Invited Review**Vascular biology in sepsis: pathophysiological and therapeutic significance of vascular dysfunction**Naoyuki MATSUDA¹ and Yuichi HATTORI¹*¹Department of Molecular and Medical Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan*

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Abstract

Sepsis is the leading cause of mortality in critically ill patients. In this pathological syndrome, septic shock and sequential multiple organ failure correlate with poor outcome. The pathophysiology of sepsis with acute organ dysfunction involves a highly complex, integrated response that includes activation of number of cell types, inflammatory mediators, and the hemostatic system. Central to this process may be alterations in vascular functions. This review article provides a growing body of evidence for the potential impact of vascular dysfunction on sepsis pathophysiology with a major emphasis on the endothelium. Furthermore, the role of apoptotic signaling molecules in the mechanisms underlying endothelial cell injury and death during sepsis and its potential value as a target for sepsis therapy will be discussed, which may help in the assessment of ongoing therapeutic strategies.

Key words: sepsis, endotoxin, microcirculation, endothelium, apoptosis

Introduction

Despite decades of efforts and significant advances in antimicrobial therapy and overall medical care, sepsis is the leading cause of death among hospitalized patients in noncoronary intensive care units (Pinner *et al.*, 1996; Riedemann *et al.*, 2003). Sepsis and its sequelae, septic shock, acute respiratory distress syndrome (ARDS), and multiple organ dysfunction syndrome (MODS), represent a continuum of a syndrome encompassing multiple pathological processes including systemic inflammation, coagulopathy, and systemic vascular collapse. The development of sepsis occurs as a result of a systemic inflammatory response syndrome (SIRS) to infection (Nyström, 1998). However, it is now generally accepted that sepsis is not the infection itself but, rather, the host response to infection that determines the outcome of sepsis (Cohen *et al.*, 2002). Future advances in sepsis therapy will require a better understanding of how the individual components of the host response interact. Central to the highly complex,

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integrated host response involving different proinflammatory molecules from immune cells would be vascular dysfunction and/or injury. Indeed, vascular dysfunction and/or injury, including endothelial cell alterations, should favor impaired perfusion, tissue hypoxia, and subsequent organ failure.

This review article provides an overview of the current knowledge about the importance of vascular derangements in septic pathophysiology. We will also focus on recent advances in the elucidation of the molecular mechanisms involved in the pathogenesis of sepsis with a major emphasis on the endothelium which may serve as a potential target for the development of sepsis therapy.

Microvascular dysfunction

Disturbed microvascular perfusion has been implicated in multiple organ dysfunction and failure associated with severe sepsis (Lush and Kvietys, 2000; De Backer *et al.*, 2002). Recent research has shown that systemic hemodynamics can be maintained at the expense of impaired microcirculatory perfusion in sepsis (Vincent, 2001). Microcirculatory perfusion is regulated by an intricate interplay of many neuroendocrine, paracrine, and mechano-sensory pathways (Lehr *et al.*, 2000). These mechanisms adapt to the balance between locoregional tissue oxygen transport and metabolic needs to ensure that supply matches demand. In sepsis, such a regulatory system is severely compromised because of decreased deformability of red blood cells with inherent increased viscosity (Astiz *et al.*, 1995), an increased percentage of activated neutrophils with decreased deformability and increased aggregability due to upregulation of adhesion molecules (Linderkamp *et al.*, 1998), activation of the clotting cascade with fibrin deposition and the formation of microthrombi (Diaz *et al.*, 1998), dysfunction of vascular autoregulatory mechanisms (Avontuur *et al.*, 1997), and finally, the secondary enhanced perfusion of large arteriovenous shunts (Cronenwett and Lindenauer, 1979). These heterogeneous processes result in tissue dysoxia, either from impaired microcirculatory oxygen delivery and/or from mitochondrial dysfunction (Fink, 1997; Ince, 2000).

Alterations in microvascular blood flow and oxygenation have been demonstrated in various models of sepsis (Cryer *et al.*, 1987; Drazenovic *et al.*, 1992; Lam *et al.*, 1994; Piper *et al.*, 1996; Farquhar *et al.*, 1996). In a normodynamic septic model using cecal ligation and puncture (CLP) in rats, reduced perfused capillary density and increased heterogeneity have been observed in striated muscles and intestinal mucosa (Lam *et al.*, 1994; Farquhar *et al.*, 1996; Madorin *et al.*, 1999; Sielenkamper *et al.*, 2001). Meanwhile, it has been shown that, for the same level of hypotension in mice, mucosal perfusion disorders are considerably larger in endotoxin-induced hypotension than in hemorrhagic hypotension (Nakajima *et al.*, 2001). Although cardiac output is frequently increased in sepsis, high lactate levels and increased pCO₂ in tissue indicate at least regional tissue dysoxia. This has been termed oxygen extraction deficit in sepsis and has been well documented in different animal models of shock (Nelson *et al.*, 1988; Cain and Curtis, 1991; Vallet *et al.*, 1994). The heterogeneity of microvascular blood flow may help explain some of the alterations in oxygen extraction capabilities that are seen in sepsis. Using a mathematical model, an increase in blood flow heterogeneity has been indicated to be associated with an

