Protective Role of Angiopoietin-1 in Endotoxic Shock

Bernhard Witzenbichler, MD; Dirk Westermann, MD; Sabine Knueppel, BS; Heinz-Peter Schultheiss, MD; Carsten Tschope, MD

Background—Angiopoietin-1 (Ang1) plays an essential role in embryonic vasculature development, protects the adult peripheral vasculature from leakage, and has antiinflammatory properties. Because endotoxin-induced shock is a condition with microvascular leakage resulting from inflammation, we examined the potential therapeutic benefit of Ang1 in a murine model of lipopolysaccharide (LPS)-induced endotoxic shock.

Methods and Results—To induce endotoxic shock, LPS was injected intraperitoneally into C57BL/6 mice. Half of the mice received an intravenous application of $1.0 \times 10^7$ plaque-forming units of an adenoviral construct expressing human Ang1 (AdhAng1); in the other half an identical vector expressing green fluorescent protein (AdGFP) was injected as a control. In the AdhAng1-treated mice, hepatic transfection and high expression of circulating Ang1 protein were observed. Whereas in LPS-treated control mice, hemodynamic function was severely depressed 12 hours after LPS injection (decrease of blood pressure from $91 \pm 3$ to $49 \pm 7$ mm Hg, dP/dt$_{max}$ from $7284 \pm 550$ to $2699 \pm 233$ mm Hg/s, cardiac output from $11.5 \pm 1.2$ to $2.8 \pm 0.8$ mL/min; $P<0.0005$), in LPS-treated AdhAng1 mice blood pressure fell only to $76 \pm 3$ mm Hg, dP/dt$_{max}$ to $5091 \pm 489$ mm Hg/s, and cardiac output to $6.7 \pm 1.4$ mL/min ($P<0.05$). This resistance to LPS-induced hemodynamic changes was reflected by an improved Kaplan-Meier survival rate of the AdhAng1 mice. Histological analysis revealed that lung injury after LPS injection was markedly attenuated in AdhAng1 mice. In addition, LPS-induced increase in lung water content and pulmonary myeloperoxidase activity was significantly reduced. Furthermore, LPS-induced increases in the expression level of vascular cell adhesion molecule-1, intracellular adhesion molecule-1, and E-selectin protein in the lungs were markedly lower in AdhAng1 mice than in control mice. Finally, in the mice overexpressing Ang1, pulmonary endothelial NO synthase (eNOS) expression and activity remained preserved after LPS challenge, providing evidence that the beneficial effect of Ang1 in endotoxic shock is mediated by eNOS-derived NO.

Conclusions—Our study demonstrates an improved mortality rate in mice with endotoxic shock pretreated with an adenoviral construct encoding Ang1. The enhanced survival rate induced by Ang1 was accompanied by an improvement in hemodynamic function, reduced lung injury, a lower expression of inflammatory adhesion molecules, and preserved eNOS activity in the lung tissue. Ang1 may therefore have utility as an adjunctive agent for the treatment of septic shock condition. (Circulation. 2005;111:97-105.)

Key Words: shock ■ genes ■ cell adhesion molecules ■ lung ■ angiogenesis

Angiopoietin-1 (Ang1) is a ligand for the endothelial-specific receptor tyrosine kinase Tie2$^2$ and has been shown to play an essential role in embryonic vasculature development.$^2$–$^4$ Unlike vascular endothelial growth factor (VEGF), which is a potent vascular permeability factor that increases microvascular permeability to blood plasma proteins, Ang1 has the ability to protect the adult peripheral vasculature from vascular leakage.$^5$–$^7$ In genetically modified mice overexpressing Ang1, it was demonstrated that blood vessels were more leakage resistant,$^5$ and Ang1, delivered by adenoviral expression, inhibited tissue edema induced by VEGF or mustard oil.$^6$ Moreover, it was reported that Ang1 can act as an antiinflammatory agent by modifying endothelial cell adhesion molecules and cell junctions.$^7$ Therefore, Ang1 might be therapeutically useful for reducing microvascular leakage in disease states in which leakage results from inflammation.

