Widespread distribution of PLTP in human CNS: evidence for PLTP synthesis by glia and neurons, and increased levels in Alzheimer's disease

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Abstract **Plasma phospholipid transfer protein (PLTP) is one of the key proteins in lipid and lipoprotein metabolism. We examined PLTP distribution in human brain using PLTP mRNA dot-blot, Northern blot, immunohistochemistry (IHC), Western blot, and phospholipid transfer activity assay analyses. PLTP mRNA of 1.8 kb was widely distributed in all the examined regions of the central nervous system at either comparable or slightly lower levels than in the other major organs, depending on the region. Cerebrospinal fluid phospholipid transfer activity represented 15% of the plasma activity, indicating active PLTP synthesis in the brain. Western blot and phosholipid transfer activity assay demonstrated secretion of active PLTP by neurons, microglia, and astrocytes in culture. IHC demonstrated PLTP presence in neurons, astrocytes, microglia, and oligodendroglia. Some neuronal groups, such as nucleus hypoglossus and CA2 neurons in hippocampus, ependymal layer, and choroid plexus were particularly strongly stained, with substantial glial and neuropil immunostaining throughout the brain. Comparison between brain tissues from patients with Alzheimer's disease (AD) and nonAD subjects revealed a significant increase ($P = 0.02$) in PLTP levels in brain tissue homogenates and increased PLTP immunostaining in AD.**—Vuletic, S., L-W. Jin, S. M. Marcovina, E. R. Peskind, T. Möller, and J. J. Albers. **Widespread distribution of PLTP in human CNS: evidence for PLTP synthesis by glia and neurons, and increased levels in Alzheimer's disease.** ***J. Lipid Res.*** **2003.** 44: **1113–1123.**

Supplementary key words brain tissue • cerebrospinal fluid • central nervous system • immunohistochemistry

The human brain is an organ of a very complex structure, function, and composition, and its rich lipid content sets it apart from other major organs and systems. Lipids represent approximately half of the brain's dry weight, with phospholipids and cholesterol among the main constituents (1, 2). Although the human central nervous system (CNS) accounts for only ~2% of the total body mass, it contains over 20% of total body unesterified cholesterol (3). While some aspects of CNS lipid metabolism are similar to the lipid metabolism of other peripheral organs (4), there are also many specific differences. Normally, adult human brain contains virtually no cholesterol esters or triglycerides (1). Distribution and quantity of the various classes and types of lipids, some of which are found exclusively in the brain, vary based on the brain region and age (1, 5). Lipids serve not only as structural components of the cell membranes but also act as precursors, biomessengers, and signal transduction molecules.

Despite the importance of lipids for brain function, our current knowledge of lipid metabolism and lipid transfer proteins in the human brain is surprisingly limited. Evidence has been provided to suggest that cholesteryl ester transfer protein (CETP) is synthesized and secreted in the brain (6), and its localization in brain astrocytes has been reported (7). However, others have not been able to demonstrate any CETP or cholesteryl ester transfer activity in human brain (8). Phospholipid transfer protein (PLTP) is

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Abbreviations: ABC, avidin biotin complex; AD, Alzheimer's disease; CNS, central nervous system; CSF, cerebrospinal fluid; DAB, 3,3′-diaminobenzidine; FFPE, formalin-fixed, paraffin-embedded; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; IHC, immunohistochemistry; MAb, monoclonal antibody; MBP, myelin basic protein; PLTA, phospholipid transfer protein activity; PLTP, phospholipid transfer protein.

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a widely expressed lipid transfer protein coded on chromosome 20q12-13.1 (9–11). In human plasma it is present both in active and inactive forms, primarily as a lipoprotein-bound protein (12). PLTP has been shown to have lipid transfer (13, 14), proteolytic (15), and fusion properties (16). It facilitates transfer of phospholipids, unesterified cholesterol, diacylglycerides, selected apolipoproteins, and α-tocopherol between various classes of lipoproteins (13, 14, 17). It has also been shown to transfer and neutralize bacterial lipopolysaccharide (18). One of the main functions of PLTP is modification of HDL, in that it facilitates formation of small, lipid-poor preβ HDL and larger HDL particles (16, 19). PLTP also plays an important role in cholesterol and phospholipid efflux from peripheral cells, thus contributing to the maintenance of cell lipid homeostasis (20). Creation of the PLTP knockout (KO) mouse model deepened our understanding of its importance in lipid metabolism. PLTP KO mouse plasma showed an absence of all phospholipid transfer and partial loss of unesterified cholesterol transfer to HDL acceptor particles (21). In vivo, phospholipid transfer from VLDL to HDL was nearly abolished. Loss of PLTP activity also caused formation and accumulation of lamellar lipoproteins rich in apolipoprotein E (apoE) and apoA-IV (22). These findings suggest that PLTP plays an essential role in bidirectional phospholipid and cholesterol transfer. The PLTP KO mouse model also offered an insight into a potential intracellular function, showing a regulatory effect of PLTP on the secretory pathway of apoB-containing lipoproteins (23).

Considering the relatively high levels of PLTP in mammalian brain (9, 24, 25) and the structural and functional complexity of the CNS, we performed an evaluation of PLTP distribution in various regions of normal and Alzheimer's disease (AD) human brains, and demonstrated PLTP synthesis in both neuronal and glial cells.

MATERIALS AND METHODS

Materials

ABC (avidin biotin complex) Elite kit, Antifade fluorescence mounting medium with 4,6-diamino-2-phenylindole, biotinylated anti-mouse and anti-rabbit secondary antibody, and 3,3′-diaminobenzidine (DAB) staining kit were purchased from Vector Laboratories Inc. (Burlingame, CA). Monoclonal antibody (MAb) PG-M1 against glial fibrillary acidic protein (GFAP) and MAb 6F2 against CD68 were purchased from DAKO Corporation (Carpinteria, CA). MAb against myelin basic protein (MBP) was obtained from Boehringer-Mannheim. Fluorescent streptavidin conjugates and fluorescent anti-mouse and anti-rabbit antibodies are from Molecular Probes (Eugene, OR). Anti-rabbit IgG for Western blot was obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Radionucleotides and C^{14}-labeled phosphatidylcholine were purchased from NEN Technologies/Perkin-Elmer Life Sciences Inc. (Boston, MA). E-Z Link and SuperSignal West Femto Maximum Sensitivity Substrate were purchased from Pierce (Rockford, IL). Polyacrylamide 4–20% gradient gels and nitrocellulose membrane were purchased from Bio-Rad Laboratories (Hercules, CA). Protease inhibitor cocktail was purchased from BD Biosciences Pharmingen (San Diego, CA). Human Cot-1 DNA was purchased from Roche Diagnostics (Indianapolis, IN). Nonradioactive phospholipids used in liposome preparation were from Sigma (St. Louis, MO).

