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van Zoelen, M.A.D.

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S100A12 and soluble RAGE levels during severe sepsis

Marieke A.D. van Zoelen¹ ², Dirk Foell³, Thomas Vogl³, Pierre-François Laterre⁴, Xavier Wittebole⁴, Michael W. Tanck⁵, Johannes Roth³ and Tom van der Poll¹ ²

Submitted

¹Center for Infection and Immunity Amsterdam (CINIMA), ²Center for Experimental and Molecular Medicine and ³Department of Clinical Epidemiology, Biostatistics and Bioinformatics; Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
⁴Institute of Immunology, University of Muenster, Muenster, Germany
⁵Department of Critical Care Medicine, St. Luc University Hospital, Université Catholique de Louvain, Brussels, Belgium
**Abstract**

**Objective**

S100A12 is highly expressed and serum levels correlate with individual disease activity in patients with inflammatory diseases. We here sought to determine the extent of S100A12 and its soluble (s) high-affinity receptor sRAGE in patients with severe sepsis stratified to the three most common infectious sources and in healthy human volunteers intravenously challenged with endotoxin.

**Design**

Observational studies in patients and healthy humans challenged with lipopolysaccharide.

**Setting**

Two intensive care and one clinical research units.

**Patients and participants**

51 patients with sepsis due to pneumonia (29), peritonitis (12) or urinary tract infection (10). In addition, 8 healthy humans were studied after intravenous injection of lipopolysaccharide (4 ng/kg).

**Interventions**

One population of healthy volunteers received lipopolysaccharide intravenously.

**Measurements and results**

Patients with severe sepsis displayed increased circulating S100A12 concentrations at day 0 and 3. All severe sepsis subgroups (sepsis due to pneumonia, peritonitis and urinary tract infection) had elevated serum S100A12 at both time points. Remarkably, patients with sepsis due to pneumonia had the highest S100A12 levels. S100A12 levels did not differ between survivors and non-survivors and were not correlated to disease severity. Intravenous lipopolysaccharide injection in healthy humans elevated systemic S100A12 levels. In contrast to S100A12, sRAGE concentrations did not change during severe sepsis or human endotoxemia.

**Conclusions**

In severe sepsis, S100A12 is released systemically irrespective of the primary source of infection. Human endotoxemia induces S100A12 as well. Concentrations of its high-affinity soluble receptor sRAGE do not change during sepsis or human endotoxemia.
INTRODUCTION

Sepsis is a common and serious problem in clinical medicine today. Although research continues on the treatment of sepsis, the understanding of the septic process remains far from complete. In sepsis, the lung is the primary source of infection (35-54%), followed by the abdomen (20-28%) and the urinary tract (8-13%) (1-3).

S100A12 is a calcium binding protein expressed in the cytoplasm of neutrophils (4), where it comprises 5% of the total protein content (5). Besides in neutrophils, S100A12 is found in monocytes (5) and lymphocytes (6). S100A12 was the first S100 protein for which a receptor was described. First, it was suggested to bind to the V-domain of the receptor for advanced glycation end products (RAGE) (7, 8); another study reported specific binding to the C1 domain of RAGE (9). Blocking the interaction between S100A12 and RAGE led to an improvement of inflammation in multiple experimental models (7, 10, 11). Interestingly, S100A12 has been suggested as a biomarker of neutrophil activation in inflammatory diseases, including sepsis and pulmonary infections (12, 13).

RAGE is a multiligand receptor of the immunoglobulin superfamily that is expressed at a high level in the lungs and at a lower level in normal adult tissues. The interaction of S100A12 and other ligands with RAGE leads to activation of pro-inflammatory signaling cascades. RAGE deficiency improved survival in a murine model of abdominal polymicrobial sepsis induced by cecal ligation and puncture (11, 14). Soluble RAGE (sRAGE) is a truncated form of the full length cell surface receptor and is composed of the extracellular ligand-binding domain (V-C-C’) only, lacking the cytosolic and transmembrane domains (i.e. the parts that transfer a signal into the cell). sRAGE can compete with full length cell-surface RAGE for ligand binding, preventing these ligands to bind to their receptors (including RAGE) and/or to exert effects otherwise; sRAGE administration decreased inflammatory responses in several animal models, including models of hepatic injury (15-17), diabetic atherosclerosis (18, 19), delayed type hypersensitivity, (11, 20), type II collagen-induced arthritis (20) and experimental autoimmune encephalomyelitis (21).

