Proteomic analysis of TNF-α-activated endothelial cells and endothelial microparticles

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Abstract. Endothelial microparticles (EMPs) are small vesicles released from endothelial cells (ECs) and found circulating in the blood. EMPs are formed by a plasma membrane surrounding a small amount of cytosol and contain a subset of cellular proteins. As the number of EMPs in the blood increases with certain diseases, they may be an attractive biomarker for clinical diagnosis. Proteomic analysis of EMPs has been previously performed by mass spectrometry. However, the proteomic information of the ECs that secrete EMPs is lacking. This study introduces an *in vitro* model of activated ECs we created for proteomic analyses and reports the changes of the protein content in the ECs and EMPs using proteomic methods. Thus, this study provides valuable information for the analysis of the highly dynamic secretion process of EMPs. There is a direct correlation between the proteins that form EMPs and tumor necrosis factor-α (TNF-α)-activated ECs. The endothelial proteins transferred by EMPs may play important roles in the interaction between EMPs and the target cells, which may lead to endothelial dysfunction.

Introduction

Sepsis and septic shock are considered the primary causes of mortality in the intensive care unit worldwide (1). It has been widely acknowledged that endothelial cells (ECs) play a key role in the pathogenesis of sepsis (2,3). The vascular endothelium is not only the barrier between blood and tissue, but also takes part in the inflammatory and coagulation responses (4). Its dysfunction or damage is a crucial link during the development of sepsis and may lead to multiorgan failure. Thus, it is important and necessary to discover the pathological mechanism of endothelial dysfunction that results in sepsis.

Endothelial microparticles (EMPs) are small vesicles released from activated, apoptotic or injured ECs ranging in size from less than 1 μm (5). EMPs were first discovered by Hamilton *et al* in 1990 (6). Under normal conditions, there is a low concentration of EMPs in the circulation. Under pathological conditions, particularly in diseases associated with endothelial dysfunction, the level of EMPs is significantly higher (5). Therefore, studying EMPs may be a practicable and effective method of studying ECs.

When ECs are stimulated by proinflammatory, prothrombotic or proapoptotic factors, or are exposed to high shear stress, EMPs will be generated and released. This may also occur in the case of cellular differentiation, senescence or apoptotic cell collapse (7). The phenotype of EMPs is distinct according to the different stimuli. In general, EMPs produced by activation factors include higher levels of endothelial inducible markers, such as CD62E (E-selectin), and EMPs produced by apoptotic factors include higher levels of Annexin V and endothelial constitutive markers, such as CD31 (PECAM) (8). The components of EMPs are also distinct according to the different stimuli. The research of Peterson *et al* (9) indicated that EMPs induced by PAI-1 and TNF-α have overlapping but distinct protein compositions.

Previous studies merely considered EMPs as inert biomarkers indicating endothelial function, but numerous studies have proven that EMPs are the vectors for intercellular information exchange (10,11). EMPs can alter the function of neighboring cells, as well as cells far away from their original cells. Their effects involve many aspects, for example inflammatory and coagulation responses, angiogenesis, cell proliferation and cell migration.

However, the mechanisms by which EMPs are released from ECs and affect the function of downstream cells remain unclear. In order to investigate the possible mechanisms, it is important to determine the protein composition of EMPs. The majority of the prior research focused on 1 or several proteins of the pathway, but these results were not comprehensive. Other studies, such as the study by Peterson *et al* (9), used proteomic analysis and discovered more valuable proteins. It is

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Abbreviations: EMPs, endothelial microparticles; ECs, endothelial cells; HUVECs, human umbilical vein endothelial cells; 2-D, two-dimensional, MS, mass spectrometry; GO, gene ontology

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clear that proteomic analysis is an ideal method to investigate the possible mechanisms of EMPs at present.

However, the existing proteomic reports still have some problems. They compared the contents of EMPs generated by different stimuli and found distinct proteins, but ignored the change of protein components that occurred in the corresponding ECs. This may be an important aspect of revealing the mechanisms associated with EMPs.

In this study, we aimed to stimulate ECs to release EMPs using tumor necrosis factor-α (TNF-α), which is a commonly used proinflammatory substance, so as to produce an *in vitro* model of activated ECs for proteomic research and then comprehensively compare the proteome of unstimulated and TNF-α-stimulated ECs using two-dimensional (2-D) gel electrophoresis followed by mass spectrometry (MS). Simultaneously, we determined the protein composition of EMPs from ECs stimulated by TNF-α using the liquid chromatography-mass spectrometry (LC-MS)/MS method. Finally, using the comparison of proteins as mentioned above, we confirmed that EMPs are capable of transferring biological information, and moreover, gain further insight into the possible functional mechanisms of EMPs generated by TNF-α.