Cerebrospinal fluid, plasma and tissue

The study was approved by the Human Subjects Review Committee of the University of Washington (UW), and written informed consent was obtained from all subjects. Cerebrospinal fluid (CSF) samples were obtained from the UW Alzheimer Disease Research Center (ADRC) CSF bank. CSF was drawn by modified lumbar puncture (26) from 28 neurologically healthy subjects, nine females and nineteen males, age range 61–86 years old. Samples were briefly centrifuged and stored at −80°C until use. The samples were assessed for blood contamination (total protein, albumin, glucose, cell count, red blood cell count, apoB), and the analyses documented that the blood-brain barrier was not compromised. Because of the large volume of the obtained CSF (35 ml from each subject in numbered, 0.5 ml aliquots), samples represent CSF from different levels of the CNS and could potentially differ depending on aliquot number. In order to test for a possible PLTP activity and mass gradient, we performed Western blot and activity assay for paired CSF samples from four subjects, representing the 5th and the 40th aliquot. We did not detect PLTP activity or mass differences between the tested aliquots, allowing us to use any aliquot for our experiments. Plasma samples from healthy subjects, stored at −80°C until assayed, were used for internal quality controls for PLTP activity (PLTA) assay.

Human brain tissue was obtained from the UW ADRC Brain Bank. Tissue specimens were obtained from subjects with no significant pathology upon neuropathological examination, and from subjects with the neuropathologically verified AD. Samples for immunohistochemistry (IHC) were obtained from twelve control subjects, six male and six female, age range 44–93 years old, and twelve AD subjects, six male and six female, age range 74–97 years old. Samples were either formalin-fixed, paraffin embedded (FFPE) sections (10 control and 10 AD subjects), or paraformaldehyde fixed sections (two control and two AD subjects). For the paraformaldehyde fixation, we used tissues obtained by rapid autopsy with a postmortem period of up to 6 h, snap frozen, and kept at −80°C until use. Frozen tissues were fixed in 4% paraformaldehyde solution for 48 h at 4°C, and cryoprotected by incubation in 30% sucrose solution at 4°C for 5 days. Tissues were sliced using a Leica cryostat and mounted on slides, or stored in PBS-0.05% NaN_3 at 4°C until use.

Control snap-frozen tissue samples for Western blot and activity assays were taken from 10 subjects, five male and five female, age range 51–93 years old. Samples from AD subjects, used in Western blot analysis, were taken from 14 subjects, six male and eight female, age range 61–92 years old. Most tissue samples for these analyses were taken from the temporal lobe, except for four sets of control samples taken from cerebellum, frontal, and temporal lobe. Tissue was homogenized in TBS buffer with addition of protease inhibitor cocktail at 4°C using an Ultra-Turrax T25 homogenizer (Janke and Kunkel, IKA Labortechnic), and centrifuged for 5 min at 4°C, 1,000 *g* in a swing-bucket centrifuge (Beckman CS-15R); the pellet was discarded, and the supernatant either used for analyses (immediately or stored at −80°C until use) or further fractionated by ultracentrifugation at 100,000 *g* in a fixed-rotor ultracentrifuge (Beckman Coulter Optima XL-100K) at 4°C into TBS soluble, SDS soluble, urea soluble, and urea insoluble fractions.

Cell culture

For preparation of fetal human astrocytes, tissue was provided by the Birth Defects Research Laboratories of UW, and the experiments were carried out in accordance with the guidelines of

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the Institution. Cultures were prepared from brains of legally aborted human fetuses (12–15 weeks gestation) as previously described (27). Conditioned serum-free medium was collected after 48 h, briefly centrifuged, and immediately used for PLTP activity assay and Western blotting. The mouse microglia cell line N9 was a kind gift of Dr. M. Righi, International School for Advanced Studies, Trieste, Italy, and was cultured in accordance with the original publication (28). The mouse microglia cell line BV-2 was a kind gift of Dr. E. Blasi, University of Perugia, Italy, and was cultured in accordance with the original publication (29). Cultured microglial cell lines N9 and BV2 were trypsinized and plated onto Primaria™ 75 cm^2 flasks (BD Biosciences, San Jose, CA) and cultured in Cellgro® Complete™ Serum-Free Medium (Herndon, VA). After 3 days, cells supernatant was collected and cells were frozen for further analysis. Primary mouse neurons, a kind gift from I. Maezava, UW Department of Pathology, from C57/BL6 mice were prepared as previously reported (30). The absence of astrocytes was confirmed by the lack of GFAP staining. The primary mouse neurons were grown in Neurobasal medium plus B27 supplement (Life Technologies, Gaithersburg, MD) on poly-D-lysine-coated dishes at a density of 5.6×10^4 cells/cm^2. The medium was collected and cells homogenized for further analyses.

mRNA expression

Human multiple tissue array (lot No. 1030450) and Northern blot for brain tissue (lot No. 0090356) were purchased from Clontech. Blots were hybridized using P-32 labeled PLTP cDNA probe (9) according to the manufacturer's instructions. Films were exposed for various times at −80°C and developed in a Konica QX-70 medical film processor. Films were scanned, and dot intensity was measured in an 80-pixel-wide central area of the dot. The data from four separate measurements were averaged and interpreted according to the manufacturer's instructions. The polyA RNA used in dot-blot is loaded based on eight housekeeping genes.

