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Comparative Proteomic Analysis of Rodent Plasma and Mesenteric Lymph

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Abstract

The lymph has long been considered as the plasma filtrate and the proteomes of the lymph have received scanty attention. Currently, mesenteric lymph is reported to play an important role in the pathogenesis of multiple organ dysfunction syndrome in some critical illnesses. A better understanding of the composition and proteomes of mesenteric lymph becomes imperative to disclose the mechanistic role of mesenteric lymph. Seven male Sprague-Dawley rats were fasted overnight, and anesthetized to collect plasma and mesenteric lymph. The specimens were subjected to proteomic analysis using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). An average of 434 and 412 protein spots were found in the gels of the plasma and mesenteric lymph respectively. Peptide mass fingerprint analysis identified 77 proteins for 212 protein spots. The 2-DE proteomic pattern of mesenteric lymph was largely similar to that of the plasma. As in the plasma, large protein spots of albumin dominated the protein pattern in mesenteric lymph. Other major proteins identified in 2-DE gels included immunoglobulin heavy and light chains, fibrinogen α-, β- and γ-chains, serotransferrin, protease inhibitors, kininogens, macroglobulins, haptoglobin, and apolipoproteins. Meanwhile, mesenteric lymph contained an array of proteins that differentiated it from the plasma. The most differentially expressed proteins in mesenteric lymph were γ-fibrinogen, protease inhibitors, and proteins related to lipid transport/metabolism. The study presents a detailed description of mesenteric lymph proteomes of a common experimental animal in physiological status using a common proteomic approach. These results provide the basis for future research.

Key Words: comparative proteomics, plasma, mesenteric lymph, two-dimensional gel electrophoresis (2-DE)

Introduction

Changes in cellular physiology lead to alterations in cellular metabolism and shedding of metabolites into the circulation, either as waste or as signals to other cells (1). For years, the analysis of plasma content has been used as a tool for disease detection and monitoring. However, some of the shed proteins and peptides are not abundant and/or are rapidly excreted, making timely and accurate traditional biochemical analysis impossible. Using the combined methodologies of biochemistry, molecular biology, and biotechnology to produce global protein expression profiles of cells, tissues, and body fluids, proteomic analysis has, over the past decade, emerged as a tool for disease detection and monitoring (10).

Lymph is usually referred to as the simple plasma filtrate and considered a necessary means of recycling excessive interstitial fluid (2, 20). Other than maintaining fluid homeostasis, mesenteric lymph also contains chylomicrons, shedding metabolites of intestinal and lymphatic cells, and an array of proteins

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and cytokines secreted by intestinal and lymphatic endothelium (5, 19). However, unlike blood plasma, the content and properties of mesenteric lymph have received scanty attention partly due to their difficult accessibility.

Recently, evidence has accumulated suggesting the important role mesenteric lymph plays in the pathophysiology of some critical illnesses (3, 4, 7, 12, 14, 21). Studies have shown that the mesenteric lymphatic route is the primary conduit for gut-derived toxic factors leading to adult respiratory distress syndrome and multiple organ failure after hemorrhagic shock, acute pancreatitis, and burn injury (3, 4, 7, 12, 14, 21). However, unlike as is the case for other body fluids, there is very little research in the literature about the proteomes of the lymph (6, 11, 15, 16, 18). Only 2 previous studies have described proteomic analysis of the lymph in physiological status (11, 15). Leak *et al*. first used a two-dimensional gel electrophoresis (2-DE) approach on the thoracic duct lymph of sheep and identified 18 proteins (11). Recently, Mittal *et al.* reported a comprehensive description of normal rodent mesenteric lymph proteomes in the fasted and fed states using the simultaneous sample measurement method of isobaric tags (iTRAQ) together with liquid chromatography tandem mass spectrometry (LC-MS/MS) identification of the component proteins (15).

This study is designed to apply the proteomic analytic method to identify and compare the proteomes of the plasma and mesenteric lymph of a commonly used experimental animal under fasted physiological condition.

Materials and Methods

Experimental Animals

Animal experiments used 7 adult male Sprague-Dawley (SD) rats, weighing 350-400 *g*, after a minimum 7-day acclimation period. All animals were housed in a central animal care facility and received chow and water ad libitum. Approval was obtained from the Institutional Animal Care and Use Committee prior to the study. All animals received humane care in compliance with the principles of laboratory animal care and use.

Experimental Model

The animals were anesthetized by intraperitoneal injection with 70-80 mg/kg of ketamine and 5-7 mg/kg of xylazine. After shaving the chest and abdomen hair, the internal jugular vein was cannulated with a silicone tube, and the femoral artery was cannulated with a polyethylene-50 tube. Midline laparotomy was then performed using aseptic technique. The main mesenteric lymphatic duct was identified and cannulated with a silicone tube (0.51/0.94 mm; HelixMark, Carpinteria, CA, USA) exiting the right flank. The abdomen was then closed. The animal was kept warm with a heat blanket and/or lamp throughout the study. Blood pressure was continuously monitored (PowerLab; ADInstruments Inc, Colorado Springs, CO, USA) from the femoral artery to ensure a physiological status throughout the study.

