Vascular endothelial growth factor is an important determinant of sepsis morbidity and mortality

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Sepsis, the systemic inflammatory response to infection, is a leading cause of morbidity and mortality. The mechanisms of sepsis pathophysiology remain obscure but are likely to involve a complex interplay between mediators of the inflammatory and coagulation pathways. An improved understanding of these mechanisms should provide an important foundation for developing novel therapies. In this study, we show that sepsis is associated with a time-dependent increase in circulating levels of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) in animal and human models of sepsis. Adenovirus-mediated overexpression of soluble Flt-1 (sFlt-1) in a mouse model of endotoxemia attenuated the rise in VEGF and PlGF levels and blocked the effect of endotoxemia on cardiac function, vascular permeability, and mortality. Similarly, in a cecal ligation puncture (CLP) model, adenovirus–sFlt-1 protected against cardiac dysfunction and mortality. When administered in a therapeutic regimen beginning 1 h after the onset of endotoxemia or CLP, sFlt peptide resulted in marked improvement in cardiac physiology and survival. Systemic administration of antibodies against the transmembrane receptor Flk-1 but not Flt-1 protected against sepsis mortality. Adenovirus-mediated overexpression of VEGF but not PlGF exacerbated the lipopolysaccharide-mediated toxic effects. Together, these data support a pathophysiological role for VEGF in mediating the sepsis phenotype.

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Abbreviations used: CLP, cecal ligation puncture; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule 1; PlGF, placental growth factor; sFlt, soluble Flt; TNFR, TNF receptor; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor.

More than 750,000 cases of severe sepsis are diagnosed each year in the United States alone (for review see reference 1). The incidence is predicted to increase by 1.5% per year, owing to aging of the population and the wider use of immunosuppressive agents and invasive procedures (2). It is widely believed that further gains in sepsis therapy will depend on the successful targeting of the host response. Thus far, efforts to block one or another component of the inflammatory or coagulation pathways have had little impact on survival. Of the many agents and drugs that have been tested, only two have demonstrated efficacy in phase 3 clinical trials: mouse monoclonal antibody to human TNF-α and human recombinant activated protein C (3, 4). However, despite these interventions, mortality rates remain high at 25–30%. Clearly, future advances in therapy will be contingent upon an improved understanding of sepsis pathophysiology.

Vascular endothelial growth factor (VEGF)/vascular permeability factor was first identified and characterized as a potent stimulator of endothelial permeability (5). VEGF was subsequently reported to promote proliferation, migration, and survival of endothelial cells (6). VEGF (also termed VEGF-A) is a member of a growing family of related proteins that include VEGF-B, -C, -D, and placental growth factor

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(PlGF; for review see reference 7). VEGF binds to two transmembrane receptors, namely Flt-1 and Flk- 1, whereas PlGF binds to Flt-1 alone. Within the vessel wall, Flk-1 is selectively expressed in endothelium. Flt-1 is present on both endothelial cells and monocytes.

In addition to its role in promoting endothelial permeability and proliferation, VEGF may contribute to inflammation and coagulation. For example, under in vitro conditions, VEGF induces the expression of cell adhesion molecules (E-selectin, intercellular adhesion molecule 1 [ICAM-1], and vascular cell adhesion molecule 1 [VCAM-1]) in endothelial cells and promotes the adhesion of leukocytes (8, 9). Moreover, VEGF signaling up-regulates tissue factor mRNA, protein, and procoagulant activity (10). These proinflammatory/procoagulant effects of VEGF are mediated, at least in part, by the activation of NF-κB, Egr-1, and NFAT transcription factors. VEGF has been implicated as a pathophysiological mediator in several human disease states, including rheumatoid arthritis, cancer, and inflammatory bowel disease (11–13). Recently, two independent studies reported an association between human severe sepsis/septic shock and elevated circulating levels of VEGF (14, 15). We designed this study to test the hypothesis that VEGF plays a pathogenic role in mediating the sepsis phenotype.

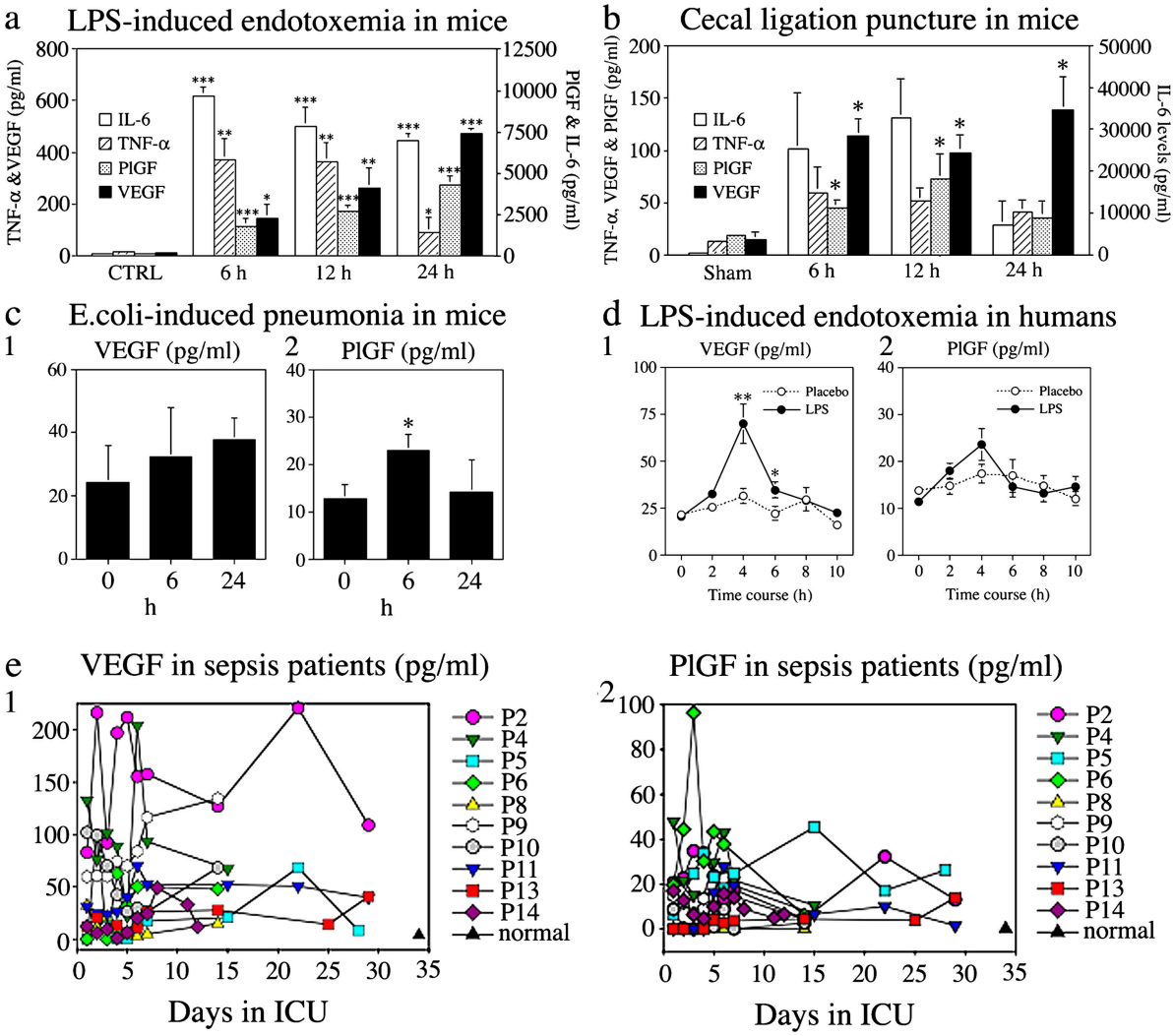


Figure 1. Circulating levels of VEGF and PlGF in mouse and human models of sepsis. (a) The levels of IL-6 (plasma), TNF-α (serum), PlGF (plasma), and VEGF (plasma) in LPS-injected mice at the time points indicated; CTRL, control normal saline-injected mice. (b) Same as in a but in a CLP mouse model; sham, sham-operated control mice. (c) PlGF and VEGF levels in a mouse pneumonia model at 6 and 24 h. (d) PlGF and VEGF levels in a human endotoxemia model at the time points indicated. Subjects were administered LPS or saline (placebo). (e) Plasma PlGF and VEGF levels in randomly chosen patients with severe sepsis plotted against time in the intensive care unit (ICU). Each line represents an individual patient (P). Mouse data are expressed as means ± SD (a and c) or as means ± SEM (b) of three independent experiments. Human data from the endotoxemia model are expressed as means ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

