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Cross-transfusion of Postshock Mesenteric Lymph Provokes Acute Lung Injury

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Abstract

Objective—Substantial investigation has implicated mesenteric lymph as the mechanistic link between gut ischemia/reperfusion (I/R) and distant organ injury. Specifically, lymph diversion prevents acute lung injury (ALI) *in vitro* and bioactive lipids and proteins isolated from postshock mesenteric lymph (PSML), maintain bioactivity *in vitro*. However, Koch's postulates remain to be satisfied via direct cross-transfusion into a naïve animal. We therefore hypothesized that real time cross-transfusion of postshock mesenteric lymph provokes acute lung injury.

Methods—One set of Sprague-Dawley rats (lymph donors) was anesthetized, with the mesenteric lymph ducts cannulated and exteriorized to drain freely into a siliconized plastic cup; concurrently, a second group of rats (lymph recipients) was anesthetized, with a cannula inserted into the animal's right internal jugular vein. Blood was removed from the donor rats to induce hemorrhagic shock (MAP of 35 mmHg $\times$ 45 minutes). The recipient rats were positioned 10 cm below the plastic cup which emptied into the jugular vein cannula. Thus, mesenteric lymph from the shocked donor rat was delivered to the recipient rat at the rate generated during shock and the subsequent 3 hours of resuscitation.

Results—Neutrophil (PMN) accumulation in the lungs was substantially elevated in the postshock lymph cross-transfusion group compared to both sham lymph cross-transfusion and instrumented control (MPO: 9.42 ± 1.55 vs. 2.81 ± 0.82 U/mg lung tissue in postshock vs. sham lymph cross-transfusion, $n=6$ in each group, $p=0.02$). Additionally, cross-transfusion of PSML induced oxidative stress in the lung (0.21 ± 0.03 vs. 0.10 ± 0.01 micromoles MDA per mg lung tissue in lymph cross-transfusion vs. instrumented control, $n=6$ in each group, $p=0.046$). Furthermore, transfusion of PSML provoked lung injury (BAL protein 0.77 ± 0.18 vs. 0.15 ± 0.02 mg/ml protein in BALF, postshock vs. sham lymph cross-transfusion, $n=6$ in each group, $p=0.004$).

Conclusion—Cross-transfusion of PSML into a naïve animal leads to PMN accumulation and provokes ALI. These data provide evidence that postshock agents released into mesenteric lymph are capable of provoking distant organ injury.

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INTRODUCTION

Multiple organ failure (MOF), the leading cause of late mortality and a major source of morbidity in trauma patients, is the net result of a maladaptive systemic inflammatory response to injury. Despite intensive investigation, the supportive care initially proposed by Eiseman nearly 35 years ago remains the standard treatment for MOF, and there is no effective pharmacologic therapy for this devastating illness.¹ Acute lung injury (ALI) is the first clinical manifestation of organ failure, followed by hepatic and renal dysfunction.² Although animal studies initially implicated gut bacterial translocation, a prospective clinical study was unable to detect endotoxin in the portal circulation of trauma patients despite a 30% incidence of MOF in this population.³ It is now understood that circulatory shock is integral in the early pathogenesis of MOF. Postshock mesenteric lymph (PSML) has been implicated as the conduit by which bioactive exudates from the ischemic gut are delivered into the circulation.^{4,5} This is supported by the fact that lymphatic diversion prior to trauma/hemorrhagic shock (T/HS) prevents remote organ injury.^{6,7} Tissue injury and hypoperfusion unleash a number of products which appear in the lymph: cytokines,⁸ inflammatory lipids, coagulation factors,⁹ and cellular breakdown products.¹⁰ The lymphatic system collects the extravasated fluid and cellular breakdown products, proteins, and lipids from the interstitial space and returns them to the blood circulation via the subclavian vein. The protein composition of lymph is nearly identical to that of interstitial fluid, and thought to be similar to, although less concentrated, than that of plasma.¹¹ An important exception is the gut, which has the ability to acquire factors from outside of the cardiovascular system and deliver them to the circulation via a mesenteric lymph conduit, giving the injured gut a unique descriptor: the “motor of MOF.”¹²

In 1882, Robert Koch published a landmark paper describing microbial pathogenicity. His methods of linking a specific bacterium to a disease, termed Koch’s postulates, are sound principles applicable to a variety of fields in translational research: 1) the microorganism is present and discoverable in every case of the disease; 2) the microorganism is to be cultivated in a pure culture; 3) inoculations from such culture must reproduce the disease in susceptible animals; 4) it must be re-obtained from such animals and again grown in a pure culture.¹³ After having satisfied Koch’s first 2 postulates in previous work, we sought to satisfy Koch’s 3rd postulate – hypothesizing that real time cross-transfusion of postshock mesenteric lymph (PSML) into a naïve animal will provoke acute lung injury.