increase in critical delivery of oxygen (Walley, 1996), and gut and muscle blood flow heterogeneity has been shown to increase together with impaired oxygen extraction after endotoxin administration or fecal peritonitis (Humer *et al.*, 1996; Ellis *et al.*, 2002). However, it is still a matter of debate whether oxygen extraction deficit can be explained only by pathologic flow heterogeneity due to dysfunctional autoregulatory mechanisms and microcirculatory dysfunction.

The gaseous molecule nitric oxide (NO) serves as a potent regulator of vascular tone, a neurotransmitter, an antioxidant, and, seemingly, a modulator of overall microvascular integrity, function, and oxygen transport. NO is synthesized from L-arginine by NO synthase (NOS). Although endothelial NOS (eNOS) is constitutively expressed and generates small amount of NO in response to physical and receptor stimuli, inducible NOS (iNOS) generates much larger amount of NO for sustained time periods and is principally implicated as the major cause for hypotension, thus contributing to many of the manifestation of septic shock as vasoplegia, myocardial dysfunction, hepatic damage, and vascular and intestinal hyperpermeability (Titheradge, 1999). Interestingly, microvascular perfusion has been shown to be restored by a redistribution within the gut wall and/or an amelioration of the cellular respiration after application of 1,400 W, an inhibitor that has partial iNOS selectivity, in pig endotoxemia model (Pittner *et al.*, 2003). Furthermore, it has been reported that, in a rat CLP model of sepsis, microvascular reactivity to acetylcholine quantified as changes in arteriolar diameter and downstream capillary red blood cell velocity, is impaired in skeletal muscle by NO and restored by neuronal NOS (nNOS) inhibition (Gocan *et al.*, 2000).

However, NO is an important factor in maintain the integrity of blood flow through microcirculation by regulating resistance vessel diameter, blood rheology, interaction between cellular blood elements and the vascular wall, and blood volume. Alternatively, increased NO production may preserve or protect microvascular blood flow. In a rat lipopolysaccharide (LPS) model of sepsis, sodium nitroprusside has been shown to attenuate the loss of perfused liver sinusoids (Gunderson *et al.*, 1998). Furthermore, it has been reported that the NO donor 3-morpholiniosydnonimine decreases critical oxygen delivery and increases critical oxygen extraction ratio in dogs treated with LPS (Zhang *et al.*, 1997), indicating that increased NO can improve the matching of microvascular oxygen delivery with oxygen demand. These findings have led to the idea that the addition of systemic NO to adequately volume resuscitated patients with septic shock may result in an improvement of microcirculatory perfusion. Sporonk *et al.* (2002) have found an improvement in sublingual microcirculatory perfusion after injection of nitroglycerin in septic shock patients. Upon administration of nitroglycerin, microcirculatory flow could increase not only in large microvessels but also in small microvessels. All patients except one, owing to late cerebral hemorrhage, were discharged from the hospital alive. This suggests that one can actively open up the microcirculatory network and keep it open by volume and vasodilator therapy. Taken together, evidence that NO donors improve microvascular hemodynamics would seem to suggest that NO overproduction may protect on microvascular blood flow and oxygen transport during sepsis, but clearly more microcirculatory research needs to be conducted to assess the role of endogenous NO during sepsis, and further studies should demonstrate whether this line of thought regarding therapy in sepsis can be guided by

microcirculatory flow patterns and might result in a better outcome.

Vascular hyporesponsiveness to vasocontractile stimuli

Hallmark clinical findings during sepsis include peripheral vasodilation with low systemic vascular resistance and high cardiac output. The reduction in peripheral vascular resistance is thought to be a key factor responsible for the death of patients with septic shock (Groeneveld *et al.*, 1986; Parrillo, 1985). However, these septic patients experience adrenergic unresponsiveness despite elevated circulating levels of catecholamines (Chernow *et al.*, 1982).

