Journal of
Proteomics & Bioinformatics

Takano et al., J Proteomics Bioinform 2014, 7:11
DOI: 10.4172/jpb.1000338

Research Article Open Access

Secretomic Analysis of Mouse Choroid Plexus Cell Line ECPC-4 Using Two-Dimensional Gel Electrophoresis Coupled to Mass Spectrometry

Masaoki Takano[1]*, Mari Uramoto[1], Mieko Otani[1], Kenji Matsuura[2], Keiji Sano[1] and Shogo Matsuyama[2]

[1]*Laboratory of Molecular Cell Biology , School of Pharmaceutical Sciences, Kobe Gakuin University, 1-1-3 Minatojima, Chuo-ku, Kobe 650-8586, Japan*
[2]*Faculty of Pharmaceutical Sciences, Himeji Dokkyo University, 7-2-1 Kamiohno, Himeji, 670-8524 Japan*

Abstract

Choroid plexus (CP), the main production site of cerebrospinal fluid (CSF), is responsible for the inflammatory mediators involved in meningitis and immune systems. The mouse choroid plexus cell line ECPC-4 is known to be useful for analyses of CP functions. In this study, we performed the secretome analysis of ECPC-4 using two-dimensional gel electrophoresis (2DE) followed by mass spectrometry. Twenty-two secreted proteins were identified in the conditioned medium of ECPC-4. They were a secreted protein acidic and rich in cysteine (SPARC), plasminogen activator inhibitor 1 (PAI-1), and others (vinculin, heat shock protein cognate 71 kDa, moesin, tubulin ß2A, fascin, calreticulin, gamma actin, alcohol dehydrogenase, malate dehydrogenase, Rho GDP dissociation inhibitor alpha, phosphoglyceratemutase 1, translationally controlled tumor protein, phosphatidylethanolamine binding protein1, peroxiredoxin1 type II, peroxiredoxin1, myosin light polypeptide 6, cofilin-1, nucleoside diphosphate kinase A, peptidyl-prolyl cis-trans isomerase A [PPI-A], and galectin1). These proteins could be classified as cytoskeleton/intermediate filament, protein folding, signal transduction, cell growth, metabolism, and redox regulation groups, suggesting that they could be related to the CP functions. Furthermore, the level of four proteins was changed in the conditioned medium of ECPC-4 treated with lipid A. Proteasome subunit alpha type-1 and nucleoside diphosphate kinase A were significantly increased and gamma-actin and galectin-1 were significantly decreased, as compared with those in the conditioned medium of non-treated ECPC-4. Also, Western Blotting also validated the changes in proteasome subunit alpha type-1, galectin-1 and nucleoside diphosphate kinase A. Thus, it is suggested that these proteins play an important role in inflammation such as meningitis. ECPC-4, like CP epithelial cells, is useful to analyze the CP functions.

Keywords: Choroid plexus; Proteome; Lipid A; Secreted proteins

Introduction

Choroid plexus (CP) located in the brain ventricles has a monolayer of epithelial cells enclosing a vascularized stroma, and is known as the main site of secretion of the cerebrospinal fluid (CSF) [1]. The CP epithelial cells are mainly responsible for the production of CSF [2]. They produce about two thirds of the total CSF volume via the secretion of water, ions, and macromolecules [3]; also synthesize and release many polypeptides and proteins [4]. Importantly, the CP epithelial cells regulate the CSF composition by producing and secreting several CSF proteins, such as transthyretin (TTR) [5], the major thyroid hormone transporter. Therefore, the CSF is not a simple ultrafiltrate of the interstitial fluids but is an active substance with many important functions. Analysis of proteins secreted by primary cultures enriched in mouse CP epithelial cells showed 43 proteins secreted through the classical vesicular pathway in CP epithelial cells-conditioned medium [6]. These cell cultures treated with lipopolysaccharide (LPS) increased the secretion of four protein species and induced the release of additional proteins [6].

Several studies have reported that the CP participates in brain inflammatory processes. Bacterial meningitis still causes high rates of neurological sequelae and mortality, despite effective antibiotic therapy. Meningitis, an inflammation of the protective membranes covering the brain and spinal cord, triggers neuronal damage, brain edema, and intracranial pressure increase [7,8]. Systemic administration of LPS causes a rapid and transient induction of interleukin-1β (IL-1 β) and tumor necrosis factor-α (TNF-α) primarily at the CP, propagating throughout the brain. Thus, it is suggested that the CP plays important roles in the transfer of information between the immune system and the brain through a coordinated local induction of proinflammatory cytokines [9].