Collection of Mesenteric Lymph

Mesenteric lymph samples were collected by direct drainage into a sterile siliconized Eppendorf tube on ice for 1 h, and the volume was measured. Maintenance normal saline was infused at 2 ml/h through the jugular vein. Before sacrificing the animal, 3 ml of blood were drawn through the jugular vein. The blood and lymph samples were centrifuged at 12,000 rpm for 15 min at 4°C, aliquoted, and immediately frozen at -80°C before testing.

Proteomic Analysis

Proteomic analysis was carried out at the Clinical Proteomics Center of Chang Gung Memorial Hospital. All experimental procedures followed the standardized protocols established in the Core Laboratory. Protein concentrations of the plasma and mesenteric lymph samples were measured using the Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA).

Two-Dimensional Gel Electrophoresis

The isoelectric focusing (IEF) was performed using Protean IEF Cell (BioRad, Hercules, CA, USA). Ready-made IPG strips (170 mm Immobiline DryStrips, Bio-Rad) were used for the first dimensional electrophoresis according to the manufacturer's instructions. Each plasma and lymph sample was solubilized for IEF in 7 M urea, 2 M thiourea, 4% w/v CHAPS, 0.001% w/v bromophenol blue (0.1%), 0.2% w/v Biolyte 3/10 (20%), and 50 mM dithiothreitol (DTT). Previous studies revealed that the optimal protein amount for mesenteric lymph samples was 600 μg for the 17 cm-IPG strip. This amount provided for optimal protein spot detection in SYPRO Ruby-stained gels without producing excessive spot overlays and the blurring effects caused by overloading of samples. Each plasma and lymph sample containing 600 μg protein was applied onto the IPG gel by in-gel rehydration using a reswelling tray, and the first dimensional IEF was conducted at 60,000 Vh at 20°C. After IEF, the IPG strip was equilibrated twice for 20 min in equilibration buffer containing 6M urea, 20%

v/v glycerol, 2% w/v sodium dodecyl sulfate (SDS) and 1.5 M Tris-HCl buffer (pH 8.8) supplemented with 2.0% w/v DTT for the first treatment and 2.5% w/v iodoacetamide (IAA) for the second treatment. After rinsing with 1 × running buffer, the IPG gel was transferred to a 12.5% polyacrylamide gel, and the second dimensional SDS-polyacrylamide gel electrophoresis (PAGE) was performed. Running conditions for horizontal SDS-PAGE were 10 mA for 30 min, followed by 45 mA for 5 h. Protein Mr and PI were assigned after calibrating 2-D gels with broad range molecular weight standard proteins and 2-D SDS-PAGE standard proteins (Bio-Rad). The 2-D SDS-PAGE was developed until the bromophenol blue dye marker reached the bottom of the gel.

Gel Staining and Imaging

The gels were stained with SYPRO Ruby (Invitrogen- Molecular Probes, Eugene, OR, USA). The gels were initially fixed in buffer containing 10% methanol and 7% acetic acid for 1 h and then stained for 6 h in a commercially available SYPRO Ruby buffer. The gels were then washed with 10% methanol in 7% acetic acid for 2 h and soaked in ddH2O overnight. Finally, the dried, stained gels were scanned with a high-resolution scanner, the ProXPRESS 2D Proteomic Imaging System (Perkin-Elmer, Waltham, MA, USA), and image analyses were performed using the Progenesis software package (Progenesis Workstation v2005; Nonlinear Dynamics, Newcastle-upon-Tyne, Tyne and Wear, UK).

In-Gel Digestion of Proteins

All the protein spots of each gel were labeled and manually excised from SYPRO Ruby-stained gels, washed, and in-gel digested with trypsin following the protocol set by the Core Laboratory. Briefly, the gel slices were cut into small pieces, destained in buffer containing 50% acetonitrile (ACN) with 25 mM ammonium bicarbonate (pH 8.5), and then washed with deionized water. The gel pieces were dehydrated in 100% ACN for 5 min, dried in a SpeedVac evaporator for 5 min, and then rehydrated in 2 µl of 5 ng/µl trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate (pH 8.5). After removing the unabsorbed solution, the gel pieces were incubated in 10-20 µl of 25 mM ammonium bicarbonate (pH 8.5) for 14-16 h at 37°C. The solution containing digested protein fragments was transferred to a new tube, and the peptide fragments remaining in the gel were extracted in 5% TFA/50% ACN for 20 min at room temperature. Crude digest mixtures were concentrated and re-dissolved in 10 µl of 50% ACN with 0.1% TFA. One µl of peptide mixture was mixed with 1 µl of matrix, and then 0.5 µl of the resulting mixture was spotted onto the AnchorChip target (Bruker Daltonics GmbH, Bremen, Germany) and allowed to air dry for approximately 5 min at room temperature.

