Skin Biopsies Demonstrate Site-Specific Endothelial Activation in Mouse Models of Sepsis

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Abstract

\textbf{Background/Aims:} Skin biopsies allow for direct phenotyping of the endothelium in clinical settings. \textbf{Objectives:} We hypothesize that in murine sepsis endothelial activation is manifested by changes in protein and mRNA expression in skin biopsies, and that such alterations differ from other organs. \textbf{Methods:} In two mouse models of sepsis [endotoxemia and cecal ligation puncture (CLP)], we measured circulating levels of endothelial biomarkers, quantitated mRNA expression of activation markers and assayed for protein expression using immunohistochemistry. \textbf{Results:} Endotoxemic mice demonstrated increased circulating levels of sE-selectin, sICAM-1, sVCAM-1 and sP-selectin at 24 h, while CLP was associated with increased levels of sE-selectin alone. In real-time PCR, mRNA levels for P-selectin, ICAM-1 and PAI-1 were increased in skin from endotoxemic mice. In CLP, mRNA levels for P-selectin, ICAM-1, E-selectin and PAI-1 were elevated, while VCAM-1 expression was reduced in skin. Most, but not all of these changes correlated with alterations in immunohistochemical staining. Expression patterns in skin differed from those in brain, heart, and lung. \textbf{Conclusions:} Skin biopsies demonstrated endothelial cell activation during sepsis. The expression patterns differed by type of sepsis model and between vascular beds of skin, brain, heart, and lung, providing a foundation for identifying skin microvascular-bed-specific molecule signatures.

Introduction

Over 750,000 cases of severe sepsis are diagnosed each year in the United States alone [reviewed in 1], incurring health care costs of USD 16.7 billion annually [2]. The hospital case mortality rate is reported to be between 30 and 50\% [2–7]. Despite significant advances in medical science, the overall survival in sepsis has not improved substantially over time [8]. Early diagnosis of sepsis is a prerequisite for improved survival. Indeed, an important goal is to develop novel diagnostic tools for sepsis that may one day aid the clinician at the bedside.

There is increasing evidence that the endothelium plays an important role in sepsis [reviewed in 1]. Endothelial activation, defined as the phenotypic response of endothelial cells to an inflammatory stimulus, is associ-
ated with changes in hemostatic balance, leukocyte trafficking, vascular permeability, inflammation, and microcirculatory flow. For example, activated endothelial cells express procoagulant properties, as manifest by increased expression of plasminogen activator inhibitor (PAI-1) and reduced expression of the natural anticoagulants, thrombomodulin and endothelial protein C receptor. Leukocyte transmigration is mediated by a multistep cascade that involves the inducible expression of selectins (E-selectin and P-selectin) and cell adhesion molecules [intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1]. Although these changes evolved as an adaptive host response to infection or injury, they may become excessive, sustained or systemically disseminated. The resulting dysfunctional phenotype is an important determinant of sepsis morbidity and mortality.

Given the central role of the endothelium in sepsis pathophysiology, there is an urgent need for endothelial assays in the clinic. In states of activation, endothelial cells release mediators into the bloodstream, including PAI-1, tissue-type plasminogen activator and von Willebrand factor (vWF). In addition, selectins and cell adhesion molecules may be cleaved from the cell surface, resulting in soluble forms of these molecules. Efforts to diagnose endothelial dysfunction have largely focused on the measurement of these circulating mediators and many studies have demonstrated that one or more of these markers are elevated in cohorts of patients with severe sepsis [9–13]. However, to date, there is no evidence that any one marker or panel of endothelial-derived markers is useful for diagnosing or staging sepsis in individual patients.