See p 2

Septic shock is caused mainly by an exaggerated systemic response to endotoxemia induced by gram-negative bacteria and their characteristic cell wall component, lipopolysaccharide (LPS).$^8$ It is characterized by systemically increased capillary permeability, interstitial and alveolar lung edema, and an increased expression of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6, and interferon-γ.$^9$–$^{10}$ Despite advances in understanding of the pathophysiological alterations and improvements in support-
ive management of patients with septic shock, the mortality rate due to systemic hypotension, decreased myocardial contractility, and tissue injury is unacceptably high. Mortality estimates for severe sepsis range as high as 85% when accompanied by shock and multiple organ dysfunction syndrome. Various novel sepsis therapies under evaluation include anticoagulant therapy and therapies directed at the neutralization of LPS and cytokines. Although the use of recombinant activated protein C has been shown to increase survival of patients, it addresses only the coagulatory aspect of sepsis. Therapies directed at the neutralization of proinflammatory cytokines or LPS with the use of TNA-α or LPS antibodies have been largely ineffective in clinical trials.

Ang1 may therefore have utility as an adjunctive agent for the treatment of septic shock. In mice, challenge with high doses of LPS results in a syndrome resembling septic shock in humans. Because Ang1 has counteracting properties on capillary permeability and endothelial inflammation, the purpose of our study was to determine a potential therapeutic benefit of chronic overexpression of Ang1 in a murine model of LPS-induced endotoxic shock. We demonstrate an improved mortality rate in mice with endotoxic shock pretreated with an adenoviral construct encoding Ang1. The enhanced survival rate induced by Ang1 was accompanied by improvement in hemodynamic function, reduced lung injury, a lower expression of inflammatory adhesion molecules on endothelial cells, and preserved pulmonary endothelial NO synthase (eNOS) activity. Ang1 may therefore have utility as an adjunctive agent for the treatment of septic shock.

Methods

Materials

LPS from Escherichia coli, serotype 055:B5, and all other chemicals were purchased from Sigma. The LPS was dissolved in 0.9% saline.

Adenoviral Constructs

Recombinant adenovirus expressing human angiopoietin-1 (AdhAng1) was generated with the use of the Adenovector System (Qbiogene) as previously described. Ang1 cDNA, derived from human adult heart tissue by reverse transcription–polymerase chain reaction and confirmed by sequencing, was inserted into the AdE1-deleted region under the control of the CMV5 promoter. An identical vector expressing green fluorescent protein (AdGFP) containing the adenoviral E1-deleted region was used as control. Recombinant viruses were propagated in 293A cells (Qbiogene). Recombinant adenovirus expressing human angiopoietin-1 from human adult heart tissue by reverse transcription–polymerase chain reaction (Qbiogene) as previously described. Ang1 cDNA, derived from human adult heart tissue by reverse transcription–polymerase chain reaction and confirmed by sequencing, was inserted into the AdE1-deleted region under the control of the CMV5 promoter. An identical vector expressing green fluorescent protein (AdGFP) containing the adenoviral E1-deleted region was used as control.

Animal Preparation

For all experiments, 3-month-old male C57BL/6 mice were used. To induce sublethal endotoxic shock, mice received an intraperitoneal injection of 80 mg/kg LPS. This dose was chosen because preliminary experiments showed that it led to a mortality rate of >90%. For all parts of the study, LPS was injected 2 days after gene transfer of AdhAng1 or AdGFP (control). To ensure optimal gene delivery, the adenoviral solutions were diluted in a 1-mL syringe with 0.9% saline to 500 μL. The mice were anesthetized by ventilation with 1.5% isoflurane, and, after a small skin incision and preparation of the femoral vein under stereomicroscopic sight, the adenoviral solutions were administered intravenously via a 29-gauge needle connected to a fine polypropylene tube. For survival studies, endotoxic shock was induced in a total of 50 mice. Twenty-five of these mice received 1.0×10^11 AdhAng1 plaque-forming units (pfu); the second group received AdGFP for control purposes intravenously 48 hours before injection of LPS. For all other studies, mice were euthanized by anesthesia with thiopental and cervical dislocation.