Peptide Mass Fingerprint by MALDI-TOF MS

Tryptic peptide mass fingerprint (PMF) analyses were performed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS analysis on an Ultraflex(tm) MALDI-TOF/TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Searches were performed without constraint by protein molecular weight or isoelectric point, and the parameters allowed for carbamidomethylation of cysteine, partial oxidation of methionine residues, and 1 missed trypsin cleavage. All spectra underwent an internal two-point calibration using two peptides derived from autodigested trypsin (m/z 842.51 and 2211.10 Da). Mass lists were then used to search the NCBI database using the MASCOT software from Matrix Science (www.matrixscience.com). Proteins with at least 5 matching peptides, and mass measurement errors of less than 100 ppm were considered a good match. Comparison of the theoretical and observed values of Mr and pI indicated that most proteins were identified at their expected position on the gels. Some different spots close to each other were identified as being different isoforms of the same protein. The MALDI-TOF MS resolution for the peptides was around 20000, and the mass accuracy was 0.01-0.02 Da. The MS/MS resolution was approximately 6000.

Statistics

The p2rogenesis software package was used to identify significantly different spots by comparing the plasma and mesenteric lymph gels of the same animal. The levels of expression of specific proteins measured by abundance were obtained. Protein spots that had 1.5 folds or more differences in abundance were considered significant. Data were analyzed by paired Student's *t*-tests using the SPSS statistical software (SPSS 13.1 for Windows; SPSS Inc, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

Results

Global View of Proteomes of the Plasma and Mesenteric Lymph

At first, 2-DE using the broad-range IPG pH 3-10 nonlinear gradient gel strip for the IEF step was

carried out. This broad-range pH gave an opportunity to obtain a global view of the constituent proteins contained in mesenteric lymph (Fig. 1A). However, limited protein spot resolution was found in the broad-range IPG pH 3-10 gels (mean, 211 protein spots). To increase protein spot resolution and the detection of single spots, a pH range of 4-7 was obtained. This pH range enhanced protein spot separation (Fig. 1B), making it easier to identify and excise individual protein spots for later PMF analysis. By employing these procedures, an average of 434 and 412 protein spots were resolved in the SYPRO Ruby-stained 2-DE gels of the plasma and mesenteric lymph respectively ($P < 0.001$ compared to those of paired pH 3-10 gels). As shown in Fig. 1, multiple protein spots appeared in single rows. Some rows presented as apparent "clusters" or "charge trains" of spots, each of which was a peptide mixture similar to those of other spots within the group (*e.g.*, rows 1-12). Some rows (*e.g.*, row 19 and spots 17, 18) revealed the presence of several different proteins with the same molecular weight but a slightly different pI.

Identification of Protein Spots in 2-DE Gels of Mesenteric Lymph

All identifiable protein spots were excised from SYPRO Ruby-stained 2-DE gels of the plasma and mesenteric lymph, using IPG gel strips of pH 3-10 and 4-7. Protein identification by PMF for 77 proteins and 212 protein spots was carried out, and the results are shown in Table 1. As in the plasma, large protein spots of albumin dominated the protein pattern in mesenteric lymph (Fig. 1B, row 22). Other major proteins identified in pH 3-10 2-DE gels included £\2-macroglobulin (Fig. 1A, row 1); fibrinogen α-, β-, and γ-chains (Fig. 1A, rows 4 and 9-10, separately); Immunoglobulin G (Ig G) heavy chains (Fig. 1A, rows 11 and 12); and serotransferrin precursor (Fig. 1A, row 7). Other major proteins identified in pH 4-7 2-DE gels of mesenteric lymph included hemopexin (Fig. 1B, row 16); kininogens (Fig. 1B, rows 20 and 21); protease inhibitors (Fig.1B, rows 23-24, and 27-29); fibrinogen γ-chain (Fig. 1B, row 30); α1-macroglobulin (Fig. 1B, row 41); haptoglobin (Fig. 1B, row 45); apolipoproteins (Apo) A-IV, A-I, and E (Fig. 1B, rows 33, 44, 46-47, and 57); and Ig light chain (Fig. 1B, row 52).

Comparative Proteomic Analysis of the Plasma and Mesenteric Lymph

The 2-DE gels of the plasma (Fig. 2) demonstrated a similar protein profile to those shown in the gels for mesenteric lymph, which were taken and processed in parallel. To compare between the plasma and mesenteric lymph gels, the volume (density × area) of each spot was calculated and the background was subtracted. The relative volume of each spot was normalized by dividing the zßlume of each spot by the sum volume of all spots present in the gel, and was used for comparison. The protein spots exhibiting a more than 1.5-fold difference in abundance were regarded as having a real variation. At a 1.5 × change threshold, more than 100 protein spots revealed either increased or decreased abundance in the plasma in comparison to mesenteric lymph (Fig. 2).

Among these differentially expressed protein spots, 15 distinct proteins were identified. These included 7 proteins that increased, and 8 proteins that decreased, in abundance in mesenteric lymph compared to those in the plasma. Table 2 shows the details of protein identification and the results of protein quantification. Apo E, γ-fibrinogen, glutathione peroxidase 3, α1-macroglobulin precursors, afamin, and serotransferrin were less abundant in mesenteric lymph. Apo A-IV, 2 protease inhibitors, cytoplasmic-1 and 2 actin (β and γ), major urinary protein (MUP) precursors, and complement C3 precursors were more abundant in mesenteric lymph. Fig. 3 shows the focal 2-DE gel images of 7 proteins that were consistently different in abundance in the samples (γ-fibrinogen, Apo E, glutathione peroxidase 3, α1-macroglobulin, Apo A-IV, cytoplasmic-1 and 2 actin, and nucleolar transcription factor 1; $P < 0.05$).

