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Comparative Proteome Analysis of Rat Brain and Coronary Microvascular Endothelial Cells

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Summary

The endothelium of different organs displays a remarkable heterogeneity, although it presents many common functional and morphological features. However, despite our knowledge of heterogeneity among endothelial cells from different sites, the differences between brain microvascular endothelial cells (BMEC) and coronary microvascular endothelial cells (CMEC) are poorly defined. The aim of this study was to investigate whether BMEC are distinct from CMEC at the protein level. Using the proteomic approach, we comparatively analyzed the proteome of cultured BMEC and CMEC. We reproducibly separated over 2000 polypeptides by using two-dimensional electrophoresis (2-DE) at pH range of 3-10. Using PDQuest software to process the 2-DE gel images, forty-seven protein spots were differentially expressed in the two-endothelial cells. Of these, thirty-five proteins are highly expressed in BMEC, whereas twelve proteins are highly expressed in CMEC. Fifteen proteins in BMEC and seven proteins in CMEC were identified with high confidence by matrix-associated laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF-MS). Our data suggested that BMEC and CMEC were different in several aspects including cytokine and growth-related molecules, stress-related proteins, metabolic enzymes, signal transduction proteins and others. The identification of a set of proteins preferentially expressed in BMEC and CMEC provided new data on the heterogeneity of the endothelium.

Key words
Brain • Endothelium • Proteomics • Heart • Microvasculature

Introduction

Endothelial cells are widely understood to play a crucial role in the development of vascular diseases in addition to serving a broad range of physiological functions in the cardiovascular system. A number of studies have shown that endothelial cells (EC) originating from distinct cardiovascular locations have unique expression patterns and differ both at the functional and the transcriptional level (Brouland *et al.* 1999, Girard *et al.* 1999). Using microarray analysis, Chi *et al.* (2003) explored EC specialization on the basis of 53 endothelial cell cultures from different locations and found tissue-specific expression patterns. Hendrickx *et al.* (2004)

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investigated the diversity of cardiac microvascular endothelium, aortic endothelium and endocardial endothelium, and identified sets of genes preferentially expressed in endocardial endothelium. Kallmann *et al.* (2002) detected 35 genes specific for brain endothelium compared with umbilical cord vascular endothelium. However, as the most easily involved organs during the occurrence of atherosclerosis, the heterogeneity of brain and heart endothelium is seldom studied.

In the brain, the endothelial layer of the blood-brain barrier forms a tight interface between blood and neuronal tissue, with active transport systems which mediate directed transport of nutrients into the CNS or of toxic metabolites out of the CNS and create a milieu essential for the function of the underlying neuronal cells. On the other hand, in the heart, coronary microvessels play a pivotal role in determining the supply of oxygen and nutrients to the myocardium by regulating the coronary flow conductance and substance transport. Many neurohumoral mediators significantly affect coronary microvascular control in an endothelium-dependent manner (Komaru *et al.* 2000). The regulation of coronary microvascular permeability is an important factor for the nutrient supply and for edema formation (Chilian and Layne 1990). Analyses of microvessels are important for understanding the pathophysiology of ischemic hearts and hypertrophied hearts. Moreover, it has been increasingly recognized that microvascular abnormalities may be central to the development of end-organ damage brought about by hypertension, including ischemic heart disease and stroke, and the endothelium is a favorite early target of cardiovascular diseases such as hypertension (Thuillez and Richard 2005).

In the present study, we use the techniques of proteomics to compare the protein expression profiles between the rat brain and heart microvascular endothelial cells. Using 2-DE and MALDI-TOF-MS, we identified 15 proteins with high abundance in rat primary cultured brain endothelial cells and 7 proteins in the coronary endothelial cells.