Antibodies

Polyclonal antibody against recombinant PLTP, raised in New Zealand white rabbits, was shown previously to react specifically with plasma PLTP (31) and neutralize plasma phospholipid transfer activity mediated by PLTP (32). The preparation and characterization of MAb 4 has been described in detail (31). A second anti-PLTP monoclonal antibody, MAb1, was prepared similarly. Competition experiments between MAb 4 labeled with horseradish peroxidase (HRP) and MAb1 indicated that MAb 4 and MAb 1 recognized different PLTP epitopes.

IHC

Areas of study included frontal, parietal, temporal, and occipital cortices, hippocampus, basal ganglia, thalamus, hypothalamus, corpus callosum, cerebellum, midbrain, pons, and medulla oblongata. Thickness of the tissue sections for standard IHC was 6 μm and 8 μm for FFPE and paraformaldehyde fixed sections, respectively. FFPE sections were deparafinized and rehydrated before immunostaining. Sections for comparative analyses (AD and control) were additionally incubated for 4 min in 88% formic acid. All sections were then quenched for peroxidase, washed, incubated with the primary antibody, 1:100 (v/v) and 1:50 (v/v) for MAb (MAb 4 or MAb 1+4 combined, and MAb 1, respectively), and 1:100 (v/v) for the polyclonal antibody, diluted in PBS-0.05% Tween 20-1% BSA. Sections were then washed and incubated with the appropriate secondary antibody in 1:200 (v/v) dilution. An ABC Elite kit was prepared according to the manufacturer's instructions and applied to the sections. Color development was obtained using a DAB staining kit and Meyer's haematoxylin counterstain. Immunofluorescent staining was performed using either FFPE or thin paraformaldehyde fixed sections mounted on slides. Double staining experiments were performed using polyclonal anti-PLTP antibody (1:50, v/v) and MAbs against MBP (1:100, v/v) as marker for oligodendrocytes, GFAP as marker for astrocytes (1:25, v/v), and CD68 (1:20, v/v) as marker for microglia, detected by goat anti-rabbit Alexa 568 and anti-mouse Alexa 488 sary antibodies (Molecular Probes). Antigen retrieval prior to primary antibody application was used for GFAP and CD68 immunostaining (incubation at 95°C for 15 min in citric buffer, pH 6.0). Slides were observed either under a Nikon Eclipse TE 200 microscope, photographed by a digital Nikon camera, and enhanced using Metamorph software, version 4.6r5 (Universal Imaging Corporation) or under a Leica DM IRBE confocal microscope, with images captured using a Leica TCSSP camera and Leica confocal software. Negative controls were performed by omitting the primary antibodies and by using nonimmune serum on representative sections (hippocampus). Under these conditions, no specific immunostaining was observed.

MAb conjugation

Conjugation of the primary antibody, MAb 4, with HRP was performed using E-Z Link (Pierce) following the manufacturer's instructions. The conjugated MAb was then used for Western blotting.

Western blot

Protein concentration was established by Lowry assay. Ten microliters of CSF, 30 μg of protein from tissue and cell homogenates, or 500 μl of cell culture serum-free media concentrated by the TCA method were loaded on 4–20% gradient gel for SDS-PAGE. Gels were transferred to a nitrocellulose membrane using either a wet or a semi-dry transfer system (Bio-Rad). After the transfer, the membrane was blocked and then incubated for 1 h with either 1:10,000 (v/v) dilution of anti-PLTP HRP-conjugated MAb or 1:100 (v/v) dilution of rabbit anti-PLTP polyclonal antibody at room temperature. The membrane incubated with the polyclonal anti-PLTP antibody was subsequently incubated with 1:100,000 (v/v) dilution of the secondary antibody, goat anti-rabbit IgG (Kirkegaard and Perry Laboratories). The detection solution (Pierce SuperSignal West Femto Maximum Sensitivity Substrate) was prepared according to the manufacturer's instructions. Films were developed in the Konica QX-70 medical film processor and scanned. Comparison between AD (n = 7) and control (n = 6) subjects was performed by densitometry scanning of samples processed at the same time.

PLTA assay

PLTA assay was performed as previously described (32). PLTA measures transfer of radioactively labeled phospholipid from liposome to the HDL_3 acceptor particle. Counts were detected using a Packard Minaxi Tri-Carb 4,000 liquid scintillation counter. Samples were done in duplicate, and the results are expressed as mean ± SD. CSF index is calculated using a plasma albumin concentration of 4,075 mg/dl and CSF albumin concentration of 26.1 mg/dl in the formula

$$\text{CSF index} = \frac{\text{CSF PLTP activity}}{\text{plasma PLTP activity}} \times \frac{\text{plasma albumin}}{\text{CSF albumin}} \qquad \textit{(Eq. 1)}$$

For cell culture media, serum-free media alone were used as a negative control.

PLTA inhibition assay

Samples were incubated overnight at 4°C, alone or with increasing concentrations of either control-purified rabbit IgG

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(Zymed Labs Inc., South San Francisco, CA) or anti-PLTP polyclonal IgG antibody. After the incubation, samples were centrifuged at 2,000 *g* for 20 min at 4°C in a Sorvall RMC centrifuge, and 300 ml of supernatant was used for the activity assay as previously described (32).

Statistical analyses

Statistical analyses were performed utilizing Statistica for Windows, StatSoft, Inc., 2000 (Tulsa, OK).

RESULTS

PLTP mRNA expression

In order to assess PLTP distribution in the brain, we determined PLTP mRNA expression in various regions of human brain and compared it to that in other selected tissues. Northern blot analysis was performed on eight representative brain regions: cerebellum, cerebral cortex, medulla, spinal cord, occipital pole, frontal lobe, temporal lobe, and putamen. Consistent with our previous reports (9, 11), the size of PLTP mRNA was ~1.8 kb in all regions, and mRNA of smaller size was not detected even after prolonged exposure of the film (5 days). In the sample derived from cerebellum, we also detected two very fine bands with approximate sizes of 7 kb and 9 kb, respectively (data not shown). PLTP mRNA expression in selected tissues by multiple tissue dot-blot analyses indicated minor differences among the high-expressing tissues: placenta, ovary, uterus, thymus, pancreas, and lung (data not shown). Average whole-brain tissue mRNA expression was somewhat lower when compared with that of the other main organs and tissues (data not shown). However, PLTP mRNA in the left cerebellum was among the highest of examined tissues (**Fig. 1**), suggesting the importance of taking into account the laterality of the brain for the assessment of mRNA expression. Evaluation of eighteen regions and structures of human brain using the dot-blot method indicates widespread distribution of PLTP in human brain (Fig. 1).

