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Laboratory Investigation

Folate receptors as potential therapeutic targets in choroid plexus tumors of SV40 transgenic mice

Todd A. Patrick[1], David M. Kranz[2], Terry A. van Dyke[3] and Edward J. Roy[1,2,4]
[1] *Neuroscience Program,* [2] *Department of Biochemistry,* [4] *Department of Psychology, University of Illinois, Urbana, IL 61801 and* [3] *Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27599, USA*

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Summary

A high affinity folate receptor is expressed in some human cancers, including choroid plexus tumors and ependymomas, and has been suggested as a target for therapeutics. In this report, the expression of folate receptors in an SV40 large T antigen transgenic mouse (SV11) was investigated. SV11 mice develop choroid plexus tumors, a property that may be related to the observation that SV40 has been isolated from human choroid plexus tumors and ependymomas. We report that SV11 choroid plexus tumors contain a high affinity folate receptor (K_D of 1 nM), detectable by ^{125}I-folate autoradiography and immunohistochemistry. Western blot analysis indicated an apparent molecular weight of 38 kDa. RT-PCR revealed the presence of transcripts for both alpha and beta isoforms of the folate receptor. Brain parenchyma has undetectable folate receptor, but normal choroid plexus has substantial levels (as does human choroid plexus). The folate receptors of the tumor are accessible from the bloodstream whereas those of the normal choroid plexus are not. Thus SV11 transgenic mice should be useful for evaluating therapeutic targeting of high affinity folate receptors, both for efficacy of specific agents and possible side effects.

Introduction

The choroid plexus is a specialized region of ependyma, the epithelial lining of the brain ventricles. The incidence of choroid plexus tumors and ependymomas is disproportionately high in young children: 40–70% of each type of tumor is diagnosed in children under two years of age [1, 2]. Pediatric ependymomas and choroid plexus tumors may have an unusual viral etiology. The genomic sequence of the SV40 viral large T antigen (Tag) was detected by PCR in nearly all pediatric ependymomas and in half of choroid plexus tumors examined [3]. The large T antigen was detectable by immunohistochemistry in these tumors. In contrast, other tumor types did not have detectable viral DNA or immunoreactivity. Furthermore, the SV40 virus has subsequently been recovered from the same tumor samples [4].

Mice that are transgenic with SV40 develop ependymal and choroid plexus tumors [5, 6]. One particular line, SV11, develops tumors exclusively in the choroid plexus, and the mice become moribund at 104 ± 12 days [7]. The mice are transgenic for the early control region and early genes of the SV40 genome encoding the large T antigen (Tag). Tag is a nuclear protein capable of binding both p53 and pRB; Tag binding of pRB is necessary for Tag-induced tumorigenesis [8]. Tag is expressed in some choroid plexus cells soon after birth and in most

cells when tumors progress to Grade III and IV at approximately 80 days of age [7]. The SV11 transgenic mice are potentially a valuable animal model for human brain tumors, because the tumors arise endogenously in the normal physiological context and the etiology mimicks that of a subset of pediatric brain tumors.

A number of therapeutic strategies require the identification of surface molecules on tumors that can serve as targets for effector mechanisms such as toxins and immune cells. The surface marker need not be unique to the tumor, but should be expressed at higher levels on tumor cells than on normal cells. One candidate surface antigen is the high affinity folate receptor (FR), a GPI-linked glycosylated monomer of approximately 38 kDa originally isolated by monoclonal antibodies to ovarian carcinomas [9–12].

FRs mediate via potocytosis the uptake of various serum folates, that serve as carbon donors for purine and thymidine synthesis. Distinct FR isoforms that exhibit greater than 70% homology have been identified in humans (FR-α, FR-β, and FR-γ) and in mice (FR-α and FR-β) [13–18]. The affinity of FRs for folate is of the order of 1 nM K_D. The exact role of the high affinity folate receptor remains unclear. A low affinity reduced folate carrier system ($K_D \approx 1$–100 μM) [19, 20] is capable of providing for the folate requirements of normal cells. Interestingly, while FR is not necessary to meet the metabolic requirements of tumor cells *in vitro*, neoplastic cells transfected with FR are capable of increased folate acquisition and enhanced cell growth [21, 22].

FR-α has been found to be over-expressed in most human ovarian carcinomas and approximately 30% of other carcinoma varieties including many mammary adenocarcinomas [9, 23, 24]. FR-β is highly expressed in some mammary carcinomas and a variety of nonepithelial tumors [24]. The isotype of FR in human choroid plexus tumors has not been characterized.

Folate receptors have been used as a possible therapeutic target in various ways. Anti-folate drugs such as 5, 10-dideazatetrahydrofolic acid, CB3717, or ICI-198,583 are believed to mediate anti-tumor activity after accumulation by FRs [25]. Other chemotherapeutics and liposomes have been specifically delivered to FR-bearing cells via chemical conjugation to folic acid [26, 27]. Bispecific antibodies that contain anti-FR antibodies linked to anti-T cell receptor antibodies have been used to target T cells to FR-positive tumor cells and are currently in clinical trials for ovarian carcinomas [28, 29]. Conjugates of folic acid and anti-T cell receptor antibodies are effective at targeting FR-positive tumors with cytotoxic T cells [30].

In this study, we analyzed the anatomical and molecular characteristics of FRs expressed by choroid plexus tumors and normal choroid plexus epithelium in SV11 transgenic mice.

