Increased death receptor pathway of apoptotic signaling in septic mouse aorta: effect of systemic delivery of FADD siRNA

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Matsuda N, Teramae H, Yamamoto S, Takano K, Takano Y, Hattori Y. Increased death receptor pathway of apoptotic signaling in septic mouse aorta: effect of systemic delivery of FADD siRNA. Am J Physiol Heart Circ Physiol 298: H92–H101, 2010. First published October 23, 2009; doi:10.1152/ajpheart.00069.2009.—Recent evidence suggests that apoptotic cell death plays an important role in the pathophysiology of sepsis. Because there is extensive apoptosis of vascular endothelial cells in sepsis, we examined whether the death receptor pathway of apoptotic signaling is altered in thoracic aortas from mice with polymicrobial sepsis, as produced by cecal ligation and puncture (CLP). In septic aorta, total and surface expression levels of the two death receptors tumor necrosis factor receptor 1 and Fas were highly upregulated. Furthermore, marked increases in the mRNA and protein levels of Fas-associated death domain (FADD), an adaptor molecule to recruit procaspase-8 into the death-inducing signal complex, were observed in septic aorta, which were strongly suppressed by systemic delivery of small interfering RNA (siRNA) against FADD. No increase in expression of death receptors and FADD was observed in endothelium-denuded aortic tissues from septic animals. Systemic administration of FADD siRNA also resulted in great attenuation of sepsis-induced increases in expression and activation of caspase-3, an effector protease in the apoptosis cascade. Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) revealed that the significant appearance of cell apoptosis in aortic endothelium after CLP-induced sepsis was eliminated when FADD siRNA was systemically applied. Light and electron microscopic examinations of septic aorta showed cell swelling, nuclear fragmentation, and partial detachment of endothelial cells from the basal membrane, which were prevented by systemic treatment with FADD siRNA. Finally, FADD siRNA administration dramatically improved survival of CLP mice, supporting the feasibility of this gene-based approach for treating septic shock.

sepsis; apoptosis; endothelium

The vascular endothelium not only provides a selective barrier between the bloodstream and solid tissues but also serves as a major regulator of vascular tone and tissue perfusion. Therefore, the endothelium plays a critical role in a large number of physical and pathological processes (6). Several studies have reported anatomic damage to the endothelium in animal models of sepsis (22, 34, 35). Importantly, evidence of endothelial cell injury has been found in postmortem biopsies obtained from patients who died of sepsis-related acute respiratory distress syndrome (29). Moreover, increased circulating endothelial cells have been observed in septic patients (30). Endothelial cell injury and/or dysfunction would favor impaired perfusion, tissue hypoxia, and subsequent organ dysfunction. This may be one of the mechanisms that ultimately lead to complications arising from sepsis, including systemic vascular collapse and multiple organ failure. Thus vascular endothelial cells are a major target of sepsis-induced events, and their damage may account for much of the pathology of the septic syndrome (1, 13, 23).

A number of studies have indicated that endothelial cells can undergo apoptosis in response to a variety of stimuli (12, 31, 40). Interestingly, in vitro studies have shown apoptotic cell death of endothelial cells in response to sepsis-related factors such as lipopolysaccharide and tumor necrosis factor (TNF)-α (11, 36). Also, aortic endothelial cell apoptosis has been observed in an in vivo model of sepsis (46). Our recent data (28) have also revealed that endothelial cell apoptosis extensively occurs in aortic tissues of mice with polymicrobial sepsis induced by cecal ligation and puncture (CLP). Moreover, we have demonstrated (23, 28) that systemic application of small interfering RNAs (siRNAs) against caspase-8 and caspase-3, a family of proteolytic enzyme proteins that are one of the main executors of the apoptotic process, is capable of preventing vascular endothelial cell injury in septic animal models. This suggests that endothelial cell apoptosis may play an important role in the development of vascular endothelial cell injury and/or dysfunction.