It was established that choroid plexus tumors developed in transgenic mice harboring the simian virus 40 large tumor antigen gene [10]. ECPC-4, which were produced from CP carcinoma in these mice, retained the characteristics of choroid plexus epithelial cells expressing TTR and α2-macroglobulin [10]. These cell lines might be useful materials for research on the CP tissue.

In the present study, we analyzed the secreted proteins in the conditioned medium of ECPC-4 and in the conditioned medium of ECPC-4 treated with lipid A, using two dimensional gel electrophoresis coupling with Matrix-assisted laser desorption/ionization mass spectrometry (MALDI TOF MS) to elucidate the various profile of the secreted proteins in CSF.

Materials and Methods

Materials

Mouse choroid plexus cell lines ECPC-4 were obtained from RIKEN cell bank (ECPC-4; Tsukuba, Japan). Lipid A was purchased

***Corresponding author:** Masaoki Takano, Laboratory of Molecular Cell Biology, School of Pharmaceutical Sciences, Kobe Gakuin University, 1-1-3 Minatojima, Chuo-ku, Kobe 650-8586, Japan, Tel (or) Fax: +81-78-974-4716; E-mail: takano@pharm.kobegakuin.ac.jp

Received August 15, 2014; **Accepted** October 14, 2014; **Published** October 20, 2014

Citation: Takano M, Uramoto M, Otani M, Matsuura K, Sano K, et al. (2014) Secretomic Analysis of Mouse Choroid Plexus Cell Line ECPC-4 Using Two-Dimensional Gel Electrophoresis Coupled to Mass Spectrometry. J Proteomics Bioinform 7: 347-352. doi:10.4172/jpb.1000338

from Sigma (Tokyo, Japan). PMSF, DNase, RNase, CBB protein assay, PVDF membrane, WIDE-VIEW® Prestained Protein Size Marker II and immuno-enhancer were purchased from Wako Junyaku (Osaka, Japan). ECL Plus was purchased from GE Healthcare (Tokyo, Japan). Mouse anti β-actin and rabbit anti SPARC were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti prostaglandin D (PGD) synthase, galectin-1 and peroxidase-conjugated antibody were purchased from Abcam (Cambridge, UK). Rabbit anti proteasome subunit alpha type-1 and nucleoside diphosphate kinase A were purchased from Protein tech (Chicago, IL, USA). Rabbit anti prostaglandin D (PGD) synthase, galectin-1 and peroxidase-conjugated antibody were purchased from Abcam (Cambridge, UK). Goat anti rabbit IgG (Fc) alkaline phosphatase conjugate, goat anti mouse IgG (H+L) alkaline phosphatase conjugate and Western Blue® stabilized substrate for alkaline phosphatase were purchased from Promega (Madison, WI, USA).

Cell culture

The ECPC-4 were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. After ECPC-4 were grown to confluence in a 100-mm culture dish, the cells were passaged twice per week.

Sample preparation of conditioned-medium

After 2 days of growth at 37°C, the cells were washed three times with phosphate buffer saline (PBS) and incubated in serum-free DME. Forty-eight hours later this conditioned serum-free medium was collected and the cell debris was removed by low-speed centrifugation. At the first concentration step of conditioned-medium, each 200 ml of conditioned medium was put into dialysis bag (Cell Sep T1: MWCO 3500); it was placed over a bed of polyethylene glycol (PEG) and covered with PEG on ice for 6 h. At the second step, the concentrated conditioned-medium was centrifuged by Amicon Ultra Centrifugal Filter 15 at 5000 g for 12 h. Then, approximately 1 ml of the concentrated conditioned-medium was mixed with four times volume of cold acetone (-20°C) and kept at -20°C for over night. This concentrated sample was centrifuged at 13000 g for 30 min, after decantation and disposal of the supernatant, the pellet was dissolved into a lysis buffer by sonication for 30 s.

Western blotting

The concentrated conditioned medium from ECPC-4 containing equal amounts of proteins (10 μg/lane) were boiled for 5 min in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue and 5% β-mercaptoethanol), and run onto a 10% SDS/polyacrylamide gel. After electrophoresis, the protein was blotted to a polyvinylidene difluoride (PVDF) membrane by use of a transblotter apparatus (Nippon Eido, Tokyo, Japan) at 1 mA/cm^2 for 1 h. Membranes were blocked for 1 h at room temperature with Tris buffered saline (TBS)-T buffer (150 mM NaCl, 20 mM Tris/HCl, pH 7.4, 0.1% Tween 20) containing 5% skim milk or 3% bovine serum albumin (BSA) for phosphoprotein. The blots were incubated over night with mouse anti beta-actin antibody (1: 1000). For the detection of beta-actin, alkaline phosphatase-conjugated antibody (diluted 1: 6700, Promega, USA) was used as secondary antibody. The reaction was detected by color development with Western Blue® stabilized substrate.

