Makino et al

Title: Receptors for Advanced Glycation End-Products (RAGE) Ligand Effects on Intestinal Ischemic Damage in Mice.

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Makino et al

(RAGE), High mobility group box 1 (HMGB-1)

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The authors have no conflicts of interest to declare.

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Study concept and design (SM); data acquisition (YM, TU,KH,SK,MT,YK,YT,KS); analysis and interpretation of data (YM, SM, TU); drafting of the manuscript (SM); critical revision of the manuscript (SM); obtained funding (SM); technical support (SM, TU).

Abstract
Object: Superior mesenteric artery ischemia and nonocclusive mesenteric ischemia are representative of the vascular emergency known as irreversible transmural intestinal necrosis (ITIN). Receptors for advanced glycation end-products (RAGE) are members of the immunoglobulin superfamily with multiple extracellular ligands such as advanced glycation end products, high mobility group box 1 (HMGB-1), which are implicated in the pathogenesis of various inflammatory disorders. This study was designed to investigate the association of RAGE and ITIN in a murine acute intestinal ischemic model.

Materials and methods: ITIN was induced by clipping the cranial mesenteric artery and the peripheral blood vessels. Blood samples were collected and analyzed for levels of S100 family of proteins. The influence of RAGE signaling on intestinal cell reproduction was investigated using an in vitro wound-healing assay, the cell scratch test. In addition to the cell scratch test,
RAGE-correlated proteins and their respective inhibitors were administered intraperitoneally to ITIN model mice to determine their effects.

**Results:** RAGE-expressing cells were located at the base of the intestinal crypts, at the stem cell position at day 0. As ITIN progressed, most of the damaged intestinal cells expressed RAGE, and ligands of RAGE such as HMGB-1, S100 A8/A9 and S100β were present in crypt cells from the bottom to the top. S100 A8/A9 and S100β showed particularly high quantities, above the levels found in other diseases. When S100A8/A9 and S100β were applied to small intestinal epithelial cells *in vitro*, regeneration was significantly impeded. Stem cell factors, such as epidermal growth factor and Rpspondin1, reversed this effect and accelerated intestinal regeneration even under the influence of S100A8/A9. However, the survival rates were no different in mice in which proteins of the S100 family and plus their inhibitors were administered *in vivo*.

**Conclusions:** This study shows that RAGE-related proteins are elevated in ITIN model mice, and these proteins impede intestinal regeneration *in vitro* but not *in vivo*. It is possible that RAGE-correlated proteins can be a new therapeutic target or new marker for ITIN.

**Introduction**

Superior mesenteric artery ischemia and nonocclusive mesenteric ischemia, which are representative of acute mesenteric ischemia in humans, are life threatening diseases combining gastrointestinal and vascular elements. The short-term mortality of acute mesenteric ischemia is high, ranging from 32 to 86% (1, 2). Mortality is closely correlated with irreversible transmural intestinal necrosis (ITIN), which often leads to extensive intestinal resection. Therefore, we focused on the receptor for advanced glycation end-products (RAGE) as a possible new marker for this disease. RAGE is a member of the immunoglobulin superfamily with multiple extracellular ligands such as advanced glycation end products, high mobility group box 1 (HMGB-1) and proteins of the S100 family, has been implicated in the pathogenesis of various inflammatory disorders, and plays a potent role in innate immunity (3). RAGE signaling is
Makino et al

required for efficient S100A8 and S100A9 expression in skin inflammation (4). The S100A8/A9 complex has been suggested as a biomarker for both rheumatoid arthritis and chronic bronchitis (5). On the other hand, S100β has demonstrated binding affinity to RAGE in microglia, astrocytes, and neurons in the CNS (6, 7). As for inflammatory cells, monocytes and macrophages that express S100A8/A9 also secrete abundant TNF-α (8), and S100A8/A9 can induce TNF-α expression in human and mouse macrophages and microvascular endothelial cells (9). Heubener et al. showed that RAGE helped promote neutrophil migration in liver necrotic tissue through HMGB1 (10).

In the present study, we investigated the clinical effects of RAGE-related proteins in the ischemic gut and found that they inhibit the regeneration of gut homeostasis

Materials and methods

Animal studies

C57BL/6 mice were purchased from SLC. Animal studies were approved by the Animal Review Board of Juntendo University (Tokyo, Japan). We anesthetized mice with 2% isoflurane in oxygen. After midline incision through the abdominal wall, the cranial mesenteric artery and the peripheral blood vessels were blocked by clip (disposable clip for animal experiment AM-1 60g/mm² (Natsume Seisakusho, Tokyo, Japan). We administered 1 µg/mouse human HMGB1 (R&D, Minneapolis, MN, USA), 1 µg/mouse S100A8/A9 (R&D), 1µg/ mouse S100β (NOVUS Biologicals, Colorado, USA) and 1mg/kg RAGE inhibitor (FPS-ZM1 (Merck Millipore, MA, USA) by intraperitoneal injection.