Apoptotic cell death in mammals can proceed by two distinct pathways that ultimately converge to activate caspases: the death receptor pathway involving activation of members of the TNF family with death ligands and the intrinsic pathway that feeds cell death signals through the mitochondrion (7, 37). In the latter pathway, the antiapoptotic proteins of the Bcl-2 family prevent mitochondrial release of cytochrome c (45), and transgenic mice overexpressing Bcl-2 family in immune effector cells display decreased apoptosis and improved survival in sepsis (17, 18, 32), suggesting a significant role in the intrinsic mitochondrial apoptotic pathway in the pathophysiology of sepsis. In the present study, we initially tested the hypothesis that the death receptor apoptotic signaling pathway is upregulated in aortic endothelial tissues of mice with CLP-induced polymicrobial sepsis. In addition to examining changes in the death receptor pathway of apoptotic signaling, we investigated the therapeutic effect of systemic application of siRNA targeting Fas-associated death domain (FADD) on aortic endothelial cell apoptosis and injury in this in vivo rodent model. FADD is an
adaptor protein that in turn recruits procaspase-8 into the death-inducing signaling complex (DISC), thereby causing its activation (21, 41).

MATERIALS AND METHODS

Animal preparation. This study was conducted in accordance with National Institutes of Health guidelines on the use of laboratory animals and with approval of the Animal Care and Use Committee of the University of Toyama. Male BALB/c mice, 8–12 wk of age, were quarantined in quiet, humidified, light-cycled rooms for at least 1 wk before use. Mice were allowed ad libitum access to food and water throughout quarantine. The surgical procedure to generate CLP-induced sepsis was carried out according to the method described in our previous study (26) with minor modification. Mice were lightly anesthetized with gaseous diethyl ether, and a middle abdominal incision was made. The cecum was mobilized, ligated, and punctured twice with a 21-gauge needle. The bowel was repositioned, and the abdomen was closed. Sham-operated control animals underwent the same procedure except for ligation and puncture of the cecum. All CLP animals were lethargic, showed lack of interest in their environment, displayed piloerection, and had crusty exudates around their eyes. In contrast, the sham-operated animals were healthy, moving freely and eating.

The CLP model, which causes peritonitis, leads to polymicrobial sepsis and represents an indirect insult similar to the pathogenesis of acute respiratory distress syndrome (42). Indeed, we have clearly demonstrated (26) that mice 10 h after CLP exhibit marked hypoxemia, increased lung vascular permeability, and histological damage in lungs, including wall thickening, inflammatory infiltrate, and hemorrhage.

Preparation and transfection of siRNAs. For silencing of gene expression, FADD siRNA oligonucleotides with the following sense and antisense sequences were designed: 5'-GCA GUC UUA UUC CUA Att-3' and 5'-UAU GGA AUA AGA GGA GUG Ctt-3'. The Silencer Negative Control #1 siRNA (Ambion, Austin, TX) was used as a negative control. In vivo transfection of synthetic siRNAs via the tail vein was performed at 10 h after CLP with Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA). We used Opti-MEM I Reduced Serum Medium (Invitrogen) to dilute synthetic siRNAs and Lipofectamine RNAiMax before complexing according to manufacturer's recommendations, by which 50 μg of FADD siRNA sequence was delivered unless otherwise noted. The time point for siRNA application was chosen so that siRNA treatment was very effective (28).

Western blot analysis. After being cleaned of adhering fat and connective tissue under a microscope, mouse aortic tissues were powdered under liquid nitrogen and solubilized in 0.5 ml of ice-cold sterile water that contained 0.1% Triton X-100. The lysates were centrifuged at 1,000 g max for 10 min at 4°C to pellet any insoluble material. Where required, the membrane fractions were prepared as described previously (25, 27). Thus the supernatant was then spun at 100,000 g max for 30 min at 4°C. The membrane pellet was resuspended in 50 μl of lysis buffer and saved. Protein concentrations were measured with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Blotting procedures, chemiluminescent detection, and densitometric analysis were performed as previously described by our laboratory (27, 28).

