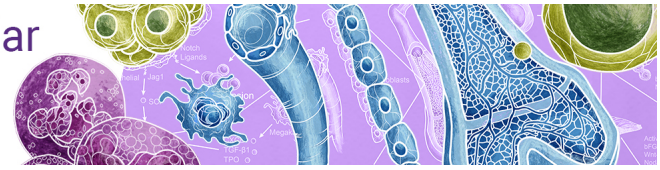

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CUTTING EDGE

Cutting Edge: An Endogenous Pathway to Systemic Inflammatory Response Syndrome (SIRS)-Like Reactions through Toll-Like Receptor 4[1]

Geoffrey B. Johnson, Gregory J. Brunn,† and Jeffrey L. Platt[2]*†‡§*

***Systemic inflammatory response syndrome (SIRS) is typically associated with trauma, surgery, or acute pancreatitis. SIRS resembles sepsis, triggered by exogenous macromolecules such as LPS acting on Toll-like receptors. What triggers SIRS in the absence of infection, however, is unknown. In this study, we report that a SIRS-like response can be induced in mice by administration of soluble heparan sulfate, a glycosaminoglycan associated with nucleated cells and extracellular matrices, and by elastase, which cleaves and releases heparan sulfate proteoglycans. The ability of heparan sulfate and elastase to induce SIRS depends on functional Toll-like receptor 4, because mutant mice lacking that receptor or its function do not respond. These results provide a molecular explanation for the initiation of SIRS. The Journal of Immunology,* 2004, 172: 20–24.**

Systemic inflammatory response syndrome (SIRS)[3] is typically seen in individuals who have suffered blunt trauma, aseptic burns, cancer, widespread surgical manipulation, or acute pancreatitis (1). The molecular events that initiate SIRS are unknown; however, the clinical and pathological features that follow mimic those of sepsis, hence the term "sepsis-like syndrome" is sometimes applied to this condition (2). Although some conditions in which SIRS is seen, such as multiorgan traumas, might lead to infection and thus to sepsis, efforts to identify infecting organisms in many patients with SIRS fail (3, 4). The absence of infection in such a large population of patients with SIRS has spurred calls to identify endogenous mediators of the syndrome (5).

The clinical manifestations of sepsis are typically observed with blood-born infection with Gram-negative bacteria and are mimicked by administration of LPS, the active component of endotoxin (6, 7). LPS activates systemic inflammatory responses through Toll-like receptor (TLR)4 (6). Stimulation of TLRs leads to secretion of "early response" cytokines, such as TNF-α, and these in turn are thought to cause the systemic changes mentioned above (6). Although TLR4 is critical for responses to LPS, LPS may not be the sole agonist for the clinical syndrome associated with sepsis (3). Nevertheless, individuals with SIRS are generally treated with antimicrobial agents, even in the demonstrated absence of infection (8).

We hypothesized that endogenous substances, particularly the degradation products of proteoglycans, might stimulate TLR4 in lieu of LPS. This hypothesis follows from the observations of Termeer et al. (9) who found that fragments of hyaluronate, a glycosaminoglycan associated with extracellular matrices and synovial fluids, stimulate TLR4. The hypothesis also follows from our own finding that fragments of heparan sulfate, a negatively charged glycosaminoglycan associated with extracellular matrices and cell surfaces, also stimulate TLR4 (10). Given the stimulatory ability of the fragments of proteoglycans, we reasoned that enzymes, such as elastase, commonly found in extracellular fluids after trauma and acute pancreatitis (11, 12), might release heparan sulfate from cellular attachments and thus initiate SIRS in the absence of pathogens. Consistent with this concept, we here report that soluble heparan sulfate and elastase (both free of contamination) trigger SIRS via TLR4. We chose to use the term "SIRS" for the SIRS-like syndrome in mice, although the strict definition of SIRS as applied in humans cannot be applied in animals. Our findings provide a molecular mechanism for the initiation of SIRS and cast new light on the functions of mammalian TLRs.

Materials and Methods

Reagents and Abs

Unconjugated monoclonal HepSS-1 (anti-heparan sulfate) was from US Biological (Swampscott, MA). Limulus anti-LPS factor (LALF) was from Associates of Cape Cod (Woods Hole, MA). CpG sequence ODN1826 (13) phosphorothioate-modified single-stranded oligonucleotide was synthesized, then quantitated spectrophotometrically. Bovine kidney-derived heparan sulfate (super special grade), and chondroitin sulfate B were purchased from Seikagaku (Falmouth, MA). *Escherichia coli*-derived LPS B4:0111, D-galactosamine, type IV porcine pancreatic elastase, zymosan A, and amebocyte lysate from *Limulus polyphemus* were from Sigma-Aldrich (St. Louis, MO). Elastase inhibitor-1 was obtained from Calbiochem (La Jolla, CA). Pharmaceutical grade heparin was from Elkins-Sinn (Cherry Hill, NJ).