Immunohistochemical Staining

Two days after gene delivery, murine livers were surgically removed, frozen in liquid nitrogen, and sectioned at 5 μm. Sections were fixed in cold acetone for 10 minutes, incubated in 0.005% hydrogen peroxide/PBS for 12 minutes, and washed with PBS before incubation with an anti-Ang1 or an anti-GFP antibody (1:50; Santa Cruz) in PBS/10% FBS or IgG, isotypic control for 2 hours at 37°C in a humidified chamber. After 2 washes, an anti-goat horseradish peroxide–conjugated secondary antibody (Dako; 1:50 in PBS/20% FBS) was added for 1 hour at room temperature. Staining was developed by incubation with 3-amin-9-ethylcarbazole (30 mg 3-amin-9-ethylcarbazole, 5 mL N,N-dimethylformamide, 17.5 mL 0.2 mol/L sodium acetate at pH 5.2, 7.5 mL 0.2 mol/L acetic acid, 50 μL H2O2, and 5 mL H2O) for 12 minutes at room temperature in the dark. After they were rinsed with PBS, sections were counterstained with Mayer’s hemaluna for 1 minute and coveredslipped with glycerin gelatin.

Measurement of Ang1 Serum Levels

For measurement of Ang1 serum levels, mice were euthanized at time intervals 12, 24, 48, and 72 hours after gene delivery (n=5 each), blood samples were obtained via cardiac puncture, and a sandwich ELISA was performed. Ninety-six–well microtiter immunoassay plates were coated with 100 μL per well of a mouse anti-human Ang1 antibody (R&D Systems) at 2 μg/mL for 24 hours at 4°C. Plates were washed 3 times in PBS and blocked with 5% dry milk powder for 2 hours, and 100 μL of different dilutions of rat Ang1 standard (R&D Systems) or 1:10 diluted murine serum samples was added in duplicate. After 2 hours, 100 μL per well of a biotinylated goat anti-human Ang1 antibody (R&D Systems) at 500 ng/mL was incubated for 1 hour. ExtrAvidin peroxidase (Sigma) at 1:1000 was added for 45 minutes, and color was developed by addition of 10 mg ortho-phenylenediamine dihydrochloride tablet dissolved in 20 mL of 0.05 mol/L citrate buffer (pH 5.0) and 10 μL of 1 mol/L hydrogen peroxide. Absorbance was then read at 492 nm.

Hemodynamic Measurements

Mice were anesthetized by intraperitoneal injection of 125 mg/kg thiopental, intubated, and ventilated with a respirator (Ugo Basile, type 7025). A 1.4F microconductance pressure catheter (ARIA SPR-719; Millar Instruments Inc) was positioned in the left ventricle (LV) via the right carotid artery for continuous registration of LV pressure-volume loops in closed chest animals. Calibration of the recorded volume signal was obtained by hypertonic (10%) saline wash-in technique. All measurements were performed while ventililation was turned off momentarily. Indices of systolic and diastolic cardiac performance were derived from LV pressure-volume data obtained at steady state. Systolic function and myocardial contractility were quantified by LV end-systolic pressure, peak rate of rise in LV pressure (dP/dtmax), ejection fraction, cardiac output, end-systolic volume, and stroke volume. Diastolic performance was measured by LV end-diastolic pressure (LVEDP), peak dP/dtmax, and time constant of isovolumic pressure relaxation.

