Roles of high-mobility group box 1 in murine experimental colitis

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Abstract. High-mobility group box 1 (HMGB1) plays a role in inflammatory and immune-mediated diseases. This study investigated the role of HMGB1 in colonic inflammation. Colitis was induced by orally feeding mice 4.5% dextran sulfate sodium (DSS) for up to 7 days. Mice were sacrificed on days 0, 3, 7 and 10, and the colon harvested for the measurement of HMGB1 and pro-inflammatory cytokines. To block HMGB1 induction, an anti-HMGB1 antibody was administered intraperitoneally 2 h before or 3 days after the induction of colitis, and disease severity was assessed by clinical and histological scoring. The colonic levels of tumor necrosis factor-α and interleukin-1β were elevated in relation to disease severity. The level of HMGB1 increased more slowly than that of the cytokines. Immunohistochemical study of the colons showed that the tissues of mice treated with DSS had a higher expression of HMGB1 and its receptor - the receptor for advanced glycation end products (RAGE) than normal controls, especially in inflammatory infiltrates. The anti-HMGB1 antibody ameliorated tissue damage. In conclusion, HMGB1 is an important mediator of colonic inflammation, and suppression of this protein partially protects against colonic inflammation.

Introduction

Inflammatory bowel disease (IBD), such as ulcerative colitis or Crohn's disease, is characterized by chronically relapsing inflammation of the bowel involving pro-inflammatory cytokines, chemotactic peptides and arachidonic acid metabolites (1-3). In particular, IBD can be exacerbated by increased secretion of pro-inflammatory cytokines. Consequently, an array of therapeutic approaches targeting pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α (4,5) or interleukin (IL)-6 (6,7), have been extensively investigated.

Materials and methods

Mice. Seven- to 8-week-old female BALB/c mice were purchased from SLC Co. Ltd. (Shizuoka, Japan) and housed in standard wire-mesh cages. The mice were fed laboratory pellet formula and tap water ad libitum. The study was approved by the Animal Research Committee of Kurume University.

Induction of colitis. Colitis was induced by drinking water supplemented with 4.5% DSS (M.W. 40,000, ICN Biomedicals, Aurora, OH) for 7 days. This model has been described in detail elsewhere (18). Control mice received plain drinking water.

Time course study of colitis. The mice were sacrificed before and at 1, 3, 7 and 10 days following DSS initiation. The colon including the cecum was removed, and the length of the colon from the colo-cecal junction to the anal verge was measured. The colon was then rinsed with ice-cold saline and used to determine cytokine and HMGB1 levels. Clinical score and colon length were also ascertained.

Administration of anti-HMGB1. We intraperitoneally injected either a neutralizing polyclonal chicken IgY anti-HMGB1 anti-

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body (200 μg/mouse, Shino-Test, Kanagawa, Japan) or control chicken IgY antibody (200 μg/mouse, Shino-Test) 2 h before and 3 days after DSS initiation. The mice were sacrificed on day 7, and colitis severity was assessed according to clinical and histological scores.

Assessment of colitis. Colitis severity was evaluated according to clinical and histological signs, and by colonic length. A clinical score was generated based on a 0-4 rating of the factors: change in body weight, stool consistency and intestinal bleeding (20). Each variable was given equal weight, with the overall clinical activity score ranging from 0 to 12. These parameters were measured by an investigator blinded to the treatment group. After randomization, a histological score was assigned by two pathologists who were also group-blinded. The histological score for each segment (cecum, proximal colon, middle colon and distal colon) ranged from 0 to 9 and represented the sum of the scores for the severity of inflammation, damage/necrosis and regeneration. The total histological score ranged from 0 to 12 and consisted of the sum of the score of the distal colon and the score of disease extent (21). We also measured the colonic length from the colo-cecal junction to the anal verge, an established inflammatory parameter in DSS colitis (18,22).

Organ culture. HMGB1 and cytokine levels in colonic tissue were measured using an organ culture technique as previously described (22). Briefly, tissue samples 3 mm in diameter were immediately taken from the colon using a dermal punch biopsy instrument (Dispopench, Stiefel Laboratories Ltd., Germany). Three tissue specimens were placed on a metal grid in the wells of a 24-well tissue culture plate (Falcon 3947; Becton-Dickinson, Lincoln Park, NJ) that contained complete medium consisting of RPMI-1640 (Nissui Pharmaceutical Co. Ltd., Japan), 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM/l L-glutamine. The specimens were cultured at 37°C in 5% CO₂ and 95% O₂. After 24 h, the supernatants were harvested for measurement of HMGB1 and cytokine levels by ELISA.

