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Protection from Lethal Cell Death in Cecal Ligation and Puncture-Induced Sepsis Mouse Model by *In Vivo* Delivery of FADD siRNA

Yuichi Hattori and Naoyuki Matsuda
Department of Molecular and Medical Pharmacology,
Graduate School of Medicine and Pharmaceutical Sciences,
University of Toyama, and
Department of Emergency and Critical Care Medicine,
Nagoya University Graduate School of Medicine,
Japan

1. Introduction

Sepsis is the leading cause of death in most intensive care units (Angus *et al*., 2001; Martin *et al*., 2003). Sepsis results from dysregulation of the normally protective anti-microbial host defense mechanism and represents a systemic inflammatory response that is associated with hypotension, insufficient tissue perfusion, uncontrolled bleeding, and multiple organ failure/dysfunction (Bone *et al*., 1992; Natanson *et al*., 1994). Accordingly, a major focus of sepsis research has been the development of anti-inflammatory strategies. In clinical trials, however, most of the therapies that may modify systemic inflammation have largely failed to reduce mortality in patients with severe sepsis (Zeni *et al*., 1997; Natanson *et al*., 1998; Marshall, 2000). These failed trials include administration of high-dose glucocorticoids; polyclonal and monoclonal antibodies against endotoxin and various inflammatory mediators such as tumor necrosis factor (TNF)-α; anti-inflammatories; nitric oxide (NO) inhibitors; anti-oxidants; and others. Hence, new understanding of the pathophysiological mechanisms underlying this complex disorder is needed to develop novel therapeutic strategies that will impact favorably on septic patient outcome.

Apoptosis is a second prominent feature of sepsis. This process is a mechanism of tightly regulated disassembly of cells caused by activation of certain specialized proteases called caspases. A number of laboratories have demonstrated that sepsis induces extensive lymphocyte apoptosis, which can impairment of immunoresponses, thereby predisposing patients to septic death (Ayala & Chaudry, 1996; Wesche *et al*., 2005; Hotchkiss *et al*., 2005; Lang & Matute-Bello, 2009; Matsuda *et al*., 2010a). Parenchymal cells, including intestinal and lung epithelial cells, also have increased apoptotic cell death in animal models of sepsis (Coopersmith *et al*., 2002a, 2002b; Perl *et al*., 2007). An autopsy study comparing samples from multiple organ systems in 20 patients who died of sepsis with those from 16 critically ill, non-septic patients has shown that gut epithelial apoptosis is increased in septic patients (Hotchkiss *et al*., 1999a). Moreover, it has been suggested that vascular endothelial cells may

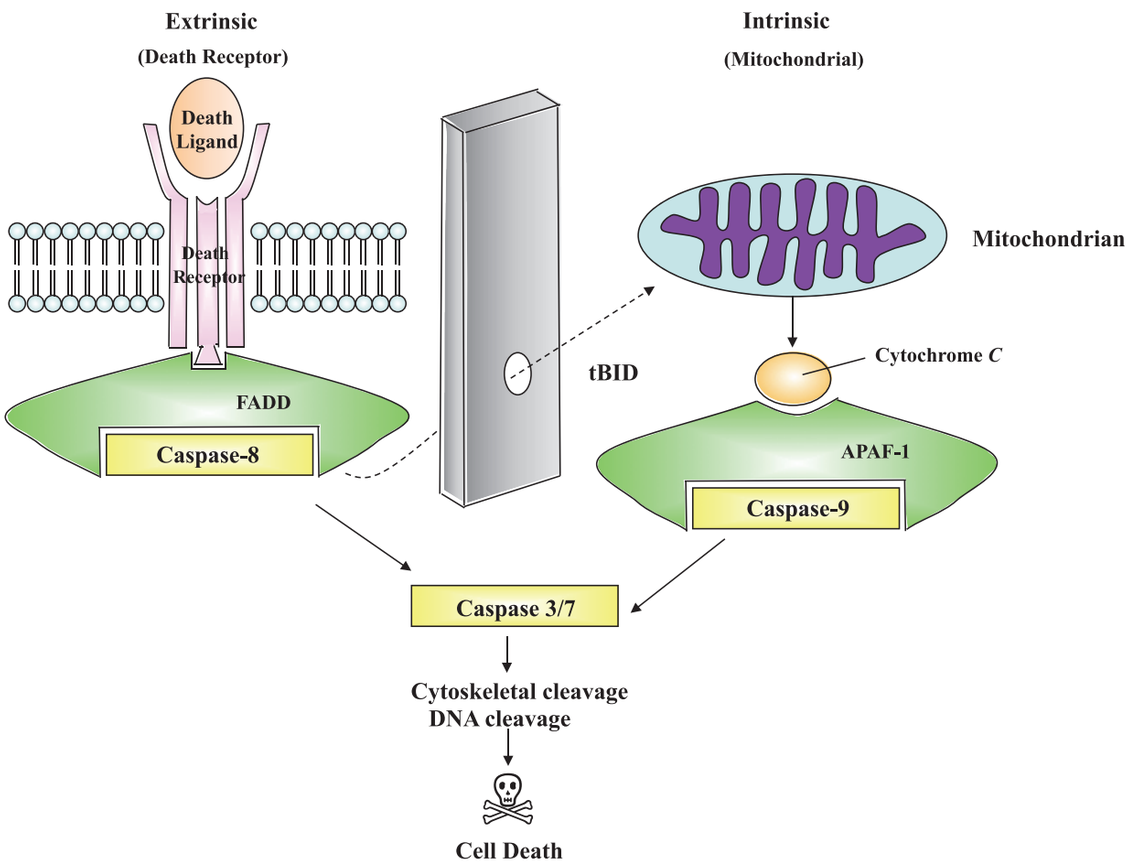
be undergoing apoptosis in sepsis (Hotchkiss *et al*., 2002). In sepsis, endothelial cell apoptosis may be associated with microvascular dysfunction with reduced perfusion and oxygen, which could result in tissue hypoxia and, ultimately, in the development of organ failure (Matsuda & Hattori, 2007). This could explain partly the disappointment in a large number of sepsis trials conducted with interventions against individual steps in the inflammatory cascade, leading investigators to the question of whether death in septic patients stems from uncontrolled inflammation (Hattori *et al*., 2010).

