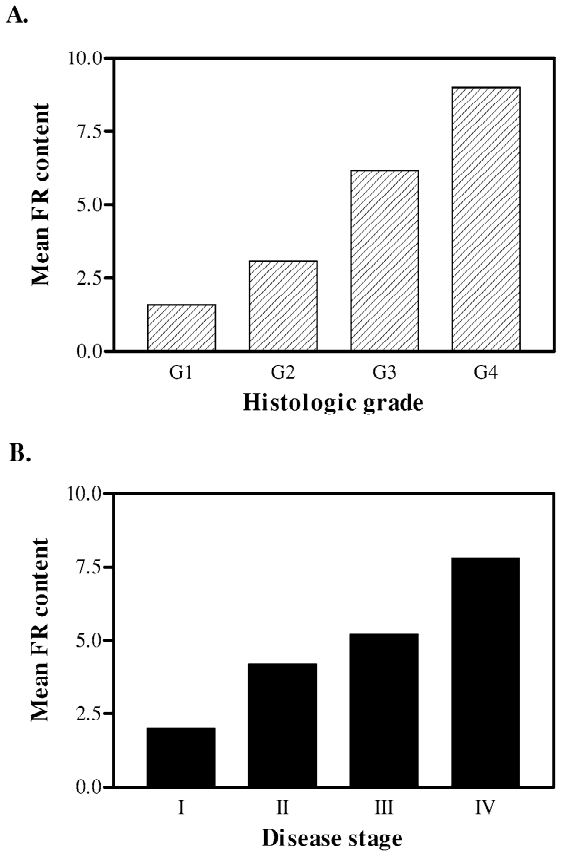

Fig. 2. Overexpression of the folate receptor in ovarian cancer is associated with a higher histologic grade (A) and more advanced stage (B) of the disease. Frozen tissue samples were mechanically disaggregated to prepare single cell suspensions for cytofluorimetric analysis using an anti-FR monoclonal antibody. The mean FR content represents receptor-associated fluorescence divided by isotopic control fluorescence (data replotted from Ref. [8]).

together, it is conceivable that the more advanced stage, higher grade, and chemotherapy-resistant cancers, i.e., the tumors that are most difficult to treat by standard procedures, comprise the population of cancers that are most readily targeted by folate-linked drugs.

FR-β, originally discovered in rat placenta [15], constitutes the isoform of the FR most commonly expressed in hematopoietic and nonepithelial cells, such as spleen and thymus [18]. FR-β is also elevated in some malignancies of nonepithelial origin, including myelogenous leukemias and sarcomas [12,23]. Importantly, while FR-β can be detected on hematopoietic stem/precursor cells and differentiated cells of myeloid lineages, it is expressed on these cell types in an inactive form, i.e., a conformation that exhibits no affinity for folates [24]. In fact, a functional FR-β has only been detected to date on activated (but not resting) macrophages [4,5]. FR-γ and -γ′ are also thought to be specific for hematopoietic tissues, particularly lymphoid cells, and are expressed only at very low levels [12]. The secreted forms of the FR may be used as potential serum markers for certain hematopoietic malignancies [12].

Because relatively high levels of FR can be measured in the proximal tubules of the kidney and the choroid plexus of the brain, some concern has arisen that therapeutic agents that target FR might prove toxic to both tissues [25]. However, as noted above, immunohistochemical techniques and ^{125}I-folate autoradiography have demonstrated a highly polarized pattern of FR distribution on these normal epithelia [25,26]. In the proximal tubules, for example, FR is seen only on the apical/lumenal or urine-facing surface of the tubule cells [25], where it probably assists in reabsorption of folates from the urine [27]. Thus, folate-targeted macromolecules should encounter kidney FR only in individuals suffering from proteinurea and other kidney dysfunctions. Similarly, FRs in the brain appear to be concentrated on the brain side of the blood brain barrier [26], where they may function to retain the vitamin within the cerebrospinal fluid. As expected, malignant choroid plexus tumor cells lose their polarized distribution patterns as demonstrated by a diffused immunohistochemical staining of FR over the entire tumor cell surface [26]. Based on these and related observations, there is currently no evidence that FR-targeted macromolecular therapeutics should damage normal tissues with elevated levels of FR expression.

2.3. Folate conjugate uptake via receptor-mediated endocytosis

Although the precise mechanism of FR transport of folic acid into cells remains unresolved, it is clear that folate conjugates are taken up nondestructively by mammalian cells via receptor-mediated endocytosis (Fig. 3) [28,29]. Nevertheless, there have