Materials and methods

Cell lines

The following DBA/2-derived tumor cell lines generously provided by Kevin Brigle were used to characterize reagents used in the analysis of SV11 FRs. Cells were maintained in RPMI 1640 containing 5 mM HEPES, 10% fetal bovine serum, 1.3 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 units/l penicillin, and 100 μg/ml streptomycin: Mel, murine erythroleukemia cell; Mel La, a subline of Mel selected on low folate; L1210, a murine leukemia line; LL3, a subline of L1210 selected on low 5-formyltetrahydrofolate; and F2-MTX^{r}A, a MTX resistant L1210 subline selected on low folate. Mel La and LL3 express primarily FR-α; F2-MTXrA expresses only the FR-β isoform.

Transgenic mice

SV11 males heterozygous for the SV40 early control region and early genes were mated to C57BL/6J females (Jackson Laboratories, Bar Harbor, ME). Progeny were screened at one month of age for the Tag gene by PCR. A 1 cm section of tail was digested in 600 μl 0.5 M Tris pH 8.0, 0.1 M EDTA, 0.1 M NaCl, 1% SDS with 100 μg of proteinase K overnight at 55° C. DNA was isolated by phenol/chloroform extraction and ethanol precipitation. DNA was resuspended in TE (10 mM Tris pH 8.0, 1 mM

EDTA) and used in PCR with primer pairs specific to regions of both Tag and transthyretin (an endogenous sequence used as a control).

Western blot analysis

Membranes were prepared by homogenizing approximately 50 mg of tissue in 5 mM Tris pH 7.4, 5 mM sucrose, 100 μg/ml phenylmethanesulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin. Homogenates were centrifuged at 1,500 × g to remove nuclei and supernatants were brought to 10 mM $CaCl_2$ followed by centrifugation at 30,000 × g for 30 min at 4° C. Membrane pellets were solubilized in extraction buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) on ice for 20 min and centrifuged at 30,000 × g to remove insoluble material. Approximately 25 μg membrane protein from tumor and 40 μg membrane protein from cortex were electrophoresed through 10% polyacrylamide gels in SDS under non-reducing conditions. Proteins were electrophoretically transferred to nitrocellulose membranes for 1 hr. Membranes were blocked with TBST (10 mM Tris base, 0.15 M NaCl, 0.05% (v/v) Tween 20) with 1% BSA for 1 hr. FRs were detected with rabbit anti-FR antiserum (generously provided by Kevin Brigle) followed by biotinylated goat anti-rabbit and ABC (Vector Elite, Burlingame, CA). The chromagen was diaminobenzidine enhanced with nickel-cobalt (DAB, Pierce, Rockford, IL).

Autoradiographic analysis of folate binding to tissue sections

Brains from tumor bearing mice were frozen in mounting medium and sections cut at 10 μm. Sections were incubated in 0.2 M sodium acetate buffer pH 4.5 to remove unlabeled folate, washed in 50 mM Tris 150 mM NaCl buffer, pH 7.6, and incubated with 100 μl of one of seven concentrations from 0.01 nM to 2.6 nM of ^{125}I-labeled folic acid (Amersham, S.A. 2000 Ci/mmol) at room temper ature in a humid chamber for 30 min. Control slides were incubated with isotope plus 100-fold excess unlabeled folic acid. Non-specific binding was less than 30% of total binding at the highest concentration. Slides were washed 3 times in Tris saline buffer, and dried in a dessicator at 4° C. After drying, slides were dipped in Kodak NTB-3 emulsion at 42° C, air dried, and stored in a dessicated box until developed 24 hrs later. Autoradiograms were analyzed using the program NIH Image to quantify silver grains.

Immunohistochemistry

Immunohistochemistry was performed using the same polyclonal rabbit antibody against the FR used in the Western blots. Mice were anesthetized and perfused with saline followed by AZF (acetic acid, zinc chloride, 10% formalin, Newcomer Supply, Middleton, WI). Similar immunoreactivity was obtained with perfusion fixation with AZF, immersion fixation with AZF, immersion fixation with AMeX (acetone, methyl benzoate, xylene), or with cryostat sections of unfixed tissue (data not shown). Sections were blocked with 5% normal goat serum and endogenous peroxidase activity quenched with 3% H_2O_2 in methanol. Six primary antibody dilutions of polyclonal anti-FR antiserum ranged from 1 : 1,000 to 1 : 50,000 (5 μg/ml – 0.1 μg/ml) in Biomeda antibody buffer. Secondary antibody (1 : 1,000) was biotinylated goat anti-rabbit IgG (Vector), followed by avidin-biotin peroxidase complex (ABC, Vector). The chromagen was metal enhanced DAB (Pierce).

RNA isolation

Total RNA was isolated from approximately 100 mg tumor tissue or 10^8 cultured cells by solubilization in guanidine hydrochloride and differential centrifugation through a CsCl gradient [31]. RNA pellets were solubilized in H_2O, ethanol precipitated, and re-solubilized in H_2O. Concentrations were calculated from A_{260} readings.

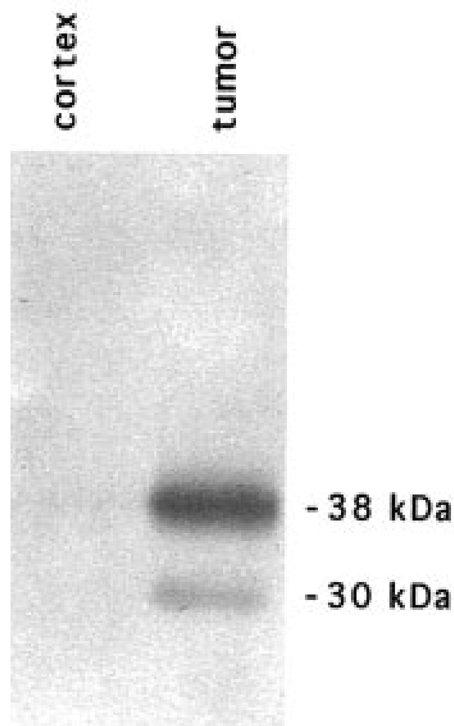

Figure 1. Western blot analysis of membrane proteins. Samples of choroid plexus tumor and surrounding brain cortex were homogenized and membranes isolated by differential centrifugation; proteins were detergent solubilized and electrophoresed through 10% polyacrylamide gels (25 μg of tumor protein and 40 μg of cortex protein). After transfer to nitrocellulose membranes, proteins were detected with a polyclonal antiserum against the mouse folate receptor.