Numerous experimental studies of sepsis have clearly documented the presence of both *in vivo* and *in vitro* vascular responsiveness to α -adrenergic stimulation (Fink *et al.*, 1985; Wakabayashi *et al.*, 1987; McKenna, 1988, 1990; Julou-Schaeffer *et al.*, 1990; Parker *et al.*, 1991; Suba *et al.*, 1992). Umans *et al.* (1993) have confirmed and extended these observations by demonstrating impaired contractile responses to angiotensin II and serotonin. Thus, sepsis changes vascular contractile responses to many vasoactive agents. Impaired vascular reactivity with an abnormal balance between vasoconstrictor and vasodilator tone should result, then, in an inability to regulate blood flow distribution between and within tissues, which can alter blood flow to vital organs such that organ failure will eventually occur. It has been shown that inhibition of NOS is beneficial to largely restoring the contractile responses to agonists (Julou-Schaeffer *et al.*, 1990). Furthermore, the restorative effect of NOS inhibition can be maintained even in vessels in which the endothelium had been removed, indicating that *in vivo* endotoxin administration may lead to high expression of iNOS within vascular smooth muscle cells, thereby impairing contractile responses (McKenna, 1988; Julou-Schaeffer *et al.*, 1990). In this regard, *in situ* hybridization and immunohistochemistry analysis of rat aorta following endotoxin administration *in vivo* and *in vitro* has suggested that aortic adventitia, in addition to the endothelium, is a potential source of iNOS (Zhang *et al.*, 1999). These changes correspond to the pathophysiological features of clinical and experimental sepsis and may account for the profound vasodilation and the limited response to the normal endogenous stimuli that can regulate blood flow distribution among organs. Finally, iNOS deficient mice have been found to be resistant to vascular hypocontractility (Gunnnett *et al.*, 1998). Accordingly, iNOS, which generates large amounts of NO, is likely to be a critical mediator of the diminished vascular contractility in sepsis (Fleming *et al.*, 1991; Griffiths *et al.*, 1995; Hom *et al.*, 1995).

We have found different expression levels of iNOS between the two types of vessels from LPS-induced septic rabbits (Matsuda *et al.*, 2003). Thus, the sepsis-induced increase in relative protein expression levels of iNOS was more marked in mesenteric (9.4-fold) than pulmonary (2.1-fold) arteries. This difference could lead to hypocontractile response to histamine in mesenteric but not in pulmonary arteries. Our findings may provide a basis for the results of past investigations that iNOS induction-associated vascular hypocontractility was not observed in the pulmonary circulation (Nelson *et al.*, 1991; Suba *et al.*, 1992; Spath *et al.*, 1994; Fullerton *et al.*, 1995) despite the presence of iNOS mRNA in pulmonary vessels *in vivo* LPS (Griffiths *et al.*, 1995).

A recent report has demonstrated that hypocontractility to methoxamine in blood vessels

from 24-h endotoxemic rats is not due to a generalized inability of the vascular smooth muscle to contract and does not appear to involve abnormalities in Ca^{2+} mobilization or entry (Farmer *et al.*, 2003). However, it has been proposed that contractile dysfunction may be primarily due to the direct deleterious effect of sepsis on vascular smooth muscle contractile mechanics/machinery, based on the observation that maximum force of contraction in response to both phenylephrine and KCl was lowered without any change in their sensitivities in the aorta from a rat model of 48-h sepsis resulting from a soft-tissue infection with *E. coli* and *B. fragilis* (Price *et al.*, 1999).

Role of vascular K_{ATP} channels in sepsis

The ATP-sensitive potassium (K_{ATP}) channel has been identified as an important modulator of arterial vascular smooth muscle tone (Standen *et al.*, 1989). Particular attention has been focused on the involvement of K_{ATP} channels in both hypotension and vascular hyporeactivity induced by endotoxemia. *In vivo* evidence for K_{ATP} channel involvement in sepsis comes mainly from anesthetized rat (Wu *et al.*, 1995; Sorrentino *et al.*, 1999), dog (Landry and Oliver, 1992), and pig (Vanelli *et al.*, 1995, 1997) models of endotoxemia. Rapid restoration of blood pressure can be achieved with glibenclamide, resulting largely from increased systemic vascular resistance rather than improved cardiac output (Landry and Oliver, 1992; Vanelli *et al.*, 1995). Glibenclamide also increases vasopressor reactivity to α_1 -adrenergic agonists in both the early phase (3 h) and the delayed phase (24 h from LPS challenge) of septic shock in the rat (Wu *et al.*, 1995; Sorrentino *et al.*, 1999). These findings strongly suggest that K_{ATP} channels preferentially open during sepsis and are important underlying cause of hypotension and vascular reactivity, since glibenclamide is without effect in control animals. This may partly explain the increased responsiveness to K_{ATP} channel opener cromakalim in LPS-treated rats (Sorrentino *et al.*, 1999; d'Emmanuele di Villa Bianca *et al.*, 2003).

