

Expression and characterization of recombinant human milk fat globule-EGF factor VIII

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Abstract. Apoptosis plays an important role in the pathobiology of sepsis. The opsonizing protein milk fat globule-EGF factor VIII (MFG-E8) is involved in apoptotic cell clearance. Our previous studies have shown that administration of rat MFG-E8-containing exosomes or recombinant murine MFG-E8 (rmMFG-E8) is protective in a rat model of sepsis induced by cecal ligation of puncture (CLP). However, one obstacle hampering the development of MFG-E8 as a therapeutic agent for septic patients is the potential immunogenicity of animal proteins in humans. The purpose of this study, therefore, was to express recombinant human MFG-E8 (rhMFG-E8) and characterize its biological activity. Using an *E. coli* system, we successfully expressed and purified the mature molecule of human MFG-E8 (Leu24-Cys387). The purity of rhMFG-E8 was over 99% and it was immunoreactive for specific anti-human MFG-E8 antibodies. Amino acid sequence analysis by LC-MS/MS identified the purified protein as human MFG-E8. Using primary rat peritoneal macrophages, we showed that rhMFG-E8 markedly increased peritoneal macrophage phagocytosis of apoptotic thymocytes, which was as effective as commercial rmMFG-E8. To determine the biological activity of rhMFG-E8 *in vivo*, male adult rats were subjected to sepsis by CLP. rhMFG-E8 or rmMFG-E8 were administered intravenously at the time of CLP. Our results demonstrated that both rhMFG-E8 and rmMFG-E8 reduced thymocyte apoptosis and plasma levels of lactate and IL-6 at 20 h after CLP, and improved the 10-day survival rate. Thus, we have successfully expressed and purified biologically active rhMFG-E8. Our newly-expressed rhMFG-E8 is highly effective in the rat model of sepsis.

Introduction

Sepsis is one of the most prevalent diseases and accounts for 20% of all admissions to intensive care units (ICUs) (1). Evidence indicates that in the US alone, more than 750,000 people develop sepsis each year with an overall mortality rate of 28.6% (1). Despite advances in the management of septic patients, a large number of such patients die of the ensuing septic shock and multiple organ failure (2-5). An analysis of hospital records indicates that the total number of patients who have died of sepsis is actually increasing (4). As the American population ages, the incidence of sepsis is projected to increase since the incidence and mortality rate of sepsis rise steadily with aging (1,4). Thus, there is an urgent unmet medical need for an effective novel therapy for patients with sepsis.

Milk fat globule-EGF factor VIII (MFG-E8), also known as lactadherin in humans, is a glycoprotein originally identified as a component of milk fat globules that bud from the mammary epithelia during lactation. It is an important milk mucin-associated defense component that inhibits enteric pathogen binding and infectivity (6). Recent studies have shown that MFG-E8 can also be secreted by activated macrophages and immature dendritic cells and has been linked to the opsonization of apoptotic cells (7-11). It promotes the engulfment of apoptotic cells by working as a bridging molecule between apoptotic cells and phagocytes.

Our previous studies have shown that administration of rat MFG-E8-containing exosomes or recombinant murine MFG-E8 (rmMFG-E8) increases phagocytosis of apoptotic cells, reduces proinflammatory cytokines, and improves survival in a rat model of sepsis induced by cecal ligation of puncture (CLP) (12,13). However, one obstacle hampering the development of MFG-E8 as a therapeutic agent for septic patients is the potential immunogenicity of animal proteins in humans. The purpose of this study, therefore, was to express recombinant human MFG-E8 (rhMFG-E8) and characterize its biological activity both *in vitro* and *in vivo*.

Materials and methods

Expression of recombinant human MFG-E8. A 1095 bp fragment encoding the mature region of human MFG-E8 (364 amino acids, R24-R387, SwissProt no. Q08431) was

