Characterization of the Amino Acid Transport of New Immortalized Choroid Plexus Epithelial Cell Lines: A Novel *In Vitro* System for Investigating Transport Functions at the Blood-Cerebrospinal Fluid Barrier

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Purpose. To establish and characterize a choroid plexus epithelial cell line (TR-CSFB) from a new type of transgenic rat harboring the temperature-sensitive simian virus 40 (ts SV 40) large T-antigen gene (Tg rat).

Methods. Choroid plexus epithelial cells were isolated from the Tg rat and cultured on a collagen-coated dish at 37°C during the first period of 3 days. Cells were subsequently cultured at 33°C to activate large T-antigen. At the third passage, cells were cloned by colony formation and isolated from other cells using a penicillin cup.

Results. Five immortalized cell lines of choroid plexus epithelial cells (TR-CSFB 1~5) were obtained from two Tg rats. These cell lines had a polygonal cell morphology, expressed the typical choroid plexus epithelial cell marker, transthyretin, and possessed Na^+, K^+-ATPase on their apical side. TR-CSFBs cells expressed a large T-antigen and grew well at 33°C with a doubling-time of 35~40 hr. [^{3}H]-L-Proline uptake by TR-CSFB cells took place in an Na^+-dependent, ouabain-sensitive, energy-dependent, and concentration-dependent manner. It was also inhibited by α-methylaminoisobutylic acid, suggesting that system A for amino acids operates in TR-CSFB cells. When [^{3}H]-L-proline uptake was measured using the Transwell device, the L-proline uptake rate following application to the apical side was five-fold greater than that following application to the basal side. In addition, both Na^+-dependent and Na^+-independent L-glutamic acid (L-Glu) uptake processes were present in TR-CSFB cells.

Conclusions. Immortalized choroid plexus epithelial cell lines were successfully established from Tg rats and have the properties of choroid plexus epithelial cells, and amino acid transport activity was observed *in vivo*.

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KEY WORDS: Choroid plexus epithelial cell; *in vitro* model; immortalized cell line; L-proline uptake transport; L-glutamic acid uptake transport.

INTRODUCTION

Immortalized cell lines are used to investigate the mechanisms governing the transport of drugs and endogenous substrates. For example, Caco-2 cells (1,2), an intestinal epithelial cell model, and MDCK (Madin–Darby Canine Kidney) (2,3), a kidney epithelial cell model, have been widely used and have been responsible for great advances in our understanding of drug transport functions.

The choroid plexus epithelial cells form a barrier between the blood and cerebrospinal fluid (CSF) that regulates concentrations of essential molecules in the CSF (4). The blood–CSF barrier has several transporters which are involved in the influx and efflux of substrates (4–6) to regulate the composition of the CSF. Therefore, in order to understand factors participating in the maintenance of CSF formation and composition, and the integrity of the central nervous system (CNS), especially with regards to protecting them from toxic effects, it is important to clarify the transport functions operating at the blood–CSF barrier. Freshly isolated samples (7) and primary cultures of choroid plexus (8–10) have been used to study drug transport. However, a choroid plexus epithelial cell line would be desirable in order to screen for the transport characteristics of drugs that act on the CNS, i.e., it would be especially suitable for high-throughput screening. Also, such a cell line would replace the need for large numbers of animals because, in most adult mammals, the choroid plexus accounts for only about 0.25% of the weight of the entire brain (4).

Recently, a transgenic rat harboring a temperature-sensitive simian virus 40 (ts SV 40) large T-antigen gene (Tg rat) has been developed as a source of a conditionally immortalized cell line (11). It is easy to establish cell lines from Tg animals since the ts SV 40 large T-antigen gene is stably expressed in all tissues, and cultured cells can be easily immortalized by the activation at 33°C (12). At this temperature, the activated large T-antigen can induce cell proliferation by interacting with the retinoblastoma gene products (Rb) and p53, which normally regulates cell proliferation, and deactivate the growth-suppression function of these two proteins (12). The large T-antigen gene is unlikely to disrupt any other critical genes in cells since the Tg rat is homozygous (11).

The purpose of the present study was to establish conditionally immortalized choroid plexus cell lines from the Tg rat and to characterize the choroid plexus epithelial markers and transport activities using an *in vitro* experimental model.

MATERIALS AND METHODS

Animals

The origin and characteristics of the male transgenic rat have been previously described (11). Briefly, transgenic rats (line # 1507-5) harboring mutants of ts SV 40 large T-antigen gene were produced by injection of BamH1 DNA fragments of SV 40 tsA58 mutant DNA (pSVtsA58ori(–)-2) into the pronucleus of fertilized eggs of Wistar rats (Charles River, Yokohama, Japan). Normal male Wistar rats, weighing 250 g

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to 300 g, were purchased from Charles River. The investigations using the rats described in this report were approved by the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University.