The following commercially available antibodies were used: anti-TNF receptor 1 (TNF-R1) (sc-7895; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Fas (Abnova, Taipei, Taiwan), anti-FADD (PA1-37674; Affinity BioReagents, Golden, CO), anti-FADD (Ser194) (Santa Cruz Biotechnology), anti-caspase-3 (BioVision, Mountain View, CA), anti-adaptin-α (Affinity BioReagents), and anti-β-actin (GeneTex, San Antonio, TX).

RT-PCR. Total RNA was extracted from aortic tissues with the use of a TRI reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA, and PCR was performed with a Takara RNA PCR kit (Takara Shuzo, Ohtsu, Japan) as described in the manufacturer's manual. Expression of the FADD gene was monitored by PCR with 5'-CCC TGT GAC GAC TGT GGT TT-3' (sense) and 5'-TTG TGT GTT TGG GAC GAA CA-3' (antisense). The PCR-amplified product was analyzed by agarose gel electrophoresis. The internal standard used was the ubiquitously expressed housekeeping genes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We have confirmed (24) that the mRNA level for β-actin remains unchanged in sepsis.

DNA agarose gel electrophoresis. DNA was isolated from aortic tissues with the DNeasy Tissue Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. After precipitation by ethanol, DNA was dissolved in Tris-EDTA (TE) buffer (pH 8.0). DNA (30 μg) was pipetted onto a 2% agarose gel containing 100 ng/ml ethidium bromide, and electrophoresis was performed.

Histological examination. For routine histology, aortas were harvested, dehydrated, paraffin embedded, and sliced into 4-μm-thick sections. After deparaffinization, hematoxylin- and eosin-stained slides were prepared by standard methods.

Immunofluorescence and confocal analysis. For immunohistoch- emical determination of target molecules, the frozen sections were exposed to the fluorescent antibody after overnight incubation with the primary antibody, according to the method in our previous study (19) with minor modification. The levels of the large fragment (17/19 kDa) of activated caspase-3 were detected with cleaved caspase-3 (Asp175) (SA1) rabbit monoclonal antibody (Cell Signaling, Danvers, MA). Anti-CD31 antibody (Chemicon, Temecula, CA) was used as an endothelial cell marker. The nucleus was counterstained with Hoechst 33258 (Nacalai Tesque, Kyoto, Japan). Immunofluorescent images were observed under an Olympus BX-51 fluorescence microscope or a Leica DMi6000 confocal system.

DNA nick end labeling. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) was performed with the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon) according to the manufacturer's instructions. In brief, paraffin sections were digested with 20 μg/ml of proteinase K (Takara Shuzo) for 10–15 min at room temperature and reacted with TdT enzyme for 60 min at 37°C. The sections were then incubated with antidigoxigenin conjugate at room temperature for 30 min, followed by incubation with diaminobenzidine (DAB) solution and counterstaining with methyl green. Apoptotic cells were observed in cross section in randomly selected microscopic fields at a final magnification of ×400.

Electron microscopic analysis. Mice were anesthetized with isoflu- rane and perfused with 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) following saline from a cannula inserted into the ascending aorta through the left ventricle of the heart, and the thoracic aorta was removed and cut into small pieces. For transmission electron microscopy, samples were washed with sodium phosphate buffer for 2 h and then postfixed in 1% OsO4 for 2 h. Samples were dehydrated through a graded series of ethanol and embedded in epoxy resin. Ultrathin sections were doubly stained with uranyl acetate and lead citrate and examined with an H7650 electron microscope (Hitachi, Tokyo, Japan). After dehydration with a graded series of ethanol, some samples were freeze dried, coated with gold-palladium with an ion coater (IB-3, Giko Engineering, Tokyo, Japan), and examined under a Hitachi S-4700 electron microscope operated at 10.0 kV for scanning electron microscopy as described previously (15).

Survival studies. Mice were randomly divided into three groups. The first group was given only saline injection at 10 h after CLP. The second group was subjected to administration of scrambled siRNA after CLP. The last group underwent FADD siRNA application after CLP. All groups of mice were followed for survival for 7 days, during which the animals were allowed free access to food and water.