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obtained by polymerase chain reaction amplification of a plasmid template containing the human MFG-E8 cDNA. The open reading frame was cloned into the *SaII* and *NotI* site of the pET-28a(+) vector (Novagen, Madison, WI) downstream of the phage T7 RNA polymerase promoter. The final protein product contained six histidines fused to the N-terminus of human MFG-E8. The plasmid was transformed into *E. coli* BL21 (DE3) cells. The cells were grown at 37°C in 2YT medium (Invitrogen) with kanamycin overnight. The rhMFG-E8 protein production was induced by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM and cell growth continued for 5 h at 25°C. The cells were harvested by centrifugation and the induced rhMFG-E8 protein was purified according to the manufacturer's instructions (Novagen). The rhMFG-E8 fractions were pooled and the endotoxin of the protein solution was removed by phase separation using Triton X-114 (14). The content of LPS in the sample was determined using the Limulus Amebocyte lysate assay (BioWhittaker, Inc., Walkersville, MD) as previously described (15). The purity of rhMFG-E8 was evaluated by SDS-PAGE on a 10-20% Tris-HCl gel and visualized using the GelCode Blue Stain Regent (Pierce, Rockford, IL). The final product was concentrated by Amicon Ultra-15 Centrifugal Filter Devices to the designed concentration and stored at -20°C.

Mass spectrometry. The amino acid sequence of the isolated and purified protein was analyzed by LC-MS/MS at the Proteomics Resource Center of the Rockefeller University (New York, NY). Briefly, the sample was reduced with 5 mM DTT and alkylated with 10 mM iodoacetamide, and then digested with sequence grade modified trypsin (Promega) in ammonium bicarbonate buffer at 37°C overnight. The digestion products were analyzed by LC-MS/MS. For LC-MS/MS analysis, the digestion product was separated by gradient elution with the Dionex capillary/nano-HPLC system and analyzed by the Applied Biosystems QSTAR XL mass spectrometer using information-dependent, automated acquisition. The acquired ms/ms spectra were converted to a Mascot acceptable format and searched using the Mascot database search algorithm. The allowed variable modifications for database searching were oxidation of methionines.

Western blot analysis of rhMFG-E8. Purified rhMFG-E8 proteins were electrophoretically fractionated on a 10-20% Tris-HCl gel under reducing conditions, transferred to a 0.45- μ m nitrocellulose membrane, and blocked with 5% non-fat dry milk in phosphate-buffered saline. Afterward, the membrane was incubated with 1:1,000 polyclonal antibody to human MFG-E8 (R&D Systems, Minneapolis, MN) overnight at 4°C. The blots were then incubated with horseradish peroxidase-linked anti-rabbit immunoglobulin G (1:10,000, Cell Signaling Technology, Beverly, MA) for 1 h at room temperature. A chemiluminescent peroxidase substrate (ECL, Amersham Biosciences, Piscataway, NJ) was applied according to the manufacturer's instructions, and the membranes were exposed briefly to radiography film.

Phagocytosis assay. This assay was conducted according to our previously described protocol (16). Briefly, freshly collected

peritoneal macrophages from normal adult Sprague-Dawley rats were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Carlsbad, CA) containing 10% heat-inactivated exosome-free fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Cells were plated at a density of 2.5x10⁴/well in a 16-well chamber slide (Nunc International, Rochester, NY). For all experiments, cells were kept at 80-90% confluence. Freshly collected thymocytes were cultured at a concentration of 10⁷ cells/ml in RPMI substituted with 10% heat-inactivated FBS, 10 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.1 μ M dexamethasone for 16-24 h at 37°C and 5% CO₂. This produced ~100% of apoptotic cells as assessed by Annexin V/propidium iodide (PI) staining and analyzed by FACS. After being washed twice with Hank's balanced salt solution (HBSS, Gibco), the apoptotic thymocytes were resuspended in OPTI-MEM (Gibco) and incubated with or without rhMFG-E8 (0.5 μ g/ml) or rmMFG-E8 (0.5 μ g/ml) for 30 min. Then the cells were incubated with 20 ng/ml pHrodo succinimidyl ester (SE) (Invitrogen) for 30 min. After washing, the cells were fed to cultured macrophages at the ratio of 4:1 (apoptotic cells/macrophages) for 1.0 h. Then, adherent macrophages were washed twice with PBS and incubated with FITC-anti-rat CD11b/c (OX42; BD Pharmingen) for 20 min. This staining provided a homogenous surface staining of macrophages and was useful to distinguish the surface of macrophages during analysis. The cells were then fixed with 1% paraformaldehyde for 15 min at 4°C, transferred to PBS and kept at 4°C until analysis by fluorescent microscopy using a Nikon Eclipse E600 fluorescent microscope (Japan). The number of apoptotic cells and of macrophages that engulfed apoptotic cells were expressed as a ratio of apoptotic cells/macrophages (phagocytosis index).