Isolation of Choroid Plexus Epithelial Cells

Choroid plexus epithelial cells were isolated from two Tg rats using a modification of the procedure described by Washington *et al.* (13). Briefly, the choroid plexus was isolated from the lateral ventricles of Tg rats. The isolated choroid plexus was washed with ice-cold phosphate buffered saline (PBS) and split into fragments using a 23-gauge needle. This was followed by dissection with microscissors, immersion in 0.5% trypsin-EDTA at 37°C with pipetting at 5 min intervals. After a treatment lasting 20 min, digestion was terminated by the addition of Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceuticals, Tokyo, Japan) containing 20 mM sodium bicarbonate, 4.5 g/L D-glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 10% fetal bovine serum (FBS; Moregate, Bulimba, Australia). The cell pellets were obtained by centrifugation for 10 min at 4°C and 230 g and seeded on to a collagen-coated dish (35 mm, Becton Dickinson, Bedford, MA). The cells were cultured in the serum-free DMEM medium described above to prevent the growth of fibroblasts at 37°C in a humidified atmosphere of 5% CO_2/air. After 3 days, the temperature was reduced from 37°C to 33°C to activate SV 40 large T-antigen, and the culture medium was changed to DMEM containing 10% FBS as described above. After two weeks, several types of colonies appeared on the dish. The colonies taking the form of polygonal-shaped cells were selected as candidates of choroid plexus epithelial cells using a penicillin cup. The colonies of other cell types, such as fibroblasts, were removed. Following two or three passages, cells were cloned from a single cell by colony formation and isolated twice from other cells using a penicillin cup.

Immunostaining Analysis

Cells were cultured on a rat tail collagen type I-coated cover-glass (Becton Dickinson) at 33°C for 48 hr. Immunostaining of transthyretin (TTR) was performed by a modification of the procedure described by Zheng *et al.* (12). After removal of the medium, cells were washed with PBS and fixed in 3% paraformaldehyde for 10 min at room temperature. Cells were then incubated with sheep anti-TTR antibody (Biogenesis, England, UK) or rabbit anti-NA^+, K^+-ATPase antibody (Upstate Biotechnology, Lake Placid, NY) as a primary antibody with blocking agent solution (Block Ace, Dainihon Pharmaceutical Co) for 1 hr at room temperature. Cells were washed with 0.05% Triton-X 100/PBS and incubated for 1 hr at room temperature with horseradish peroxidase conjugated antisheep IgG for TTR, or fluorescein isothiocyanate conjugated antirabbit IgG for Na^+, K^+-ATPase, as a secondary antibody. For TTR, cells were stained with 3,3′-diaminobenzidine tetrahydrochloride (0.4 mg/ml 30% H_2O_2/PBS) and viewed under a microscope (IX 70, Olympus, Tokyo, Japan). For Na^+, K^+-ATPase, cells were subsequently stained with propidium iodide and viewed by a Leica confocal laser scanning microscopy (TCS SP, Heidelberg, Germany).

L-Proline and L-Glutamic Acid Uptake by TR-CSFB Cells

TR-CSFB cells were seeded on the rat tail collagen type I-coated 24-well culture plate (Becton Dickinson) or a collagen-coated Transwell (12 well, pore size 0.4 mm, Corning Coster, Cambridge, MA) at a density of 1×10^5 cells/well and cultured for 40 hr at 33°C. Cells were washed with 1 ml extracellular fluid (ECF) buffer consisting of 122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 10 mM D-glucose, and 10 mM Hepes (pH 7.4, 290 mOsm/kg) at 37°C. Na^+-free ECF buffer was prepared by equimolar replacement of NaCl and $NaHCO_3$ with choline chloride and choline bicarbonate. Uptake was initiated by applying ECF buffer containing 2.5 μCi L-[2,3-^{3}H]-proline ([^{3}H] L-proline, 40 Ci/mmol, NEN Life Science Products, Boston, MA) or L-[2,3-^{3}H]-glutamic acid ([^{3}H] L-Glu, 24 Ci/mmol, NEN Life Science Products) and 0.5 μCi [^{14}C] carboxy-inulin ([^{14}C] inulin, 2.64 mCi/g NEN Life Science Products) to estimate the volume of water adhering at 37°C. After a predetermined time, the uptake was terminated by removing the applied solution and cells were immersed in ice-cold ECF buffer. Following this, the cells were solubilized in 1 ml 1% Triton-X 100/PBS. The radioactivity was measured using a liquid scintillation counter equipped with an appropriate channel crossover correction for [^{3}H] and [^{14}C] (LS6500, Beckman, Fullerton, CA), and protein content was determined by a DC protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as a standard.

Data Analysis

For kinetic studies, the Michaelis–Menten constant (K_m) and the maximal rate (V_{max}) of L-proline or L-Glu uptake were calculated from equation (1) or (2) using a nonlinear least-squares regression analysis program (MULTI) (14):

$$V = \frac{V_{max1} \times [S]}{(K_{m1} + [S])} + \frac{V_{max2} \times [S]}{(K_{m2} + [S])} \quad (1)$$

$$V = \frac{V_{max1} \times [S]}{(K_{m1} + [S])} + \frac{V_{max2} \times [S]}{(K_{m2} + [S])} + \frac{V_{max3} \times [S]}{(K_{m3} + [S])} \quad (2)$$

where V, $V_{max1\sim3}$, $K_{m1\sim3}$, and [S] are the uptake rate, the maximum uptake rate of 1 to 3 sites, the K_m value of 1 to 3 sites, and the concentration of L-proline or L-Glu, respectively.

