Silencing of caspase-8 and caspase-3 by RNA interference prevents vascular endothelial cell injury in mice with endotoxic shock

Naoyuki Matsuda\textsuperscript{a}, Yasuo Takano\textsuperscript{b}, Shun-ichiro Kageyama\textsuperscript{a}, Noboru Hatakeyama\textsuperscript{c}, Kiyoshi Shakunaga\textsuperscript{c}, Isao Kitajima\textsuperscript{d}, Mitsuaki Yamazaki\textsuperscript{c}, Yuichi Hattori\textsuperscript{a,*}

\textsuperscript{a} Department of Molecular and Medical Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan
\textsuperscript{b} Department of Diagnostic Pathology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan
\textsuperscript{c} Department of Anesthesiology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan
\textsuperscript{d} Department of Clinical Laboratory Medicine, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan

Received 19 December 2006; received in revised form 22 May 2007; accepted 24 May 2007
Available online 5 June 2007
Time for primary review 26 days

Abstract

Objectives: Septic shock and sequential multiple organ failure remain the cause of death in septic patients. Vascular endothelial cell apoptosis may play a role in the pathogenesis of the septic syndrome. Caspase-8 is presumed to be the apex of the death receptor-mediated apoptosis pathway, whereas caspase-3 belongs to the “effector” protease in the apoptosis cascade. Synthetic small interfering RNAs (siRNAs) specifically suppress gene expression by RNA interference. Therefore, we evaluated the therapeutic efficacy of caspase-8/caspase-3 siRNAs in a murine model of polymicrobial endotoxic shock.

Methods: Polymicrobial endotoxic shock was induced by cecal ligation and puncture (CLP) in BALB/c mice. In vivo delivery of siRNAs was performed by using a transfection reagent (Lipofectamine 2000) at 10 h after CLP. As a negative control, animals received non-sense (scrambled) siRNA.

Results: Marked increases in caspase-8 and caspase-3 protein expression in CLP aortic tissues were strongly suppressed by treatment with caspase-8/caspase-3 siRNAs. This siRNA treatment prevented DNA ladder formation and less phosphorylation of the pro-apoptotic protein Bad seen in CLP aortic tissues. Transferase-mediated dUTP nick end labeling (TUNEL) revealed that the appearance of apoptosis in aortic endothelium after CLP was eliminated by this siRNA treatment. Although all of the control animals subjected to CLP died within 2 days, administration of caspase-8/caspase-3 siRNAs indefinitely (>7 days) improved the survival of CLP mice.

Conclusions: Gene silencing of caspase-8 and caspase-3 with siRNAs provided profound protection against polymicrobial endotoxic shock. The prevention of vascular endothelial cell apoptosis appears to be, at least in part, responsible for their beneficial effects in endotoxic shock.

© 2007 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: Akt; Apoptosis; Endothelium; Endothelial nitric oxide synthase; Phosphatidylinositol 3-kinase; Endotoxic shock; Small interfering RNA

1. Introduction

Sepsis is a subset of the systemic inflammatory response syndrome, but therapeutic efforts aimed at eliminating the inflammatory response have shown only modest clinical benefit [1]. Despite recent advances in critical care therapy, sepsis is the leading cause of death in intensive care units, and its prognosis is as poor as ever [2]. Septic shock and sequential multiple organ failure/dysfunction syndrome (MOF/MODS) correlate with poor outcome [3]. Vascular endothelial cells play a key role in the regulation of tissue perfusion by releasing vasoactive mediators, including nitric oxide (NO) [4]. Endothelial dysfunction at macro- and microcirculation levels may lead to the development of MOF/MODS in sepsis [5]. Impaired endothelium-dependent vascular relaxation has been shown in blood vessels from endotoxemic animals [6–8]. In agreement with this impaired...
endothelial function, our recent data have demonstrated that endothelial NO synthase (eNOS) expression is greatly reduced in blood vessels from the rabbit lipopolysaccharide (LPS)-induced endotoxic shock model [9,10].

The role of apoptotic cell death has been implicated in the pathophysiology of sepsis [11]. Transgenic mice overexpressing Bel-2, a potent anti-apoptotic protein, in T cells displays improved survival in endotoxic shock [12]. Although lymphocytes and parenchyma cells increase apoptotic cell death in animal models of endotoxic shock [13,14], studies have shown that endothelial cells can undergo apoptosis in response to various physiologic and pathologic stimuli [15–18]. LPS has been found to induce endothelial cell apoptosis both in vivo and in vitro [19,20]. Furthermore, vascular endothelial cell apoptosis has been observed in rats with polymicrobial endotoxic shock induced by cecal ligation and puncture (CLP) [21]. Therefore, it is conceivable that endothelial cell apoptosis may play an important role in the development of vascular endothelial dysfunction during sepsis.