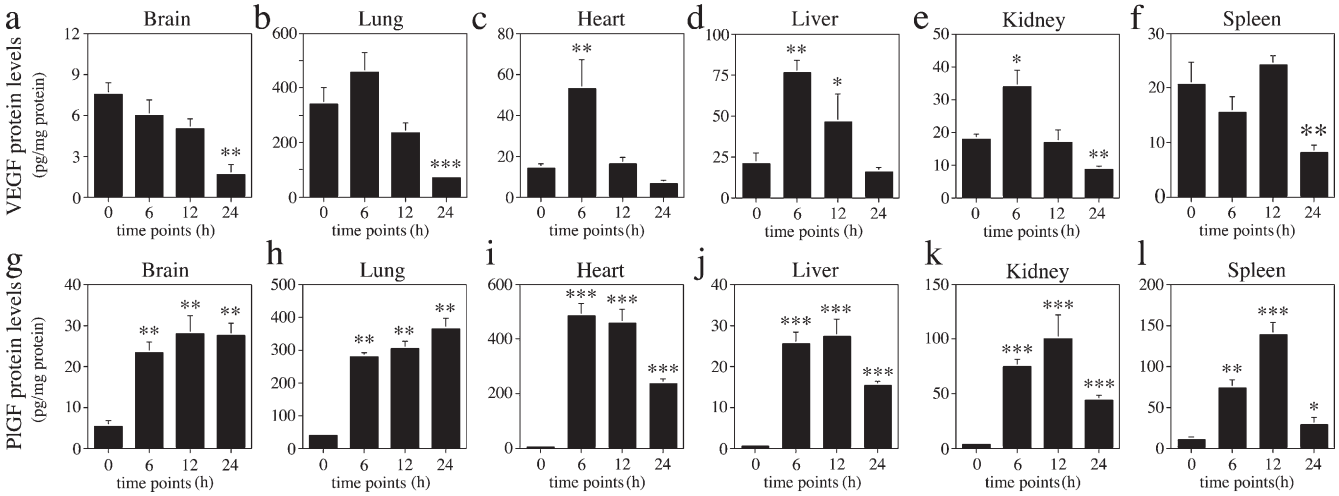


Figure 2. VEGF and PlGF protein levels in a mouse model of endotoxemia. Results of ELISA for VEGF (top) and PlGF (bottom) in tissues from mice injected with or without LPS at the time points indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$. Error bars represent SD.

RESULTS

Circulating levels of VEGF and PlGF in animal and human models of sepsis

To confirm the association between sepsis and circulating VEGFs, we assayed plasma levels of VEGF (and the related growth factor PlGF) in both mouse and human models of infection. As shown in Fig. 1 a, i.p. administration of LPS in mice resulted in a time-dependent increase in plasma VEGF and PlGF concentrations, with peak levels (477 and 4311 pg/ml, respectively) occurring at 24 h. In contrast, circulating levels of IL-6 and TNF-α were maximal at the earliest time point measured (6 h). In a cecal ligation puncture (CLP) model of sepsis, peak levels of VEGF (137.26 pg/ml) and PlGF (71.25 pg/ml) occurred at 24 and 12 h, respectively (Fig. 1 b). In a mouse model of *Escherichia coli* pneumonia, plasma VEGF levels were not significantly altered (Fig. 1 c [1]), whereas PlGF levels were increased (23.01 pg/ml) at 6 h (Fig. 1 c [2]). In human subjects, the systemic administration of LPS resulted in elevated circulating levels of VEGF and PlGF (Fig. 1 d and Table S1, available at http://www.jem.org/cgi/content/full/jem.20060375/DC1), with peak levels (70 and 23.5 pg/ml, respectively) occurring at 4 h in contrast to TNF-α and IL-6, which peaked at 1.5 and 2.5 h, respectively (not depicted). Plasma levels of VEGF and PlGF were measured in 10 patients with severe sepsis and 10 healthy volunteers. At study entry, VEGF levels in the patients (mean and SD = 46.49 ± 46.17 pg/ml) were significantly higher than in the healthy volunteers (mean and SD = 3.83 ± 3.16 pg/ml; $P = 0.009$). Similarly, PlGF levels in the patients at study entry (mean and SD = 13.52 ± 14.55 pg/ml) were significantly higher compared with healthy volunteers (mean and SD = 0.18 ± 0.58 pg/ml; $P = 0.009$). In most cases (8/10), the VEGF and PlGF levels remained elevated during their intensive care unit (ICU) stay (in some cases, up to 29 d).

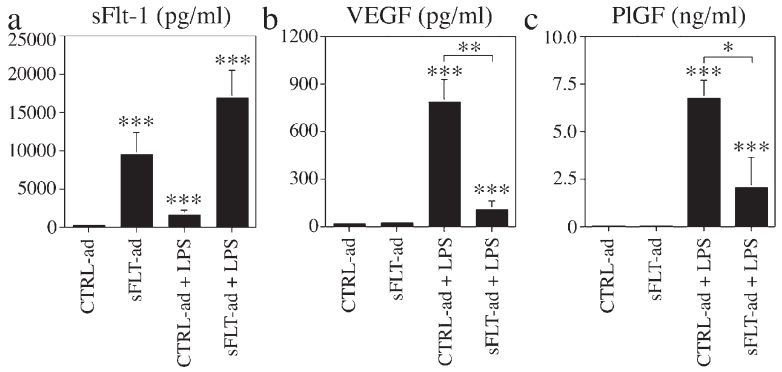


Figure 3. Effect of sFlt-1 overexpression on circulating levels of sFlt, VEGF, and PlGF in a mouse model of endotoxemia. Mice were injected with adenovirus overexpressing GFP (CTRL-ad) or sFlt-1 (sFlt-ad). 3 d later, the animals were administered saline (control) or LPS i.p. Blood samples were taken 24 h later and assayed for plasma levels of sFlt-1, free VEGF, and free PlGF. Data are expressed as means ± SD (error bars) of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