Unless otherwise indicated, all data represent the mean ± S.E.M. An unpaired, two-tailed Student's t-test was used to determine the significance of any differences between two group means. Statistical significance among means of more than two groups was determined by a one-way analysis of variance (ANOVA) followed by the modified Fisher's least squares difference method.

RESULTS

Characterization of Immortalized Rat Choroid Plexus Epithelial Cell Line (TR-CSFB)

Five immortalized rat choroid plexus epithelial cell lines were obtained from two Tg rats and named TR-CSFB1~5. These cell lines had a polygonal-shaped morphology as indicated, for example, with TR-CSFB3 cells (Fig. 1A). Strong staining was observed following incubation with an anti-TTR antibody (Fig. 1B) compared with a control (Fig. 1C), indi-

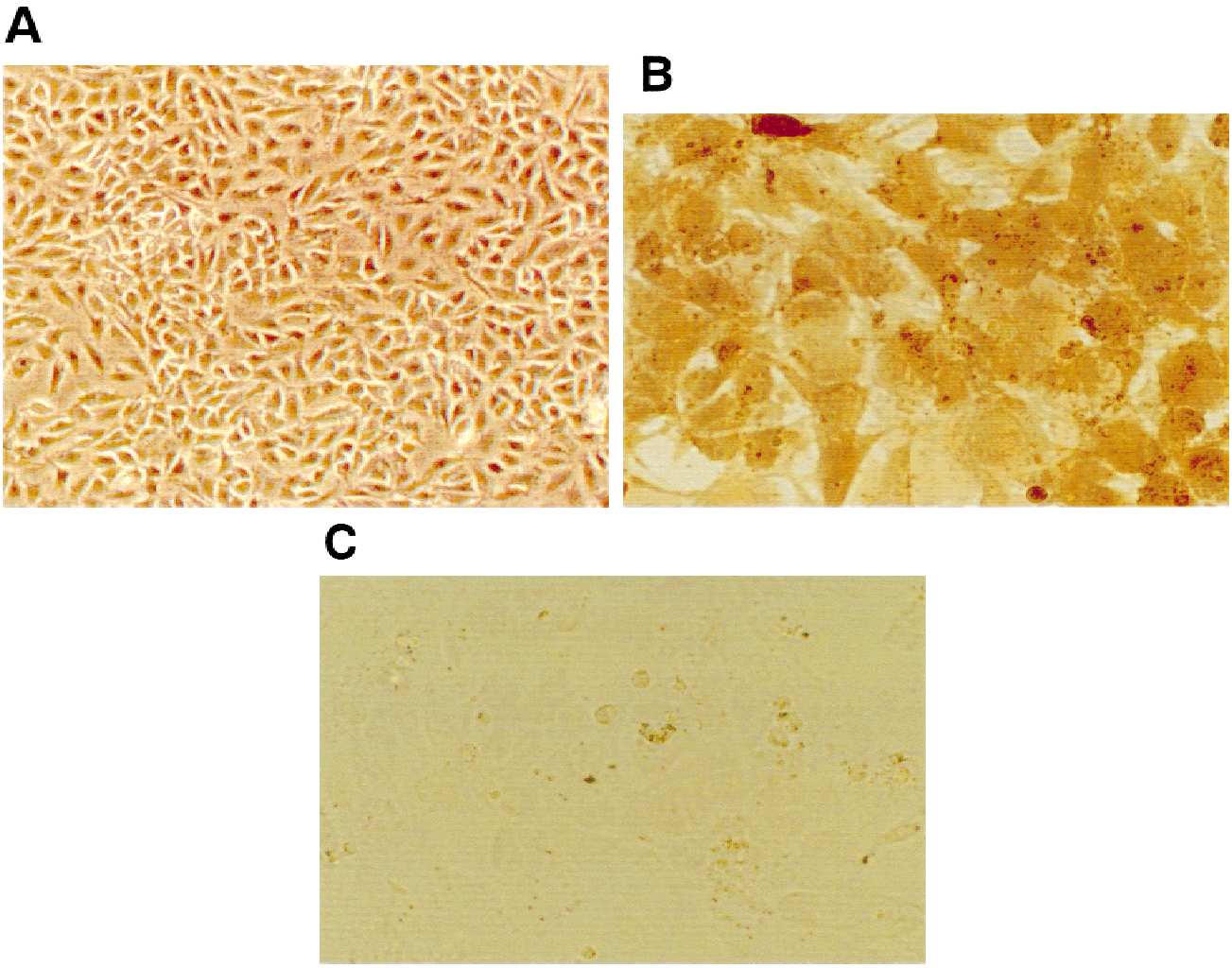

Fig. 1. Phase microscopic image (A) and immunostaining of transthyretin (TTR) in TR-CSFB3 cells (B and C). A: magnification; ×40; B,C: TR-CSFB3 cells were immunostained by anti-TTR antibody (B) and secondary antibody alone (C). magnification; ×200.

cating TTR expression in TR-CSFB3 cells. Na^+, K^+-ATPase localization was examined using a confocal laser scanning microscopy (Fig. 2). The fluorescence following incubation with an anti-Na^+, K^+-ATPase antibody (Figs. 2A and B) was much stronger than that with nonimmune IgG (Figs. 2C and D). An X-Z section showed that fluorescein (green) was predominantly located under the cell nucleus (red) (Fig. 2B), providing supporting evidence that Na^+, K^+-ATPase is localized on the apical side of TR-CSFB3 cells.