Caspases are proenzymes of the aspartate-specific cysteine protease family and activation of caspases plays a central role in the execution of apoptosis [22,23]. Depending on the nature of the stimuli and the cell types, two caspase activation pathways have been described, including the receptor-initiated caspase-8-dependent pathway and the mitochondria-initiated caspase-9-mediated pathway [23]. Activated caspase-8 or caspase-9 initiates a downstream cascade of effector caspases, such as caspase-3, which cleaves various substrates such as D4-GDI, and leads to the execution of cell death [23]. In the present study, we examined the therapeutic efficacies of caspase-8 and caspase-3 gene silencing with small interfering RNAs (siRNAs), which were delivered by systemic injection, in a CLP endotoxic shock mouse model. RNA interference is one of the most recent and promising gene transfer technologies, allowing specific silencing of genes by delivering highly homologous RNA into cells [24]. In this regard, transfection of cells with double-stranded, synthetic siRNAs, 21–23 nucleotides in length, can specifically suppress expression of endogenous and heterologous genes by RNA interference.

2. Materials and Methods

2.1. Animal preparation

This study was conducted in accordance with the National Institutes of Health guidelines on the use of laboratory animals and with approval of the Animal Care and Use Committee of University of Toyama. Male BALB/c mice, 8–12 weeks of age, were quarantined in quiet, humidified, light-cycled rooms for 2–3 weeks before use. Mice were allowed ad libitum access to food and water throughout quarantine. Endotoxic shock was induced by a standard CLP technique [25]. Briefly, under gaseous diethyl ether anesthesia, the cecum was delivered through a midline incision, ligated at 3 mm from its top, and then perforated in two locations with a 23-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.

2.2. Preparation and transfection of siRNAs

For silencing of gene expression of caspase-8 and caspase-3, siRNA oligonucleotides with the following sense and antisense sequences were designed: caspase-8 siRNA, 5′-AAC CUC GGG AUA AUA UCU GAtt-3′ (sense) and 5′-UCA GAC AGU AUC CCG AGG UUt-3′ (antisense); caspase-3 siRNA, 5′-UGA GGU AGC UUC AUA GUG Gtt-3′ (sense) and 5′-CCA CUA UGA AGC UAC CUC Att-3′ (antisense). The Silencer Negative Control #1 siRNA (Ambion, Austin, TX, USA) was used as a negative control. In vivo transfection of synthetic siRNAs was performed by using Lipofectamine 2000 and Opti-MEM media (Invitrogen, Carlsbad, CA, USA) according to manufacturer recommendations, by which 50 μg of each siRNA sequence was delivered into tail vein with a 30-gauge needle at 10 h after CLP unless otherwise noted.

In a clinical situation, caspase-8/caspase-3 siRNAs would be applied after the onset of sepsis. We thus assessed a time point for siRNA application after mice were subjected to CLP in preliminary survival studies. We found that siRNA treatment was very effective 10 h after CLP.

2.3. 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 which contained 0.1% Triton X-100. The lysates were centrifuged at 100,000 × gmax for 10 min at 4 °C to pellet any insoluble material. Where required, the membrane fractions were prepared as described previously [26]. Thus, the supernatant was then spun at 100,000 × gmax for 30 min at 4 °C. The membrane pellet was resuspended in 50 μl of lysis buffer and saved. Protein concentrations were measured using a Bio-Rad protein assay kit.

The following commercially available antibodies were used: anti-human caspase-8 (Chemicon, Temecula, CA, USA), anti-mouse caspase-3 (BioVision, Mountain View, CA, USA), anti-mouse Bad (BioVision), anti-human phospho-Bad (Ser-112) (Stressgen, Ann Arbor, MI, USA), anti-human phospho-Bad (Ser-136) (Signal Antibody Technology, Sunnyvale, CA, USA), anti-human endothelial nitric oxide synthase (eNOS) (Acros Antibodies GmbH, Hiddenhausen, Germany), anti-human phospho-eNOS (Ser-1177) (Upstate Cell Signaling, Lake Placid, NY, USA), anti-rat phosphatidylinositol 3-kinase (PI3-K) (Upstate Cell Signaling), anti-Akt (Rockland Immunochimicals, Gilbertsville, PA, USA), anti-phospho-Akt (Thr-308) (GeneTex, San Antonio, TX, USA), anti-phospho-Akt (Ser-473) (GeneTex), and anti-mouse actin (GeneTex).
Immunoblotting was performed as described in our previous report [9,10]. Samples (2–5 μg) were run on a 7.5–12.5% SDS-polyacrylamide gel and electrotransferred to a polyvinylidene difluoride filter membrane. The membrane was then blotted with the indicated antibody and processed via chemiluminescence.

