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Proteomic Analysis of Human Mesenteric Lymph

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Abstract

Extensive animal work has established mesenteric lymph as the mechanistic link between gut ischemia/reperfusion (I/R) and distant organ injury. Our trauma and transplant services provide a unique opportunity to assess the relevance of our animal data to human mesenteric lymph under conditions that simulate those used in the laboratory. Mesenteric lymph was collected from eleven patients; with lymphatic injuries, during semi-elective spine reconstruction, or immediately before organ donation. The lymph was tested for its ability to activate human neutrophils in vitro, and was analyzed by label-free proteomic analysis. Human mesenteric lymph primed human PMNs in a pattern similar to that observed in previous rodent, swine, and primate studies. A total of 477 proteins were identified from the 11 subject's lymph samples with greater than 99% confidence. In addition to classical serum proteins, markers of hemolysis, extracellular matrix components, and general tissue damage were identified. Both tissue injury and shock correlate strongly with production of bioactive lymph. Products of red blood cell hemolysis correlate strongly with human lymph bioactivity and immunoglobulins have a negative correlation with the pro-inflammatory lymph. These human data corroborate the current body of research implicating post shock mesenteric lymph in the development of systemic inflammation and multiple organ failure. Further studies will be required to determine if the proteins identified participate in the pathogenesis of multiple organ failure and if they can be used as diagnostic markers.

Keywords

mesenteric lymph; neutrophil; superoxide; proteomics; trauma; hemorrhagic shock; multiple organ failure; acute lung injury

INTRODUCTION

Post injury multiple organ failure (MOF) is the net result of a dysfunctional immune response to injury characterized by a hyperactive innate system and a suppressed adaptive

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system. Acute lung injury (ALI) is the first clinical manifestation of organ failure, followed by renal and hepatic dysfunction. Circulatory shock is integral in the early pathogenesis of MOF, and the gut has been invoked as the “motor of MOF” (1). Mesenteric ischemia reperfusion (IR), subsequent to trauma/hemorrhagic shock (T/HS), is central in the pathogenesis of post-injury organ dysfunction (2). Previous work in our own laboratory has documented PMN priming occurring in the early post-injury period contributes to MOF (3).

The lymphatic system was first described during ancient times, but its significance remained obscure until the Renaissance period with the realization that lymphatics were different than veins (4). The lymphatic system collects extravasated fluid, proteins, and lipids from the interstitial space and returns them to the blood circulation via the subclavian vein. An important difference is mesenteric lymph contains a large amount of fat and other molecules resorbed from the intestine, giving it a unique descriptor, *chyle*. Lymph is formed due to a filtration process of the plasma in the capillaries. It contains a large number of lymphocytes, macrophages, and number of plasma proteins including coagulation factors and albumin. In one of the first studies to use modern approaches to characterize lymph, Leak *et al.* examined the protein expression profiles of normal ovine lymph (5).

Post shock mesenteric lymph (PSML) serves as the conduit by which exudates from the ischemic gut are delivered into the circulation, and lymphatic diversion prior to T/HS attenuates the severity of organ injury (6–8). Investigations to identify the specific agents contained in PSML have implicated both proteins and lipids (9–10). The physiologic response to trauma has been inferred by a number of specific proteins, including acute phase components, cytokines and contents of cellular lysates, yet the proteome of human PSML has not been characterized. A prior biochemical study by Kaiser *et al.* reported that the level of a 24 amino acid, N-terminal fragment of serum albumin was significantly increased in lymph collected from rat subjected to trauma with hemorrhagic-shock (10). Initial studies in our laboratory characterized the protein fraction of rodent mesenteric lymph in the context of an experimental trauma/hemorrhagic shock model using differential gel electrophoresis and mass spectrometry analysis (11). In addition, Mittel *et al.* as well as Fang *et al.* have published proteomic studies of rodent mesenteric lymph during fasted and fed states, acute pancreatitis and hemorrhagic shock models (12–15). At the present time, our active trauma and transplant surgical services provide a unique opportunity to assess the relevance of the animal models to the human condition. The aim of this study is to provide the first proteomic description of human mesenteric lymph collected from critically ill or injured patients using a label-free semi-quantitative mass spectrometry based approach.