Statistical analysis. All values are expressed as means ± SE. Statistical assessment of the data was made by one-way ANOVA, and
then differences among groups were analyzed by Tukey’s multiple comparison test, unless otherwise noted. Analyses were performed with PRISM software (version 4; Graph Pad, San Diego, CA). A significant difference was assumed to exist if the \( P \) value was \( < 0.05 \).

RESULTS

Death receptor pathway of apoptotic signaling in sepsis.

When sepsis was induced by CLP, a time-dependent increase in the total expression level of the death receptor TNF-R1 was observed in mouse aorta (Fig. 1). Furthermore, sepsis induction significantly increased the TNF-R1 level in the membrane fraction (Fig. 1), indicating an increase in surface expression of TNF-R1. Similar to TNF-R1, another death receptor, Fas, was highly expressed in mouse aortic tissues after sepsis (Fig. 2). In addition, sepsis induction resulted in a time-dependent increase in translocation of Fas to the membrane pool in vascular tissues (Fig. 2). When aortic tissues were denuded mechanically, the sepsis-induced increases in surface expression of TNF-R1 and Fas were not observed (Figs. 1C and 2C), suggesting that these death receptors are upregulated mainly on endothelial cells during sepsis.

After ligand binding, death receptors recruit the adaptor protein FADD through homophilic interaction of their death domain with the death domain of FADD, and then FADD can recruit procaspase-8 to the DISC, thereby causing its activation (21, 41). When RT-PCR was used to evaluate the mRNA expression level of FADD, aortic expression of FADD mRNA was strikingly upregulated at 24 h after CLP-induced sepsis (Fig. 3). Systemic delivery of siRNA targeted against FADD, when given at 10 h after CLP, nearly completely eliminated aortic expression of its mRNA, suggesting the successful efficacy of systemically administered siRNA for silencing...
aortic gene expression of FADD. Administration of scrambled siRNA did not affect sepsis-induced upregulation of gene expression of FADD in aortic tissues. In addition, induction of sepsis by CLP led to a time-dependent increase in FADD protein expression in aortic tissues (Fig. 4). No increase in FADD protein expression was seen in endothelium-denuded aortic tissues from septic animals (data not shown). The increased level of FADD protein was significantly phosphorylated by sepsis induction. Systemic delivery of FADD siRNA strongly suppressed the phosphorylated level of FADD in septic aortas (Fig. 4).

Activation of caspase-8 as a consequence of recruitment into the DISC triggers activation of the downstream effector caspase-3, which is the proteolytic engine for cell death (8, 37). Expression and activation of caspase-3 were evaluated with an antibody that reacts with both pro- and cleaved forms of the enzyme. On immunoblots, only inactive caspase-3 of 32 kDa was detectable in control aortic tissues (Fig. 5). In addition to increased expression of procaspase-3, caspase-3 activity was strikingly increased in aortic tissues at 24 h after CLP, as indicated by the marked increase in catalytically active caspase-3 of 17 kDa after cleavage. In septic mouse aorta, a 12-kDa band showing another subunit of activated caspase-3 was notably detected. The marked increases in procaspase-3 and the active form of caspase-3 were greatly attenuated when septic animals were systemically given FADD siRNA.

Effect of FADD siRNA on endothelial cell apoptosis in sepsis. DNA agarose gels from aortas of control mice had no evidence of DNA fragmentation, i.e., ladder formation by the electrophoresis procedure. Aortas from mice at 24 h after CLP displayed significant ladder formation, a characteristic of apoptosis (Fig. 6). However, the DNA degradation in septic aorta was prevented when mice were systemically given FADD siRNA. The preventive effect of FADD siRNA on sepsis-induced DNA degradation in aortic vessels was dose dependent. In contrast, aortas from CLP mice treated with nonsense siRNA had ladder formation, and this was not different from the untreated CLP group.