S100A12 - a high-affinity ligand for RAGE - is one possible candidate to be targeted by sRAGE in these diseases and inhibition of S100A12 might be partially responsible for the observed effects of sRAGE. Of note, although many RAGE ligands are promiscuous with regard to receptor use, S100A12 has only been shown to bind to RAGE.

In this study, we sought to determine the extent of systemic S100A12 and sRAGE levels in patients with severe sepsis stratified to the three most common primary sources of infection (lungs, abdomen and urinary tract) and in human endotoxemia.
Chapter 12

**Materials and methods**

**Patients and design**
All studies were approved by the scientific and ethics committees of the Academic Medical Center (Amsterdam, the Netherlands), the St. Luke University Hospital (Brussels, Belgium) and the St. Pierre’s Hospital (Ottignies, Belgium). Written informed consent was obtained from all subjects or their relatives. The study included 51 patients with severe sepsis, defined as a known or suspected infection plus a systemic inflammatory response syndrome and failure of at least one organ (22). These patients were identical to those in a previous report (23); they were admitted to the intensive care unit of either St. Luke University Hospital or St. Pierre’s Hospital at the day the diagnosis of severe sepsis was made and serum was obtained on inclusion (“day 0”) and at 3 days thereafter (“day 3”). 31 healthy subjects served as controls. In addition, eight healthy males (22.6 ± 0.6 years) were studied after intravenous injection of lipopolysaccharide (from *Escherichia coli*, lot G, United States Pharmacopoeial Convention, Rockville, MD) at a dose of 4 ng/kg. In these subjects EDTA anticoagulated blood was obtained before and 0, 3, 6, 8 and 24 hours (hrs) after challenge.

**Measurements and assays**
Data were collected prospectively from patient records, patient data management system (at the intensive care unit) and hospital information system. The following variables were collected when appropriate: date of birth, gender, APACHE-II and sepsis-related organ failure assessment (SOFA) scores, presence of septic shock, of organ dysfunction (both defined according to the consensus published in Crit Care Med 1992 (24)), length of intensive care stay and of hospital stay, blood culture results, date of death. S100A12 concentrations were analyzed by an enzyme-linked immunosorbent assay as described before (25, 26). sRAGE levels were determined using a commercially available enzyme-linked immunosorbent assay kit (Quantikine, R&D systems, Minneapolis, Minn.) according to the manufacturer’s protocol. Briefly, a monoclonal antibody against sRAGE was used to capture sRAGE. Captured sRAGE was detected with a polyclonal antihuman sRAGE antibody. After washing, plates were incubated with streptavidin-HRP, developed with appropriate substrate, and OD$_{450}$ was determined using an enzyme-linked immunosorbent assay plate reader.

**Statistical analysis**
Data are presented as mean ± SEM. Differences between sepsis groups (and healthy controls) were performed with non-parametric repeated measures analysis of variances of Kruskal-Wallis. Differences in time after intravenous lipopolysaccharide in healthy volunteers were compared using non-parametric repeated measures of variances.
Differences between time points within groups were compared using the Wilcoxon signed-rank test. S100A12 levels between survivors and non-survivors were compared with a Mann-Whitney U test. Correlations were calculated using Spearman’s rho test. Values of $p < .05$ were considered to represent a statistically significant difference.

