

Reversing Established Sepsis in Rats with Human Vasoactive Hormone Adrenomedullin and its Binding Protein

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We recently demonstrated that early administration of rat adrenomedullin (AM), a vasoactive peptide, in combination with its binding protein (human AMBP-1) produces various beneficial effects in sepsis. Human AM is a 52-amino acid peptide, but rat AM differs from human AM, having only 50 amino acid residues, with two amino acid deletions and six substitutions. It remains unknown whether a combination of human AM and human AMBP-1 (AM/AMBP-1) is also beneficial in sepsis and, if so, whether human AM/AMBP-1 reverses established sepsis in rats. To test the effects of human AM/AMBP-1, we induced sepsis in male adult rats by cecal ligation and puncture (CLP). At 10 h after CLP (i.e., severe sepsis), human AM (12–48 µg/kg body weight) was administered in combination with human AMBP-1 (40–160 µg/kg body weight). Vehicle-treated animals received a nonspecific human plasma protein (albumin). Blood and intestinal samples were collected at 20 h for various measurements. In additional groups of septic animals, the gangrenous cecum was surgically excised at 20 h after CLP. The 10-day survival was recorded. Our results showed that tissue injury, as evidenced by increased levels of transaminases and lactate, was present at 20 h after CLP. Proinflammatory cytokines tumor necrosis factor- α and interleukin-6 were significantly elevated. Gut barrier dysfunction, manifested by increased mucosal permeability to hydrophilic macromolecules and increased bacterial translocation to mesenteric lymph nodes, also occurred at 20 h after CLP. Administration of human AM/AMBP-1 in established sepsis markedly attenuated tissue injury, reduced proinflammatory cytokine levels, ameliorated intestinal-barrier dysfunction, and improved the survival rate from 47% to 67%–80%. Thus, human AM/AMBP-1 can be further developed as a safe and effective therapy for patients with established sepsis.

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INTRODUCTION

Sepsis remains a critical problem, leading to significant morbidity and mortality even in the modern era of critical care management (1–4). Sepsis is the second leading cause of death among patients in noncoronary intensive care units (ICU), and the 10th leading cause of death overall in the United States (5–8). Evidence indicates that in the United States alone, more than 750,000 people develop sepsis each year, with an overall mortality of 28.6% (9). Seven years ago the reported average cost per case was more than \$22,100, and the annual total cost was

more than \$16 billion nationally (9). The total cost of treating septic patients is even higher today. Given the intensive and prolonged care necessary to treat patients with sepsis, the economic burden is profound. Thus, there is an urgent unmet medical need for an effective novel therapy for septic patients.

Adrenomedullin (AM), a 52-amino acid vasoactive peptide, was originally isolated by Kitamura *et al.* in 1993 from a human pheochromocytoma (10). Recently, a novel specific AM-binding protein, AMBP-1, was identified in human plasma, and the purified protein was re-

ported to be identical to human complement factor H (11,12). AMBP-1 potentiates AM-induced vascular relaxation in the aorta under normal as well as pathophysiological conditions (13). Our recent studies have shown that upregulation of AM plays a major role in initiating the hyperdynamic response during the early stage of sepsis and the reduced vascular responsiveness to AM, due to decreased AMBP-1, appears to be responsible for the transition from the hyperdynamic phase to the hypodynamic phase during the progression of polymicrobial sepsis (13,14).

We have recently demonstrated that early administration of rat AM in combination with human AMBP-1 attenuates tissue injury, inhibits inflammatory responses, and reduces mortality in a rat cecal ligation and puncture (CLP) model of sepsis (14,15). However, the antigenic-

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ity of animal proteins in humans prevents the use of this complex in humans. Therefore, human proteins must be tested in animal models before these findings can be verified in humans. Although we have shown that early administration of rat AM plus human AMBP-1 in sepsis is protective, it remains unknown whether a combination of human AM and human AMBP-1 (AM/AMBP-1) is also beneficial and, if so, whether human AM/AMBP-1 reverses established sepsis in rats. Although less potent than rat AM, human AM has been shown to increase organ perfusion in rats (16). We therefore hypothesize that the administration of human AM/AMBP-1 late after the onset of sepsis attenuates tissue injury and improves survival. The aim of this study therefore was to determine the efficacy of human AM/AMBP-1 on sepsis-induced organ injury, inflammation, and mortality in the rat.