Experimental dextran sulfate sodium (DSS) colitis was induced by administering 2% DSS (molecular weight, 36,000–50,000 Daltons; ICN Biomedicals Inc, Ohio, GA) via drinking water on days 0–7.

Histology

We fixed small intestine in 10% buffered formalin, embedded them in paraffin, cut them into 3-mm sections and stained them with H&E.
Immunohistochemistry

Ischemic tissues were snap-frozen in liquid nitrogen and cut into 5-μm sections. They were stained with antibody RAGE antibody (Abcam, Cambridge, MA, USA), anti-HMGB1 antibody (clone EPR3507; Abcam), anti-S100A8/A9 antibody (clone MAC 387; Thermo Fisher Scientific, Waltham, MA, USA) and anti-S100β antibody (clone EP1576Y; Abcam), respectively. The peroxidase activity was visualized using 3,3'-diaminobenzidine-tetrahydrochloride (IVIEW Roche Tissue Diagnostics, Indianapolis, IN, USA).

Immunoassay

All assays were done using enzyme-linked immunosorbent assay (ELISA) from commercially available kits. HMGB1 were assayed using HMGB1 ELISA Kit II (Shino-Test Corporation, Tokyo). The quantitative determination of mouse S100A8/A9 and S100β was done using ELISA kits (LSBio, Seattle, WA, USA).

Cell lines

The IEC-6 (RIKEN, Tokyo, Japan) rat small intestinal cell line was maintained at 37°C in 5% CO2 in 10-cm dishes with DMEM (Wako Pure Chemicals, Tokyo, Japan) supplemented with 10% fetal bovine serum. Before the start of each experiment, the cells were incubated overnight in medium.

In Vitro scratch wounding assay

An IEC-6 cell monolayer was scratched along a straight line using a P200 pipette tip, and recombinant proteins of rat S100β (Abcam), rat S100A8/A9 (Cusabio Biotech, College Park, MD) and human HMGB1 (R&D Systems) were sprinkled on the cells at concentrations of 100 nM, 1 μM, 10 μM with or without the stem cell factors: respective inhibitors: 50 ng/ml murine EGF (Thermo Fisher Scientific), 100 ng/ml murine Noggin (PEPROTECH, Rocky Hill, NJ),
and 500 ng/ml human R-Spondin 1 Protein (R&D Systems). After 6 hour incubation, the length of the line was evaluated by microscope BZ-X700 (KEYENCE, Osaka, Japan), with the original scratched distance assigned 5 points. The interval between two points of scratches was measured and divided by the result after 6 hours and calculated as the growth rate. (11)

**Cell migration assay**

A myeloid cell migration assay was established using chemotaxis cell units with a 5-μm pore size (Kurabo, Osaka, JAPAN). Myeloid cells were collected from the bone marrow of healthy mice. Migrated cells were quantified by counting within each culture dish of transmigrated cells. Into the culture dishes, 100 nM S100β (Abcam), 100 nM S100A8/A9 (Cusabio Biotech) or 1 μM HMGB1 (R&D) was administered. Cell counts were performed using FACS Verse (BD Biosciences, San Jose, CA).

**Quantification of messenger RNA expression in ischemic tissues by Reverse-Transcription Polymerase Chain Reaction**

Total RNA was isolated from normal and ischemic tissue using a Nucleospin RNA Plus (TaKaRa Bio, Otsu). Real-time PCR was performed using SYBR® Green PCR Master Mix (Toyobo, Osaka) on a 7500 Fast Real-Time PCR System (Applied Biosystems, CA). The relative mRNA expression was calculated using the 2^ΔΔCt method. PCR was performed with the following specific forward and reverse primer pairs, respectively: 

- hmgbl: 5'-GGCGAGCATCCTGGCTTATC-3' and 5'-GGCTGCTTGTCATCTGCTG-3'; S100a8:
- 5'-CCCCTCTTCAAGACATCGTGTGTTG-3' and 5'-ATATCCAGGGACCAGCCCTAG-3';
- S100a9 : 5'-CCCTGACACCCCTGAGCAAGAAG-3' and
- 5'-TTTCCCAGAAAGGAAGCATTGAG-3'; S100b : 5'-CTGGAGAAGCCATGGTTGC-3' and 5'-CTCCAGGAAGTGAGAGAGCT-3' 

*Actb*: 5'-GGCTGTATTTCCCCCTCCATCG3' and 5'-CCAGTTGGTAACATGCCATGT-3'.