2.4. DNA agarose gel electrophoresis

DNA was isolated from aortic tissues using the DNeasy Tissue Kit (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions. After precipitation by ethanol, DNA was dissolved in 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.

2.5. Histologic 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 using standard methods.

2.6. TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) was performed using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon) according to the manufacturer’s instructions.

2.7. Survival studies in sepsis

Additional groups of mice underwent CLP and were included in survival studies. Mice were randomly divided into two groups. At 10 h after CLP, one group of the animals was given only saline injections. The other group received intravenous administration of siRNAs targeted to caspase-8 and/or caspase-3. The animals were allowed free access to food and water, and the survival time of each animal was recorded for 7 days.

2.8. Statistical analysis

Data are expressed as mean±SD. Statistical assessment of the data was made by Student t test or a repeated-measures...
one-way ANOVA followed by Bonferroni’s multiple comparison test, where appropriate. A significant difference was assumed to exist if the $P$ value was less than 0.05.

3. Results

3.1. Silencing of caspase-8 and caspase-3 in aortas of endotoxic shock mice

Initial experiments were conducted to evaluate the efficacy of systemically administered siRNAs targeted to caspase-8 and caspase-3 in silencing vascular gene expression. Caspase-8 and caspase-3 protein levels were determined in aortic tissues by Western blot analysis. Polyclonal antibodies against caspase-8 used in this study detected the entire pro-caspase (approx. 55 kDa), as well as an activation-associated cleavage product that corresponds to 43 kDa. As shown in Fig. 1A, induction of septic shock by CLP resulted in time-dependent increases in the entire pro-caspase-8 level and activation of caspase-8. The increased levels of inactive and activated caspase-8 proteins were strongly inhibited by systemic application of siRNAs targeted to caspase-8/caspase-3. In contrast, no decrease in caspase-8 expression and activity was demonstrated in animals given non-sense siRNA. On immunoblots, inactive pro-caspase-3 of 32 kDa and catalytically active caspase-3 of 17 kDa after cleavage were detected. As shown in Fig. 1B, caspase-3 activity was markedly increased in aortic tissues at 24 h after CLP, as indicated by the striking increase in the level of the p17 fragment. In CLP mouse aorta, a 12-kDa band showing another subunit of activated caspase-3 was notably detected. Systemic delivery of caspase-8/caspase-3 siRNAs, but not of scrambled siRNA, resulted in significant down-regulation of caspase-3 protein expression and activity in aortic tissues after induction of endotoxic shock.

3.2. Effect of siRNAs on endotoxic shock-induced DNA fragmentation by agarose gel electrophoresis

DNA agarose gels from aortas of sham-operated mice had no evidence of DNA fragmentation, i.e., ladder formation by the electrophoresis procedure. Aortas from mice at 24 h after CLP exhibited significant ladder formation, a characteristic of apoptosis (Fig. 2). However, the DNA degradation was not present in aortas from CLP mice given caspase-8/caspase-3 siRNAs. In contrast, aortas from CLP mice treated...
Fig. 4. Normalization of reduced Bad phosphorylation in aortic tissues from CLP-induced endotoxic shock mice by systemic administration of caspase-8/caspase-3 siRNAs. (A) Representative Western blots of phospho-Bad at Ser-112 (top) and at Ser-136 (middle) and of total Bad (bottom). (B) Densitometric analysis of levels of Bad phosphorylated at Ser-112 (upper trace) and at Ser-136 (lower trace), and the data represent phospho-Bad/Bad, expressed relative to the respective sham control result. Mean of data from four animals in each group is presented, with SD shown by vertical lines. **Significant difference from control (P<0.001). ###Significant difference from 24-h CLP alone (P<0.001).