MATERIALS AND METHODS

Study population and data collection

Injured patients admitted to the Rocky Mountain Regional Trauma Center surgical ICU (SICU) at Denver Health Medical Center (DHMC) and patients undergoing brain-dead organ donation at Colorado hospitals were evaluated for inclusion into a study from 2008 through 2010. DHMC is a state-designated level I trauma center verified by the American College of Surgeons Committee on Trauma and the academic trauma center for the University of Colorado Denver. The data collection and storage processes are in compliance with Health Insurance Portability and Accountability Act regulations. The local institutional review board approved the study. Patients with various injury mechanisms were included in the study: trauma/hemorrhagic shock, brain death, mesenteric lymphatic injury, and elective spine surgery.

Mesenteric lymph collection

After obtaining informed consent from patients undergoing semi-elective spine injury reconstruction, the exposure of the patient's distal thoracic and proximal lumbar vertebral bodies is achieved via a left thoraco-abdominal incision. The cisterna chyli is visualized between the aorta and spine and lymph aspirated using a 27-gauge needle. During the donor operation, before cold preservation, a right medial visceral rotation is performed to expose the vena cava and the aorta just inferiorly to the take off of the superior mesenteric artery. At this level, the left renal vein is easily identified anteriorly crossing the aorta. Running along the retroperitoneal small bowel mesentery and crossing anterior and perpendicular to the left renal vein are typically large distended lymphatic vessels. These are cannulated with a 21-gauge angiocatheter to procure the mesenteric lymph. Patients are NPO (*nil per os*) overnight in preparation for semi-elective surgery and all nutrition (enteral and parenteral) is discontinued at the time of consent for organ donation. Consent for organ donation as well as lymph collection is obtained by the organ procurement organization, from the deceased (based on a document of gift: donor card, living will, or driver's license) or from the next of kin.

Mesenteric lymph (100 μ l – 1 mL) was collected and placed into an EDTA containing tube. Samples were centrifuged at $3500 \times g$ for 10 min to remove cellular components, and stored in a freezer at -80°C . Protein concentration was quantified using the Bradford assay, and lymph bioactivity measured by superoxide dismutase-inhibitable *cytochrome c* reduction in human neutrophils. Patients were categorized based on the predominant illness or mechanism of injury. The Injury Severity Score (ISS), a numerical method to describe the overall magnitude of injury, was calculated for trauma patients.

Human PMN Isolation

Human polymorphonuclear neutrophils (PMNs) were isolated from the heparinized blood of healthy donors with the use of dextran sedimentation followed by Ficoll density gradient, as previously described (8). Briefly, 10 mL from the samples was centrifuged at $200 \times g$ for 15 min. The upper layer was removed and the buffy coat was mixed with phosphate-buffered saline (PBS). This mixture was then placed on 5 mL of Ficoll and was centrifuged at $400 \times g$ for 30 min. Hypotonic lysis was performed twice at 4°C to eliminate contaminating red blood cells. The resulting PMNs were washed once and resuspended in Kreb's Ringer phosphate with dextrose buffer (KRP-d) at pH 7.35 to a final concentration of 2.5×10^7 cells/mL. The final cell population was more than 95% PMNs as determined by cell sorting.

Superoxide Production Assay

Lymph was tested for bioactivity using the spectrophotometric assay for superoxide dismutase (SOD) as described previously (8). Briefly, isolated PMNs (3.75×10^5) were incubated with either Kreb-Ringer phosphodextrose buffer (control), PAF (1 μM) or lymph mesenteric (10% vol/vol) for 5 minutes at 37°C then centrifuged for 3 minutes at $400 \times g$ and resuspended in fresh Kreb-Ringer phosphodextrose at 37°C . All priming assays were completed at 37°C in duplicate with a separate superoxide dismutase blank and an unstimulated control, to assure that the resting cells were not primed before beginning the experiment. The respiratory burst was initiated with the addition of 1 mM *N*-formylmethionyl-leucyl-phenylalanine (fMLP) to experimental wells. The fMLP, a known chemoattractant and granulocyte activator, was used as an internal standard. The maximal rate of superoxide dismutase inhibitable superoxide anion production (V_{max}) was determined by the slope of the absorbance curve using Softmax software (Molecular Devices, Silicon Valley, CA). The data were recorded as the maximal rate of superoxide anion production ($\text{nM O}_2^-/3.75 \times 10^5$ cells per minute).

