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Bladder pain relief by HMGB1 neutralization and soluble thrombomodulin in mice with cyclophosphamide-induced cystitis

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ABSTRACT

High mobility group box 1 (HMGB1), one of damage-associated molecular patterns (DAMPs), plays roles in not only inflammation but also processing of somatic pain. Given that no evidence for roles of HMGB1 in visceral pain signaling is available, we asked if HMGB1 participates in bladder pain accompanying cystitis caused by cyclophosphamide in mice, using the anti-HMGB1 neutralizing antibody and recombinant human soluble thrombomodulin (rhsTM) that sequesters HMGB1 and promotes its degradation by thrombin. Cyclophosphamide, administered i.p., caused bladder pain-like nociceptive behavior and referred hyperalgesia accompanying cystitis symptoms including increased bladder weight, an indicator of edema, in mice. The cyclophosphamide-induced bladder pain and referred hyperalgesia, but not increased bladder weight, were prevented by i.p. preadministration of the anti-HMGB1 neutralizing antibody or rhsTM. HMGB1, given i.p., facilitated the bladder pain and referred hyperalgesia caused by a subeffective dose of cyclophosphamide, an effect blocked by rhsTM. In the cyclophosphamide-treated mice, HMGB1 levels greatly decreased in the bladder tissue, particularly in the urothelial cells, but did not change in the plasma. Low molecular weight heparin, known to inhibit the receptor for advanced glycation end products (RAGE), but not lipopolysaccharide from *Rhodobacter sphaeroides*, an inhibitor of toll-like receptor 4 (TLR4), blocked the cyclophosphamide-induced bladder pain and referred hyperalgesia. Thus, our data indicate involvement of HMGB1 in the cyclophosphamide-induced bladder pain signaling, but not cystitis itself, and suggest that targeting HMGB1 with rhsTM or blocking RAGE might serve as a novel therapeutic strategy for the management of bladder pain.

1. Introduction

High mobility group box 1 (HMGB1), a nuclear architectural chromatin-binding protein, is passively released to the extracellular space from necrotic cells or damaged cells and actively secreted by certain cells such as macrophages or hepatic cells (Evankovich et al., 2010; Sims et al., 2010). The extracellular HMGB1 activates the receptor for advanced glycation end products (RAGE) and toll-like receptor 4 (TLR4), thereby facilitating inflammation (Malarkey and Churchill, 2012; Yanai et al., 2012). Several studies employing the anti-HMGB1 neutralizing antibody suggest involvement of HMGB1 in neuropathic pain and bone cancer pain (Feldman et al., 2012; Otoshi et al., 2011; Ren et al., 2012; Shibasaki et al., 2010; Tong et al., 2010). On the other hand, the role of HMGB1 in processing of visceral nociception including bladder pain has yet to be investigated.

Thrombomodulin (TM), known to be expressed in vascular endothelial cells, forms a complex with thrombin that converts protein C into activated protein C, playing anti-coagulant and anti-inflammatory roles (Ito and Maruyama, 2011). TM is composed of five domains: an N-terminal lectin-like domain (D1), an epidermal growth factor (EGF)-like domain (D2), an O-glycosylation-rich domain (D3), a trans-membrane domain (D4) and a C-terminal cytoplasmic domain (D5). D2 is responsible for thrombin binding and formation of activated protein C. Recombinant human soluble thrombomodulin (rhsTM, thrombomodulin alfa, Recomodulin®) consisting of D1, D2 and D3 has been approved as a medicine for treatment of disseminated intravascular coagulation (DIC) in Japan

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(Ito and Maruyama, 2011). Exogenously applied rhsTM is capable of causing D2-dependent production of activated protein C in humans or monkeys, but not non-primate animals including rodents and rabbits (Mohri et al., 1997). Nonetheless, rhsTM still exhibits anti-inflammatory activity through D1-dependent effects such as sequestration of HMGB1 followed by its degradation with thrombin in rodents (Ito and Maruyama, 2011; Li et al., 2012). In this context, it is likely that rhsTM is capable of modulating HMGB1-dependent pain/hyperalgesia.