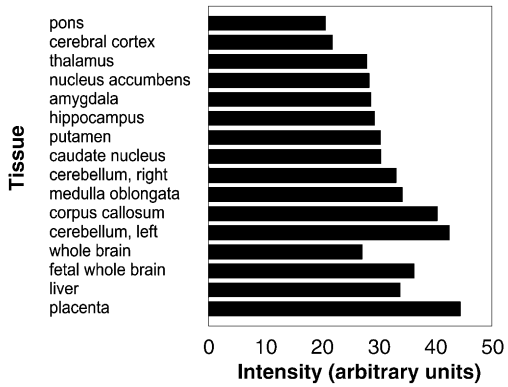

Fig. 1. Phospholipid transfer protein (PLTP) mRNA in different brain regions and structures. PLTP mRNA levels in liver and placenta, organs with high PLTP expression, are added as a comparison. Clontech Multiple Tissue Expression array mRNA was hybridized with the P-32-labeled PLTP cDNA. The film was exposed for 3 days at −80°C and scanned. Each intensity value represents the mean of four intensity measurements of a defined area adjusted for background intensity. The results are normalized for eight housekeeping genes. All tissues are adult unless specified as fetal.

The 25% difference in PLTP mRNA expression between fetal and adult brain (Fig. 1) is second only to that detected between fetal and adult spleen, where PLTP mRNA expression level was decreased by ~50% (data not shown). The fact that the adult spleen no longer functions as a haematopoietic organ suggests that PLTP plays a functional role in haematopoiesis. Other major organs (thymus, lung, liver, kidney, heart, in order of expression) showed only minor differences in PLTP mRNA expression levels between adult and fetal tissues (data not shown).

PLTP distribution in brain tissue

Regional and cellular distribution of PLTP in the healthy human brain was assessed by immunohistochemical analyses. Neuropathological examinations of the control brain tissue samples revealed no pathology except age-associated changes within normal limits. All the examined areas (see Materials and Methods) of the CNS revealed the presence of PLTP by immunostaining. Although variability in tissue immunostaining intensity occurred among individual subjects, primarily due to variation in the number of glial cells, there was a consistent PLTP cellular distribution pattern among comparable regions from different individuals, demonstrated by both MAbs and polyclonal antibodies (data not shown).

Cortical neurons throughout the brain showed variable granular intracytoplasmic staining that extended toward the axons (**Fig. 2A**). Intracytoplasmic staining was also present in glial cells, which are involved in the formation and metabolism of lipoprotein particles in the CNS. White matter showed strong staining along the axons of myelinated tracts (Fig. 2B), as well as in glial cells (Fig. 2C). PLTP immunostaining was particularly strong in selected groups of neurons in the subcortical gray matter, such as those in basal ganglia, thalamus, hypothalamus, and neurons of the nucleus hypoglossus (data not shown), as well as in epithelial cells of choroid plexus (Fig. 2D) and the ependymal cells (Fig. 2E). The epithelial cells of the choroid plexus and ependymal cells actively regulate CSF composition (33). Strong PLTP presence in these cells suggests that PLTP plays a role in lipoprotein uptake, modification, and assembly. In the hippocampus, dentate granule neurons showed moderate staining (Fig. 2F), CA2 pyramidal neurons (Fig. 2G) showed intense PLTP immunostaining, while hippocampal CA1 neurons (Fig. 2H) presented with much weaker staining. PLTP was also detected in pigmented neurons of substantia nigra and locus coeruleus (not shown). PLTP immunoreactivity was detected in cerebellar glial cells, Purkinje cells, basket cells, some granule cells (Fig. 2I), and neurons in the deep gray matter of the cerebellum (Fig. 2J). Neuropil throughout the brain contained well-defined, dot-like staining finely delineating numerous cell processes. The significant presence of PLTP immunostaining in neuropil implies the presence of PLTP in extracellular space around terminals, as would be ex-

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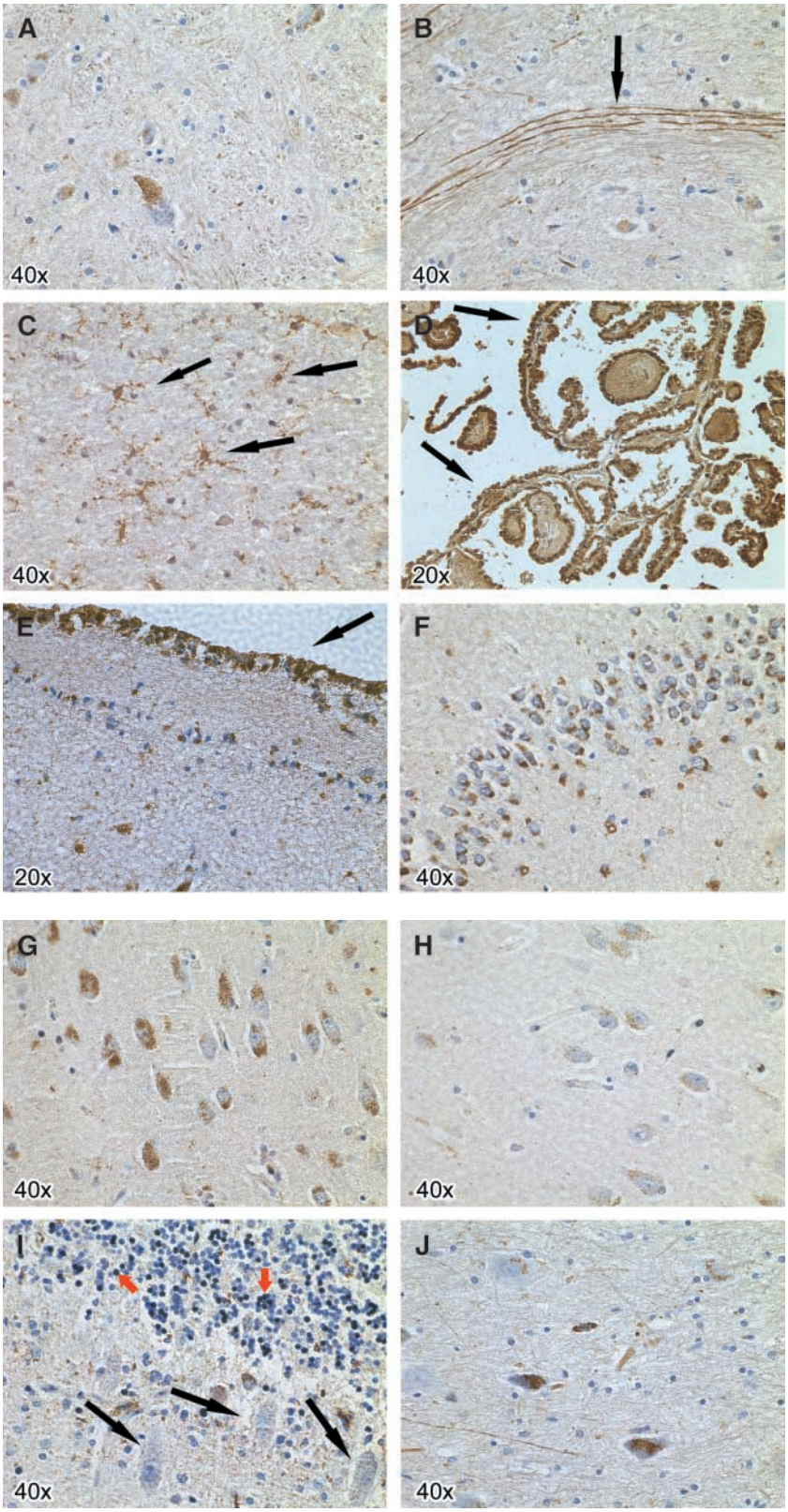