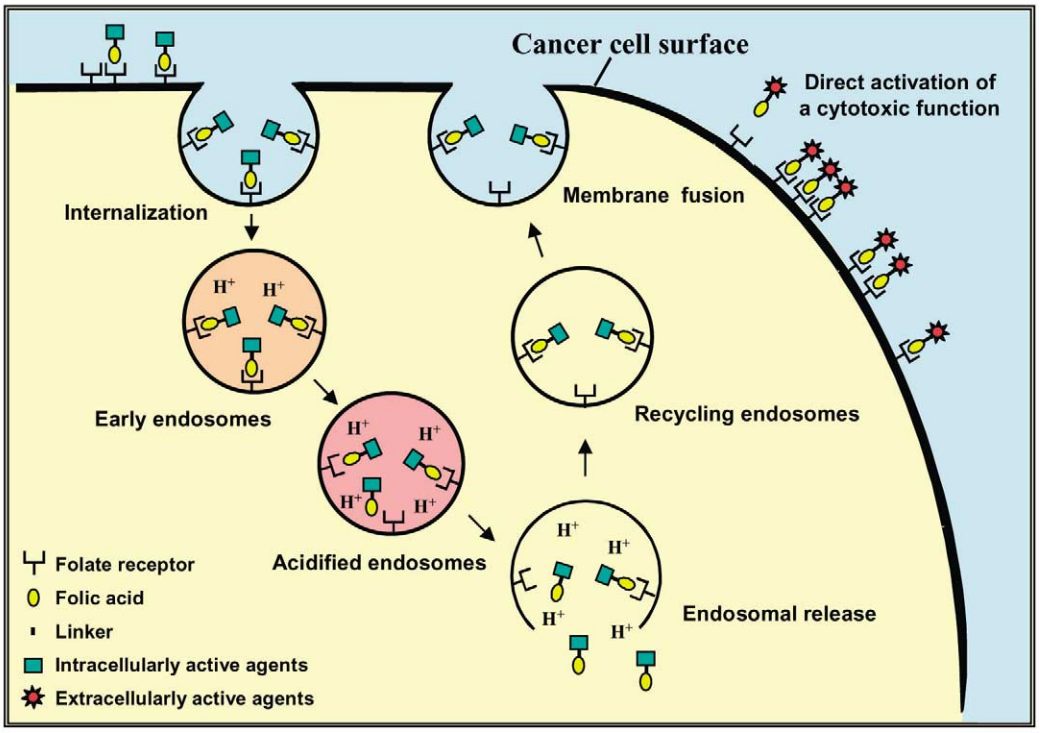

Fig. 3. Folate-mediated delivery of therapeutic agents to folate receptor-positive cancer cells. Because a fraction of the FR-associated folate–drug conjugates will traffick into the cancer cells by receptor-mediated endocytosis (left side of diagram), while the remainder will remain on the cell surfaces (right side of diagram), two types of therapeutic strategies can be envisioned. Drugs that require access to intracellular targets can be delivered in substantial quantities to cytosolic locations by the endocytic pathway, while drugs that can or must function from an extracellular location will be enriched on cancer cell surfaces by the stationary population of the FR. See text for details.

been conflicting reports on the mechanism or pathway involved in the internalization and trafficking of GPI-anchored FR [30,31]. Thus, early studies suggested that FR is not associated with clathrin-coated pits [32], but organized into submicron domains at the cell surface. These studies also suggested that the GPI anchor might be responsible for mediating receptor clustering in association with flask-shaped membrane structures called caveolae [30]. It was further proposed that FR is internalized via the pinching off of caveolae in a process termed potocytosis [33]. Later studies, however, concluded that multimerization of the GPI-anchored FR does not occur in caveolae, and that the receptor may remain diffusely distributed over the plasma membrane until folate ligation [31]. More recent results now seem to suggest that FR is organized by its GPI anchor into ‘‘lipid rafts’’ or receptor rich complexes in the membrane and that these submicron domains (<70 nm in diameter) are devoid of caveolae but rich in sphingolipid and cholesterol [34,35]. Regardless of the route of entry, physiologic folates clearly move across the plasma membrane into the cytoplasm via a specialized endocytosis pathway mediated by the FR [36].

After binding to FR on the cancer cell surface, folate conjugates, regardless of size, are seen to internalize and traffick to intracellular compartments called endosomes [29]. Folate conjugate-containing endosomes have been shown to have pH values between 4.3 and 6.9 (most frequently, pH ~ 5.0) due to a process called endosome acidification [37]. Since the binding of folic acid to its receptor is pH dependent, decreasing dramatically at pH values <5 [14], it can be anticipated that some of the folate conjugates will dissociate from their receptors and

remain inside the cell. However, direct measurements of the efficiency of folate conjugate unloading reveal that only 15 to 25% of the receptor bound conjugates are released inside the cell (Reddy, Paulos and Low, unpublished observations), the remainder apparently recycling back to the cell surface attached to FR. Related studies also indicate that the total number of folate conjugates internalized is roughly proportional to the number of FR expressed by a cell, and that an average FR-expressing cancer cell may internalize folate conjugates at a rate of $\sim 1–2 \times 10^5$ molecules/cell/h.

2.4. Tumor selectivity of folate conjugates in vivo

The tumor selectivity of folate conjugates in vivo has been well documented in tumor-bearing mice using low molecular weight folate-linked radiopharmaceuticals [38–40]. In fact, following examination of several generations of such folate-based radioimaging agents complexed to a variety of radionuclides (^{125}I, ^{67}Ga, ^{111}In, ^{99m}Tc), two water-soluble conjugates, ^{111}In-diethylenetriamine pentaacetic acid (DTPA)–folate and a ^{99m}Tc-based folate conjugate (EC20, Endocyte, West Lafayette, IN, USA), were qualified for additional evaluation in human clinical trials [41]. In women suspected of having ovarian cancer, intravenously administered ^{111}In-DTPA–folate was found to concentrate in abdominal masses that were subsequently confirmed to be malignant (Fig. 4B). The accuracy and detection sensitivity of the imaging agents were very encouraging, since little uptake was ever observed in patients with benign tumors (Fig. 4A). Further, except for malignant masses, only the kidneys and in some patients the liver displayed significant retention of ^{111}In-DTPA-folate. The uptake in the kidneys was obviously anticipated due to the known FR expression on the apical membrane of the proximal tubules (the low molecular weight imaging agents are rapidly excreted into the urine where they can easily access the kidney FR). While uptake in the liver of a fraction of the patients was not predicted, it has subsequently been shown to derive from FR on the surface of activated macrophages (i.e., Kuppfer cells) [5] that presumably became activated in response to some type of inflammatory stimulus (Hilgenbrink and Low, unpublished observations). Because resting macrophages do not bind folate or folate conjugates, and since activated macrophages that do take up folate conjugates can be replaced from the resting macrophage population, delivery of folate conjugates into activated macrophages may not constitute a serious health hazard.

3. Folate-mediated delivery of macromolecular therapeutics

As illustrated in Fig. 3, applications of folate targeting for delivery of macromolecular therapeutic agents to cancer cells may be classified into two categories. For drugs that require intracellular release to exert their cytotoxic/regulatory functions, FR offers a ligand-activated endocytosis pathway for transport into the cytoplasm of cancer cells [28]. Examples of macromolecules that fall into this category include most protein toxins [42–44], drug-encapsulating liposomes [45–47], oligonucleotides [48–50], gene therapy vectors [51–53] and many other colloidal drug carriers [54–56]. Although few studies have examined the mechanism and intracellular trafficking of folate-conjugated macromolecules, it has been suggested that endosomal release may not follow the mechanism of free folic acid, but rather may depend on some type of slow nonspecific escape during cycling/breakdown of the organellar membranes [29]. On the other hand, for drugs that do not require intracellular unloading, but are capable of mediating their cytotoxic functions on the surface of a target cell, FR can simply act as a tumor marker that allows concentration of the drug on the tumor cell surface. Examples of this latter class of therapeutic agents might include prodrug-activating enzymes [57] and immunotherapeutic agents that stimulate or redirect the immune system to the cancer cell [58,59]. Importantly, the continuous recycling of only a fraction of cell surface FR allows for both types of targeting strategies to be exploited. More importantly, since folate–macromolecule conjugates are not rapidly degraded following internalization, delivery systems for even the most hydrolytically sensitive macromolecules (e.g., proteins and gene therapy vectors) can potentially be developed.