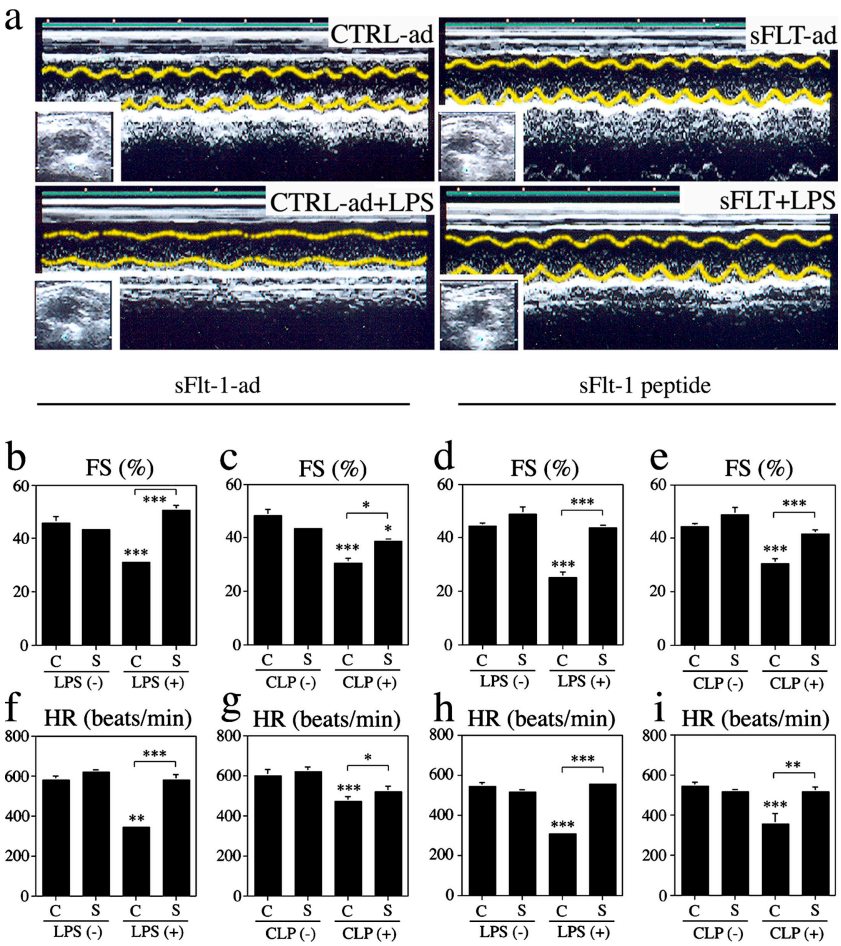


Figure 4. Effect of sFlt-1 overexpression on cardiac function in mouse models of sepsis. Mice were injected with adenovirus overexpressing GFP (CTRL-ad; C) or sFlt-1 (sFlt-ad; S) in a–c, f, and g. 3 d later, the animals were administered saline (control) or LPS i.p. or were subjected to CLP. Alternatively, mice were injected i.v. with PBS (C) or sFlt-1 peptide (S) 1 h after LPS injection or CLP procedure (d, e, h, and i). An echocardiogram and electrocardiogram were performed 24 h after saline or LPS injections or 26 h after CLP. (a) Representative M modes and two-dimensional images (insets) from an echocardiogram in an endotoxemia model. (b and d) Quantitative analysis of the fractional shortenings (FS) from an echocardiogram in an endotoxemia model. (f and h) Heart rate (HR) measurements on an electrocardiogram in an endotoxemia model. (c and e) Quantitative analysis of the fractional shortenings from an echocardiogram in a CLP model. (g and i) Heart rate measurements on an electrocardiogram in a CLP model. Analysis of variance was used for statistic analysis. Error bars represent SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

The maximum VEGF and PlGF levels were 221 and 96 pg/ml, respectively (Fig. 1 e).

To determine the source of elevated growth factor concentrations, VEGF and PlGF levels were assayed in various tissues using ELISA. Systemic administration of LPS in mice resulted in increased VEGF protein levels at 6 h in the liver (3.8-fold), kidney (1.9-fold), and heart (3.7-fold) but decreased levels in the brain, lung, and spleen (Fig. 2). PlGF protein was increased in all tissues examined, including the brain (4.8-fold), lung (7.1-fold), heart (71.8-fold), liver (35.2-fold), kidney (28.5-fold), and spleen (28.4-fold; Fig. 2). There were no differences in VEGF protein in the serum compared with plasma of LPS-treated or control mice, arguing against an important contribution of platelets to the plasma VEGF levels in endotoxemia (not depicted). Based on the aforementioned findings, we conclude that sepsis is associated with increased expression and circulating levels of VEGF and PlGF.

Effect of sFlt-1 on endotoxemia-mediated induction of circulating VEGF and PlGF levels

To determine whether increased levels of VEGF and/or PlGF are important determinants of sepsis pathophysiology, mice were injected i.v. with adenovirus-overexpressing soluble Flt-1 (sFlt-1) or GFP (control) before treatment with LPS. sFlt-1 is a natural splice variant of the cell surface receptor Flt-1 that binds to free VEGF-A, VEGF-B, and PlGF, thus blocking their interaction with cell surface receptors (16). Previous studies have shown that Ad–sFlt-1 (adenovirus–sFlt-1) transduces liver hepatocytes in vivo and results in high circulating levels of sFlt-1 for up to 2 wk after injection

(17, 18). As shown in Fig. 3, Ad–sFlt-1–treated mice displayed elevated levels of sFlt-1 in the serum compared with Ad-GFP–treated animals. Importantly, Ad–sFlt-1 significantly inhibited LPS-mediated induction of free VEGF ($P < 0.01$) and PlGF ($P < 0.05$), whereas control adenovirus had no such effect. Interestingly, in control animals, LPS increased circulating levels of endogenous sFlt-1 (from 159.62 to 1,585.40 pg/ml) and further increased total sFLT-1 in Ad–sFlt-1–treated animals (Fig. 3).

Effect of sFlt-1 on endotoxemia-mediated cardiac dysfunction and barrier dysfunction

Systemic administration of LPS or CLP in mice resulted in a marked depression of cardiac function (Fig. 4), as indicated by reduced fractional shortening (Fig. 4, b–e) and heart rate (Fig. 4, f–i) on echocardiography, and was associated with PR interval prolongation on an electrocardiogram (not depicted). These effects were completely blocked by adenovirus-mediated overexpression of sFlt-1 (Fig. 4, b and f) but not GFP (control) or PBS (control) injection. Similar results were demonstrated in the CLP model (Fig. 4, c and g). LPS administration resulted in an organ-specific loss of barrier function, with increased extravasation of Evans blue dye in the lungs, liver, and kidney but not in the brain, heart, or spleen (Fig. 5). Overexpression of sFlt-1 completely blocked LPS-induced vascular leak in the liver and lung but only partially inhibited the effect of LPS on kidney permeability.

Effect of sFlt-1 on endotoxemia-mediated changes in mRNA expression of inflammatory and coagulation mediators

Exogenous sFlt-1 had no effect on the initial 6-h peak of plasma cytokines induced during endotoxemia (Fig. 6). However, cytokine levels fell more rapidly in animals that had received Ad–sFlt-1 compared with control adenovirus. Ad–sFlt-1 significantly blunted LPS-mediated induction of TNF-α, IL-1β, IL-6, and D-dimers at 12 and/or 24 h ($P < 0.05$ for TNF-α and D-dimers at 12 h; $P < 0.01$ for IL-1β and IL-6 at 12 h and D-dimers at 24 h; $P < 0.0001$ for IL-6 at 24 h; Fig. 6, a–f). LPS administration in triple mutant mice that were null for IL-1RI and the two TNF-α receptors (TNFRs), TNFR1 and TNFR2, resulted in a significantly lower induction of VEGF ($P < 0.05$) and PlGF ($P < 0.01$) compared with wild-type controls at 24 h (Fig. 6, g–i). Together, these findings suggest that VEGF and PlGF lie downstream of commonly implicated sepsis-induced cytokines.