**HMGB1 and cytokine ELISA.** ELISA for HMGB1 was performed using monoclonal antibodies to HMGB1 and recombinant HMGB1 as the standard (23). Black polystyrene microtiter plates (Corning, New York, NY) were coated with 100 μl anti-HMGB1 polyclonal antibody (peptide antigen: KPDAAKKVGVKAEK) in phosphate-buffered saline (PBS). The unbound antibodies were removed by washing the plate 3 times with PBS containing 0.05% Tween-20 (washing buffer). The remaining binding sites in the wells were blocked by incubating the plates for 2 h with 400 μl/well PBS-1% BSA. After washing, 100 μl of each dilution of the standard and samples [1:1 dilutions in 0.2 mol/l Tris (pH 6.5), 0.15 mol/l NaCl containing 1% BSA] was added to the wells. The microtiter plates were incubated for 24 h at room temperature. After washing, 100 μl/well of anti-human HMGB1 peroxidase-conjugated monoclonal antibody was added and the plates were incubated at room temperature for 30 min. Followng another washing step, 100 μl of lumigene PS-atto was added to each well. Luminescence was measured with a 9000D microplate luminescence reader (Dia-Iatron Co. Ltd., Tokyo, Japan). Immunoreactive TNF-α and IL-1β were quantified in duplicate using commercially available ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Immunohistochemical analysis.** Immunohistochemistry was performed in 5-μm paraffin sections using an avidin-biotin peroxidase procedure (Vector Laboratories, Burlingame, CA). To detect HMGB1 and RAGE, the samples were incubated with primary antibodies (polyclonal rabbit anti-HMGB1 antibody, Shino-test and monoclonal rat anti-RAGE antibody, R&D Systems). The sections were incubated with biotinylated IgG for 30 min, washed with PBS, incubated with avidin/biotinylated horseradish peroxidase complex for 1 h and washed with PBS. Slides were stained with diamobenzidine tetrahydrochloride substrate for 2 min at room temperature, rinsed in tap water for 5 min, counterstained with hematoxylin and eosin and dipped in saturated lithium carbonate solution for bluing. Normal blocking serum without primary antibody was used as the negative control.

**Statistical analysis.** Where appropriate, results are presented as the means ± SEM. Comparative assessment was performed using the Student’s t-test and one-way ANOVA followed by...
Scheffé’s (post-hoc) test for the statistical significance level of each measured item and score. P<0.05 was considered statistically significant.

Results

Time course of colonic damage during dextran sulfate sodium-induced colitis. The ingestion of 4.5% DSS resulted in severe colon injury as assessed by clinical score and colon length (Fig. 1). These gradually increased, peaked on day 7 and declined thereafter.

Kinetics of cytokine and HMGB1 levels during dextran sulfate sodium-induced colitis. IL-1β and TNF-α levels began to increase on day 3, peaked on day 7 and declined thereafter (Fig. 2), in parallel with the degree of colon injury. In contrast, HMGB1 levels peaked on day 10, after IL-1β and TNF-α, corresponding to the start of injury recovery.

Discussion

HMGB1 influences animal models of inflammation such as endotoxemia (14), peritonitis (16), hepatic injury (17) and lung injury (15), where the blockade of HMGB1 with specific antibodies improves survival and inflammation and diminishes circulating levels of pro-inflammatory cytokines. However, little is known regarding the role of HMGB1 in colonic injury.

IL-1β and TNF-α are elevated in experimental colitis and reflect inflammation levels. HMGB1 also increased in the colon, but more slowly than IL-1β and TNF-α. Similarly, HMGB1 production in monocytes was induced by IL-1β and TNF-α as well as by lipopolysaccharide (14), suggesting that HMGB1 expression in DSS-induced colitis results from enhanced IL-1β and TNF-α production. HMGB1 also induces the synthesis of cytokines by activated monocytes and
Figure 4. Anti-HMGB1 antibody treatment improves (A) the body weight loss and (B) clinical score during dextran sulfate sodium (DSS)-induced colitis in mice. Two hours before and 3 days after ingesting 4.5% DSS, mice were injected with either control chicken IgY (Hem+Cont antibody, 200 μg/mice, n=10) or polyclonal chicken IgY anti-HMGB1 antibody (200 μg/mice, n=10). Data are representative of two independent experiments. P-values vs. control antibody-treated mice are shown.

Figure 5. (A) Histological scores in mice treated with anti-HMGB1 antibody 2 h before and 3 days after ingesting 4.5% DSS. Mice were injected with either control chicken IgY (Hem+Cont antibody, 200 μg/mice, n=10) or polyclonal chicken IgY anti-HMGB1 antibody (200 μg/mice, n=10). The severity of colitis was evaluated 7 days after the initiation of DSS ingestion according to its histological score. (B) Representative H&E staining of mouse colonic mucosa exposed to DSS. Note that microscopic inflammation was improved by anti-HMGB1 antibodies. Original magnification, x100.
macrophages (24), potentially leading to a feed-forward inflammatory loop. Animal models of endotoxemia and sepsis show persistent HMGB1 elevation, even after other pro-inflammatory cytokines can no longer be detected (15). These data point to a role for HMGB1 as a late-acting mediator of inflammation and organ dysfunction. HMGB1 acts as a cytokine by signaling via RAGE, a transmembrane receptor of the immunoglobulin superfamily (11-13). RAGE staining was barely detectable in the colonic tissues of normal mice, but was elevated in the colonic tissues of the DSS mice. This finding suggests that the up-regulation of RAGE is involved in the colonic injury caused by DSS. Although the role of RAGE in mediating inflammatory responses to HMGB1 requires further investigation, our results suggest that RAGE accumulation accompanies the up-regulation of its ligand, HMGB1, associated with DSS-induced colitis.

The results of this study provide compelling evidence for the participation of endogenous HMGB1 in the development of DSS-induced colitis. Specifically, they show that HMGB1 is produced at inflammatory sites, and that its inhibition reduces tissue damage. However, the effect of anti-HMGB1 was not complete, perhaps due to an inadequate dose or the presence of other factors contributing to inflammation. Administration of antibodies against IL-1 (25), TNF-α (26) or the macrophage migration inhibitory factor (27) can ameliorate DSS-induced colitis.

In summary, we found HMGB1 to be up-regulated in DSS-induced colonic lesions, and that an anti-HMGB1 antibody ameliorated tissue damage. This suggests that HMGB1 is an important mediator of colonic inflammation. Therefore, strategies to manipulate HMGB1 expression may constitute a realistic approach to the treatment of colonic inflammation such as human inflammatory bowel disease.

References