Abnormal opening of K_{ATP} channels and, to a lesser extent, large conductance Ca^{2+} -activated K^+ (BK_{Ca}) channels, appears to be responsible for an increase in membrane hyperpolarization reported in mesenteric arteries and aortas from rats with endotoxic shock (Chen *et al.*, 2000; Wu *et al.*, 2004). PNU-37883A and barium, both of which close K_{ATP} channels via pore inhibition, could significantly reverse hyporeactivity to phenylephrine in rat mesenteric arteries incubated with LPS (O'Brien *et al.*, 2005). Likewise, relaxations to K_{ATP} channel openers are potentiated in both *in vitro* and *ex-vivo* models of LPS-induced hyporeactivity (Sorrentino *et al.*, 1999; Chen *et al.*, 2000; Wilson and Clapp, 2002), indicative of up-regulation of K_{ATP} channel function in vascular smooth muscle by endotoxin. However, a somewhat surprising but consistent finding in *in vitro* model is the lack of effect of sulfonylurea receptor inhibitors such as glibenclamide on vascular hyporeactivity and in some cases contractions can be even further reduced (Wu *et al.*, 1995; Sorrentino *et al.*, 1999; Preiser *et al.*, 2003; O'Brien *et al.*, 2005). An intriguing possibility is that endotoxin may alter K_{ATP} channel pharmacology such that channel inhibition via sulfonylurea receptors would become dysfunctional. It has been shown that glibenclamide become significantly less effective at inhibiting levcromakalim- and iNOS-induced vascular relaxation in the presence of endotoxin, while the vascular selective pore inhibitor PNU-37883A

remains effective (Wilson and Clapp, 2002). Perhaps less intuitive may be the reason why glibenclamide is efficacious in *in vivo* animal studies. Glibenclamide has been used at much higher dosages in *in vivo* compared to *in vitro* studies, at which concentrations it may be also acting on the pore-forming subunit (Bryan and Aguilar-Bryan, 1999). Moreover, since these *in vivo* doses (10-40 mg/kg) are far in excess of those given to humans (<0.25 mg/kg), the dose of glibenclamide required to effectively block vascular K_{ATP} channels would never be given to patients.

The hyporeactivity to phenylephrine, the increased hypotensive effect of cromakalim, and glibenclamide-induced hypertension seen in LPS-treated rats could be prevented by dexamethazone treatment (d'Emmanuele di Villa Bianca *et al.*, 2003). This may be related to dexamethazone either inhibiting K_{ATP} channel subunit expression and/or synthesis of a mediator regulating expression. Therefore, the clinical beneficial effect of glucocorticoids in septic patients may be linked not only to the well-known anti-inflammatory properties (Annane, 2005), but also to an improvement of vascular hyporeactivity by reducing vascular expression of the K_{ATP} channel subunit. Notably, both mRNA and protein levels of Kir6.1 gene expression have been reported to be substantially increased in the diaphragm of endotoxin-treated rats, with levels peaking over 24-48 h (Czaika *et al.*, 2000). Moreover, Kir6.1 gene expression is up-regulated 22-fold in experimental colitis (Jin *et al.*, 2004). However, knockout of the *KCNJ8* gene encoding the vascular Kir6.1 K_{ATP} channel pore predisposes to an early and profound survival disadvantage in a mouse model of septic shock with LPS (Kane *et al.*, 2006), suggesting a vital role for adequate Kir6.1-mediated K_{ATP} channel function in cardiovascular response to endotoxic challenge.

Excessive activation of K_{ATP} channels is clearly implicated in a number of crucial mechanisms in sepsis, including vascular hyporeactivity. However, channel opening may afford a degree of cellular protection. Furthermore, whether up-regulation of vascular expression of K_{ATP} channels is beneficial or detrimental in sepsis remains to be determined. It should be kept in mind that the involvement of K_{ATP} channels in vascular disturbances associated with sepsis is questioned in a recent placebo-controlled study in septic shock patients where glibenclamide has no effect on blood pressure or norepinephrine requirements (Warrillow *et al.*, 2006). However, targeting of the vasculature with specific pore rather than glibenclamide might appear more appropriate in sepsis. Clearly further research is needed to better delineate the protective and harmful roles of K_{ATP} channels in sepsis.

