Alterations in Cell Signaling in Sepsis

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Multiple intracellular signaling pathways involving kinases, transcriptional factors, and the expression of immunoregulatory mediators are altered in sepsis. Recent data have shown stable patterns of activation among peripheral blood mononuclear cells and neutrophils in healthy human subjects. Although polymorphisms in Toll-like receptors play a contributory role in determining cellular activation, other factors are involved as well. Increased activation of the mitogen-activated protein kinase protein 38, Akt, and nuclear factor (NF)–kB in neutrophils and other cell populations obtained at early time points in the clinical course of sepsis-induced acute lung injury or after accidental trauma is associated with a more-severe clinical course, suggesting that a proinflammatory cellular phenotype contributes to organ system dysfunction in such settings. Identification of patients with cellular phenotypes characterized by increased activation of NF-kB, Akt, and protein 38, as well as discrete patterns of gene activation, may permit identification of patients with sepsis who are likely to have a worse clinical outcome, thereby permitting early institution of therapies that modulate deleterious signaling pathways before organ system dysfunction develops, reducing morbidity and improving survival.

Multiple intracellular signaling pathways involving kinases, transcriptional factors, and the expression of immunoregulatory mediators are altered in cell populations that contribute to organ system dysfunction and mortality in sepsis. Activation of such intracellular events is initiated by interaction of microbial products with Toll-like receptors (TLRs) and other receptors, including G protein–coupled receptors. In addition, circulating and locally released mediators of inflammation, including cytokines, complement fragments, and components of activated coagulation and fibrinolytic systems, that are generated in increased amounts during severe infection also interact with membrane-based receptors, leading to activation of intracellular pathways capable of further accelerating proinflammatory cascades. For example, the expression of cytokines such as TNF-α and IL-1β is increased in sepsis, and engagement of TNF-α with type I (p55) and type II (p75) TNF receptors or IL-1β with IL-1 receptors belonging to the TLR/IL-1 receptor family produces activation of kinases (including Src, p38, extracellular signal-regulated kinase, and phosphoinositide 3-kinase) and transcriptional factors (such as nuclear factor [NF]–kB) important for further up-regulation of inflammatory proteins [1–9]. Similarly, association of complement fragments, such as C5a, or fibrinolytic molecules, such as urokinase plasminogen activator, with their receptors has potent proinflammatory effects [10–13].

Circulating and organ-specific cell populations are activated to produce proinflammatory mediators during sepsis. Neutrophils and PBMCs bear TLR2 and TLR4, as well as other receptors, such as G protein–coupled receptor, that induce increased generation of cytokines and other immunoregulatory proteins, as well as enhance release of proinflammatory mediators, including reactive oxygen species, after engagement by microbial products from bacteria, fungi, and viruses [14–16]. Similar receptors and activation patterns are demonstrated in fixed tissue populations, such as macrophages and endothelial and epithelial cells [7, 17–20].

Genetic polymorphisms lead to alterations in TLR conformation that are accompanied by decreased cellular activation after exposure to bacterial products. In mice, mutations in tlr4, as present in the C3H/HeJ strain, are associated with resistance to challenge with endotoxin [19, 21, 22]. Approximately 6% of humans have amino acid substitutions in TLR4 at Asp299Gly.
Figure 1. Nuclear concentrations of nuclear factor (NF)-κB in peripheral blood mononuclear cells are higher in nonsurvivors (black bars) than survivors (white bars) at varying time points after diagnosis of severe sepsis. Adapted from Bohere et al. [48] with permission.

and Thr399Ile that render them hyporesponsive to inhaled lipopolysaccharide (LPS) [23–26]. These TLR4 polymorphisms also appeared to be associated with a predisposition to gram-negative bacteremia and septic shock but did not demonstrate any survival advantage or disadvantage in patients with sepsis [25, 27, 28]. However, such genetic alterations in TLR and other receptors appear to account for only a relatively small percentage of the variability demonstrated in humans when their cells are exposed to bacterial products.