MATERIALS AND METHODS

Animal Model of Sepsis

Male Sprague-Dawley rats (275–325 g) were housed in a temperature-controlled room on a 12-h light/dark cycle and fed a standard Purina rat chow diet. Prior to the induction of sepsis, rats were fasted overnight, but allowed water *ad libitum*. Rats were anesthetized with isoflurane inhalation and the ventral neck, abdomen, and groin were shaved and washed with 10% povidone iodine. CLP was performed as we previously described (17–19). Briefly, a 2-cm midline abdominal incision was performed. The cecum was exposed, ligated just distal to the ileocecal valve to avoid intestinal obstruction, punctured twice with an 18-gauge needle, squeezed slightly to allow a small amount of fecal matter to flow from the holes, and then returned to the abdominal cavity, following which the abdominal incision was closed in layers. Sham-operated animals (controls) underwent the same procedure with the exception that the cecum was neither ligated nor punctured. The animals were resuscitated with 3 mL/100 g body weight

(BW) normal saline subcutaneously immediately after surgery. The animals were then returned to their cages. All experiments were performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. This project was approved by the Institutional Animal Care and Use Committee of The Feinstein Institute for Medical Research.

Administration of Human AM/AMBP-1

At 10 h after the onset of sepsis (i.e., severe sepsis), a femoral vein was cannulated with a PE-50 tubing under anesthesia (isoflurane inhalation). Human AM (12–48 $\mu\text{g}/\text{kg}$ BW) in combination with human AMBP-1 (40–160 $\mu\text{g}/\text{kg}$ BW) in a volume of 1 mL normal saline was administered via the femoral venous catheter over a period of 60 min. Vehicle-treated animals received a non-specific human plasma protein (albumin, 208 $\mu\text{g}/\text{kg}$ BW) at 10 h after CLP. We purchased synthetic human AM with a purity of more than 99% (by high-performance liquid chromatography) from Phoenix Pharmaceuticals (Belmont, CA, USA). Human AMBP-1, isolated from human serum/plasma, had a purity of more than 98% (by SDS-PAGE), and was purchased from Cortex Biochem (San Leandro, CA, USA). The human AMBP-1 preparation was tested at serum/plasma donor level by FDA-approved methods and found negative for HIV I/II, and human cytomegalovirus antibodies and hepatitis B surface antigens. Blood samples were collected at 20 h after sham operation or CLP and placed on ice to allow clotting. The samples were then centrifuged at 1200g for 10 min at 4°C, and the serum samples were stored at –80°C until assayed.

Determination of Serum Levels of Transaminases and Lactate

Serum concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate were determined by using assay kits according to the manufacturer's instructions (Pointe Scientific, Lincoln Park, MI, USA).

Determination of Serum Levels of Tumor Necrosis Factor- α (TNF- α) and Interleukin-6 (IL-6)

The concentrations of TNF- α and IL-6 in the serum were quantified by using commercially obtained enzyme-linked immunosorbent assay (ELISA) kits specific for rat-TNF- α and IL-6 (BioSource International, Camarillo, CA, USA). The assay was carried out according to the instructions provided by the manufacturer.

Determination of Intestinal Mucosal Permeability

To determine whether human AM/AMBP-1 ameliorates structural and functional damage to the intestinal mucosa induced by the progression of polymicrobial sepsis, human AM (24 $\mu\text{g}/\text{kg}$ BW) and AMBP-1 (80 $\mu\text{g}/\text{kg}$ BW) in combination or vehicle were administered intravenously over a period of 60 min at 10 h after CLP. At 10 h after AM/AMBP-1 treatment (i.e., 20 h after CLP), intestinal barrier function was assessed by measuring translocation of the fluorescent tracer, fluorescein isothiocyanate dextran with a molecular weight of 4000 Da (FD4, Sigma, St. Louis, MO, USA) by the everted gut sac method as previously described by others (20–23) and recently used by us (24).