Fig. 5. Recovery effect of systemic administration of caspase-8/caspase-3 siRNAs on reduced Akt phosphorylation (A), membrane translocation of p85 subunit of PI3-K (B), and phosphorylation and protein expression of eNOS (C) in aortic tissues from CLP-induced endotoxic shock mice. In the top trace of each panel, representative Western blots are shown. In the bottom trace, densitometric analyses of levels of phosphorylated Akt (A), PI3-K membrane translocation (B), and phosphorylated and total eNOS (C) are shown, and the data represent phospho-Akt/Akt (A), PI-3K p85 in membrane/total fractions (B), and phospho-eNOS/eNOS and eNOS/actin (C), expressed relative to the respective sham control result. Mean of data from four animals in each group is presented, with SD shown by vertical lines. **Significant difference from control (P<0.001). ###Significant difference from 24-h CLP alone (P<0.001).
with scrambled siRNA had ladder formation, and this was not different from the untreated CLP group (data not shown).

3.3. Histopathologic analysis after CLP in aortic vessels of animals treated with siRNAs

Histology sections of aortas in animals at 24 h after CLP showed partial detachment of endothelial cells from the basal membrane. Such endothelial damage was drastically prevented by systemic treatment with caspase-8/caspase-3 siRNAs (Fig. 3A–C).

Induction of endotoxic shock by CLP led to a significant appearance of TUNEL-positive endothelial cells in mouse aortas. Endothelial cells in aortas of CLP mice treated with caspase-8/caspase-3 siRNAs were completely negative for TUNEL staining, indicating complete protection from apoptosis mediated by endotoxic shock (Fig. 3D–E).

3.4. Effect of siRNAs on septic shock-induced decrease in phosphorylation of Bad

We turned our attention to the pro-apoptotic protein Bad. This pro-apoptotic function can be cancelled via phosphorylation on distinct serine residues [27,28]. Using antibodies specific to the forms phosphorylated on Ser-112 and Ser-136 residues, we evaluated changes in Bad phosphorylation in aortic tissues. As shown in Fig. 4, both phosphorylated forms of Bad were strikingly reduced after CLP as compared with those obtained from sham-operated controls. The reduced levels were significantly normalized by treatment with caspase-8/caspase-3 siRNAs of animals subjected to CLP but remained unchanged by treatment with scrambled siRNA. We detected no change in the total amount of Bad following CLP regardless of whether siRNAs were treated.

3.5. Effect of siRNA on upstream events to cell apoptotic regulators

The serine/threonine kinase Akt is a key regulator of cell survival, and potential downstream targets of Akt include pro-apoptotic proteins such as Bad [29] and caspase-8 [30]. Akt is activated by dual phosphorylation of Ser-473 and Thr-308 [31,32]. The results of Western blot analysis showed that the expression levels of Akt dually phosphorylated at Ser-473 and Thr-308 in aortic tissues were reduced in a time-dependent manner after induction of endotoxic shock by CLP (Fig. 5A). Systemic delivery of caspase-8/caspase-3 siRNAs completely prevented the marked reduction in Akt phosphorylation caused by 24 h of CLP. Scrambled siRNA treatment was without effect on the CLP-induced reduction in Akt phosphorylation. Akt is activated following PI3-K recruitment to the inner surface of the plasma membrane [33]. Although PI3-K is a heterodimer lipid kinase consisting of a p85 regulatory subunit bound to a p110 catalytic subunit [34], the antibody which recognizes the p85 subunit of PI3-K was used in the current study. Furthermore, this antibody appears to detect multiple isoforms of p85 subunit. CLP-induced endotoxic shock resulted in a time-dependent decrease in translocation of PI3-K p85 to the membrane pool in aortic tissues without any change in the total amount of PI3-K p85 (Fig. 5B). CLP animals given caspase-8/caspase-3 siRNAs presented a complete recovery of the reduced membrane translocation of PI3-K p85. No difference was demonstrated in CLP mice given scrambled siRNA compared with untreated CLP animals.

It has been shown that the PI3-K pathway that activates Akt causes direct phosphorylation of eNOS, leading to increased production of NO [35–37]. We found a strikingly lower level of eNOS phosphorylated at Ser-1177 in aortic tissues from CLP-induced endotoxic shock mice, as determined by immunoblotting with the phospho-specific antibody (Fig. 5C). Systemic delivery of caspase-8/caspase-
3 siRNAs strongly prevented the reduction in eNOS phosphorylation caused by endotoxic shock. Moreover, caspase-8/caspase-3 siRNAs greatly improved an endotoxic shock-induced reduction in total eNOS expression from 54 ± 10% to 112 ± 16% of control. Such recovery effects on eNOS phosphorylation and protein expression were not observed with non-sense siRNA.