Western Blot Analysis

Tissues (mural livers or lungs) were homogenized in RIPA buffer (1% NP-40, 0.5% Na-deoxycholic acid, 0.1% SDS in PBS, pH 7.4; 1 μmol/L leupeptin; 5 μmol/L aprotonin; 1 mmol/L phenylmethylsulfonyl fluoride; 1 μmol/L pepstatin; Sigma Chemical Co), and protein concentration was determined with the use of BCA assay (Pierce). Tissue extracts were separated on 10% SDS-PAGE and transferred to a 0.2-μm polyvinylidene difluoride membrane (BioRad). The membranes were blocked in 10% nonfat dry milk, 0.2%PBS, and incubated with a 1:500 dilution of anti-Ang1 antibody (R&D Systems) or 1:10 diluted murine serum samples was added in duplicate. After 2 hours, 100 μL per well of a biotinylated goat anti-human Ang1 antibody (R&D Systems) at 500 ng/mL was incubated for 1 hour. ExtrAvidin peroxidase (Sigma) at 1:1000 was added for 45 minutes, and color was developed by addition of 10 mg ortho-phenylenediamine dihydrochloride tablet dissolved in 20 mL of 0.05 mol/L citrate buffer (pH 5.0) and 10 μL of 1 mol/L hydrogen peroxide. Absorbance was then read at 492 nm.
Tween-20 in PBS, pH 7.4, and then incubated with specific rabbit antibodies as indicated at room temperature for 4 hours. Blots were washed with Tween-20 in PBS and incubated with horseradish peroxidase–linked goat anti-rabbit antibody (Santa Cruz) for 45 minutes. Immunocomplexes were visualized with the use of ECL reagent (Amersham). Ang1, VEGF, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule (ICAM), E-selectin, eNOS (NOS3), and inducible NO synthase (iNOS) antibodies were purchased from Santa Cruz and used at 1:200 dilutions. After they were stripped, blots were incubated with an anti-tubulin antibody (1:200, Santa Cruz) to ensure equal loading.

### Lung Myeloperoxidase Activity

The left lung of mice 12 hours after administration of LPS was surgically removed, rinsed with saline, blotted dry, and weighed.25 The lung tissue was homogenized in 2 mL of 50 mmol/L potassium PBS (pH 7.4), containing 0.5% hexadecyl-trimethylammonium bromide (Sigma). The homogenate was centrifuged at 25 000g for 20 minutes at 4°C, the supernatant was decanted for myeloperoxidase measurements, and the pellet was resuspended in 1 mL of hexadecyl-trimethylammonium bromide PBS. This extraction procedure was repeated 3 times, and the supernatants were collected. The supernatants were mixed 1:30 (vol/vol) with 50 mmol/L Tris-HCl (pH 7.4)/10 mmol/L EDTA/10 mmol/L EGTA and centrifuged at 12 000 rpm for 5 minutes at 4°C. The supernatants were transferred to new tubes and separated by centrifugation at 100 000g for 60 minutes at 4°C. The pellets, containing membrane-associated eNOS, were resuspended in homogenization buffer, and protein contents were determined. Samples were then incubated in 10 mmol/L NADPH and 1 μCi/μL [3H]arginine in reaction buffer containing 6 mmol/L CaCl2 according to the manufacturer’s instructions. The radioactivity of the sample eluates was measured by liquid scintillation counting, and enzyme activity was expressed as citrulline production in picomoles per milligram protein per hour.

### Statistical Analysis

Results are presented as mean ± SEM. Statistical significance was evaluated by ANOVA followed by post hoc Student t test for unpaired observations and Bonferroni correction for multiple comparisons. Survival data were analyzed with the Kaplan-Meier test. A value of P<0.05 was interpreted to denote statistical significance.

### Results

#### Ang1 Protein Expression After Adenoviral Gene Transfer

Intravenous administration of adenoviral vectors leads to specific viral gene uptake and expression in the liver.28–30 We thus analyzed murine livers for expression of Ang1 protein 48 hours after intravenous injection of 1.0×109 pfu AdhAng1 or AdGFP (each n=4 animals). Immunohistochemical staining for Ang1 detected only a faint staining of hepatocytes, indicating little endogenous Ang1 protein expression in the AdGFP-treated mice (Figure 1A). Immunostaining for GFP demonstrated successful adenoviral gene expression of AdGFP (containing a nuclear localization sequence) into the murine livers, as shown by a strong nuclear staining of hepatocytes (Figure 1B). Similarly, in the AdhAng1-injected animals, the cytosol of a large number of hepatocytes was strongly stained (Figure 1C). Note the nuclear staining of GFP. C, Ang1 staining shows strong expression of Ang1 protein in a large number of hepatocytes after injection of AdhAng1. Note the cytosolic staining of Ang1.