To assess whether sepsis results in apoptotic cell death in the aortic endothelium, the tissue sections were labeled with an in situ TUNEL assay. As shown in Fig. 7, TUNEL-positive apoptotic endothelial cells were undetectable in sham-operated control aortas. In contrast, a significant number of apoptotic endothelial cells was found in aortas of mice at 24 h after sepsis induction by CLP. Endothelial cells in aortas of CLP mice treated with FADD siRNA were completely negative for TUNEL staining, while scrambled siRNAs failed to inhibit
endothelial apoptotic death in sepsis and FADD siRNA

Fig. 5. Immunoblot analysis of caspase-3 expression in aortic vessels of mice subjected to sham operation (control, C), sepsis (10 and 24 h after CLP), or FADD siRNA-treated sepsis. A: representative Western blots of caspase-3 (top) depicting the procaspase of 32 kDa as well as activation-associated cleavage products of 17 and 12 kDa. β-Actin (bottom) served as loading control. B: bar graphs comparing the immunostained bands for caspase-3 in the 4 groups of vessels. Bands were quantified in arbitrary units by densitometry. Values are procaspase-3/β-actin (top) and active caspase-3 of 17 kDa/β-actin (bottom), expressed relative to the respective sham-operated control result. Means of data from 4 animals/group are presented with SE shown by vertical lines. *P < 0.05, ***P < 0.001 vs. control value; ###P < 0.001 vs. value obtained in 24-h CLP vessels without any treatment.

apoptosis, indicating complete protection from apoptosis caused by sepsis. Similar results were obtained in spleens (Fig. 8). A marked increase in TUNEL-positive cells, morphologically identical to lymphocytes phagocytosed by macrophages, was observed at 24 h after CLP. In spleens from CLP mice given FADD siRNA but not its scrambled form, TUNEL-positive cells were drastically reduced, in line with sham-operated control mice.

As depicted in Fig. 9B, immunofluorescent staining for the cleaved form of caspase-3, a member of the caspase superfamily that initiates cell apoptotic events, showed that its protein expression was clearly detectable in endothelial cells rather than in smooth muscle cells of aortic tissues from CLP-induced septic mice. In addition, it should be noted that septic endothelial cells showed discontinuous staining with anti-CD31 antibody, in contrast with the continuous staining in controls.

Effect of FADD siRNA on sepsis-induced changes in light and electron micrographs of aortic endothelium. Histology sections of aortas of mice at 24 h after CLP showed partial detachment of endothelial cells from the basement membrane. Such endothelial cellular damage was drastically prevented by systemic treatment with FADD siRNA but not with scrambled siRNA (Fig. 9A).

Figure 10 displays electron micrographs of aortic endothelium in the sham-operated control group. Electron microscopic analysis indicated that the structure of aortic endothelium from septic or scrambled siRNA-treated septic mice exhibited a remarkable morphological abnormality. Thus scanning electron micrographs revealed that most endothelial cells were swollen (Fig. 10A), and nuclear fragmentation was found with transmission electron microscopy (Fig. 10B). Systemic treatment with FADD siRNA evidently reduced the endothelial damage induced by sepsis.

Effect of FADD siRNA on animal survival after CLP. Finally, we examined whether systemic application of FADD siRNA improves survival of mice with CLP-induced polymicrobial sepsis (Fig. 11). All animals subjected to CLP without treatment died within 2 days. Treatment of CLP mice with scrambled siRNA was without effect on survival. The animals that received systemic administration of FADD siRNA after CLP exhibited a striking improvement of survival. A significant survival benefit was still evident even at the end of 7 days.

DISCUSSION

Previous animal studies have provided increased evidence that apoptosis serves as an important mechanism of cell death in sepsis (3, 4, 43). In this setting, lymphocytes and parenchymal cells, including intestinal and lung epithelial cells, as well as vascular endothelial cells undergo apoptotic death (3, 14, 16, 46). The facts that both overexpression of the antiapoptotic protein Bcl-2 and genetic manipulation of the defective pathways of apoptotic signaling (5, 17) had an improvement in sepsis survival suggest that cell apoptosis may be potentially detrimental in sepsis. In support of this, our recent work (28) demonstrated profound protection against polymicrobial sepsis when gene transcripts for caspase-8 and caspase-3 are silenced when their siRNAs in septic mice.