3.6. Effect of siRNAs on animal survival after CLP

Finally, we examined whether systemic application of caspase-8 and/or caspase-3 siRNAs improves survival of mice with CLP-induced polymicrobial endotoxic shock. All animals subjected to CLP without treatment died within 2 days. Interestingly, a significant survival benefit was evident in animals given caspase-8 siRNA or caspase-3 siRNA alone at 10 h after CLP. However, when mice were injected with both caspase-8 siRNA and caspase-3 siRNA after CLP, its survival advantage was more striking. No deaths occurred in the animals that received systemic administration of caspase-8/caspase-3 siRNAs following CLP. Even at the end of 7 days, there was a 100% survival in the group treated with caspase-8/caspase-3 siRNAs (Fig. 6A). The effect of caspase-8/caspase-3 siRNAs on animal survival after CLP was strongly dependent on the dose of siRNAs given to animals. Percentages of survival (>7 days) of mice receiving 1, 10, and 50 μg of caspase-8/caspase-3 siRNAs after CLP were 0, 40, and 100%, respectively (Fig. 6B).

4. Discussion

Apoptosis may play an important role in the pathophysiology of sepsis. In the present study, we targeted caspase-8 gene expression with siRNA, because caspase-8 is an essential component in the signal transduction of all known death receptors (e.g., tumor necrosis factor (TNF)-α, Fas, TNF-related apoptosis-inducing ligand). In addition, to block the apoptosis pathway triggered by mitochondrial dysfunction, we also attempted to down-regulate caspase-3, a downstream effector component of the apoptotic process. Herein, we demonstrated that intravenously injected siRNAs targeted to caspase-8 and caspase-3 greatly inhibited up-regulation of vascular expression levels of caspase-8 and caspase-3 in the mouse model of polymicrobial endotoxic shock by CLP. This indicates that siRNAs targeting our genes of interest, caspase-8 and caspase-3, were effectively delivered to target tissues in the whole animal and silenced or suppressed these gene transcripts necessary for apoptosis in endotoxic shock.

This study showed that CLP-induced polymicrobial endotoxic shock caused cell death in aortic tissues. Cell death was characterized as apoptotic cell death by DNA ladder formation on agarose gel electrophoresis. However, none of aortas from CLP mice treated with caspase-8/caspase-3 siRNAs had ladder formation, suggesting that in vivo delivery of caspase-8/caspase-3 siRNAs was capable of preventing apoptotic cell death in vascular tissues of animals with endotoxic shock.

The anti-apoptotic protein Bcl-2 gene and protein expression levels have been shown to be reduced in aortic tissues from CLP rats [21]. In this study, we turned our attention to the pro-apoptotic protein Bad. Bad can bind to anti-apoptotic proteins and promote apoptosis, but this ability is impaired by the mechanisms that control the state of phosphorylation of this pro-apoptotic protein [38]. We found that the level of phosphorylated Bad protein was markedly decreased in aortas from CLP mice which was prevented by caspase-8/caspase-3 siRNA treatment. The preventive effect of caspase-8/caspase-3 siRNAs on septic shock-induced decrease in Bad phosphorylation may contribute to its inhibition of cell apoptosis. Because activation of Akt stimulates phosphorylation of Bad, thereby inhibiting apoptosis execution [16,29], changes in the PI3-K/Akt signal transduction pathway may determine the phosphorylation levels of Bad in endotoxic shock when untreated and treated with caspase-8/caspase-3 siRNAs.

In accordance with our recent study with a rabbit LPS model [10], induction of endotoxic shock led to marked reductions in translocation of PI3-K to the membrane and Akt phosphorylation in vascular tissues. Since activation of Akt occurs downstream of PI3-K [39,40], reduced activity of PI3-K may result in impaired phosphorylation of Akt in vascular tissues during endotoxic shock. Much to our surprise, systemic application of caspase-8/caspase-3 siRNAs showed a recovery effect on the endotoxic shock-induced reductions in membrane translocation of PI3-K and phosphorylation of Akt. Akt is a key regulator of cell survival and reduces activation of caspase-8 and caspase-3 due to impaired recruitment of pro-caspase-8 to the death-inducing signaling complex [30]. Conversely, up-regulation of caspases as seen in endotoxic shock might regulate negatively Akt activation. We thus assume that the recovery effect of caspase-8/caspase-3 siRNAs on the PI3-K/Akt pathway was possibly the result of down-regulation of these caspases; however, further investigations are needed to prove this point.