A limitation of circulating mediators is that they are surrogate markers for the intact endothelium. Although their measurement provides information about the net biomarker response to sepsis, circulating levels do not directly reflect endothelial phenotypes. One approach for directly surveying the endothelium is to obtain biopsies from patients with sepsis. Skin biopsies provide sufficient material to carry out protein and/or gene expression analyses. An important caveat when approaching a single organ such as the skin is that endothelial cells are heterogeneous in health and disease [reviewed in 14–17]. Therefore, any changes observed in the vasculature of the skin may differ from those found in other vascular beds. The goal of the present study was to explore the diagnostic potential of skin biopsies in sepsis and to further study pathophysiology of the endothelial cell response in sepsis. Specifically, we tested the hypotheses that (1) murine sepsis-induced endothelial activation is manifested by demonstrable alterations in protein and mRNA expression in skin biopsies and (2) the endothelial response varies between the vascular beds of the skin and other vital organs.

Methods

**Mouse Models of Endotoxemia and Cecal Ligation Puncture**

All animal studies were carried out in accordance with guidelines established by the Beth Israel Deaconess Medical Center Animal Care and Use Committee. Mouse models of endotoxemia and cecal ligation puncture (CLP) were performed as previously described [15], using 22- to 24-gram 8-week-old male C57BL/6 mice. For endotoxemia, mice were injected intraperitoneally (i.p.) with lipopolysaccharide (LPS; 18 mg/kg weight) from *Escherichia coli* serotype 0111:B4 (Sigma-Aldrich, St. Louis, Mo., USA) phenol extracted or an equal volume of saline (control). To perform the CLP model, mice were anesthetized with isoflurane. After shaving the abdomen, a 2-cm midline incision was created under aseptic conditions to expose the cecum and adjoining intestine. Approximately 25–30% of the cecum was ligated distal to the ileocecal valve with a 4-0 vicryl suture, and punctured with a 21-gauge needle. The cecum was then gently squeezed to extrude a small amount of feces to ensure patency of the perforation sites and returned to the peritoneal cavity. Mice received 1 ml of saline subcutaneously for fluid resuscitation at the time of closure and 0.1 mg/kg buprenorphine subcutaneously every 12 h to minimize distress.

**Circulating Marker Assays**

sE-selectin, sP-selectin, ICAM-1, VCAM-1, VEGF, and interleukin (IL)-6 were assayed in heparinized plasma using commercially available ELISA kits (R&D Systems, Minneapolis, Md., USA), according to the manufacturer’s instructions. Blood was obtained via cardiac puncture at 24 h following LPS injection or CLP.

**Tissue Harvesting**

Mice were anesthetized 24 h following LPS injection or CLP. After shaving the dorsal hair, the skin, heart, lung and brain were removed and either snap-frozen for real-time PCR analyses or embedded in OCT compound for histological analyses.

**Tissue RNA Isolation and Real-Time PCR**

Tissue RNA isolate and quantitative real-time PCR were carried out as previously described [18]. Tissue RNA was isolated using Trizol (Invitrogen, Carlsbad, Calif., USA), and RNA mini preparation kit (Qiagen, Valencia, Calif., USA). For quantitative real-time PCR, total RNA was prepared using the RNeasy RNA extraction kit with DNase I treatment following the manufacturer’s instructions (Qiagen). To generate cDNA, total RNA (100 ng) from each of triplicate samples was mixed and converted into cDNA using random primers and Superscript III reverse transcriptase (Invitrogen). All cDNA samples were aliquotted and stored at −80°C. Primers were designed using the Primer Express oligo design software (Applied BioSystems, Foster City, Calif., USA).
USA), and synthesized by Integrated DNA Technologies (Coralville, Iowa, USA). All primer sets were subjected to rigorous database searches to identify potential conflicting transcript matches to pseudogenes or homologous domains within related genes as described previously [18]. The SYBR Green I assay and the ABI Prism 7500 Sequence Detection System were used for detecting real-time PCR products from the reverse-transcribed cDNA samples using a master template. PCR reactions for each sample were performed in duplicate and copy numbers were measured as described previously. The level of target gene expression was normalized against the 18S rRNA expression in each sample and the data presented as mRNA copies per 10^6 18S rRNA copies.