Our results may help to understand the mechanism of endothelial dysfunction and sepsis pathogenesis. Thus we may open up a new field of research for diagnosis and therapy of sepsis.

Materials and methods

HUVEC culture and EMP generation. Primary cultures of human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cord veins using collagenase type I (Sigma, St. Louis, MO, USA) and maintained in medium 200 (Cascade Biologics, Carlsbad, CA, USA) supplemented with low serum growth supplement (LSGS; Cascade Biologics). Cell passages were performed using 0.05% trypsin in 0.02% ethylenediaminetetra-acetic acid. Cells were incubated in a humidified incubator with 5% carbon dioxide at 37˚C. The medium was renewed every 48 h until confluence occurred. The HUVECs used for this research were from the second passage.

HUVECs were equally divided into 1 control group and 9 TNF-α-stimulated groups. The control group was incubated in fresh medium 200 with LSGS. The TNF-α-stimulated groups were incubated in serum-free medium for 2 h and then incubated in fresh medium 200 with LSGS containing 10, 100 or 200 ng/ml TNF-α (PeproTech, Rocky Hill, NJ, USA) for 1, 3 or 24 h, respectively.

Following incubation, the cell-conditioned medium of each group was harvested and centrifuged at 200 x g for 5 min at room temperature to remove cell debris. The supernatant was collected and ultracentrifuged at 100,000 x g for 2 h at 4˚C. The supernatant was discarded. The sediment was washed once with PBS and ultracentrifuged in the same way. The EMP pellet was used for flow cytometry and LC-MS/MS and it was stored at 4˚C for no more than 72 h.

HUVECs from each group were washed 3 times with PBS, digested by trypsin, and then centrifuged at 200 x g for 5 min at room temperature. The supernatant was discarded and cells were collected for 2-D electrophoresis.

EMP detection. Flow cytometry was used for EMP detection. The EMP pellet of each group was resuspended in 200 μl PBS and labeled by CD62E (BD Biosciences, Franklin Lakes, NJ, USA). For counting, a known amount of 1 μm fluorescent latex beads (Sigma) was added to samples as an internal standard. Using these latex beads as gating parameters, EMPs were defined as particles <1 μm in size. EMPs were counted from the gate corresponding to PE-CD62E+ events, so that other small granules in the medium, such as the composition of LSGS, could be excluded. The results are expressed as the number of EMPs/1x10^6 cells.

Statistical analysis was performed using ANOVA. $P<0.05$ was considered to indicate a statistically significant difference.

According to the EMP detection results, the group that generated the most EMPs among the 9 TNF-α groups was determined. The EMPs of this group were used for LC-MS/MS. HUVECs of this group and the control group were used for 2-D electrophoresis.

HUVEC 2-D electrophoresis. Two groups of HUVECs were respectively suspended in a solution containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% w/v DTT (all from Sigma), 2% IPG buffer (3-10), 40 mM Tris-base (both from Bio-Rad, Hercules, CA, USA) and protease inhibitor cocktail EDTA-free (Pierce, Rockford, IL, USA). The suspension was sonicated 5 times for 10 sec at 4˚C. The samples were centrifuged at 16,000 x g for 30 min to pellet cell debris. The concentration of sample proteins were detected by RCDC Protein Assay (Bio-Rad). IPG strips (Bio-Rad) used for isoelectric focusing (IEF) were 17 cm with pH 3 to 10. In total, 100 μg of protein from each sample was added into rehydration solution. IPG strips were put into individual grooves in the reswelling tray. IEF was carried out using a horizontal electrophoresis apparatus (Bio-Rad). After the rehydration at 50 V for 12 h at 20˚C, focusing was started at 250 V and the voltage was progressively increased to 10,000 V until a maximum of 88,700 Vh. When IEF was finished, strips were equilibrated twice for 15 min in equilibration buffer containing 6 M urea, 30% v/v glycerol (Sigma), 2% w/v SDS (Bio-Rad) and 1.5 M Tris-HCl buffer (pH 8.8) supplemented with 5 mg/ml DTT for the first treatment and 45 mg/ml iodoacetamide (Sigma) for the second treatment. Polyacrylamide gels (12.5%) were used for SDS polyacrylamide gel electrophoresis. The running conditions were 16 mA/gel for 30 min followed by 24 mA/gel for 6 h at 20˚C. The gels were stained by silver, according to the method of Westermeier *et al* (12). 2-D electrophoresis was repeated 3 times. Stained gels were scanned and the images were analyzed by PDQuest Image Analysis software (Bio-Rad) in order to find the differentially expressed protein spots and ratio level of the TNF-α stimulated group to the control group.