**Statistical analyses.**
All data are presented as means ± standard errors of the mean (SEMs). Student’s t-tests were performed. Survival curves were plotted using Kaplan-Meier estimates with log rank. Values of P < 0.05 were considered to indicate statistical significance.

Results

**RAGE is activated during the early phase of ITIN.**

RAGE-expressing cells were located at the base of the intestinal crypts at day 0. As ITIN progressed, almost all damaged intestinal cells were found to express RAGE (Fig. 1A), and ligands of RAGE such as HMGB-1 and S100A8/A9 were present in crypt cells from the bottom to the top of the crypt (Fig. 1B). As far as S100A8/A9 is concerned, in a steady state, it was only stained at the top of the crypt, but as ischemia progressed, all damaged cells expressed it. We next examined the expression of these factors in intestine extracts and serum samples (Fig. 2A). The total expression of RAGE-related protein mRNA was higher in ITIN model mice. In particular, RAGE-related proteins S100A8/A9 and S100β showed extremely high quantities even in serum data (Fig. 2B). Compared with the other models, namely the DSS-induced colitis model and sham operation, S100A8/A9 and S100β were extremely high in the ITIN model. This data suggest that S100A8/A9 and S100β would be reliable diagnostic markers for early detection of ischemic change (Fig. 2C).

**Ligands of RAGE hinder intestinal cell regeneration.**

We investigated how RAGE signaling influenced intestinal cell reproduction using an *in vivo* wound-healing assay. There was a significant difference in growth suppression when S100A8/A9 and S100β were applied to the small intestinal epithelium (Fig. 2D). Regarding HMGB1, there was growth promotion at the low concentration of 100 nM, but an inhibitory effect was recognized as well (1 uM) (Fig. 2D).

The data indicated that RAGE-associated proteins generally inhibit intestinal regeneration. However, RAGE inhibition [FPS-ZM1 (1 mg/kg, i.p.)] could not ameliorate the ischemic disease progression in mice (Fig 3A). Furthermore, the administration of S100A8/A9 and S100β clinically did not significantly worsen the survival curve (Fig3A).
The inflammatory cells were mobilized in RAGE related abundance site.

Some in vivo studies have also suggested a role for S100A8/A9 in leukocyte migration. S100A8 and S100A9 are exceptionally abundant in neutrophils and are upregulated in activated macrophages (12). On the other hand, S100β participates in inflammatory processes in the brain by enhancing microglia activation and by stimulating microglia migration (13). We investigated whether RAGE-related proteins might be involved in myeloid cell migration. Bone marrow cells were confirmed to migrate toward S100A8/A9 but not toward HMGB-1 or S100β (Fig. 3B). These data show that S100A8/A9 enhances inflammatory myeloid cell influx.

Stem cell factor correlated with the epithelial regeneration without RAGE related proteins.

Studies have shown that stem cell factors (R-spondin1, Noggin, and epidermal growth factor (EGF)) are all essential to small intestine culture (14). Intestinal stem cells and neighbor cells were first reported in the bottom of the epithelium, similar to RAGE (15). As confirmed in in vivo wound healing assay, stem cell factors, Rspodin1 and EGF, but not Noggin, promoted the regeneration of the epithelium (Fig. 4A). The gut epithelium regeneration in mice supplemented by EGF and Rspodin1 overcame the deleterious effect of RAGE-related proteins. In particular, EGF and Rspodin1 suppressed the function of S100A8/A9 (Fig. 4B).

Discussion

Destruction due to ischemic injury of the gut has been associated with RAGE-related proteins such as HMGB-1, S100A8/9 and S100β. Inhibition of the RAGE pathway using its Antibodies has been reported as an effective approach for treatment of sepsis in mice (16). Unfortunately, EGF and Rspodin1 cultures overcame (“overwhelmed”) the effect of RAGE proteins, in vitro but this ITIN model was extremely lethal; if used clinically after resection of ischemic tissue by operation, these sorts of RAGE inhibitors may have benefit on the patient’s postoperative course.

As for the stem cell treatment, Yui et al showed engraftment of organoids derived from a single Lgr5+ cells could repair superficially damaged tissue (17). The growth of these Lgr5+ intestinal stem cells at the bottoms of intestinal crypts has been shown to require EGF, R-spondin1 and Noggin (14). Chen et al indicated that heparin binding EGF preserves Wnt/β-catenin signaling in
intestinal stem cells after intestinal ischemia/reperfusion injury (18). EGF, R-spondin1 and Noggin overcame the RAGE-related proteins’ action of dampening in vitro regeneration in our studies.