Transepithelial electrical resistance (TEER) was measured at 33°C using Millicell-ERS equipment (Millipore, Bedford, MA). The TEER in TR-CSFB1~5 cells was 139~143 ohm · cm^2 at 24~72 hr after seeding (1×10^5 cells/cm^2) on collagen-coated Transwell device. The electrical resistance of the Transwell membrane without cells was about 90 ohm · cm^2 (data not shown).

Cell Growth and Expression of Large T-Antigen

TR-CSFBs cells cultured at 33°C expressed a large T-antigen with a molecular weight of 94 kDa (data not shown); this was the same molecular weight as a large T-antigen of the COS1 cells used as a positive control (15). Growth of TR-CSFBs cells was measured at 33°C. When TR-CSFBs cells were seeded at 5.0×10^3 cells/cm^2, cells exhibited logarithmic growth with a doubling-time of 35.2~40.1 hr and contact inhibition after 7 days. TR-CSFB1 cells grew fastest with a doubling-time of 35.2 ± 0.8 hr, and TR-CSFB3 cells had a doubling-time of 40.1 ±1.9 hr (mean ± S.D.) (data not shown).

L-Proline Uptake by TR-CSFB Cells

The uptake rate of [^{3}H]L-proline was compared in TR-CSFBs cells at 37°C for 5 min when cells were cultured on 24-well cell culture plates. TR-CSFB3 cells had the highest rate (20.9 ± 1.8 μl/(5 min · mg protein)), followed by TR-CSFB5 cells (17.6 ± 3.2 μl/(5 min · mg protein)), TR-CSFB4 cells (13.0 ± 1.0 μl/(5 min · mg protein)), TR-CSFB1 cells (5.97 ± 0.69 μl/(5 min · mg protein)), and TR-CSFB2 cells (1.72 ± 0.41 μl/(5 min · mg protein)). Therefore, TR-CSFB3 cells were selected for the following uptake experiments. As TR-CSFB3 cells are cultured at 33°C, the effect of the incubation period at 37°C on [^{3}H] L-proline uptake was examined. The uptake of [^{3}H] L-proline by TR-CSFB3 cells was not significantly different over the period 10 min to 5 hr, indicating that the uptake of L-proline does not change during incubation at 37°C, at least over a 5 hr period (data not shown).