**Results**

**Severe sepsis results in elevated systemic S100A12 levels irrespective of the source of infection, while sRAGE concentrations do not change**

In total 51 patients were included with an overall in–hospital mortality of 45%. These patients were described in detail previously (23) (and chapter 11). The primary sources of infection were the lungs in 29 patients (52%), the abdomen in 12 patients (24%) and the urinary tract in 10 patients (20%). Sepsis patients showed increased circulating S100A12 levels both at day 0 and day 3 (both $p < .0001$, Figure 1A). All severe sepsis subgroups, with peritonitis, pneumonia or urinary tract infection as primary infection, displayed elevated serum S100A12 concentrations at day 0 and day 3 (all $p < .05$, Figure 1B-D). Remarkably, patients with sepsis due to pneumonia had the highest S100A12 serum levels. There was no apparent correlation between serum S100A12 concentrations and the severity of disease: serum S100A12 did not correlate with either APACHE II scores ($r = -.185$, $p = .19$) or SOFA scores ($r = -.194$, $p = .17$). In addition, serum S100A12 levels did not differ between survivors and non-survivors ($706.4 \pm 116$ ng/ml and $495.9 \pm 82.0$ ng/ml, respectively, $p = .24$).

S100A12 is a high-affinity ligand for (s)RAGE (9, 27). We next measured sRAGE in these severe sepsis patients. Relative to healthy controls, neither the total sepsis population nor any of the subgroups with different infectious sources demonstrated changes in sRAGE concentrations at day 0 or day 3 (Figure 2A-D). Together, these data suggest that sepsis results in a sustained elevation of serum S100A12 concentrations irrespective of the source of the infection and that this response is not related to the severity of disease.

**Human endotoxemia is associated with systemic S100A12 release but does not influence circulating sRAGE levels**

To investigate whether intravenous lipopolysaccharide induces S100A12 release in humans *in vivo*, peripheral blood was obtained from healthy volunteers intravenously injected with endotoxin and S100A12 was measured before and 3, 6, 8, 12 and 24 hrs after lipopolysaccharide. Infusion of lipopolysaccharide was associated with a rapid increase in S100A12 concentrations (Figure 3A), reaching peak levels after 3 hrs. At 24 hrs, S100A12 concentrations had returned to baselines levels (Figure 3A). To determine whether human endotoxemia affects systemic sRAGE concentrations, we measured sRAGE levels in concurrently obtained samples. Unlike S100A12, sRAGE levels were not influenced by intravenous lipopolysaccharide injection (Figure 3B).
Discussion

Sepsis remains a life threatening condition associated with bacterial systemic dissemination and systemic inflammation. With a mortality rate of 20-50% sepsis remains a major challenge for clinicians. S100A12 is highly expressed at sites of local inflammation and serum concentrations correlate with individual disease activity in patients with inflammatory diseases (25, 28). We here sought to determine the extent of S100A12 release and its (soluble) receptor sRAGE in patients with severe sepsis. The main finding of our study is that patients with severe sepsis and healthy humans intravenously injected with lipopolysaccharide have elevated circulating S100A12 levels, while sRAGE levels do not change. In sepsis patients, the increase of S100A12 concentrations was irrespective of the source of infection.
S100A12 in severe sepsis

Figure 2. Systemic sRAGE levels in patients with severe sepsis. Serum sRAGE concentrations from patients with severe sepsis (all patients, panel A, n = 51) and subgroups of sepsis patients with different infectious sources; abdomen (peritonitis, panel B, n = 12), lungs (pneumonia, panel C, n = 29) and urinary tract (UTI, panel D, n = 10) and from healthy controls (n = 31). UTI = urinary tract infection. Data are mean ± SEM.