METHODS

Trauma/Hemorrhagic Shock Model

Sprague-Dawley rats weighing 350g to 400g (Harlan Labs, Indianapolis, IN) were housed in a climate-controlled barrier facility with 12 hr light/dark cycles and free access to food and water for a period of at least one week prior to experimental procedures. General anesthesia was performed with an intraperitoneal injection of 50 mg/kg sodium pentobarbital, and local anesthesia with a subcutaneous injection of 1% lidocaine. Body temperature was monitored rectally and euthermia maintained with a heat lamp. The femoral artery and vein were cannulated with PE 50 tubing and blood pressure monitored using a ProPac invasive monitoring device (Welch Allyn Inc., Skaneateles Falls, NY). A separate skin incision was created to tunnel the femoral catheters prior to closure of the groin incision. A 3 cm midline laparotomy was performed, simulating tissue injury with trauma. The bowel was eviscerated and rotated to the left to expose the mesenteric duct and accessory duct (located adjacent to the superior mesenteric artery) which were isolated by blunt dissection. The main lymphatic duct was cannulated with PE 100 tubing, secured with 7-0 prolene suture, and maintained in place with Super Glue (Rancho Cucamonga, CA). The tubing was then exteriorized via a right flank stab wound, and the accessory lymph duct was then ligated. The laparotomy

incision was closed in a two-layer fashion, and the mesenteric lymph transfused directly into the right internal jugular vein of the anesthetized naïve rat (Figure 1). In the donor rat, shock was induced by controlled hemorrhage to a MAP of 35 mmHg which was sustained for 45 min by the withdrawal or return of shed blood (SB). Hypotension was maintained until the base deficit was >20 meq/L.

Resuscitation of the donor rat was performed by infusing twice the SB volume in NS over 30 min, half the volume of the shed blood over 30 min, and then twice the SB volume in NS over 60 min via the femoral vein. Lymph cross-transfusion continued for 3 hrs (paralleling the 2 hr resuscitation period followed by the 1 hr observation period in the hemorrhagic shock or sham shock donor rat). A bronchoalveolar lavage was performed in the recipient rat; both animals were euthanized via a pentobarbital overdose. In the sham lymph cross-transfusion group, the donor rat received a laparotomy without hemorrhagic shock, with transfusion of sham lymph for 3 hours. In the instrumented control group, the internal jugular vein was cannulated with infusion of normal saline, at an equivalent volume and rate (3 ml/hr). Plasma and BAL samples were centrifuged at 400g at 4° C for 10 minutes, and snap frozen in liquid nitrogen and stored at -80° C.

Tissue and BAL Fluid Analysis

A shock state leads to sequestration of large numbers of polymorphonuclear leukocytes in the lungs.¹⁴ When provoked, these neutrophils provoke acute lung injury via release of toxic metabolites.¹⁵ Therefore, lung PMN accumulation was evaluated using the myeloperoxidase (MPO) assay. Lung tissue stored at -80° C, was thawed, weighed, and homogenized in 10 mL of 20 mmol/L potassium phosphate buffer (PPB) and centrifuged at 40,000g for 30 minutes. The pellet was sonicated for 90 seconds in 4 mL of 50 mmol/L PPB containing 0.5 g/dL hexadecyltrimethyl ammonium bromide, incubated at 60 C and centrifuged. The supernatant (5 µL) was added to 145 µL of 50 mmol/L PPB containing 0.167 mg/mL O-dianisidine with 0.0005% hydrogen peroxide; the absorbance at 460 nm was measured with a spectrophotometer (Molecular Devices Corp, Sunnyvale, Calif) to determine MPO activity.¹⁶ Oxygen metabolites generated by PMNs in the lung have been shown to provoke acute lung injury.¹⁷ Therefore, malondialdehyde (MDA), a product of lipid peroxidation, was used to measure oxidative stress in the lung tissue (TBARS assay kit, Cell Biolabs, Inc., San Diego, CA) using a colorimetric assay.