Endotoxemia in mice was associated with increased mRNA expression of inflammatory and procoagulant molecules (Fig. 7 A and Table S2, available at http://www.jem.org/cgi/content/full/jem.20060375/DC1). As a negative control, VE-cadherin mRNA levels did not increase in any tissue examined (not depicted). In immunofluorescent studies, most of these inflammatory mediators were localized primarily in the endothelium (Fig. 7 B). In the heart, E-selectin, P-selectin, ICAM-1, VCAM-1, and Cox-2 were induced in small venules. ICAM-1 and VCAM-1 were also increased in capillary endothelium. PAI-1 was induced in capillaries alone. In the brain, all of the aforementioned mediators were induced in the endothelium of venules but not capillaries. In the lung, E-selectin, P-selectin, ICAM-1, VCAM-1, and Cox-2 were increased in venular endothelium. ICAM-1 and PAI-1 were increased in lung parenchyma. The immunohistochemical analyses demonstrated that the endothelial expression of these activation markers during endotoxemia was abrogated by the administration of sFlt-1 (Fig. 7 B).

To gain further insights into the mechanisms by which VEGF mediates sepsis morbidity and mortality, human

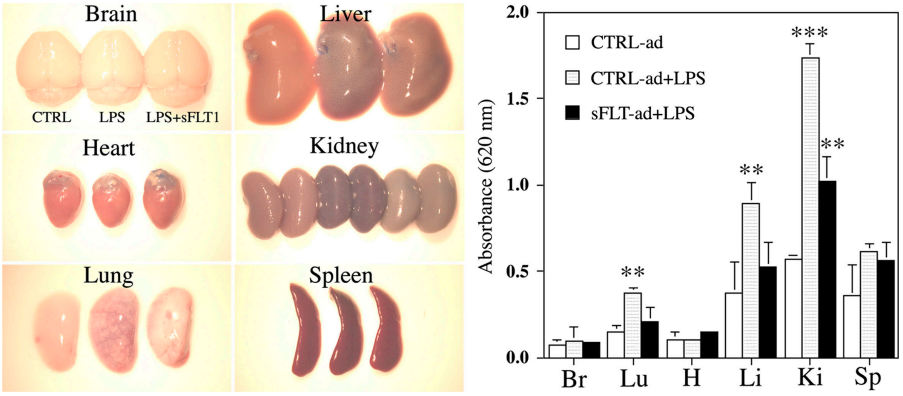


Figure 5. Effect of sFlt-1 overexpression on vascular permeability in a mouse model of endotoxemia. Mice were injected with adenovirus overexpressing GFP (CTRL-ad) or sFlt-1 (sFlt-ad). 3 d later, the animals were administered saline (control) or LPS i.p. 24 h later, the animals were injected i.v. with 0.1 ml of 1% Evans blue dye. After 40 min, the mice were perfused, and the brain (Br), lung (Lu), heart (H), liver (Li), kidney (Ki), and spleen (Sp) were harvested and incubated in formamide for 3 d to elute Evans blue dye. (left) Whole mount photomicrographs of organs. In each group, the left-most specimen is from control untreated mice, the middle specimen is from control adenovirus-treated endotoxemic mice, and the right specimen is from Ad–sFlt-1–treated endotoxemic mice (in the case of the kidney, two specimens are shown for each condition). (right) Quantitation of Evans blue extravasation (OD at 620 nm). Error bars represent SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

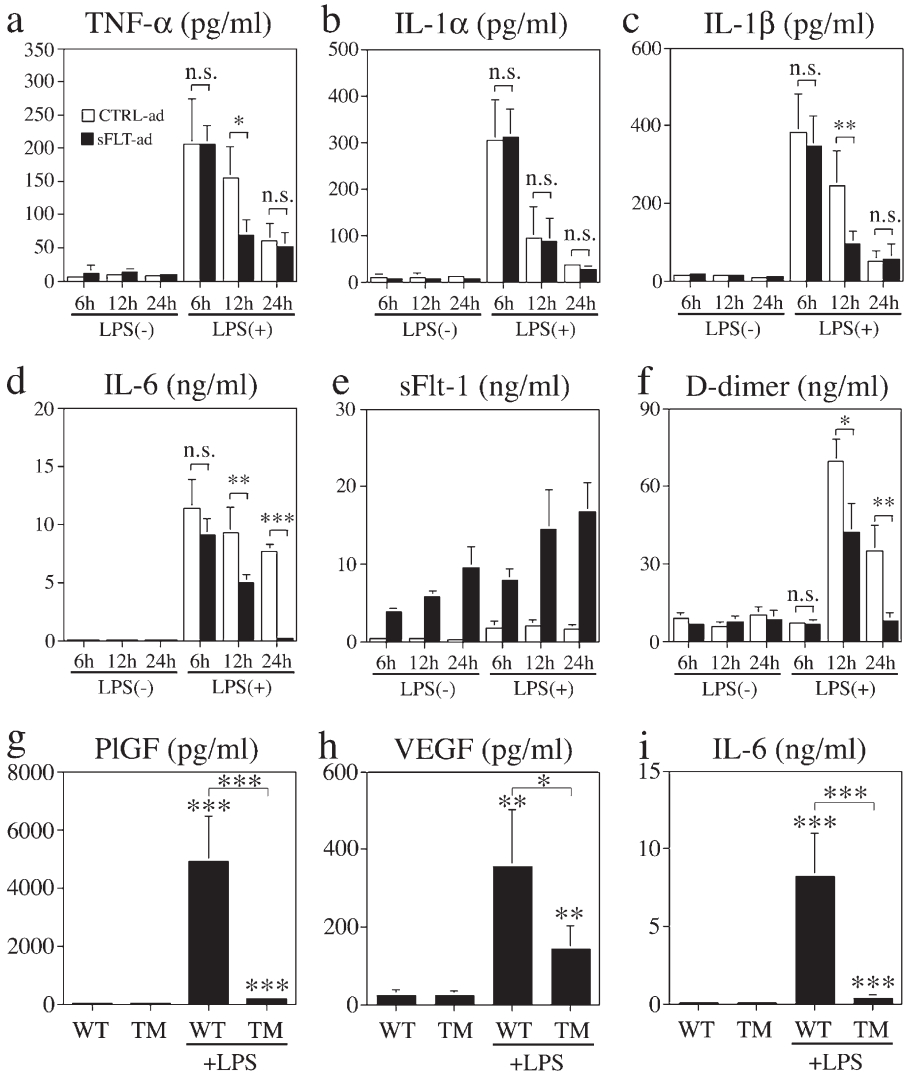


Figure 6. Effect of sFlt-1 overexpression on circulating cytokine levels and D-dimers in a mouse model of endotoxemia. (a–f) Mice were injected with adenovirus overexpressing GFP (CTRL-ad) or sFlt-1 (sFlt-ad). 3 d later, the animals were administered saline (control) or LPS i.p. Serum or plasma levels of TNF-α, IL-1α, IL-1β, IL-6, sFlt-1, and D-dimer were measured at 6, 12, and 24 h. (g–i) Circulating levels of VEGF and IL-6 levels in triple mutant mice (IL-1−/−, TNFR1−/−, and TNFR2−/−) with LPS-induced endotoxemia. Plasma levels of VEGF, PlGF, and IL-6 in LPS-injected wild-type (WT) or triple mutant (TM) mice 24 h after i.p. injection of saline (control) or LPS. Error bars represent SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

umbilical vein endothelial cells (HUVECs) were incubated for 4 h with TNF-α, VEGF, and PlGF either alone or in combination. VEGF but not PlGF induced the expression of several genes, including VCAM-1 (9.3-fold), Cox-2 (2.77-fold), and tissue factor (16.37-fold; Fig. 8). Endothelial cells treated with 0.1 ng/ml TNF-α alone demonstrated a 29.35-fold increase in VCAM-1, a 2.13-fold increase in tissue factor, and no change in Cox-2 mRNA levels. In contrast, 0.1 ng/ml TNF-α plus 100 ng/ml VEGF resulted in a significantly increased expression of VCAM-1, Cox-2, and tissue factor over and above the effects of VEGF alone ($P < 0.01$). These data suggest that VEGF sensitizes endothelial cells to low concentrations of TNF-α.