Methods

Cell cultures

Brain microvascular endothelial cells (BMEC)

Rat brain capillary fragments were isolated and endothelial cells cultured using a modification of methods introduced by Abbott *et al.* (1992) and Lin and Rui (1994). Briefly, fresh rat brains were obtained from 6-week-old Wistar rats, dropped into an ice-cold Buffer A (HEPES 10 mM, $NaHCO_3$ 11.9 mM, NaCl 140 mM, KCl 10 mM, BSA 0.1 %), and the cerebellum, brain stem, choroid plexus, and the meninges were carefully removed. The cortices were rolled on dry lint to remove adherent surface cells, then chopped with a scalpel for <1 min into uniform 2-3 mm pieces in Buffer A, digested in a collagenase/dispase solution (0.1 %, Boehringer, Mannheim, in Ca, Mg-free HBSS, 20 units/ml DNase I was added) for 1 h at 37 °C to separate microvessels from other components, and then was centrifuged in 15 % Dextran (4500 x *g*, 4 °C for 20 min). The pellet containing crude microvessels was further digested in a second collagenase/dispase solution for 2 h at 37 °C. Microvascular endothelial cell were purified by a Percoll gradient (stock solution prepared from 50 ml Percoll plus 5.5 ml 10×HBSS +45 ml HBSS, centrifuged at 25000 x *g* at 4 °C for 1 h) at 1000 x *g* for 10 min.

Coronary microvascular endothelial cells (CMEC)

CMEC were isolated from 6-week-old Wistar rats by collagenase digestion according to the method of Nishida *et al.* (1993) with slight modification. Briefly, after the rats were anesthetized and heparinized, the hearts were removed and placed in an ice-cold solution (in mmmol/l, Buffer 1): NaCl 118, KCl 4.7, NaH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, and glucose 11, pH 7.4, at 37 °C (gassed with 95 % O_2/5 % CO_2), then perfused 5 min through the ascending aorta to remove blood cells. Epicardial mesothelial cells were devitalized with 70 % (vol/vol) ethanol. After removal of the connective tissue, the atria, right ventricular tissue and the outer one-fourth of the left ventricular free wall and septum, the remaining heart tissue was minced finely in 0.2 % collagenase (Sigma type) in buffer with added $CaCl_2$ (0.25 mmol/l) and incubated for 30 min at 37 °C (0.7 mg/ml). Trypsin (0.02 %) was added and incubated for another 30 min. The dissociated cells were filtered through a 100-μm mesh filter and washed with Buffer 1, followed by the same buffer containing calcium and centrifugation at 150x*g* for 5 min to remove myocytes, and then the supernatant was further centrifuged at 1000x*g* for 10 min and washed. Further purification of CMEC was accomplished by sequential filtration through a series of 90-, 45-, 25- and 15-μm nylon screens.

Both cells were plated on rat-tail-collagen-coated dishes and were cultured in minimal essential medium containing D-valine (to inhibit growth of non-endothelial cells, US Biological), 20 % FBS (Gibco),

endothelial cell growth supplement (100 μg/ml), heparin (120 U/ml), L-glutamine (2 mM), sodium pyruvate (2 mM), nonessential amino acids, vitamins, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cultures were incubated at 37 °C in a humid atmosphere of 5 % CO_2. At 48 h after seeding, BMEC were purified by selectively lysing the contaminating astrocytes and pericytes by antibody/complement treatment. BMEC were incubated with an antibody directed against Thy 1.1 antigen (1:500, Santa Cruz) followed by incubation with rabbit complement serum (1:10, Sigma) for another 2 h (Risau *et al.* 1990).

For the experiments, primary cultured cells were used at confluence at 7 days. Morphological and immunocytochemical analyses of cells monolayers were performed with cells grown to confluency on collagen-coated coverslips and were examined with an Olympus microscope with a phase contrast. Specific marker studies were carried out as previously described. Briefly, cells were grown on collagen-coated glass coverslips, washed with Hanks balanced salt solution (HBSS), fixed in cold acetone-methanol (1:1, vol/vol) for 15 min, washed with phosphate-buffered saline containing 0.1 % bovine serum albumin and 0.01 % Tween, preincubated with 10 % normal goat serum for 15 min, and incubated with the appropriate antibody for 1 h at room temperature. After incubation with the primary antibody, the monolayers were washed with phosphate-buffered saline containing 0.1 % bovine serum albumin and 0.01 % Tween, incubated with peroxidase-labeled secondary antibody for 30 min, and mounted on slides with glycerol. Specimens were viewed in a Nikon microscope. Endothelial cells were identified by using rabbit anti-rat factor VIII related antigen (dilution 1:200). Pericytes were identified by using mouse anti-smooth muscle actin (1:400).