Northern blot analysis

Forty μg total RNA was denatured in 0.5 X formaldehyde gel-running buffer (1X : 0.02 M 3-(N-morpholino)propanesulfonic acid pH 7.0, 8 mM sodium acetate, 1 mM EDTA), 2.2 M formaldehyde, and 50% (v/v) deionized formamide and the sample was electrophoresed through a 1.5% agarose gel containing 0.66 M formaldehyde and 1 X formaldehyde gel-running buffer. Molecular weight marker lanes were stained separately in 5 μg/ml ethidium bromide, 0.1 M ammonium acetate for 15 minutes and destained in 0.1 M ammonium acetate for two hrs. RNA was transferred to nylon membranes by capillary action in 20 X SSC (1 X SSC: 0.15 M NaCl, 0.015 M Na_3 citrate) for a minimum of 16 hrs. Membranes were washed with 2 X SSC and dried prior to baking at 80° C for 2 hrs.

Probes for FR-α and FR-β were made from isolated cDNAs of each (provided by Kevin Brigle). DNA was labeled using a random primer kit (Gibco BRL, Gaithersburg, MD) and [α-^{32}P]-dATP (ICN, Costa Mesa, CA) according to the manufacturer's instructions. Membranes were pre-hybridized for 1 hr at 65° C in 0.52 M sodium phosphate (pH 7.2), 15 mM EDTA, 0.25 M NaCl, 6.4% SDS, and 0.01% BSA with 4 μl/ml denatured sonicated salmon sperm DNA. 20 μl heat-denatured [α-^{32}P]-labeled probe and an additional 4 μg/ml denatured sonicated salmon sperm were added to the hybridization buffer and allowed to hybridize for a minimum of 12 hrs at 65° C. Membranes were washed successively in 2 X SSC, 0.1% SDS at room temperature, 2 X SSC, 0.1% SDS at 65° C, and 0.2 X SSC, 0.1% SDS at 65° C. The sizes of the major transcripts were calculated by a linear regression plot derived from the marker lane (0.16–1.77 Kb RNA Ladder, Gibco BRL).

PCR of FR-α and FR-β cDNA

Plasmid containing cDNAs for FR-α or FR-β was serially diluted by ten-fold increments (5 ng/μl – 500 fg/μl). 1 μl aliquots of each dilution were added to 100 μl of 1 X PCR buffer (50 mM KCl, 10 mM Tris pH 8.4, 1.5 mM $MgCl_2$, 50 mM dNTPs) containing primer pairs (200 nM each) specific to either FR-α or FR-β and 1 unit of Taq polymerase (Fisher). Primer pairs for the FRs amplified the entire coding regions (765 bp for FR-α and 753 bp for FR-β) of each respective cDNA. Each respective set of FR primer pairs flank introns such that amplification of genomic DNA is readily distinguished from cDNA. Sequences of primer pairs were as follows:

FR-α5′ ATGGCTCACCTGATGACTGTGCAG
FR-α3′ GCTGATCACCCAGAGCAGCACTAA
FR-β5′ ATGGCCTGGAAACAGACACCACTC
FR-β3′ GCCAGGGAGCCATAATGACAGCAC

PCR was carried out a total of 35 cycles with a denaturing temperature of 94° C and elongation tem-

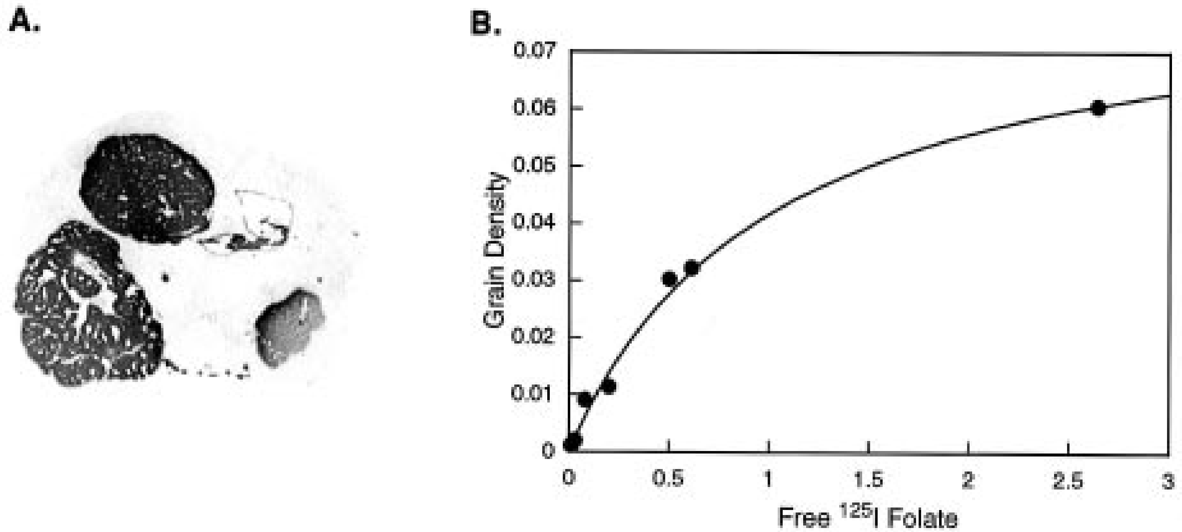

Figure 2. Autoradiographic detection of folate receptor binding using ^{125}I-folate on tissue sections. Cryostat sections of unfixed brain and tumor were incubated with varying concentrations of labeled folate (0.01 nM to 2.6 nM) for 30 min. Slides were dipped in photographic emulsion and exposed for 24 hrs. Sections were counterstained with hematoxylin. *A*. Labeling at 2.6 nM ^{125}I-folate. *B*. For each concentration of labeled folate, silver grains were counted using the computer program NIH Image. Nonlinear regression analysis of the binding isotherm yielded a K_D of 1 nM.

perature of 72° C for each set of primers. Annealing temperature for FR primer pairs was 57° C.