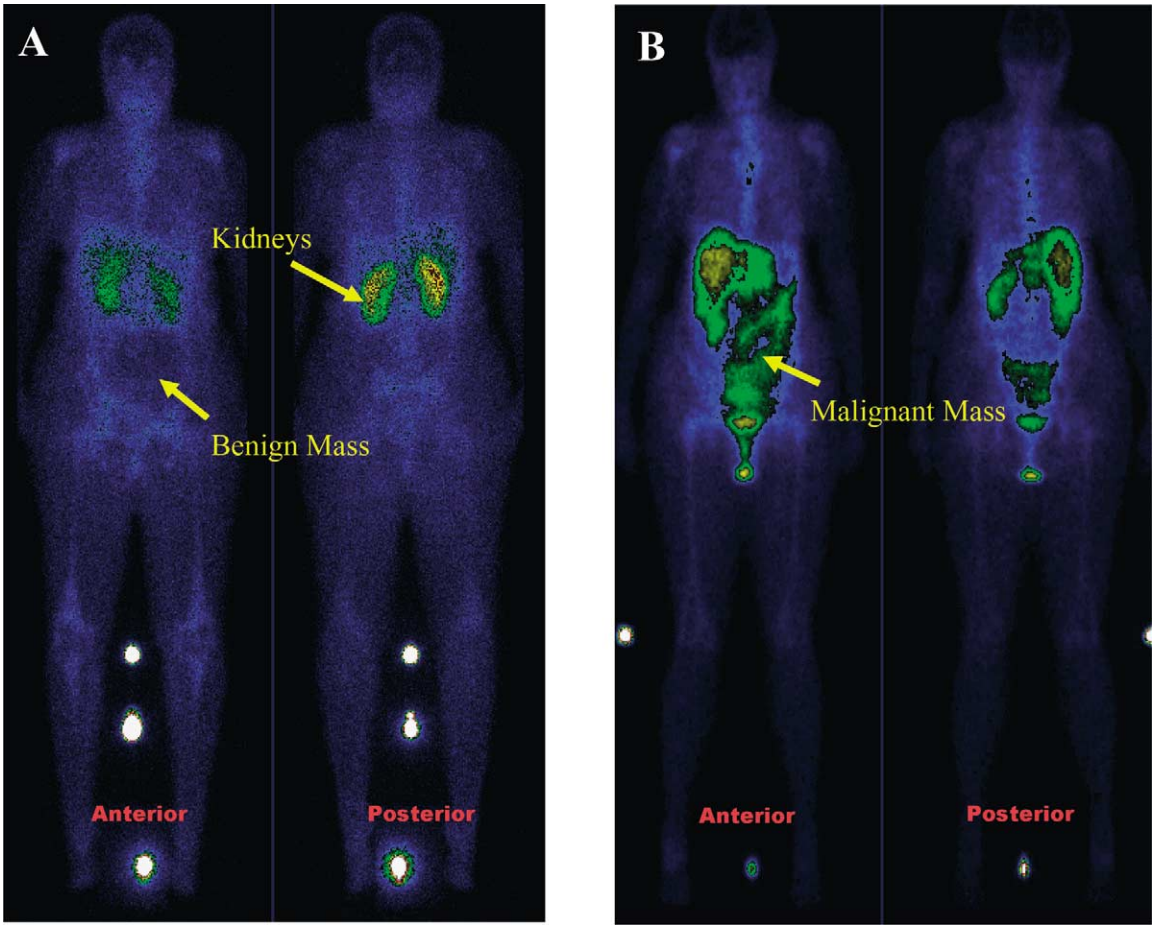

Fig. 4. Anterior and posterior gamma scintigraphic images of a patient with a benign mass (A) or multiple disseminated malignant masses (B) 4 h following intravenous administration of 2 mg ^{111}In-DTPA–folate. Both patients were admitted to the clinical trial following ultrasound diagnosis of an abdominal mass. One patient was subsequently diagnosed with a benign ovarian cyst (A), while another patient was later shown to have stage IIIc ovarian cancer (B). Other than uptake in the cancerous masses, the ^{111}In-DTPA–folate distribution is primarily limited to the kidneys.

3.1. Drugs that require intracellular delivery

3.1.1. Protein toxins

Ribosome inactivating proteins such as the plant-derived toxin, momordin, and the bacteria-derived protein, *Pseudonomous* exotoxin, were among the first macromolecular drugs to be successfully delivered into FR-positive tumor cells by FR-mediated endocytosis [42,43]. Since both momordin and the recombinant form of *Pseudonomous* exotoxin lack a cell surface binding domain, they are essentially inactive unless attached to a ligand that mediates their intracellular transport. In the case of FR-expressing cells, the IC_{50} of the two toxins decreases from $>10^{-5}$ M for the underivatized toxins to $<10^{-9}$ M for the folate-modified toxins. For cells that lack an FR, however, they remain innocuous regardless of derivatization. Indeed, folate-conjugated toxins have demonstrated a highly quantitative and tumor-specific killing of FR-positive human cancer cell lines (HeLa, KB and Caco-2 cells) when cultured together with FR-negative cell types (WI38 and Hs67 cells), which as anticipated remained completely unharmed [43,60].

Folate–protein conjugates can be easily prepared by reacting NHS (*N*-hydroxysuccinimide)- or EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide]-activated folate with the protein of interest to generate a stable amide linkage to the protein's accessible lysine side chains [10]. In most cases, ~three folates per protein have proven sufficient to achieve good FR targeting without compromising the protein's biological activity. In some cases, however, direct protein derivatization has resulted in protein inactivation. Thus, Atkinson et al. observed a 225-fold decrease in gelonin activity following conjugation to folic acid via amino groups [44]. To circumvent this obstacle, the authors modified the plant toxin with a thiol-derivatized folate that was attached to gelonin via carbohydrate residues. The resulting protein product retained 99% of its original activity. In another study, however, Leamon et al. compared the potencies of a disulfide and amide-linked folate conjugate of *Pseudomonas* exotoxin [43]. They determined that both forms of the toxin exhibited an IC_{50} value of $\sim 10^{-11}$, suggesting that attachment to either a cysteine or lysine side chain preserved the protein's full activity. Apparently, the optimal chemistry of protein conjugation may depend on the individual protein's characteristics.