2-DE and analysis

When primary cultures attain confluence, cellular monolayers were washed three times in PBS, harvested and suspended at a concentration of ~10^7 cells/ml in a sample solution containing 6 M urea, 2 M thiourea, 2 % CHAPS, 0.5 % DTT and 2 mM PMSF (all from Sigma), then centrifuged at 18 000 rpm for 15 min at 4 ^oC. The electrophoretic separation of proteins was performed as previously described (Neuhoff *et al.* 1988). Extracts containing 500 μg proteins were first separated by using non-linear gradient IPG-strips, pH 3~10 (Bio-Rad, USA), after which proteins were separated according to their size using 10 % polyacrylamide gels. Protein extracts were included in the in-gel rehydration step, which proceeded over night. The rehydration solution contains: 6 M urea, 2 M thiourea, 2 % CHAPS, 0.5 % DTT and 2 mM PMSF, 0.5 % biolyte (pH 3~10) and a trace of bromophenol blue 0.01 % (w/v). Gels were stained using silver nitrate and colloidal Coomassie Brilliant Blue (CBB) according to previous reference.

CBB-stained gels were scanned using GS-800 Calibrated Densitometer (Bio-Rad) and gel images were analyzed using PDQuest 2D-image-analysis software (Bio-Rad). For a between-gel comparison, a set of spot-generation conditions (weakest spot, smallest spot, size of the largest spot, and a selected region of the background) were used. The total density in a gel image was used to normalize each spot volume in the gel image to minimize the effect of experimental factors on spot volume. Routine statistical analyses available within the software package were used to identify up or down expressed spots. At least three independent sets of cultures of ECs were grown to extract proteins. All samples were run on three replicate gels in the Protein Dodeca Cell (Bio-Rad). Only differences that were found to be present in all of the analyzed gel pairs were considered. Protein spots of interest were subject to MALDI-TOF-MS.

Protein preparation for mass spectrometry

The in-gel digestion was performed as described earlier (Rosenfeld *et al.* 1992). Protein spots were excised from the CBB-stained gel and were cut into small pieces. Pieces of gel were distained twice using 60 μl 200 mmol/l NH_4HCO_3 / ACN (50:50 v/v), shrunk by dehydration in 60 μl ACN twice then completely dried at 37 ºC for about 20 min. The samples were then swollen in a digestion buffer containing 100 mmol/l NH_4HCO_3 and 12.5 ng/μl trypsin (sequencing grade, Roche Diagnostics, USA) at 4 ºC after 30 min incubation, and replaced with 20 μl 20 mmol/l NH_4HCO_3 without trypsin. Samples were incubated more than 12 h at 37 ºC. Peptides were then extracted twice using 5 % FA in 50 % CAN with sonication and concentrated to about 5 μl with protection of N_2.

Peptide mass fingerprint by MALDI-TOF-MS and database searching

In MALDI-TOF-MS analysis, the peptide mixtures from the tryptic digests were desalted and concentrated using 10 ml C18 ZipTipse (Millipore). In brief, samples were eluted in 1 ml of 50 % (v/v)