Recent data have shown relatively stable patterns of cytokine production in PBMCs from some healthy humans after stimulation with LPS [29]. In that study, volunteers were identified who consistently demonstrated either decreased or increased release of TNF-α and other cytokines when their blood was cultured with LPS. We have found similar patterns of high or low response among neutrophils obtained from human subjects and stimulated with LPS, a TLR4 ligand, peptidoglycan, which interacts with TLR2, or high mobility group box protein–1 (HMGB1), a dual TLR2 and TLR4 ligand. Similarly, in gene array experiments, we have also demonstrated stable patterns of gene expression over time in neutrophils obtained from healthy volunteers.

Despite the stable variability in cellular activation that is present among the genetically heterogeneous human population, only a limited number of studies have examined how such patterns may correlate with clinical outcome in sepsis or other critical illnesses in which the risk of organ dysfunction due to severe infection is high. Examination of such issues is hampered, in part, by the difficulty in obtaining cells for analysis at early time points in a patient’s clinical course before pathophysiological events, such as hypotension or severe hypoxemia, intercede and provide clear indications of subsequent outcome. Nevertheless, a number of studies have examined the transcriptional factor NF-κB and kinases, including p38 and Akt, and provide insights into how heterogeneity in cell signaling may contribute to subsequent clinical course.

NF-κB

The transcriptional regulatory factor NF-κB is a central participant in modulating the expression of many immunoregulatory mediators involved in the acute inflammatory response [30–35]. NF-κB/rel transcription factors function as dimers held latent in the cytoplasm of cells by inhibitory IκB proteins [3, 6, 30, 31, 34–39]. There are 5 known mammalian NF-κB/Rel proteins: Rel (c-Rel), p65 (RelA), RelB, p50, and p52. Signaling pathways initiated by engagement of TLRs, such as TLR2 and TLR4, by microbial products and other inflammatory mediators lead to nuclear accumulation of NF-κB and enhanced transcription of genes responsible for the expression of cytokines, chemokines, adhesion molecules, and other mediators of the inflammatory response associated with infection. Association of NF-κB with the inhibitory protein IκB-α in the cytoplasm blocks the nuclear localization sequence of NF-κB, inhibiting its movement into the nucleus [40]. Exposure of cells to inflammatory stimuli, including LPS, peptidoglycan, and proinflammatory cytokines (such as TNF-α or IL-1β), results in phosphorylation of IκB-α on serines 32 and 36, leading to its subsequent ubiquitination and degradation by the 26S proteasome. Phosphorylation of IκB-α is mediated by the kinases IKKα and IKKβ, which are catalytically active components of the IκB kinase complex (IKK) [34]. Upstream kinases that participate in the phosphorylation and activation of IKK include those that are directly associated with TLRs, such as IL-1 receptor–associated kinase (IRAK)-1 and IRAK-4, as well as kinases that can be activated through either TLR or other receptors, including p38 and Akt [5, 6, 41–43]. Phosphorylation events, in addition to those involving IKKα/β and IκB-α, and
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Figure 2. Nuclear levels of nuclear factor (NF)-κB are significantly increased in neutrophils obtained within 24 h of initiation of mechanical ventilation in patients whose clinical course from sepsis-induced acute lung injury is more severe (as defined by death or ventilation for >14 days—that is, ≤14 ventilator-free days [VFD]), compared with patients with a less-severe course (as defined by mechanical ventilation for <14 days, or >14 VFD). Neutrophil activation, as shown by nuclear translocation of NF-κB in response to culture with lipopolysaccharide (LPS), was also significantly greater in patients who died or required mechanical ventilation for prolonged periods (≤14 VFD). Note that baseline nuclear concentrations of NF-κB were lower in healthy volunteers than in patients with sepsis-induced acute lung injury, regardless of subsequent clinical course, demonstrating baseline activation of NF-κB in association with sepsis. *P < .05, vs. volunteers. †P < .05, vs. >14 VFD. Adapted from Yang et al. [55] with permission.

Involving NF-κB subunits (such as p65) and nuclear coactivator proteins (such as TATA box binding protein or cAMP-responsive element–binding protein) are mediated by p38, Akt, and other kinases and play an important role in regulating the transcriptional activity of NF-κB [44–47]. IκB-α also is important in terminating NF-κB–mediated transcription through migrating into the nucleus and associating with NF-κB, with this interaction exposing a nuclear export signal, leading to movement of NF-κB from the nucleus back to the cytoplasm.