Effect of sFlt-1 and anti-VEGF receptor antibodies on sepsis mortality

Survival studies were performed in 20 mg/kg LPS-treated mice overexpressing GFP (control), VEGF, PlGF, or sFlt (Fig. 9 a). In the control group, 13/24 (54.2%) mice died from endotoxemia. sFlt-1 resulted in a significant reduction in mortality (2/24 animals died; $P < 0.0008$). Overexpression of VEGF (mean plasma levels of 5.08 ng/ml) resulted in a marked increase in LPS sensitivity (100% mortality), whereas overexpression of PlGF (mean plasma levels of 28.23 ng/ml) had no such effect. sFlt-1 also improved survival in the CLP model of sepsis (Fig. 9 b). A total of 4/15 (27%) mice overexpressing sFlt-1 died from CLP-induced sepsis compared

with 12/16 (75%) in the GFP-expressing control group ($P = 0.0063$; Fig. 9 b). To further verify a primary role for VEGF, animals were pretreated with neutralizing antibodies against Flt-1 or Flk-1. In these experiments, anti–Flk-1 antibodies but not anti–Flt-1 antibodies reduced mortality in the mouse endotoxemia model (Fig. 9, c and d, respectively). Together, these findings suggest that VEGF-A is a critical determinant of the sepsis phenotype.

Therapeutic effect of sFlt-1 peptide on sepsis morbidity and mortality

To determine whether VEGF inhibition after the onset of sepsis results in improved outcome, mice were injected i.v. with 1 μg of human recombinant sFlt-1 peptide or an equal volume of PBS (control) every 3 h (four doses) beginning 1 h after LPS administration or CLP procedure. sFlt-1 peptide completely blocked the effects of LPS or CLP on fractional shortening (Fig. 4, d and e) and heart rate (Fig. 4, h and i). In survival studies, recombinant sFlt-1 improved survival in both endotoxemia and CLP models of sepsis. Mortality in LPS was reduced from 80 to 20% ($P = 0.0091$; Fig. 10 a), whereas CLP-mediated mortality was decreased from 83.3 to 25% ($P = 0.0061$; Fig. 10 a).

DISCUSSION

Despite decades of intense research, severe sepsis continues to be associated with an unacceptably high rate of mortality. It has been estimated that 500–1,000 patients die every day from this condition in the United States (2). An important goal is to identify novel therapeutic targets. However, further advances in therapy will be critically dependent on an improved understanding of sepsis pathophysiology.

Using several different animal and human models, we have demonstrated that sepsis is associated with increased circulating levels of VEGF and PlGF. Levels were highest in endotoxemia models, intermediate in CLP, and lowest in pneumonia. Importantly, the findings were confirmed in human models of endotoxemia and severe sepsis. In all cases, the induction of VEGF and PlGF levels occurred at a later time point compared with TNF-α, IL-1, and IL-6. sFlt-1 blocked free VEGF and PlGF but had no effect on the early increase of TNF-α, IL-1β, and IL-6. Moreover, triple mutant mice that lack the ability to respond to IL-1 and TNF-α did not demonstrate LPS-mediated increases in VEGF and PlGF levels. Together, these findings strongly support the notion that VEGF and PlGF are late markers of sepsis and lie downstream of early response cytokines.

The administration of sFlt-1 attenuated LPS- and CLP-mediated morbidity and mortality. Although sFlt-1 binds both VEGF and PlGF, several findings point to the primary importance of VEGF in mediating sepsis pathophysiology. First, PlGF concentrations were lower than circulating VEGF concentrations in human sepsis. Second, PlGF binds to Flt-1 at a lower affinity than VEGF. Third, VEGF but not PlGF sensitized endothelial cells to the effects of low TNF-α concentrations. Fourth, the overexpression of VEGF but not PlGF resulted in a marked increase in LPS sensitivity (100% mortality). Finally, and most importantly, antibodies against Flk-1 but not Flt-1 attenuated sepsis mortality.

All organs examined displayed increased levels of PlGF protein, whereas the heart, liver, and kidney were the major sources of VEGF induction. The finding that VEGF levels were similar in serum and plasma (both in control and LPS-treated mice) argues against a substantial contribution of platelets to the circulating pool of VEGF. The mechanisms underlying the induction of VEGF and PlGF remain be determined. Although hypoxia is known to induce the expression of both growth factors, hypoxemia is not a universal finding in mouse and human models of sepsis. Previous studies have shown that inflammatory mediators, including IL-1, IL-6, and Cox, may increase the mRNA expression of VEGF in various cell types (19–21). Under in vitro conditions, VEGF and glucose have been shown to induce PlGF mRNA and protein levels in endothelial cells (22). Thus, it is possible that the cytokine storm associated with sepsis contributes to the increase in VEGF and PlGF levels.

The observation that VEGF sensitizes endothelial cells to the effects of low TNF-α concentrations suggests that the high VEGF levels observed in sepsis may serve to accentuate the activation phenotype. In addition, it is well established that VEGF induces endothelial permeability, an effect that may contribute to the morbidity and mortality in sepsis. However, the precise mechanisms by which VEGF contributes to disease progression will require ongoing studies, including the generation of lineage-specific conditional knockouts of VEGF receptors and/or the specific/selective attenuation of one or another VEGF effects.

The finding that endotoxemia is associated with increased circulating levels of sFlt-1 is novel. Previous studies have demonstrated that circulating levels of sFlt-1 are increased in the third trimester of pregnancy and are abnormally elevated in patients with preeclampsia (18). These latter changes are associated with a reduction in circulating free VEGF and PlGF. Interestingly, TNF-α has been shown to induce the release of sFlt-1 from normal placental villous explants (23). Together, these data suggest that sepsis-associated changes in circulating sFlt-1 may represent an endogenous compensatory anti-inflammatory mechanism.

In summary, our data support an association between sepsis and increased circulating levels of VEGF, PlGF, and sFlt-1. More importantly, the results suggest a pathophysiological role for VEGF in mediating the sepsis phenotype. Further studies are required to determine the diagnostic and/or prognostic value of these markers as well as the therapeutic potential of anti-VEGF strategies in patients with the sepsis syndrome.