Restrictions on the site of derivatization of the vitamin, folic acid, are less cumbersome. Whereas linkages to atoms within the pterin ring or *p*-aminobenzoic acid moieties abrogate FR binding, attachment via either the α- or γ-carboxyl group of folic acid generally allows retention of receptor affinity [61]. Because these carboxyl groups are easily activated for subsequent attachment to proteins, and since the chemistry is well worked out for regiospecific selection of either the α- or γ-carboxyl group for derivatization [61], preparation of highly functional proteins modified with folate at a predetermined site is no longer problematic. In this latter regard, it should be noted that a γ-carboxyl linkage is thought to yield a higher affinity protein conjugate.

Unfortunately, even with optimal conjugation, the efficiency of folate-mediated protein targeting in vivo is limited by the ability of the protein to passively penetrate the tumor [62]. In general, enrichment of folate–protein conjugates in tumor tissues exceeds passive targeting (EPR effect) to the same tissue by only 3- to 12-fold (unpublished observations). Since low molecular weight folate conjugates are often enriched in FR-expressing cancers >100-fold over their nontargeted counterparts, the obstacle in protein targeting is obviously not the absence of functional FR. Further, since a folate–bovine serum albumin (BSA)–fluorescein isothiocyanate (FITC) conjugate can aggressively target tumor cells in unmodified ascites fluid freshly isolated from ovarian cancer patients [63], the presence of serum proteins and other physiological solutes also cannot account for the reduced folate–protein uptake. Therefore, we assume that the lower tumor specificity seen in protein targeting probably reflects the protein's hindered passive penetration into solid tumors. In this regard, it is conceivable that the first protein conjugates to extravasate and enter a malignant mass might bind avidly to FR on the cancer cells directly adjacent to the capillary bed, and thereby block diffusion of subsequent folate–protein conjugates past this ''protein roadblock'' to cells deeper within the tumor. Regardless of the cause, the data suggest that simple protein–folate conjugates do not reach every active FR within a solid tumor. While multiple solutions to this problem can be envisioned, a relatively simple and successful solution is summarized in the section on folate-targeted immunotherapy (see below).

3.1.2. Drug-encapsulating liposomes

Liposomes are attractive vehicles for drug delivery due to their ability to encapsulate and deliver large quantities of an unmodified drug in a single container. Intravenously administered liposomal drugs tend to accumulate naturally in tumor tissues next to capillaries due to the previously mentioned passive targeting (EPR) effect. For active targeting, however, various types of ligands have been used for selective delivery of liposomes to epitope or receptor-positive tumor cells, including antibodies, asialoglycoproteins, oligosaccharides, transferrin, and various hormone analogs [64,65]. The first use of folate to deliver liposomes into cancer cells was achieved by covalently conjugating the headgroup of phosphatidylethanolamine (PE) to folic acid via an intervening spacer. After incorporating the modified phospholipid into calcein encapsulating liposomes (~66 nm diameter), the folate-tethered liposomes were seen to enter cultured KB cells by FR-mediated

endocytosis [66]. Analysis of the spacer requirement for the above derivatized phospholipid demonstrated that optimal targeting was observed with a polyethyleneglycol (PEG) spacer of $M_r \sim 3350$ that in its extended conformation can be calculated to be ~250 Å long [66]. It was assumed that this lengthy spacer was necessary to permit the folate to penetrate cell surface obstructions in its search of an unoccupied FR. Methods for preparing various types of folate-tethered liposomes have been recently reviewed by Stephenson et al. [46], and procedures for loading various classes of drugs into liposomes have also been described [67].

The therapeutic potential of folate-targeted liposomes was initially demonstrated by encapsulating the anticancer drug, doxorubicin, in liposomes comprised of 0.1 mol% folate–PEG–distearoylphosphatidylethanolamine (DSPE), 58.3 mol% distearoylphosphatidylcholine (DSPC) and 41.6 mol% cholesterol [45]. Uptake of the folate–PEG–liposomal doxorubicin by KB cells was found to be 45- and 1.6-times higher than that of nontargeted liposomal doxorubicin and free doxorubicin, respectively, while the cytotoxicity was 86- and 2.7-times higher, respectively [45]. This greater than anticipated increase in cytotoxicity was later shown by Goren et al. to be due to a more efficient transport of doxorubicin into the nucleus [47] when the liposomes are taken into cancer cells by FR-mediated endocytosis [68]. The specificity of liposomal doxorubicin for cancer cells was also shown to be enhanced when the properties of targeted and nontargeted liposomal doxorubicin were compared in co-cultures of FR-positive (HeLa) and FR-negative (WI38) tumor cell lines [45]. Thus, at the same drug concentrations, HeLa cells were completely killed upon exposure to folate–PEG–liposomal doxorubicin, while adjacent WI38 cells were left unharmed. In contrast, the same concentration of nontargeted liposomes was largely ineffective against both cell lines. These results were interpreted to suggest that anticancer drugs can be safely targeted to cancer cells without damaging normal cells via encapsulation in folate-targeted liposomes.

An essential step in FR-mediated liposomal drug delivery is the unloading of the encapsulated contents following endocytosis by the target cell. Normally, there is an unforgiving trade-off between liposome stability in circulation and liposome unloading efficiency following endocytosis by a target cell. Thus, to achieve a long circulation time, liposomal formulations must be both highly stable and relatively small (≤ 150 nm in diameter) in order to avoid opsonization by serum proteins and the consequent removal by the reticuloendothelial system (RES uptake). Modifications to avoid these pitfalls have included derivatization of the liposome surface with sterically protecting polymers such as PEG [69], or assembly of the liposomes from saturated long chain lipids and cholesterol [70]. Unfortunately, such stabilizing factors can also block receptor–ligand interactions and prevent unloading of the encapsulated drugs following uptake by the target cell. Although the ligand recognition problem may be solved by attaching the ligand to a PEG spacer that is longer than the underlying PEG coating, the intracellular unloading problem is considerably more problematic. Nevertheless, several very encouraging strategies have been explored for building a pH-triggered release mechanism that can enable cargo escape after sterically stabilized liposome uptake into acidic endosomes. One such method was to incorporate a pH-sensitive fusogenic peptide (e.g., the EALA peptide) into the liposome to catalyze liposome fusion with the endosome at the low endosomal pH [71]. Alternatively, pH-sensitive fusogenic lipids have been constructed that increase liposome permeability and promote content release only following uptake into acidic endosomal compartments [72,73]. Major improvements in cell killing with folate-targeted liposomal formulations (>60-fold) have been reported upon the use of each of these pH dependent release mechanisms [72], confirming that delivery across the plasma membrane constitutes only one of several hurdles in liposomal drug delivery.