Endothelial dysfunction

Impaired endothelium-dependent vascular relaxations

Endothelial cells produce vasoactive molecules that regulate arteriolar tone and contribute to blood pressure control. These include the vasodilators, including NO and prostacyclin, and the vasoconstrictors, including endothelin, thromboxane A_2 , and platelet-activating factor (Wanecek *et al.*, 2000). Impaired endothelium-dependent relaxations have been shown in blood vessels from endotoxemic animals (Parker and Adams, 1993; Umans *et al.*, 1993; Myers *et al.*,

1995; Wiel *et al.*, 2000). Apart from anatomical injuries, such impairment observed in endotoxemic blood vessels may result from several mechanisms: alteration in endothelial cell surface receptors; modified signal transduction pathways such as receptor-eNOS uncoupling; altered function and expression of eNOS; changes in the pathways that lead to release of NO; and changes in mechanisms that participate in subsequent degradation of NO. We have shown that eNOS expression is obviously diminished in blood vessels from rabbits (Matsuda *et al.*, 2003) and in lung tissues from mice (Matsuda *et al.*, 2004) following induction of sepsis with LPS. In our recent work, furthermore, it has been demonstrated that sepsis causes a progressive and profound reduction in phosphorylation of eNOS in rabbit mesenteric arteries (Matsuda *et al.*, 2006), suggesting less production of NO by eNOS in sepsis. We have also found that both phosphorylation of Akt and membrane translocation of phosphatidylinositol 3-kinase (PI3-K) are markedly decreased in mesenteric arteries from LPS-induced septic rabbits (Matsuda *et al.*, 2006). The PI3-K/Akt pathway is known to signal eNOS activation in response to shear stress (Fulton *et al.*, 1999; Dimmeler *et al.*, 1999). We thus interpret our findings to indicate that reduced phosphorylation of eNOS in septic vessels is possibly the result of the impaired PI3-K/Akt pathway.

Vascular hyperpermeability

In the intact vasculature, the endothelium forms a continuous, semipermeable barrier that varies in integrity and control for different vascular beds (Stevens *et al.*, 2000). During septic shock the breakdown of endothelial barrier function occurs. Thus, a central feature of the endothelium in sepsis is an increased permeability or loss of barrier function. The loss of fluid into the extravascular space leads to life-threatening edema in the lungs, kidney, and brain of septic patients. There is evidence that LPS directly contributes to endothelial barrier dysfunction through a caspase-mediated cleavage of junctional proteins involved in regulating transport of material between the vascular space and tissue (Bannerman *et al.*, 1998). Furthermore, the contribution of structural damage to endothelial cells to skeletal muscle edema has been shown in a pig model of septic shock (Hauptmann *et al.*, 1994). More importantly, the increase in endothelial permeability can be induced by a number of sepsis-related factors. Indeed, the cytokine tumor necrosis factor- α (TNF- α) induces an increase in endothelial cell permeability both *in vivo* and *in vitro* (Johnson *et al.*, 1989; Goldblum *et al.*, 1993; Ferro *et al.*, 1997, 2000). Under *in vitro* conditions, thrombin also increases endothelial cell permeability, while TNF- α and thrombin act synergistically to induce barrier dysfunction *in vitro* (Tiruppathi *et al.*, 2001). We have clearly demonstrated that *in vivo* transfection of nuclear factor- κ B (NF- κ B) decoy oligodeoxynucleotide results in a dramatic improvement of increased pulmonary vascular permeability in septic mice (Matsuda *et al.*, 2004, 2005). In contrast, the agents to inhibit iNOS nonselectively and selectively are only partially effective. This suggests that a number of molecules that could be actually generated by activation of the NF- κ B signaling pathway are involved in the development of vascular hyperpermeability during sepsis.

Abnormal coagulation and fibrinolysis

Normally the endothelium possesses anticoagulant/antithrombotic properties in expressing

e.g. tissue factor (TF) pathway inhibitors, thrombomodulin, NO, and prostacyclin (PGI₂) (Pearson, 1999). Thrombomodulin is responsible for inhibition of thrombin activity. Thrombomodulin, when bound to thrombin, forms a potent protein C activator complex, which leads to anticoagulant behavior (Owen and Esmon, 1981). In severe meningococcal sepsis, thrombomodulin and endothelial protein C receptors are lacking, resulting in impaired protein C activation (Faust *et al.*, 2001). In a mouse model of endotoxemia, administration of LPS results in reduction in total tissue thrombomodulin antigen in the lung and brain, but not in the kidney (Weiler *et al.*, 2001), suggesting that sepsis-associated changes in thrombomodulin expression may vary between organs. During the pathogenesis of sepsis, alterations in expression of coagulation-involved factors occur (Levi *et al.*, 2003). A procoagulant glycoprotein TF may be released by endothelial and subendothelial cells, and a dysregulated balance of tissue-type plasminogen activator (TPA) and plasminogen activator inhibitor-1 (PAI-1) would lead to increased coagulation and suppressed fibrinolytic activity. Despite increased levels of PAI-1 in sepsis (Mavrommatis *et al.*, 2001; Green *et al.*, 2002), however, an endothelial source of PAI-1 has not been established. Furthermore, sepsis studies with few exceptions (Drake *et al.*, 1993; Todoroki *et al.*, 2000) have consistently failed to demonstrate TF in the intact endothelium.