Studies have shown that greater nuclear accumulation of NF-κB in PBMCs is accompanied by higher mortality and worse clinical course in patients with sepsis (figure 1) [48, 49]. In general, these clinical series demonstrated that persistent activation of NF-κB was found in nonsurvivors, with surviving patients having lower nuclear concentrations of NF-κB at early time points in their septic course than did nonsurvivors as well as more rapid return of nuclear accumulation of NF-κB to levels found in control, unactivated PBMCs.

Although NF-κB heterodimers consisting of the p50:p65 subunits are capable of transactivating genes when bound to κB promoter sequences, p50:p50 homodimers appear to be inhibitory and capable of blocking transcription. Endotoxin tolerance was shown to be dependent on p50 expression, because cells from p50-deficient mice could not be made tolerant to LPS [50]. Given the inhibitory properties of p50:p50 homodimers, it is interesting that nuclear concentrations of this NF-κB homodimer are increased in PBMCs from nonsurvivors of sepsis relative to survivors [51]. Similarly, nonsurvivors demonstrated significantly decreased p65:p50/p50:p50 ratios in PBMCs, compared with those in survivors.

Although studies of patients with sepsis have generally shown that nuclear concentrations of NF-κB are higher in nonsurvivors than in survivors, an unresolved issue is whether such changes occur early and, therefore, define the subsequent course of sepsis or whether pathophysiological changes that result in poor clinical outcome also produce NF-κB activation as a secondary event, so that such changes in NF-κB are simply associated with more severe organ system dysfunction but do not contribute directly to outcome. In preclinical models of sepsis, blockade of NF-κB improves survival, indicating that NF-κB activation does contribute to cellular and organ dysfunction [36, 52, 53]. A study of surgical patients without sepsis supports the hypothesis that neutrophil phenotypes defined by NF-κB activation patterns predict clinical outcome [54]. In that clinical series of patients undergoing repair of aortic aneurysms, higher preoperative levels of NF-κB in peripheral neutrophils were...
Patients with a more-severe clinical course from sepsis-induced acute lung injury (as defined by death or mechanical ventilation for >14 days—that is, ≤14 ventilator-free days [VFD]) show greater activation of Akt in peripheral neutrophils stimulated with lipopolysaccharide (LPS) than do patients with a less-severe course (as defined by ventilation for <14 days, or >14 VFD). Neutrophils were collected within 24 h of initiation of mechanical ventilation. At this point, there were no differences in oxygenation or other physiological parameters that differentiated patients whose subsequent clinical courses were more or less severe. Of note, phosphorylated Akt (p-Akt) levels were higher in unstimulated neutrophils from patients with acute lung injury than in cells from healthy volunteers, regardless of their subsequent clinical course, demonstrating activation of Akt in such patients. However, the ability to further induce Akt phosphorylation after exposure to LPS differentiated patients with a more-severe clinical course from those whose subsequent clinical course was less severe. Such findings indicate that the presence of neutrophils with a greater proinflammatory phenotype appears to be an early indicator of worse clinical outcome in sepsis-induced acute lung injury. *P < .05, vs. volunteers. †P < .05, vs. >14 VFD. t-Akt, total Akt. Adapted from Yang et al. [55] with permission.

Recent data from our laboratory support the contention that early alterations in NF-κB contribute to the severity of organ failure and clinical outcome [55]. In that study, peripheral blood neutrophils were collected within the first 24 h of intubation from patients with sepsis-induced acute lung injury. Increased nuclear levels of NF-κB in unstimulated neutrophils were associated with a worse clinical outcome, as defined by death or mechanical ventilation for >14 days, compared with surviving patients who required mechanical ventilation for ≤14 days (figure 2). Of note, at the time neutrophils were collected, the severity of pulmonary dysfunction, as defined by the PaO2/FiO2 ratio, as well as the overall severity of illness, as defined by APACHE II scores, was similar in the patients who subsequently had a more or less severe clinical course. In addition, neutrophils from patients whose subsequent clinical course was more severe showed increased nuclear translocation of NF-κB after being cultured with LPS. Because neutrophils play an important role in the development and severity of acute lung injury, particularly at early time points [56, 57], such data suggest that early alterations in NF-κB activation contribute to outcome in sepsis. This clinical information, coupled with the above-mentioned studies that showed stable high and low responder phenotypes in the healthy population, implies that the presence of a preexistent high responder neutrophil phenotype, as characterized by increased nuclear translocation of NF-κB after stimulation with TLR2 or TLR4 ligands, would be associated with more severe pulmonary inflammatory response and clinical course in response to infection. Conversely, persons whose neutrophils have diminished activation of NF-κB after stimulation would be expected to have less-intense neutrophil-driven inflammation, as well as organ dysfunction.