**Immunohistochemistry**

Immunohistochemistry for inflammatory and coagulation markers was carried out on 5-μm frozen sections from heart, lung and liver of control and LPS-treated mice, as previously described [15]. Antibodies included rabbit polyclonal anti-mouse P-selectin antibody (Chemicon International, Temecula, Calif., USA), rabbit polyclonal anti-mouse PAI-1 antibody (Innovative Research, Southfield, Mich., USA), rabbit polyclonal anti-mouse Cox-2 antibody (Cayman Chemical, Ann Arbor, Mich., USA), rat monoclonal anti-mouse E-selectin antibody (BD Biosciences Pharmingen, San Diego, Calif., USA), rat monoclonal anti-mouse VCAM-1 antibody (Chemicon International), and rat monoclonal anti-mouse ICAM-1 antibody (Serotec, Oxford, UK). Anti-rat IgG antibody conjugated with FITC and anti-rabbit IgG antibody conjugated with Cy3 (Invitrogen) were used as secondary antibodies. For colocalization studies, a rat monoclonal anti-mouse CD31 antibody (BD Biosciences Pharmingen) was used in double immunofluorescent stains with rabbit polyclonal antibodies (P-selectin, PAI-1 and Cox-2), and a rabbit polyclonal anti-mouse vWF antibody (Abcam, Cambridge, Mass., USA) was combined with the rat monoclonal anti-mouse antibodies (E-selectin, VCAM-1 and ICAM-1). Secondary antibodies included anti-rabbit IgG antibody conjugated with Cy3 (Invitrogen), anti-rat IgG conjugated with FITC (Jackson Immunoresearch Laboratories, West Grove, Pa., USA) and anti-mouse IgG conjugated with FITC (Sigma). Representative sections are shown for experimental models.

**Statistical Analysis**

Due to the small sample size, we used the nonparametric median test for statistical analysis in mouse cytokine and gene expression studies. We also report the descriptive statistics of mean, standard deviation, and fold change.

**Results**

**LPS and CLP Result in Differential Induction of Circulating Mediators**

Mice were injected i.p. with 18 mg/kg LPS or subjected to CLP (n = 6/group). Twenty-four hours later, blood was collected by cardiac puncture and processed for plasma. ELISA was used to measure circulating markers that are derived, at least in part, from endothelial cells, including sE-selectin, sICAM-1, sVCAM-1, and sP-selectin. As a control for severity of illness, we assayed for circulating levels of IL-6, VEGF and sFlt-1 [18]. Compared with saline-injected controls, LPS-treated mice demonstrated significantly elevated circulating levels of sE-selectin (5.0-fold), sICAM-1 (6.1-fold), sVCAM-1 (6.1-fold), and sP-selectin (17-fold) at 24 h (p < 0.05 for all comparisons) (fig. 1). Consistent with our previous findings, circulating levels of VEGF, sFlt-1 and IL-6 were also increased [18]. Compared with sham-operated controls, CLP resulted in significantly increased levels of sE-selectin, but not sICAM-1, sVCAM-1 or sP-selectin. Similar to endotoxemia, CLP was associated with increased levels of VEGF, sFlt-1 and IL-6. Interestingly, IL-6 levels were induced to a greater extent in CLP, whereas E-selectin levels were higher in endotoxemia. These data suggest that LPS and CLP result in distinct patterns of induction of circulating endothelial-derived mediators.