It has been observed that the use of glycoprotein IIb/IIa inhibitor can attenuate endotoxin-induced monocyte TF expression through decreased platelet activation, leading to a marked reduction in endothelial injury, increased endothelium-dependent relaxations, and improved survival rates in the treated animals (Pu *et al.*, 2001). This suggests that monocyte activation and TF expression may be of importance in sepsis-associated endothelial injury. Thus, coagulation activation may itself contribute to endothelial injury seen during sepsis. Endothelial injury, in turn, exacerbates sepsis-induced coagulation abnormalities. Indeed, release of endothelium-derived factors such as NO and PGI₂ is impaired. Because NO and PGI₂ not only control vascular tone but also have antiadhesive and TPA-like properties, loss of NO and PGI₂ release facilitates leukocyte and platelet aggregation, and aggravation of coagulopathy. Furthermore, when the endothelium is viewed in the context of its native environment, additional properties emerge that may contribute to a procoagulant state. When activated endothelial cells generate adhesion molecules, including E-selectin, P-selectin, intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), during endotoxemia that bind leukocytes and monocytes, they favor enhancement in local procoagulant reactions. The importance of adhesion molecules in mediating the sepsis phenotype is supported by studies in knockout mice (Munoz *et al.*, 1997; Steeber *et al.*, 1999; Matsukawa *et al.*, 2002). The relationship between activation of innate immunity and coagulation in sepsis is phylogenetically ancient (Opal, 2000). However, generalized intravascular coagulation, as a generalized inflammatory response, is detrimental to the host, favoring widespread fibrin deposition and altered tissue perfusion during sepsis.

Endothelial cell apoptosis

Endothelial cell apoptosis is a highly regulated process (Stefanec, 2000). Normally, only a small percentage (<0.1%) of endothelial cells are apoptotic. Under *in vitro* conditions, certain

pathogens are capable of inducing endothelial cell apoptosis (Winn and Harlan, 2005). *In vitro* incubation of cultured bovine and ovine endothelial cells with LPS has been reported to induce apoptosis (Hoyt *et al.*, 1995, 1996; Bannerman *et al.*, 1998; Frey and Finlay, 1998). Evidence that LPS-induced endothelial cell death is apoptotic in nature has been confirmed by several criteria, including morphological changes (Frey and Finlay, 1998), DNA laddering (Bannerman *et al.*, 1998), and transferase-mediated dUTP nick-end labeling (TUNEL) (Hoyt *et al.*, 1995, 1996; Bannerman *et al.*, 1998). However, LPS alone fails to induce apoptosis in cultured human endothelial cells (Pohlman and Harlan, 1989; Hu *et al.*, 1998). Furthermore, sodium arsenite has been required for induction of apoptosis by LPS in cultured porcine endothelial cells (Hotchkiss *et al.*, 2002). The ability of endotoxin to induce apoptosis may be cell specific. Alternatively, the apoptosis effect of endotoxin in *in vitro* system may be dependent on whether the transduction molecules for the LPS signal are indispensably present in the endothelial cell line.

In *in vivo* animal models, purified LPS has been reported to elicit endothelial cell injury and apoptosis. Endothelial cell injury and detachment from the vascular wall have been demonstrated when LPS was injected into mice (Koshi *et al.*, 1993), rats (Reidy and Schwartz, 1983; Sutton *et al.*, 1993), rabbits (Cybulsky *et al.*, 1988; McCuskey *et al.*, 1996), and sheep (Meyrick, 1986b). In a mouse model of endotoxemia, intraperitoneal delivery of LPS from *Salmonella typhimurium* could result in widespread apoptosis of the endothelium in a process mediated via ceramide generation (Haimovitz-Friedman *et al.*, 1997). In other study, intravenous administration of LPS in mice has been shown to induce endothelial cell apoptosis in the lung, but not the liver, pointing to organ-specific differences in programmed cell death (Fujita *et al.*, 1998). However, liver sinusoidal endothelial cells obtained from LPS-treated rats display enhanced activation of caspase-3, a central apoptotic effector protease (Deaciuc *et al.*, 1999). Interestingly, evidence of endothelial cell injury has been provided in postmortem biopsies obtained from patients who had died of sepsis-related ARDS (Meyrick, 1986a). Moreover, an increase in circulating endothelial cells has been found in septic patients, and the magnitude of this increase correlates negatively with survival (Mutunga *et al.*, 2001).