HUVEC matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF)/TOF-MS. Protein spots were excised from the preparative gels, and destained with 100 mM NH_4HCO_3 in 30% ACN. After removing the destaining buffer, the gel pieces were lyophilized and rehydrated in 30 μl of 50 mM NH_4HCO_3 containing 50 ng trypsin (Promega, Madison, WI, USA). Following overnight digestion at 37˚C, the peptides were extracted 3 times with 0.1% TFA in 60% ACN. Extracts were pooled together and lyophilized. The resulting lyophi-

lized tryptic peptides were maintained at -80˚C until mass spectrometric analysis. A protein-free gel piece was treated as above and used as a control to identify autoproteolysis products derived from trypsin.

MS and MS/MS spectra were obtained using the ABI 4800 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, Carlsbad, CA, USA) operating in a result-dependent acquisition mode. Peptide mass maps were acquired in positive ion reflector mode (20 kV accelerating voltage) with 1000 laser shots per spectrum. Monoisotopic peak masses were automatically determined within the mass range 800-4000 Da with a signal-to-noise ratio minimum set to 10 and a local noise window width of 250 m/z. Up to 5 of the most intense ions with minimum signal-to-noise ratio of 50 were selected as precursors for MS/MS acquisition, excluding common trypsin autolysis peaks and matrix ion signals. In MS/MS-positive ion mode, spectra were averaged, collision energy was 2 kV and default calibration was set. Monoisotopic peak masses were automatically determined with a signal-to-noise ratio minimum set to 5 and a local noise window width of 250 m/z. The MS together with MS/MS spectra were searched against the UniprotKB/Swiss-Prot database using the software GPS Explorer (Applied Biosystems) and MASCOT (Matrix Science, Boston, MA, USA) with the following parameter settings: trypsin cleavage, one missed cleavage allowed, carbamidomethylation set as fixed modification, oxidation of methionines allowed as variable modification, peptide mass tolerance set to 100 ppm, fragment tolerance set to ±0.3 Da and minimum ion score confidence interval for MS/MS data set to 95%.

EMP LC-MS/MS. EMPs were disrupted by lysis buffer with protease inhibitor cocktail EDTA-free (Pierce). The suspension was sonicated 5 times for 10 sec at 4˚C. The sample was then centrifuged at 16,000 x g for 30 min to pellet the insoluble protein fraction. The supernatant containing soluble proteins was used for LC-MS/MS.

Ettan™ MDLC system (GE Healthcare, Piscataway, NJ, USA) was applied for desalting and separation of tryptic peptide mixtures. In this system, samples were desalted on RP trap columns (Zorbax 300SB-C18, Agilent Technologies, Santa Clara, CA, USA), and then separated on a RP column (150 μm i.d., 100 mm length, Column Technologies Inc., Downers Grove, IL, USA). The mobile phase A (0.1% formic acid in HPLC-grade water) and mobile phase B (0.1% formic acid in acetonitrile) were selected. A total of 20 μg of tryptic peptide mixtures of EMPs were loaded onto the columns, and separation was performed at a flow rate of 2 μl/min by using a linear gradient of 4-50% B for 120 min. A Finnigan™ LTQ™ linear ion trap MS (Thermo Electron, Waltham, MA, USA) equipped with an electrospray interface was connected to the LC setup for detection of eluted peptides. Data-dependent MS/MS spectra were obtained simultaneously. Each scan cycle consisted of 1 full MS scan in profile mode followed by 5 MS/MS scans in centroid mode with the following Dynamic Exclusion™ settings: repeat count 2, repeat duration 30 sec, exclusion duration 90 sec. Each sample was analyzed in triplicate.

The MS/MS spectra were automatically searched against the non-redundant International Protein Index (IPI) human protein database (version 3.26, 67687 entries) using the BioworksBrowser (Thermo Electron). Protein identification results were extracted from the SEQUEST files with BuildSummary (13).