To reduce sepsis-induced apoptosis, caspase inhibitors have been examined in mice with cecal ligation and puncture (CLP)-induced sepsis. It has been reported that the pan-caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp (*O*-methyl) fluoromethyl ketone (*z*-VAD) decreases lymphocyte apoptosis in the thymus and spleen, decreases blood bacterial counts, and improves survival in sepsis (Hotchkiss *et al*., 1999b). In a similar study, the pan-caspase inhibitor L-826,920 (M-920) and the selective caspase-3 inhibitor L-826,791 (M-791) have shown a survival benefit being the result of the rescue of lymphocytes from apoptosis (Hotchkiss *et al*., 2000). Furthermore, Kawasaki *et al*. (2000) have demonstrated that *z*-VAD decreases apoptosis on pulmonary endothelial cells and epithelial cells and prolongs the survival rate in a lipopolysaccharide (LPS)-induced acute lung injury (ALI) mouse model. However, successful anti-apoptotic therapy in sepsis with caspase inhibitors may be challenging, because caspase inhibitors themselves can have detrimental effects within the cell when large amounts of them must reach the cell cytosol to inhibit apoptosis that is initiated only by small amounts of caspases. In fact, survival in *z*-VAD high-dose group after CLP returns toward the level of the control (Hotchkiss *et al*., 1999b), indicating that there appears to be close-limiting toxicity associated with the administration of *z*-VAD.

Small interfering RNA (siRNA) is another potential reversible inhibitor of the apoptotic death pathways. siRNA therapy may offer a unique alternative sepsis treatment to shorten the apoptotic arm of sepsis, revealing a number of targets within the apoptotic death pathways, which may be useful in designing stand-alone and/or adjuvant therapies that would have a significant impact on septic mortality. Although the causative agents of sepsis vary widely as do their traditional anti-microbial treatments, siRNA therapy targeted toward salvaging immune effector cells, vascular endothelial cells, and parenchymal cells from apoptosis has the potential to be beneficial in sepsis regardless of the source. We have generated synthetic double-stranded siRNA targeting Fas-associated death domain (FADD) and examined the therapeutic effect of systemic administration of the siRNA in the CLP mouse model, regarded as a highly clinically relevant animal model of polymicrobial sepsis. As described below, FADD is an essential component of the death-inducing signaling complex (DISC) for all death receptors (Thorburn, 2004; Lavrik *et al*., 2005). Here we present that this RNA interference-mediated gene silencing *in vivo* is effective as gene therapy of the septic syndrome.

2. Apoptotic cell death pathways

Two major pathways are involved in the initiation of apoptotic cell death (Figure 1) (Roy & Nicholson, 2000). The first apoptotic pathway is mediated by specific ligands and surface receptors, which are capable of delivering a death signal from the microenvironment and can activate the execution of apoptosis in the cell cytoplasm and organelles (Herr & Debatin,



The extrinsic, death receptor-mediated apoptotic pathway involves the binding of TNF-α or FasL to its cell surface receptor, resulting in the activation of caspase-8 and, subsequently, caspase-3. FADD serves as a docking protein for caspase-8, tethering the enzyme to activated death receptor. The intrinsic, mitochondria-mediated apoptotic pathway results in the release of cytochrome *c*, which binds to APAF-1 and caspase-9, thus forming the apoptosome. This complex activates caspase-3, which is involved in the final common pathway of the cell death program. The interaction of these two cell death pathways *via* tBID are presented.

Fig. 1. Two major pathways involved in initiation of apoptosis.

2001). This pathway is termed the extrinsic pathway. The second apoptotic pathway called the intrinsic pathway is activated by mitochondrial injury (Korsmeyer, 1999). The two apoptotic signaling pathways ultimately converge into a common pathway causing the activation of effector enzymes termed caspases.