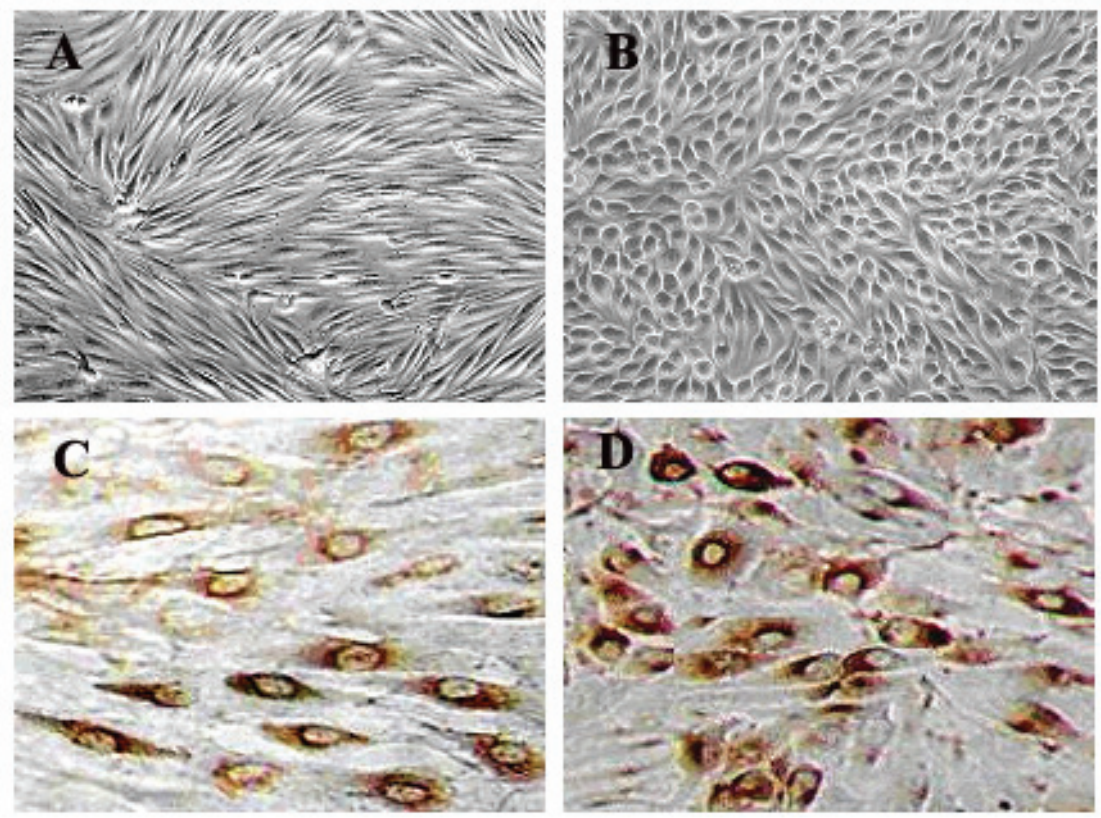

Fig. 1. Cutured rat microvascular EC. **A**, **B**, constant-phase micrograph (×100) of a confluent monolayer of BMEC and CMEC, respectively. **C, D**, positive staining for the factor Ⅷ/von Willebrand factor antigen (×400) specific for BMEC and CMEC, respectively.

acetonitrile/0.1 % trifluoroacetic acid and mixed 1:1 with saturated solution of α-cyano-4-hydroxycinnamic acid in 50 % (v/v) acetonitrile/0.3 % trifluoroacetic acid. Samples were then added into a stainless steel 96×2 target MALDI plate and air-dried before analysis in the mass spectrometer. Mass spectrometry analysis was performed using a PerSeptive Biosystems Voyager DE-PRO equipped with a nitrogen laser (337 nm, 3 ns pulse width, 20.0 Hz REP Rate). Peptide mass fingerprint spectra were acquired in the reflection positive mode with accelerating voltage of 20 KV, grid voltage setting of 72 %, and a 100-ns delay using approximately 150 laser shots and calibrated using trypsin autolysis peaks as internal standards (842.5100, 2211.1046 Da). Collision-activated dissociation (CAD) spectra in micro-HPLC-MS data and Peptide mass fingerprinting (PMF) data from MALDI-TOF-MS were analyzed by searching against an NCBInr database using MASCOT (Matrix Science, London) search software and ProteinProspector software, respectively. The Mammalia sub-database was used and one missing cleavage point of trypsin was added to the database searching.

Results

BMEC and CMEC were grown under identical culture conditions and displayed a typical cobblestone appearance and were free of contamination. The cell purity was assayed by staining with factor VIII /von Willebrand factor antibody and the anti-smooth muscle actin (Fig. 1). Samples that exhibited significant non-EC cells contamination were excluded from further analysis.

Two-dimensional protein maps of BMEC and CMEC

In this work, we established the 2-DE protein patterns of both BMEC and CMEC. More than 2000 protein spots were detected on the 2-DE gels that showed similar protein patterns with silver staining of 100 μg of total proteins in both BMEC and CMEC. Figure 2 displays the overall 2-DE patterns of protein extracts of the two cell lines. Figure 3 showed a representation of the comparison of protein spots between BMEC and CMEC. More than 1200 protein spots had alkaline pIs and 759 spots fell within the acidic region with molecular masses ranging from 17 to 96 kDa. In the assessing of differential expression, we included only those proteins differentially