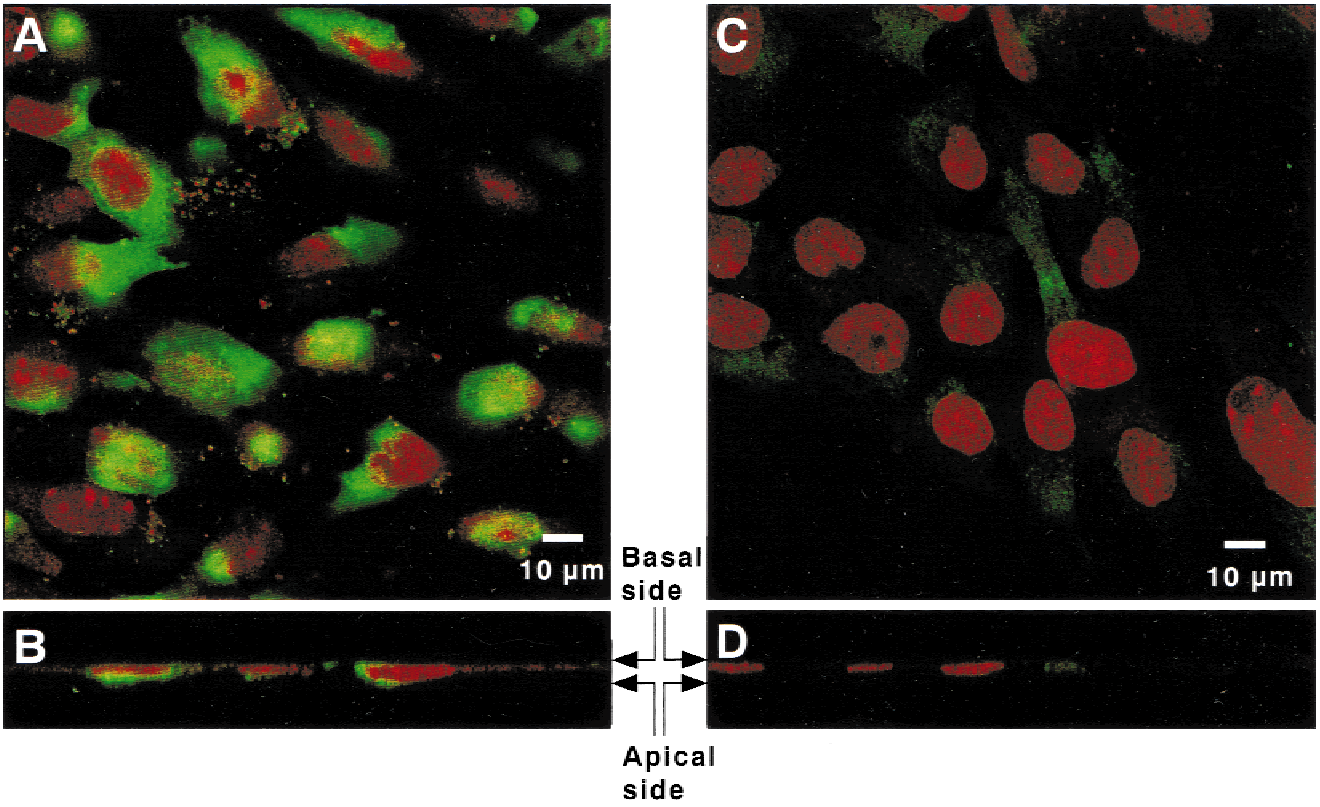

Fig. 2. Localization of Na^+, K^+-ATPase in TR-CSFB3 cells. TR-CSFB3 cells were immunostained by anti-Na^+, K^+-ATPase antibody (A and B) and nonimmune IgG (C and D). A and C indicate an X-Y section; B and D indicate an X-Z section.

The time-courses of [^{3}H] L-proline uptake by TR-CSFB3 cells are shown in Fig. 3A following application of [^{3}H] L-proline to the apical or basal sides using the Transwell device. [^{3}H] L-Proline uptake following application to the apical side was greater than that following application to the basal side. At 10 min, the cell-to-medium (C/M) ratio of [^{3}H] L-proline uptake from the apical side was 41.0 ± 7.2 μl/mg protein and fivefold greater than that from the basal side (8.72 ± 2.67 μl/mg protein), demonstrating that L-proline uptake is predominantly from the apical side. Therefore, the following uptake experiments were performed using TR-CSFB3 cells cultured on the 24-well culture plate. [^{3}H]L-proline uptake increased linearly by 87.6 ± 18.2 μl/mg protein for 30 min at 37°C (Fig. 3B). In contrast, at 4°C, [^{3}H]L-proline uptake was 6.39 ± 0.85 μl/mg protein for 30 min, a reduction of 93.2% compared with that at 37°C (Table 1).

Kinetic analysis showed the existence of both high and low affinity processes for [^{3}H]L-proline uptake by TR-CSFB3 cells (Fig. 4). The corresponding K_{m1} and K_{m2} values were 5.27 ± 2.40 μM and 1.57 ± 0.05 mM, respectively, and the V_{max1} and V_{max2} values were 0.0158 ± 0.0030 nmol/(min · mg protein) and 2.40 ± 0.03 nmol/(min · mg protein) (mean ± S.D.), respectively. Under Na^+-free conditions and in the presence of 1 mM ouabain, there was a significant reduction in [^{3}H] L-proline uptake by TR-CSFB3 cells, by 97.8% and 56.4%, respectively (Table 1). The presence of 1 mM 2,4-

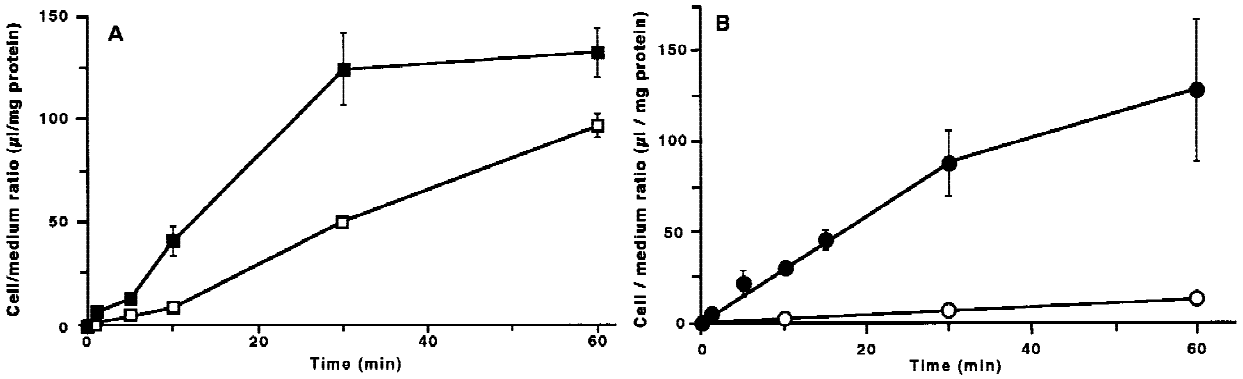

Fig. 3. Time-courses of [^{3}H]L-proline uptake by TR-CSFB3 cells cultured on Transwell (A) and on 24-well culture plates (B). Each point represents the mean ± S.E.M. (n = 3–4). A: [^{3}H]L-Proline uptake following application to the apical side, ■; [^{3}H]L-proline uptake following application to the basal side, □. B: [^{3}H]L-Proline uptake at 37°C, ●; [^{3}H]L-proline uptake at 4°C, ○.