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[3] Abbreviations used in this paper: SIRS, systemic inflammatory response syndrome; TLR, Toll-like receptor; LALF, *Limulus* anti-LPS factor; PAEC, porcine aortic endothelial cell.

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Animals

Mice used in this study included TLR4-deficient C57BL/10ScNCr (National Cancer Institute, Bethesda, MD), TLR4-mutant C3H/HeJ, and their TLR4 wild-type control strains C57BL/10SnJ and C3H/HeSnJ, respectively (The Jackson Laboratory, Bar Harbor, ME). Mayo's internal Institutional Animal Care and Use Committee approved all animal procedures.

Enzyme purification

Enzymes were repurified before use by passage over a polymyxin B cross-linked 6% agarose column (Pierce Biotechnology, Rockford, IL). Fractions of each enzyme preparation were boiled and tested to have only trace LPS contamination (<1% of limiting concentration needed to evoke responses) by *Limulus* amebocyte lysate assay gel clot method (Seikagaku, Falmouth, MA). Human platelet heparanase was purified as previously described (14), then dialyzed into PBS to a final concentration of 3.4 mg/ml and stored at −80°C until used. Heparanase activity was measured as previously described (14) to be 0.19 μg of heparan sulfate released per microgram of heparanase per hour.

Cell isolation and culture

Dendritic cells were generated from murine bone marrow cultures as previously described (15). At days 6 or 7 of culture, nonadherent cells and loosely adherent proliferating dendritic cell aggregates were harvested for analysis or stimulation. Primary porcine aortic endothelial cells (PAEC) were cultured to confluence and their identity was confirmed as previously described (16).

Cell culture stimulation

Dendritic cells (2×10^6 per ml) cocultured with confluent monolayers of PAECs in 96-well plates were stimulated with 10 μg/ml chondroitin sulfate, 500 ng/ml CpG DNA, 10 ng/ml LPS, 10 μg/ml heparan sulfate, 50 μg/ml zymosan or PBS, unless otherwise indicated. To control for purity, agonists were pretreated with END-X B15, or mixed with *Limulus* anti-LPS factor before stimulation of cells, as indicated. In some experiments, agonists were boiled before use for 60 min at 100°C as indicated.

Cytokine quantification

Age-matched female mice were injected in the peritoneum with 0.5 U of elastase, 5 mg of heparan sulfate, 200 U of heparin, 150 μg of CpG DNA or PBS with a total volume of 250 μl. One and 3 h after injection, 100-μl blood samples were collected from the tail vein. Cell supernatants and serum samples were immediately frozen at −20°C until analyzed. Concentrations of TNF-α were analyzed by enzyme-linked sandwich ELISA (R&D Systems, Minneapolis, MN).

Immunopathology

After i.p. injection of ketamine and xylazine, murine spleens were directly visualized through an incision in the lateral abdominal wall and injected with 100 μl of PBS containing 0.1 U of elastase or PBS alone. Five hours later, the spleen was harvested and pieces snap-frozen. Tissue sections were prepared and stained as previously described (17) with the following modifications. Secondary and tertiary Abs were mouse serum (Jackson ImmunoResearch Laboratories, West Grove, PA) preabsorbed and diluted in M.O.M. diluent (Vector Laboratories, Burlingame, CA). Fluorescent images were converted to grayscale using SPOT software (Diagnostic Instruments, Sterling Heights, MI).

Experimental shock model

Age- and sex-matched mice were injected with 5 mg of heparan sulfate, 5 μg of LPS, 5 μg of *Limulus* anti-LPS factor, 5 mg of chondroitin sulfate, 200 U (~5 mg) heparin, 150 μg of CpG DNA, 1.5 U of elastase, or PBS mixed with 20 mg of D-galactosamine in a total volume of 500 μl of PBS by i.p. injection as previously described (18). Concentrations of heparan sulfate and elastase were calculated by weight of lyophilized powder, and the doses used were near the LD_{50}, based on dose-response experiments. In some experiments, the agonist was mixed with 5 or 20 μg of *Limulus* anti-LPS factor before injection. For some injections, enzymes were boiled at 100°C for 60 min and vortexed vigorously, or preincubated with elastase inhibitor-1 for 4 h at room temperature, and then mixed with D-galactosamine before injection. Mice were monitored every hour for 48 h, and then euthanized.