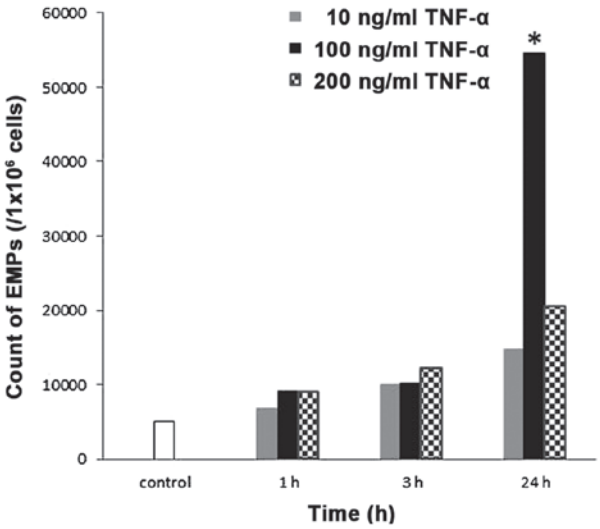

Figure 1. TNF-α dose and time-dependency of EMP generation. *The group treated with 100 ng/ml TNF-α for 24 h had the most EMPs. Significant differences (P<0.05, ANOVA): 10 ng/ml TNF-α 1 vs. 3 h, 1 vs. 24 h, 3 vs. 24 h; 100 ng/ml TNF-α 1 vs. 24 h, 3 vs. 24 h; 200 ng/ml TNF-α 1 vs. 3 h, 1 vs. 24 h, 3 vs. 24 h; 1 h 10 ng/ml TNF-α vs. 200 ng/ml TNF-α; 3 h 10 ng/ml TNF-α vs. 200 ng/ml TNF-α, 100 ng/ml TNF-α vs. 200 ng/ml TNF-α; 24 h 10 ng/ml TNF-α vs. 100 ng/ml TNF-α, 100 ng/ml TNF-α vs. 200 ng/ml TNF-α. TNF-α, tumor necrosis factor-α; EMP, endothelial microparticles.

The peptides were constrained to be tryptic and up to 2 missed cleavages were allowed. Carbamidomethylation of cysteines was treated as a fixed modification, whereas oxidation of methionine residues was considered as a variable modification. The mass tolerance allowed for the precursor ions was 2.0 Da and fragment ions was 0.2 Da, respectively. The protein identification criteria were based on Delta CN (≥0.1) and cross-correlation scores (Xcorr, 1 charge ≥1.9, 2 charges ≥2.2, 3 charges ≥3.75).

Protein analysis. All accession IDs of the proteins identified in HUVECs via MALDI-TOF/TOF-MS and in EMPs via LC-MS/MS were unified to Swiss-Prot IDs at http://www.uniprot.org/ and the protein names were also discovered. The endothelial differentially expressed proteins were compared with the proteome of EMPs in order to determine any existing correlations. The Swiss-Prot IDs of the proteins we were interested in were used to annotate these proteins with their corresponding Gene Ontology (GO) annotations including the cellular components, molecular functions and biological processes involved. In addition, KEGG pathway annotations of these proteins were also found at http://www.genome.jp/kegg/.

Results

TNF-α dose and time-dependency of EMP generation. All 9 TNF-α groups generated more EMPs than the control group, which generated 5123 EMPs/1x10^6 cells. Fig. 1 indicates clearly that the group treated with 100 ng/ml TNF-α for 24 h had the most EMPs (54588 EMPs/1x10^6 cells). Differences