The extrinsic pathway involves activation of members of the TNF receptor (TNF-R) family with an intracellular death domain (DD), including TNF-R1, Fas, DR3, DR4, DR5, and DR6. These death receptors transmit apoptotic signals initiated by specific ligands such as TNF-α, Fas ligand (FasL), and TRAIL. Thus, once activated, death receptors recruit the adaptor molecule FADD (plus others in some cases) through the homophilic interaction of their own DD to the DD of the adaptor molecule. FADD can then recruit the apoptosis initiator enzyme procaspase-8 into the DISC as a consequence of the death effector domain-mediated homophilic interaction. Subsequently, procaspase-8 is activated proteolytically into caspase-8 and further activates the apoptosis effector enzymes caspase-3 and other execuitioner

caspases (caspase-6 and caspase-7) (Thorburn, 2004; Lavrik *et al*., 2005; Green & Kroemer, 2005).

The intrinsic pathway is initiated by stress signals through the release of apoptogenic factors such as cytochrome *c* from the mitochondrial intermembrane space. Upon release into the cell cytoplasm, cytochrome *c* promotes the formation of a complex between the caspase adaptor molecule APAF-1 and the apoptosis initiator enzyme termed procaspase-9 in a caspase-activating structure known as the apoptosome, and consequently triggers the apoptotic cascade by activating procaspase-9. Thus, the apoptosome, through newly activated caspase-9, activates the effector caspases along the common pathway of apoptosis (Korsmeyer, 1999).

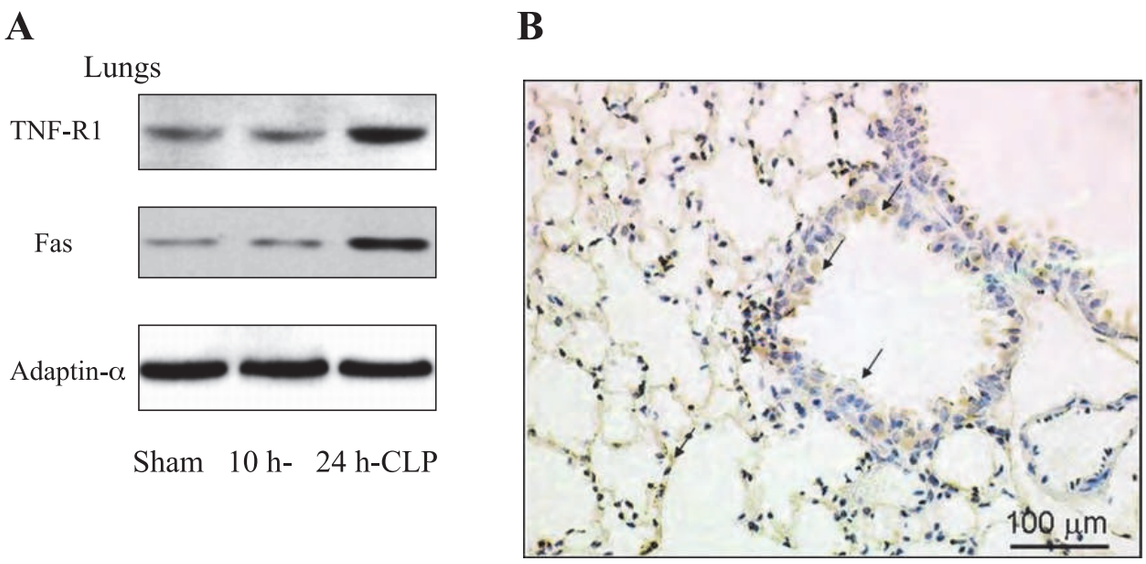
In certain types of cells, there is extensive cross-talk that occurs between the extrinsic and intrinsic apoptotic pathways (Roy & Nicholson, 2000). Thus, the extrinsic and intrinsic apoptotic pathways are intimately connected. This appears to occur via the proteolysis of BID, which normally serves an anti-apoptotic role within the intrinsic mitochondrial-mediated pathway. BID is truncated to receptor pathway, whereupon tBID promotes activation of Bax and Bak and thereby induces cytochrome *c* release, leading to formation of the apoptosome (Esposti, 2002).

3. Impact of the FADD gene silencing with siRNA in sepsis therapy

3.1 Sepsis-induced up-regulation of death receptors

We initially verified the hypothesis that tissue expression of death receptors is up-regulated in sepsis. Polymicrobial sepsis was induced by CLP in BALB/c mice (Matsuda *et al*., 2005). A middle abdominal incision was performed under anesthesia. The cecum was mobilized, ligated at 5 mm from its top, and then perforated in two locations with a 21-gauge needle, allowing expression of feces. The bowel was repositioned, and the abdomen was closed. Sham-operated animals underwent the same procedure except for ligation and puncture of the cecum. This model has high clinical relevance to humans, because it reproduces many hallmarks of sepsis that occur in patients (Hubbard *et al*., 2005).