Discussion

Rodents, including mice and rats, are widely used in research as the animal model evaluation for human disease. A better understanding of the physiological condition of these animals helps us to more accurately interpret the changes observed in these animal experiments. This study provides the first detailed description of the mesenteric lymph proteomes of SD rats in fasted physiological status using 2-DE and MALDI-TOF MS analysis. Previously published attempts at proteomic analysis of lymph used a 2-DE approach on thoracic duct lymph in the sheep and identified only 18 proteins (11). In this study, 77 proteins for 212 protein spots were identified. The results of this study provided valuable information about rodent mesenteric lymph proteomes for use in future studies.

The lymph is traditionally regarded as the filtrate of the plasma. It has long been accepted that the plasma and mesenteric lymph should have the most content in common. A great similarity in the pattern of 2-DE gels between the two is not surprising. However, the proteomic content of mesenteric lymph comes from not only filtered plasma, but also the

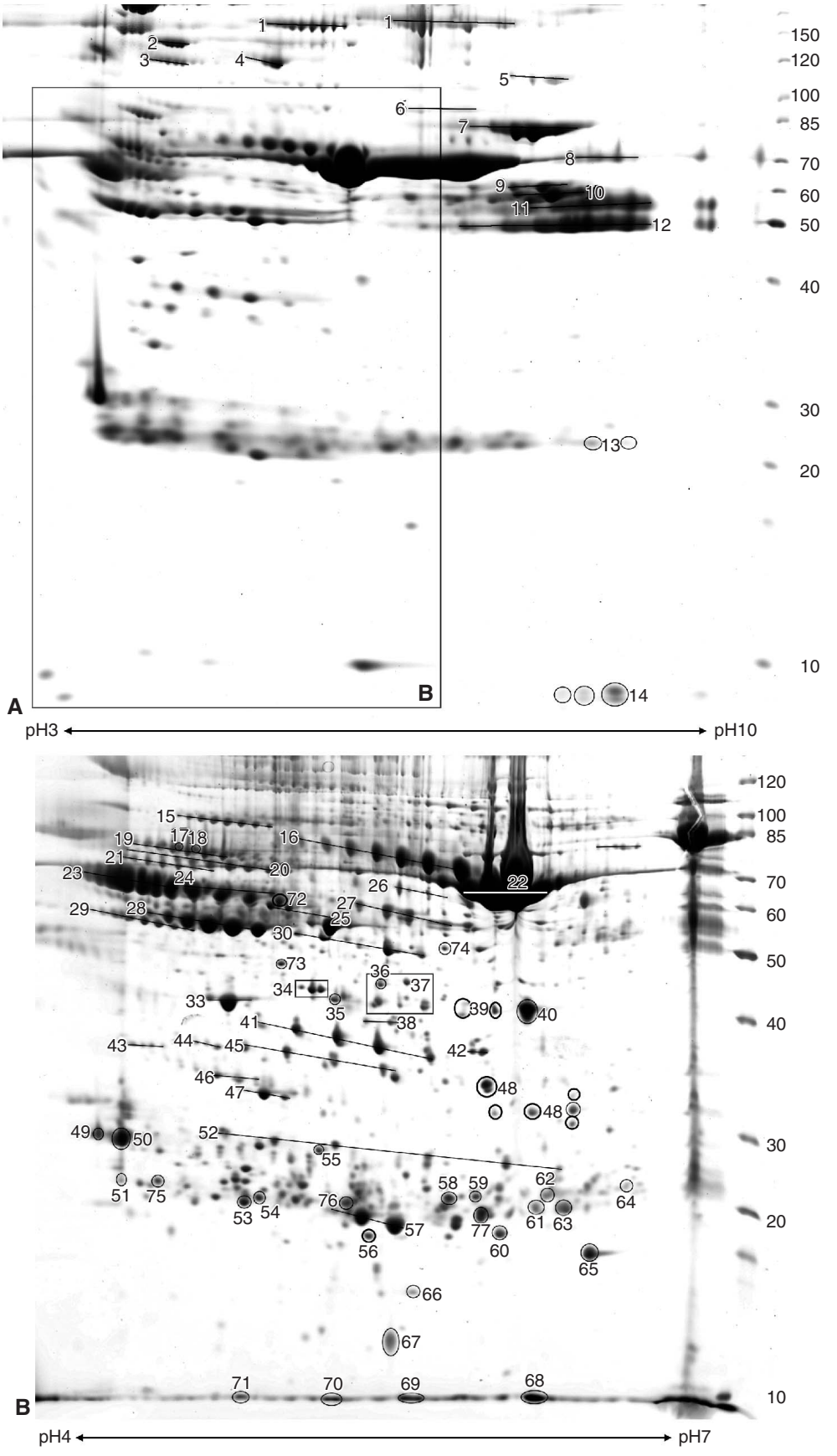

Fig. 1. Images of pH 3-10 and pH 4-7 two-dimensional electrophoresis gels of mesenteric lymph.