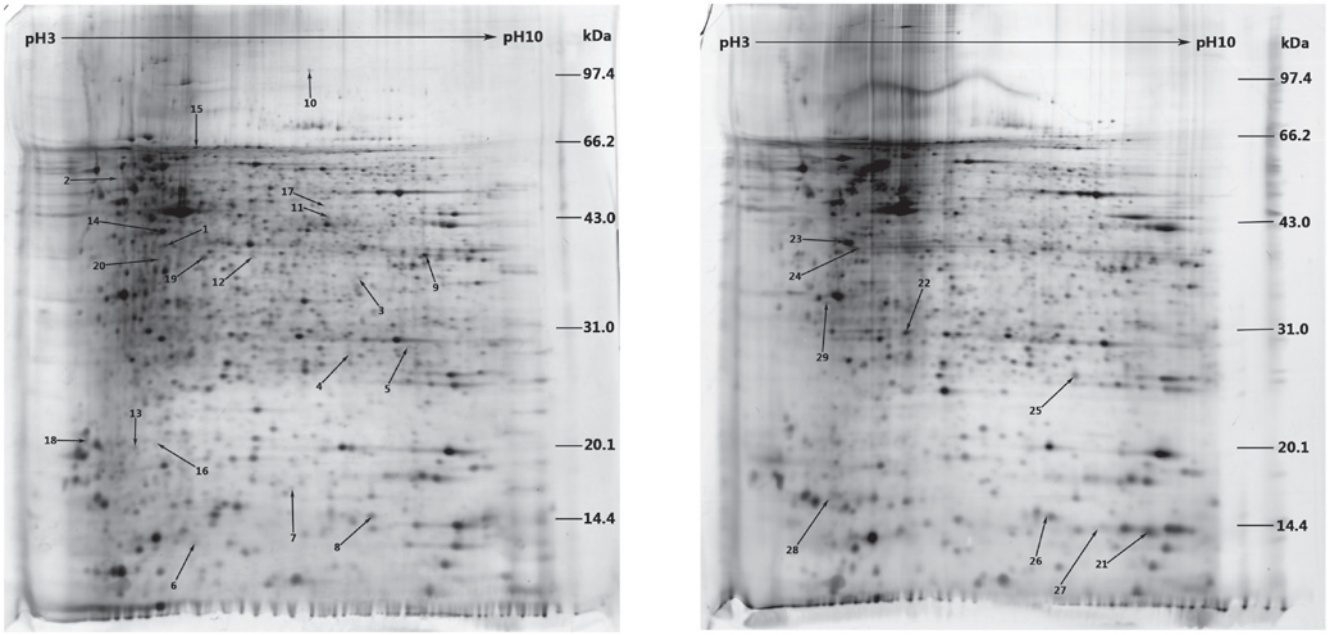

Figure 2. 2-D patterns of HUVECs. Left panel, unstimulated HUVECs; right panel, TNF-α-stimulated HUVECs. IPG strip of pH 3 to 10 for isoelectric focusing (IEF) in combination with SDS-PAGE (12.5%) and stained by silver. The spots of 29 differentially expressed proteins are indicated with black arrows and spot numbers. Left panel, nos. 1-8 represent the no-longer expressed proteins and nos. 9-20 represent the proteins that are downregulated. Right panel, no. 21 represents the freshly generated protein and nos. 22-29 represent the proteins that are upregulated. The name and information for each protein is shown in Table I according to its corresponding spot number. HUVECs, human umbilical vein endothelial cells.

among 9 groups under different TNF-α doses and time conditions were statistically analyzed (Fig. 1).

2-D patterns of unstimulated HUVECs and TNF-α-stimulated HUVECs. 2-D gel electrophoresis was used to respectively separate all proteins from unstimulated HUVECs and TNF-α-stimulated HUVECs and repeated 3 times. Fig. 2 shows these 2 patterns of silver stained 2-D polyacrylamide gels based on protein molecular weight (MW) and isoelectric point (pI), each of which contained 100 μg of proteins. pH ranged from 3 to 10 and MW ranged from 14.4 to 97.4 kDa. The patterns were generally consistent with the pattern reported previously (14).

The protein spots were analyzed by PDQuest Image Analysis software in order to detect the differentially expressed proteins. We found 47 differentially expressed proteins. Each protein level ratio of TNF-α-stimulated HUVECs to unstimulated HUVECs was calculated. Among these 47 proteins, the expression of 11 proteins was upregulated, 21 proteins were downregulated, 13 proteins were no longer expressed, and 2 proteins were freshly generated after the stimulation.

Identification of differentially expressed proteins in HUVECs by MALDI-TOF/TOF-MS. In total, 29 of these 47 differentially expressed proteins were successfully identified through MALDI-TOF/TOF-MS and every protein score C.I.% was higher than 95%. Among these 29 proteins, the expression of 8 proteins was upregulated, 12 proteins were downregulated, 8 proteins were no longer expressed, and 1 protein was freshly generated (Fig. 2). Protein name, protein level ratio and the other information are shown in Table I. The highest ratio of upregulation was approximately 3.5-fold and the highest ratio of downregulation was approximately 3.18-fold.

Proteome identification of EMPs generated from TNF-α-stimulated HUVECs by LC-MS/MS and protein analysis. A total of 83 proteins were identified in EMPs generated from TNF-α-stimulated HUVECs. Comparing these proteins with 29 differentially expressed proteins as noted previously, we observed 8 common proteins within EMPs, of which 1 protein was no longer expressed, the expression of 4 proteins was downregulated, and the expression of 3 proteins was upregulated in TNF-α-stimulated HUVECs. GO annotation and KEGG pathway analysis data of these 8 proteins is shown in Table II.

Discussion

In EMP research, TNF-α is widely used to activate ECs to produce EMPs and mimic acute inflammation. However, there are scarce details about the TNF-α dose and time-dependency of the EMP generation. Our results showed that TNF-α enhanced EMP release compared with unstimulated HUVECs. When treated with 100 ng/ml TNF-α for 24 h, HUVECs generated the most EMPs. According to these findings, the *in vitro* model of activated ECs could be used in order to generate enough EMPs for proteomic research.

Our study indicates that a direct correlation exists between the proteins comprising EMPs and the proteins expressed by ECs induced by TNF-α. We confirmed that endothelial proteins of various pathways will change when TNF-α, as an important proinflammatory factor during sepsis, stimulates HUVECs. These changes take place not only in quantity but also in type of proteins. According to our results, there were 29 differentially expressed EC proteins activated by TNF-α compared to the unstimulated ECs, 8 of which were upregulated, 12 of which were downregulated, 8 of which were no longer expressed, and 1 of which was freshly generated.