Of note, in the case of an ischemic brain, bone marrow stem cells were mobilized in ischemic tissue. BM-derived cells such as hematopoietic stem cells, mesenchymal stem cells, and endothelial progenitor cells have therapeutic benefits of regeneration (19). On the other hand, during hind limb ischemia, tissue-plasminogen activator promotes neoangiogenesis by mobilizing angiopotent myeloid cells (20). Likewise, we showed that bone marrow cells migrated faster toward wells that contained S100A8/9 but not HMGB-1 or S100β.

In fact, though intestinal infarction is strongly associated with mortality (21), acute intestinal ischemia is difficult to diagnose because the early clinical signs are non-specific. Plasma biomarkers for intestinal ischemia have been proposed, including D-dimer, D-lactate, intestinal fatty acid binding globulin (I-FABP), alkaline phosphatase, creatine kinase and lactate dehydrogenase (22, 23). I-FABP is the only biomarker that is specific to the small bowel membrane, and it had the best diagnostic performance. However, I-FABP levels can be raised in other types of small bowel disease, such as acute enterocolitis, Crohn’s disease and simple bowel obstruction (24). As far as the S100 family is concerned, it is possible that in the near future, fecal S100A8/9 and S100A12 levels may serve as essential components in a novel, updated activity index for Inflammatory Bowel Disease. Interestingly, the level of S100A8/9 and S100β in serum was shown to have significantly higher correlation with disease in an ITIN a normal_model than in a DSS-induced colitis model (25). S100A8/9 and S100β did not correlate with DSS-induced colitis and sham operations in mice. The S100A8/9 and S100β may be a useful marker to diagnose ITIN.

In conclusion, our data introduces RAGE-related proteins as a novel diagnostic markers and therapeutic targets to ameliorate ITIN progression.

Figure legends
Makino et al

Figure 1. RAGE is activated during the early phase of ITIN.  
An ischemic model was induced surgically using clips. Representative macroscopic pictures and images of H&E stained ischemic areas are shown (0—6 hours). (B) Representative receptors for advanced glycation end-products (RAGE), the ligands of RAGE, HMGB-1, S100A8/A9 and S100β-stained sections of ischemic tissue are shown. Scale bar, 100 um. Values represent means ± SEMs. \( *P < .05, **P < .01, ***P < .001 \), determined by 2-tailed Student’s t-test.

Figure 2. The ligands of RAGE are elevated in ITIN.  
(A) Gene expressions of hmbgl, S100a8/a9, and S100b in small intestine homogenates of clipped C57BL/6 mice, as determined by polymerase chain reaction (normalized to the expression of Actb). \( n = 3 \) / group.  
(B) RAGE-related proteins HMGB1, S100A8/A9, and S100β were assayed in serum samples of mice with an ITIN model as determined by enzyme-linked immunosorbent assay. \( n = 5 \) / group.  
(C) The RAGE-related proteins S100A8, S100A9S100, and S100β were assayed in serum samples of an ITIN model, DSS model and sham-operation mice, as determined by enzyme-linked immunosorbent assay. \( n = 3 \) / group.  
(D) Ligands of RAGE including S100A8/A9 and S100β inhibited wound healing in a 6-hour regeneration assay. HMGB-1 also dampened cell proliferation at the low concentration of 100 nM, but promoted cell regeneration at 1 uM in vitro.

Figure 3. Inflammatory cells were mobilized in sites with abundant RAGE-related proteins  
The ligands of RAGE promoted the influx of myeloid cells into the ischemic tissue.  
(A) Kaplan-Meier survival curve of ITIN-induced mice in the following treatment groups:  
ITIN+treated with 0.03/kg (n=5) of RAGE inhibitor, \( n = 5 \), ITIN+inhibitors, or treated with S100A8/A9, \( n = 5 \), ITIN+S100β, \( n = 5 \), ITIN+β and HMGB,\( n = 5 \), ITIN + phosphate-buffered saline.\( n = 10 \). Administered together, RAGE inhibitors and ligands of
Makino et al

RAGE showed no significant difference from control results. Values represent means ± SEMs. *P < .05, **P < .01, ***P < .001, determined by 2-tailed Student’s t-test.

(B) Transmigration index of bone marrow myeloid cells migrated toward medium with S100A8/A9 in vivo. Values represent means ± SEMs. *P < .05, **P < .01, ***P < .001, determined by 2-tailed Student’s t-test.

Figure 4. Stem cell factors promoted epithelial regeneration with RAGE-related proteins.

(A) In the wound-healing assay, EGF and R-spondin1 promoted IEC-6 proliferation. (B) Under the influence of the RAGE-related proteins S100A8/A9, S100β and HMGB-1, the inhibitor EGF & R-spondin strongly ameliorated wound healing in vitro in case of S100A8/A9. Values represent mean ± SEM. *P < .05, **P < .01, ***P < .001, determined by 2-tailed Student’s t-test.

References


Figure 3

A

CTL

S100A8

B

Area covered with cells (% of initial wound area)

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