RT-PCR

5 μg of total RNA isolated from cell lines or tumor was heat denatured and added to 1 X cDNA reaction buffer (50 mM KCl, 10 mM Tris pH 8.4, 5 mM DTT, 2.5 mM $MgCl_2$, 0.5 mM dNTPs) with 0.3 μl RNAsin (Promega, Madison, WI), 5 mM oligo-dT, and 45 units AMV reverse transcriptase (Seikagaku, Japan) in a total reaction volume of 20 μl. Reverse transcription reactions were carried out at 42° C for 1 hr. 1 μl of the resulting cDNA reaction mixture was aliquoted into PCR buffer containing either FR-α or FR-β primers and amplified as described.

For RT-PCR analysis of FR isoform expression in tumor and choroid, 0.5 μg of total RNA isolated from normal choroid plexus or tumor was examined as described above to evaluate relative FR isoform expression between tissue samples. Normal choroid plexus was dissected from C57BL/J6 mice 2 hrs after intravenous injection of 0.1 ml 5% (w/v) Evan's Blue dye to facilitate visualization of the choroid plexus. Two-fold dilutions of cDNA reaction mixtures (ranging from 2 μl – 1/256 μl) were used in PCR. β-actin primers, amplifying a 193 bp fragment of β-actin, served as a control for the level of intact mRNA in different samples. Similar to FR primers, β-actin primers flanked at least one intron. Sequences of β-actin primers were as follows:

β-actin 5′ TGCTCTAGACTTCGAGCAGGAG
β-actin 3′ CATGATGGAATTGAATGTAGTT

Southern blot analysis of RT-PCR products

Ten μl of each PCR reaction mixture was electrophoresed on a 1.5% agarose gel containing 5 μg/l ethidium bromide. Gels were washed in 0.2 M HCl for 30 min followed by 0.2 M NaOH, 0.6 M NaCl for 30 min, and finally 0.5 M Tris pH 7.5, 1.5 M NaCl for 30 min. DNA was transferred by capillary action to Genescreen nylon membranes in 20 X SSC for a minimum of 12 hrs. Membranes were pre-treated