Results

Soluble heparan sulfate induced responses in TLR4 wild-type and mutant mice

During sepsis, Gram-negative bacteria shed LPS, which activates TLR4 on cells that then release inflammatory cytokines mediating systemic inflammation and death (19). To address the question of whether soluble heparan sulfate can induce SIRS via activation of TLR4, we administered heparan sulfate by i.p. injection to TLR4 wild-type and mutant mice in a model system previously used to study shock and the sepsis-like syndrome in response to microbial toxins (20). Eighty percent of TLR4 wild-type mice injected with heparan sulfate died, whereas no TLR4-mutant mice injected with heparan sulfate died (Fig. 1). As expected, wild-type mice injected with LPS died (Fig. 1). The TLR4-mutant mice were capable of undergoing SIRS as the condition could be induced in these mice by administration of CpG DNA, a TLR9 agonist (21) (Fig. 1). The response is specific for heparan sulfate, because mice injected with heparin which is structurally related to heparan sulfate (22) but does not stimulate TLR4 (10), survived, and all but one mouse injected with chondroitin sulfate, which has the same charge density as heparan sulfate (23), also survived (Fig. 1).

It would be reasonable to ask whether the responses we observed represented contamination of heparan sulfate by LPS. However, the heparan sulfate used was not contaminated with LPS (10), and *Limulus* anti-LPS factor had no impact on the ability of heparan sulfate, but greatly diminished the ability of LPS, to induce death in treated mice (Fig. 1).

Elastase-induced responses in TLR4 wild-type and mutant mice

We next asked whether endogenous stores of heparan sulfate could trigger SIRS. To address this question, we injected pancreatic elastase into the peritoneal cavity of mice. Elastase cleaves heparan sulfate from cell surfaces and extracellular matrixes in vitro (24, 25), thus liberating endogenous heparan sulfate. Fifty percent of wild-type mice injected with elastase died, whereas no TLR4 mutant mice died, suggesting that injection of elastase leads to activation of TLR4 and thus to death (Table I). The elastase solution was not contaminated with LPS as it

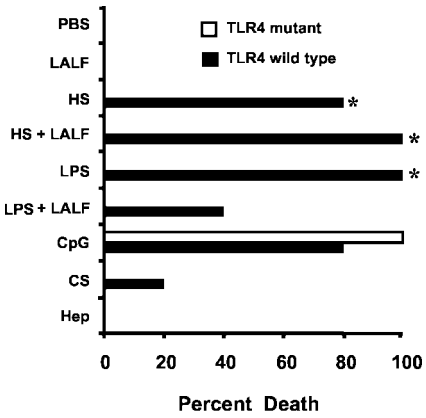

FIGURE 1. Induction of systemic inflammatory response by heparan sulfate via TLR4. C57BL/10SnJ (TLR4 wild type) or C57BL/10HeJ (TLR4 nonsignaling mutant) were injected with D-galactosamine plus TLR4 agonists (heparan sulfate (HS) or LPS), or the TLR9 agonist CpG DNA. Control injections included D-galactosamine plus the PBS vehicle, chondroitin sulfate (CS), heparin (Hep) or LALF, a protein that specifically binds to and neutralizes LPS but not HS. Each group included four or five animals and when no deaths occurred, no bar is indicated on the graph. Results are representative of two experiments. *, $p < 0.05$ using Fisher's exact test when compared with TLR4-negative controls. These data illustrate that the systemic inflammatory response and its sequelae may be initiated by an endogenous TLR4 ligand such as soluble heparan sulfate.

Table I. *Percent death after injection of elastase in SIRS shock model*

	TLR4 Mutant (%)	TLR4 Wild Type (%)
Elastase	0	50
Boiled Elastase	0	0

was passed through a polymyxin B column and confirmed to contain <1% of a limiting dose of LPS by *Limulus* amebocyte lysate assay. Boiling elastase, which does not inactivate LPS but denatures elastase, eradicated the response (Table I). Moreover, elastase inhibitor-1, a specific inhibitor of pancreatic elastase, reduced the death rate by 50%, similar to boiling elastase. These results indicate that the enzymatic activity of elastase is required for activation of TLR4.