It has been shown that injection of a broad spectrum caspase inhibitor decreases endothelial cell apoptosis in the lung after LPS administration and improves survival in a murine model of acute lung injury (Kawasaki *et al.*, 2000). We very recently assessed the impact of endothelial cell apoptosis on mortality in mice with CLP-induced sepsis. Because 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 (Fig. 1), synthetic small interfering RNAs (siRNAs) which specifically suppress gene expression for caspase-8 and caspase-3 were tested in that model for their demonstrated ability to treat aortic endothelial injury and mortality (Matsuda *et al.*, 2007). We found that systemic treatment with caspase-8/caspase-3 siRNAs prevented a significant appearance of TUNEL-positive aortic endothelial cells, which was strongly induced at 24 h after the onset of sepsis by CLP (Fig. 2). Furthermore, electron microscopic analysis revealed partial detachment of some endothelial cells from basal membrane at 10 h after CLP. The structure of aortic endothelium from mice at 24 h after CLP exhibited a more remarkable morphologic abnormality: most endothelial cells were swollen and appeared to be partially

Fig. 1. Regulation of endothelial cell apoptosis during sepsis. There are two major pathways involved in initiation of apoptotic death, both of which ultimately converge to activate executioner caspases. The death receptor pathway involves activation of members of TNF receptor (TNF-R) family with an intracellular death domain including Fas, TNF-R1, DR3, and the receptors for TRAIL. After ligand binding to death receptors, the death domain recruits an adaptor protein FADD. FADD can then recruit pro-caspase-8 to the “death-inducing signal complex,” thereby causing its activation. Caspase-8 then activates caspase-3. The second apoptotic death pathway is mitochondrial-mediated pathway. Following mitochondrial release of apoptogenic compounds such as cytochrome *c* (CytoC), caspase-9 activation occurs at the hands of the oligomerization mediator APAF-1, resulting in amplification of the death receptor-mediated pathway.

detached from the basal membrane. Systemic treatment with caspase-8/caspase-3 siRNAs prevented the sepsis-induced endothelial damage seen with electron microscopy, although leukocytes and platelets appeared to accumulate on the endothelial layer (Fig. 3). Finally, *in vivo* delivery of caspase-8/caspase-3 siRNAs conferred a dramatic survival advantage to septic mice (Matsuda *et al.*, 2007). We propose that the prevention of vascular endothelial apoptosis may be, at least in part, responsible for the greatly beneficial effect of caspase-8/caspase-3 siRNAs in sepsis, but the possibility cannot be entirely excluded that the ability of the siRNAs to lead to survival advantage in sepsis involves the effect to prevent apoptosis of other cell types, including lymphocytes and gastrointestinal epithelial cells.

A key marker of endothelial cell activation is NF- κ B activation and nuclear translocation, a requisite event for many endothelial cell responses, including expression of cytokines and adhesion molecules. In addition to its role in promoting expression of proinflammatory gene products, NF- κ B has been implicated in both pro- (Ryan *et al.*, 2000; Heimberg *et al.*, 2001) and antiapoptotic signaling (Van Antwerp *et al.*, 1996; LaCasse *et al.*, 1998). Blocking NF- κ B activation has been shown to sensitize human endothelial cells to direct TNF- α -induced apoptosis in the absence of cycloheximide, suggesting an antiapoptotic role for NF- κ B (Zen *et al.*, 1999). Evidence has been provided that this sensitization is conferred by inhibition of NF-

Fig. 2. Detection of apoptotic endothelial cells in mouse aorta by TUNEL staining. Note that TUNEL-positive apoptotic cells (arrows) observed in mice at 24 h after sepsis induction with CLP (B) are undetectable with systemic application of caspase-8/caspase-3 siRNAs (C). No histological evidence for apoptosis is consistently found in sham-operated control. All images are at $\times 400$ magnification (scale bar = 50 μm).