Immunoblot analysis showed that surface expression of the two death receptors TNF-R1 and Fas were up-regulated in lung tissues with time after CLP induction of sepsis (Figure 2A). Immunohistochemical studies indicated more abundant TNF-R1 expression in the inner wall of microvessels from septic mouse lungs (Figure 2B). Meanwhile, Fas was detected mainly in alveolar epithelial Type II cells (Matsuda *et al*., 2009). Similar to these death receptors, DR4 and DR5, both of which mediate TRAIL-induced cell death, were up-regulated in septic mouse lungs (Matsuda *et al*., 2009). We also found time-dependent increases in surface expression of TNF-R1 and Fas in mouse aortic tissues after CLP sepsis (Figure 3A). These death receptors are likely to be up-regulated mainly on endothelial cells, because the sepsis-induced up-regulation of TNF-R1 and Fas expression in aortic tissues was abolished when the tissues were denuded mechanically. Previous works from other laboratory have demonstrated that Fas expression is increased in hepatocytes and in selected gastrointestinal-associated lymphoid tissues (Chung *et al*., 2001, 2003). Moreover, splenocytes harvested 24 hours after CLP and stimulated with the T cell mitogen concavalin A showed an increase in $CD4^+$ T-cell apoptosis as compared to sham controls, which was associated with an increase in Fas expression (Ayala *et al*., 1999). Based on the findings of

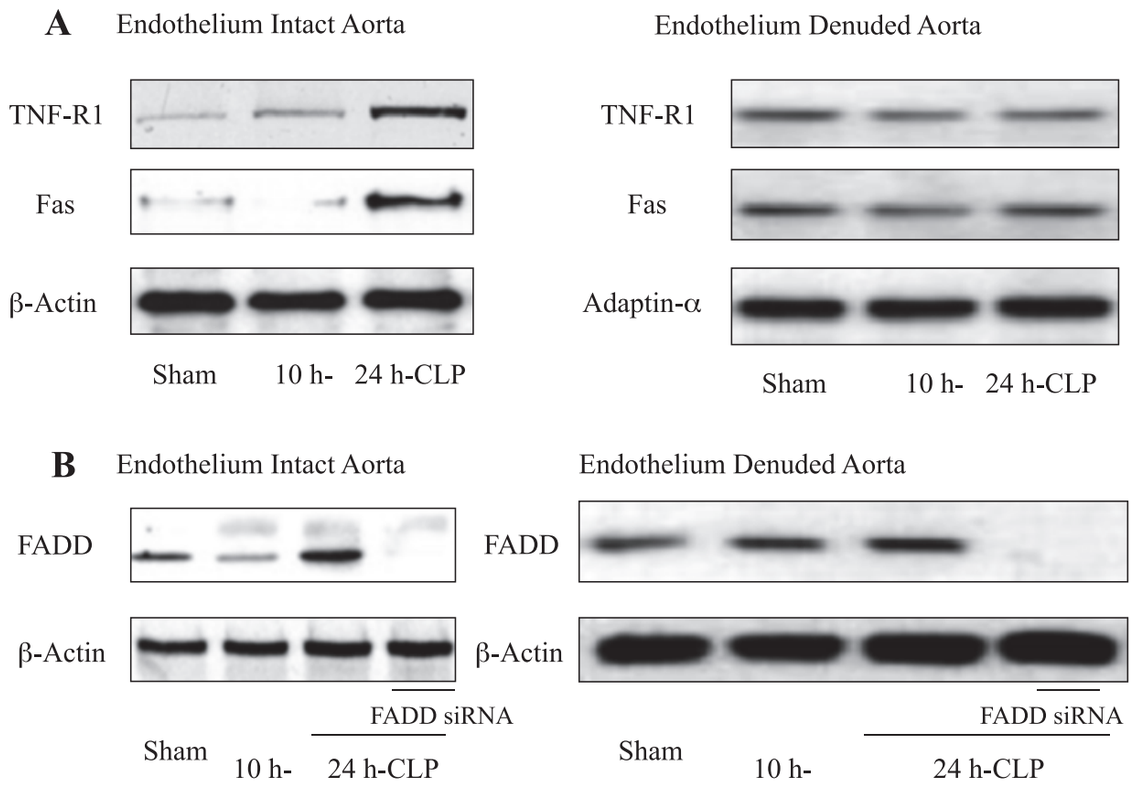


(A) Western blots of TNF-R1 and Fas in the membrane fractions of lung tissues from mice that were subjected to sham operation (control) and sepsis (10 and 24 hours after CLP). Adaptin-α served as loading control. (B) Immunohistochemical finding for TNF-R1 in the lung section from the mouse subjected to sepsis (24 hours after CLP). Positive staining is indicated by arrows.

Fig. 2. Increased death receptor expression in lung tissues of septic mice.

increased death receptor expression in tissues of septic mice, we suggest the importance of the extrinsic death receptor pathway in apoptotic cell death in sepsis, although a preeminent role for the intrinsic mitochondrial pathway has often been noted (Exline & Crouser, 2008).

