Hypothesis

Simultaneous activation of apoptosis and inflammation in pathogenesis of septic shock: a hypothesis[1]

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Abstract Sepsis, a widely prevalent disease with increasing morbidity and mortality, is thought to result from uncontrolled inflammatory responses to microbial infection and/or components. However, failure of several experimental anti-inflammatory therapies has necessitated re-evaluation of the paradigm underlying the pathogenesis of this complex disorder. Apoptotic cell death forms a second dominant feature of septic shock in patients and animal models. Anti-apoptotic strategies may protect animals from septic death. However, simultaneous occurrence of apoptosis and inflammation is necessary for septic death. At the cellular level, apoptosis plays a central role in the development of the lymphoid system and regulation of immune responses. Immune activation renders cells refractory to apoptosis while apoptosis of activated lymphocytes is an important immunoregulatory mechanism. Factors such as complement factor 5a, caspase-1 and mitogen-activated protein kinase, which participate in apoptosis as well as pro-inflammatory pathways, may be responsible for simultaneous activation of apoptosis and inflammation in sepsis. Further identification of other similar biochemical events capable of co-activating inflammation and apoptosis may provide new targets for therapy of this hitherto untreatable disease.

Key words: Apoptosis; Inflammation; Sepsis

1. Introduction

Sepsis results from dysregulation of the normally protective anti-microbial host defense mechanisms, and is characterized by a systemic inflammatory response manifested by fever, mental confusion, hypotension, coagulopathy, cell and multi-organ injury [1,2]. Sepsis remains a major cause of morbidity and mortality in the intensive care unit. Its incidence in the USA is approximately three cases per 1000 population, out of which nearly 50% die due to a more severe form of disease called septic shock. The fact that activated protein C alone, from the range of experimental anti-inflammatory therapies developed to treat sepsis (Table 1) was approved, albeit for restricted use, for treatment of sepsis, emphasizes the continuing necessity to understand the patho-physiological mechanism(s) underlying this complex disorder.

Recent studies have shown an increased incidence of apoptosis during septic shock. Hotchkiss et al. [3] reported extensive apoptosis of T and B lymphocytes in sepsis patients. Apoptotic lymphocyte death can cause impairment of immune responses [4,5] and anergy and predispose patients to septic death [6]. Increased apoptosis is also observed in animal models of sepsis and its inhibition protects animals from lethality [7–10]. However, the exact relevance of cell death to the pathogenesis of septic shock is poorly understood. Here, we identify apoptosis and inflammation as dual pathogenic factors involved in various animal models of septic shock and hypothesize that simultaneous activation of these two processes may represent a critical pathogenic step in septic shock. Identification of condition(s) co-activating these processes may provide useful therapeutic targets for sepsis.

2. Inflammation and apoptosis in animal models of sepsis

Research in sepsis has relied heavily on a number of animal models such as bacterial endotoxin (lipopolysaccharide, LPS)-induced mortality, D-galactosamine+LPS-induced lethality (DGL), cecal ligation and puncture (CLP) [11–17] and colon ascendent stent peritonitis (CASP) [18]. While LPS- and DGL-induced lethality models are more popular, CLP is considered clinically the more relevant model of septic shock. All these models exhibit little similarity and differ with respect to the cytokines involved in development of sepsis (Table 2).

Systemic inflammation is a common feature of all these

Table 1
Experimental therapies for sepsis

Target	Therapy
Bacterial endotoxin	Endotoxin neutralizing antibodies
TNFα, IL-1	Cytokine neutralizing agents
PAF, thromboxane synthase	PAF antagonists, ketoconazole
Reactive oxygen species	*N*-Acetylcysteine
NO scavenger/iNOS inhibitor	PHP, L-NAME
Immune modulation	Glucocorticoids, pentoxifylline, IFNγ, G-CSF
Coagulation pathway	Factor IXa, Xa, XIa, XIIa, TFPI

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Table 2
Neutralizing various cytokines protects animals from lethality