**LPS and CLP Result in Organ-Specific Changes in mRNA Expression of Endothelial-Derived Inflammatory and Procoagulant Mediators**

We next wished to determine whether the altered patterns of circulating markers in septic mice correlated with changes in mRNA expression in the skin. Skin biopsies were performed on LPS-treated mice or mice subjected to CLP (n = 3/group) at 24 h and assayed for mRNA expression by real-time PCR. In addition to measuring mRNA levels for E-selectin, ICAM-1, VCAM-1, and P-selectin, we also assayed for expression of the activation markers, PAI-1 and cyclooxygenase (COX)-2 [18]. Both LPS and CLP were associated with increased expression of P-selectin (10.3-fold and 7.9-fold, respectively), ICAM-1 (10-fold and 7.3-fold, respectively), and PAI-1 (4.6-fold and 4.5-fold, respectively) (fig. 2; online suppl. table 1, www.karger.com/doi/10.1159/000210662). All changes were statistically significant (p < 0.03). CLP alone resulted in increased expression of E-selectin mRNA (6.7-fold, p < 0.03) and decreased expression of VCAM-1 (5-fold, p < 0.03). COX-2 mRNA levels did not change in either model of sepsis (1.6-fold and 1.1-fold, p = 0.46 for both LPS and CLP). These findings demonstrate that the skin is involved in the host response to infection and that the response differs according to the nature of the underlying insult.

Next, we sought to determine whether the expression patterns in the skin were similar to those in other organs of septic mice. To that end, we compared expression levels of E-selectin, P-selectin, ICAM-1, VCAM-1,
COX-2 and PAI-1 in the heart, lung and brain. Compared with saline-injected controls, LPS-treated mice demonstrated increased expression of E-selectin, P-selectin, ICAM-1 and PAI-1 in all three organs (p < 0.03 for all measurements) (fig. 2; online supplement table 1, www.karger.com/doi/10.1159/000210662). LPS induced expression of VCAM-1 in the heart and lung, but not the brain. COX-2 was increased in the heart and brain, but not the lung of LPS-treated mice. Similar to LPS, CLP resulted in increased expression of E-selectin, P-selectin, ICAM-1, and COX-2 in all three organs (p < 0.03). CLP was not associated with changes in VCAM-1 or PAI-1 in the heart, lung or brain. Thus, sepsis is associated with model- and organ-specific changes in vascular markers.

Immunohistochemistry of Dermal Microvascular Demonstrates Evidence for Site-Specific Endothelial Activation

To correlate mRNA with protein expression and to localize the various markers in the skin, biopsies from septic animals were processed for immunofluorescent staining. Consistent with the real-time PCR results, P-selectin and ICAM-1 were increased in both LPS-treated mice and in CLP, while E-selectin was increased in CLP only (fig. 3). In contrast with real-time PCR, PAI-1 was increased in CLP, but not in endotoxemia, and there was no obvious reduction in the expression of VCAM-1 in CLP. All markers were colocalized with endothelial cell-specific vWF or CD31.

Discussion

We have shown that murine sepsis is associated with endothelial cell activation as assayed by skin biopsies. However, expression of activation markers in the skin did not always correlate with changes in circulating levels or with expression in other organs. Moreover, the patterns of expression differed between LPS and CLP models. These data are consistent with our knowledge that endothelial phenotypes differ in space and time and they provide a foundation for identifying skin microvascular-bed-specific molecule signatures inherent in the host response to infection.

Previous studies in animal and human models of sepsis have demonstrated elevated circulating levels of sE-selectin, sICAM-1, sVCAM-1, and sP-selectin [19, 20]. Our findings are consistent with these data, with the exception that CLP was not associated with increased sICAM-1 or sVCAM-1 at 24 h. It is possible that circulat-
ing sICAM-1 and sVCAM-1 levels were increased at different time points in the CLP model. Alternatively, the discordant findings between endotoxemia and CLP may reflect fundamental differences in the two sepsis models.

It is noteworthy that circulating levels of soluble mediators did not always reflect local changes in mRNA or protein expression. For example, while ICAM-1 protein and/or mRNA were increased in the skin, heart, lung and brain of mice subjected to CLP, circulating levels of sICAM-1 levels were unaltered. As another example, LPS-mediated induction of sE-selectin was associated with elevated mRNA levels of E-selectin in the heart, lung and brain, but not the skin. One explanation for these latter results is that the release, half-life and/or clearance of the soluble molecules are uncoupled from the mechanisms controlling mRNA and/or protein expression. Another possibility is that dermal endothelial cells do not contribute to the circulating pool of sE-selectin.