For the detection of PGD synthase and galectin-1, proteasome subunit alpha type-1 and nucleoside diphosphate kinase A, Western Blotting was performed using rabbit antibodies against PGD synthaseand galectin-1, proteasome subunit alpha type-1, nucleoside diphosphate kinase A and SPARC (diluted 1: 1000). Peroxidase-conjugated antibody (diluted 1: 5000) was used as secondary antibody. The reaction was detected by chemiluminescence with ECL reagents.

Two-dimensional electrophoresis

2DE was carried out by the method as previously described [11] with minor modifications. Approximately 500 μg of protein was applied to Immobiline Dry Strip pH 3-10 NL (7 cm) gels (GE Healthcare UK Ltd., England) and separated at 50 V for 6 h, at 100 V for 6 h, and at 2000 V for 6 h. The immobilised pH gradient (IPG) strips were equilibrated for 15 min in 50 mM Tris-HCl, pH8.8, 6 M urea, 30% (v/v) glycerol, 1% SDS, and 1% (v/v) DTT, and then for 15 min in the same buffer with 2.5% (w/v) iodoacetamide instead of DTT. After equilibration, the IPG strips were set onto a 12.5% acrylamide gel and SDS-polyacrylamide gel electrophoresis was performed at 5 mA/gel for 7 h.

SYPRO Ruby staining

Proteins on SDS-polyacrylamide gels were detected using the SYPRO Ruby Protein Gel Stain (Molecular Probes). Gels after 2DE were fixed in a solution containing 10% acetic acid/50% methanol for 30 min, then 7% acetic acid/10% methanol for 30 min. After fixing, the gels were incubated in the undiluted stock solution of SYPRO Ruby for 90 min, and destained with 7% acetic acid/10% methanol for 30 min. After rinsing with H_2O for 10 min, digital images were acquired using a Fluorophorestar 3000 image capture system (Anatech, Japan) with 470 nm excitation and 618 nm emission for SYPRO Ruby detection.

Image analysis

Following image acquisition, 2DE gel imaging and analysis software Prodigy Same Spots (Nonlinear Dynamics, UK) version 1.0 was used for gel-to-gel matching and identifying differences between the conditioned medium from the non-treated and lipid A treated ECPC-4. Each of four sets of samples was represented by two independent gels biological replicates of 2DE gels. The gel images were normalized in the Prodigy Same Spots software to even out differences in staining intensities among gels. Analysis of variance (ANOVA) was performed with 95% significance level to determine which proteins were differentially expressed between the conditioned medium from the non-treated and lipid A treated ECPC-4.

MALDI-TOF MS/MS and protein identification

Proteins of interest were excised and digested in gel using trypsin (Gold, Promega), as previously described [12]. Aliquots (1.0 μl) of digest products and matrix were spotted consecutively in a 1: 1 ratio on a stainless steel target and dried under ambient conditions. All spectra acquired by MALDI-TOF MS were externally calibrated with peptide calibration standard II (Bruker Daltonics, Germany). An MS condition of 2500 shots per spectrum was used. Automatic monoisotopic precursor selection for MS/MS was done using an interpretation method based on the 12 most intense peaks per spot with an MS/MS mode condition of 4000 laser shots per spectrum. Minimum peak width was one fraction and mass tolerance was 80 ppm. Adduct tolerance was (m/z) ± 0.003. MS/MS was performed with a gas pressure of 1×10^{-6} bar in the collision cell. Ambient air was used as collision gas. Data analyses were performed using Data Explorer version 4.9 (Applied Biosystems) software, and proteins were identified through the search engine Mascot (www.matrixscience.com; Matrix Science, Boston, MA) (peptide mass tolerance: 60 ppm; MS/MS tolerance: 0.3 Da; maximum missed cleavages: 1) using the protein database NCBInr. Proteins

identified by MALDI-TOF MS with a score of 79 or higher were considered significant ($p<0.05$). Single peptides identified by MALDI-TOF/TOF MS/MS with individual ions scores greater than 47 indicate identity or extensive homology ($p<0.05$).