Fig. 2. PLTP immunostaining in selected areas of the histologically normal human central nervous system. Staining was performed using 10 μg/ml of combined anti-PLTP monoclonal antibodies (MAbs) 1 and 4 on 6 μm formalin-fixed, paraffin-embedded (FFPE) sections unless otherwise stated. A: Neurons and axonal tracts, medulla oblongata. B: Axonal tract in midbrain (arrow). C: Glial cell morphology and staining (arrows) were much more visible when using paraformaldehyde fixation on 8 μm tissue sections, as shown in corpus callosum. D: Choroid plexus. E: Ependymal cells (arrow), as presented in a hypothalamic tissue section. F: Hippocampal granular layer neurons. Variability in staining of different neuronal groups is best illustrated with hippocampal CA2 (G) and CA1 (H) neurons, stained on the same tissue section. I: Cerebellum, Purkinje cells (long black arrow), and granular layer neurons (short red arrows). J: Cerebellum, deep gray matter neurons. All images are digital.

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pected for a protein associated with lipoprotein particles. Negative control samples (see Materials and Methods) showed no staining (data not shown). Wide PLTP expression in the human brain is suggestive of its importance for internal lipid recycling, which is essential for CNS structural and functional integrity (3).

To determine the identity of PLTP-positive glial cells, double staining experiments using specific markers for individual glial types were performed. **Figure 3** demonstrates that PLTP was present in glial cells positive for astrocytic marker GFAP (Fig. 3A–C), microglial marker CD68 (Fig. 3D–F), and oligodendroglial marker MBP (Fig. 3G–I).

The presence of PLTP in brain tissue as well as in different classes of brain cells was further supported by Western blot analyses. Western blots of brain tissue homogenate fractions (TBS-soluble, SDS-soluble, urea-soluble, and urea-insoluble fractions) indicated the presence of PLTP in the TBS-soluble fraction (not shown). Tissue homogenate Western blot analyses revealed a typical ~80 kDa band (**Fig. 4**). However, the majority of PLTP from the brain tissue homogenates primarily contained species of smaller molecular weight. A similar pattern was observed in homogenates of the brain-derived cells in culture (not shown). Unlike the tissue and cell homogenates, PLTP from CSF migrated almost exclusively as an ~80 kDa band, with a minor portion migrating as an ~30 kDa fragment (Fig. 4). Western blot analysis of the cell culture media from primary human astrocytes, two mouse microglial cell lines (BV-2 and N9), and primary mouse neurons all exhibited PLTP with an apparent molecular weight of ~80 kDa (not shown).

The analyses of AD and nonAD brain tissue homogenates from temporal cortex with a minimal amount of white matter by Western blot revealed a significant increase in PLTP in AD subjects (**Fig. 5**). Median relative intensity for the major band was 84.3 in AD, nearly double that of the median relative intensity of 44.2 obtained for controls.

The significant increase in PLTP levels in AD subjects compared with age-matched controls as shown by Western blot analysis may reflect the general increase in astroglia and microglia in AD brains. Therefore, immunohistochemical analyses of sections obtained from subjects with histologically verified AD were performed. The intensities of staining in affected regions among AD cases were variable. Significantly, in cases with no apparent or only mild neuronal loss, there is an increase of PLTP staining