Previous studies have demonstrated a similar discordance between circulating levels and gene/protein expression at the tissue level. For example, Faust et al. [21] obtained skin biopsies in a series of patients with meningococcemia. Histology demonstrated evidence of thrombosis with sICAM-1 and sVCAM-1 levels increased at different time points in the CLP model. Alternatively, the discordant findings between endotoxemia and CLP may reflect fundamental differences in the two sepsis models.

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Fig. 3. Immunofluorescent detection of endothelial-associated inflammatory and hemostatic markers in the skin of septic mice. Mice were administered saline (control, CTL) or LPS i.p. Alternatively, they were subjected to CLP or sham surgery (sham). Skin biopsies were taken at 24 h and processed for immunofluorescent detection of P-selectin (a–d), ICAM-1 (e–h), and E-selectin (i–l). For colocalization, the sections were counterstained with antibodies to vWF or CD31 to colocalize in endothelium. Scale bars = 120 μm (a, c, e, g, i, k) and 40 μm (b, d, f, h, j, l).
bosis and inflammation, and reduced staining of thrombomodulin and the endothelial protein C receptor [21]. The downregulation of dermal thrombomodulin expression contrasted with an increase in circulating levels of soluble thrombomodulin [21]. In a study of critically ill patients, circulating levels of sE-selectin, sP-selectin, sICAM-1 and sVCAM-1 were markedly elevated in patients with septic shock, whereas only sP-selectin was increased in the context of trauma hemorrhage [22]. However, skin biopsies revealed comparable upregulation of ICAM-1, VCAM-1 and E-selectin in both groups [22]. Malaria and/or sepsis was associated with an induction of ICAM-1 in dermal vessels and the focal appearance of E-selectin and VCAM-1 in a subset of endothelial cells [23]. Although the circulating levels of these markers correlated with the severity of malarial disease, the endothelial phenotype in situ did not. The discordance between levels of circulating markers and mRNA/protein expression is not unique to the skin. For example, in tumor necrosis factor (TNF)-treated mice, elevated levels of sICAM-1 are not reflected by changes in the density of membrane-bound ICAM-1 in the lung, small intestine and spleen [24].

The pattern of mRNA and protein expression in the skin differed from that of other tissues. These findings are consistent with previous studies in mice demonstrating organ-specific differences in basal and LPS- and/or TNF-inducible expression of E-selectin, P-selectin, ICAM-1, and VCAM-1 at both a protein and mRNA level [18, 25, 26]. In a mouse model of CLP membrane expression of E-selectin and P-selectin was also heterogeneous between organs [27, 28]. Our finding that the skin, like other organs, displays an overlapping but distinct activation phenotype underscores the potential limitations of extrapolating results from skin biopsies to other vascular beds in the setting of sepsis.

Caution is required in extrapolating the results of animal models of sepsis to humans [reviewed in 29]. Although the endotoxemia model is simple, and highly reproducible, it is an imperfect surrogate for clinical infection. CLP mimics only one form of human sepsis, namely ruptured appendicitis or perforated diverticulitis. The severity of endotoxemia and CLP may be varied according to the dose of LPS and the size of the needle puncture, respectively. However, we chose to assay for endothelial response using a single dose of LPS and a constant puncture in CLP. Moreover, we assayed blood and tissues for a limited number of activation markers at a single time point (24 h). In the future, it will be important to determine the effect of disease severity and time course on a broader panel of endothelial-derived molecules.

We have shown that sepsis is associated with endothelial activation in the dermal microvasculature and that
the response varies according to the nature of the insult. One avenue for further investigation is to identify specific molecular signatures that will allow the clinician to predict the underlying pathogen and/or assess the severity of illness in sepsis. Additionally, skin biopsies may prove useful in identifying those patients who will benefit most from endothelial sparing strategies (e.g. VEGF inhibition).

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References