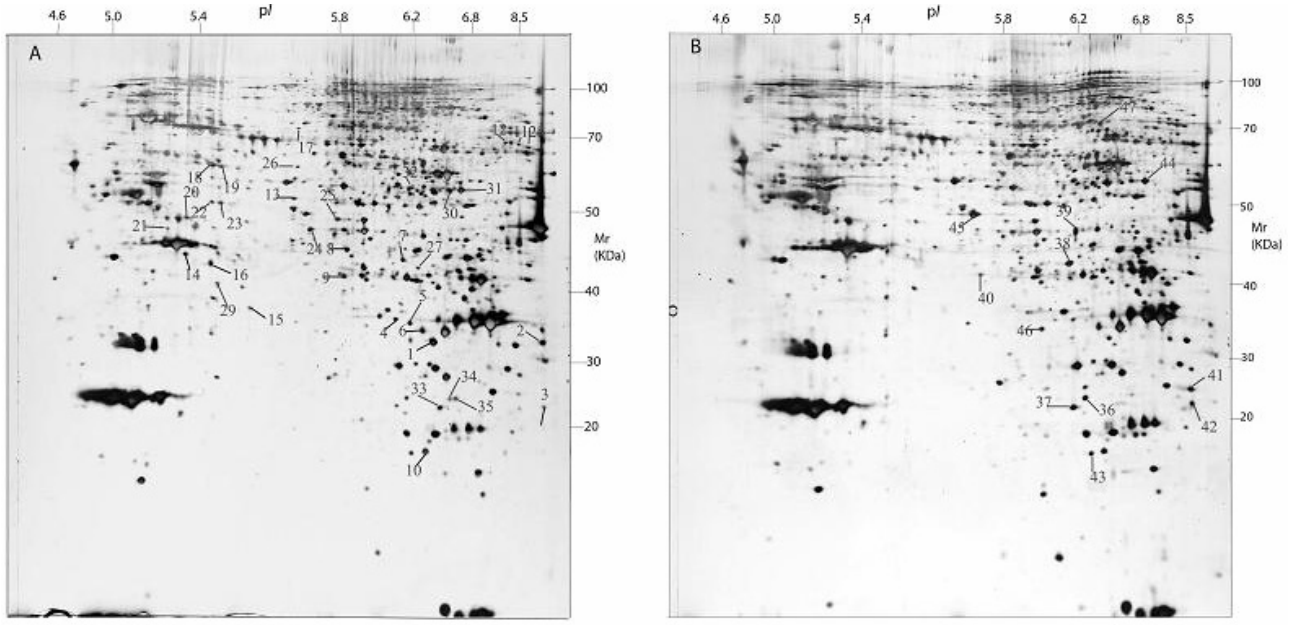

Fig. 2 . The two-dimensional protein maps of the BMEC **(A)** and CMEC **(B)**. The proteins were separated using isoelectric focusing (IPG: 17 cm, non-linearly covering a pH range of 3-10) in the first dimension, and 10% SDS-PAGE gel in the second dimension. Gels were stained by CBB. Spots whose locations are indicated by numbers were identified and are outlined in Tables 1 and 2.