BAL protein was used as a direct marker of lung damage. Normal saline (5 ml) was injected into the trachea (three times) and collected, with the return of bronchoalveolar lavage fluid (BALF) consistently greater than 12 mL. The BALF was then centrifuged at 400g at 4° C for 10 minutes and protein quantification performed using the BCA protein analysis method with bovine serum albumin standards. Data are reported as mean ± SEM. Student's t-test was used to determine statistical significance.

RESULTS

Lung neutrophil accumulation following lymph cross-transfusion

Neutrophils, critical in the pathogenesis of ALI, were significantly elevated following lymph cross-transfusion. PMN accumulation in the lungs as measured by myeloperoxidase (MPO) activity was substantially higher in the lymph cross-transfusion group is shown in Figure 2: (MPO: 9.42±1.55 vs. 2.81±0.82 U/mg lung tissue in postshock vs. sham lymph cross-transfusion), n=6 in each group, p=0.02). The sham lymph cross-transfusion group levels were comparable to the instrumented controls (MPO: 2.81±0.82 vs. 2.47±0.02 U/mg lung tissue, p=0.35).

Cross-transfusion induces PMN-generated oxidative stress in the lung

Malondialdehyde (MDA), a product of lipid peroxidation, was used to measure oxidative stress in the lung tissue following cross-transfusion of PSML into a naïve rat. Transfusion of postshock mesenteric lymph induced oxidative stress in the lung (Figure 3: 0.21 ± 0.03 vs. 0.10 ± 0.01 micromoles MDA per mg lung tissue in lymph cross-transfusion vs. sham, $p = 0.046$).

Postshock lymph transfusion provokes an increase in lung vascular permeability

The primary study endpoint was ALI, determined by protein extravasation into the alveolar space at 3 hours postinjury. As illustrated in Figure 4, transfusion of mesenteric lymph provoked substantial acute lung injury (BAL protein 0.77 ± 0.18 vs. 0.15 ± 0.02 mg/ml protein in BALF, postshock lymph vs. sham lymph cross-transfusion, $n=6$ in each group, $p=0.004$). The sham lymph cross-transfusion group levels were comparable to the instrumented controls (0.15 ± 0.02 vs. 0.14 ± 0.06 mg/ml, $p=0.38$).

DISCUSSION

Robert Koch was the first person to demonstrate a strict causative relationship between a microorganism and disease. Before his pioneering work, the diagnosis of tuberculosis was a matter of judgment and dispute, described by a group of syndromes: miliary tuberculosis, caseous bronchitis, phthisis, scrofula, and bovine tuberculosis, with no universal agreement as to whether these were manifestations of the same condition. Since that time, Koch's postulates have been applied to a variety of research fields, including atherosclerosis,¹⁸ microbial genetics,¹⁹ and the gut lymph hypothesis.²⁰

Acute lung injury, ARDS, and multiple organ failure are a group of syndromes caused by systemic hyperinflammation, in which morbidity is high and treatment remains supportive. The combined insults of trauma and hemorrhagic shock lead to the release of toxic inflammatory products from the gut into the circulation. Initially, loss of gut barrier function and bacterial translocation was the predominant explanation for the development of MOF.¹² In a prospective clinical study exploring this hypothesis, however, our group did not find endotoxin in the portal circulation of trauma patients with MOF.³ Subsequent clinical studies have supported this finding.^{21,22} After the bacterial translocation mechanism failed to satisfy Koch's postulates, the lymphatic system became the target of investigation as the primary conduit for delivering inflammatory factors from the ischemic gut into the circulation.