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 access to water *ad libitum*. Rats were anesthetized with isoflurane inhalation and the ventral neck, abdomen and groin were shaved and washed with 10% povidone iodine. Cecal ligation and puncture (CLP) was performed as 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. Immediately after CLP, a femoral vein was cannulated with a PE-50 tubing under anesthesia (isoflurane inhalation). The animal received a bolus injection of rhMFG-E8 (20 μ g/kg BW) in a volume of 1 ml normal saline via the femoral venous catheter. Positive control animals received commercial rmMFG-E8 (20 μ g/kg BW). Vehicle-treated animals received a non-specific human plasma protein (i.e., human albumin) at the time of CLP. Sham-operated animals (i.e., control animals) underwent the same procedure with the exception that the cecum was neither ligated nor punctured. The animals were resuscitated with

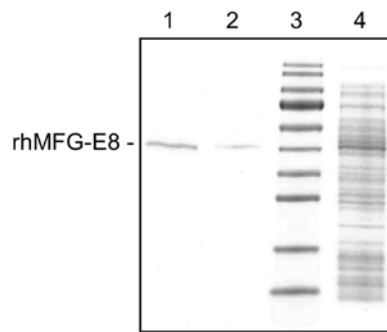


Figure 1. SDS-PAGE analysis of the expressed and purified rhMFG-E8. Lane 1, purified rhMFG-E8 (1 μ g); lane 2, purified rhMFG-E8 (0.5 μ g); lane 3, marker; lane 4, unpurified bacterial lysis.

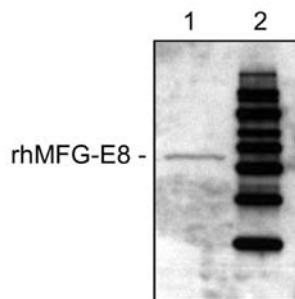


Figure 2. Western blot analysis of the expressed and purified rhMFG-E8. The specific anti-human antibody recognizes MFG-E8 by Western blot analysis. Lane 1, purified rhMFG-E8 (1 μ g); lane 2, marker.

3 ml/100 g 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 (IACUC) of the Feinstein Institute for Medical Research.

Determination of thymocyte apoptosis. Thymocyte apoptosis was assessed by Annexin V/PI staining and Western blot analysis of cleaved caspase-3 protein expression. Briefly, the fresh thymus was harvested at 20 h after CLP or sham operation. Thymocytes were isolated as previously described (16). The cells were stained using the Annexin V Fluos staining kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instruction and analyzed by flow cytometry with FACSCalibur (BD Biosciences). The Annexin V⁺-PI⁺ cells were considered as apoptotic cells. Cleaved caspase-3 protein expression was measured by Western blot analysis similar to the method for rhMFG-E8 protein analysis, as described above. Specific antibodies against cleaved caspase-3 protein (Cell Signaling, Danvers, MA) were used. β -actin was used as the loading control.

Determination of serum levels of lactate and IL-6. Serum concentrations of lactate were determined by using the assay kit according to the manufacturer's instructions (Pointe Scientific, Lincoln Park, MI). Serum levels of IL-6 were measured using

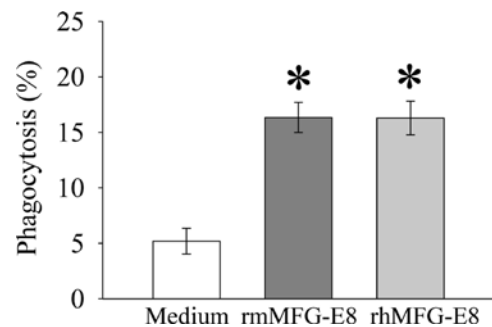


Figure 3. rhMFG-E8 potentiates phagocytosis of apoptotic cells. Fluorescent intensity of pHrodo-SE-labeled apoptotic thymocytes increases after engulfment by macrophages. Splenic macrophages were labeled with FITC anti-CD11b/c and thymocytes with pHrodo-SE. Cells were co-incubated for 60 min, collected and fixed with 1% PFA prior to fluorescent microscopy. The phagocytosis index of CD11b/c+pHrodo+ cells is shown. Data are presented as means \pm SE (n=6-9/group) and compared by one-way ANOVA and the Student Newman-Keuls test, *P<0.05 vs. medium alone.

a commercially available enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Camarillo, CA) according to the manufacturer's instruction.