Table I. Differentially expressed proteins between unstimulated HUVECs and TNF-α-stimulated HUVECs identified by MALDI-TOF/TOF-MS.

Spot no.	Protein name	Swiss-Prot	Predicted MW (Da)	Predicted pI	Protein score	Protein score CI (%)	Ratio[a]
1	Far upstream element-binding protein 1	Q96AE4	67689.5	7.18	309	100	
2	Vimentin[b,c]	P08670	53738.1	5.03	69	98.646	
3	Four and a half LIM domains protein 2	Q14192	34166.4	7.80	76	99.717	
4	LDLR chaperone MESD	Q14696	26231.4	7.60	402	100	
5	3-Hydroxyacyl-CoA dehydrogenase type-2	Q99714	20738.8	7.93	92	99.992	
6	Galectin-1	P09382	14868.3	5.34	171	100	
7	Superoxide dismutase [Cu-Zn]	P00441	16023.0	5.70	72	99.221	
8	40S ribosomal protein S12	P25398	14904.6	6.81	200	100	
9	Annexin A2[b,c]	P07355	38779.9	7.57	423	100	-3.17897
10	Vinculin	P18206	124292.0	5.50	719	100	-2.31222
11	Septin-2	Q15019	47068.8	6.95	380	100	-2.10470
12	Four and a half LIM domains protein 3	Q13643	33210.3	5.67	191	100	-1.98458
13	Protein canopy homolog 2	Q9Y2B0	20981.3	4.81	246	100	-1.94463
14	Heterogeneous nuclear ribonucleoproteins C1/C2	P07910	32003.7	5.10	176	100	-1.87902
15	Heat shock cognate 71 kDa protein	P11142	71082.3	5.37	77	99.759	-1.80698
16	T-complex protein 1 subunit β	P78371	22924.2	5.88	146	100	-1.80438
17	Actin, cytoplasmic 1[b]	P60709	40536.2	5.55	65	96.271	-1.67332
18	Protein disulfide-isomerase[b,c]	P07237	57479.8	4.76	141	100	-1.59406
19	Tubulin β chain[b,c]	P07437	48135.1	4.70	411	100	-1.59367
20	Elongation factor 1-δ	P29692	31216.8	4.90	445	100	-1.55537
21	Profilin-1	P07737	15084.6	8.48	221	100	
22	Heat shock protein β-1[b]	P04792	22825.5	5.98	371	100	+3.50088
23	Nucleophosmin[b]	P06748	31090.3	4.71	633	100	+2.09525
24	Cathepsin Z	Q9UBR2	27758.9	5.48	168	100	+1.96644
25	β-hexosaminidase subunit β	P07686	6829.4	6.81	94	99.996	+1.86827
26	Ran-specific GTPase-activating protein	P43487	23395.6	5.19	89	99.986	+1.84756
27	Putative nucleoside diphosphate kinase	O60361	15690.0	8.76	359	100	+1.81783
28	Myosin light polypeptide 6[b,c]	P60660	17090.2	4.56	276	100	+1.61712
29	Clathrin light chain B	P09497	23281.2	4.63	244	100	+1.52998

[a]Protein level ratio of TNF-α-stimulated HUVECs to unstimulated HUVECs. [b]Proteins also included in EMPs. [c]Proteins also reported in the study by Sander *et al* (21). Nos. 1-8, proteins are no longer expressed; nos. 9-20, protein expression is downregulated; no. 21, protein is freshly generated; and nos. 22-29, protein expression is upregulated. +, Upregulation; -, downregulation; EMPs, endothelial microparticles; HUVECs, human umbilical vein endothelial cells.

The probable causes of the modification of proteins are as follows. The upregulation and new generation occur due to the augmentation of protein synthesis or the conversion from other proteins. The downregulation and the lack of expression occur due to the decrease of protein synthesis, transforming to other proteins, protein degradation or the release out of the cells, which may include the direct release to the extracellular space and the release via EMPs. Further study revealed that EMPs contained 8 of the 29 proteins, of which the expression of 3 were upregulated, 4 were downregulated, and 1 was no longer expressed. We consider that the shedding of EMPs is an important cause of endothelial protein modification. It should be noted that the shedding of EMPs did not only occur during protein downregulation and lack of expression, but also occurred during upregulation, as we found 3 upregulated proteins in EMPs. The fact that the quantity of these proteins still increases is perhaps due to the protein synthesis exceeding the release, or the release having a negative feedback effect, which makes the synthesis augment.