Table 1. Effect of Several Inhibitory Conditions on [^{3}H]L-Proline and [^{3}H]L-Glutamic Acid Uptake by TR-CSFB3

Inhibitory conditions	Percentage of control (%)	
	L-Proline	L-Glutamic acid
Control	100 ± 4.1	100 ± 8.4
Na^+-free condition	2.20 ± 0.40**	48.8 ± 4.3*
1 mM Ouabain	43.6 ± 8.2*	35.9 ± 2.4**
4°C	6.80 ± 1.00**	2.30 ± 0.40**
1 mM 2,4-Dinitrophenol (DNP)	49.8 ± 1.8*	42.8 ± 8.7*
25 μM Rotenone	36.6 ± 2.8*	ND

Note: [^{3}H]L-Proline and [^{3}H]L-glutamic acid uptake was performed at 30 min and 5 min, respectively. Each value represents the mean ± S.E.M. (n = 4).
* $p < 0.05$, ** $p < 0.01$, significantly different from control. ND, not determined.

dinitrophenol (DNP) and 25 μM rotenone caused significant reduction by 50.2% and 63.4%, respectively (Table 1).

Small neutral amino acids (L-proline, L-cysteine, L-alanine, N-methyl-L-alanine, α-methylaminoisobutylic acid [MeAlB], glycine, and hydroxyproline) significantly reduced [^{3}H] L-proline uptake (Table 2) by up to 93.7% ($p < 0.01$). In contrast, large neutral amino acids (L-valine and L-phenylalanine), a basic amino acid (L-arginine), and an acidic amino acid (L-aspartic acid) did not affect [^{3}H] L-proline uptake ($p > 0.05$).

L-Glutamic Acid (L-Glu) Uptake by TR-CSFB Cells

The uptake of [^{3}H] L-Glu by TR-CSFB3 cells increased up to 10 min and reached a steady-state of about 200 μl/mg protein at 37°C (Fig. 5A). In contrast, at 4°C, [^{3}H] L-Glu uptake was 1.89 ± 0.30 μl/mg protein for 5 min, a reduction of 97.7% compared with that at 37°C (Table 1).

L-Glu uptake was concentration-dependent in the presence and absence of Na^+, indicating the existence of Na^+-dependent and Na^+-independent saturable uptake processes (Fig. 5B). In the presence of Na^+, an Eadie–Scatchard plot exhibited 3 saturable processes (Fig. 5B inset). On the other hand, in the absence of Na^+, the Eadie–Scatchard plot exhibited a single saturable process (data not shown). The corresponding K_m and V_{max} were 119 ± 11 μM and 0.871 ± 0.022 nmol/(min · mg protein) (mean ± S.D.). These parameters were fixed and substituted for K_{m1} and V_{max1} in equation (2). The K_{m2} and K_{m3} were 165 ± 85 μM and 11.5 ± 2.4 mM, respectively, and the V_{max2} and V_{max3} were 0.374 ± 0.101 nmol/(min · mg protein) and 10.7 ± 1.8 nmol/(min · mg protein) (mean ± S.D.), respectively. Under Na^+-free conditions, the presence of 1 mM ouabain significantly reduced the [^{3}H]

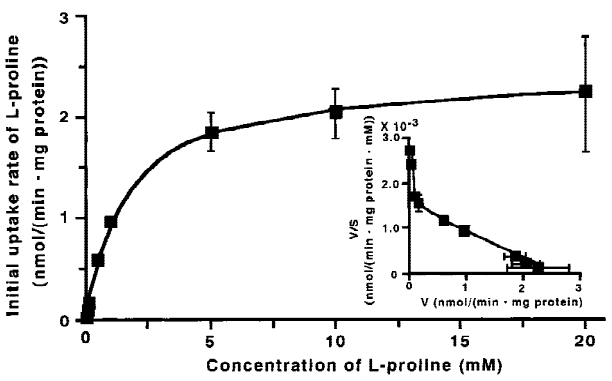

Fig. 4. Concentration-dependence of L-proline uptake by TR-CSFB3 cells cultured on 24-well culture plates. [^{3}H]L-Proline uptake was performed at 30 min and 37°C. Each point represents the mean ± S.E.M. (n = 4). Inset: Eadie–Scatchard plot for L-proline uptake by TR-CSFB3.

Table 2. Effect of Several Amino Acids on [^{3}H]L-Proline Uptake by TR-CSFB3

Inhibitors	Percentage of control (%)
Control	100 ± 11
L-Proline	6.97 ± 0.84**
L-Cysteine	6.29 ± 0.80**
L-Alanine	6.41 ± 0.77**
N-Methyl-L-alanine	14.4 ± 1.6**
α-Methylaminoisobutylic acid (MeAlB)	24.3 ± 1.9*
Glycine	25.2 ± 1.6*
Hydroxyproline	30.6 ± 6.5*
L-Valine	65.4 ± 2.8
L-Phenylalanine	66.6 ± 7.4
L-Arginine	77.9 ± 16.6
L-Aspartic acid	128 ± 10

Note: [^{3}H]L-Proline uptake was performed at 30 min. Each amino acid concentration was 10 mM. Each value value represents the mean ± S.E.M. (n = 4–8).
* $p < 0.01$, ** $p < 0.001$, significantly different from control.