As presented in Figure 1, death receptors, after ligand binding, recruit the adaptor protein FADD through hemophilic interaction of their DD with the DD of FADD, and then FADD can recruit procaspase-8 to the DISC, thereby causing its activation (Thorburn, 2004; Lavrik et al., 2005). When FADD protein levels were assessed by Western blotting, induction of sepsis by CLP led to a time-dependent increase in FADD protein expression in aortic tissues (Figure 3B). This increase occurred on endothelial cells since FADD protein expression was not increased in endothelium-denuded aortic tissues from septic animals. For silencing of gene expression of FADD, siRNA oligonucleotides with the following sense and antisense sequences were designed: 5′-GCA GUC UUA UUC CUA Att-3′ and 5′-UUA GGA AUA AGA GGA GUA Ctt-3′ (Matsuda et al., 2009, 2010b). In vivo transfection of synthetic siRNAs via tail vein was performed at 10 hours after CLP with Lipofectamine RNAiMAX (Invitrogen). We used Opti-MEM I Reduced Serum Medium (Invitrogen) to dilute siRNAs and Lipofectamine RNAiMAX before complexing, by which 50 μg of FADD siRNA sequence was usually delivered. Systemic delivery of FADD siRNA nearly completely eliminated aortic protein expression of FADD (Figure 3B). We also confirmed that the increased levels of FADD mRNA and protein in lungs after CLP induction of sepsis were strongly suppressed by systemic application of FADD siRNA but not of scrambled siRNA (Matsuda et al., 2009). These findings suggested the successful efficacy of systemically administered siRNA for silencing tissue expression of FADD in septic mice.

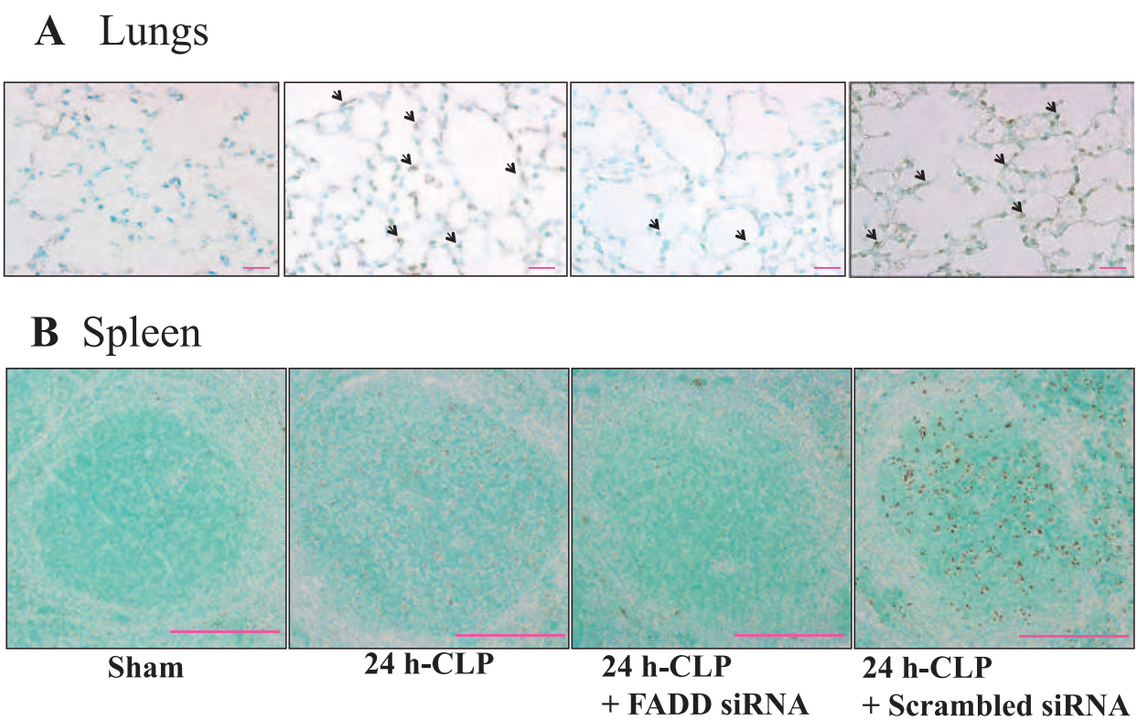


Western blots of TNF-R1 and Fas in the membrane fractions (A) and FADD in the total fractions (B) of aortic vessels from mice that were subjected to sham-operation (control) and sepsis (10 and 24 hours after CLP). β-Actin and adaptin-α served as loading control. In B, sepsis-induced up-regulation of FADD protein expression was eliminated by systemic delivery of FADD siRNA. Note that no increase in death receptors and FADD was observed when the endothelium was removed by gently rubbing the intimal surface.

Fig. 3. Endothelium-dependent increases in expression of death receptors and FADD in aortic vessels of septic mice.

3.2 Effect of FADD siRNA on cell apoptosis in sepsis

To assess whether FADD siRNA treatment has a beneficial effect on sepsis-induced apoptotic cell death in lungs, the tissue sections were labeled with an *in situ* TUNEL assay (Figure 4A). Apparently, no TUNEL-positive cells were observed in sham control mice. Induction of sepsis by CLP resulted in a striking appearance of TUNEL-positive cells. Apoptotic cells were identical morphologically to endothelial cells of capillary vessels in the alveolar septa and to epithelial type II cells (Matsuda *et al*., 2009). In agreement with this finding, our recent immunofluorescence studies showed that the cleaved form of caspase-3 was present in cell types other than CD31-positive endothelial cells in lungs (Takano *et al*., 2011). In lungs from CLP mice treated with FADD siRNA, but not with scrambled siRNA, TUNEL-positive cells were decreased sharply, providing a protective effect of FADD siRNA treatment on pulmonary cell apoptosis mediated by sepsis.