Koch's postulates (adapted to the gut lymph hypothesis):

1. The inflammatory mediator must be found in all organisms with the disease, and is not present in animals without the disease. *A factor (or factors) in the PSML provokes acute lung injury following hemorrhagic shock, and in the absence of PSML due to ligation of the mesenteric duct, acute lung injury does not occur.*^{6,7}
2. The inflammatory mediator must be isolated from a diseased organism and maintain activity in vitro. *PSML provokes PMN priming*^{23,24} *and is toxic to human endothelial cells in vitro.*^{25,26}
3. The inflammatory mediator should cause disease when introduced into a healthy organism. *Injection of PSML provokes acute lung injury when transfused into a naïve animal.*
4. The inflammatory mediator must be re-isolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative

agent. With Koch's 3rd postulate satisfied, future experiments are designed to test Koch's 4th and final postulate.

Infusion of stored PSML into naïve rats has been shown to provoke ALI;²⁷ however, lymph contains volatile peptides and lipids, sensitive to storage, freeze/thaw cycles, including clotting factors prone to contact activation. Concerned that processing the lymph may have altered its bioactivity, a method was developed to deliver PSML directly from the mesenteric duct from a T/HS rat into the jugular vein of a naïve rat, which provoked ALI. Real time transfusion of sham lymph resulted in lung injury similar to instrumented controls given normal saline only. This observation supports previous work that preshock lymph does not cause inflammation, and may even be protective against ALI.²⁸

Because the lymphatic system is highly permeable, there is little, if any, exclusion of interstitial molecules.¹¹ Mesenteric lymph contains a number of inflammatory products including arachidonic acid,²⁹ serine proteases,³⁰ cytokines,^{8,31} cellular breakdown products,^{32,33} coagulation factors,^{9,10} and modified albumin.³⁴ Bacteria and bacterial products are noticeably absent in PSML.³⁵ Additionally, PSML demonstrates increased levels of anti-inflammatory cytokines²¹ and depletion of protease inhibitors.¹⁰

Phospholipase A₂ (PLA₂), an enzyme abundant in the mammalian gut has been implicated as the source of lipid mediators in the setting of hemorrhagic shock and sepsis.³⁶ PLA₂ produces arachidonic acid, a precursor to a variety of inflammatory products found in the lymph, leukotrienes, thromboxane, and prostaglandins. Arachidonic acid is abundant in PSML, and leukotrienes are increased in the lungs of animals following T/HS, making these lipids the current focus of intensive investigation in our lab.³⁷ Postshock mesenteric lymph may be toxic through a variety of mechanisms, including endothelial inflammation,^{6,26} neutrophil priming,^{38,39,40} enhanced apoptosis,⁴¹ pulmonary epithelial inflammation,⁴² and RBC hemolysis.^{43,32}

In this study, we demonstrate that postshock mesenteric lymph is necessary and sufficient to provoke ALI in the naïve rat. These data further support a central mechanistic role of PSML in the pathogenesis of ALI. Although the postshock lung may also play a role, the cross-transfusion model will facilitate mechanistic investigation, compartmentalizing the gut-lymph versus ischemic lung contributions.

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Figure 1.
The method of real-time cross-transfusion of mesenteric lymph from a donor rat undergoing trauma and hemorrhagic shock to a naïve recipient is illustrated.

Figure 2.

PMN accumulation in the lungs was substantially elevated in the postshock lymph cross-transfused group (9.42 ± 1.55 vs. 2.81 ± 0.82 U/mg lung tissue in postshock vs. sham lymph cross-transfusion, indicated by an asterisk, $p=0.02$). The sham lymph cross-transfusion group levels were comparable to the instrumented controls (2.81 ± 0.82 vs. 2.47 ± 0.02 U/mg lung tissue, indicated by a number sign, $p=0.35$).

Figure 3. Oxidative lung injury was increased in the lymph transfused animals (MDA 0.21 ± 0.03 vs. 0.10 ± 0.01 micromoles MDA per mg lung tissue in lymph cross-transfusion vs. instrumented control, $p = 0.046$)

Figure 4.

Transfusion of mesenteric lymph provoked acute lung injury (BAL protein 0.77 ± 0.18 vs. 0.15 ± 0.02 vs. 0.14 ± 0.06 mg/ml protein in BALF, postshock lymph vs. sham lymph cross-transfusion, indicated by an asterisk, $p=0.004$). The sham lymph cross-transfusion group levels were comparable to the instrumented controls (0.15 ± 0.02 vs. 0.14 ± 0.06 mg/ml, indicated by a number sign, $p=0.38$).