Determination of Bacterial Translocation

The mesenteric lymph-node complex was harvested, and an equal amount of wet tissues was homogenized and briefly centrifuged to remove gross particulate matters. Serial log dilutions of tissue homogenates were applied. We then plated 500 μL of each dilution on chocolate agar plates (Fisher Scientific, Pittsburgh, PA, USA) and incubated at 37°C for 24 h under aerobic conditions. The colony-forming units (CFU) were counted and results were expressed as CFU/g tissue.

Survival Study

In additional groups of animals, human AM/AMBP-1 or vehicle was administered as described above at 10 h after CLP. At 20 h after CLP, the necrotic

cecum was excised, and the abdominal cavity was washed twice with 40 mL of warm, sterilized normal saline solution. The abdominal incision then was closed in layers. The procedure of cecal excision in CLP animals was performed to mimic the clinical situation in which septic focus is removed whenever possible. The animals then were allowed food and water *ad libitum* and were monitored for 10 d to record survival.

Statistical Analysis

Results are expressed as means \pm SE. One-way analysis of variance and the Student-Newman-Keuls method were used to compare different groups of experimental animals. The survival rate was estimated by Kaplan–Meier method and compared by the log-rank test. Differences in values were considered significant if $P < 0.05$.

RESULTS

Human AM/AMBP-1 Attenuates Organ Injury in Sepsis

Consistent with previous studies (19), our study revealed significant increases in liver enzymes (AST, ALT) at 20 h after CLP (Figure 1), indicating hepatic injury. All three doses of human AM/AMBP-1 treatment significantly attenuated the increased levels of AST and ALT ($P < 0.05$). However, human AM/AMBP-1 did not affect hepatic enzymes in a clearly dose-dependent manner. Serum levels of lactate, a marker for systemic hypoxia, increased by 382% at 20 h after CLP, and administration of all three doses of human AM/AMBP-1 decreased lactate levels significantly (Figure 2). Similarly, human AM/AMBP-1 did not affect lactate in a clearly dose-dependent manner.

Human AM/AMBP-1 Downregulates Proinflammatory Cytokines in Sepsis

Serum levels of TNF- α were more than tripled at 20 h after CLP ($P < 0.05$) (Figure 3A). Human AM/AMBP-1 treatment decreased TNF- α levels in a dose-dependent manner ($P < 0.05$, Figure 3A). Similarly, circulating levels of IL-6 in-

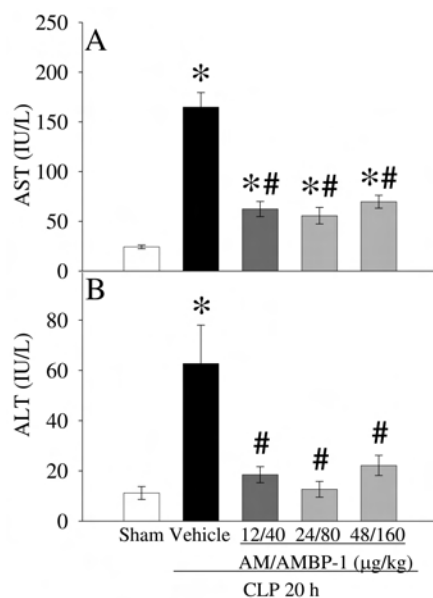


Figure 1. Alterations in circulating levels of (A) aspartate aminotransferase (AST) and (B) alanine aminotransferase (ALT) in sham-operated animals (Sham) and septic animals treated with human albumin (Vehicle) or human adrenomedullin (AM)/human adrenomedullin binding protein (AMBP)-1 (12/40, 24/80, or 48/160 $\mu\text{g}/\text{kg}$ BW) at 20 h after cecal ligation and puncture (CLP). Data are presented as means \pm SE ($n = 4\text{--}6/\text{group}$) and compared by one-way analysis of variance and Student-Newman-Keuls test: * $P < 0.05$ versus sham group; # $P < 0.05$ versus vehicle group.

creased by 15-fold at 20 h after CLP; AM/AMBP-1 treatment significantly reduced IL-6 levels in a dose-dependent manner ($P < 0.05$, Figure 3B).