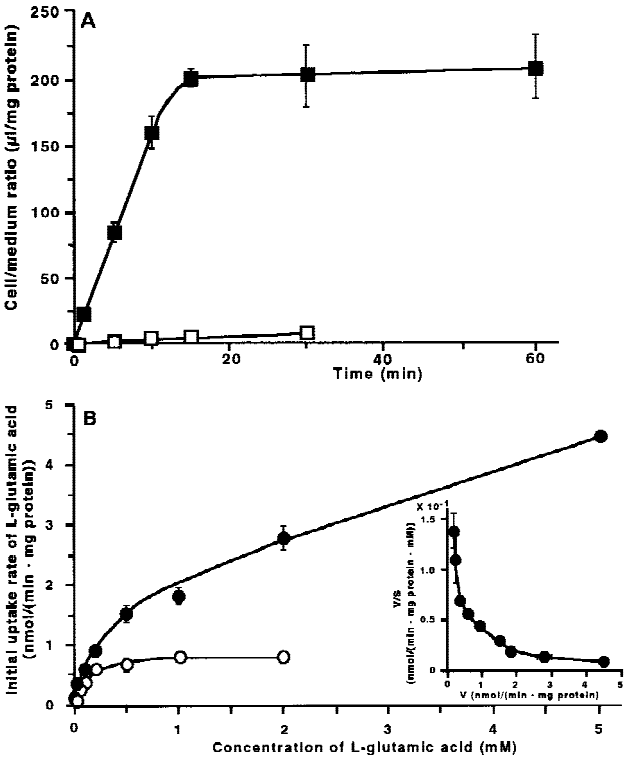

Fig. 5. Time-courses (A) and concentration-dependence of L-glutamic acid (L-Glu) uptake by TR-CSFB3 cells cultured on 24-well culture plates. Each point represents the mean ± S.E.M. (n = 4). A: [^{3}H]L-Glu uptake at 37°C, ■; [^{3}H]L-Glu uptake at 4°C, □. B: [^{3}H]L-Glu uptake in the presence of Na^+, ●; [^{3}H]L-Glu uptake in the absence of Na^+, ○. [^{3}H]L-Glu uptake was performed at 5 min and 37°C. Inset: Eadie–Scatchard plot for L-Glu uptake by TR-CSFB3 cells.

L-Glu uptake by TR-CSFB3 cells by 51.2% and 64.1%, respectively (Table 1). The presence of 1 mM (DNP) produced a significant reduction of 57.2% (Table 1).

Acidic amino acids (L-Glu, L-aspartic acid, D-aspartic acid, L-trans-pyrrolidine-2, 4-dicarboxylate, threo-β-hydroxyasparate, dihydrokainate, quisqualate) and a neutral amino acid (L-cystine) significantly reduced the [^{3}H] L-Glu uptake by up to 94.9% (Table 3, $p < 0.01$). In contrast, basic amino acids (L-arginine and L-lysine) did not affect the [^{3}H] L-Glu uptake ($p > 0.05$).

DISCUSSION

In the present study, immortalized choroid plexus epithelial cell lines (TR-CSFB) were established from transgenic rats harboring the ts SV 40 large T-antigen gene. TR-CSFBs cells grew well at 33°C due to the expression of large T-antigen and exhibited contact inhibition of growth after reaching confluence (data not shown). TR-CSFBs cells have a polygonal-shaped morphology, characteristic of an epithelial cell origin (Fig. 1A). TTR was expressed in TR-CSFB3 cells (Fig. 1B) and Na^+, K^+-ATPase was predominantly localized in the apical side (Fig. 2). TTR is a choroid plexus-specific thyroxine transport protein in the brain (16). Na^+, K^+-ATPase is specifically localized on the brush border side (CSF side, apical side) in choroid plexus epithelial cells (9) and retinal pigmented epithelial cells (17), but not on the basolateral side (blood side), like other epithelial cells. Therefore, this evidence suggests that TR-CSFBs cells are choroid plexus cell lines having the characteristics of TTR expression and Na^+, K^+-ATPase localization.

The net membrane resistance of TR-CSFBs cells was about 50 ohm · cm^2. Although this value is not close enough to that in the transcellular transport study, it is close to that of rabbit (43 ohm · cm^2) (8) and rat choroid plexus epithelial cell's primary culture (80 ohm · cm^2) (10). TR-CSFB3 cells are functionally polarized since the L-proline uptake on the apical side was fivefold higher than that on the basal side (Fig. 3A). This is because L-proline transport is localized on the apical side (CSF side) and governs the efflux of L-proline (18). TR-CSFB3 cells exhibit L-proline uptake activity involving both high and low affinity processes with K_m values of 5.27 μM and 1.57 mM, respectively (Fig. 4). These processes are Na^+- and energy-dependent, as well as being ouabain-sensitive (Table 1), suggesting that the processes represent active transport. The K_m value for the low affinity process is similar to that in the primary culture of rabbit choroid plexus (1.1 mM) (18). L-Proline uptake by TR-CSFB3 cells was significantly inhibited by small neutral amino acids such as MeAlB, hydroxyproline, L-cystein, L-alanine, and glycine (Table 2). Moreover, MeAlB, which is a specific substrate for system A for amino acids (19), reduced L-proline uptake by 75% (Table 2). The low affinity process for L-proline uptake is likely involved in system A since the K_m value for system A is about 1 mM at the blood–brain barrier (20). System A plays a role in the efflux of amino acids from the CSF to the circulating blood in order to maintain a constant concentration of amino acids in the CSF (18). On the other hand, the K_m value of the high affinity process is very close to that of the L-proline transporter in the rat hippocampus (K_m = 4.6 μM) (21). The L-proline concentration in the CSF under normal conditions is 3.8 μM and much lower than that in plasma (182 μM) (22). This transport process is necessary in order to maintain a low level since L-proline is known to produce L-ornithine in the brain (23) which has neurotoxic effects (24). The high affinity process may play a role in the efflux of L-proline under normal physiologic conditions. The low affinity process, which is most likely system A, may play a role in the efflux of L-proline when, in the genetic disorder of hyperprolinemia type II, the L-proline concentration in the CSF is generally tenfold higher than that under normal conditions (25).