**KINASE ACTIVATION AND OUTCOME FROM SEPSIS**

Although activation of the p38 and phosphoinositide 3–kinase/Akt kinase pathways has been demonstrated to contribute to pulmonary neutrophil accumulation and the development of organ dysfunction in preclinical models of sepsis [58–60], there is only limited information suggesting that these kinases con-
tribute to outcome in patients with overwhelming infection. As was the case for NF-κB, we have found that neutrophils from healthy volunteers demonstrate stable high and low responder phenotypes for phosphorylation of p38 and Akt after culture with LPS or HMGB1. Of note, persons who demonstrate consistently increased activation of p38 after LPS exposure are not necessarily the same as those who show enhanced phosphorylation of Akt.

In a study by Rosengart et al. [61] of uninfected trauma patients, increased progression to multiple organ dysfunction was found in the group with high baseline levels of p38 activation among cells obtained by bronchoalveolar lavage. Of note, there were no differences in terms of demographic characteristics, hypotension, injury severity score, number of blood transfusions, or severity of illness scores at the time samples were collected among patients with low or high baseline p38 activation patterns. Such data suggest that enhanced cellular activation, as determined by p38, at early time points after injury not only identifies patients who are likely to have a more severe subsequent clinical course but also may contribute to the development of organ system dysfunction.

To examine further the relationship between neutrophil phenotypes and subsequent clinical course in sepsis, we obtained peripheral blood neutrophils from patients with sepsis-induced acute lung injury within 24 h of the initiation of mechanical ventilation and then determined baseline phosphorylation of p38 and Akt, as well as the increase in levels of phosphorylated p38 and Akt, after culture of the neutrophils with LPS for 1 h [55]. Patients whose subsequent clinical course was characterized by either death or mechanical ventilation for >14 days showed significantly greater activation of Akt, but not p38, after LPS-induced neutrophil stimulation than did the patients who required mechanical ventilation for ≤14 days (figure 3). Of note, at the time the neutrophils were isolated, there were no differences in terms of demographics, severity of illness, or oxygenation abnormalities in the 2 groups of patients. Such results, as well as those described by Rosengart et al. [61] among trauma patients, suggest that persons whose neutrophils have a proinflammatory phenotype, as defined by increased activation of kinases, are more likely to have severe clinical course associated with their critical illness. Because the cellular alterations precede many of the organ system dysfunctions that contribute to mortality in this setting, these studies also suggest that identification and correction of alterations in cellular signaling pathways at early time points in a patient’s clinical course may improve outcome.

CONCLUSIONS

Modulation of intracellular signaling cascades involving kinases, such as p38 or Akt, or transcriptional factors, such as NF-κB, through specific inhibitory approaches has shown their pathophysiological importance in experimental models. However, the role of specific intracellular pathways in contributing to clinical outcomes in patients with sepsis remains incompletely determined, primarily because such alterations in cellular activation patterns have not been examined at early time points before the onset of multiple organ dysfunction. Recent information shows that alterations in p38, Akt, and NF-κB among neutrophils and other cell populations not only precede the development of organ system dysfunction but also has predictive value in identifying patients with a more severe subsequent clinical course. Such results suggest that stable responder phenotypes that are independent of TLR2 or TLR4 polymorphisms are present among humans and may correlate with clinical outcome in the setting of sepsis. Identification of patients with proinflammatory cellular phenotypes at early time points may permit institution of therapies that interrupt detrimental signaling cascades before organ system dysfunction develops, reducing morbidity and improving survival.

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