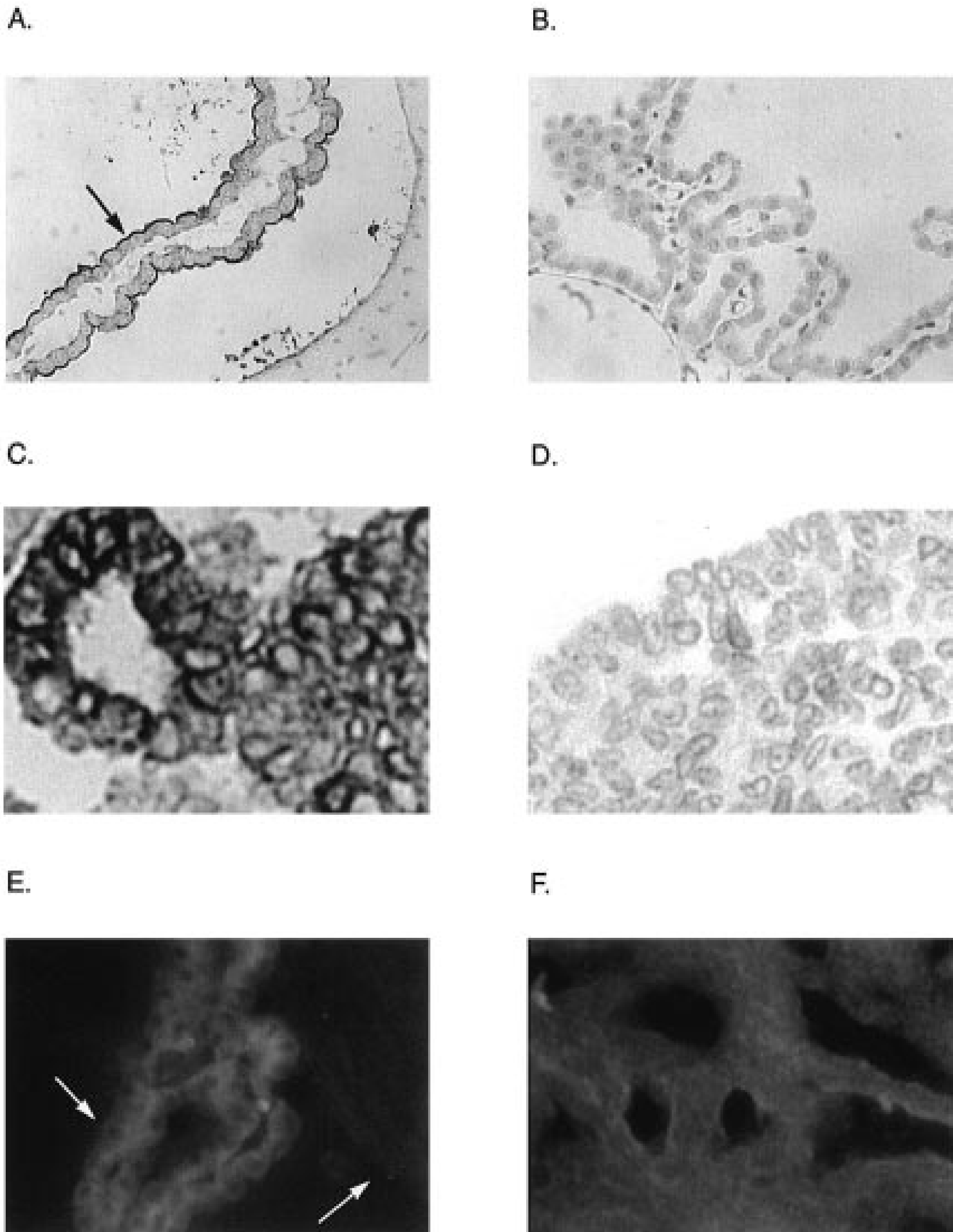

Figure 3. Immunohistochemistry of folate receptor in normal choroid plexus and choroid plexus tumor (A–D). A polyclonal rabbit antibody against the mouse folate receptor was used on AZF-fixed, paraffin-embedded tissue sections with the ABC method and metal-enhanced DAB as chromagen. A. Normal choroid plexus (1 : 5,000 dilution of primary antibody). Arrow indicates strong reactivity of apical surface of choroid plexus. B. Normal choroid plexus processed as in A. but with pimary antibody omitted as a control for nonspecific binding. C. Choroid plexus tumor (1 : 5,000 dilution of primary antibody). D. Tumor section with primary antibody omitted. E. Evans Blue dye analysis of accessibility of tissue by blood-borne agent. Staining is primarily in stromal side of normal choroid plexus. Arrows indicate apical surface of choroid plexus and normal brain parenchyma, which are unstained. F. Choroid plexus tumor of same mouse as in E., showing intense staining throughout tumor.

and hybridized with FR-specific probes as described above. Probe for β-actin was isolated from the product of an RT-PCR reaction on murine erythroleukemia cells (Mel). Membranes were washed as described above. Membranes were exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, CA) and visualized on a Phosphorlmager (Molecular Dynamics).

Blood-Cerebrospinal Fluid (CSF) barrier assessment

Evans Blue is a vital dye commonly used to assess the intactness of the blood brain barrier [32]. To compare the access of blood-borne substances to tumor versus components of normal choroid plexus, 100 μl of 5% (w/v) Evans Blue (Sigma Chemical, St. Louis, MO) in 10 mM sodium phosphate 150 mM NaCl (PBS) was injected intraperitoneally. Twenty-four hrs later mice were perfused with heparinized saline followed by AZF fixative for 10 min. Brains were stored in AZF for 2 hrs, then placed in cold 15% sucrose overnight. Brains were sectioned on a cryostat at 6–20 μm, coverslipped and immediately examined by fluorescence microscopy using a rhodamine filter.

Results

To determine whether choroid plexus tumors express a protein with the properties of folate receptors, tumor membranes were examined by Western blotting with an antiserum that was raised against purified mouse FRs [33]. Flow cytometric analysis was used to confirm the specificity of this antiserum, which binds to FR-positive cell lines (LL3, F2-MTXrA, and Mel La) but not FR-negative cell lines (L1210 and Mel) (data not shown).

A major immunoreactive protein of 38 kDa was identified in the tumor but not in brain cortex that surrounded the tumor (Figure 1). This component corresponds to the 38 kDa FR previously identified on mouse cell lines by affinity labelling with NHS-[^{3}H]-folic acid [33]. A less prominent band of ap-