Protein Sample preparation

The two most abundant proteins, albumin and IgG, were removed from the lymph samples using ProteoPrep[®] spin columns (Sigma-Aldrich) according to manufacturer's protocol. Total protein content was determined by the bicinchoninic acid assay (BCA) and absorbance read at 595 nm. A portion of the sample (30 µg) was diluted into SDS-PAGE sample buffer, heated at 70 °C for 10 min and loaded in a single lane on a 1-mm-thick 4–12% Bis-Tris gel (Invitrogen). After separation, the gel was stained with Coomassie Blue stain (Invitrogen). Each lane of the gel was divided into 10 equal-sized bands and proteins in the gel were digested as follows. Bands were destained in 200 µl of 25 mM ammonium bicarbonate in 50 % v/v ACN for 15 min, and then 200 µl of 100% ACN was applied for 15 min at room temperature. Dithiothreitol (DTT) was added to a final concentration of 10 mM and incubated at 65 °C for 30 min to reduce the disulfide bonds. The reduced cysteines were then alkylated with the addition of iodoacetamide (IAM) at a final concentration of 20 mM and incubated at room temperature in the dark for 30 min. The iodoacetamide was then removed, and washes were performed with 200 µl of distilled water followed by addition of 100 µl of ACN. Then ACN was removed, and 50 µl of the 0.01 µg/µl trypsin solution was added to each plug and allowed to rehydrate the gel plugs at 4 °C for 30 min and then incubated at 37 °C overnight. The tryptic mixtures were acidified with formic acid up to a final concentration of 1%. Peptides were extracted three times from the gel plugs using 50% ACN, 1% FA, concentrated by SpeedVac to a desired volume (~18 µl), and subjected to LC-MS/MS analysis.

Liquid Chromatography-Tandem Mass Spectrometry

Nano-flow reverse phase LC-MS/MS was performed using a capillary HPLC system (Agilent 1200, Palo Alto, CA) coupled with a linear ion trap mass spectrometer LTQ-FT Ultra Hybrid ion cyclotron resonance mass spectrometer (Thermo Fisher; San Jose, CA) through an in-house built nanoelectrospray ionization source. Eight microliters of the tryptic peptides were preconcentrated and desalted onto a C18 trap column ZORBAX 300SB-C18, (5 µm i.d. × 5 mm, Agilent Technologies, Santa Clara, CA) with 5% ACN, 0.1% FA at a flow rate of 15 µL/min for 5 min. The separation of the tryptic peptides was performed on a C18 reverse phase column (75 µm ID × 360 µm OD × 100 mm length) packed in-house with 4 µm 100 Å pore size C18 reversed-phase stationary phase (Synergy, Phenomenex, Torrance, CA) kept at a constant 40 °C using an in-house built column heater at a flow rate of 380 nL/min. The mobile phases consisted of 5% acetonitrile with 0.1% formic acid (A) and 95% acetonitrile with 0.1% formic acid (B), respectively. A 90-min linear gradient from 5 to 50% B was typically used. Data acquisition was performed using the instrument supplied Xcalibur (version 2.0.6) software. The LC runs were monitored in positive ion mode by sequentially recording survey MS scans (m/z 400–2000), in the ICR cell, while three MS² were obtained in the ion trap via CID for the most intense ions.

Mass Spectrometry Data Analysis

MS/MS spectra were extracted from raw data files and converted into mascot generic files (mgf) using an in-house script. Mascot (version 2.2; Matrix Science Inc., London, UK) was used to perform database searches against the human subset SwissProt database of the extracted MS/MS data. Peptide tolerance was set at ± 10 ppm with MS/MS tolerance set at ± 0.6 Da. Trypsin specificity was used allowing for 1 missed cleavage. The modifications of Met oxidation, protein N-terminal acetylation, and peptide N-terminal pyroglutamic acid formation were allowed for, and Cys carbamidomethylation was set as a fixed modification.

Scaffold (version 2, Proteome Software, Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. All mascot. DAT files, for each subject's lymph

(10 bands each) were loaded together as one “biological sample” within Scaffold. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified unique peptides.

Both unique peptides and total peptides per protein were calculated for each patient and normalized to total unique and total peptides per sample. These values were used for a semi-quantitative comparison between subjects. Linear regression (R-squared method) was used to identify proteins with abundance patterns that correlated with lymph bioactivity as measured by PNM priming. The correlation coefficients and p values are uncorrected for multiple comparisons and represent nominal values.