To the best of our knowledge, S100A12 protein levels during sepsis have not been reported before. Payen et al. found that mRNA S100A12 expression by circulating leukocytes in patients with septic shock is diminished during the recovery phase (29). Other studies found that S100A12 protein is strongly expressed in inflammatory diseases such as Crohn’s disease, cystic fibrosis, atherosclerosis and rheumatoid arthritis (12, 25, 30-33). We here demonstrate for the first time that systemic S100A12 concentrations are elevated in patients with sepsis. There was no correlation between S100A12 and the severity of sepsis as reflected by APACHE II and SOFA scores, or mortality. The observation that systemic administration of lipopolysaccharide to healthy volunteers induces a rise in circulating S100A12 levels implicates that lipopolysaccharide might be in part responsible for this upregulation during gram-negative infection.
Since S100A12 is not present in rodents (34), the functional role of S100A12 during sepsis cannot be easily investigated by inhibiting/deleting S100A12 in animals. One possible function in host defense of S100A12 during infection and sepsis might be to warn the host for eminent danger by exerting pro-inflammatory effects. Recently, such endogenous proteins that are released by activated or damaged cells under conditions of cell stress have been called damage associated molecular patterns (DAMPs, “endokines” or “alarmins”) (35). Indeed, NF-κB mediated expression of pro-inflammatory cytokines, such as tumor necrosis factor-α has been documented after S100A12 stimulation (7). Also, in vitro experiments indicate that S100A12 can enhance the expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 on endothelium (36). These pro-inflammatory effects of S100A12 could help to kill or contain pathogens during infection. Furthermore, S100A12 could be of benefit for the host during infection and sepsis due to its (more direct) antibacterial activity. Cole et al. documented that S100A12 (or calcitermin) has activity primarily against gram-negative bacteria, including E. coli (37). On the other hand, S100A12 induced pro-inflammatory effects may underlie the tissue injury/damage associated with the infection and/or inflammation and therefore be (net) detrimental for the host. The pro-inflammatory effects of S100A12 might in part be mediated by interaction with RAGE. Taken together, the role of S100A12 during sepsis (or infection) - either beneficial or detrimental for the host - has still to be investigated using non-murine models.

Knowledge about (s)RAGE involvement in host defense during sepsis or infection is limited. Bopp et al. showed that sRAGE plasma levels are increased in patients with
sepsis and that non-survivors had higher circulating sRAGE concentrations than survivors (38), suggesting that sRAGE is related to severity and outcome of sepsis. Here we did not find an upregulation of sRAGE in blood during severe sepsis, nor during human endotoxemia. Inflammatory stimuli as lipopolysaccharide can increase transcription of RAGE via NF-κB, resulting in increased expression of full length RAGE (39). Therefore, full length RAGE can be upregulated during sepsis. Indeed, we found that RAGE expression is enhanced in lungs and livers in mice during abdominal sepsis (chapter 2). Possibly there is a regulated equilibrium between membrane-bound and s(oluble) RAGE in which sRAGE might function as a decoy receptor for pro-inflammatory ligands such as S100A12 in patients with sepsis. The subsequent S100A12-sRAGE complexes might be cleared and not be measured in our study. Since we do not have data about the clearance of sRAGE, this possibility remains speculative.

RAGE knockout mice (deficient in the full length RAGE as well as sRAGE) displayed a reduced mortality after induction of polymicrobial sepsis produced by CLP (11, 14). Moreover, anti-RAGE therapy conferred a survival advantage even when administered 24 h after CLP in mice receiving antibiotic treatment (14). Since mice do not express S100A12 and other RAGE ligands have been described to play a role in sepsis (23), non-S100A12 mediated RAGE effects play an important role too.

One limitation of our study is that we measured all sRAGE isoforms. These include isoforms produced by truncation of full-length RAGE as well as by alternative splicing from the single rage gene. The rage gene has been demonstrated in humans to generate ~20 alternative splice variants (7, 40-42). They have in common that they contain the ligand-binding domain of the full-length RAGE protein, but not its transmembrane and signaling domains. Splice variants of RAGE may affect the S100A12-binding domain by insertion of removal of parts of the V or C1 domain (43, 44). The sRAGE ELISA we here used does not distinguish between the different isoforms. Further experiments are needed to 1) investigate separate activities of different isoforms of sRAGE and 2) develop sRAGE ELISA assays which distinguish between these (possibly also functionally) different forms of sRAGE.

The present study is the first to document that S100A12 release occurs in severe sepsis, as well in human endotoxemia. Its high-affinity soluble RAGE sRAGE is not elevated in concurrently obtained blood samples.
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