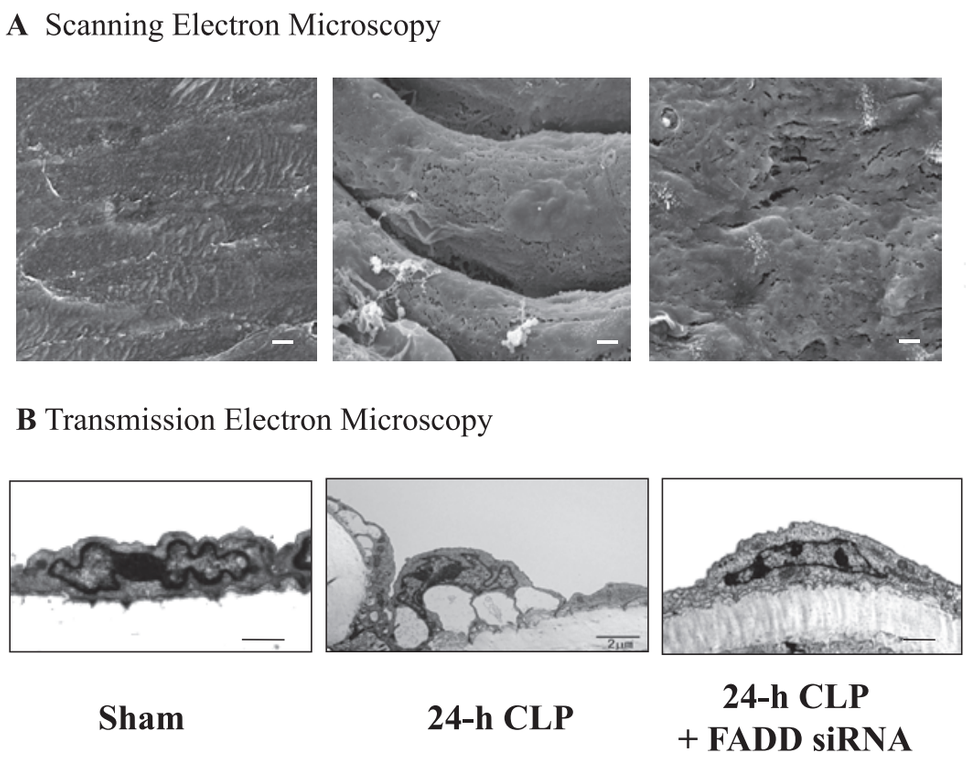


Sections of the lung (A) and spleen (B) were prepared from mice that were underwent sham procedure, CLP, CLP and FADD siRNA administration, or CLP and scrambled siRNA administration. Tissues were harvested 24 hours after surgery. In A, TUNEL-positive apoptotic cells are indicated by arrows. Scale bars = 20 μm (A) and 200 μm (B).

Fig. 4. Mouse lung and spleen tissue sections showing apoptotic cells by an *in situ* TUNEL assay.

Physiologic TUNEL-positive cells, morphologically identical to lymphocytes (Matsuda *et al*., 2009, 2010a), were sporadically present in the spleen tissues from sham control mice (Figure 4B). In spleens 24 hours after septic insult, marked apoptosis of follicular lymphocytes were observed. Most apoptotic lymphocytes were located in the white pulp of the spleen. TUNEL-positive lymphocytes in spleen follicles were greatly reduced when FADD siRNA was systemically given after CLP. Administration of scrambled siRNA to septic mice showed more frequent TUNEL positivity than no treatment.

Light microscopic studies of aortic tissue sections from septic mice at 24 hours after CLP showed partial detachment of endothelial cells from the basal membrane (Matsuda *et al*., 2007, 2010b). When the tissue sections were labeled with an *in situ* TUNEL assay, a significant number of apoptotic endothelial cells was found in aortas of septic mice (Matsuda *et al*., 2007, 2010b). Furthermore, scanning electron microscopic analysis indicated that the structure of aortic endothelium displayed a remarkable morphological abnormality: most endothelial cells were badly swollen (Figure 5A). Such an endothelial histological injury was strikingly prevented by systemic treatment with FADD siRNA but not with scrambled siRNA.