Polymetric clustering analysis

PermutMatrix (version 1.9.3, Montpellier, FR) was used for clustering analysis for proteins that were identified in seven or more of the eleven patient's samples. The multiple-fragment heuristic (MF) seriation rule was used for the ordering of individual proteins. The sum of all pairwise distances of neighboring rows was $S=13.294$ and $R=0.487$ for the objective function. Patients were clustered using McQuitty's criteria for the linkage rule (path length $S=10.198$, objective function $R=0.546$). The Euclidean distance method was used to calculate dissimilarity (16).

RESULTS

Lymph samples were collected from eleven subjects in accordance with local and federal regulations. A summary of the patient profiles are listed in Table 1. The diverse group covers a wide range of ages and medical conditions, with recovery of lymph during semi-elective spine reconstruction, lymphatic injuries or organ donation (Figure 1). A neutrophil priming assay was used to evaluate each of the samples for bioactivity (Figure 2). The priming ranges from slightly greater than platelet-activating factor (PAF) for patient #1 to slightly less than the control (fMLP), for patient #11. Of the patients samples collected, two were classified as having high bioactivity (> 2 fold priming over fMLP), three medium (> 1.5 fold over fMLP) and six with low bioactivity (< 1.5 fold over fMLP). All trauma patients and brain dead organ donors in this study generated lymph that had bioactivity in our PMN priming assay. Although two patients without shock had the highest lymph bioactivity, all of the samples from patients with a vasopressor requirement (5/5) at the time of collection had lymph with bioactivity of 1.4 fold higher priming over fMLP.

Proteomics analysis

Using a 1D-PAGE separation step prior to nanocapillary LC-MS/MS analysis, we analyzed the protein profiles of mesenteric lymph from the eleven human subjects. To improve proteome coverage, we performed single step depletion of the two most abundant serum proteins, albumin and IgG. While this approach is likely to also deplete specific proteins that are bound to albumin or possibly non-specifically to the depletion column, depletion of these highly abundant proteins has proven to be a particularly beneficial step in achieving greater proteome coverage. The depleted protein samples were separated by 1D SDS-PAGE followed by slicing gel lanes into 10 bands (gel fractions). Each band was subjected to tryptic digestion and the resulting peptides were analyzed by LC-MS/MS. A total of 477 proteins with 99% confidence ($FDR < 1\%$) were identified from the eleven lymph samples (30 μ g of total protein each). The proteins identified in our study include extracellular matrix components, secreted proteases and proteases inhibitors, mitochondrial and cytoskeleton proteins, other intracellular proteins, in addition to classical blood proteins.

Representative examples from several classes of proteins are listed in Table 2. Most abundant proteins, such as complement proteins and apolipoproteins, were identified in all patients. Rare proteins such as neutrophil serum deprivation-response protein and Xaa-Pro dipeptidase were only found in single patient's samples. The protein results were ranked based on the total number of samples a protein was identified in and the average number of unique peptides per protein (complete results in Supplementary Table S1). This rank gives a crude evaluation of relative protein abundance in lymph and more importantly allows us to compare the proteins identified to other proteomic datasets, such as the Human Plasma Proteome dataset, which has been collected by 35 collaborating laboratories.

Bioactivity trends

We found that several proteins were strongly associated with bioactivity, as measured by the ability of lymph to stimulate superoxide production by human neutrophils. Several of the proteins that had high correlation with bioactivity were markers of hemolysis. We compared the proteomic analysis of erythrocytes by Kakhniashvili *et al.* to identify potential protein contributions to the lymph from hemolysis (18). Table 3 shows the top RBC proteins and their rank in our dataset. Of the top 25 proteins in the RBC dataset 21 were found in human lymph, 14 with a correlation coefficient of greater than 0.60, and 17 with a P value of less than 0.1 when comparing high and medium to low bioactivity samples.

Despite having used immuno-affinity depletion to remove IgG, roughly 75% of the immunoglobins found in serum, some IgG peptides were identified in addition to a large number of the additional proteins in this class of abundant serum proteins. It is not unexpected to find an abundant amount of IgGs in lymph fluid based on the immune-surveillance function of this organ system. Spectral counts of the IgG sequences identified were one of the best predictors of lymph having lower PMN priming activity as shown (Figure 3). Interestingly polymetric clustering shows that lymph from patient #11, that had the lowest bioactivity in the priming assay, was an outlier. It has been shown that several antibodies have immuno-modulatory properties such as those that can prime PMNs (19), to that those that neutralize pro-inflammatory cytokines, examples include autoantibodies that bind IL-8 (20), IL-6 (21), IFN- γ (22).