Survival study. In additional groups of animals, vehicle (human albumin), rhMFG-E8 or rmMFG-E8 (20 μ g/kg BW) was administered immediately after CLP as described above. At 20 h after CLP, the gangrenous cecum was surgically excised and the peritoneal cavity was irrigated twice with 20 ml warm, sterile saline solution. The abdominal incision was then closed in layers, and rats received 3 ml/100 g BW saline subcutaneously. The animals were then returned to their cages and allowed food and water *ad libitum*. The changes in survival were monitored for 10 days.

Statistical analysis. All data are expressed as means \pm SE and compared by one-way analysis of variance (ANOVA). When the ANOVA was significant, post-hoc testing of differences between groups was performed using the Student Newman-Keuls test. The survival rate was estimated by Kaplan-Meier method and compared by the log-rank test. A P-value <0.05 was considered statistically significant.

Results

Expression and purification of rhMFG-E8. Using the *E. coli* system, we successfully expressed and purified rhMFG-E8. The SDS-PAGE analysis showed a single band at approximately 46 kDa (Fig. 1). The purity of rhMFG-E8 was >99% according to SDS-PAGE method (Fig. 1). The endotoxin level in the recombinant protein sample was not detectable as measured by the Limulus Amebocyte Lysate method (data not shown). Western blot analysis showed that purified rhMFG-E8 was immunoreactive for specific anti-human MFG-E8 antibodies (Fig. 2). Amino acid sequence analysis by LC-MS/MS showed that the purified protein was identified as human MFG-E8 with more than 95% confidence.

rhMFG-E8 increases the phagocytosis of apoptotic cells *in vitro*. Using peritoneal macrophages isolated from normal

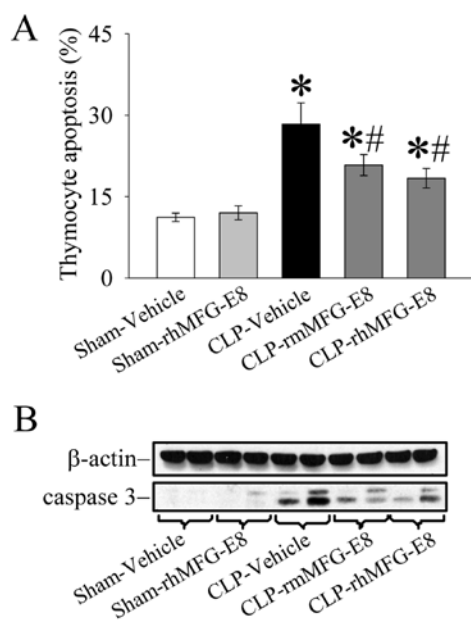


Figure 4. rhMFG-E8 reduced thymocyte apoptosis after CLP. Rats underwent CLP to induce experimental sepsis and were treated with human albumin (vehicle), rmMFG-E8 (20 μ g/kg BW), or rhMFG-E8 (20 μ g/kg BW) immediately after CLP. (A) Thymocyte apoptosis was assessed 20 h after CLP by Annexin V/PI staining and FACS analysis. Data are presented as means \pm SE (n=4/group) and compared by one-way ANOVA and the Student Newman-Keuls method: *P<0.05 vs. the sham group; #P<0.05 vs. the vehicle group. (B) Alterations in cleaved caspase-3 in the thymus were determined by Western blot analysis at 20 h after CLP. Representative gels of two independent observations are presented.

rats, we have shown that rhMFG-E8 (0.5 μ g/ml) markedly increased peritoneal macrophage phagocytosis of apoptotic thymocytes as compared to medium control (P<0.05) (Fig. 3). Moreover, our test indicated that rhMFG-E8 is as effective as commercial rmMFG-E8 in the rat (Fig. 3). Thus, the purified rhMFG-E8 effectively increases the clearance of apoptotic cells *in vitro*.

rhMFG-E8 reduces apoptosis and tissue injury in a rat model of sepsis. To determine the biological activity of the newly-expressed rhMFG-E8 *in vivo*, we examined its effect in a rat model of CLP. As shown in Fig. 4A, thymocyte apoptosis at 20 h after CLP increased by 153% relatively to vehicle-treated animals. Administration of rmMFG-E8 or rhMFG-E8 decreased sepsis-induced thymocyte apoptosis by 27 and 35%, respectively, relative to the CLP-vehicle (P<0.05). However, rhMFG-E8 had no effect on thymocyte apoptosis in sham-operated animals. These findings were confirmed by protein levels of cleaved caspase-3 (an indicator of cell apoptosis) in the thymus (Fig. 4B). Serum levels of lactate, a marker for systemic hypoxia, increased by 60% at 20 h after CLP. Administration of rhMFG-E8 decreased lactate levels by 19% (P<0.05) (Fig. 5A). Similarly, serum levels of IL-6, an organ injury indicator as well as a marker for inflammation, increased by 457% at 20 h after CLP. Administration of rmMFG-E8 or rhMFG-E8 decreased IL-6 levels by 46 and 38%, respectively (P<0.05) (Fig. 5B).