MATERIALS AND METHODS

Mouse models of endotoxemia, CLP, and pneumonia. All animal studies were performed in accordance with guidelines established by the Beth Israel Deaconess Medical Center Animal Care and Use Committee or the Harvard Medical Area Standing Committee on Animals. LPS injections, CLP, and pneumonia models were performed in male 8-wk-old C57BL/6 (22–24-g body weight). For the endotoxemia model, mice were i.p. injected

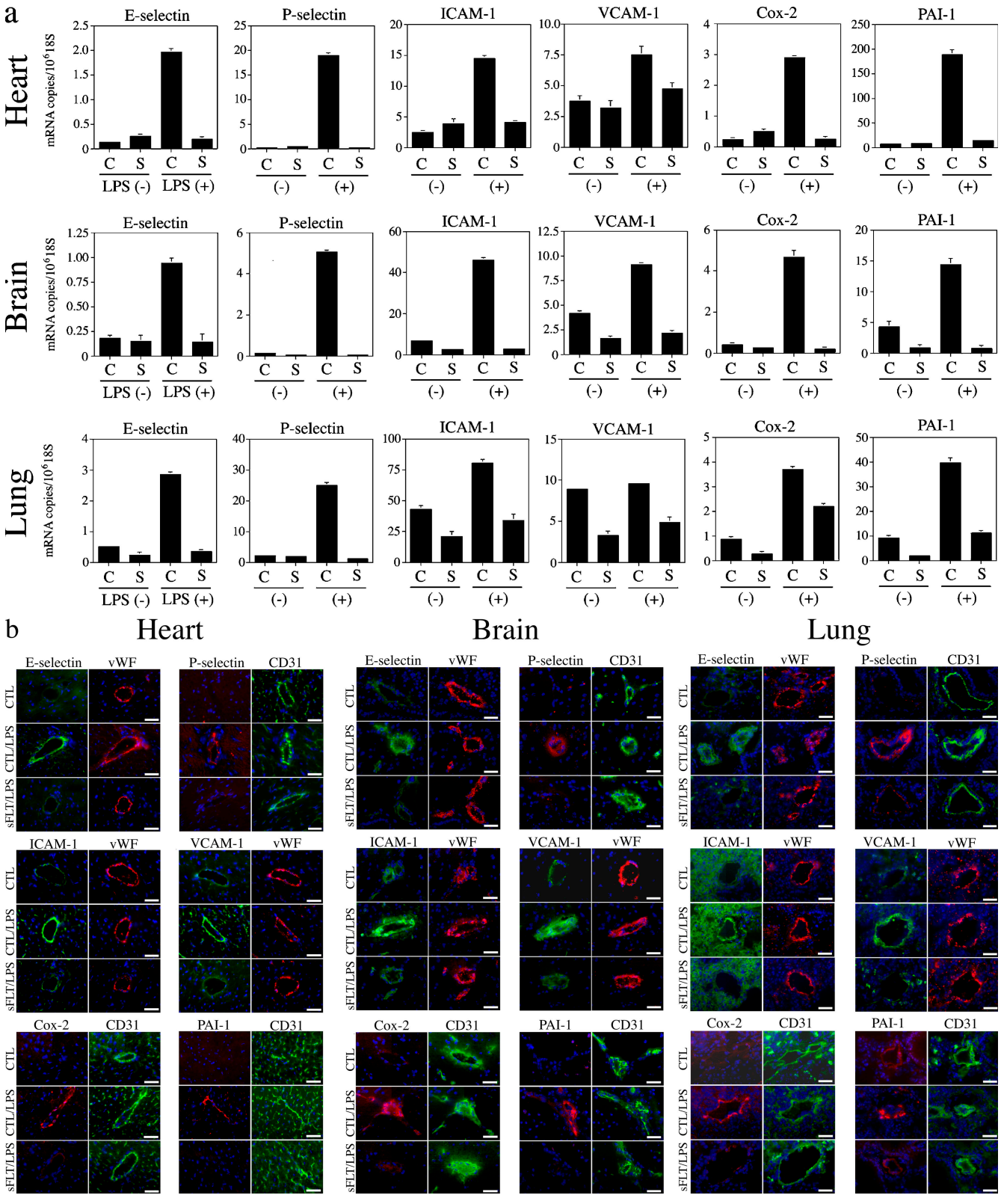
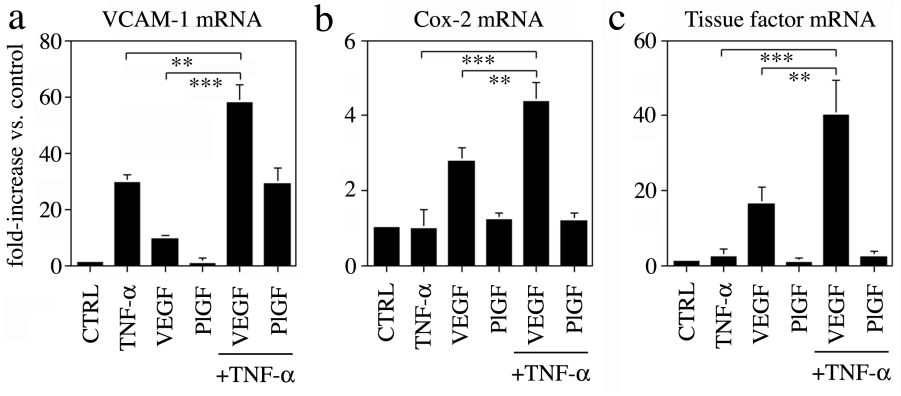


Figure 7. Effect of sFlt-1 overexpression on tissue mRNA/protein levels of inflammatory and hemostatic markers in a mouse model of endotoxemia. (a) Results of quantitative TaqMan analyses (mRNA copy number per 10^6 copies of 18S) of E-selectin, P-selectin, ICAM-1, VCAM-1, Cox-2, and PAI-1 in the heart, brain, and lung of mice overexpressing GFP (C) or sFlt-1 (S) 24 h after i.p. injection with (LPS+) or without LPS (LPS−). Error bars represent SD of triplicate PCR reactions using pooled cDNA from three mice in each treatment group. The results are



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	CTRL	0.1 ng/ml TNF-α	100 ng/ml VEGF	200 ng/ml PlGF	0.1 ng/ml TNF-α 100 ng/ml VEGF	0.1 ng/ml TNF-α 200 ng/ml PlGF
E-selectin	1.00	10.63	2.24	0.53	22.46	15.38
P-selectin	1.00	0.52	1.18	0.95	0.54	0.58
ICAM-1	1.00	9.00	1.41	1.03	6.50	8.25
VCAM-1	1.00	29.35	9.30	0.79	57.97	29.17
Cox-2	1.00	0.99	2.77	1.21	4.37	1.19
PAI-1	1.00	1.51	1.07	0.88	1.73	1.36
Tissue factor	1.00	2.13	16.37	0.62	40.20	2.24

fold-increase vs. control

Figure 8. Effect of VEGF and PlGF on the cytokine responsiveness of primary human endothelial cells. Results of quantitative TaqMan analyses (mRNA copy number per 10^6 copies of 18S) of VCAM-1 (a and d), Cox-2 (b and d), tissue factor (c and d), E-selectin (d), P-selectin (d), ICMA-1 (d), and PAI-1 (d) in serum-starved HUVECs treated for 4 h in the absence (CTRL; control) or presence of TNF-α, VEGF, and PlGF alone or in combination. (a–c) Data are expressed as means ± SD (error bars) of three independent experiments. **, $P < 0.05$; ***, $P < 0.0001$.

with normal saline (control) or LPS (18- mg/kg weight) from *E. coli* serotype 0111:B4 (Sigma-Aldrich). Where indicated, LPS was administered to triple mutant mice that were null for TNFR1, TNFR2, and the type I IL-1 receptor or wild-type controls on a matched C57BL/6 × 129/Sv background. Triple mutant mice were maintained in a full barrier facility under specific pathogen-free conditions (24). CLP was performed as previously described with minor modifications (25). In brief, mice were anesthetized with isofluorane. 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 ileo-cecal 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 was returned to the peritoneal cavity. Mice received 1 ml of saline i.p. for fluid resuscitation at the time of closure and 0.1 mg/kg buprenorphine s.c. every 12 h to minimize distress. For the pneumonia model, mice were anesthetized by i.m. injection of 100 mg/kg ketamine hydrochloride, 5 mg/kg acepromazine maleate, and 0.1 mg/kg atropine. The trachea was surgically exposed, and *E. coli* (strain 19138; American Type Culture Collection) were intratracheally instilled at 10^6 CFU/mouse via angiocatheter through the trachea to the left bronchus. After 6 or 24 h, mice were killed by halothane inhalation.