Table 1. Identification of protein spots in the mesenteric lymph under physiologic status with MALDI-TOF MS

Spot No.	Accession No.	Protein Name	MASCOT Score	Category/Molecular Function
Identified Proteins				
1	P06238	α-2 macroglobulin	145	Protease inhibitor
2	O35802	Inter-α-inhibitor H4 heavy chain	215	Protease inhibitor
3	P01048	T-kininogen 1 precursor	86	Protease inhibitor; Vasodilator
4	P08932	T-kininogen 2 precursor	107	Protease inhibitor; Vasodilator
5	P05545	Contrapsin-like protease inhibitor 1 or 3	219	Protease inhibitor
6	P24090	α-2-HS-glycoprotein	51	Protease inhibitor
7	Q9QX79	Fetuin-B precursor	129	Protease inhibitor
8	P17475	α-1 antiproteinase (antitrypsin)	110	Protease inhibitor
9	P09006	Contrapsin-like protease inhibitor 6	227	Protease inhibitor
10	Q63041	α-1 macroglobulin precursor	88	Protease inhibitor
11	P12346	Serotransferrin precursor	102	Carrier protein
12	P36953	Afamin	103	Carrier protein
13	P02768	Albumin	186	Carrier protein
14	P04276	Vit. D binding protein	114	Carrier protein
15	P02770	Serum albumin precursor	186	Carrier protein
16	P02770	Serum albumin precursor	186	Carrier protein
17	P02767	Transthyretin precursor	74	Carrier protein
18	P02770	Serum albumin precursor	84	Carrier protein
19	P23680	Serum amyloid P-component	54	Carrier protein
20	P02770	Serum albumin precursor	84	Carrier protein
21	O35141	Ras asso. domain family 1 isoform	60	Carrier protein
22	P04916	Plasma retinol-binding protein (PRBP)	136	Carrier protein
23	P02767	Transthyretin precursor	74	Carrier protein
24	P02625	Parvalbumin alph	105	Carrier protein
25	P09216	Protein kinase C epsilon type	45	Carrier protein
26	P01024	C3 α chain	184	Innate immunity
27	P01026	Complement C3	82	Innate immunity
28	Q62930	Complement component C9 precursor	80	Innate immunity
29	P01026	Complement C3 precursor	74	Innate immunity
30	P01026	Complement C3 precursor	104	Innate immunity
31	P020760	Immunoglobulin α 2A	177	Immunoglobulin
32	P020761	Immunoglobulin α 2B	164	Immunoglobulin
33	P20767	Immunoglobulin λ-2 chain C region	65	Immunoglobulin
34	P01835	Immunoglobulin κ chain C region	51	Immunoglobulin
35	P48199	CRP precursor	100	Immune related
36	P02680	Fibrinogen γ chain	162	Coagulation/Clotting
37	Q01177	Plasminogen	105	Coagulation/Fibrinolysis
38	P06399	Fibrinogen α chain	129	Coagulation/Clotting
39	P14480	Fibrinogen β chain	154	Coagulation/Clotting
40	P02680	Fibrinogen γ chain	101	Coagulation/Clotting
41	P02680	Fibrinogen γ chain	101	Coagulation/Clotting
42	P06399	Fibrinogen α-chain precursor	73	Coagulation/Clotting
43	P01946	Hemoglobin α chain	125	Oxygen transport
44	P20059	Hemopexin precursor	141	Oxygen transport
45	P02651	Apolipoprotein A-IV	254	Lipid transport/Metabolism
46	P04639	Apolipoprotein A-I	170	Lipid transport/Metabolism
47	P02650	Apolipoprotein E precursor	98	Lipid transport/Metabolism
48	P02650	Apolipoprotein E precursor	110	Lipid transport/Metabolism
49	P02650	Apolipoprotein E precursor	123	Lipid transport/Metabolism
50	P04639	Apolipoprotein A-I	170	Lipid transport/Metabolism
51	Q6IG01	Keratin, Type II cytoskeletal Ib	53	Cytoskeleton
52	P60711	Actin-β, γ	185	Cytoskeleton
53	Q63608	Tropomyosin I, α isoforms	64	Cytoskeleton

Table 1 (Coutinued)