In the present study, we found that surface expression levels of death receptors TNF-R1 and Fas were highly upregulated in aortic tissues with intact endothelium from
polymicrobial septic mice. In previous reports, it has been shown that Fas mRNA expression displays a strong increase in lung tissues of mice challenged with hemorrhagic shock and sepsis (33), that Fas and Fas ligand (FasL) are elevated at both mRNA and protein levels in a mouse model of lipopolysaccharide-induced acute lung injury (20), and furthermore, that soluble FasL and Fas are present in bronchoalveolar lavage fluid from patients with acute respiratory distress syndrome, a condition that often develops during sepsis or septic shock (2). Here, we demonstrated that upregulation of Fas was evident in vascular tissues of septic animals. To the best of our knowledge, moreover, this study represents the first report that another death receptor, TNF-R1, was also greatly upregulated at the surface level of septic animal tissues.

Fig. 7. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) analyses of aortic sections at 24 h after CLP in mice given FADD siRNA. Mice underwent sham procedure, CLP, CLP and FADD siRNA treatment, or CLP and scrambled siRNA treatment. A: representative micrograph showing apoptotic cells (brown) by in situ TUNEL assay. B: counts of apoptotic cells were made in the sections at a final magnification of ×400, and the average of apoptotic cell number in 3 high-power fields (HPF) per sample was calculated. Means of data from 3 animals/group are presented with SE shown by vertical lines. **P < 0.01 vs. control value; ##P < 0.01 vs. value obtained in 24-h CLP vessels without any treatment.

Fig. 8. TUNEL analyses of spleen sections at 24 h after CLP in mice given FADD siRNA. Mice underwent sham procedure, CLP, CLP and FADD siRNA treatment, or CLP and scrambled siRNA treatment. A: representative micrograph showing apoptotic cells (brown) by in situ TUNEL assay. B: counts of apoptotic cells were made in the sections at a final magnification of ×400, and the average of apoptotic cell number in 3 fields per sample was calculated. Means of data from 3 animals/group are presented with SE shown by vertical lines. *P < 0.05 vs. control value; #P < 0.05 vs. value obtained in 24-h CLP vessels without any treatment.
FADD is an essential component of the DISC for all death receptors (21, 41). Thus, after ligand binding to death receptors, the DISC is formed by recruitment of FADD and initiator procaspase-8, which provides a molecular scaffold to induce the death signal with the activation of effector caspase-3. We showed that sepsis induced a marked increase in FADD expression in the mouse aorta. This modulation suggests that possible apoptotic signals by sepsis through death receptor upregulation would be assisted through high expression of FADD. Interestingly, FADD displays, outside the death domain, a single serine phosphorylation site (human: Ser194; mouse: Ser191), which is crucial for its nonapoptotic activity (38). The phosphorylated form of FADD was increased by sepsis induction. However, this may simply be the result of sepsis-induced high expression of total FADD. The significance of the elevation of phosphorylated FADD during sepsis is uncertain. In the present study, we clearly demonstrated that siRNA targeted to FADD could be delivered effectively into target tissues through the use of the transfection agent Lipofectamine 2000 in Opti-MEM when being introduced into the whole animal. We thus found that intravenously injected FADD siRNA led to a strong inhibition of the high expression levels of its mRNA and protein in aortic tissues of the mouse model of polymicrobial sepsis by CLP. In contrast, we noted no significant change in FADD mRNA in aortas of septic mice receiving nonsense siRNA.

Previous studies have shown that transgenic mice overexpressing Bcl-2, an inhibitor of the mitochondria-mediated pathway, in immune effector cells decreases apoptosis and improves survival in sepsis (17, 18, 32). This may suggest that the mitochondria-mediated pathway plays a significant role in the process of apoptotic death in sepsis. In the present study, however, the active forms of caspase-3, one of the downstream effector caspases, virtually waned in aortic tissues of septic animals treated with FADD siRNA. Furthermore, gene silencing of FADD with siRNA resulted in a striking reduction in the appearance of aortic endothelial cell apoptosis caused by sepsis, as indicated by DNA ladder assay and TUNEL assay. It is possible that the two apoptotic death pathways, the death receptor pathway and the mitochondria-mediated pathway, are linked under certain pathological conditions, such as sepsis. Recent work using mice with defective Bid has indicated the existence of truncated Bid-mediated cross talk between the two apoptotic death pathways in sepsis (5). The proapoptotic factor Bid is a Bcl-2 family member that translocates to mitochondria on cleavage after being activated by caspase-8 derived from the death receptor pathway (10). Thus Bid cleavage, which leads to its translocation into the mitochondria, may promote cytochrome c release and subsequent activation of the mitochondria-mediated apoptotic death pathway.