Human AM/AMBP-1 Ameliorates Intestinal Barrier Dysfunction in Sepsis

As indicated in Figure 4A, ileal mucosal permeability to the fluorescent macromolecule, FD4, was significantly increased at 20 h after CLP in vehicle treated animals as compared with sham controls ($P < 0.05$). Similarly, bacterial translocation to mesenteric lymph nodes was minimal in the sham group, but was extensive in the CLP vehicle-treated group ($P < 0.05$, Figure 4B). Treatment with human AM/AMBP-1 (24/80 $\mu\text{g}/\text{kg}$ BW)

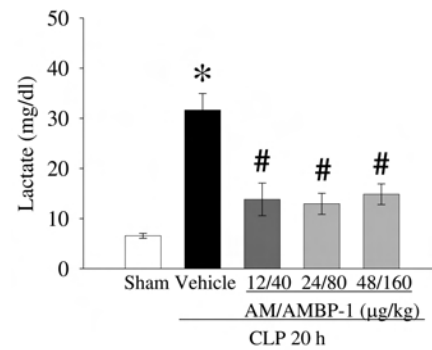


Figure 2. Alterations in circulating levels of lactate in sham-operated animals (Sham) and septic animals treated with human albumin (Vehicle) or human adrenomedullin (AM)/human adrenomedullin binding protein (AMBP)-1 (12/40, 24/80, or 48/160 $\mu\text{g}/\text{kg}$ BW) at 20 h after cecal ligation and puncture (CLP). Data are presented as means \pm SE ($n = 5\text{--}6/\text{group}$) and compared by one-way analysis of variance and Student-Newman-Keuls test: * $P < 0.05$ versus sham group; # $P < 0.05$ versus vehicle group.

at 10 h after CLP, however, significantly ameliorated the development of both ileal mucosal hyperpermeability and bacterial translocation, respectively ($P < 0.05$, Figure 4).

Human AM/AMBP-1 Improves Survival in Sepsis

To determine the long-term effect of human AM plus human AMBP-1 in sepsis, we conducted a 10-d survival study. The survival rate after CLP and cecal excision in vehicle (albumin) treated animals was 60% at d 2, and decreased to 47% at d 5–10 (Figure 5). Administration of 12/40 or 48/160 $\mu\text{g}/\text{kg}$ BW human AM/AMBP-1 improved the survival rate to 67% and 73%, respectively. However, these increases were not statistically significant. When 24/80 $\mu\text{g}/\text{kg}$ BW human AM/AMBP-1 was administered, the survival rate was significantly increased to 80% ($P < 0.05$, Figure 5).

DISCUSSION

AM was originally isolated from a human pheochromocytoma because of its ability to increase cyclic adenosine