Table 3. Effect of Several Amino Acids on [^{3}H]L-Glutamic Acid Uptake by TR-CSFB3

Inhibitors	Percentage of control (%)
Control	100 ± 8
L-Glutamic acid	12.3 ± 1.2**
L-Aspartic acid	29.6 ± 1.7**
D-Aspartic acid	36.9 ± 0.8**
L-Trans-pyrrolidine-2,4-dicarboxylate	32.0 ± 2.9**
Threo-β-hydroxyasparate	45.0 ± 3.9*
Dihydrokainate	46.6 ± 5.3*
Quisqualate	5.14 ± 0.6**
L-Cystine	20.4 ± 2.8**
L-Arginine	110 ± 11
L-Lysine	98.9 ± 10.7

Note: [^{3}H]L-Glutamic acid uptake was performed at 5 min. Each amino acid concentration was 10 mM. Each value value represents the mean ± S.E.M. (n = 4–8).
* $p < 0.01$, ** $p < 0.001$, significantly different from control.

Carrier-mediated L-Glu uptake by TR-CSFB3 cells is indicated by the temperature-, Na^+-, and energy-dependence, as well as the ouabain-sensitivity (Table 1), saturability (Fig. 5B), and the nature of its inhibition by amino acids (Table 3). The kinetic analysis revealed that L-Glu uptake by TR-CSFB3 cells has two Na^+-dependent high and low affinity processes and one Na^+-independent process (Fig. 5B). The corresponding K_m values for the Na^+-dependent high and low affinity process were 165 μM and 11.5 mM, respectively. In the isolated miniswine choroid plexus, L-Glu uptake is principally energy-dependent, ouabain-sensitive, and saturable with a K_m of 196~264 μM. This is inhibited by L-aspartic acid in a concentration-dependent manner (26), suggesting that it appears to be system X_{AG}^-, which may play a role in efflux from CSF to the circulating blood. L-Glu uptake by TR-CSFB3 cells is inhibited by D-aspartic acid, L-trans-pyrrolidine-2, 4-dicarboxylate, threo-β-hydroxyasparate, and dihydrokainate. Therefore, the high affinity process of L-Glu uptake by TR-CSFB3 cells is consistent with the existence of system X_{AG}^- as reported previously (26), although the details of the low affinity process in the choroid plexus remain unclear. The L-Glu concentration in CSF is maintained at a lower level (0.7~10 μM) than that in plasma (150 μM) (27), since L-Glu has neurotoxic effects (28). The uptake clearance (V_{max}/K_m) for the high and low affinity processes is 2.27 and 0.93 μl/(min · mg protein), respectively. Therefore, the high affinity process may play the main role in the efflux transport of L-Glu from the CSF. Segel *et al.* (29), using the isolated perfused choroid plexus in sheep, reported that L-Glu uptake from the blood side is an Na^+-independent process with a K_m of 2.59 μM, suggesting that an Na^+-independent L-Glu uptake process exists on the basolateral side. However, there is

a lack of agreement between the K_m value for L-Glu uptake by TR-CSFB3 cells and in this article. L-Glu uptake by TR-CSFB3 cells was inhibited by quisqualate and L-cystine, suggesting that the Na^+-independent process is most probably system x_c^-. System x_c^- with a K_m of 160 μM, which has been recently cloned in mouse macrophages, is inducible under oxidative stress (30). Although it is possible to induce system x_c^- in TR-CSFB3 cells throughout the culture period, the Na^+-independent L-Glu transport system is the only system x_c^- known at the present time. Further studies are needed to identify both the Na^+-dependent and Na^+-independent L-Glu transport processes in TR-CSFB cells, and in the choroid plexus *in vivo*.

In conclusion, immortalized choroid plexus epithelial cell lines (TR-CSFBs) were established from Tg rats. These TR-CSFBs cells grew well at 33°C and exhibit the typical properties of choroid plexus epithelial cells, such as TTR expression and an apical Na^+, K^+-ATPase localization. TR-CSFB cells have Na^+-dependent L-proline uptake processes, which are predominantly located on the apical side, and are both Na^+-dependent and Na^+-independent L-Glu uptake processes. TR-CSFB cell is a good *in vitro* model for investigating carrier-mediated transport functions at the blood–CSF barrier.

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