Human models of endotoxemia and sepsis. The human endotoxemia study was approved by the Institutional Review Board for the Protection of Human Subjects at the Beth Israel Deaconess Medical Center. In brief, after informed consent, the subjects underwent comprehensive screening and were included if they had no current or past history of psychiatric, neurological, immune, cardiovascular, or sleep disorders; no history of drug dependence/abuse, including cigarette smoking during the last 6 mo; normal blood chemistry (complete and differential blood counts, T cell subsets, glucose, creatinine, sodium, potassium, and thyroid-stimulating hormone); and negative blood and urine toxicology. Subjects received an i.v. injection of either placebo or a host-response challenge with 2 ng/kg *E. coli* endotoxin (O:113; Lot EC-5). The human sepsis study was approved by the Research Ethics Board of the Hamilton Health Sciences (Henderson General Hospital). Patients with severe sepsis were identified in the ICUs of the Henderson General Hospital, St. Joseph's Healthcare, and Hamilton General Hospital using the inclusion and exclusion criteria as previously described (26). Patients who received Xigris were excluded from the analysis. Baseline characteristics of the patients were collected, including demographic information, acute physiology and chronic health evaluation (APACHE II) admission scores, multiple organ dysfunction scores, comorbidities, organ function, site and type of infection, and hematologic tests (Table S3, available at http://www.jem.org/cgi/content/full/jem/20060375/DC1).

Measurement of cytokine levels in plasma, serum, or tissue lysates. TNF-α and IL-1 were measured in serum. IL-6, VEGF, and PlGF were measured in plasma. To isolate plasma from mice, blood samples were collected by heart puncture into heparinized tubes, centrifuged, and the supernatant was saved as plasma. To obtain mouse serum, blood samples were incubated overnight at 4°C, centrifuged, and the supernatant was saved as serum. In the human endotoxemia study, blood samples were drawn at regular intervals in

representative of two independent experiments. (b) Immunofluorescent studies of E-selectin, P-selectin, ICAM-1, vWF, Cox-2, and PAI-1 in the heart, brain, and lung of mice overexpressing GFP (CTL) or sFlt-1 24 h after i.p. injection with or without LPS. Serial sections were stained for vWF or CD31 to colocalize in endothelium. Bars, 50 μm.

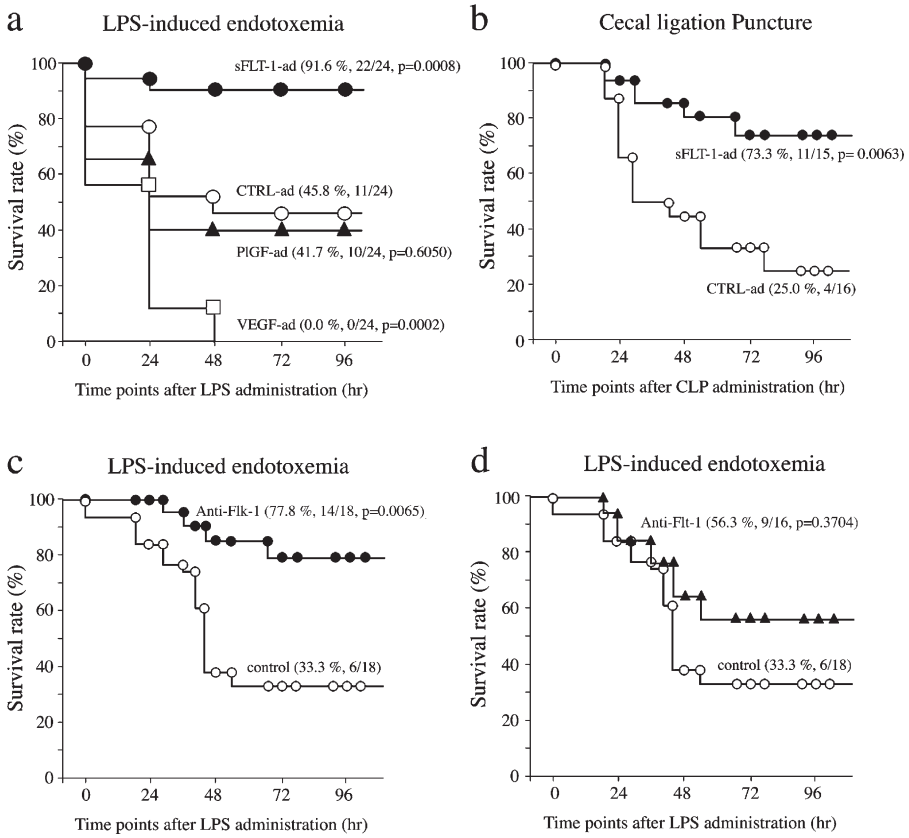

Figure 9. Survival studies in mouse models of sepsis. (a) Survival curves for mice overexpressing GFP (CTRL-as), sFlt-1 (sFlt-ad), PlGF (PlGF-ad), and VEGF (VEGF-ad) and injected i.p. with LPS. Circulating VEGF, PlGF, and sFlt-1 at 24 h after LPS injection was 5.08 ± 1.41 ng/ml, 28.23 ± 5.84 pg/ml, and 20.64 ± 5.20 ng/ml, respectively. (b) Survival curves for mice overexpressing GFP (CTRL-as) or sFlt-1 (sFlt-ad) and subjected to CLP. (c and d) Survival curves for endotoxemic mice pretreated with i.p. injection of antibodies against Flk-1 (c) or Flt-1 (d).

EDTA tubes and set on ice for 5 min before centrifuging at 2,600 *g*, and plasma was pipetted into aliquots and stored at −80°C until subsequent assay. In patients with severe sepsis, blood was collected within 24 h of meeting the definition of severe sepsis. Blood was collected daily for the first 7 d and once a week thereafter for the duration of the patients' stay in the ICU. 9 ml venous blood was drawn via indwelling catheters from the patients. As controls, 9 ml venous blood was drawn via venipuncture from healthy adult volunteers. For each patient or volunteer, 4.5 ml of the collected blood was immediately transferred to 15 ml polypropylene tubes containing 0.5 ml of 0.105 M buffered trisodium citrate, pH 5.4, and the remaining 4.5 ml was transferred into 15-ml polypropylene tubes containing 0.5 ml of 0.105 M buffered trisodium citrate, pH 5.4, and 100 μl of 1 M benzamidine HCl (20 mM benzamidine final). The blood was spun at 1,500 *g* for 10 min at 20°C, and the plasma was stored as aliquots at −80°C. To prepare mouse organ tissue lysates, mice were perfused with PBS containing 2 mM EDTA. Organs were removed and snap frozen in liquid nitrogen. Frozen tissues were homogenized in lysate buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% protease inhibitor cocktail (Sigma-Aldrich) and were centrifuged to obtain the supernatant (tissue lysate). Mouse sFlt-1, VEGF, TNF-α, IL-6, IL-1α, and IL-1β were assayed using the Quantikine ELISA kit (R&D Systems). Mouse D-dimer was measured using Asserachrom DI-D, an enzyme immunoassay of D-dimer (Diagnostica Stago). Human plasma PlGF and VEGF-A were measured using human PlGF and VEGF Quantikine ELISA kits (R&D Systems).