To date, no folate-targeted liposomal drugs have been tested in vivo. Nevertheless, the biodistribution of radiolabeled folate-derivatized liposomes has been compared with that of nontargeted liposomes in a murine tumor model [74]. Importantly, both targeted and nontargeted liposomal formulations showed enhanced uptake in the tumor, however, no difference was observed in tumor accumulation between targeted and nontargeted liposomes. It was presumed that passive targeting due to the EPR effect domi-

nated the biodistribution of both formulations and that if any advantage to folate targeting existed in vivo, it would have to be found in the ability of folate derivatization to mediate internalization of the liposome and its contents. As will be discussed below, this advantage can in fact be demonstrated in vivo for liposomal gene therapy vectors.

3.1.3. Gene therapy vectors

Along with efforts to develop folate-conjugated anticancer drugs, progress has been made in the field of folate-targeted gene therapy, where both viral and nonviral (liposomal or polylysine-based) vectors have been examined [10,11]. As might be anticipated, when liposomal vectors are used for targeted gene delivery, they encounter the same obstacles as drug-encapsulating liposomes, including problems with serum stability, tumor penetration, vector internalization, and endosomal escape following tumor cell uptake. The solutions to these problems, however, are very different from those for targeted liposome-encapsulated drugs. First, encapsulation of bulky, negatively charged polynucleotides requires a very different set of components and methods than those used with low molecular weight drugs. Second, unloading of liposome-entrapped genes following cell surface binding and endocytosis requires formation of pores much larger than those needed for escape of small molecules. And third, genes (unlike many drugs) must gain access to the nucleus before their therapeutic activities can be expressed. As a result, folate-targeted liposomal vectors must also include features that enable transfer of the genetic material from the cytoplasm into the nucleus.

Whereas low molecular weight drugs can be encapsulated in liposomes of virtually any size, naked DNA is too bulky to entrap into small liposomes. This size limitation is critical, since the well-characterized routes for particle endocytosis may all have size limits near 100 nm [75]. As a consequence, compaction of DNA becomes necessary for its delivery into cells by receptor-mediated endocytosis. DNA compaction is generally achieved by complexation with high molecular weight polycations (polylysine, polyethylenimine, and polyamidoamine dendrimers) in ratios that can allow retention of electrostatic charge, if desired [76]. For example, a slight excess of positive charge has been found useful for encapsulation of DNA · polylysine particles into folate-targeted anionic liposomes [51,77]. The net anionic character of the final vector complex has been shown to reduce nonspecific binding to mammalian cell surfaces, thereby allowing transgene expression to be determined primarily by the distribution of FR.

Endosomal escape mechanisms have also contributed significantly to the efficiency of folate-targeted gene therapy. Unlike cationic liposomes and lipoplexes, which can fuse with most plasma membranes and release their contents directly into the cytoplasm, FR-targeted vectors enter endosomal compartments from which they must escape for transfection to occur. For this purpose, mixtures of DOPE and cholesterol hemisuccinate (CHEMS) have proven useful in formulating liposomes that are stable at neutral or basic pH, but fusogenic at acidic/endosomal pH values [51]. Folate-targeted liposomal vectors constructed from these fusogenic components transfect cells orders of magnitude better than nonfusogenic lipids of similar composition. Similarly, the use of a ''caged'' pH-sensitive lipid, *N*-citraconyl-dioleoylphosphatidylethanolamine (C-DOPE), that releases its headgroup at endosomal pH values and thereby becomes a fusogenic DOPE, also augments folate-mediated gene expression [77]. Since an improvement in folate-targeted gene therapy is also seen after incorporation of a pH-dependent fusogenic peptide into liposomal vectors [78], it can be concluded that some type of pH-triggered endosomal unloading mechanism must be included to enhance the efficiency of folate-targeted gene therapy [79]. Finally, incorporation of a nuclear localization sequence into the encapsulated polynucleotide can also modestly increase the transfection activity of an FR-directed vector, suggesting that facilitated transport of the genetic material from the cytoplasm into the nucleus may also contribute to the efficiency of targeted gene therapy [77].

Although ligand-targeted liposomes do not display greater tumor accumulation than nontargeted liposomes [74,80], folate-targeted gene therapy vectors have been found to promote much higher levels of tumor-specific gene expression than nontargeted vectors. Presumably, as noted above, the folate derivatization enhances vector internalization, without significantly affecting deposition/retention of the

large particles in the tumor. Thus, Xu et al. [53] observe a manifold increase in β-galactosidase gene expression in solid tumors following intravenous administration of folate-targeted liposomal vectors (60–70 nm diameter). Not only was transgene expression limited to malignant tissues, but most cells in each tumor mass were observed to express the gene. Furthermore, systemic delivery of a folate-targeted p53 cationic gene therapy vector was found to greatly improve the therapeutic efficacy of conventional chemo- and radiotherapeutic agents against FR-positive human tumor xenografts, yielding complete cures of subcutaneous cancers of the breast, prostate, and head and neck where the chemo- and radiotherapeutic agents alone exerted little effect [53]. As expected from studies with other cationic liposomes, the major limitations associated with the above liposomal preparations were low stability during storage, high RES uptake, and fast plasma clearance. To circumvent these problems, a folate–PEG coating method was proposed where the precondensed DNA-cationic lipid structure would be protected by a layer of PEG, with folic acid at the distal ends of the PEG to facilitate tumor cell targeting [53]. Leamon et al. have also observed significantly improved tumor-specific transgene expression following derivatization of their liposomal vectors with a PEG–tethered folic acid (manuscript submitted). Not only was tumor expression greatly enhanced, but with one particular vector composition, gene expression in other tissues was either low or absent. Although many variables were examined in these latter studies, vector size and charge emerged as the most critical parameters to optimize for folate-mediated gene expression.

The lack of tumor specificity of viral vectors has also presented a challenge for workers in the gene therapy field. Attempts to clone cell-targeting sequences into viral envelope proteins have generally led to disappointing results, largely due to nonspecific uptake of the transformed viral particles by nontargeted cells [81]. We have observed, however, that folate derivatization does allow avid binding of both ecotropic retroviruses and adenoviruses to FR-positive KB cells [82]. The cell-associated viruses, however, were found to remain almost exclusively on the cell surface, possibly because their sizes were too large (>120 nm) for FR-mediated endocytosis. As a consequence, transgene expression was not observed. Interestingly, the binding of the folate-derivatized adenoviral vectors to the target cell's FR actually prevented normal viral transfection of the same cell. That is, since viral transfection could be restored by blocking FR with excess free folic acid, it could be concluded that the folate-linked adenovirus was fully active, but unable to infect cells by its usual pathway because it was forced to dock at the very high affinity FR rather than its usual receptor.