Due to this correlation, we proved that EMPs are not only markers reflecting the condition of ECs but also vectors exchanging intercellular information. The cellular component of these 8 common proteins tells us that EMPs could carry proteins from any component out of the ECs (Table II). Among these 8 proteins, there were 2 upregulated proteins associated with anti-apoptosis [heat shock protein β-1 (HspB1) and nucleophosmin] and 3 proteins associated with cytoskeletal structure (vimentin, actin cytoplasmic 1 and tubulin β chain). TNF-α is

Table II. GO and KEGG pathway analysis of common proteins between the proteome of EMPs and differential proteins of HUVECs.

	Gene Ontology			
Protein name	Cellular component	Molecular function	Biological process	KEGG pathway
Vimentin	Cytosol Intermediate filament	Protein C-terminus binding Structural constituent of cytoskeleton	Cellular component disassembly involved in apoptosis	
Annexin A2	Soluble fraction	Calcium ion binding Calcium-dependent phospholipid binding Cytoskeletal protein binding Phospholipase inhibitor activity	Cellular component movement Positive regulation of vesicle fusion	
Actin, cytoplasmic 1	Nucleus Cytoskeleton Cytosol Ribonucleoprotein complex	ATP binding Kinesin binding Nitric-oxide synthase binding Structural constituent of cytoskeleton	'*De novo*' posttranslational protein folding Adherens junction organization Blood coagulation Cell junction assembly Cellular component movement	Phagosome Focal adhesion Adherens junction Tight junction Leukocyte transendothelial migration Regulation of actin cytoskeleton *Vibrio cholerae* infection Pathogenic *Escherichia coli* infection Shigellosis
Protein disulfide-isomerase	ER-Golgi intermediate compartment Cell surface Endoplasmic reticulum lumen Extracellular region Plasma membrane	Procollagen-proline 4-dioxygenase activity Protein binding Protein disulfide isomerase activity	Cell redox homeostasis Lipid metabolic process Lipoprotein metabolic process Peptidyl-proline hydroxylation to 4-hydroxy-L-proline	Protein processing in endoplasmic reticulum
Tubulin β chain	Cytosol Microtubule	GTP binding GTPase activity MHC class I protein binding	G2/M transition of mitotic cell cycle Cellular component movement Microtubule-based movement Natural killer cell mediated cytotoxicity Protein polymerization	Phagosome Gap junction Pathogenic *Escherichia coli* infection
Heat shock protein β-1	Cell surface Cytosol Nucleus Spindle	Identical protein binding	Anti-apoptosis Cell death Cellular component movement mRNA metabolic process Regulation of translational initiation Response to heat Response to unfolded protein Response to virus	MAPK signaling pathway VEGF signaling pathway Amoebiasis

Table II. Continued.

Protein name	Gene ontology			KEGG pathway
	Cellular component	Molecular function	Biological process	
Nucleophosmin	Spindle pole centrosome	NF-κB binding Tat protein binding Histone binding Protein binding Protein heterodimerization activity Protein homodimerization activity Ribosomal large subunit binding Ribosomal small subunit binding Transcription coactivator activity Unfolded protein binding	CenH3-containing nucleosome assembly at centromere DNA repair Anti-apoptosis Centrosome cycle Intracellular protein transport Negative regulation of cell proliferation Negative regulation of centrosome duplication Positive regulation of NF-κB transcription factor activity Protein oligomerization Regulation of endodeoxyribonuclease activity Regulation of endoribonuclease activity Ribosome assembly Signal transduction	
Myosin light polypeptide 6	Cytosol Unconventional myosin complex	Actin-dependent ATPase activity Calcium ion binding Motor activity	Muscle filament sliding	Vascular smooth muscle contraction

EMPs, endothelial microparticles; HUVECs, human umbilical vein endothelial cells.

capable of causing endothelial apoptosis by activating ECs (15). Therefore the generation of anti-apoptotic proteins rises to a higher level. The knowledge of EMP production and release comes from research on platelet microparticles (16). Briefly, the mechanism concerns the alteration of the cytoskeleton. Thus the related endothelial proteins change. The proteome of EMPs may provide a detailed statement about the situation of the original ECs, such as what stimulated the cells and how the cells responded. However, EMPs as vesicles transferring biological signals and information should contain proteins playing a role in essential biological processes. Among these 8 proteins, for example, there were proteins (actin cytoplasmic 1 and tubulin β chain) involved in cell adhesion, cell junction or leukocyte transendothelial migration, which are key proteins in the processes of endothelial high permeability and capillary leak.

Furthermore, when these 8 proteins were investigated, we found that both the protein with the highest increase in expression and that with the lowest among the 29 differentially expressed endothelial proteins examined, were within the EMPs. This does not mean that the greatest change is the most important change, but it is evident that these 2 proteins, HspB1 and annexin A2, are significant proteins.