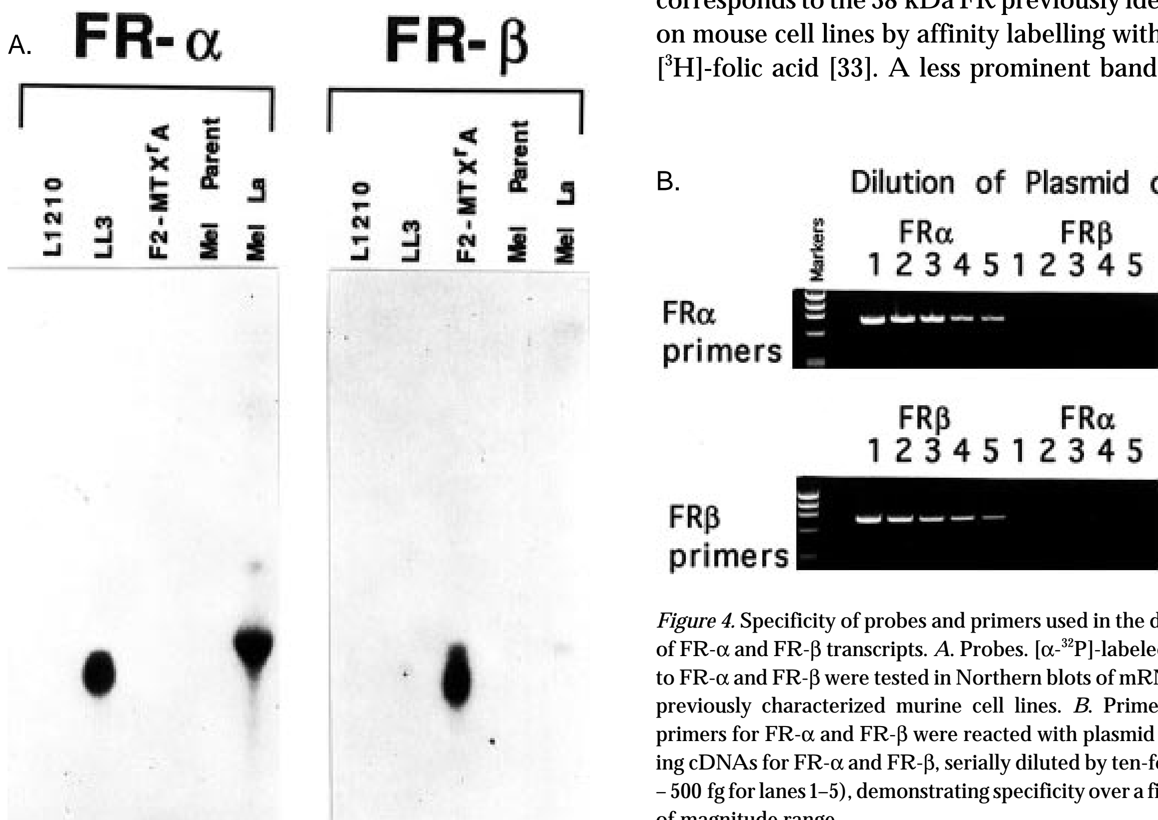

Figure 4. Specificity of probes and primers used in the detection of FR-α and FR-β transcripts. *A.* Probes. [α-^{32}P]-labeled probes to FR-α and FR-β were tested in Northern blots of mRNA from previously characterized murine cell lines. *B.* Primers. PCR primers for FR-α and FR-β were reacted with plasmid containing cDNAs for FR-α and FR-β, serially diluted by ten-fold (5 ng – 500 fg for lanes 1–5), demonstrating specificity over a five order of magnitude range.

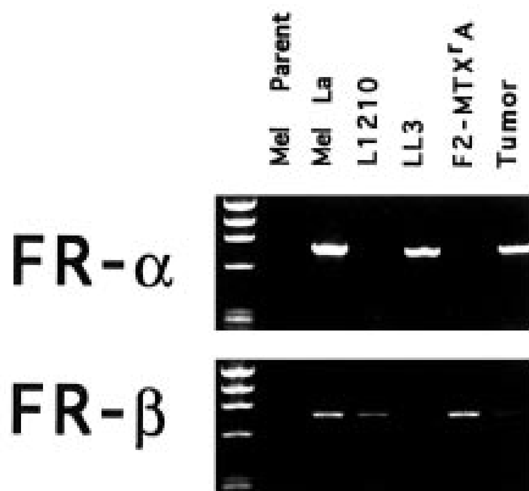

Figure 5. Detection of FR-α and FR-β transcripts in mouse tumor cell lines and tumor by RT-PCR. Total RNA from cell lines and tumor (5 μg from each) was reverse transcribed to generate cDNA. An aliquot from each sample cDNA reaction (1 μl) was amplified for FR-α or FR-β by PCR (35 cycles) using primers specific to each respective isoform. FR-α transcript is detectable by RT-PCR in L1210, LL3, Mel La and tumor as a 765 bp band. FR-β transcript is present in L1210, LL3, F2-MTX^{r}A, Mel La, and tumor as a 753 bp band.

proximately 30 kDA was also detected in tumor samples. This component corresponds to the non-glycosylated form of the FR, as previously identified by Brigle et al. [33].

Autoradiographic analysis of [^{125}I]folic acid binding to tissue sections of tumor and normal brain parenchyma showed that binding of labeled folic acid was high in the tumor (Figure 2A). Quantitation of silver grain density over tumor tissue and nonlinear regression analysis of the saturation curve indicated that the folate binding by the tumor is of high affinity, with a Kd of 1 nM (Figure 2B).

Regions of normal choroid plexus epithelium were also observed to bind significant amounts of labeled folic acid in both tumor bearing SV11 animals and C57 controls. Normal brain parenchyma had very little binding of labeled folic acid, with less than 1% of the silver grain density of the tumor or normal choroid plexus.

Immunohistochemical staining of normal murine choroid plexus with anti-FR antibody showed a highly polarized distribution of FR (Figure 3A). Reactivity was much more intense on the apical surface of the choroid plexus epithelium. In addition, a dilution of primary anti-FR antibody also demonstrated a marked disparity in the density of FR on the apical surface compared to the basal surface. For example, staining of the basal surface was barely noticeable at a 1 : 5,000 dilution of primary antibody (Figure 3A), yet staining of the apical surface persisted even at a 1 : 50,000 dilution of antibody (data not shown). The normal ependymal lining of the ventricles was also lightly stained, but FR was not detectable in normal brain parenchyma.

Immunohistochemical analysis of tumor tissue indicated a diffuse staining of FRs over the entire cell surface (Figure 3C). Since tumors in the SV11 mice arise from choroid epithelium, the FR staining pattern suggests that the individual neoplastic cells lose their original polarity. Qualitative analysis at various antibody concentrations indicated that FR density is greater on the apical surface of normal choroid epithelium than on the tumor cells (for example, at a 1 : 5,000 dilution, nearly all tumor cells were stained, but at 1 : 50,000 only rare tumor cells were stained even though the apical surface of choroid plexus was stained).

Determination of FR probe and primer specificity

The approaches described above do not distinguish between the α and β isoforms of the FR. To examine whether either α or β or both forms are expressed by tumor, RT-PCR of tumor and normal choroid plexus was performed. The specificities of the primers for PCR and the probes used in the post-PCR Southern blots were evaluated. Northern blot analysis was performed with FR cDNA probes to establish probe specificities. Transcripts for FR-α were detected in the LL3 and Mel La cell lines (Figure 4A). LL3 and Mel La are cell lines known to express the FR-α isoform [13]. Tanscripts from the two cells lines were of expected size (approximately 1700 bp for LL3 and $\sim$ 2000 bp for Mel La). The transcript for FR-β was detected in the cell line F2-MTX^{r}A at an expected size of approximately 1600 bp (Figure 4A).