Hemolysis

A significant increase of hemolysis products such as hemoglobin alpha, beta, gamma and delta (Table 3 and 4) correlates with bioactivity from human mesenteric lymph samples. Released hemoglobin may represent a common factor resulting from multiple pathways such as oxidative stress, nitric oxide (NO) depletion and platelet activation and aggregation (23). These processes may aggravate ongoing inflammation to produce vascular damage and lung injury and also drive damage in other susceptible organs, such as the kidneys. Decreased levels of haptoglobin also correlated with bioactivity from human mesenteric lymph samples. Haptoglobin is an acute phase glycoprotein that binds free hemoglobin with high avidity during hemolysis, protecting organs from iron-generated reactive oxygen species. In addition to sequestration of hemoglobin, haptoglobin is an alpha-2-glycoprotein with anti-inflammatory properties (24). Thus, the depletion of haptoglobin and increase in free hemoglobin found in post shock mesenteric lymph may indicate a loss of protection from inflammation and free radical damage, ultimately contributing to remote organ injury.

An additional product of hemolysis, the cytosolic carbonic anhydrase (CA) isoenzymes 1, 2 and 3 abundant in the RBC proteome and in our dataset, increased proportionally to human mesenteric lymph bioactivity. Carbonic anhydrase, a zinc metalloenzyme, catalyzes the reversible hydration of carbon dioxide, a product of cellular aerobic energy production. It participates in various biological processes including acid-base regulation, gas transport,

bone resorption, ureagenesis, gluconeogenesis, cell proliferation, oncogenesis and lipogenesis. In addition to these physiological functions, CA hydrolyzes various esters (25).

Tissue damage

Several cellular proteins associated with tissue injury, such as creatine kinase (MB isoenzymes), myoglobin, cytoplasmatic malate dehydrogenase, and fatty acid binding protein, were identified in human mesenteric lymph. Liver type fatty acid-binding protein (L-FABP) correlates positively with bioactivity from human mesenteric lymph samples. This relatively small (14 kDa) cytoplasmic protein is abundantly expressed in the cytoplasm and the nucleus of hepatocytes. L-FABP has high affinity and capacity to bind long-chain fatty acid oxidative products. Additionally, L-FABP has been identified as a marker of acute liver injury (26).

Cytosolic malate dehydrogenase (MDH) is another protein correlating with human mesenteric lymph bioactivity. MDH was identified in all high and medium priming samples and conversely, was not presented in any of the samples with low bioactivity. This enzyme plays a crucial role in the malate–aspartate shuttle and the citric-acid cycle in all aerobic tissues of mammals. Previous work from our laboratory reported that MDH levels increased in rat post-shock mesenteric lymph as well (11). The levels of other glycolytic pathway proteins also correlate with the pro-inflammatory activity of mesenteric lymph. These proteins include fructose-1,6-bisphosphatase (FBP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and triosephosphate isomerase (TPI). A substantial increase of fructose 1,6-bisphosphatase has been observed in the early phase of hemorrhagic shock (27). FBP is a critical regulatory enzyme in glycolysis that catalyzes the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate.

Cytoskeletal proteins

We identified several cytoskeletal proteins including actin, tubulin, cofilin-1, spectrin, gelsolin and profilin-1. Among these markers of tissue damage, cofilins are the principal actin filament depolymerizing proteins and cofilin-1 has been implicated in regulation of ischemia-induced endothelial cell alteration, leading to cell damage and microvascular dysfunction (28). A significant increase in the relative abundance of actin, cofilin-1 and profilin has been previously reported in rodent mesenteric lymph following hemorrhagic shock (12).

Mitochondrial proteins

Sixteen mitochondrial proteins were identified in the human mesenteric lymph samples. There have been reports showing that circulating mitochondrial DNA and formyl peptides can mediate organ injury through PMN activation (29). While we did not identify specific mitochondrial DNA binding proteins or formylated peptides that target the formyl peptide receptor (a GPCR) (30), our data suggests the presence of lysed mitochondria, presumably from cell death.