Cytokine/chemokine	Animal model	Reference
IL-1	LPS-induced lethality in mice	[24,25]
IL-6	LPS-induced lethality in mice	[29]
IFNγ	LPS-induced lethality, CLP, murine Gram-negative sepsis	[26–28]
IL-12	LPS-induced lethality in mice, Schwarzman reaction	[83]
IL-18	*Propionibacterium acnes*/LPS-induced lethality, LPS-induced lethality	[21,84]
TNFα	*Escherichia coli*-induced death in mice and baboons	[22–24]
MIP1α	Murine endotoxemia, CLP	[85,86]
HMGB-1	LPS-induced lethality in mice	[87]
MIF	TSST-1-induced shock	[33–35]
C5a	LPS-induced lethality in rats	[49,80]

animal models. Various proinflammatory cytokines including tumor necrosis factor-α (TNFα), interleukin (IL) 6, interferon-γ (IFNγ), IL-1β, and IL-18 [19–21] are overexpressed during sepsis and their inhibition suppresses the pathological response and protects animals from septic death (Table 2). For instance, TNFα depletion by either pharmacological strategies or genetic disruption significantly improves animal survival after endotoxin challenge [22,23]. Similarly, inhibition of activities of several other pathogenically relevant cytokines including IL-1β [24,25], IFNγ [26–28], IL-6 [29], transforming growth factor-β (TGF-β) [30] and high mobility group 1 (HMG-1) protein [31,32] (Table 2) protects animals from sepsis [33–35]. In addition, protective effects of endogenous anti-inflammatory phospholipids have also been reported [36]. However, evidence suggests that unlike the traditionally held view, the inflammatory milieu in septic animals and individuals is more complex. Septic patients also exhibit features consistent with immunosuppression and high levels of anti-inflammatory cytokines such as IL-4 or IL-10. Reversal of this Th2 response [37] and immunostimulation using granulocyte colony-stimulating factor (G-CSF) [38], which increases neutrophils and IFNγ, a potent macrophage activator that restores HLA-DR expression and TNFα production [39], have shown beneficial results. However, excessive elimination of cytokines can be harmful, e.g. co-neutralization of TNFα and IL-1β enhanced rather than reduced mortality in a neutropenic rat model of sepsis while IL-18 gene deletion enhanced LPS-induced mortality of *Propionibacterium acnes*-treated mice [40]. Cytokines therefore have protective and pathogenic roles in septic death and their optimal levels (and activity) are necessary for survival of septic animals [21].

Apoptosis is a second prominent feature of sepsis. Increased apoptosis is observed in all these models of sepsis. LPS induces apoptosis in glomerular [41], heart [42] and other cells. Co-administration of D-galactosamine and LPS is associated with liver damage through apoptosis [43]. CLP leads to apoptosis in thymus, spleen, lung, and gut [44] while CASP causes bone marrow apoptosis [18] and inhibition of apoptosis was shown to protect animals from lethal shock (see below). Thus, like inflammation, apoptosis forms an important feature of all above models of septic shock.

Apoptosis is a mechanism of controlled disassembly of cells brought about by activation of certain specialized proteases called caspases (cas). Inhibitors of cas-1 and cas-3 protect mice from death induced by LPS [7,8,20], DGL [9] and CLP [10] and rats from LPS-induced lethality [7]. Since cas-1 and cas-3 also mediate proteolytic maturation of cytokines IL-1β and IL-18 [45] and IL-18 and IL-16 [46] respectively, the protective effect of caspase inhibition could be a result of inhibition of cytokine maturation.

If apoptosis is really central to sepsis, other anti-apoptotic strategies, which are devoid of anti-inflammatory activity, should also protect animals from septic shock. Indeed, transgenic expression of the anti-apoptotic protein Bcl-2 and hepatocyte growth factor-induced Bcl-2 expression protect mice from LPS-induced hepatic failure and DGL- [43] and CLP-induced death [10] respectively.

Clearly, apoptosis is a necessary pathogenetic factor and its inhibition may be a useful therapeutic strategy for sepsis [6]. In agreement, treatment with agents like gadolinium chloride [47], depletion of glutamyl-cysteinyl-glycine [48], complement factor C5a blockade [49] and *N*-acetylcysteine (Joshi and Cross, unpublished observations) which prevent apoptosis, albeit indirectly, can protect from sepsis.

3. Apoptosis and inflammation

Apoptosis plays a central role in the generation of the lymphoid system. Developing lymphocytes are destined to die unless a functional antigen receptor is produced through gene rearrangements to trigger a rescue signal. Lymphocytes bearing 'self-reactive' receptors are eliminated by apoptosis to