Results

Comparison of proteins between conditioned medium and cell lysate of ECPC-4

We compared proteins between the conditioned medium and cell lysate of ECPC-4 by Coomassie Blue staining. To examine intracellular proteins contamination, we analyzed the secretome collected from 48-h serum-free culture media. Coomasie brilliant blue (CBB)-stained gels showed no changes between the non-treated conditioned medium and the conditioned medium treated with lipid A (Figure 1A: lane 1; non-treated, 2; lipid A-treated), but a quite different pattern as compared with the cell lysate (1-A: lane 3). Next, we examined the presence of β-actin as the intracellular protein marker. Western blot analysis showed no β-actin in the conditioned medium (1-A: lane 4; non-treated, 5; lipid A-treated) but β-actin in the cell lysate of ECPC-4 (1-A: lane 6). Furthermore, we examined the presence of leaked intracellular proteins in serum-free culture media, using the 2DE gels stained by SYPRO Ruby (Figure 1B). The 2DE gel from the conditioned medium of ECPC-4 showed over 180 unique spots detected by the Prodigy Same Spots software, which did not match to those in the 2DE gel from the cell lysate. In addition, we detected PGD synthase in conditioned medium of ECPC-4 by Western blotting (Figure 1C), as CSF richly contains PGD synthase [13].

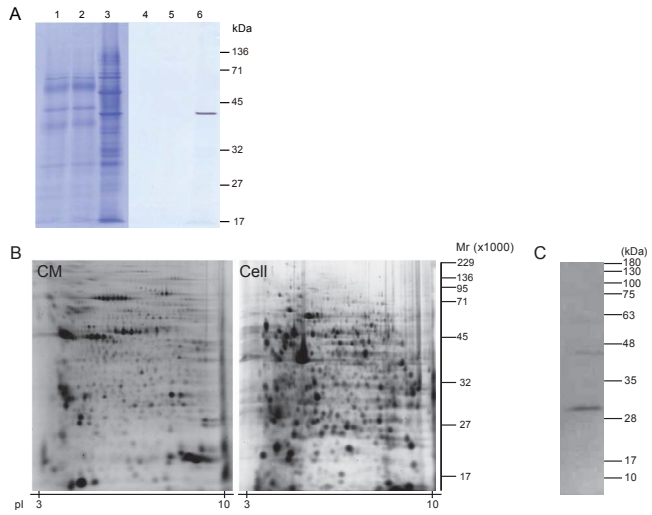

Figure 1: Comparison of the conditioned medium and the cell lysate of ECPC-4.

(A) Monodimensional protein patterns were detected by Coomassie Blue staining of the secretome (A: lane 1; non-treated, 2; lipid A-treated) and the proteome (A: lane 3). Western blot analysis showed no ß-actin expression in the conditioned medium (A: lane 4; non-treated, 5; lipid A-treated) and ß-actin expression in the cell lysate (A: lane 6).

(B) The secretome from the conditioned medium and the proteome from the cell lysate of ECPC-4. Two-dimensional electrophoresis analysis of the conditioned medium for 48 h (B, left: CM) and the cell lysate of ECPC-4 (B, right: Cell). Two-dimensional electrophoresis gels were stained by SYPRO Ruby.

(C) Western Blotting of PGD synthase in the conditioned medium of ECPC-4.

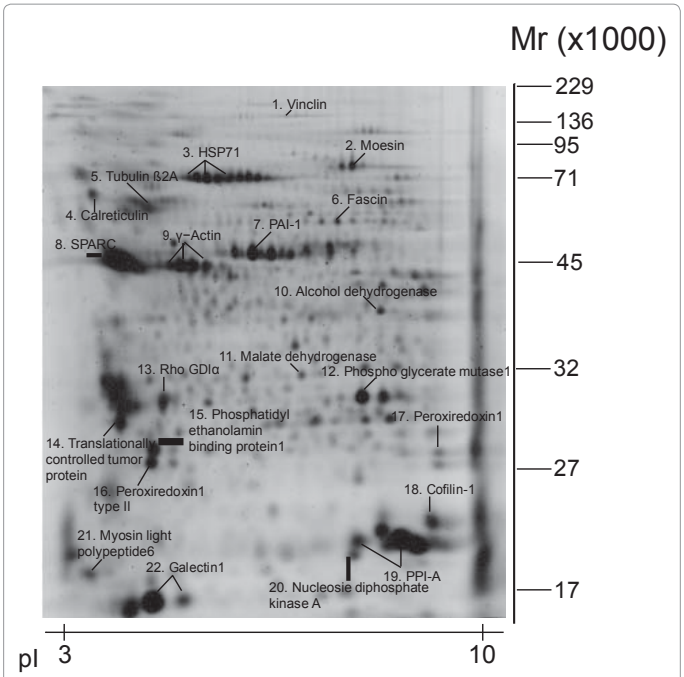

Figure 2: Proteins in the conditioned medium of ECPC-4 identified by mass spectrometry after Two-dimensional gel electrophoresis and SYPRO Ruby staining.