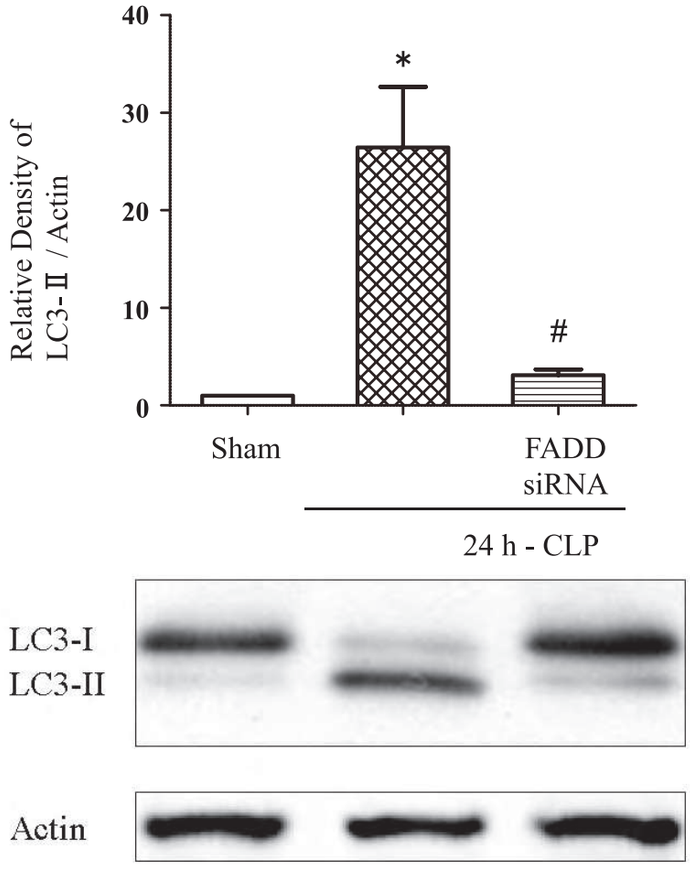
Mice were underwent sham procedure, CLP, or CLP and FADD siRNA administration. Aortic endothelial cells were evaluated by scanning electron microscopy (SEM, A) and by transmission electron microscopy (TEM, B) 24 hours after surgry. Septic mice showed anomalous swelling of cells (SEM) and autophagy-related vacuolation in the cytoplasm (TEM). These electron-microscopical changes were prevented by systemic treatment with FADD siRNA. Scale bars = 2 μm.

Fig. 5. Electron microscopic analysis of mouse aortic endothelial cells.

3.3 Effect of FADD siRNA on sepsis-induced autophagy in endothelial cells

A non-apoptotic and non-oncotic type of cell death has been recognized (Clarke, 1990). This type of cell death is characterized by the appearance of double- or multi-membrane cytoplasmic vesicles engulfing bulk cytoplasm and cytoplasmic organelles, such as mitochondria and endoplasmic reticulum, and their delivery to and subsequent degradation by the lysosomal system of the same cell (Gozuacik & Kimchi, 2004). This type of cell death is referred to as autophagic cell death, but it is still unsettled whether autophagy is the direct primary cause of cell death or a compensatory mechanism that tries to rescue a cell from dying. In starvation, autophagy provides an internal source of nutrients for energy generation, promoting cell survival. Defects in autophagy have been implicated in the pathophysiology of cancer and neurodegenerative diseases (Rabinowitz & White, 2010). On the other hand, systemic inflammatory response syndrome and multiple organ dysfunction syndrome are suggested to be accompanied by increased cell death, including autophagy, in the affected organs (Yasuhara *et al*., 2007). A recent report has shown that LPS induces autophagy in human umbilical vein endothelial cells (Meng *et al*., 2010). Moreover, LPS-induced systemic inflammation has been demonstrated to exert autophagy of hepatocytes in streptozotocin-induced diabetic rats (Hagiwara *et al*., 2010).

Our ultrastructural analysis using transmission electron microscopy indicated the formation of autophagy-like vesicles in aortic endothelial cells of CLP septic mice (Figure 5B). Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a soluble protein with a molecular mass of ~17 kDa that is distributed ubiquitously in mammalian tissues. Cleavage of LC3 at the carboxyterminus immediately following synthesis yields the cytosolic LC3-I form. LC3-I form is converted to LC3-II during autophagy. Thus, LC3-II is widely used as an indicator of autophagy (Kabeya *et al.*, 2000). Western blot analysis revealed significantly elevated aortic LC3-II levels in the CLP septic group (Figure 6). Very interestingly, these autophagy-related changes were prevented by systemic application of FADD siRNA (Figures 5B and 6).