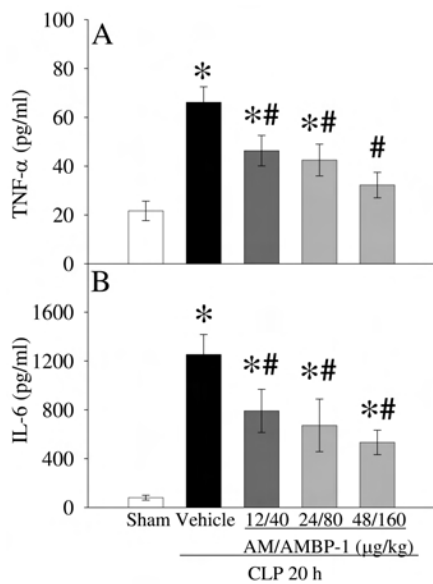


Figure 3. Alterations in circulating levels of (A) TNF- α and (B) IL-6 in sham-operated animals (Sham) and septic animals treated with human albumin (Vehicle) or human adrenomedullin (AM)/human adrenomedullin binding protein (AMBP)-1 (12/40, 24/80, or 48/160 $\mu\text{g}/\text{kg}$ BW) at 20 h after cecal ligation and puncture (CLP). Data are presented as means \pm SE ($n = 6/\text{group}$) and compared by one-way analysis of variance and Student-Newman-Keuls test: * $P < 0.05$ versus sham group; # $P < 0.05$ versus vehicle group.

3'-5'-monophosphate (cAMP) levels in platelets and to cause strong hypotension (25). Since then, AM has attracted the interest of investigators in the cardiovascular field because of AM's potent and long-lasting vasoactive properties. Infusion of AM causes vasodilatation, diuresis, and natriuresis and inhibits aldosterone secretion in normal animals (26). In addition to AM's well-known vasodilatory effects, AM also modulates production of inflammatory cytokines. Studies have shown that AM suppresses proinflammatory cytokine expression in various cell types (27,28). Mice heterozygous for adrenomedullin exhibit a more extreme inflammatory response to endotoxin-induced septic shock (29). Various studies have demonstrated that circulating levels of AM increase in patients with sepsis and systemic inflammatory response syn-

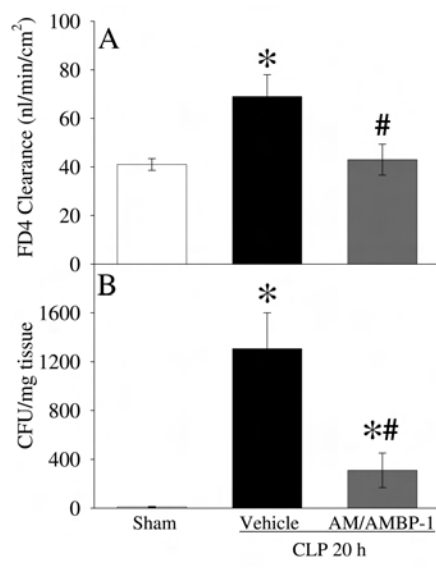


Figure 4. Alterations in intestinal mucosal permeability (A) to fluorescein isothiocyanate dextran with a molecular weight of 4000 Da (FD4) and bacterial translocation to mesenteric lymph nodes (B) in sham-operated animals (Sham) and septic animals treated with human albumin (Vehicle) or human adrenomedullin (AM)/human adrenomedullin binding protein (AMBP)-1 (24/80 $\mu\text{g}/\text{kg}$ BW) at 20 h after cecal ligation and puncture (CLP). Data are presented as means \pm SE ($n = 4-8/\text{group}$) and compared by one-way analysis of variance and Student-Newman-Keuls test: * $P < 0.05$ versus sham group; # $P < 0.05$ versus vehicle group.

drome (30,31). Using the CLP model of sepsis in the rat, we have also shown that the upregulation of AM occurs early after the onset of sepsis (32). The increased levels of AM appear to be a protective mechanism under such conditions. In this regard, AM is an excellent candidate for the treatment of septic patients. As we previously reported, however, administration of low-dose of AM failed to maintain cardiovascular stability in the CLP model of sepsis in the rat (14).