In accordance with our prior study (28), histopathological examination of aortic endothelium revealed that a number of endothelial cells were about to detach from the basal membrane after sepsis induction by CLP. The partially detached endothelial cells in septic aorta were more clearly demonstrated by our present electron microscopic analysis. This morphological damage is considered to result from vascular endothelial cells that were undergoing apoptosis under polymicrobial septic conditions, because the partially detached endothelial cells were all positive for TUNEL staining. Such endo-

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**Fig. 9.** Histopathological changes and activation of caspase-3 in aortic tissues from mice at 24 h after CLP. A: sections stained with hematoxylin and eosin. Visibly, CLP mice had endothelial cells about to detach from the basement membrane. Such endothelial cellular damage was prevented by FADD siRNA but not by scrambled siRNA. B: immunofluorescent images for cleaved caspase-3 (green) in aortic tissues from control (left) and CLP (right) mice. Endothelial cells stained with anti-CD31 antibody (red) and nuclei stained with Hoechst (blue) are shown as an overlaid image. Shown are representative micrographs from 2 independent experiments in which the same results were obtained.
Endothelial cell damage would lead to microvascular dysfunction with reduced perfusion and oxygen, which could result in tissue hypoxia and, ultimately, in the development of multiple organ failure (23). It is striking that endothelial cell detachment from the basal membrane was not observed when septic mice were systemically treated with FADD siRNA.

Importantly, we observed that systemic application of FADD siRNA dramatically improved survival of CLP mice. Interestingly, this survival benefit was evident despite FADD siRNA administration as late as 10 h after CLP, suggesting that the pathologically detrimental effect of FADD-associated apoptotic events may take time to develop during sepsis. In addition, it should be noted that the effect of FADD siRNA on septic mortality was more pronounced than that of Fas siRNA that was reportedly given 12 h after CLP (42). This would suggest the importance of TNF-R1-mediated apoptotic cell death in sepsis. The greatly beneficial effect of FADD siRNA on survival in sepsis may be, at least in part, related to its prevention of vascular endothelial apoptosis, but the possibility cannot be entirely excluded that the ability of the siRNA to lead to survival advantage in sepsis involves the effect of preventing apoptosis of other cell types, including lymphocytes and gastrointestinal epithelial cells. Indeed, our present study showed that FADD siRNA prevented apoptosis of spleen lymphocytes as well as vascular endothelial cells in CLP mice. Thus which cells are protected by systemic treatment with FADD siRNA in this murine model of sepsis that is enabling the animal to survive remains the subject of ongoing studies. Caution is required, furthermore, in extrapolating results in animal studies to humans. Although the CLP model is believed to reflect the conditions that occur in patients, the findings obtained from many previous animal studies of sepsis were not duplicated in clinical trials, because of fundamental differences in the response to sepsis among species (9).

In conclusion, the present study is the first to describe that surface expression of death receptors TNF-R1 and Fas is upregulated and the death adaptor FADD is highly expressed in aortic tissues of mice with CLP-induced polymicrobial sepsis. Systemic application of FADD siRNA not only prevented sepsis-induced vascular endothelial cell apoptosis but also resulted in successful improvement of survival in mice during ongoing sepsis. This study confirms the pathophysiological significance of the death receptor apoptotic signaling pathway in the septic syndrome and provides potential usefulness of the specific siRNA targeted to FADD for gene therapy of septic shock and sequential multiple organ failure.
ENDOTHELIAL APOPTOSIS IN SEPSIS AND FADD siRNA

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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