κB -dependent expression of members of cellular inhibitor of apoptosis protein (cIAP) gene family (Stehlik *et al.*, 1998). In addition, LPS and $\text{TNF-}\alpha$ each up-regulate expression of another antiapoptotic proteins, including A1 (Karsan *et al.*, 1996; Hu *et al.*, 1998) and A20 (Dixit *et al.*, 1990; Hu *et al.*, 1998; Zen *et al.*, 1998), in endothelial cells. Conversely, recent studies demonstrate that several signaling molecules originally described as mediators of apoptosis can contribute to the regulation of NF- κB activation. For example, transient overexpression of Fas-associated death domain (FADD), FLICE-like inhibitor protein (FLIP), or caspase-8 augments basal levels of NF- κB activation (Chaudhary *et al.*, 2000; Hu *et al.*, 2000; Kataoka *et al.*, 2000). FADD is an adaptor protein which can recruit pro-caspase-8 to the “death-inducing signal complex,” thereby causing its activation (Fig. 1), and FLIP is an antiapoptotic protein with significant homology to caspase-8. Moreover, evidence that apoptotic signaling molecules can affect the ability of LPS to induce NF- κB activation has been shown by the finding that overexpression of Bcl-2 and Bcl-x_L, antiapoptotic members of the Bcl-2 family, inhibits LPS-induced NF- κB activation and NF- κB -dependent gene expression in endothelial cells (Badrichani *et al.*, 1999). This inhibition of NF- κB activation corresponds with Bcl-2-mediated inhibition of I $\kappa\text{B}\alpha$ degradation. Further evidence for a role for apoptotic signaling molecules in promoting NF- κB activation has been provided in a recent report showing that FADD down-regulates LPS-induced NF- κB activation (Bannerman *et al.*, 2002). However, additional work will be needed to delineate pathophysiological significance of cross talk between apoptotic and NF- κB signaling molecules.

LPS up-regulates expression of iNOS and increases NO production in endothelial cells

Fig. 3. Electron microscopic analysis of mouse aortic endothelium. Mice were subjected to sham operation (A), CLP alone 10 h (B) and 24 h later (C), or CLP followed by systemic administration of caspase-8/caspase-3 siRNAs (D). Endothelium-intact aortic segments measuring 3–5 mm were fixed for 5 h at room temperature with 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Samples were rinsed in PBS and then stained with a 3% platinum blue aqueous solution for 15 min at pH 9.0. After being rinsed in distilled water, the sections were examined with an electron microscope (Hitachi Tabletop Microscope TM-1000; Hitachi, Tokyo, Japan).

(Laskin *et al.*, 1994; Rockey and Chung, 1996). It has been demonstrated that suppression of iNOS induction and inhibitors of NOS activity protect against LPS-elicited endothelial cell injury (Palmer *et al.*, 1992; Sato *et al.*, 1995; Higaki *et al.*, 2001), suggesting that iNOS-derived NO promotes LPS-induced apoptosis of endothelial cells. The mechanism by which NO overproduction contributes to LPS-elicited endothelial cell apoptosis remains unknown, but the generation of large amounts of NO following iNOS induction by LPS may result in NO reaction with superoxide anion to form peroxynitrite, a potent oxidizer. Endothelial cell injury resulting from the generation of peroxynitrite may synergistically enhance endothelial cell apoptosis elicited by LPS. Alternatively, high concentrations of NO can inhibit protein synthesis (Wolkow, 1998). Because protein synthesis inhibition has been established to sensitize human endothelial cells to LPS-induced apoptosis by inhibiting expression of the antiapoptotic protein FLIP (Bannerman *et al.*, 2001), one may speculate that high levels of NO may result in a decrease in expression of this cytoprotective protein. On the contrary, NO has been shown to inhibit LPS-induced apoptosis in endothelial cells. Increased production of NO due to iNOS overexpression could block LPS-elicited endothelial cell apoptosis (Ceneviva *et al.*, 1998; Tzeng *et al.*, 1997). The differential effects of NO on mediating LPS-induced endothelial cell apoptosis may be dependent on its concentrations, since it has been reported that moderate concentrations of NO

confer protection, whereas higher concentrations of NO enhance LPS-induced apoptosis in endothelial cells (DeMeester *et al.*, 1998).

Conclusions

Despite new understanding of the molecular basis and much progress in therapies that specifically interfere in an interesting target, sepsis and related syndromes remain the leading causes of death in patients critically ill, with an unacceptably high mortality rate. In sepsis, microvascular dysfunction with reduced perfusion and oxygen could result in tissue hypoxia and, ultimately, in the development of organ failure. The precise mechanisms underlying microvascular dysfunction remain unclear, but include altered vasomotor tone, reduced functional capillary density, reduced red blood cell deformability, increased numbers of activated neutrophils, and endothelial cell dysfunction with increased permeability and apoptosis. The endothelium is the key in initiating, perpetuating, and modulating sepsis pathophysiology. A deeper understanding of the regulation of survival and death pathways in endothelial cells may facilitate the development of novel therapies to avoid the pathological events associated with endothelial injury during sepsis. Knockout of proapoptotic molecules with siRNAs to rescue endothelial cells may represent a novel therapeutic strategy in sepsis. However, further studies should demonstrate whether this line of thought regarding therapy of sepsis can be guided by potential endothelial cell apoptosis in septic patients and might result in a better outcome clinically.

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