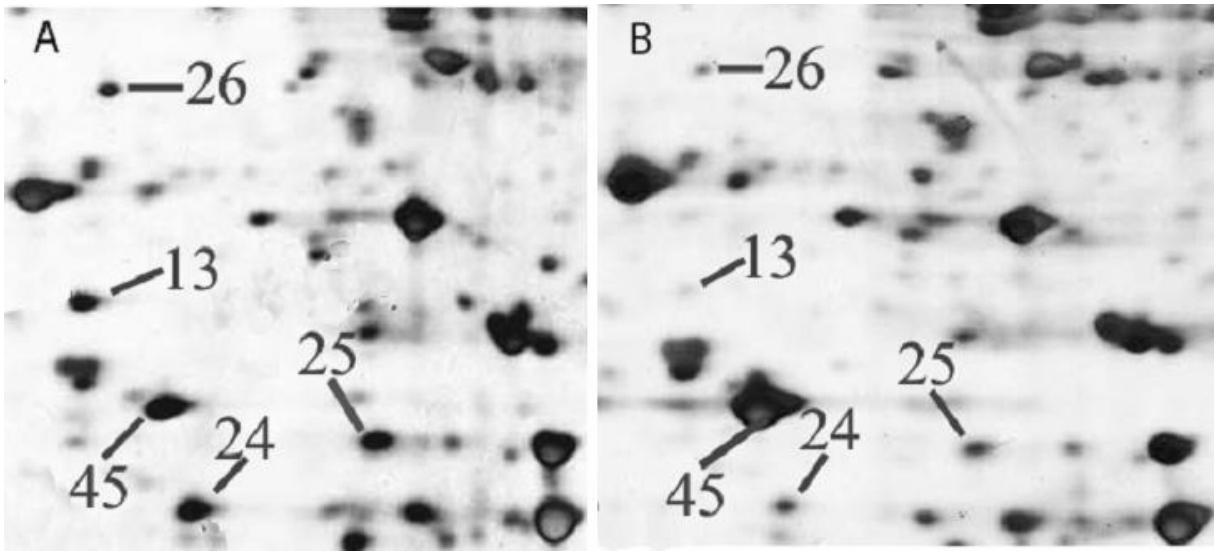

Fig. 3. Close-up of portions of the 2-DE gel images, shown in Fig. 1. The location of spots that significantly differ in BMEC **(A)** and CMEC **(B)** are indicated by numbers.

expressed consistently upon repeated analysis. Processed with PDQuest 2D-image-analysis software, 47 protein spots were differentially expressed between BMEC and CMEC. Among all the detected spots, 35 were highly expressed in BMEC and 12 were highly expressed in CMEC.

Differential protein expression profiles identified by MALDI-TOF-MS and database searching

Keeping the silver-staining gels as a reference, we excised the differentially expressed proteins from CBB stained 2-D gels and digested them with trypsin. Of these markedly changed proteins, 22 were successfully identified after MALDI-TOF-MS peptide mass fingerprinting, while 15 were up-regulated in BMEC and 7 up-regulated in CMEC. Database searches with ProteinProspector software were performed. The results had high confidence if the protein was ranked as the best hit with a significant score. The identified 15 highly-expressed proteins in BMEC are listed in Table 1,

Table 1. Identification of 15 proteins highly expressed by BMEC

No.	Protein name	NCBInr Index code	MW	PI	Score	Spots Intensities	
						BMEC	CMEC
2	VAMP-associated protein of 33 kDa	gi|3320446	27263	8.27	143	1538	709
3	interleukin-6	gi|6841045	23630	9.01	210	659	85
4	similar to glyceraldehyde-3-phosphate dehydrogenase	gi|23617935	31753	6.96	303	1453	156
5	macrophage capping protein	gi|729023	39215	6.73	203	5066	85
10	similar to chloride intracellular channel protein 4	gi|28491107	15989	6.84	243	2543	1297
13	selenium binding protein 2	gi|18848341	52576	5.78	160	2487	67
17	calcium/calmodulin-dependent serine protein kinase membrane-associated guanylate kinase	gi|8101954	99120	6.21	162	1652	163
20	chaperonin subunit 8 (theta)	gi|6753328	59517	5.44	141	677	55
24	caldesmon 1	gi|21704156	60416	6.97	239	2009	341
25	ornithine aminotransferase	gi|8393866	48323	6.19	171	1870	342
27	peptidylprolyl isomerase D (cyclophilin D)	gi|13385854	40716	7.08	236	2366	300
30	acetyl-coenzyme A dehydrogenase, long-chain	gi|20071667	47877	8.53	226	3097	1890
31	similar to hypothetical protein FLJ12618	gi|21703884	50866	8.59	253	2121	340
32	cytosol aminopeptidase	gi|18202835	52716	6.58	321	2433	1000
33	epidermal growth factor	gi|22022644	7514	12.3	133	1980	685

Table 2. Identification of 7 proteins highly expressed by CMEC

No.	Protein name	NCBInr Index code	MW	PI	Score	Spots Intensities	
						BMEC	CMEC
37	guanosine diphosphate (GDP) dissociation inhibitor 3	gi|6679987	31229	6.75	283	2786	609
39	sarcolemma associated protein	gi|6005874	52252	5.13	150	3907	79
40	immune associated nucleotide 1 isoform a	gi|28416440	30558	5.94	327	439	21
42	unknown (protein for IMAGE:5066189)	gi|19353651	19028	8.90	184	1764	99
44	tissue inhibitor of matrix metalloproteinase-2	gi|14091321	11216	6.10	151	5112	69
45	vesicle amine transport protein 1 homolog	gi|23623337	52576	5.95	239	6704	1230
46	PDZ and LIM domain 1 (elfin)	gi|13435939	23630	6.38	215	1553	108

whereas 7 highly-expressed proteins in CMEC are listed in Table 2. In order to assess the functional relevance of changes in the identified proteins, the proteins were classified into five groups according to their primary functions.