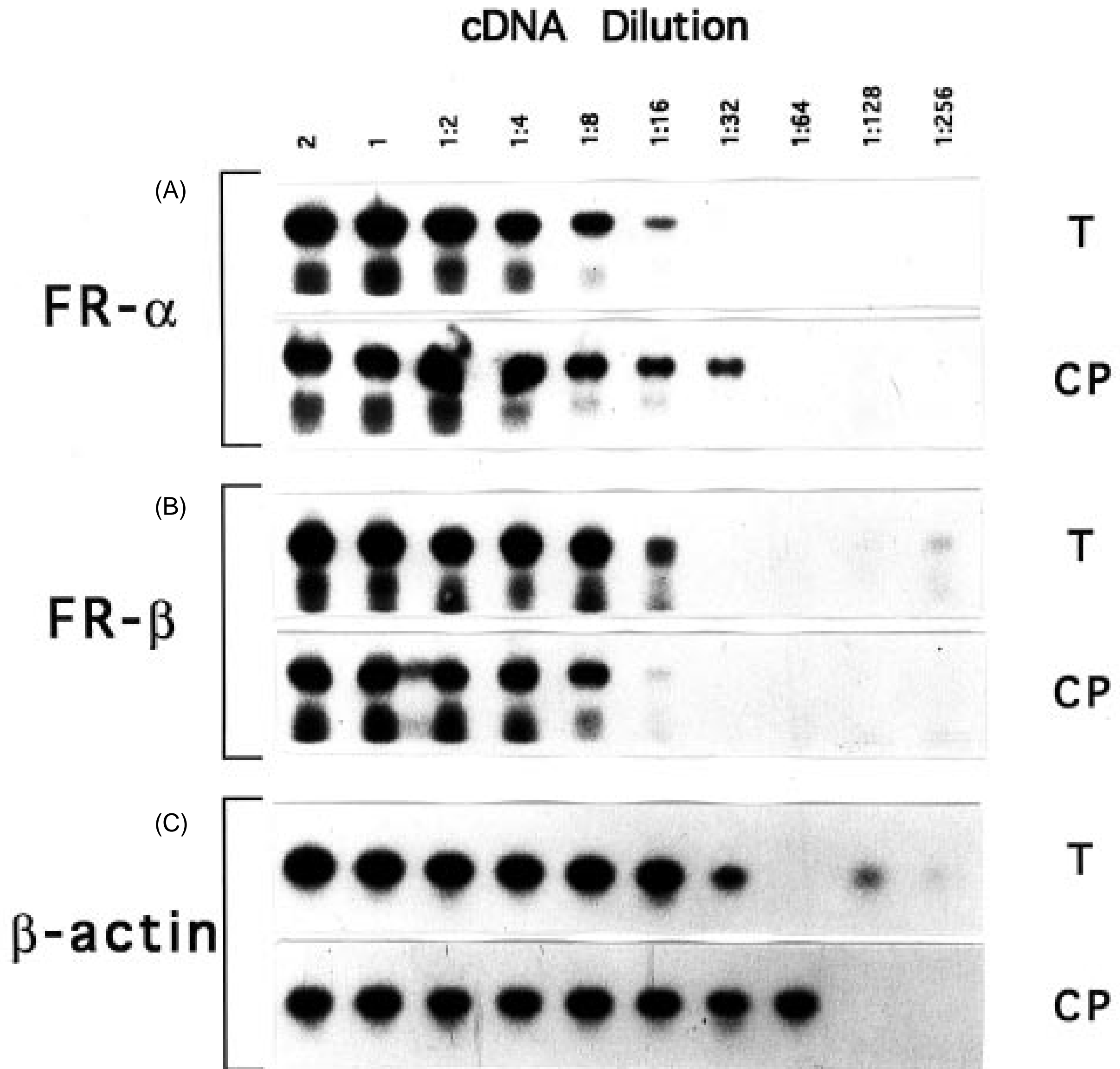

Figure 6. Detection of FR-α and FR-β transcripts in mouse choroid plexus tumor and normal choroid plexus by RT-PCR and Southern blot. Total RNA was isolated from choroid plexus (tissue pooled from eight mice) and tumor (0.5 μg RNA from each) by differential centrifugation, and reverse transcribed to generate cDNA. Aliquots of the cDNA reactions (2 μl and dilutions to 1/256 μl) were amplified for FR-α, FR-β, or β-actin by PCR (35 cycles). Amplified products (10 μl) were electrophoresed on a 1.5% agarose gel, transfered to a nylon membrane, and detected with an [α-^{32}P]-labeled probe to FR-α (*A*), FR-β (*B*), or β-actin (*C*). Membranes were exposed for 44 hrs. Counts were quantitated on a PhosphorImager following a 12 hr exposure.

For PCR, primer pairs for the coding region of each FR isoform were synthesized and their sensitivity and specificity examined. Various amounts of plasmid containing cDNA for each FR isoform (5 ng – 500 fg) were amplified by PCR in the presence of each set of FR primers. An aliquot of each

reaction product was electrophoresed in an agarose gel and visualized by ethidium bromide staining. Each primer pair was found to be sensitive to at least 500 fg and specific to amplifying its respective isoform at 5 ng (Figure 4B). Additional experiments have shown these primers to be specific up to 1 μg of cDNA-containing plasmid (data not shown). Thus the specificity of the primers extends over at least six orders of magnitude.