Identification of proteins secreted in the conditioned-medium of ECPC-4

Over 300 spots were detected in the 2DE gel from the conditioned medium of ECPC-4. We picked up the highly expressed 28 spots in SYPRO Ruby-stained gel from the conditioned medium of ECPC-4, and 22 proteins were successfully identified by MALDI TOF MS/MS, which were vinculin, HSP71, moesin, tubulin β2A, fascin, calreticulin, SPARC, gamma actin, PAI-1, alcohol dehydrogenase, malate dehydrogenase, Rho GDI alpha, phosphoglyceratemutase 1, translationally controlled tumor protein, phosphatidylethanolamine binding protein1, peroxiredoxin 1 type II, peroxiredoxin-1, myosin light polypeptide 6, cofilin-1, nucleoside diphosphate kinase A, PPI-A, and galectin1 (Figure 2, Table 1). They could be classified as cytoskelton, protein folding, signal transduction, cell growth, metabolism, and redox regulation groups.

Identification of proteins altered in the conditioned medium of ECPC-4 treated with Lipid A

We previously reported that LPS induced the expression of T-kininogen in choroid plexus [14] and enhanced the expression of inflammation mediators like bradykinin, cyclooxygenase-2, IL-1 β, and TNF-α in ECPC-4 [15]. Thus, we analyzed the levels of proteins significantly changed in the conditioned medium of ECPC-4 treated with lipid A, compared with the conditioned medium of non-treated ECPC-4. The levels of proteins were significantly decreased for gamma actin and galectin-1 and increased for proteosome subunit alpha type-1 and nucleoside diphosphate kinase A (Figure 3, Table 2).

Western blotting for the altered proteins in the conditioned medium of ECPC-4 treated with Lipid A

We performed Western Blotting to validate the identity

Spot number	GI accession number	Identified protein name	Theoretical Mass/PI	Score	Sequence coverage	Number of peptides	Functions/	Cellular localization
1	309533	Vinculin	117303/5.72	66	5	3	intermediate filament	C
2	70778915	Moesin	67839/6.22	461	12	8	cytoskeleton	C
3	13242237	Heat shock cognate 71 kDa protein	71055/5.37	308	14	6	protein folding	C
4	6680836	Calreticulin precursor	48136/4.33	187	11	4	signal transduction	C
5	4507729	Tubulin beta-2A chain	50274/4.78	113	8	3	cytoskeleton	C
6	497775	Fascin	55112/6.21	74	3	1	intermediate filament	C
7	129577	Plasminogen activator inhibitor 1	45198/6.17	164	18	6	signal transduction	S
8	6678077	SPARC precursor	35283/4.77	269	19	5	cell growth	S
9	809561	Gamma-actin	41335/5.56	223	15	4	cytoskeleton	C
10	10946870	Alcohol dehydrogenase	36792/6.9	107	12	3	metabolism	C
11	387129	Cytosolic malate dehydrogenase	36625/6.16	117	8	3	metabolism	C
12	10179944	Phosphoglyceratemutase 1	29033/6.19	60	21	3	metabolism	C
13	13435747	Rho GDP dissociation inhibitor alpha	23434/5.12	106	23	3	signal transduction	C
14	6678437	Translationally-controlled tumor protein	19564/4.76	209	21	4	cytoskeleton	C
15	84794552	Phosphatidylethanolamine-binding protein 1	20988/5.19	247	36	5	signal transduction	M
16	3603241	Type II peroxiredoxin 1	21949/5.2	64	18	5	redox regulation	C
17	6754976	Peroxiredoxin-1	22390/8.26	76	14	3	redox regulation	C
18	6680924	Cofilin-1	18776/8.22	64	18	2	cytoskeleton	C
19	6679439	Peptidyl-prolyl cis-trans isomerase A	18131/7.74	91	21	2	metabolism	C
20	37700232	Nucleoside diphosphate kinase A	17311/6.84	145	38	5	metabolism	C
21	17986258	Myosin light polypeptide 6 isoform 1	17090/4.56	77	13	2	cytoskeleton	C
22	6678682	Galectin-1	15198/5.32	337	39	5	signal transduction	C

The proteins of conditioned medium were separated by 2DE and identified by MALDI-TOF MS/MS, following in-gel digestion with trypsin. The spots representing the identified proteins are indicated in Figure 2 and are designated by gene ID accession numbers (GI acc. no.) in the Swiss Prot database. Score relates to the probability assignment, theoretical molecular weight, and pI, and sequence coverage (SC) values are given. Score and sequence coverage were calculated via the MASCOT search engine (http://www.matrixscience.com). Note that all MASCOT scores are greater than 60 (p<0.05). Cellular localization of proteins is also indicated (S, secreted; C, cytosolic; M, membrane).

Table 1: Proteins identified in the supernatant of ECPC-4 cell cultures.