HspB1 was 3.50-fold more abundant in the TNF-α-induced ECs than in resting ECs. According to GO analysis, HspB1 is involved in anti-apoptosis, cellular component movement, mRNA metabolic processes, regulation of translational initiation and other biological processes. A recent report clarified that ECs with upregulated HspB1 could survive apoptosis (17). Another report revealed that HspB1 could have a protective effect on the cytoskeleton and preserve EC integrity, which could protect against acute kidney injury after hepatic ischemia and reperfusion (18). Moreover, KEGG pathway analysis showed that HspB1 is associated with the MAPK and VEGF signaling pathways. Thus, once activated by a proinflammatory factor such as TNF-α, ECs will upregulate HspB1 to protect themselves. This condition could be detected through EMPs and EMPs may carry HspB1 to affect other cells.

Annexin A2 was 3.18-fold less abundant in TNF-α-induced ECs than in resting ECs. The endothelial-related information of GO analysis shows that annexin A2 positively regulates the vesicle fusion. We know that ECs liberate EMPs so they need to downregulate proteins leading to vesicle fusion, such as annexin A2. Reflecting this condition, EMPs with annexin A2 tend to fuse with other cells and then play biological roles. In addition, it was confirmed that annexin A2 is capable of assembling plasminogen and has a positive effect on vascular fibrinolysis (19,20).

We consider that perhaps the proteins within EMPs shedding from ECs have 2 different effects. Firstly, they are useful to regulate the function of other cells, no matter whether the cells are neighboring or remote, or whether the cells are the same type or a different type, for example HspB1. Secondly, they are harmful to the original ECs, so they are released to protect the cells themselves. However, these proteins, for example annexin A2, may have a positive effect on downstream cells. Therefore, EMPs are not inert cellular debris, but vital vectors.

Compared with the previous proteomic reports of EMPs, 5 of these 8 proteins included in the TNF-α-derived EMPs in our study were the same as the proteins in the plasminogen activator inhibitor type 1 (PAI-1)-derived EMPs in the study by Sander *et al* (21) (indicated in Table I). However, all of these 8 proteins in our study were not found in proteins identified as unique to control EMPs, PAI-1 EMPs, or TNF-α EMPs in the study by Peterson *et al* (9). This is an interesting finding. The various proteomic methods used in these 3 studies is a possible cause for these differences since Sander *et al* used 2-D/MALDI-TOF/TOF-MS, Peterson *et al* used LC-MS/MS, and in our study, we used both 2-D/MALDI-TOF/TOF-MS and LC-MS/MS. 2-D/MALDI-TOF/TOF-MS is the classical method for finding differentially expressed proteins and LC-MS/MS is suitable for detecting substances with lower quantities of proteins, so we used the former to find differentially expressed proteins of HUVECs and the latter to detect the proteome of EMPs. Another possible cause is that the previous studies used commercially available HUVECs and our study used cells from fresh human umbilical cord veins. The HUVECs we used are more in accord with the actual situation found in the human body. Putting aside the factors of sensitivity and heterogeneity, it appears that there are proteins in all types of EMPs and these common proteins could account for the mechanisms of formation and function of EMPs.

It should be noted that the 8 no-longer expressed and 1 freshly generated endothelial proteins we found could be the consequence of the relatively low sensitivity of the 2-D electrophoresis method. It means that these 9 proteins do exist in ECs but they could not be detected in the 2-D gels. In other words, the no-longer expressed proteins could be downregulated proteins and the freshly generated protein could be an upregulated one. Even so, the conclusion of our study is meaningful as the changes of these endothelial proteins did occur. In addition, the number of proteins found within EMPs was less than that reported in previous proteomic research. This is probably due to fewer EMPs released by HUVECs from the fresh human umbilical cord veins we used than by commercially available HUVECs. However, if we consider the proteome of EMPs as a whole, this study of the proteins with relatively great changes will clearly indicate the important effect of EMPs.

In conclusion, we created an *in vitro* model of activated ECs for proteomic research with cultured HUVECs treated with 100 ng/ml TNF-α for 24 h. We found the differentially expressed proteins between the control ECs and the ECs stimulated by TNF-α and confirmed that EMPs could carry endothelial proteins out of the cells. GO and KEGG pathway analysis indicated that the common proteins included in EMPs have effects on vital biological processes, which may lead to endothelial dysfunction. Furthermore, we concluded that EMPs play roles not only in protecting their original ECs but also in affecting other cells. In ongoing studies in our laboratory, we are focusing on each important protein of ECs or EMPs according to this study to make clear the whole pathway through which the protein alters downstream cell function. We believe that EMPs could be diagnostic biomarkers and therapeutic targets in diseases associated with endothelial dysfunction, particularly in sepsis.

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