Finally, small antisense oligodeoxyribonucleotides (ODN) have also been non-destructively delivered into cultured KB cells by encapsulation in egg phosphatidylcholine/cholesterol/folate–PEG–PE liposomes [48]. Furthermore, antisense ODN and even naked plasmid DNA have been efficiently delivered into tumor cells via direct conjugation to folic acid [49,50], or via complexation with folate– [83] or folate–PEG-conjugated polycations [83,84]. Regarding the latter, it has recently been observed that introduction of a PEG spacer of appropriate size ($M_r \sim 3400$) between the polycation and the folate can not only provide a barrier against nuclease digestion of the cation condensed DNA, but also greatly improve particle binding to the cancer cell FR (similar to folate–PEG-conjugated liposomes) [84,85]. Thus, while the properties of these latter constructs remain poorly understood, they appear to constitute fertile areas for future research, since their small sizes allow for improved extravasation and penetration of tumor masses [50].

3.1.4. Other macromolecular drug carriers

There are obviously many other types of macromolecular drug carriers that could be candidates for FR-mediated delivery. Currently available or under development are biodegradable nanospheres (nanoparticles) [86], water-soluble *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers [55], and polymeric micelles [56]. Such biocompatible drug delivery systems are often designed to include a drug release mechanism that discharges the therapeutic agent in its free form as the carrier degrades in vivo. While some of these carriers have shown enhanced tumor uptake when linked to tumor-specific monoclonal antibodies [55], very few have been examined as conjugates of folic acid. However, in

one interesting report, folic acid was covalently linked to a PEG-coated nanoparticle composed of the novel copolymer, poly[aminopoly(ethylene glycol)cyanoacrylate-*co*-hexadecyl cyanoacrylate] [54]. When ~15% of the total PEG ($M_r \sim 3400$) chains on the surface of the nanoparticles were modified with folic acid, the resulting derivatized nanoparticles demonstrated strong multivalent avidity towards soluble FR immobilized on a sensorchip (surface plasma resonance analysis). Obviously, more comprehensive studies will have to be conducted in order to evaluate the targetability of nanoparticles in vivo, but the basic formulation may be worthy of further scrutiny.

In collaboration with Dr. Jindrich Kopecek and Dr. Pavla Kopeckova at The University of Utah, we have attempted to target HPMA copolymers to FR-expressing tumor cells. Two folate-derivatized HPMA copolymers ($M_r \sim 24{,}000$) were synthesized to contain either a fluorescent marker (FITC) or the anticancer drug, doxorubicin (Fig. 5A). Designed for intracellular cleavage by lysosomal enzymes, a biodegradable oligopeptide (Gly–Phe–Leu–Gly) sequence was used to covalently link folic acid and doxorubicin to the HPMA copolymer in the folate–HPMA–doxorubicin construct. In contrast, a physiologically stable spacer (Gly or Lys) [87] was used to link folic acid or FITC to HPMA in the folate–HPMA–FITC construct. Both constructs were then tested in vitro for FR-dependent cellular uptake and/or cytotoxicity. In comparison to the nontargeted HPMA–FITC control, folate–HPMA–FITC demonstrated strong cell-associated FITC fluorescence that was totally competable by excess free folic acid (Fig. 5B), confirming that the cell uptake was FR-mediated. In contrast, neither FR-specific uptake nor cytotoxicity was detected for the folate–HPMA–doxorubicin construct (data not shown). In fact, underivatized HPMA–doxorubicin copolymer was shown to undergo nonspecific cellular uptake regardless of the level of FR expression. Although a reason for the failure to achieve folate targeting with the doxorubicin-containing copolymer was never determined, it is conceivable that the folate ligand may have been inaccessible to cell surface FR in its location on the HPMA copolymer surface. That is, as we have observed with other hydrophobic conjugates of folic acid, the folate may have been buried within the hydrophobic interior of the copolymer when the HPMA was substituted with doxorubicin. While the data with the folate–HPMA–FITC copolymer demonstrate that HPMA constructs are clearly targetable, innovations must still be developed to ensure proper ligand presentation and reduced copolymer uptake in the absence of ligand–receptor interactions.

Finally, polymeric micelles represent a third category of novel drug carriers that are potentially targetable with folic acid. To date, a number of poorly water-soluble drugs have been entrapped within the hydrophobic cores of spherical micelles comprised of amphiphilic block co-polymers [88,89]. Because of their hydrophilic surfaces and small sizes (<100 nm), these polymeric micelles exhibit long circulation times in vivo and can selectively accumulate in malignant tissues via passive targeting. And, as with liposomal gene therapy vectors, it is conceivable that tumor-targeting/intracellular delivery could be improved by attachment of folate to the surface of the micelle. Although no studies of such targeted constructs have been reported to date, we would assume that the same methods and potential pitfalls would arise in this targeting application as seen previously with other polymers and liposomal formulations.

3.2. Drugs that do not require intracellular delivery

3.2.1. Prodrug-activating enzymes

The concept of enzyme/prodrug therapy involves the localization of an activating enzyme on cancer cells and the subsequent conversion of inactive prodrugs into active therapeutic agents by the tumor-localized enzyme. In view of this tumor localization requirement, an obvious application of the folate targeting strategy would be to use folate to concentrate the activating enzyme within malignant tissues [57]. In this application, the vitamin would simply serve the role of the antibody in the more familiar two-step procedure, termed ADEPT, or antibody-directed enzyme prodrug therapy [90]. Although folate is certainly not capable of targeting all cancer types, folate targeting would seem to offer several improvements over most versions of ADEPT, in that the targeted enzyme complex would be much smaller (better capable of tumor penetration), less immuno-