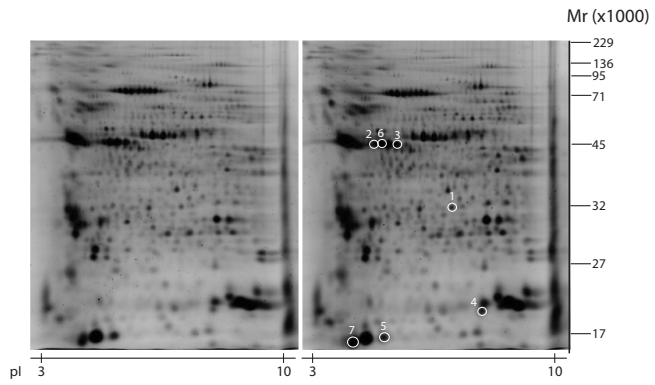

Figure 3: SYPRO Ruby-stained 2DE gels (pH range 3-10) from the conditioned medium of non-treated ECPC-4 (Figure 3: left) and ECPC-4 treated with lipid A (500 ng/ml) (Figure 3: right) for 48 h. For detection of protein expression, 500 µg of protein was loaded to each gel. Protein molecular mass standards (y-axis) and pHs (x-axis) are shown.

of galectin-1, proteasome subunit alpha type-1 and nucleoside diphosphate kinase A as the altered proteins, and SPARC as the unchanged protein (as internal control) in the conditioned medium of ECPC-4 treated with Lipid A. The level of galectin-1 tended to decrease, and the level of proteasome subunit alpha type-1 or nucleoside diphosphate kinase A tended to increase (Figure 4A and 4B).

Discussion

In this study, using 2DE coupled with MALDI TOF MS/MS, we identified 22 secreted proteins from the conditioned medium of ECPC-4 and 4 secreted proteins showing differential levels in the conditioned medium of ECPC-4 treated with lipid A. These proteins were classified into the following groups: cytoskeleton, protein folding, signal transduction, cell growth, metabolism, and redox regulation, on a functional basis, by using MOTIF (http://www.genome.jp/tools/motif/) database, as shown in Tables 1 and 2.

The choroid plexus epithelial cells have the several key roles in CNS. Especially, one of the important functions is the production of CSF, which includes the filtration and the selection of blood, and the paracrine secretion by CP epithelial cells [1,16]. Importantly, the secretome analysis of mouse CP epithelial cells showed that secreted classical vesicles included transport proteins, cell matrix proteins, proteases, protease inhibitors, and neurotrophic factors [6]. However, this secretome analysis of CP epithelial cells is insufficient to clarify the CP function. The reasons why we used choroid plexus cell line ECPC-4 in this study were that ECPC-4 possesses the characters of choroid plexus epithelial cells owing to the expression of TTR and α2-macroglobulin [10] and that the contamination of other cells such as fibroblasts in the brain are excluded. Also, we showed that the ECPC-4 secreted PGD synthase (Figure 1C), which is richly contained in the CSF [13]. Taken together, it is suggested that the ECPC-4 retains the function of choroid plexus, including proteins secretion into CSF.

We identified PAI-1 and SPARC precursor/osteonectin in secretome analysis of ECPC-4, which were also identified in primary mouse choroid plexus cells [6]. PAI-1 [17] and SPARC precursor/osteonectin [18] were classified as the secreted proteins via the classical vesicular pathway. PAI-1 was the serine protease inhibitor, which was secreted in the CSF and related with neurological diseases [19]. SPARC precursor protein was identified as a glycoprotein in the bone binding calcium and was secreted by osteoblasts during bone formation [18]. In addition, cultured leptomeningeal cells secreted SPARC into the

Spot number	GI accession number	Identified protein name	Theoretical Mass/PI	Score	Sequence coverage	Number of peptides	Fold increase/decrease	Functions	Cellular localization
1	33563282	Proteasome subunit alpha type-1	29813/6	70	9	2	1.3	proteolysis	C
2	809561	Gamma-actin	41335/5.56	160	10	3	0.77	cytoskeleton	C
3	809561	Gamma-actin	41335/5.56	223	15	4	0.77	cytoskeleton	C
4	37700232	Nucleoside diphosphate kinase A	17311/6.84	145	38	4	1.2	metabolism	C
5	6678682	Galectin-1	15198/5.32	235	33	4	0.83	signal transduction	C
6	809561	Gamma-actin	41335/5.56	221	15	3	0.83	cytoskeleton	C
7	6678682	Galectin-1	15198/5.32	337	39	4	0.83	signal transduction	C

The proteins of conditioned medium were separated by 2DE and identified by MALDI-TOF MS/MS, following in-gel digestion with trypsin. The spots representing the identified proteins are indicated in Figure 3 and are designated by gene ID accession numbers (GI acc. no.) in the Swiss Prot database. Score relates to the probability assignment, theoretical molecular weight, and pI, and sequence coverage (SC) values are given. Score and sequence coverage were calculated via the MASCOT search engine (http://www.matrixscience.com). Data were analyzed by ANOVA (p<0.05). Cellular localization of proteins is also indicated (S, secreted; C, cytosolic; M, membrane).