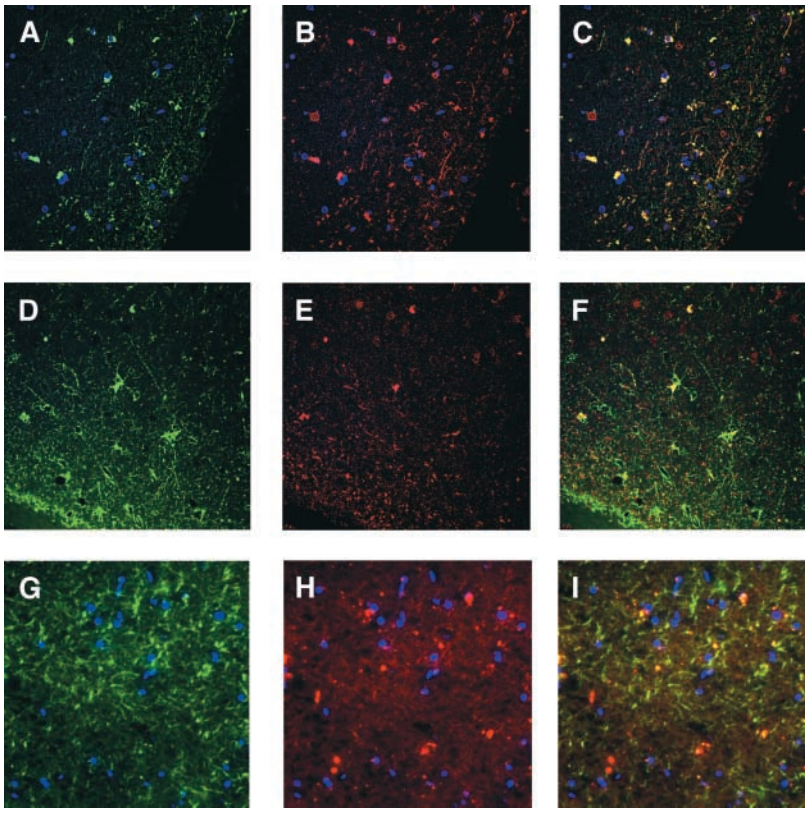

Fig. 3. PLTP in different types of glial cells. Confocal images of hippocampal tissue sections stained for PLTP, using 20 μg/ml of rabbit anti-PLTP IgG, and microglial marker, CD68, using 50 μg/ml of PG-M1 monoclonal anti-CD68 IgG (A–C), astrocytic marker, glial fibrillary acidic protein (GFAP), using 40 μg/ml of 6F2 monoclonal anti-GFAP IgG (E–F), respectively, were imaged under a Leica confocal microscope. Co-staining with oligodendroglial marker, myelin basic protein (MBP), using 4 μg/ml of monoclonal anti-MBP IgG (G–I), was imaged using a Nikon fluorescent microscope. A, D, and G: Present staining with the respective cellular markers; B, E, and H represent PLTP staining, while C, F, and I represent merged images where colocalization of PLTP with the specific cell marker appears as yellow. All samples were counter-stained with 4,6-diamino-2-phenylindole. All images are digital.

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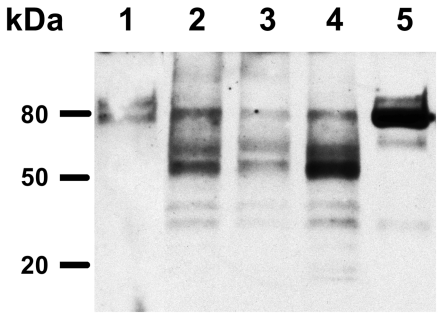

Fig. 4. PLTP Western blot. A representative blot containing 10 μl of cell culture medium containing recombinant PLTP (lane 1), 30 μg of total protein from temporal cortex (lane 2), frontal cortex (lane 3), and cerebellum (lane 4), and 10 μl of cerebrospinal fluid (lane 5) separated on 4–20% gel by SDS PAGE, transferred to the nitrocellulose membrane by semi-dry transfer system, and incubated with 0.1 μg/ml MAb 4 labeled with horseradish peroxidase (HRP). The blot was developed using Pierce West Femto Maximum Sensitivity Substrate. The film was exposed for 5 min, developed, and scanned.

intensity, particularly in neurons (**Fig. 6E**) and in the hippocampal subependymal layer (Fig. 6B). Thus, neuronal PLTP may also contribute to the increased PLTP levels seen in AD homogenates. However, in those cases in which we observed severe neuronal loss, the white matter staining by PLTP is very pale, probably reflecting general axonal loss (not shown). The intensity of PLTP staining in the remaining neurons (Fig. 6F) and of the hippocampal subependymal layer (Fig. 6C) appears decreased, with overall reduction in neuropil staining when compared with control subjects (Fig. 6A, D). More detailed immunochemical studies to delineate the changes of PLTP in relation to lesions (neuritic plaques and neurofibrillary tangles) and altered markers (apoE, β-amyloid, tau protein) in AD are underway in our laboratories.

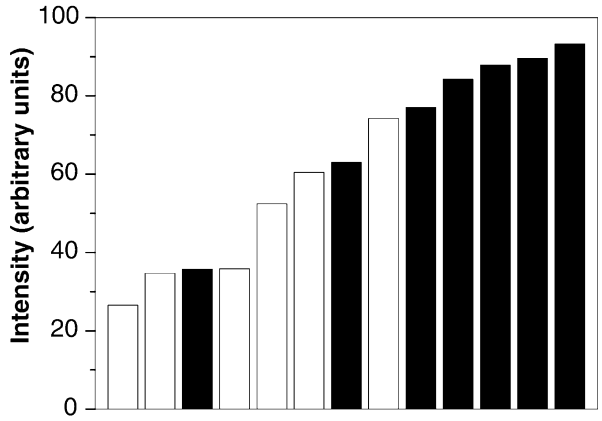

Fig. 5. PLTP in Alzheimer's disease (AD) and control temporal lobe tissue homogenate. Western blot containing 30 μg of total protein from AD (n = 7, black bars) and control (n = 6, empty bars) temporal cortex samples processed at the same time by SDS PAGE on 4–20% gel, transferred to the nitrocellulose membrane by wet transfer system, and incubated with 0.1 μg/ml MAb 4 labeled with HRP. Films were developed and scanned, and intensity measured by densitometry. Mean intensity for an ~55 kDa band in AD was significantly higher than in control (75.8 vs. 47.4, $P = 0.02$).