Spot No.	Accession No.	Protein Name	MASCOT Score	Category/Molecular Function
Identified Proteins				
54	P23764	Glutathione peroxidase 3	109	Antioxidant
55	P14141	Carbonic anhydrase	209	Antioxidant
56	P13635	Ceruloplasmin	178	Antioxidant; Ion transport
57	P23764	Glutathione peroxidase 3	109	Antioxidant
58	P23764	Glutathione peroxidase 3	109	Antioxidant
59	B0K008	rCG33041	61	Signal molecule
60	P0C8E4	Mitogen-activated protein kinase	52	Signal molecule
61	Q6PDQ2	Chromodomain helicase DNA 4 binding protein	69	Signal molecule
62	Q8VII0	Ferm domain-containing protein 650	Binding	
63	P26644	β2 glycoprotein 1	55	Heparin binding
64	P07335	Creatine kinase B-type	156	ATP binding
65	P06866	Haptoglobin precursor	80	Hemoglobin binding
66	Q66HC0	EF-hand calcium-binding domain-containing protein	57	Calcium ion binding
67	P84083	ADP-ribosylation factor 5	60	GTP binding protein
68-69	P02761	Major urinary protein precursor	109	Pheromone binding
70	Q3KR56	GRAM domain containing 1A	63	Unclear
71	P25977	Nucleolar transcription factor 1	60	Transcription regulation
72a	P31936	59 kDa BSP	51	Biomineralization; Cell adhesion
				Uncertain Proteins
72	O35814	Hsc70/Hsp90 organizing protein	49	Binding protein
73	Q07116	Sulfite oxidase, mitochondrial precursor	42	Heme and metal binding
74	P83006	Platelet activating factor acetylhydrolase	42	Anti-apoptosis; Lipid metabolism
75	Q4V8A8	Zinc finger protein 90	44	Transcription regulator
77	Q63548	Semaphorin 3A	47	Differentiation; Neurogenesis

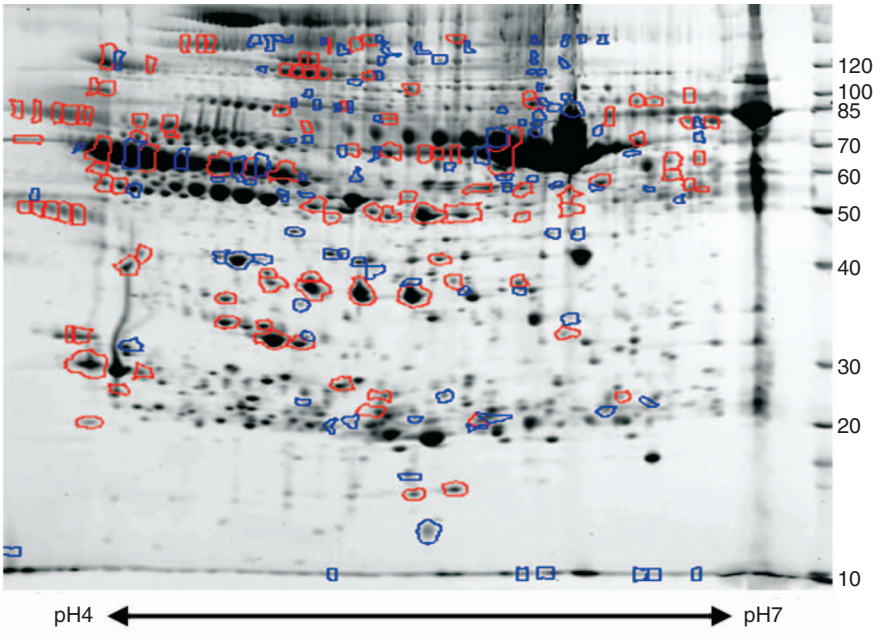

Fig. 2. Images of pH 4-7 two-dimensional electrophoresis gel of the plasma. Relative protein abundance was compared to paired gel of mesenteric lymph. Areas where a given protein with a 1.5-fold increase in relative abundance were circled in red color, and areas where a given protein with a 1.5-fold decrease in relative abundance were circled in blue color.

Table 2. Identification and quantification of protein spots that are differentially expressed in the plasma and mesenteric lymph

Accession No.	Protein Name	Category/Molecule Functions	Peak Volume Ratio
Decreased abundance in mesenteric lymph			
P02680	Fibrinogen γ-chain precursor (5/5)[#]	Coagulation/Clotting	2.21 ± 1.29[@]
P02650	Apolipoprotein E precursor (5/5)[#]	Lipid transport/Metabolism	2.09 ± 0.81[@]
P23764	Glutathione peroxidase 3 (5/5)[#]	Antioxidant	2.38 ± 0.75[@]
Q63041	α-1 macroglobulin precursor (5/5)[#]	Protease inhibitor	1.91 ± 1.01[@]
P36953	Afamin (3/5)[#]	Carrier proteins	1.67 ± 0.77
Q9QX79	Fetuin B (2/5)[#]	Protease inhibitor	1.58 ± 0.65
P12346	Serotransferrin (2/5)[#]	Carrier proteins	1.59 ± 0.92
Increased abundance in mesenteric lymph			
P02651	Apolipoprotein A-IV (5/5)[#]	Lipid transport/Metabolism	2.29 ± 1.02[@]
P60711	Actin-β, γ(5/5)[#]	Cytoskeleton	2.17 ± 1.06[@]
P25977	Nucleolar transcription factor 1 (5/5)[#]	Transcription regulation	2.35 ± 0.56[@]
P02770	Serum albumin precursor (4/5)[#]	Carrier proteins	1.84 ± 0.64[@]
P02761	Major urinary protein precursor (4/5)[#]	Pheromone binding	1.76 ± 0.51[@]
P01026	Complement C3 precursor (3/5)[#]	Immune related	1.64 ± 0.056
P05545	Contrapsin-like protease inhibitor (2/5)[#]	Protease inhibitor	1.61 ± 0.68
P24090	Fetuin A or 59 kDa BSP (2/5)[#]	Protease inhibitor	1.57 ± 0.58

[#]Data in parentheses represent number of rats showing a more than 1.5-fold change in abundance of the protein spots.
[@]With a P value < 0.05.