Following induction of polymicrobial endotoxic shock by CLP, strikingly diminished eNOS phosphorylation was observed in mouse aortas, with a significant decrease in eNOS expression. This observation is basically in line with our recent finding obtained in mesenteric arteries from LPS-induced endotoxic shock rabbits [10]. In the light of the requirement of eNOS phosphorylation for NO production, our results would suggest less production of NO by eNOS in endotoxic shock. Because the relatively small amount of NO generated by eNOS provides important physiologic regulations, including vascular tone, neurotransmission, and immune defense [41,42], the impairment of expression and activation of eNOS could be involved in the development of tissue/organ injury in endotoxic shock. The diminished expression and phosphorylation levels of eNOS in CLP
aortas were dramatically improved when the animals were given caspase-8/caspase-3 siRNAs. This impairment seemed to be associated with the impaired PI3-K/Akt pathway. It is well established that Akt phosphorylates eNOS, leading to a persistent, Ca\(^{2+}\)-independent enzyme activation and thus endothelial NO synthesis [35,36].

Our histopathologic examination of aortic endothelium showed that a number of endothelial cells were about to detach from the basal membrane at 10 h after CLP. This morphologic damage is most likely to result from vascular endothelial cells that were undergoing apoptosis under polymicrobial endotoxic shock conditions, because significant aortic endothelial cell apoptosis in this model was analyzed by TUNEL. Moreover, systemic treatment with caspase-8/caspase-3 siRNAs completely prevented the endotoxic shock-induced partial detachment and apoptosis occurrence of aortic endothelial cells.

From an outcome perspective, we found that, importantly, in vivo delivery of caspase-8/caspase-3 siRNAs conferred a dramatic survival advantage to CLP mice as compared with controls. Notably, this survival benefit was observed despite administration of caspase-8/caspase-3 siRNAs as late as 10 h after CLP. However, treatment with caspase-8 or caspase-3 siRNA alone had a significant but less marked effect on survival of animals subjected to CLP. One previous study has reported that treatment with caspase-8 siRNA alone can significantly improve survival of mice with polymicrobial endotoxic shock when the animals received this siRNA only at 30 min following the performance of CLP [43]. We thus confirm that gene silencing of both caspase-8 and caspase-3 can provide a more effective survival benefit to polymicrobial endotoxic shock mice than that of each caspase alone. The question remains, however, as to what cells are protected by caspase-8/caspase-3 siRNA treatment in this murine model of endotoxic shock that is enabling the animal to survive. Blocking apoptosis of vascular endothelial cells may be important in preventing septic mortality, because endothelial cell damage could develop hypoxic conditions in tissues due to the loss of blood flow regulation and of anticoagulant/antithrombotic properties, contributing decisively to MOF. On the other hand, it should be noted that extensive apoptosis of lymphocytes and intestinal epithelial cells has been detected in patients who have died of MODS [44].

Although siRNAs have proven to very potently suppress gene expression and allowed for the elucidation and better understanding of gene functions, there is little information on the duration and degree of in vivo silencing of genes using siRNA. It has been shown that after hydrodynamic delivery of caspase-8 siRNA, silencing in the liver lasts for up to 10 days [44]. Furthermore, it has been reported that a single intranasal instillation of 10 \(\mu\)g of GAPDH siRNA results in a 50% reduction in pulmonary GAPDH at day 1 and remains notably depressed (67%) up to day 7 after instillation [45]. Transfection of siRNA with viral vectors might represent a further longer half-life of siRNA and promise a clinical relevance as a powerful tool for gene therapy.

Our results demonstrate that systemic application of siRNAs targeted to caspase-8 and caspase-3 provides significant protection against polymicrobial endotoxic shock and results in successful improvement of survival in mammals during ongoing endotoxic shock. The mechanisms of the protective effect of caspase-8/caspase-3 siRNAs may be, at least in part, related to the prevention of vascular endothelial cell apoptosis. The use of caspase-8/caspase-3 siRNAs not only identifies these caspases as potential upstream and downstream therapeutic targets in sepsis but indicates a significant role of activation of the caspase-8 and caspase-3 pathways in the septic syndrome.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Megumi Matsui for expert secretarial assistance.

References

Lizcano JM, Morrice N, Cohen P. Regulation of BAD by cAMP-