The recent discovery of a specific AM-binding protein in mammalian blood has greatly expanded understanding of the regulation of AM activity in sepsis (11,14,33). Discovery of this protein is partly attributable to the specific binding of ^{125}I -AM to a 120-kDa band on a blot ob-

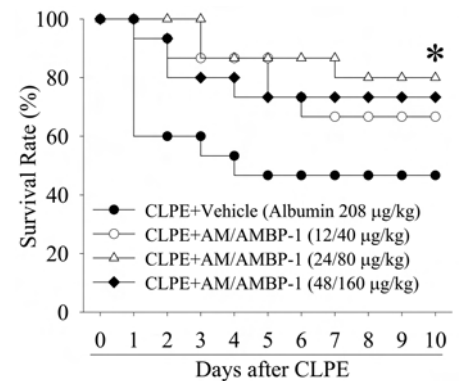


Figure 5. Alterations in the survival rate at 10 d after cecal ligation and puncture and cecal excision with human albumin treatment (CLPE+Vehicle) or human adrenomedullin (AM)/human adrenomedullin binding protein (AMBP)-1 (12/40, 24/80, or 48/160 $\mu\text{g}/\text{kg}$ BW) treatment. There were 17-21 animals in each group. The survival rates were estimated by the Kaplan-Meier method and compared by using the log-rank test. * $P < 0.05$ versus vehicle group.

tained from a nonreducing, electrophoretic gel separation of serum proteins from several species including humans. Pío *et al.* purified this binding protein, named it AMBP-1, and discovered that AMBP-1 is identical to complement factor H (12). The rat cDNA-derived coding sequence of AMBP-1 has an identity of 74% to the human AMBP-1 nucleotide sequence. The translation product of rat AMBP-1 mRNA was 1236 amino acids in length (leader sequence included), with an identity of 63% to human AMBP-1 (34). The presence of AMBP-1 in tissues may affect the autocrine/paracrine actions of AM. AMBP-1 binding increases receptor-mediated effects of AM but suppresses its receptor-independent antimicrobial activity (35). AMBP-1 does not change the affinity of AM receptors for AM, but AMBP-1 has sequences that may allow it to bind to cell-surface adhesion molecules and therefore may bring AM near its receptors and raise the effective concentration of AM (12). Further investigation is required to confirm that this process occurs *in vivo*, however. AM binding with AMBP-1 may also create a locally contained reservoir of AM at high concentrations. Through this pro-

cess, AMBP-1 may increase AM effectiveness without modifying the affinity of its receptor. It is also possible that AMBP-1 inhibits the degradation of the biologically active AM. Thus, circulating AMBP-1 can regulate the bioactivity of AM under normal and possibly pathologic conditions.

The finding that AMBP-1 potentiates AM-induced cAMP accumulation in cultured Rat-2 fibroblast cells (12) suggests that AMBP-1 plays an important role in AM-induced vascular relaxation. A study from our laboratory showed that AMBP-1 in an organ bath at concentrations of 2 and 5 nM enhanced AM-induced relaxation of aortic rings taken from normal rats (13). AMBP-1 alone is associated with only minimal vascular relaxation (13). AMBP-1 also potentiates AM's downregulatory effect on LPS-induced cytokine production (36). In a primary culture of Kupffer cells, AM or AMBP-1 alone inhibited LPS-induced TNF- α production by 52% and 44%, respectively. However, AM in combination with AMBP-1 reduced TNF- α production by 90% (36). The direct antiinflammatory effect of AM/AMBP-1 is mediated through both the cAMP-dependent pathway and proline-rich tyrosine kinase-2 (Pyk-2)-ERK1/2-dependent induction of peroxisome proliferator-activated receptor γ (37). Thus, AM and AMBP-1 target both cardiovascular and inflammatory responses at the same time.

A deficiency of AMBP-1 in humans is associated with higher susceptibility to recurrent infections (38). To investigate this association, we measured AMBP-1 expression in the CLP model of sepsis in the rat (39). Our results showed that both plasma levels of AMBP-1 and its gene expression in the liver, the major source of circulating AMBP-1 (40-42), decrease significantly at 20 hours after CLP (i.e., late sepsis). Moreover, vascular levels of AMBP-1 and plasma AMBP-1 binding capacity were significantly reduced at the same time point (13). Therefore, AMBP-1 deficiency occurs under septic conditions. Our previous study, however, indicated that administration of AMBP-1 alone is insufficient for preventing organ injury in sepsis (14). On the other hand, treatment