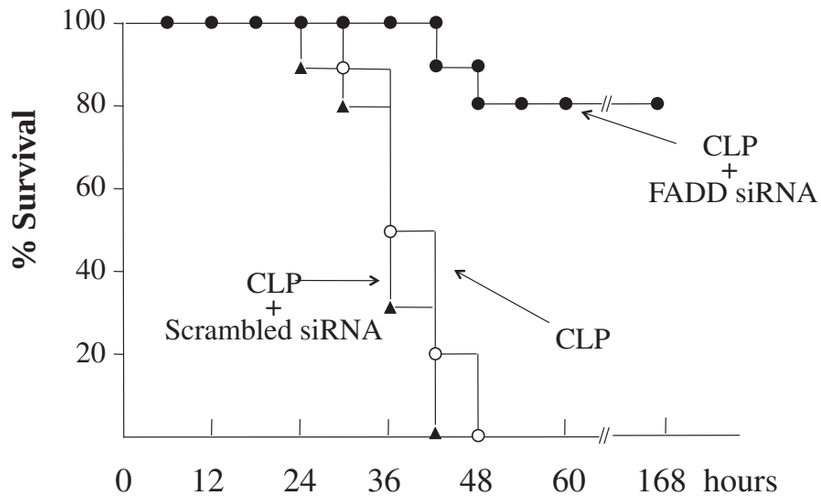


Immunoblotting of LC3 usually reveals two bands: the cytosolic form LC3-I and the membrane-bound form LC3-II. Sepsis (24 hours after CLP) resulted in a significant conversion of LC3-I to LC3-II. Tracking the conversion of LC3-I to LC3-II is indicative of autophagic activity. Systemic administration of FADD siRNA prevented this conversion. Summarized data are presented as the mean ± S.E. of five different experiments. *P<0.05 vs. sham control. #P<0.05 vs. CLP alone.

Fig. 6. Immunoblot analysis of LC3 in mouse aortic vessels.

There are several lines of experimental evidence that apoptosis and autophagy may be interconnected in some settings, and in some cases even simultaneously regulated by the same trigger resulting in different cellular outcomes (Gozuacik & Kimchi, 2004). Previous data supporting the interconnection between the two types of cell death have come from

gene expression profiles during steroid-triggered developmental cell death in the *Drosophila* system where several apoptosis-related genes are up-regulated together with autophagy-related genes (Gorski *et al*., 2003; Lee *et al*., 2003). In other cellular settings, autophagy may antagonize apoptosis and inhibition of autophagy may increase the sensitivity of the cells to apoptotic signals (Gozuacik & Kimchi, 2004). Moreover, apoptosis and autophagy may manifest themselves in a mutually exclusive manner. Inhibition of autophasic activity in cells may switch responses to death signals from autophagic to apoptotic cell death (Gozuacik & Kimchi, 2004). In addition, there are numerous reports showing a direct physical interaction between autophagy-inducing proteins and proteins involved in apoptosis, especially anti-apoptotic Bcl-2 family members (Liang *et al*., 1998; Vande Velde *et al*., 2000; Yanagisawa *et al*., 2003).



Mortality was monitored 4 times daily, and survival time was recorded for 7 days. Systemic delivery of FADD siRNA, but not scrambled siRNA, resulted in a significant survival benefit. Percentage survival of mice after CLP is plotted. Ten mice for each group underwent CLP.

Fig. 7. Kaplan-Meier survival curves after CLP in mice given FADD siRNA.

3.4 Effect of FADD siRNA on animal survival after CLP

To evaluate the impact of FADD siRNA on survival benefit in sepsis, we examined mortality in mice subjected to CLP (Figure 7). After CLP, mice exhibited signs of sepsis. Thus, they showed lack of interest in their environment, displayed piloerection, and had crusty exudates around their eyes. Finally, all animals subjected to CLP without treatment died within 2 days. Treatment of CLP mice with scrambled siRNA was without effect on survival. However, when FADD siRNA was administered to CLP mice, its survival advantage was very striking (P<0.0001; Log rank test). Even at the end of 7 days, a greater proportion of the animals given FADD siRNA survived CLP with 80%.

4. Conclusions

Despite recent advances in antibiotics and critical care therapy, sepsis treatment remains clinical conundrum, and its prognosis is still poor, especially when septic shock and/or

multiple organ failure develop. Although a host of promising candidates for therapeutic intervention in sepsis have been propelled, almost all of these trials have failed to demonstrate a mortality benefit for patients suffering from sepsis. Ongoing research into this highly lethal disorder has shown that apoptosis is fully associated with an unfavorable outcome of sepsis and its inhibition may provide useful therapies for treatment of sepsis. Here we propose that FADD siRNA therapy may offer a unique alternative sepsis treatment to shorten the apoptotic arm of sepsis. This therapy salvaged immune effector cells, vascular endothelial cells, and parenchyma cells from apoptosis, which would arrest the development of complications arising from sepsis, including multiple organ failure, and ultimately have a beneficial impact on septic mortality. While appreciating that additional work is required to optimize preclinical and possibly clinical application, treatment with FADD siRNA will hopefully provide novel potential usefulness for gene therapy that could improve the survival of critically ill septic patients.

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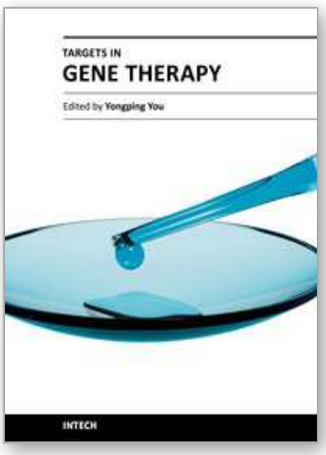

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This book aims at providing an up-to-date report to cover key aspects of existing problems in the emerging field of targets in gene therapy. With the contributions in various disciplines of gene therapy, the book brings together major approaches: Target Strategy in Gene Therapy, Gene Therapy of Cancer and Gene Therapy of Other Diseases. This source enables clinicians and researchers to select and effectively utilize new translational approaches in gene therapy and analyze the developments in target strategy in gene therapy.

InTech Europe

University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
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