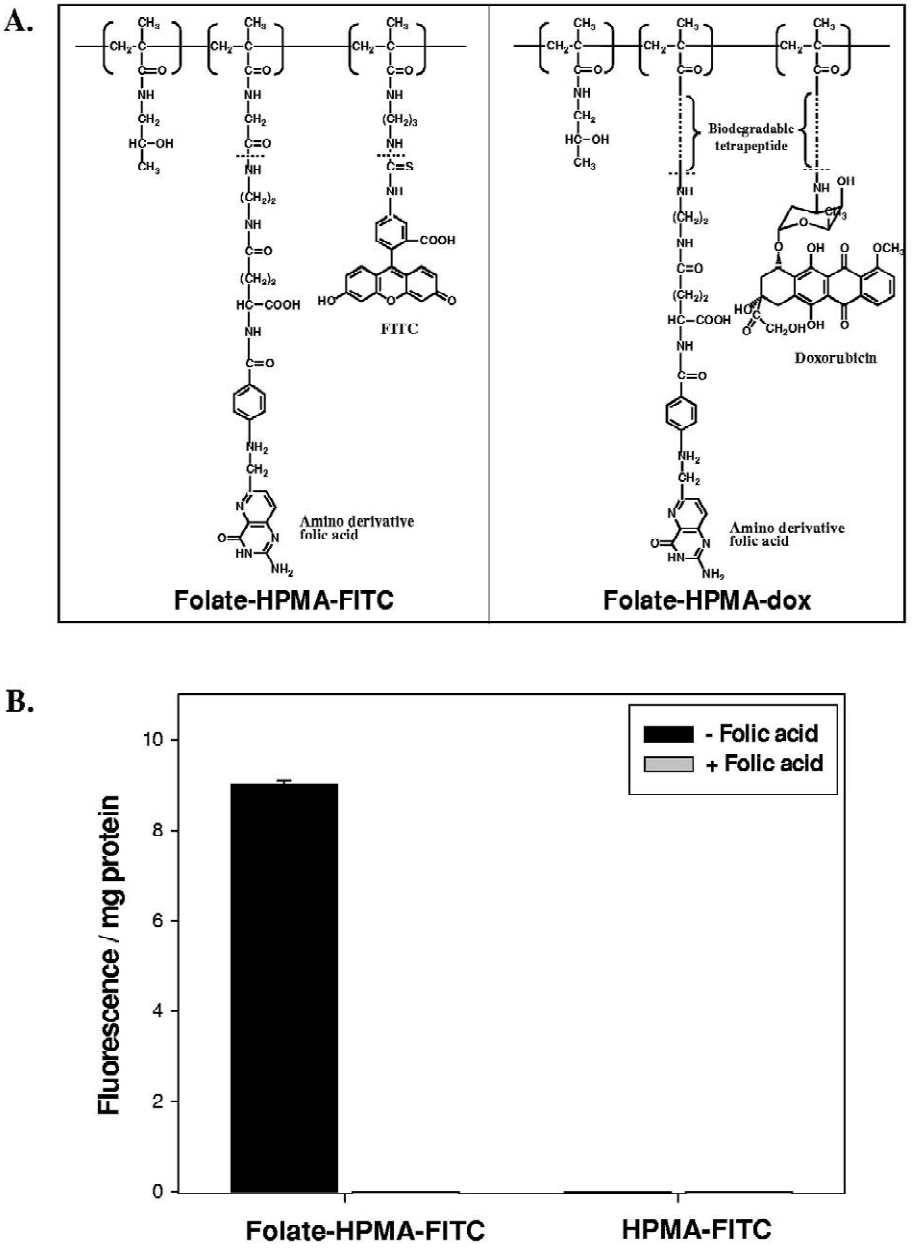

Fig. 5. Folate-conjugated HPMA copolymers. (A) Structures of folate–HPMA–FITC and folate–HPMA–doxorubicin; (B) folate–HPMA–FITC binds specifically to FR-positive tumor cells. Cultured KB cells were incubated with folate–HPMA–FITC or HPMA–FITC at 37 °C for 2 h in the presence (+) or absence (−) of excess free folic acid. The cells were then washed, dissolved in detergent and evaluated in a fluorescence spectrophotometer for FITC fluorescence. Cellular uptake of each HPMA copolymer is expressed as mean cell-associated fluorescence divided by total cell protein.

genic, more easily prepared and stored, and more avidly attracted to the tumor cells than its antibody-linked counterparts.

To demonstrate that selective tumor cell delivery of a prodrug-activating enzyme could be achieved using folate as the targeting ligand, penicillin-V amidase (PVA), a fungal enzyme that hydrolyzes the amide bond between doxorubicin and *p*-hydroxyphenoxyacetamide (DPO) and thereby releases active doxorubicin, was conjugated to folic acid [57]. While the prodrug alone had no apparent cytotoxic activity towards FR-positive tumor cells, the combination of folate–enzyme conjugate and DPO prodrug generated cytotoxicity at a level that was comparable to that of free doxorubicin (IC_{50}, ~0.6 μM). Further, the enhanced cytotoxicity was completely reversed upon addition of free folic acid, which blocks the binding of the folate–enzyme conjugate to cell surface FR. Based on these data, it was concluded that specific killing of FR-positive tumor cells can be achieved by folate targeting of a prodrug-activating enzyme followed by administration of its prodrug substrate. However, as with all ADEPT-related strategies, the immunogenicity of the activating enzyme must be addressed, since enzymes not represented in the human genome are generally preferred in order to avoid activation of prodrug in nontarget tissues by an endogenous enzyme.

3.2.2. Immunotherapeutic agents

In perhaps its most exciting application, cell surface FR can also serve to concentrate immunotherapeutic agents on cancer cell surfaces. For example, folic acid has been recently exploited to redirect T cells to tumor cells in an effort to force the immune system to recognize and destroy the tumor cells [58,91]. In this application, folate was conjugated to a single chain variable fragment (scFv) of an anti-T-cell receptor/anti-CD3 monoclonal antibody. The resulting conjugate led to tumor cell killing in vitro at folate–scFv concentrations 1000-fold lower than those necessary to even detect the conjugate analytically (1 pM) [58]. In live animal studies, administration of the folate–scFv conjugate resulted in a 10- to 20-fold increase in T-cell infiltration into FR-positive brain tumors [92] and either a complete cure (for freshly implanted tumors) or a significantly prolonged survival (for established tumors) of the tumor-bearing mice [93].