In the present study, we wished to clarify the role of HMGB1 in processing of bladder pain signals and to evaluate the therapeutic usefulness of rhsTM. Therefore, we investigated effects of removing HMGB1 with the anti-HMGB1 neutralizing antibody or rhsTM on bladder pain in mice with cyclophosphamide-induced cystitis in mice (Laird et al., 2002; Olivar and Laird, 1999), a model for interstitial cystitis (Wantuch et al., 2007).

2. Materials and methods

2.1. Experimental animals

Female ddY mice (18–22 g, 4–5 weeks old) were purchased from Kiwa Laboratory Animals Co., Ltd. (Wakayama, Japan). The animals were housed in a temperature-controlled room under a 12-h day per night cycle at about 24 °C and had free access to food and water. All experimental protocols were approved by Kinki University's Committee for the Care and Use of Laboratory Animals and were in accordance with the *Guiding Principles approved by The Japanese Pharmacological Society* and with the *Guide for the care and Use of laboratory Animals published by the US national Institutes of Health*.

2.2. Major chemicals

Cyclophosphamide was purchased from Sigma–Aldrich Chemical (St Louis, MO). High-mobility group box 1 protein (HMGB1), chicken anti-HMGB1 polyclonal antibody (neutralizing antibody) and chicken IgY (control) were obtained from SHINO-TEST Corporation (Kanagawa, Japan). Recombinant human soluble thrombomodulin (rhsTM, also known as ART-123) was provided by Asahi Kasei Pharma (Tokyo, Japan). Low molecular weight heparin (LMWH; molecular weight, 4500–6500; 79.5 U/mg) was kindly provided by Fuso Pharmaceutical Industries, Ltd. (Osaka, Japan). Lipopolysaccharide from *Rhodobacter sphaeroides* (LPS-RS), an inhibitor of TLR4, was purchased from InvivoGen (San Diego, CA, USA). Cyclophosphamide, HMGB1, LMWH and LPS-RS were dissolved in saline, and rhsTM was in 0.002% Tween 80-containing saline. The neutralizing antibody and chicken IgY were dissolved in 0.2 M phosphate-buffered saline (PBS).

2.3. Creation of a mouse model for cyclophosphamide-induced cystitis

As described previously (Miki et al., 2011; Olivar and Laird, 1999), cystitis in mice was created by intraperitoneal (i.p.) administration of cyclophosphamide at 150–300 mg/kg. Observation of bladder pain-like nociceptive behavior and evaluation of referred hyperalgesia were performed 3.5–4 h after i.p. cyclophosphamide, and then the mice were killed by cervical dislocation under anesthesia with i.p. urethane at 1.5 g/kg. The bladder was isolated and weighed in order to evaluate the bladder edema following the development of cystitis. In some experiments, the isolated bladder tissue was used for morphological observation by staining with hematoxylin and eosin.

2.4. Assessment of bladder pain-like nociceptive behavior and referred hyperalgesia in mice treated with cyclophosphamide

Assessment of bladder pain-like nociceptive behavior and referred hyperalgesia in mice was carried out as described previously (Laird et al., 2002; Olivar and Laird, 1999), with minor modifications (Miki et al., 2011). Each mouse was placed on raised wire-mesh floor under a clear transparent plastic box (23.5 × 16.6 × 12.4 cm), and acclimated to the experimental environment for 1 h. Bladder pain-like nociceptive responses, such as licking and biting of the skin of the lower abdomen close to the bladder (Laird et al., 2002; Olivar and Laird, 1999), were counted for a 30-min period starting 3.5 h after i.p. administration of cyclophosphamide. Immediately after the observation of nociceptive behavior, to determine referred hyperalgesia, essentially on the basis of the previous report (Laird et al., 2002), mechanical stimulation was applied to the skin region between the anus and urethral opening of mice, using 4 distinct von Frey filaments with strengths of 0.008, 0.07, 0.4, and 1.0 g, in ascending order of strength. The mechanical stimulation with each filament was applied 10 times at intervals of 5–10 s. To prevent "wind-up" effects or desensitization, repetitive stimuli of the same skin point was avoided. Scoring of nociceptive behavior was defined as follows: score 0 = no response; score 1 = licking or biting of the external urethral opening and/or the surrounding area, leaving the position, bending of the trunk, raising the upper half of the body, and thrashing limbs; score