### Measurement of eNOS Activity

For determination of eNOS enzyme activity, calcium-dependent L-arginine to L-citrulline conversion was assayed in the membrane-associated fractions of the murine lung extracts (NOSdetect Assay Kit, Stratagene). Briefly, lung samples were homogenized in 250 mmol/L Tris-HCl (pH 7.4)/10 mmol/L EDTA/10 mmol/L EGTA and centrifuged at 12 000 rpm for 5 minutes at 4°C. The supernatants were transferred to new tubes and separated by centrifugation at 100 000g for 60 minutes at 4°C. The pellets, containing membrane-associated eNOS, were resuspended in homogenization buffer, and protein contents were determined. Samples were then incubated in 10 mmol/L NADPH and 1 μCi/μL [3H]arginine in reaction buffer containing 6 mmol/L CaCl2 according to the manufacturer’s instructions. The radioactivity of the sample eluates was measured by liquid scintillation counting, and enzyme activity was expressed as citrulline production in picomoles per milligram protein per hour.

#### Lung Wet-to-Dry Weight Ratio

Mouse lung wet-to-dry weight ratios (W/D ratio) were used as a parameter of lung water accumulation after LPS injection.27 LPS-treated mice were studied 12 hours after LPS injection. Lung wet weight was determined immediately after removal of the right lung. Lung dry weight was determined after the lung had been dried in an oven at 50°C for 24 hours. The W/D ratio was calculated by dividing the wet weight by the dry weight.

#### Histopathological Examination

Mouse lungs were excised at various times after LPS injection and inflated at a pressure of 20 cm H2O with formalin. Organs were fixed overnight, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.
positive for Ang1 protein (Figure 1C). Western blot analysis from adenoviral-transduced livers confirmed high expression of Ang1 protein in a dose-dependent manner (Figure 2A). Although not detectable in AdGFP-transduced control mice, Ang1 protein was also highly expressed in the lungs of these mice (Figure 2C). As an additional control experiment for successful transgene expression, mice were intravenously injected with AdVEGF165, which led to a dramatic increase in VEGF protein expression (Figure 2B). To ensure systemic expression of Ang1 protein, we measured circulating serum levels of Ang1 at different time intervals after gene delivery (Figure 2D). We observed very high serum levels of Ang1 starting at 12 hours and lasting at least 72 hours, similar to previously published data. All mice expressing Ang1 seemed to be normal and healthy. In contrast, those expressing VEGF became moribund because of generalized edema formation within 24 hours, which is in agreement with previous reports. Thus, this animal model ensures high systemic blood levels of Ang1 protein secreted from the liver for several days.

**Ang1 Improves Hemodynamic Changes Induced by LPS Injection**

Hemodynamic measurements were performed 12 hours after LPS injection. There were no significant hemodynamic differences between control mice and mice pretreated with AdGFP or between LPS-treated mice and LPS-treated/AdGFP-injected mice (not shown). Compared with control mice (n=6), the hemodynamic parameters in LPS-treated mice (n=6) were severely depressed. Systolic blood pressure fell from 91±3 to 49±7 mm Hg (P<0.0005), dP/dt max decreased from 7284±550 to 2699±233 mm Hg/s (P<0.0001), cardiac output decreased from 11.3±1.2 to 2.8±0.8 mL/min (P<0.0005), and LVEDP increased from 6.3±1.2 to 9.1±1.1 mm Hg (P<0.05; Figure 3A). In contrast, systolic blood pressure in LPS-treated mice that were preinjected with AdhAng1 (n=6) fell only to 76±3 mm Hg (P<0.01 versus LPS-treated mice), dP/dt max to 5091±489 mm Hg (P<0.005), and cardiac output to 6.7±1.4 mL/min (P<0.05); LVEDP (5.8±0.8 mm Hg) did not significantly differ from controls but differed from LPS-treated mice (P<0.05; Figure 3A). Representative LV pressure-volume loops of control, LPS-, and AdhAng1/LPS-treated mice are shown in Figure 3B. Thus, mice overexpressing Ang1 were significantly more resistant to LPS-induced hemodynamic alterations than control mice.

**Ang1 Attenuates LPS-Induced Lung Damage**

Light microscopic examination of lungs taken from LPS-treated mice 24 hours after LPS injection demonstrated an infiltration of numerous polymorphonuclear leukocytes and macrophages in the interstitial spaces, hemorrhage, and marked swelling of the alveolar walls, as described before (Figure 4C). These changes were attenuated in AdhAng1/LPS-treated mice (Figure 4D). Injection of AdhAng1 by itself did not result in any apparent change of lung morphology compared with untreated controls (Figure 4A and 4B; 72 hours after injection).