In an unrelated effort to improve tumor immunogenicity, we have developed a two-step strategy of folate-targeted immunotherapy that forces thorough scrutiny of cancer cells by various components of the immune system [59]. In the first step, FR-positive tumor cells are converted from a poorly immunogenic state to a highly immunogenic state by the folate-targeted enrichment of their cell surfaces with a hapten. If a humoral immunity has already been elicited against this hapten (as is the case for haptens against which we have been immunized, such as those derived from tetanus, diphtheria, measles virus, Bordetella pertussis, etc., or for de novo immunization against an antigenic hapten like fluorescein, dinitrophenol, or muramyl peptide), then anti-hapten antibodies are seen to rapidly opsonize the cancer cell surface, rendering it ''marked'' for removal by the immune system. In the second step, the immune system, which can now easily recognize the opsonized tumor cells, is stimulated with nontoxic levels of immunostimulatory cytokines (Interleukin-2 and Interferon-α) to assure that F_c-expressing immune cells mediate removal of all antibody-marked tumor cells. Using immune-competent syngeneic mouse tumor models, we have demonstrated that even well established tumors can be completely eradicated by the above protocol, and that long-term protective immunity against the same tumor cell lines (but not against unrelated tumor lines) develops in the process. The strategy has been shown to be dependent on folate targeting, since administration of the same protocol with a nontargeted hapten shows no therapeutic effect. Most importantly, because the anti-hapten antibodies that decorate the tumor cells and induce their destruction do not gain access to FR on normal epithelial cells [25,26] (e.g., kidney proximal tubules and choroid plexus of the brain), no toxicity has been detected to normal cells. While additional mechanistic studies must still be conducted to fully understand the therapeutic mechanisms involved, it is possible to suggest a pathway responsible for folate–hapten mediated tumor cell destruction (Fig. 6). Thus, following anti-hapten antibody opsonization, tumor cells become primed for antibody-dependent cellular cytotoxicity (ADCC). In this pathway, F_c receptor-expressing immune cells, such as natural

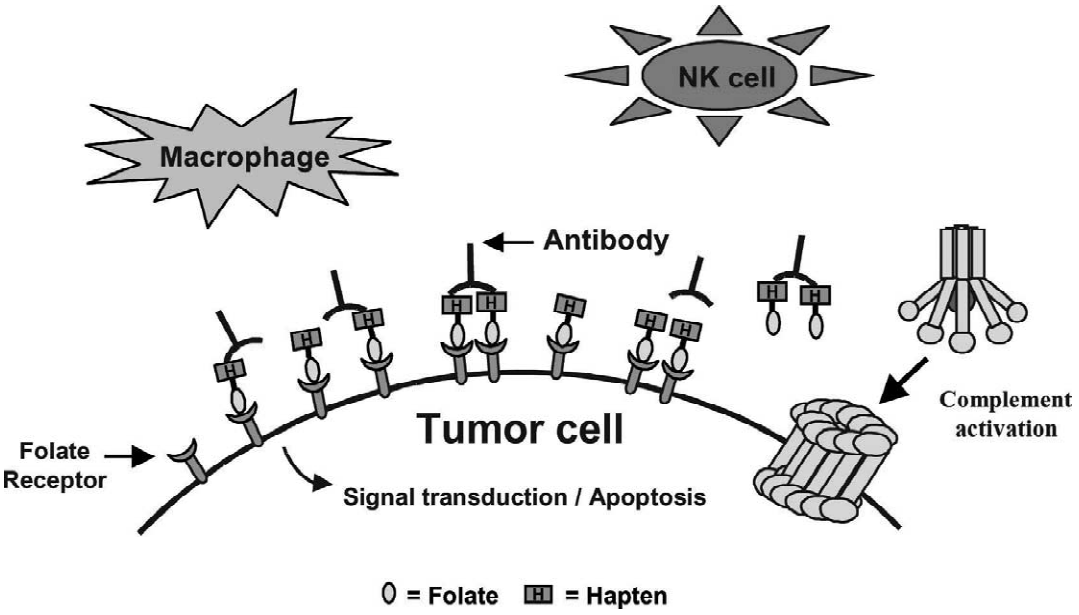

Fig. 6. Proposed mechanism of cancer cell eradication using a two-step strategy of folate–hapten targeted immunotherapy. Following immunization against a potent hapten (e.g., fluorescein, dinitrophenol, or a tetanus peptide), the cancer patient is injected intravenously with a folate conjugate of the same hapten. Folate-mediated decoration of the cancer cell surface with the hapten then promotes opsonization of the cancer cell with anti-hapten antibodies. The cancer cell is then eliminated much like an antibody-coated virus or bacterium, as diagrammed in the figure. Such antibody-mediated immune effector mechanisms generally involve participation of complement proteins, natural killer cells and macrophages, with the latter two collectively termed antibody-dependent cellular cytotoxicity (ADCC).

killer cells and macrophages, recognize opsonized cancer cell surfaces and initiate killing/phagocytosis of the marked cells. Complement, if present, can also enhance the killing/removal process. Long-term protective immunity might then arise through subsequent presentation of tumor cell components to T cells that recognize a natural tumor antigen among the presented material. Expansion of tumor-specific $CD8^+$ T cell memory clones could then protect the host from relapsed tumor growth in the absence of any further treatment.

While the above folate-mediated immunotherapy may not appear to teach any general principles, there is one take-home lesson that should not go unnoticed. That is, it is easier to concentrate a protein in tumor tissue by first enriching the tumor in its low molecular weight ligand and then allowing the protein to passively follow (i.e., as in targeting a folate–hapten to tumor cells and allowing the antibody to follow) than it is to actively target the protein directly (e.g., as in directly targeting the antibody by conjugating it to folic acid). Thus, future strategies to enrich a tumor mass in a macromolecular drug may benefit by first enriching the malignant mass with a high affinity tumor-targeted ligand and then permitting diffusive forces to slowly drive macromolecule docking on the previously positioned ligand.

4. Conclusion and closing remarks

The discovery of high levels of folate receptor expression on many human cancer cells has rendered the folate binding protein an attractive candidate for development of tumor-specific therapeutics. In this role, the folate receptor can effectively serve either of two distinct functions: one as a vehicle for the non-destructive trafficking of extracellular therapeutic agents into the cytoplasm of targeted tumor cells, and the other as a simple tumor marker that allows ligand-mediated enrichment of therapeutic macro-

molecules on tumor cell surfaces. Applications that exploit the former property of FR include the delivery of toxins, polymers, gene therapy vectors, and liposome-encapsulating drugs into cancer cells. Applications that exploit the latter include folate-targeted enzyme-prodrug therapy and folate-targeted immunotherapeutics. Because folate-linked macromolecules do not appear to target normal tissues, development of the technology may be primarily limited by poor penetration of macromolecular conjugates into solid tumors. Solutions to these problems might include: (1) a two-stage targeting strategy, as seen with the folate–hapten therapy, (2) a reduction in size of the targeted complex to facilitate better tumor penetration, (3) application of strategies to increase the permeability of solid tumors, or (4) the use of more potent therapeutic agents that are effective at the lower doses that fortuitously penetrate the tumor masses. Clearly, folate targeting shows considerable promise for development of tumor-specific therapeutic agents, but obstacles must still be overcome before it can reach its full potential.

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