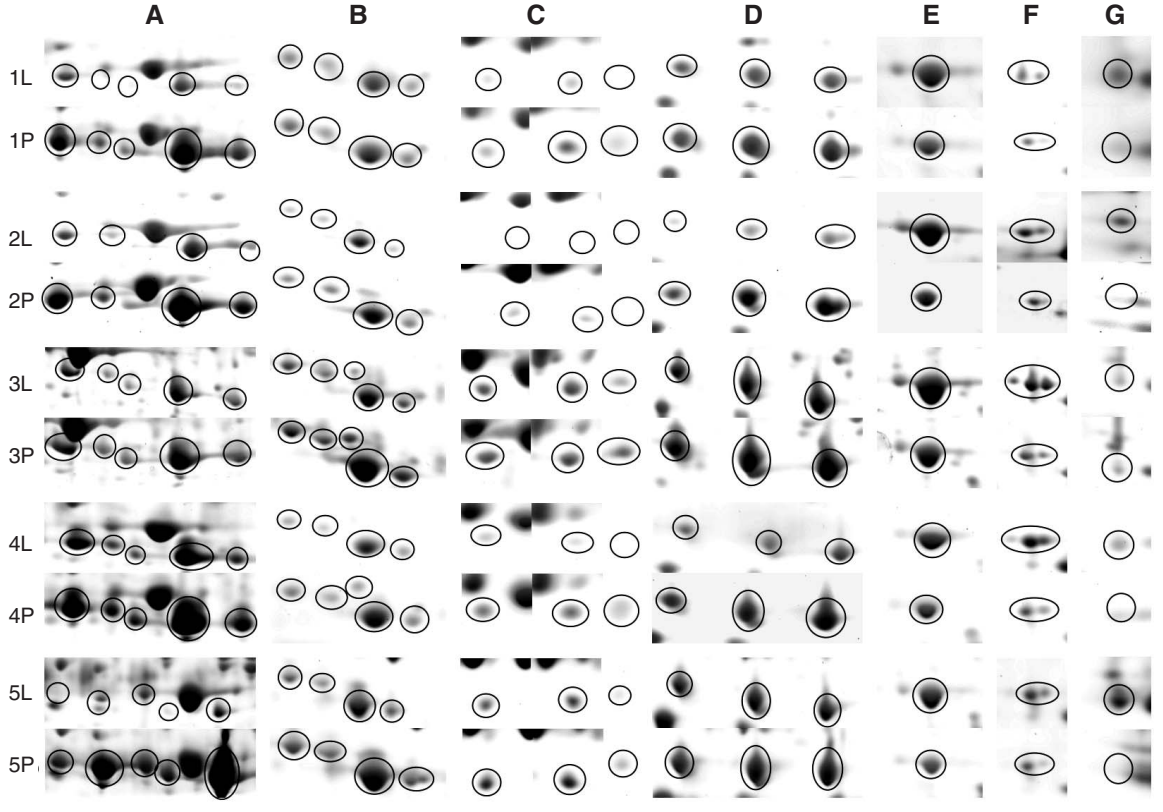

Fig. 3. The focal paired two-dimensional electrophoresis gel images demonstrate the differential expression of proteins among mesenteric lymph (upper) and the plasma (lower) of 5 rats under fasted status. (A: γ-fibrinogen; B: apolipoprotein E; C: glutathione peroxidase 3; D: α-1 macroglobulin; E: apolipoprotein A-IV; F: two isoforms of actin; G: nucleolar transcription factor 1).

secretions of intestinal and lymphatic cells. After exhausting spot-to-spot comparisons, we found that the proteins that were more abundant in the plasma were those with a larger molecular weight (*e.g.*, Afamin and serotransferrin), those related to coagulation functions (*e.g.*, γ-fibrinogen), and those related to inter- or intra-cellular activity (*e.g.*, glutathione peroxidase 3). In contrast, proteins more abundant in mesenteric lymph were those with a smaller molecular weight (*e.g.*, major urinary protein) and those related to lipid transport/metabolism (*e.g.*, apolipoproteins). This specific compositional profile demonstrates that mesenteric lymph has a unique protein profile and thus is more than a simple filtrate of the plasma.

The reasons for the differential expression of certain proteins in mesenteric lymph to those in the plasma are not clear. Coagulation is an important function of the plasma. Fibrinogen binds, through its gamma chain, to cell surface receptors, growth factors, and coagulation factors (8). It is vital to maintain a stable coagulation protein concentration in the plasma to assure this function. In contrast, mesenteric lymph does not have to carry out this function. This may explain why γ-fibrinogen is less abundant in mesenteric lymph than in the plasma.