The first group consisted of 5 proteins related with cytokine and growth. Three of them, interleukin-6 (IL-6), epidermal growth factor (EGF) and selenium binding protein 2 (SBP2), were highly expressed in BMEC. Two proteins, namely a tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) and immune-associated nucleotide 1 isoform a (IAN-1), appeared to have a higher level in CMEC. The second group comprised collection of proteins involved in stress with one highly-expressed in CMEC and two highly-expressed in BMEC. For example, capping protein (actin filament), gelsolin-like (CapG) were found to be expressed mainly in BMEC, whereas PDZ and LIM domain 1 (elfin) was expressed mainly in CMEC. The third group of proteins included a series of metabolic enzymes such as cytosol

aminopeptidase and others. Another group was involved in signal transduction. Calcium/calmodulin-dependent serine protein kinase membrane-associated guanylate kinase (CASK) in this group had a high level in BMEC. The final group consisted of some hitherto unclassified proteins.

Discussion

This study compared whole-cell proteins from BMEC and CMEC by a large-scale method of proteomics that involved fractionation of proteins by 2D-PAGE, PDQuest image analysis of CBB-stained gels, and identification of target proteins by MS. Expression of 47 polypeptides was consistently altered in the two cell lines. In addition, we identified 22 protein spots and they had multiple functions and were involved in several metabolic pathways.

Although we found the proteomics-based approach to be valuable in generating novel information, 2D-PAGE bears some of limitations in identifying some membrane proteins and other low-abundance proteins. Due to these limitations, it is not difficult to understand that some well-known characteristic proteins in BMEC such as γ-glutamyl transpeptidase (GGTP), alkaline phosphatase and glucose transporter (Glut-1) were not readily detected in our analysis.

The current study demonstrated that BMEC and CMEC were different in several aspects including cytokine and growth-related molecules, stress-related proteins, signal transduction proteins, metabolic enzymes and others.

Cytokine and growth-related molecules

Interleukin-6, highly expressed in BMEC, is a multifunctional cytokine with both protective and destructive actions. Some reports have demonstrated that IL-6 plays positive roles in cerebral ischemia and acts as anti-inflammatory cytokine in endotoxemia and bacterial meningitis by inhibiting leukocyte recruitment in CNS (Loddick *et al.* 1998, Xing *et al.* 1998). Further research of the function of IL-6 in brain will help to understand the pathophysiology of brain inflammatory diseases. Other highly expressed proteins in BMEC were EGF and selenium binding protein 2 (SBP2). SBP2 is believed to play a crucial role in the growth inhibitory and anticarcinogenic effects of selenite by acting as growth regulatory proteins (Chu *et al.* 2004). EGF is a neurotrophic peptide produced in the central nervous system (Peng *et al.* 1998, Kallmann *et al.* 2002). High level of EGF may prevent ischemic and free radical-induced hippocampal damage (Baugnet-Mahieu *et al.* 1990).

There were two high expression proteins in CMEC in this group: TIMP-2 and IAN-1. Our findings were in accordance with the studies of Harkness *et al.* (2000) who found that level of TIMP-2 expression in aortic endothelial cell is significantly greater than in CNS-derived endothelia. This may demonstrate functional differences between various vascular endothelia. The functions of IAN-1 are currently not known and may participate in the control of apoptosis (Poirier *et al.* 1999).