TNF-α secretion in response to enzymatic degradation of heparan sulfate proteoglycan

We next asked whether specific shedding of heparan sulfate induced by the action of heparanase, an endoglycosidase that specifically cleaves heparan sulfate (26), would also trigger TLR4 activation. The studies were conducted in vitro because heparanase is tightly regulated by pH (14) and because complete depolymerization of heparan sulfate abolishes agonist activity (15). To address this question, we added heparanase purified from human platelets (27) to 24-h-old cocultures of PAECs and murine APCs that were TLR4-positive or -negative, and then assayed TNF-α. The heparanase was passed over polymyxin B columns, and confirmed by *Limulus* amebocyte lysate assay to lack LPS (data not shown). Aortic endothelial cells express an abundance of heparan sulfate proteoglycans (28) that are released into solution by elastase (24) and heparanase (29). In response to soluble heparan sulfate, elastase, or heparanase, the APC secreted TNF-α in a TLR4-dependent manner and responded to control stimulants as expected (Fig. 2).

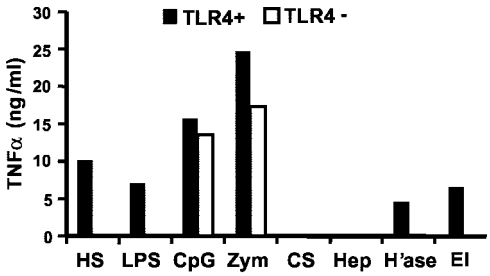

FIGURE 2. Mobilization of extracellular matrix components by elastase induces release of TNF-α by dendritic cells. Immature dendritic cells cultured from C57BL/10ScNCr (TLR4$^-$) or C57BL/10SnJ (TLR4 wild type) female mice were added to 96-well plates containing confluent cultures of PAECs. The cocultures were stimulated for 24 h with TLR4 agonists or control buffer, or with the TLR2 agonist zymosan (Zym) or the TLR9 agonist CpG DNA. Alternatively, the cocultures were treated with the extracellular matrix-mobilizing enzymes heparanase (H'ase) or elastase (El). All conditions were tested in triplicate wells and the mean concentrations of TNF-α from the supernatant, as measured by ELISA, are shown. TNF-α concentrations below assay detection limits are set at 0 and no bar is indicated on the graph. These data are representative of two experiments and support the idea that degradation of extracellular matrix by elastase or heparanase may initiate the systemic inflammatory response in part by stimulating a TLR4-dependent increase in TNF-α expression.

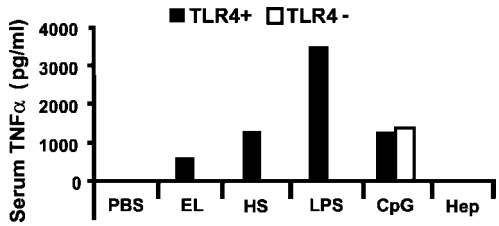

FIGURE 3. Serum TNF-α concentration in wild-type or TLR4-deficient mice. C57BL/10SnJ (TLR4$^+$) and C57BL/10ScNCr (TLR4$^-$) mice were injected with elastase (El), heparan sulfate (HS), LPS, CpG DNA, heparin (Hep), or the PBS vehicle only. Mean serum TNF-α concentrations 1 h following treatment are shown. TNF-α concentrations were measured in triplicate and the results shown are representative of two experiments. Serum TNF-α levels below the sensitivity of the assay were assigned a value of 0, and no bar is indicated on the graph.

Effects of heparan sulfate and elastase on serum TNF-α levels in TLR4 wild-type and mutant mice

Because TNF-α is thought to be one of the key mediators of the systemic inflammation in sepsis and SIRS (30), we next asked whether heparan sulfate or enzymes that release heparan sulfate lead to high serum levels of TNF-α via TLR4, typically seen in these conditions. To address this question, we administered heparan sulfate, elastase, or PBS to wild-type and mutant mice and measured serum TNF-α 1 and 3 h later. Wild-type, but not TLR4-mutant, mice treated with heparan sulfate or elastase had high serum levels of TNF-α 1 h after treatment (Fig. 3). As expected, TNF-α was not detectable by 3 h (data not shown). TLR4-mutant mice responded to the TLR9 agonist CpG DNA, used as a positive control, by producing TNF-α (Fig. 3).