Examination of VCAM-1, ICAM-1, and E-selectin protein expression revealed a very low expression level of all 3 adhesion molecules in the lungs of normal control mice (Figure 5). LPS challenge substantially increased the expression of lung VCAM-1, ICAM-1, and E-selectin protein. In contrast, the expression levels of all 3 proteins in AdhAng1/LPS-treated mice were attenuated compared with LPS-treated mice (Figure 5).

Because LPS induces pulmonary leukostasis and edema, we assessed pulmonary granulocyte infiltration by measuring lung myeloperoxidase activity and edema by measuring lung W/D ratio. At baseline, only low levels of myeloperoxidase activity were measured. Twelve hours after LPS treatment, myeloperoxidase activity (A absorbance per minute per gram tissue) strongly increased from 12±1.9 to 150±17 (P<0.001), whereas the increase in AdhAng1 mice was significantly lower (to 83±10; P<0.05 versus LPS-treated control mice; Figure 6A). Moreover, the lung W/D ratio as a
parameter of lung water accumulation increased from $4.2 \pm 0.3$ to $4.9 \pm 0.2$ after LPS injection ($P<0.01$). However, in the AdhAng1/LPS-treated mice, the increase in W/D ratio was markedly reduced (to $4.4 \pm 0.08$; $P<0.05$ versus AdGFP/LPS-treated mice; Figure 6B).

**Effects of Ang1 on Pulmonary eNOS and iNOS Expression**

In accordance with previous studies, LPS treatment led to a decrease in pulmonary eNOS and a marked increase in iNOS protein expression (Figure 7A) within 12 hours. Although in the LPS-treated mice overexpressing hAng1 no apparent change in the level of iNOS protein compared with AdGFP could be detected, the decrease in eNOS protein expression was clearly attenuated. Moreover, measurement of eNOS activity demonstrated a significant decrease of eNOS activity 12 hours after LPS administration (Figure 7B; from $10.2 \pm 1.2$ to $5.7 \pm 1.1$ pmol/mg protein per hour; $P<0.05$). In contrast, in the AdhAng1/LPS-treated mice, eNOS activity was restored ($9.1 \pm 0.7$ versus $5.7 \pm 0.9$ in AdGFP/LPS mice; $P<0.05$). Thus, in the mice overexpressing Ang1, eNOS expression and activity remained preserved after LPS challenge.

**Ang1 Improves Survival in LPS-Induced Endotoxic Shock**

LPS-treated mice showed signs of severe sepsis such as reduced mobility, apathy, conjunctivitis, diarrhea, and fur ruffling within a few hours. AdhAng1/LPS-treated mice exhibited fewer signs of sickness than the control mice. To investigate whether Ang1 gene transfer could also improve survival in LPS-induced sublethal endotoxic shock, we followed the mortality rate of mice over a period of 3 days. By 24 hours after LPS injection, 62% of LPS-treated control mice died, in contrast to 35% of AdhAng1/LPS-treated mice. Within 60 hours after LPS injection, 91% of control mice, but only 60% of AdhAng1-mice, died at this time point. The Kaplan-Meier survival curves of both groups are shown in Figure 8. Therefore, these results demonstrate that adenoviral overexpression of hAng1 in mice significantly increases survival from LPS-induced endotoxic shock ($P<0.05$).

**Discussion**

From gene targeting experiments it is known that Ang1, along with VEGF, plays an essential role in vascular development during embryogenesis. Transgenic overexpression of Ang1
in mouse skin resulted in increased dermal vascularization. It became clear that Ang1 is able to mediate a multitude of biological effects. It can act to promote the integrity of the endothelial cell barrier against a variety of challenges. In this context it was shown that Ang1 is able to block the permeability effect of serotonin, platelet-activating factor, VEGF, and mustard oil. Moreover, Ang1 exhibited antiinflammatory properties on endothelial cells by targeting cell adhesion molecules and cell junctions, raising the question of its therapeutic use in inflammatory diseases. It is potentially able to interfere with the coagulation cascade by suppressing tissue factor expression and activity in endothelial cells. It was therefore intriguing for us to investigate whether Ang1 could be of beneficial use in endotoxin-induced shock, a condition of systemic vascular inflammation and hyperpermeability. The present study indeed demonstrates that mice overexpressing Ang1 were more resistant to endotoxic shock. Because Ang1 is able to mediate multiple biological effects on endothelial cells, different explanations for this improvement in survival are possible.