ECM components

The extracellular matrix (ECM) is a complex protein network composed of collagens, connective tissue glycoproteins, elastic fibers, hyaluronan and proteoglycans. The ECM provides a molecular scaffold and can serve as a repository for cytokines. Several identified ECM components such as fibrinogen, von Willebrand factor, and fibulin-1 are abundant in blood. Collagens, laminins and ECM-1 are structural matrix components found in human mesenteric lymph and known substrates for the gelatinases. The presence of these ECM components suggests tissue damage either by the trauma itself or by degradation of other

matrix by activated proteases. One such protease identified in human mesenteric lymph was matrix metalloproteinase 9 (MMP-9). Several clinical studies have shown that neutrophil-derived MMPs (specifically MMP-2 and MMP-9) are involved in the development of acute lung injury both experimentally and in clinical ARDS (31).

Oxidative Stress

Additional proteins that appeared to correlate with the pro-inflammatory activity of mesenteric lymph are associated with oxidative stress. These include glutathione S-transferase P/Mu4/omega1, peroxiredoxin-2 and -6, catalase, Cu/Zn and Mn superoxide dismutase, extracellular superoxide dismutase and protein DJ-1. Animal models have indicated that the GSTs function as an early and sensitive indicator of hepatocyte damage caused by various adverse conditions, including hemorrhagic shock (32). Peroxiredoxin-6 (PRDX6) is another protein that correlates with bioactivity from human mesenteric lymph samples and has a major role in lung phospholipid metabolism. Manevich and Fisher found that PRDX6 may offer cellular protection from oxidative stress (33). The DJ-1 protein, an oxidative stress response protein, correlates positively with inflammatory activity of mesenteric lymph. DJ-1 affects cell survival, in part, by modulating cellular signaling cascades such as PTEN/phosphatidylinositol 3-kinase/Akt (34) and altering p53 activity. Additionally, a number of studies have shown that DJ-1 can have protective functions against oxidative stress-induced injury. It was found that DJ-1 is required for the activity of Nrf2 (nuclear factor erythroid 2-related factor), a master regulator of response to oxidative stress (35).

Other Proteins

Lipopolysaccharide (LPS)-binding protein (LBP) had negative correlation with pro-inflammatory activity of human mesenteric lymph. It is a type I acute phase protein which markedly enhances cellular responses to LPS. LBP binds LPS and then interacts with membrane CD14 receptor and TLR4 to initiate a downstream signaling cascade, culminating in the activation of transcription factors such as NF- κ B and the IFN regulatory factors (36). Elevated levels of LBP have been reported in infections and systemic inflammatory response syndrome (37). Macrophage migration inhibitory factor (MIF) was observed in lymph samples with high pro-inflammatory activity. MIF is a conserved 12.5 kDa pro-inflammatory cytokine secreted by immune and endocrine cells, and also by the epithelial lining of tissues in direct contact with the external environment upon inflammatory and stress stimulation. MIF participates in the pathogenesis of acute and chronic inflammatory and autoimmune disorders, such as sepsis, asthma, rheumatoid arthritis, and inflammatory bowel disease. It has been demonstrated that MIF can mediate inflammatory lung injury in acute respiratory distress syndrome (ARDS). The role of MIF as a promoter of intrapulmonary inflammation in ARDS was suggested by the observations that MIF augmented TNF- α and IL-8 secretion from alveolar cells obtained from ARDS patients (38).

DISCUSSION

The present study provides a description of the human mesenteric lymph proteome from eleven patients. We applied a strategy that combines immuno-affinity depletion, 1D SDS-PAGE separation and LC-MS/MS to increase the dynamic range of detection. We have identified, with greater than 99% confidence, a total of 477 proteins. Several of the proteins identified in our study are markers of hemolysis and oxidative stress, matrix degradation, and general tissue damage. Our lab and others have demonstrated the pro-inflammatory activity of post shock mesenteric lymph in a variety of animal models. While the detailed

proteome for rodent mesenteric lymph has been reported to date (11–15) studies using human samples have been limited.

Out of the top 200 proteins in our dataset, several are not found in the validated 889 human plasma proteome project (HPPP) dataset (17). This finding is consistent with work by Leak *et al.* that showed ovine lymph contains plasma proteins and additional metabolic products of connective tissue cells and proteins released from lymphatic endothelium (5).

Our previous proteomic analysis of mesenteric lymph was from a well-studied rodent model of shock. We used the differential gel electrophoresis (DIGE) technique and MS to identify proteins that change in relative abundance. Not surprisingly, most of the identified proteins in rodent lymph were also identified in human mesenteric lymph. However, the rodent lymph proteome contains a population of proteins such as major urinary protein, alpha-1-inhibitor 3, murinoglobulin-1 that are not presented in the human lymph dataset.