Adenovirus-mediated overexpression of sFlt, VEGF, and PlGF. Mice were injected i.v. with adenovirus overexpressing GFP (2×10^8 pfu of control), 10^8 pfu of mouse sFlt-1, 2×10^8 pfu of mouse VEGF-A (isoform 120), or 2×10^8 pfu of mouse PlGF-2. The construction of these viruses has been described previously (17, 18, 27). The dose of Ad–sFlt-1, Ad-VEGF, and Ad-PlGF was titrated to achieve plasma levels of ~10–25 ng/ml. Commercial Quantakine ELISA kits (R&D Systems) that measure mouse sFlt-1, VEGF, and PlGF were used to assay circulating levels of these cytokines from mouse plasma.

Antibody administration. 16 h before LPS administration, mice were injected i.p. with an injection of 800 μg of anti–mouse Flk-1 antibody (DC101), 1,200 μg of anti–mouse Flt-1 antibody (MF-1), or control PBS. Both antibodies were provided by D.J. Hicklin (ImClone Systems, Inc., New York, NY).

sFlt-1 peptide administration. Mice were injected i.v. with 1 μg of human recombinant sFlt-1 domain D1-3 (Cell Sciences, Inc.) or an equal volume of PBS (control) every 3 h for 12 h, beginning 1 h after LPS administration or CLP.

Cardiac physiological analysis. Echocardiographic examination of mice was performed as previously described (28, 29). In brief, mice were anesthetized with i.p. injection of 50 mg/kg ketamine HCl and 10 mg/kg xylazine. A sector scanner (Sonos 1500; Hewlett-Packard) equipped with a 12-MHz transducer was used to record two-dimensionally guided M-mode tracings to assess left ventricle wall thickness, left ventricle dimensions, and fractional shortening. Electrocardiogram recordings were acquired on anesthetized mice with a multichannel amplifier and were converted to digital signals for analysis (PowerLab system; ADInstruments).

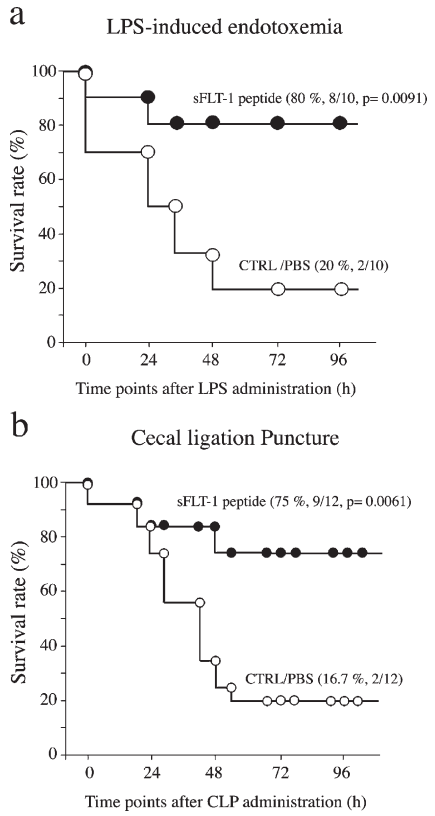

Figure 10. Therapeutic effect of sFlt-1 peptide on sepsis mortality. Survival curves for mice injected i.v. with sFlt-1 peptide or the same volume of PBS (control) 1 h after LPS injection (a) or CLP (b).

Permeability assay. Mice were anesthetized by i.p. injection of 0.5 ml avertin. 100 μl of 1% Evans blue dye in PBS was injected into the tail vein. 40 min later, mice were perfused via heart puncture with PBS containing 2 mM EDTA for 20 min. Organs (brain, heart, lung, liver, kidney, and spleen) were harvested and incubated in formamide for 3 d to elute Evans blue dye. OD of formamide solution was measured using a 620-nm wavelength.

Tissue RNA isolation and quantitative TaqMan PCR analysis. Tissue RNA was isolated using TRIzol (Invitrogen) and the RNA mini preparation kit (QIAGEN). For quantitative real-time PCR, total RNA was prepared using the RNeasy RNA extraction kit with DNase I treatment according to the manufacturer's instructions (QIAGEN). To generate cDNA, 100 ng of total RNA from each of triplicate samples was mixed and converted into cDNA using random primers and Superscript III reverse transcriptase (Invitrogen). All cDNA samples were aliquoted and stored at −80°C. Primers were designed using the Primer Express oligo design software (Applied BioSystems) and synthesized by Integrated DNA Technologies. All primer sets (Table S4, available at http://www.jem.org/cgi/content/full/jem.20060375/DC1) were subjected to rigorous database searches to identify potential conflicting transcript matches to pseudogenes or homologous domains within related genes. Amplicons generated from the primer set were analyzed for melting point temperatures using the first derivative primer melting curve software supplied by Applied BioSystems. The SYBR Green I assay and the ABI Prism 7500 Sequence Detection System (Applied Biosystems) 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 triplicate, and copy numbers were measured as described previously (30). The level of target gene expression was normalized against the 18S rRNA expression in each sample and, the data were presented as mRNA copies per 10^6 18S rRNA copies.

Immunohistochemistry. Immunohistochemistry was performed on 5-μm frozen sections from the heart, brain, and lung of control and LPS-treated mice. Antibodies included rabbit polyclonal anti–mouse P-selectin antibody (Chemicon International), rabbit polyclonal anti–mouse PAI-1 antibody (Innovative Research Inc.), rabbit polyclonal anti–mouse Cox-2 antibody (Cayman Chemical), rat monoclonal anti–mouse E-selectin antibody (BD Biosciences), rat monoclonal anti–mouse VCAM-1 antibody (Chemicon International), and rat monoclonal anti–mouse ICAM-1 antibody (Serotec Ltd). Anti–rat IgG antibody conjugated with FITC and anti–rabbit IgG antibody conjugated with Cy3 (Zymed Laboratories) were used as secondary antibodies. For colocalization studies, a rat monoclonal anti–mouse CD31 antibody (BD Biosciences) 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 Inc.) was combined with the rat monoclonal anti–mouse antibodies (E-selectin, VCAM-1, and ICAM-1).

Cell culture. HUVECs were cultured in EGM medium (Cambrex Bio Science). Once cells reached 70% confluence, they were serum starved in 0.5% FBS EGM for 20 h and incubated with 0.1 ng/ml TNF-α, 100 ng/ml VEGF, and 200 ng/ml PlGF alone or in combination for 4 h. The cells were harvested for RNA and processed for mRNA expression as described in Tissue RNA isolation…analysis.

Survival study. 3 d before LPS injection or CLP, C57BL/6 male mice were treated with the control (GFP), sFlt-1, PlGF, and/or VEGF-A adenoviruses. Alternatively, animals received the aforementioned anti–Flk-1 antibody, anti–Flt-1 antibody, or sFlt-1 peptide. Survival was assessed at 24, 48, 72, and 96 h after 20-mg/kg weight LPS injection or CLP.

Statistical analysis. Student *t* test was used for statistical analysis in mouse cytokine and gene expression studies. Analysis of variance was used for statistical analysis of cardiac physiology data. General linear model repeated measures were used to assess differences between human subject groups (placebo vs. endotoxin). The Greenhouse-Geisser correction of degrees of freedom was applied where appropriate. In cases of condition or interaction effects, simple contrasts were used to specify which time points significantly differed from each other. Survival data were analyzed by the construction of Kaplan-Meier plots and use of the log-rank test.

Online supplemental material. Table S1 shows VEGF and PlGF time course in a human model of endotoxemia. Table S2 shows the expression profile of genes involved in inflammation and coagulation in organs from control or LPS-treated mice. Table S3 describes baseline characteristics and the 28-d mortality rate of patients with severe sepsis. Table S4 provides the DNA sequences for the forward and reverse primers used in TaqMan analyses. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20060375/DC1.

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