First, the improvement in hemodynamic and pulmonary function of mice with endotoxic shock is likely due to a direct antipermeability effect of Ang1 on capillaries, protecting them from systemic leakage. In fact, water accumulation was clearly reduced in the lungs of Ang1-pretreated mice with endotoxic shock, and the swelling of the interstitial spaces and alveolar walls was markedly reduced. Second, we showed that overexpression of Ang1 led to a pronounced decrease in expression of endothelial cell adhesion markers ICAM-1, VCAM-1, and E-selectin in the lungs of LPS-treated mice. These adhesion molecules are essential for the regulation of leukocyte trafficking across the vascular endothelium and are critically involved in vascular inflammatory responses. Lower expression of these adhesion markers should result in less attachment of leukocytes to endothelium and consequently less tissue infiltration by leukocytes. In fact, we demonstrated that leukocyte infiltration into lung alveoli was reduced in Ang1-expressing mice, as seen by histology and measured by reduced myeloperoxidase activity. Our observations are in agreement with previously published in vitro data demonstrating that Ang1 can reduce leukocyte adhesion to endothelial cells by reducing expression of endothelial cell adhesion markers. Moreover, in a rodent model of diabetic retinopathy, Ang1 treatment led to a 50% reduction in permeability as a result of an Ang1-induced decline in the level of ICAM-1 expression in the retinal vasculature.

Figure 4. Representative photomicrographs of paraffin sections (magnification ×200) from lungs of untreated mice (top) and mice 24 hours after LPS treatment (bottom), stained with hematoxylin and eosin. A, Untreated control mouse. B, Control mouse, 72 hours after intravenous injection of AdGFP. C, AdGFP/LPS mouse, 72 hours after intravenous injection of AdGFP, 24 hours after LPS treatment. Note extensive interstitial edema, infiltration of inflammatory cells, and hemorrhage. D, AdhAng1/LPS mouse, 72 hours after intravenous injection of AdhAng1, 24 hours after LPS treatment. Note that LPS-induced lung damage is markedly reduced in mice overexpressing Ang1 compared with control mice. Photographs show representative examples from 5 experiments per treatment group.

Figure 5. Western blot analysis for adhesion molecules ICAM-1, VCAM-1, and E-selectin. Protein extracts from untreated mouse lungs (Co), AdGFP/LPS-treated lungs, and AdhAng1/LPS-treated lungs (n=3 each), taken 12 hours after LPS challenge, were separated by SDS gel electrophoresis and probed with antibodies as indicated. Intensity of β-tubulin band is used as a loading control between samples.
It was shown before that LPS induces certain cytokines such as interleukins and TNF-α.9 These cytokines stimulate the expression of adhesion molecules and therefore most probably mediate the inflammatory changes in the lung, resulting in neutrophil-dependent pulmonary edema.30 Given that it has been published that Ang1 is able to directly inhibit TNF-α–induced transmigration of neutrophils through endothelial cell monolayers,7 it is likely that Ang1 additionally exerts its protective effect toward leukocyte infiltration via strengthening endothelial cell junctions. Taken together, this decrease of leukocyte infiltration might be one reason for the observed attenuation of lung injury in Ang1-overexpressing LPS-treated mice. In this context, it is noteworthy that the adult lung exhibits one of the highest expression levels of the Ang1 receptor Tie2 of any organ.41