Stress-related proteins

This study demonstrated that two stress-related proteins, CapG and CLIC4, were highly expressed in BMEC. CapG is a member of the gelsolin/villin family of actin-regulatory proteins. Pellieux *et al.* (2003) demonstrated that CapG is a good candidate to participant in the differential EC sensitivity to shear stress and is considered to play critical roles against the formation of atherosclerotic plaques. It is well known that atherosclerosis is less extensive in brain vessels than in extracranial vessels while the mechanisms involved remain to be explored. Although atherosclerosis occurs mainly in larger vessels, microvascular endothelial dysfunction is associated with early atherosclerosis and atherosclerosis may also be a microvascular disease (Zeiher *et al.* 1991, Chen and Henry 1997). Therefore, the present observation that brain endothelium expressed high levels of CapG compared with heart endothelium suggests that brain vessels may be more resistant to atherosclerotic plaques by the anti-shear stress role of CapG. Our study first demonstrated that CLIC4 expressed in endothelial cells, with especially high levels in BMEC. Previous reports have indicated that CLIC4 colocalizes with the tight junction protein ZO-1 in the apical region of polarized epithelial cells, and may play an important role in regulating the blood-brain barrier (BBB) organization (Suh *et al.* 2004). Other findings concluded that CLIC4 interacts with signaling proteins involved in cell membrane remodeling, and participates in the stress-induced apoptotic response in several cellular compartments (Suginta *et al.* 2001).

On the contrary, both BMEC and CMEC expressed elfin, but CMEC appeared to have a higher level of this protein. It has been shown that elfin is a

protein most abundantly expressed in the heart, and is involved in myofibrillogenesis and heart development (Kotaka *et al.* 2001).

Metabolic enzymes

In our study, we found a series of metabolic enzymes highly expressed in BMEC, including cytosol aminopeptidase, acetyl-coenzyme A dehydrogenase, long-chain, ornithine aminotransferase, peptidylprolyl isomerase D (cyclophilin D) and 5'-methylthioadenosine phosphorylase. Cyclophilin D has been proved to play pivotal role in the development of ischemic brain damage (Uchino *et al.* 2003). The presence of cytosol aminopeptidase in BMEC may present an enzymatic barrier to the passage of peptides from the blood into the brain. These highly expressed enzymes meet the high metabolic requirements of the central nervous system tissue (CNS) and maintain the unique characteristics of brain capillaries (Johansson 1990).

Signal transduction proteins

CASK is Ca^{2+}-dependent signaling protein with structural similarity to tight junction (TJ) proteins in zona occludens-1 (ZO-1)/ZO-2, whereas TJ between brain endothelial cells forms the structural basis of the BBB. Moreover, junctional adhesion molecule (JAM) localized in TJ is found to the colocalized with CASK at intercellular contacts along the lateral surface of the plasma membrane in human epithelial cells (Martinez-Estrada *et al.* 2001), suggesting that JAM association with CASK is dynamically regulated during junction assembly when CASK in partially released from its cytoskeletal links. We thus propose that the high expression of CASK in BMEC indicates its active participation in BBB formation.

Others

In this investigation, we encountered some differentially expressed unclassified proteins. Caldesmon 1 (CaD), VAMP-associated protein of 33 kDa (VAP-33) had a higher level in BMEC. The overexpression of CaD in capillary endothelial cells inhibits the focal adhesion and leads to a loss of actin stress fibers (Helfman *et al.* 1999). CaD can also control contractility in endothelial cells and consequently affect adhesion-dependent signaling (Numaguchi *et al.* 2003). It has been shown that VAP-33 and occlusion are co-localized at the tight junction (Lapierre *et al.* 1999), suggesting that VAP-33 and occlusion interact. Though the functional implication of an interaction between VAP-33 and occlusion is not clear, VAP-33 may regulate the induction of occlusion at the tight junction. The role of vesicle amine transport protein 1 in CMEC needs to be further determined.

In conclusion, our findings suggest that BMEC and CMEC are associated with a distinct pattern of protein expression. It has appeared from this study that CLIC4, CASK and VAP-33 were widely associated with BMEC, whereas three proteins were found to be colocalized with some tight junction proteins. We speculated that they act as both structural support and a barrier-forming element and that they cooperate with the tight junctions in maintaining the blood-brain barrier. Our data also suggest that BMEC are actively involved in neuroprotection and metabolism. Some of the proteins identified have multiple functions and are involved in several metabolic pathways. Identification and further functional characterization of proteins specifically expressed by these two ECs will have an important impact on our understanding of endothelial functions of different origin.

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