We have demonstrated that many of the proteins amongst the 477 identified appear to correlate with the PMN priming activity of mesenteric lymph and therefore may correlate with MOF and critical illness. While the polymetric analysis used here did not reveal a clear relationship between patient samples when using the entire dataset, analysis on subsets of related proteins resulted in patient sample clustering that correlated with bioactivity. Interestingly, both tissue injury and shock correlate strongly with production of bioactive lymph. While the two patients that generated lymph with the highest bioactivity were not in shock, each patient with a vasopressor requirement generated bioactive lymph samples, and all of the trauma patients and brain dead organ donors generated lymph that primed PMNs greater than fMLP. This indicates that lymph from critically ill patients has the capacity to initiate a strong inflammatory response, and that shock is sufficient but not a necessary condition for neutrophil priming. Because the lymphatic system is permeable, there is little, if any, exclusion of interstitial molecules (39). In addition, the increased microvascular permeability following severe injury, suggests that markers of MOF identified in mesenteric lymph could lead to the identification of plasma biomarkers.

While the methods employed in this study allowed for the identification of several hundred proteins, our dataset is not a comprehensive list of all proteins in human mesenteric lymph. The limited sample, high dynamic range of protein concentrations in lymph, and inherent sensitivity of the analytical platform used precludes the identification of many bioactive components such as cytokines. Due to the heterogeneous patient population in this study, it is not possible to conclude that the correlations observed are a direct consequence of trauma, shock, or brain death. It is likely that the priming of neutrophils observed is mediated, at least in part, by additional factors not identified in our dataset i.e. endotoxin, cytokines, etc. However, the diverse collection of immunomodulatory proteins identified in this paper provides insight and offer direction for future studies.

Our human findings substantiate the current body of research implicating post shock mesenteric lymph in the development of systemic inflammation and MOF. Further studies are required to determine if, and how, the proteins identified here provide a mechanistic link between the ischemic gut and remote organ injury, serving as potential biomarkers for early identification of patients at risk for the development of multiple organ failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Mesenteric lymph was obtained from the efferent lymph ducts in patients undergoing organ donation. Typically, these ducts were found at the junction of the IMV (inferior mesenteric vein) and the LRV (left renal vein). Also pictured are the aorta and the IVC.

Figure 2.
Parametric analysis of immunoglobulin entries.

Figure 3.
The bioactivity of human mesenteric lymph was assessed by superoxide production in human PMNs. PAF (platelet activating factor) was used as a PMN NADPH oxidase priming agent. FMLP (formyl-methionyl-leucyl-phenylalanine) was employed as an activator.

Table 1

Patient profiles.

Patient	Mechanism / Injury	Sex	Age	Trauma (ISS)/ Brain Death/ No Trauma	Shock	Bioactivity
1	Auto vs. pedestrian	M	18	T (57)	N	High
2	Cardiopulmonary arrest - asthma	M	65	BD	N	High
3	Auto vs. bicycle	F	15	T (75) + BD	Y	Med
4	Meningitis	F	37	BD	Y	Med
5	Motor vehicle collision	M	21	T (66) + BD	Y	Med
6	Cardiopulmonary arrest - overdose	F	47	BD	Y	Low
7	Gunshot wound to neck	M	18	T (45)	Y	Low
8	Cerebrovascular Accident	M	63	BD	N	Low
9	Cardiopulmonary arrest - AMI	M	76	BD	N	Low
10	Cardiopulmonary arrest - AMI	F	62	BD	N	Low
11	Compression fracture due to osteoporosis	F	53	NT	N	Low

Table 2

Representative proteins identified in human lymph.