Effects of elastase on endogenous heparan sulfate proteoglycan in vivo

To determine whether elastase liberates heparan sulfate in vivo, we harvested spleen tissues from mice that had been injected intrasplenically with the enzyme and tested the tissues for the presence of heparan sulfate. Mice injected with pancreatic elastase lost heparan sulfate from blood vessels at the injection site within 5 h of injection (Fig. 4). These data show that pancreatic elastase can induce loss of heparan sulfate proteoglycan from tissues in vivo.

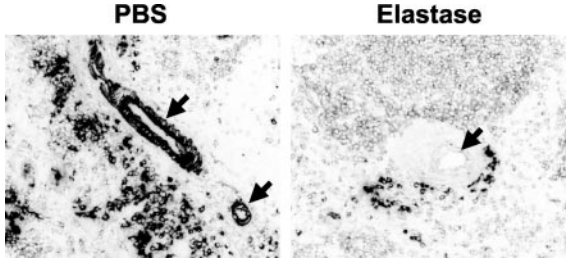

FIGURE 4. Elastase mobilizes local stores of heparan sulfate proteoglycan in vivo. Elastase or PBS was injected into the spleen of mice and heparan sulfate was then localized 5 h later by immunopathology. Arrows indicate blood vessels. Images shown are representative of tissue sections of two independent experiments. Anti-mouse IgM Abs labeled B cells directly, but not heparan sulfate proteoglycans in blood vessels. This result supports a model by which the systemic inflammatory response syndrome may be initiated in a response to mobilization of an endogenous ligand, such as heparan sulfate proteoglycan, by elastase.

Discussion

In this study, we show that SIRS can be induced by signaling through TLR4, a member of an ancient receptor system known to detect molecules from microorganisms (19), but in SIRS the agonists may include the breakdown products of heparan sulfate proteoglycan. Heparan sulfate proteoglycan, a component of all cell surfaces and extracellular matrices, is degraded by proteases in immune, inflammatory, and traumatic conditions (31). In this study, we show that rapid enzymatic degradation of heparan sulfate activates TLR4, and when this activation reaches a threshold, the pathophysiology of the syndrome of sepsis and ensuing death can occur.

One observation that supports the specific link between SIRS and heparan sulfate is our finding that platelet heparanase, an enzyme that is highly specific for cleaving heparan sulfate polysaccharide chains (26), can generate fragments of heparan sulfate capable of acting on TLR4. Despite these results, we could not induce the sepsis syndrome by administration of heparanase (data not shown). The failure of systemically administered heparanase to induce SIRS may reflect the stringent control of heparanase activity by pH (14). Thus the function of platelet heparanase requires a local acidic environment (14) (as might be found in the conditions commonly associated with SIRS); however, systemic acidification is lethal to mice (data not shown). Whether under some conditions heparanase might induce systemic inflammation remains to be seen.

A variety of endogenous substances, including fibrinogen (32), fibronectin extra domain A (33), hyaluronic acid (9), and perhaps heat shock proteins (34) can trigger TLR4 and thus might be considered potential mediators of the SIRS. We have focused on degraded heparan sulfate proteoglycan as a potential trigger of sepsis syndrome, because, among these substances, only heparan sulfate proteoglycan is degraded by elastase into a soluble form that might activate TLR4. However, the release of elastase certainly must cleave other proteins, which might potentially interact with TLR4.

In addition to other endogenous agonists, disruption of the integrity of the gut during stress may allow LPS-laden bacteria to enter the serum and give rise to SIRS in the absence of "true" infection (35). However, this scenario does not explain most cases of SIRS (35). In our experiments, intestinal blood vessels retained their integrity, and fully expressed heparan sulfate in the presence of injected enzymes (data not shown), suggesting that the injected elastase is not active at that site. In addition, SIRS in response to elastase cannot be ascribed to loss of intestinal barrier because LALF did not reduce the responses to injected elastase (data not shown).

The finding that the SIRS induced by heparan sulfate can be recapitulated by administration of elastase explains the origin of the syndrome in pancreatitis and certain inflammatory conditions. Both pancreatitis and systemic inflammation lead to release of elastase into the blood and tissues (36, 37). In murine models, pancreatic and neutrophil elastase induce the systemic inflammatory effects and respiratory distress syndrome seen in acute pancreatitis (38, 39). In rat models, elastase inhibitors reduce the severity of symptoms associated with acute pancreatitis (40, 41). In humans, serum elastase is a prognostic indicator for the severity of multiple organ failure as a result of acute pancreatitis (12). Our results thus suggest the possibility that blockade of elastase or of heparan sulfate might improve the outcome of SIRS.

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