Detection of FR-α and FR-β transcripts in tumor and choroid plexus by RT-PCR

Due to the very small quantities of total RNA derived from choroid plexus (2 μg) and the relative insensitivity of Northern blots, RT-PCR was utilized to examine FR isoform expression in choroid plexus and tumor. The RT-PCR assay was tested on cDNA derived from five cell lines expressing varying amounts of the FR isoforms. Consistent with the Northern blot analysis, FR-α was present in LL3 and Mel La (Figure 5). In addition, L1210 also contained a detectable amount of FR-α transcript (Figure 5). FR-β was detected in all cell lines examined except the parental Mel line which is known not to express FRs of either isoform (Figure 5). Both FR-α and FR-β were detectable in tumor (Figure 5). RT-PCR followed by Southern blotting of the PCR reaction products detected both α and β FR isoform transcripts in tumor and in normal choroid plexus (Figure 6).

Analysis of the blood-CSF barrier

Because the immunoreactive FR staining of the apical surface of normal choroid plexus brought into question the usefulness of the FR as a target on tumor cells, we investigated the status of the blood-CSF barrier in SV11 mice. In non-tumor-bearing mice, macroscopic and microscopic observation of Evans Blue dye indicated that the blood-brain and blood-CSF barriers were intact (data not shown). The median eminence of the hypothalamus, a region of the brain where the BBB is incomplete, showed intense staining.

In tumor-bearing SV11 mice, fluorescence microscopic analysis of the blood-CSF barrier revealed that the stroma of normal choroid plexus stained intensely with Evans Blue, reflecting the rich supply of capillaries. The choroid epithelium itself displayed a moderate degree of staining, but the apical surface was not stained (Figure 3E). CSF and adjacent brain regions lacked staining, indicating that the blood-CSF barrier is still intact in nonaffected regions of the choroid plexus. Microscopic analysis showed intense staining throughout the tumors (Figure 3F).

Discussion

In this report, endogenously arising brain tumors of SV40 transgenic mice were evaluated for the expression of a recently described tumor-associated antigen, the high affinity folate receptor. Various independent approaches were used to show that these tumors express high affinity folate receptors with properties that are similar to the human receptor. These properties included its molecular weight (∼ 38 kDa) and a binding affinity (K_D) for folate of ∼ 1 nM. RNA transcripts of both FR-α and FR-β isoforms were detected in the tumor.

The interest in FR expression by the SV-40-induced tumors stems from several recent findings regarding the use of FR as a target for therapy. First, human choroid plexus tumors and ependymomas, which may have a similar etiology to the transgenic mouse tumors, express FR at substantial levels [11, 12]. Thus, it seemed possible that mouse choroid plexus tumors may also express FR. Second, monoclonal antibodies to the human FR have recently been studied as possible therapeutic agents either as immunotoxins, radiolabelled antibodies, or bispecific antibodies that target tumor cells for lysis by cytotoxic T lymphocytes [28, 29]. Some of these agents have already reached clinical trials with ovarian carcinomas, of which approximately 98% express FR. We have also recently developed a novel targeting agent that consists of folic acid directly conjugated to an anti-T cell receptor antibody [30]. Finally, the endogenously derived-SV40 tumors can be used as a model that more closely mimics the human disease

than do animal studies that involve human tumor transplantation into immune deficient mice.

As with other tumor-associated antigens, the usefulness of FR as a therapeutic target depends in part on its expression in normal tissue. Earlier work with human tissues has indicated that FR-α is expressed to some degree in placenta, epithelia of the choroid plexus, epididymus, lung, thyroid, and kidney proximal tubules, ductal epithelia of the breast and pancreas, and acinar cells of the breast and salivary gland [11, 12, 24]. In the present study, *in situ* analysis revealed high levels of folate binding activity and FR not only in tumor cells but in normal choroid plexus of both control mice and SV11 mice.

One of the findings described here lends support to the notion that FR on tumor cells may be a useful target despite the presence of FR in normal choroid plexus. Immunohistochemistry shows that the FR expressed by choroid plexus is highly polarized. The protein is present on the apical surface of choroid plexus cells on the CSF side of the blood-CSF barrier. This surface may be less accessible than the tumor to blood-borne therapeutic agents. This possibility is supported by evidence that Evans Blue dye was able to stain cells throughout tumor and the stroma of the choroid plexus, but not the apical aspect of the choroid plexus epithelium.

Another possible approach to targeting tumor cells, rather than normal choroid plexus, would involve identifying whether the FR isoforms are differentially expressed. In this regard, the present results are the first characterization of isoforms of FR in any normal murine tissue. FR-α and FR-β were detected in both tumor and normal choroid plexus, but there appeared to be at least a several-fold higher expression of FR-β mRNA in tumor than FR-β mRNA in choroid plexus, based on densitometry of the probe signal and standardization to the β-actin signal. The levels of cell surface FR-α and FR-β protein on tumor cells and choroid plexus remain to be determined when appropriate monoclonal antibodies that distinguish the murine isoforms become available. However, the suggestion that FR-β may be relatively higher in some tumor cells may warrant the search for agents that target this form specifically.

Recently there have been a number of possible therapeutic agents developed that could target cells that bear folic acid receptors without the use of monoclonal anti-FR antibodies. These include folic acid analogs and folic acid conjugates that are directly cytotoxic or that deliver cytotoxic agents [26, 27, 30]. Preliminary results with a folic acid/antibody conjugate injected intravenously into SV11 mice showed that it accumulated preferentially in the tumor as opposed to other regions of the brain (E.J.R., T.A.P., D.M.K., unpublished data). This agent, along with other folic acid conjugates [34], can be used in the SV11 mice to test the relative effectiveness and potential toxicity of various therapeutic regimens.

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Address for offprints: E.J. Roy, Department of Biochemistry, University of Illinois, 600 S. Mathews, Urbana, IL 61801, USA