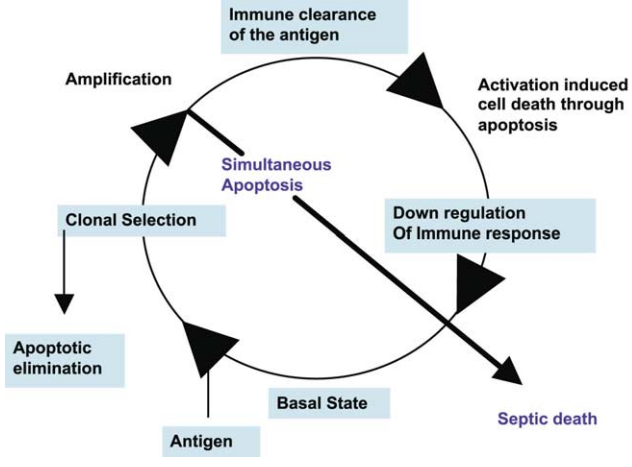

Fig. 1. Simultaneous activation of apoptosis and pro-inflammatory events may precipitate septic death. Apoptosis of antigen non-reactive cells and activated cells plays a central role in stimulation and downregulation of the immune response. Simultaneous activation of pro-inflammatory and apoptotic events can cut short the immune response, disrupt immunoregulatory networks and precipitate septic death.

protect the organism from inappropriate assault. Similarly, mitogenic activation of neutrophils and monocytes inhibits the naturally active apoptotic pathways in these cells, through upregulation of Bcl-2 family member genes like Bfl-1 [50–52]. Immunostimulation activates the transcription factor NF-κB which mediates expression of TRAF-1, TRAF-2, cIAP-1 and cIAP-2 genes that block the activation of cas-8, a key initiator of apoptosis, and inhibit apoptosis [53]. The enhanced survival of the inflammatory cells helps in amplification and sustenance of inflammatory responses. On the other hand, upregulation of apoptosis-inducing gene products like Cardinal [54], the death receptor Fas and the Bcl-2 family protein Bim stimulates the death of activated lymphocytes through activation-induced death pathways, leading to downregulation of ongoing immune responses [55,56]. Thus apoptosis is involved in a constant interplay with the inflammatory system that is critical to the development and homeostasis of immune responses [55,56] and its premature induction concomitantly with inflammation may cut short the immune response, disrupt the immunoregulatory processes and lead to septic death (Fig. 1).

4. Sepsis-related lethality is dependent upon dual activation of apoptosis and inflammation

Although important, neither apoptosis nor inflammation alone is sufficient for lethality in animal models. Apoptotic hepatocyte death due to inhibition of RNA synthesis caused by D-galactosamine-induced reduction of hepatocyte UTP levels [43] sensitizes mice to LPS-induced lethality [57]. Inhibition of this hepatocyte apoptosis by treatment with exogenous uridine or UTP protects mice from DGL-induced lethality [57] indicating the importance of apoptosis as a necessary pathogenetic factor for DGL-induced lethality. Similarly, apoptosis resulting from inhibition of RNA synthesis by agents like actinomycin D and α-amanitine also renders mice hypersensitive to LPS-induced death [58]. Thus, apoptosis appears to be a necessary sensitizing step for endotoxin-induced lethality in mice.

Apoptosis has to be complemented by immunoreactivity to bacterial components for septic death. Freudenberg et al. [59] demonstrated that C3H/HeJ mice, which lack the functional receptor of LPS called TLR-4, are resistant to DGL-induced death. TLR-4 belongs to a family of 10 Toll-like receptors (TLR) which recognize different pathogen-associated molecular patterns and stimulate anti-microbial innate immune responses. And the absence of TLR-4 renders animals resistant to death in response to CLP [60], CASP [61] and endotoxemia.

Therefore extrapolating from the findings in the DGL model, the protective effect of caspase inhibition and the importance of apoptosis and inflammation in different models of sepsis, we hypothesize that simultaneous activation of apoptotic cell death and inflammation is essential for pathogenesis of septic shock. Simultaneous activation of pro-inflammatory processes and apoptosis may disrupt the normal immunoregulatory mechanism(s) and make the host susceptible to septic death (Fig. 1).

This hypothesis of synergistic lethality may also be supported by an earlier observation that superantigens such as staphylococcal enterotoxin B and toxic shock syndrome toxin 1 (TSST-1), which are efficient inducers of apoptosis of T and B cells [62,63], induce LPS hypersensitivity. For instance, pretreatment with TSST-1 renders rabbits 50 000-fold more susceptible to LPS-induced lethality [64]. This requirement of two signals for induction of septic shock-dependent death is summed up in the 'two-hit' hypothesis by Bannan and coworkers [66]. They explain the synergism between Gram-negative bacterial LPS and Gram-positive bacterial superantigen to produce lethal shock [65] in terms of a necessity for two episodes of infection – one with Gram-negative bacteria and the other with Gram-positive bacteria, for pathogenesis of sepsis [66]. Our hypothesis may provide a possible molecular mechanism underlying this two-hit hypothesis.