Table 2: Differentially expressed proteins in the supernatant of lipid A-treated ECPC-4 cell cultures when compared with untreated (n=3).

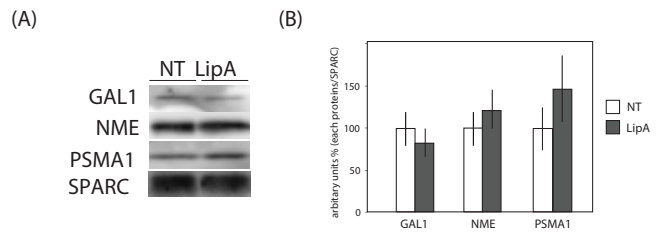

Figure 4: Western Blotting of galectin-1, nucleoside diphosphate kinase A and proteasome subunit alpha type1 in the conditioned medium of non-treated and lipid A-treated ECPC-4. NT, medium of non-treated ECPC-4 conditioned for 48 h; LipA, medium of ECPC-4 with lipid A (500 ng/ml) conditioned for 48 h. GAL1: galectin-1, NME: nucleoside diphosphate kinase A, PSMA1: proteasome subunit alpha type1. SPARC was used as the internal control protein. (A) The levels of GAL1, NME, PSMA1 and SPARC in individual samples of each group were detected. (B) Graphical representation of the semi quantitative analysis. (mean ± S.E.M. of O.D. of bands). Data are presented as mean ± S.E.M. of 3 samples from each group.

conditioned medium [20]. Both proteins were known as the secreted proteins in CSF [21], although their physiological functions are still unknown.

We identified metabolic system proteins and signal transduction proteins in the conditioned medium from ECPC-4, which included alcohol dehydrogenase, cytosolic malate dehydrogenase, phosphoglyceratemutase 1, peroxiredoxin 1, PPI-A and nucleoside diphosphate kinase A. As cytoskeletal and intermediate proteins, vinculin, moesin, tubulin β-2A, fascin, gamma-actin, translationally-controlled tumor protein, cofilin-1, and myosin light polypeptide 6 were identified. These proteins were originally classified as intracellular proteins, but not secreted proteins. However, the secretome analysis of CSF showed that the cell part proteins were secreted into CSF [21]. Interestingly, the increased levels of non-secreted proteins such as α-tubulin and tubulin β 2C were identified in the CSF of patients with Alzheimer's disease (AD) by two dimensional fluorescence difference gel electrophoresis (2D-DIGE) analysis, suggesting that these proteins might become candidates of biomarker for diagnosis of AD [22].

We hypothesized that the choroid plexus epithelial cells have roles for innate immunity responses and host defense in the brain. We previously reported that the intracerebro ventricular administration of LPS evoked the expression of T-kininogen in the rat choroid plexus [14]. In addition, the secreted inflammation mediators IL-1α, IL-6, and TNF-β were expressed in ECPC-4 and detected by RT-PCR; a low-molecular weight kininogen was secreted into the conditioned medium by LPS addition in the ECPC-4 by Western blotting [15]. The expression of IL-1α, IL-6, and TNF-β as secreted inflammation mediators of ECPC-4 treated with LPS were detected by RT-PCR, and a low-molecular weight kininogen secreted into the conditioned medium of ECPC-4 treated with LPS was also detected by Western blotting [15]. Therefore, to elucidate the immune response in ECPC-4, we performed the secretome analysis of ECPC-4 treated with lipid A. This secretome analysis showed that proteasome subunit alpha and nucleoside diphosphate kinase A were significantly increased, and gamma-actin and galectin-1 were significantly decreased (Figure 3 and Table 2). Contrary to expectation, lipid A caused a few change in the secreted proteins of ECPC-4. These results let us guess that the sensitivity of fluorescence SYPRO Ruby staining is insufficient to detect cytokines or inflammatory proteins, as compared with RT-PCR or Western blotting.

In conclusion, these findings suggest that the secretomic analysis using ECPC-4 could be useful to elucidate the CP functions.

Acknowledgement

This work was supported by grants from the Smoking Research Foundation, Kobe Gakuin University for Collaborative Research B, and the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (no. 18023033, 21500352, 21390271, 20023027).