Apolipoprotein E has long been thought to be made exclusively in the liver of humans and experimental animals. However, studies showed that numerous peripheral tissues synthesize Apo E and secrete it into the circulation (9). Measurements of Apo E synthesis indicate that Apo E is a moderately abundant product of many peripheral tissues. The relative rates of Apo E synthesis in the testis and adrenal gland are approximately the same as in the liver, synthesis rates in the spleen and lung are somewhat lower, and in the mesenteric lymph node and kidney are considerably lower (9). Apo E is reportedly involved in the transport of cholesterol from peripheral tissues via interstitial fluid. Apo E was elevated in concentration in peripheral lymph from dogs fed with a high cholesterol diet (22). In the current study, mesenteric lymph was collected after overnight fasting; this may also have contributed to the low abundance of Apo E in the mesenteric lymph.

Protease inhibitors are reported to be one of the most abundant protein classes identified in mesenteric lymph (15). In the current study, various protease inhibitor spots showed different abundances in mesenteric lymph when compared to those of the plasma. The only protease inhibitor that was consistently less abundant in mesenteric lymph than in the plasma was α1-macroglobulin. It is a broad-spectrum proteinase inhibitor that inactivates all known classes of proteinase. Rat plasma contains 2 α-macroglobulins (13). Normal adult rat serum was reported to have a high α1-macroglobulin concentration (23), which may explain the differential expression of this protein between the plasma and mesenteric lymph.

Cells continuously produce free radicals and reactive oxygen species (ROS) as a part of their normal metabolic process. In general, the body has adequate antioxidant reserves to cope with this oxidative species under physiological conditions. Glutathione peroxidase 3 serves as an antioxidant defense system to preserve homeostasis in normal cell functions. Glutathione peroxidase 3 plays several protective roles-simultaneously acting as an antioxidant enzyme, which scavenges ROS, and as a repair enzyme, which eliminates damage. In the current study, several spots identified as glutathione peroxidase 3 were less abundant in mesenteric lymph.

Mesenteric lymph is rich in fat and is considered to play an important role in lipid transport/metabolism. A previous study showed that there is a circadian rhythm in the serum and lymph Apo A-IV (24). In 24-h fasted rats, the serum Apo A-IV concentration maintains a circadian rhythm, and is high in dark conditions. With mesenteric lymph diversion, serum Apo A-IV concentration diminishes and the circadian rhythm is abolished. This may explain the finding in the current study that Apo A-IV is more abundant in mesenteric lymph than in the plasma. Actin typically constitutes the framework of cytoskeletal machinery. Surgical manipulation of the mesenteric lymphatic duct for the harvest of mesenteric lymph may cause some tissue injury, which leads to selective intestinal actin cytoskeleton disruption-resulting in increased abundance of actin in the mesenteric lymph.

In this study, all identifiable protein spots were excised from the 2-DE gels and subjected to tryptic digestion and analysis. However, a large number of the protein spots could not be identified. Two-DE fights with extremes of isoelectric point, and proteins of high hydrophobicity. It is also difficult to provide conclusive data with 2-DE for the changes in specific protein levels, since numerous proteins may be represented in the same spots or overlap with other spots, and single proteins may be distributed across several gel spots. There are also other limitations to this approach: [1] 2-DE gels is not very compatible for hydrophobic proteins, [2] 2-DE gel cannot provide equivalent separation resolution for proteins with high and low molecular weights while using fixed percentage polyacrylamide gel, [3] the successful rate of protein identification depends on staining method and the technique of in-gel digestion. In order to have higher yield, we choose SYPRO Ruby staining method; it was believed providing three orders of dynamic work range. New and more modern techniques such as LC-MS/MS or iTRAQ may lead to

better results than can be achieved with the 2-DE gel method (15, 17).

Another concern is the impact of not using an abundant protein depletion. Most of the study about plasma proteome in literature is to seek potential biomarker which is specific for a certain disease. Therefore they usually will do abundant protein depletion in order to prevent mask effect to scanty protein and to get high yield. However, this study is different in purpose. This study is to compare the global difference of protein expression between mesenteric lymph proteome and plasma proteome. It also provides proof that mesenteric lymph proteins is a filtrate of plasma proteins. So we did not perform abundant protein depletion. Another reason is that volume and protein concentration of lymph is relative lower then plasma. So lymph sample collection is more limited. If we performed abundant protein depletion, the rest of lymph sample will not be enough for further 2D PAGE study.

However, this study provided a basic detailed description of mesenteric lymph proteomes of a common experimental animal (SD rats) in physiological status using a common proteomic approach.

In conclusion, although our study is based on a physiological condition, there are still some differences in protein amount noted between the plasma and mesenteric lymph in rat. And these changes seem physiologically reasonable. Our experiment definitely provides a practical and probable animal model in the study of mesenteric lymph. In the future, we should use this successful animal model for further extensive studies to investigate the effect of mesenteric lymph in different diseases.

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