rhMFG-E8 decreases sepsis-induced mortality in rats. To determine the long-term effect of rhMFG-E8 in sepsis, a 10-day

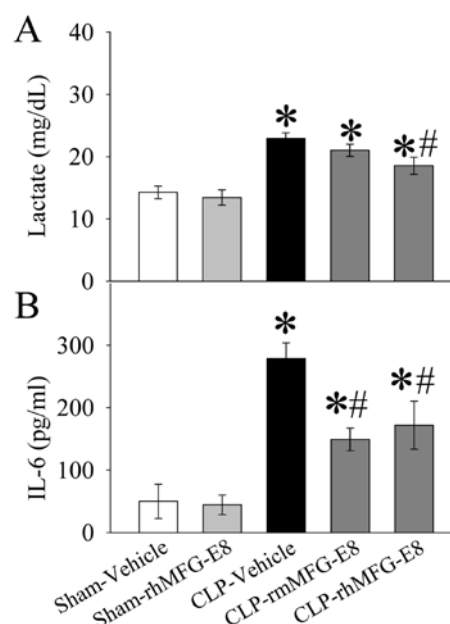


Figure 5. rhMFG-E8 attenuated organ injury after CLP. Rats underwent CLP to induce experimental sepsis and were treated with human albumin (vehicle), rmMFG-E8 (20 μ g/kg BW) or rhMFG-E8 (20 μ g/kg BW) immediately after CLP. Serum levels of (A) lactate and (B) IL-6 were measured at 20 h after CLP. Data are presented as means \pm SE (n=4-6/group) and compared by one-way ANOVA and the Student Newman-Keuls method: *P<0.05 vs. the sham group; #P<0.05 vs. the vehicle group.

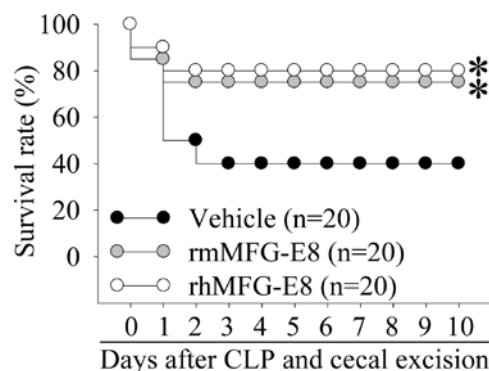


Figure 6. Alterations in the survival rate at 10 days after cecal ligation and puncture and cecal excision with human albumin treatment (vehicle), rmMFG-E8 (20 μ g/kg BW) or rhMFG-E8 (20 μ g/kg BW) treatment. There were 20 animals in each group. The survival rates were estimated by the Kaplan-Meier method and compared by using the log-rank test. *P<0.05 vs. the vehicle group.

survival study was conducted. As shown in Fig. 6, the survival rate after CLP and cecal excision in vehicle (albumin)-treated animals was 50% on Day 2, and decreased to 40% on Days 3-10. Administration of rmMFG-E8 or rhMFG-E8 improved the survival rate to 75 and 80%, respectively (P<0.05) (Fig. 6).

Discussion

Sepsis is a common, expensive, and frequently fatal condition. Although a great deal of preclinical and clinical trials have been carried out testing the efficacy and safety of various anti-sepsis agents (e.g., anti-cytokine and anti-endotoxin