with rat AM plus human AMBP-1 improved cardiovascular function, attenuated tissue injury and inflammatory responses, and reduced mortality in the rat CLP model of sepsis (14,15), suggesting that AM/AMBP-1 may be a beneficial treatment approach in human sepsis. AM/AMBP-1's beneficial effects in sepsis appear to be related with their vasoactive (14) and antiinflammatory (15) properties. In addition, AMBP-1, i.e., complement factor H, inhibits activation of the alternative pathway of the complement system. AM influences the complement regulatory function of factor H by enhancing the cleavage of C3b via factor I (12). Activation of the complement cascade is known to play a key role in the adverse immune consequences of sepsis (43-45). Therefore, inhibition of the alternative complement pathway might also contribute to AM/AMBP-1's beneficial effect in sepsis.

The immunogenicity of therapeutic proteins or peptides is an area of concern for the pharmaceutical industry. Human AM is a 52-amino acid peptide that has a carboxy-terminal amidated residue and a 6-residue ring structure formed by an intramolecular disulfide bridge. However, rat AM differs from human AM, having 50 amino acid residues with two amino acid deletions and six substitutions (46). Because the sequence variation and the length of amino acids are important factors controlling the immunogenicity, the major obstacle hampering the development of AM/AMBP-1 as a therapeutic agent for sepsis is the potential immunogenicity of rat AM in humans. Even though rat and human AM differs by only two amino acid deletions and six substitutions, it is highly unlikely that rat AM would ever have been considered for human use. As an important and necessary step in developing AM/AMBP-1 as a safe and effective treatment for patients with sepsis and septic shock, we therefore tested the efficacy of human AM/AMBP-1 in a rat model of polymicrobial sepsis in the current study. Our results have clearly demonstrated the beneficial effect of human AM in combination with human AMBP-1 in established sepsis in rats. Sim-

ilar to rat AM plus human AMBP-1 (14,15), human AM plus human AMBP-1 decreased serum levels of ALT, AST, and lactate; downregulated circulating levels of proinflammatory cytokine TNF- α and IL-6; ameliorated functional damage to the intestinal mucosa; and reduced mortality even when they were administered at 10 hours after CLP (i.e., severe sepsis). Moreover, it appears that human AM/AMBP-1 at the dose of 24/80 $\mu\text{g}/\text{kg}$ BW provides the best protection.

Our recent study has shown that the half-lives of human AM and human AMBP-1 to be 35.8 minutes and 1.68 hours, respectively (47). Apparently, human AM/AMBP-1's effect on the survival rate of septic animals is due to the blockade of the inflammatory cascade, thus leading to a long-term protection. As shown in Figure 5, although the majority of the deaths occurred within 48 hours after CLP, some septic animals died at 3-7 days after CLP. In this regard, multiple administration of human AM/AMBP-1 may produce even better protection. The ratio of human AM and human AMBP-1 was chosen based on our previous experience in rat AM and human AMBP-1 (14,15). However, it remains to be determined whether this is the best ratio. Moreover, the exact treatment window of human AM/AMBP-1 in sepsis and the pharmacokinetic characterization of human AM/AMBP-1 will be assessed in our future studies.

In summary, tissue injury as evidenced by increased levels of transaminases and lactate was present at 20 hours after CLP. Moreover, proinflammatory cytokines TNF- α and IL-6 were also significantly elevated. Administration of human AM in combination with human AMBP-1 at 10 hours after CLP markedly attenuated tissue injury, reduced cytokine levels, ameliorated intestinal barrier dysfunction, and improved survival. Thus, administration of human AM/AMBP-1 may provide a novel approach to the treatment of sepsis. We believe that the current study is an important step toward clinical use of human AM/AMBP-1 as a novel therapeutic approach for patients with sepsis.

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DISCLOSURE

One of the authors (P Wang) is an inventor of issued United States Patent #6,884,781, "Treatment of shock using adrenomedullin binding protein-1." This patent covers the fundamental concept of using human AM/AMBP-1 for the treatment of sepsis.

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