PLTP activity in CNS

To verify that PLTP in the human brain is not only present but also exhibits phospholipid transfer activity, we performed a PLTA assay on CSF, cell culture media, brain tissue, and neuronal cell homogenates. CSF composition reflects both normal and pathological brain metabolism, which makes it a good medium for gaining insight into brain tissue lipid metabolism. PLTA of CSF samples (n = 28) was 2.5 ± 0.5 μmol/ml/h (**Table 1**), and represented 15.3 ± 2.9% of plasma PLTA, with the range between 11% and 23%. PLTP-mediated phospholipid transfer activity in CSF from male subjects was slightly higher than that obtained from female subjects ($P < 0.05$). In general, CSF is fairly protein-poor compared with plasma, which is a much richer and more complex environment than CSF. Very efficient blood-brain and blood-CSF barriers allow only a very small quantity of plasma proteins to reach the CNS. CSF index, as a measure of local synthesis, relies on established values for protein diffusion through the blood-CSF barrier using albumin concentration in plasma and CSF as the base. An index higher than one is indicative either of local synthesis or of active transport of the protein through the blood-CNS barriers. As a comparison, apoE in CSF, which is essentially all brain derived (34), has an index of five (6), while the average PLTA CSF index for our samples was about 24 (Table 1), which provides strong evidence that active PLTP is synthesized in the brain. PLTA of tissue homogenates from cerebellum, temporal, and frontal cortex from four subjects showed relatively low activity, slightly greater than 1% of the average CSF transfer per microgram of total protein per hour. To verify that the observed phospholipid transfer activity is indeed due to PLTP-facilitated phospholipid transfer, we performed PLTA before and after incubation with polyclonal anti-PLTP IgG (see Materials and Methods). Twenty five micrograms per milliliter of polyclonal anti-PLTP antibody inhibited >98% of the CSF PLTA, while control preimmune rabbit IgG did not inhibit the transfer activity, indicating that phospholipid transfer in CSF in that assay system is mediated almost exclusively by PLTP. By contrast, tissue phospholipid transfer activity was inhibited ~37% by the anti-PLTP antibody, suggesting that only a portion of its PLTA is mediated by PLTP. This is not surprising in view of the fact that brain has been shown to contain other intracellular phospholipid transfer proteins (35) that could have contributed to the PLTA values and would not be inhibited by the antibody against PLTP. PLTP-mediated phospholipid transfer activity was also detected in serum-free cell culture media from cultured human primary astrocytes, mouse microglial cell lines BV-2 and N9, and primary mouse neurons, as well as in cell homogenates (not shown).

DISCUSSION

Many studies have demonstrated the importance of PLTP-mediated lipid transfer in plasma [(20–23); for review see ref. (36)]. Despite the major relevance of the brain lipid

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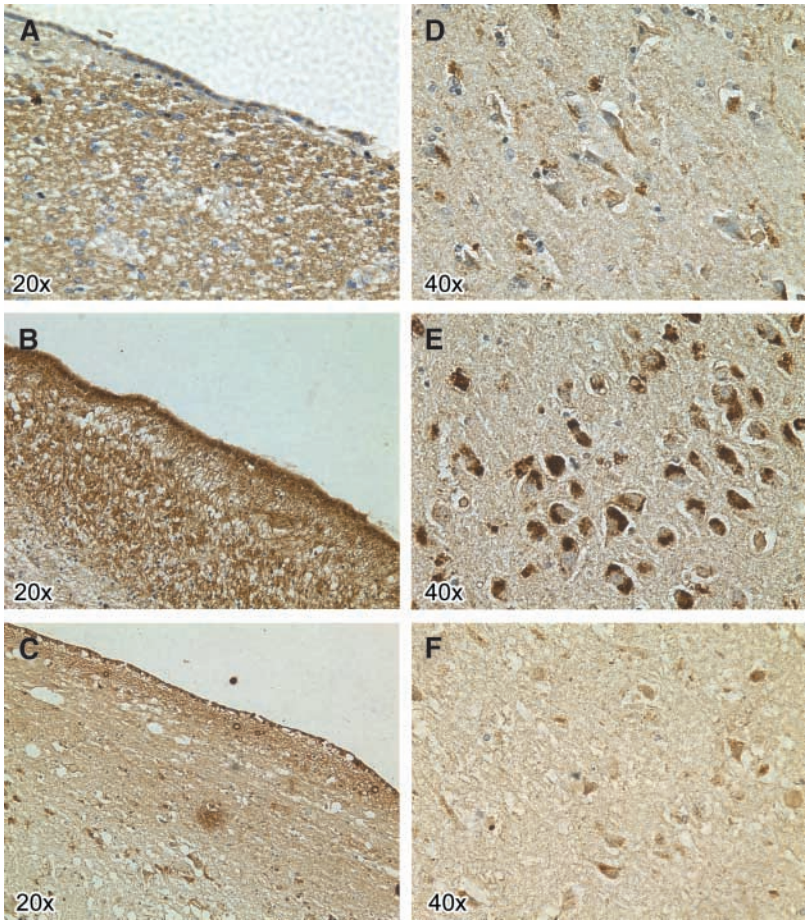

Fig. 6. PLTP immunostaining in AD brain tissue. Immunohistochemistry was performed using 10 μg/ml of combined anti-PLTP MAb 1 and 4 on 6 μm FFPE sections, with antigen retrieval in 88% formic acid. A: Ependymal layer in control hippocampus. B: Hippocampal subependymal layer in mild neuronal loss, AD. C: Hippocampal subependymal layer in significant neuronal loss, AD. D: Cortical neurons, control. E: Cortical neurons in mild neuronal loss, AD. F: Cortical neurons in significant neuronal loss, AD. All images are digital, and photographs are taken under the identical exposure conditions.

metabolism for its function and structure, very little is known about PLTP, or lipid transfer in general, within the human CNS (4).

Maturation of the brain and synaptogenesis depends on the availability of cholesterol and phospholipids to cell membranes, and would require transport of significant amounts of these lipids to neurons (37–39). Although neurons synthesize cholesterol, the rate of neuronal cholesterol synthesis is not sufficient for the formation of synapses, requiring additional cholesterol transport from glial cells to neurons via brain lipoproteins (37). Lack of PLTP or its dysfunction during this crucial period of neural development may not be lethal, as shown by the existence of the PLTP KO mouse model (21), but might affect some subtle subcellular structural details as well as brain functioning at a later stage. Such findings are not atypical. KO mouse models for some important molecules for neurophysiological processes, such as apoE (40), beta site amyloid precursor protein cleaving enzyme (41), and postnatal prion protein (42), also appear to have normal brain macrostructure and function under basal conditions when compensatory mechanisms may be adequate to maintain apparently normal structure and function. Abnormalities could become evident, however, in states of increased metabolic demand, such as recovery from neuronal injury or disease, or when accumulation of subtle defects due to aging overcomes compensatory mechanisms. PLTP abnormalities may be combined with other metabolic deficiencies and thereby potentially contribute to observed neuronal defects, as has been proposed for Down syndrome (43, 44).