In a previous study, cell-based Ang1 gene transfer into the lungs of rats led to a marked upregulation of eNOS gene expression in an animal model of pulmonary hypertension.42 On the other hand, transgenic overexpression of eNOS in the endothelium of mice resulted in resistance to LPS-induced endotoxic shock.25 In this study, the extent of VCAM-1 and ICAM-1 expression was less in LPS-treated eNOS transgenic mice than in LPS-treated control mice. In a recent report, Ang1-induced angiogenesis in subcutaneous Matrigel implants was dependent on the availability of endothelial-derived NO.43 In agreement with these findings, our results indicate that Ang1 preserved pulmonary eNOS expression and activity after LPS challenge, providing direct evidence that the beneficial effect of Ang1 in our endotoxic shock model is likely to be mediated by eNOS-derived NO, particularly because in lungs NO made by eNOS is attributed to play a protective role against the toxic effects of LPS-released mediators.25,44,45

There is well-founded evidence that Ang1 acts as a survival factor for endothelial cells,46,47 which is attributed to Ang1-dependent activation of phosphatidylinositol 3-kinase and the Akt pathway.48,49 Apoptosis, like inflammation, forms an important feature of septic shock.50 LPS induces apoptosis in lymphocytes and gastrointestinal epithelial cells,51 glomerular endothelial cells,52,53 and heart.54 Although not generally accepted,55 it was reported that LPS induced disseminated endothelial cell apoptosis in a mouse model.56 Furthermore, activation of the phosphatidylinositol 3-kinase/Akt pathway by a poly(ADP-ribose) polymerase-1 (PARP) inhibitor attenuated sepsis-induced detrimental alterations in murine57 and pig models,58 and mice deficient in PARP were more resistant to acute septic peritonitis.59 It is therefore possible that the antiapoptotic activity of Ang1 via Akt activation contributes to the observed beneficial effect of Ang1 in our endotoxic shock model. Additional experiments treating mice with a phosphatidylinositol 3-kinase inhibitor are necessary to prove this hypothesis.

As a limitation of our study, it must be emphasized that the mice were injected with LPS once the Ang1 protein was overexpressed, and the effects of LPS were examined 12 to 60 hours later. Under these conditions, Ang1 prevented rather than treated the deleterious effects of LPS. It must be proven whether Ang1 can reverse LPS-induced multiorgan failure once established or at least decrease its severity if administered during sepsis. Moreover, in our present study, we only evaluated the beneficial effect of Ang1 in a model of endotoxic shock, and whether or not overexpression of Ang1 after LPS challenge. Values indicate mean±SEM of 5 experiments per group. *P<0.05 vs control mice; †P<0.05 vs AdGFP/LPS group.
is effective in models of gram-negative bacterial sepsis still remains to be determined.

For this animal study, overexpression of Ang1 via gene delivery is clearly advantageous compared with administration of recombinant Ang1 protein because administration of the latter at large doses is costly, cannot be given for longer desired periods of time, and is unable to maintain a constant high systemic level because of the short half-life of recombinant protein in vivo. In contrast, administration of a single dose of an adenoviral construct is able to achieve a high sustainable level of the transgene product for 10 to 14 days. A high systemic level because of the short half-life of recombinant Ang1 protein because administration of an adenoviral construct is able to achieve a high sustainable level of the transgene product for 10 to 14 days.

Our results demonstrate an improved survival rate of Ang1-pretreated mice in LPS-induced shock. Because septic shock therapy in patients is still unsatisfactory, there have been continued efforts to investigate new and effective means to improve outcome by modifying the inflammatory response, coagulation pathway, or other aspects of sepsis. Because of its wider range of biological functions, Ang1 has the theoretical advantage of influencing not only a single facet but multiple facets of the septic cascade. In principle, Ang1 may therefore have utility as an adjunctive treatment modality for septic shock, although the results presented here must be confirmed in other clinically relevant animal models.

Acknowledgments

This work was supported by a grant of the German Research Foundation (DFG Wi-1415/3-1) and university research funds (FG 341).

References


Protective Role of Angiopoietin-1 in Endotoxic Shock
Bernhard Witzenbichler, Dirk Westermann, Sabine Knueppel, Heinz-Peter Schultheiss and Carsten Tschope

Circulation. 2005;111:97-105; originally published online December 20, 2004;
doi: 10.1161/01.CIR.0000151287.08202.8E
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/111/1/97

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/