5. Simultaneous activation of cell death and inflammation in sepsis

Several mechanisms may be involved in simultaneous activation of apoptosis and inflammatory processes in sepsis. Proteins participating in biochemical pathways shared by inflammation as well as apoptosis may be responsible. For instance, we have shown that overexpression and activation, during lethal response to LPS, of cas-1, which is an important mediator of apoptosis and proteolytic maturation of cytokines IL-1 and IL-18 [39,40], leads to endotoxic shock and its inhibition protects mice from death [20]. Similarly, cas-3, which mediates cell death [67] and maturation of IL-18 and IL-16 [46], may also be responsible for triggering the pathological events in septic shock. We have also shown that during protective immune responses to LPS, type I interferons (IFNα/β) play an important IL-1-regulatory role which is disturbed during lethal endotoxemia (Joshi et al., manuscript submitted). IFNα/β-induced signaling molecules like dsRNA-dependent protein kinase, which mediate NF-κB activation and MHC gene expression along with caspase-dependent cell death, may be important to septic death [68]. Another protein called p38 mitogen-activated protein (MAP) kinase plays an important role in synthesis of cytokines like TNFα and IL-1β and its inhibitors are anticipated to provide useful therapies for treatment of inflammatory disorders. MAP kinase pathways consist of sequential phosphorylation and activation of a three-kinase cascade: MEKK-MEK-MAPK, and are also involved in differential regulation of apoptosis and survival in thymocytes. This dual effect of MAP kinases is mediated by two proteins, ERK1 [69,70] and p38 [71,72], which regulate T cell receptor-induced multiplication and apoptosis respectively. The simultaneous activation of these two kinases may lead to co-activation of cell replication and apoptosis. Recent studies have shown that transient activation of extracellular signal-regulated kinases (ERKs) stimulates multiplication, while their sustained activation promotes cell cycle arrest in multiple cell types and death [73]. The systemic inflammatory reaction associated with septic shock may also lead to a sustained activation of ERKs which in turn may cause cell cycle arrest of immune cells and lead to apoptotic death. Secondly, improved efficacy of host anti-microbial responses stimulated by presentation of microbial peptides scavenged by dendritic cells from apoptotic infected macrophages [74] can dictate coexistence of inflammatory and apoptotic processes. Thirdly, macrophages which are responsible for phagocytosis and elimination of apoptotic cells exhibit enhanced responses [75] or express anti-inflammatory cytokines like TGF-β [76]. Irrespective of the underlying mechanism, the deleterious effects of

simultaneous activation of apoptosis and inflammation have been exploited effectively by microbes like *Shigella flexneri* [77] and myxoma virus [78].

Thus, multiple mechanisms may be responsible for the simultaneous activation of apoptosis and inflammation and identification of the responsible factors may help design useful therapies for sepsis. For example, complement factor 5a (C5a), which is critical for lethality in CLP, stimulates the expression of the pro-inflammatory cytokines IL-6 [79] and TNFα [79,80] as well as thymocyte apoptosis through activation of cas-3, -6, and -8 [81] and its blockade protects mice from CLP-induced mortality [49]. On the other hand, this hypothesis may also explain the failure of TNFα-neutralizing therapies in the treatment of septic shock [82]. Interaction of TNFα, a pro-inflammatory pathogenic cytokine in septic shock, with its receptor (TNF-R1) induces recruitment of the death domain-containing cytosolic adapter proteins TRADD (TNF-R1-associated death domain) and FADD (Fas-associated domain), and procaspase-8, which triggers the caspase activation cascade and apoptosis. However, NF-κB activation by TNFα through the TNF receptor interacting protein- and TNF receptor-associated factor-2 (TRAF-2)-mediated signaling events inhibits the apoptotic pathways. Thus, although capable of inducing both pro-apoptotic and pro-inflammatory signaling pathways, TNF probably cannot activate both these processes simultaneously and may not be the pathologically relevant factor of sepsis, as suggested by the recent failure of TNF-directed therapies [64,82].

6. Conclusion

In summary, the simultaneous occurrence of inflammation and apoptosis is an important pathogenic event in septic shock and their inhibition may provide useful therapies for treatment of sepsis. In agreement, inhibition of pro-inflammatory cytokine synthesis as well as T cell apoptosis by C5a blockade protects mice from CLP-induced mortality. Further extension of our studies with the lethal and non-lethal immune responses to LPS will help confirm the role(s) of C5a, caspases, MAP kinases and IFNα/β-stimulated signaling events in inflammation and apoptosis during septic death.

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