antibodies, steroids, antithrombin, insulin and inhibition of apoptosis), these investigations have not resulted in the development of effective clinical treatments (2-5). Apoptosis plays an important role in the pathobiology of sepsis (20-25). Reduction of apoptosis by overexpression of the anti-apoptotic Bcl-2 protein or inhibition of pro-apoptotic molecules such as caspases, Fas-ligand, TNF-R or TRAIL has been proven to be beneficial in septic animals (26-31). In addition to the increased incidence of apoptosis, the phagocytic function is impaired in sepsis (32-35). Our previous studies have shown that down-regulation of MFG-E8 is responsible for the reduced phagocytosis of apoptotic cell in sepsis (12,13). Administration of rat MFG-E8-containing exosomes or rmMFG-E8 increases phagocytosis of apoptotic cells, reduces proinflammatory cytokines, and improves survival in a rat model of sepsis (12,13). The biological effect of this molecule has been confirmed using the MFG-E8 knockout animal model (13). Similarly, Bu *et al* (36) have shown that sepsis-triggered intestinal injury was associated with a down-regulation of intestinal MFG-E8 and treatment with rmMFG-E8 promoted mucosal healing in septic mice. Thus, MFG-E8 appears to be a leading candidate for treating septic patients.

Human MFG-E8 shares only 59, 57 and 53% amino acid (aa) sequence identity with porcine, rat and mouse MFG-E8, respectively (<http://blast.ncbi.nlm.nih.gov>). In order to move forward this technology into the preclinical and clinical development, human MFG-E8 is required. However, the extremely high cost of commercial human MFG-E8 (using the murine myeloma cell line by R&D Systems) limits its further development. In the current study, we have successfully expressed and purified rhMFG-E8 using an *E. coli* system at a much lower cost (>95% less expensive). The human MFG-E8 gene is located on chromosome 15q25 and is composed of eight exons. Human MFG-E8 protein is synthesized as a 387 aa precursor that contains a 23 aa signal sequence and a 364 aa mature region. The protein we have expressed is the mature molecule of human MFG-E8 (i.e., Leu24-Cys387) with an N-terminal 6-His tag. Native MFG-E8 is a glycoprotein. Since our rhMFG-E8 was expressed in an *E. coli* system, it has no glycosylation. As demonstrated by this study, our *E. coli*-derived rhMFG-E8 is as effective as the rmMFG-E8 expressed in the murine myeloma cell line (R&D Systems). Our *E. coli*-derived rhMFG-E8 markedly increased peritoneal macrophage phagocytosis of apoptotic thymocytes and reduced thymocyte apoptosis and plasma levels of lactate and IL-6 at 20 h after CLP. Most importantly, administration of our *E. coli*-derived rhMFG-E8 improved the survival rate after CLP. Apparently, the glycosylation may not be essential for the biological function of MFG-E8.

The mature molecule of human MFG-E8 contains four N-linked glycosylation sites, an amino-terminal EGF-like domain, plus C1 and C2 Ig-like domains which are related to discoidin I and are homologous to those of human coagulation factors V and VIII (37,38). The EGF like domain contains the 'RGD-motif' (the amino acid sequence: Arg-Gly-Asp), which is strategically placed in a hairpin loop between two antiparallel β strands (37,38). In this way, the EGF like domain serves as a scaffold for the RGD sequence, which is proposed to promote cell adhesion by binding cell surface integrin receptors, such as $\alpha\beta 3$ or $\alpha\beta 5$ (39-41). The coagulation factor V/VIII like

domains bind to phosphatidylserine (PS) exposed on the surface of apoptotic cells (42). Binding of MFG-E8 to PS on apoptotic cells opsonizes them for a complete engulfment by macrophages via $\alpha\beta 3$ - or $\alpha\beta 5$ -integrins. Without MFG-E8, full engulfment and the removal of apoptotic cells cannot be completed (8). Apoptosis has been considered as an orderly process of cell suicide that does not elicit inflammation (43). However, recent discoveries have shown that apoptotic cells eventually undergo secondary necrosis and stimulate an inflammatory response if they are not removed by phagocytosis (44,45). The lack of clearance of apoptotic B-cells in the spleen potentially leads to autoimmune diseases (7,8). Similar phenomena were also reported in the acute inflammatory environment such as sepsis (13,46). In a recent study, pre-treatment of animals with apoptotic splenocytes worsens the outcome of sepsis (46), pointing out the detrimental effect of apoptotic cells in the septic organism. Our current study also confirms the importance of apoptotic cell clearance in the pathogenesis of sepsis.

In summary, we have successfully expressed and purified biologically active rhMFG-E8. Our newly-expressed rhMFG-E8 is highly effective in the rodent model of sepsis. To the best of our knowledge, we are the only group that has investigated the potential role of the promotion of apoptotic cell clearance by MFG-E8 on the treatment of sepsis.

Acknowledgements

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