References

1. Strazielle N, Ghersi-Egea JF (2000) Choroid plexus in the central nervous system: biology and physiopathology. J Neuropathol Exp Neurol 59: 561-574.
2. Speake T, Whitwell C, Kajita H, Majid A, Brown PD (2001) Mechanisms of CSF secretion by the choroid plexus. Microsc Res Tech 52: 49-59.
3. Johanson CE, Duncan JA 3rd, Klinge PM, Brinker T, Stopa EG, et al. (2008) Multiplicity of cerebrospinal fluid functions: New challenges in health and disease. Cerebrospinal Fluid Res 5: 10.
4. Chodobski A, Szmydynger-Chodobska J (2001) Choroid plexus: target for polypeptides and site of their synthesis. Microsc Res Tech 52: 65-82.
5. Sousa JC, Cardoso I, Marques F, Saraiva MJ, Palha JA (2007) Transthyretin and Alzheimer's disease: where in the brain? Neurobiol Aging 28: 713-718.
6. Thouvenot E, Lafon-Cazal M, Demettre E, Jouin P, Bockaert J, et al. (2006) The proteomic analysis of mouse choroid plexus secretome reveals a high protein secretion capacity of choroidal epithelial cells. Proteomics 6: 5941-5952.
7. Tunkel AR, Scheld WM (1993) Pathogenesis and pathophysiology of bacterial meningitis. Clin Microbiol Rev 6: 118-136.
8. Koedel U, Bernatowicz A, Paul R, Frei K, Fontana A, et al. (1995) Experimental pneumococcal meningitis: Cerebrovascular alterations, brain edema, and meningeal inflammation are linked to the production of nitric oxide. Annals of neurology 37: 313-323.
9. Strazielle N, Ghersi-Egea JF (2000) Choroid plexus in the central nervous system: biology and physiopathology. J Neuropathol Exp Neurol 59: 561-574.
10. Enjoji M, Iwaki T, Hara H, Sakai H, Nawata H, et al. (1996) Establishment and

characterization of choroid plexus carcinoma cell lines: connection between choroid plexus and immune systems. Jpn J Cancer Res 87: 893-899.

11. Otani M, Taniguchi T, Sakai A, Seta J, Kadoyama K, et al. (2011) Phosphoproteome profiling using a fluorescent phosphosensor dye in two-dimensional polyacrylamide gel electrophoresis. Appl Biochem Biotechnol 164: 804-818.

12. Takano M, Maekura K, Otani M, Sano K, Nakamura-Hirota T, et al. (2012) Proteomic analysis of the brain tissues from a transgenic mouse model of amyloid β oligomers. Neurochem Int 61: 347-355.

13. Watanabe K, Urade Y, Mäder M, Murphy C, Hayaishi O (1994) Identification of beta-trace as prostaglandin D synthase. Biochem Biophys Res Commun 203: 1110-1116.

14. Takano M, Horie M, Yayama K, Okamoto H (2003) Lipopolysaccharide injection into the cerebral ventricle evokes kininogen induction in the rat brain. Brain Res 978: 72-82.

15. Takano M, Satoh C, Kunimatsu N, Otani M, Hamada-Kanazawa M, et al. (2008) Lipopolysaccharide activates the kallikrein-kinin system in mouse choroid plexus cell line ECPC4. Neurosci Lett 434: 310-314.

16. Falcão AM, Marques F, Novais A, Sousa N, Palha JA, et al. (2012) The path from the choroid plexus to the subventricular zone: go with the flow! Front Cell Neurosci 6: 34.

17. Pannekoek H, Veerman H, Lambers H, Diergaarde P, Verweij CL, et al. (1986) Endothelial plasminogen activator inhibitor (PAI): a new member of the Serpin gene family. EMBO J 5: 2539-2544.

18. Fisher LW, Hawkins GR, Tuross N, Termine JD (1987) Purification and partial characterization of small proteoglycans I and II, bone sialoproteins I and II, and osteonectin from the mineral compartment of developing human bone. J Biol Chem 262: 9702-9708.

19. Akenami FO, Koskiniemi M, Färkkilä M, Vaheri A (1997) Cerebrospinal fluid plasminogen activator inhibitor-1 in patients with neurological disease. J Clin Pathol 50: 157-160.

20. Ohe Y, Ishikawa K, Itoh Z, Tatemoto K (1996) Cultured leptomeningeal cells secrete cerebrospinal fluid proteins. J Neurochem 67: 964-971.

21. Thouvenot E, Urbach S, Dantec C, Poncet J, Séveno M, et al. (2008) Enhanced detection of CNS cell secretome in plasma protein-depleted cerebrospinal fluid. J Proteome Res 7: 4409-4421.

22. Belohlavek M, Jiamsripong P, Calleja AM, McMahon EM, Maarouf CL, et al. (2009) Patients with alzheimer disease have altered transmitral flow: Echocardiographic analysis of the vortex formation time. J Ultrasound Med 28: 1493-500.