Extracellular matrix	Mitochondrial
Lumican	Superoxide dismutase [Mn]
Von Willebrand factor	Malate dehydrogenase
Gelectin-3-binding protein	Carbamoyl-phosphate synthase
SPARC-like protein 1	ATP synthase subunit alpha and beta
Multimerin-2	Aldehyde dehydrogenase
Fibulin-2	3-ketoacyl-CoA thiolase
Laminins ($\alpha 2$, $\alpha 4$, $\beta 1$, $\beta 2$, $\gamma 1$)	Aspartate aminotransferase
Collagens I, III, V and VI	Thioredoxin-dependent peroxide reductase
Extracellular matrix protein I	Glutamate dehydrogenase 1
Mimecan	Hydroxyacyl-coenzyme A dehydrogenase
Proteases	Cytoskeleton
Complement factor I	Gelsolin
Complement C2	Actin, cytoplasmic 2
Complement C1s subcomponent	Profilin-1
Complement C1r subcomponent	Tubulin α -1B chain
Complement factor D	Tubulin β -2A chain
Carboxypeptidase N catalytic chain	Tubulin β -2C chain
Hepatocyte growth factor activator	Filamin-A
Plasma kallikrein	Actin, α cardiac muscle 1
Carboxypeptidase B2	α -actinin-4
Carboxypeptidase A1	Titin
Proteasome subunit alpha type-4	Filamin-C
Protease inhibitors	
Inter- α -trypsin inhibitor heavy chain H2, H3 and H4	
α -1-antitrypsin	
α -1-antichymotrypsin	
Antithrombin-III	
Plasma protease C1 inhibitor	
Kininogen-1	
Protein AMBP	
Heparin cofactor 2	
α -2-antiplasmin	
Kallistatin	
Cystatin-C	
Pregnancy zone protein	

Table 3

Comparison of lymph and erythrocyte proteomes (17).

ID Rank	Uniprot ID	Identified Proteins	RBC Protein Rank	Unique Peptides	Corr. Coef.	H/M vs L (P value)
37	P68871	Hemoglobin subunit beta	1		0.18	
62	P69905	Hemoglobin subunit alpha	2		0.38	**
76	P00915	Carbonic anhydrase 1	3		0.61	***
241	P04040	Catalase	4		0.61	*
124	P32119	Peroxiredoxin-2	5		0.60	***
113	P00918	Carbonic anhydrase 2	6		0.87	***
263	Q06830	Peroxiredoxin-1	7		0.54	*
207	P30043	Flavin reductase	8		0.91	***
105	P62937	Peptidyl-prolyl cis-trans isomerase A	9		0.72	***
380	P00441	Superoxide dismutase [Cu-Zn]	10		0.50	*
138	P69892	Hemoglobin subunit gamma-2	12		0.61	***
452	P26447	Protein S100-A4	13		0.52	*
123	P04075	Fructose-bisphosphate aldolase A	15		0.48	
251	Q99497	Protein DJ-1	16		0.82	**
362	P07738	Bisphosphoglycerate mutase	17		0.60	
95	P04406	Glyceraldehyde-3-phosphate dehydrogenase	18		0.68	***
172	P62988	Ubiquitin	19		0.64	*
199	P00558	Phosphoglycerate kinase 1	21		0.63	*
109	P60174	Triosephosphate isomerase	22		0.84	***
136	P30041	Peroxiredoxin-6	23		0.86	***
100	P07195	L-lactate dehydrogenase B chain	25		0.54	

Of the top 25 proteins identified in the non-depleted red blood cell proteome by Kakniashvili, Bulla, and Goodman (17) as determined by unique peptides identified, 21 were identified in our human lymph dataset. Unique peptide bar graph represents unique peptides identified for each sample and normalized to the maximum. The order is patient #1 on the right to #11 on the left.

* A student's *t*-test was used to determine significance between high and medium vs. low priming samples: $P < 0.1$,
 ** < 0.05 ,

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Table 4

Additional proteins discussed.

ID Rank	Uniprot ID	Identified Proteins	MW [kDa]	Unique Peptides	Corr. Coef.	H/M vs L (P value)
85	P02042	Hemoglobin subunit delta	16		0.40	**
175	P07451	Carbonic anhydrase 3	30		0.42	*
13	P00738	Haptoglobin	45		-0.46	*
157	P07148	Fatty acid-binding protein, liver	14		0.66	***
144	P40925	Malate dehydrogenase, cytoplasmic	36		0.84	***
139	P09467	Fructose-1,6-bisphosphatase 1	37		0.65	**
215	P23528	Cofilin-1	19		0.78	**
130	P09211	Glutathione S-transferase P	23		0.87	***
279	Q03013	Glutathione S-transferase Mu 4	26		0.66	**
200	P78417	Glutathione S-transferase omega-1	28		0.67	**
313	P04179	Superoxide dismutase [Mn], mitochondrial	25		0.53	
117	P08294	Extracellular superoxide dismutase [Cu-Zn]	26		0.58	
137	P18428	Lipopolysaccharide-binding protein	53		-0.75	**
252	P14174	Macrophage migration inhibitory factor	12		0.81	***