TABLE 1. PLTP-mediated phospholipid transfer in cerebrospinal fluid

Subjects	Age	PLTA	CSF Index[a]
	years	*μmol/ml/h*	
All (n = 28)	73.4 ± 6.7	2.5 ± 0.5	23.9 ± 4.5
Male (n = 19)	75.0 ± 6.8	2.6 ± 0.5	24.9 ± 5.1
Female (n = 9)	69.9 ± 5.3	2.3 ± 0.2	21.7 ± 1.5

CSF, cerebrospinal fluid; PLTA, phospholipid transfer protein activity.

[a] $\text{CSF index} = \frac{\text{CSF PLTP activity}}{\text{plasma PLTP activity}} \times \frac{\text{plasma albumin}}{\text{CSF albumin}}$

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Immunohistochemical analyses of control brain tissue elucidated the details of the regional and cellular PLTP distribution. The observation that PLTP immunostaining is stronger in some groups of neurons than others suggests a particular role of PLTP in certain neuronal pathways. Relatively weak staining of hippocampal CA1 neurons compared with the neurons in the other hippocampal layers is particularly interesting, since CA1 neurons are known to be more vulnerable to ischemia (45, 46), various toxic substances, and some pathological processes, such as epilepsy and AD (46) than other neurons within the same region. It is tantalizing to speculate that, in view of the PLTP function in cholesterol transport, the relative lack of PLTP in CA1 neurons may be related to their vulnerability to various insults that demand efficient repair. The widespread PLTP immunostaining present in virtually all brain cells represents a distinctively different pattern from that observed for other apolipoproteins in human brain, such as apoE (47), apoD (48), and apoJ (49, 50). Staining along the axons suggests that PLTP functions in their maintenance, which significantly depends on lipoprotein metabolism (38). PLTP might also be involved in regulation of axonal sprouting or axonal guidance.

Despite the fact that individual neurons exhibited a significantly stronger immunostaining than individual glial cells, the observed overall difference in the intensity of PLTP staining within a given region or structure among different control subjects was primarily due to the number of glial cells. This is of practical importance, since conditions that give rise to an increase in the number of glial cells, such as brain injury or tumors of glial origin, would likely greatly increase the amount of PLTP in the brain, as we observed in a case of hippocampal sclerosis (unpublished observations). The consequences of an increase might be beneficial. For example, PLTP transfers α-tocopherol, a potent antioxidant (17, 51). Facilitation of an influx of antioxidants could be as relevant as the contribution of free radicals and oxidation to pathophysiological processes in the brain (51–55). Increased PLTP-mediated lipid transfer might also contribute to the repair of the damaged neurons affecting lipoprotein delivery of cholesterol and phospholipids from glial cells to neurons, a process necessary for repair to occur (38, 56).

Our data indicate that PLTP is significantly altered in AD brain tissue. An increase in PLTP levels in brain tissue obtained from AD subjects, as shown by Western blot data, might represent a functional response to the metabolic processes underlying the development of AD. At the same time, in CSF obtained from subjects with probable AD, there was no evidence of higher PLTP-mediated phospholipid transfer activity or PLTP levels (unpublished observations). Immunohistochemical data showing a marked increase in neuronal staining in affected areas with no apparent, or only a mild, neuronal loss might reflect a molecular response to specific alterations found in AD brain. In contrast, significant neuronal loss in AD led to a decrease in PLTP immunostaining both in the gray and the white matter of the affected regions. Such a loss in PLTP synthesis might profoundly affect the repair and functioning of the remaining neurons and contribute to the functional and structural deterioration in the latter stages of the disease. Further elucidation of PLTP function and molecular interactions in control and AD brain is warranted.

The average phospholipid transfer activity mediated by PLTP in CSF obtained from neurologically healthy subjects represented ~15% of the PLTP activity measured in plasma. Although some of the CSF PLTP could be of plasma origin, our results suggest that plasma contribution to PLTP levels in the brain is fairly minor. This notion is further supported by the cell culture Western blot and PLTA data, indicating that all classes of brain cells synthesize and secrete active PLTP. Other investigators have reported lower PLTP activity in human CSF (8). The higher PLTP activity we obtained in CSF cannot be explained by a compromised blood-brain barrier, because our CSF samples were carefully screened prior to PLTP analysis to exclude CSF contamination by plasma PLTP. Different methodological approaches may explain quantitative differences between studies.

The Western blot analysis of the brain tissue and brain-derived cell homogenates revealed the existence of smaller-molecular-weight forms of PLTP. The calculated molecular weight of PLTP based on its amino acid sequence is about 55 kDa (11). Homogenized brain cells are not unique in displaying smaller molecular forms of PLTP. Homogenized HepG2, BeWo cells, and macrophages all can exhibit smaller molecular forms than the full-length 80 kDa fully glycosylated mature protein (unpublished observations). Other authors have also reported smaller-molecular-weight immunoreactive PLTP forms in cell homogenates exhibiting increased phospholipid transfer activity (57). Our group also reported the existence of several lower-molecular-weight forms of PLTP in human plasma fractions (31). Therefore, the smaller-molecular-weight PLTP immunoreactive species might represent either the characteristic proteolytic PLTP fragments or newly synthesized intracellular PLTP that is not fully glycosylated.

Three PLTP mRNA-size isoforms in brain tissue, one full-length and two shorter mRNA molecules, have been reported (35). Our Northern blot results could not confirm this finding, even after prolonged exposure of the film. The observation of shorter mRNA forms could be due to mRNA fragmentation during extraction, or alternatively, could relate to the type of probe used for detection. This does not mean that shorter forms of PLTP mRNA do not exist in some pathological states of the brain, but healthy adult human brain tissue did not have any of the reported shorter mRNA forms.

The PLTP mRNA, IHC, Western blot, and phospholipid transfer assay data strongly suggest that most of the PLTP found in human CNS is of local origin. Our detailed observations of the variations in PLTP among distinct cell groups, in addition to the regional differences within the CNS, suggest that PLTP has more specific functions in brain lipid and lipoprotein metabolism than that of pure maintenance. These findings, combined with our current knowledge of the central role PLTP plays in lipid metabolism, underline the importance of further research on

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PLTP function in both healthy and diseased human brain.

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