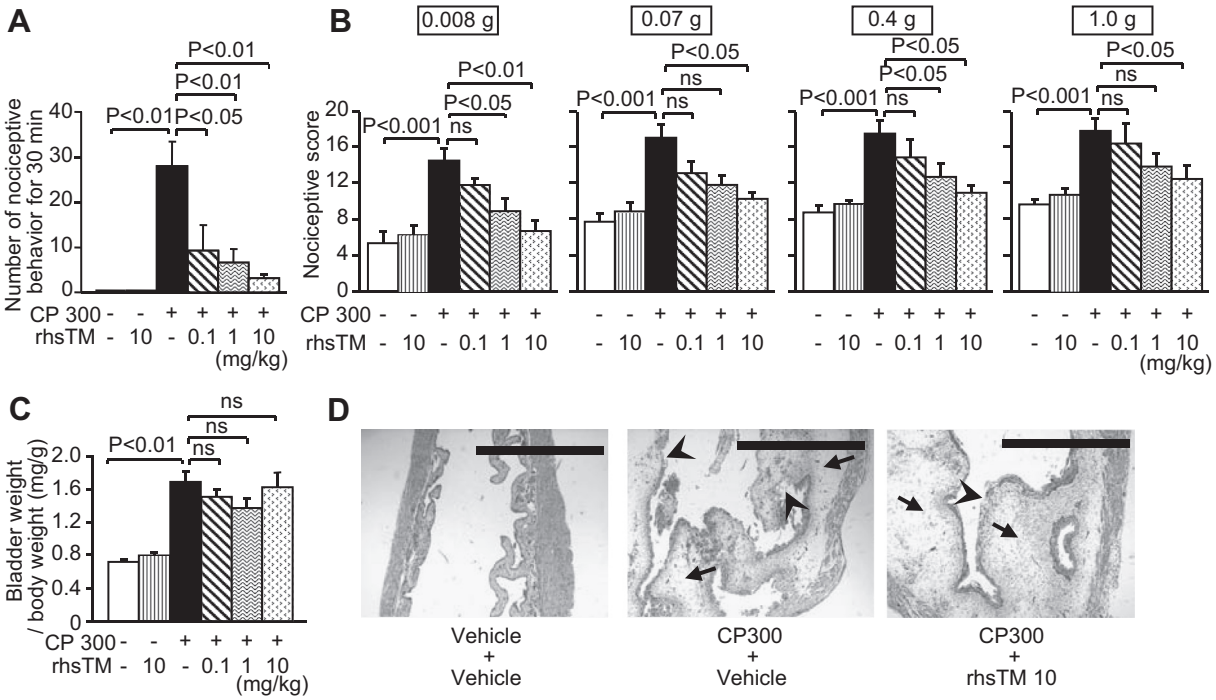

Fig. 1. Effects of rhsTM on the cyclophosphamide-induced bladder pain-like nociceptive behavior and referred hyperalgesia accompanying cystitis in mice. rhsTM at 0.1, 1 and 10 mg/kg or vehicle was administered i.p. 30 min before i.p. administration of cyclophosphamide (CP) at 300 mg/kg or vehicle. Bladder pain-like nociceptive behavior (A) was observed 3.5–4 h after CP treatment, and then referred hyperalgesia (B) was evaluated by the von Frey test. The bladder isolated after the von Frey test was weighed as an indicator of edema (C). Data show the mean with S.E.M. from 4 to 9 mice, and "ns" means not significant (A–C). (D) In the histological examination, the bladder tissue was stained with hematoxylin and eosin. Bars show 1 mm. Arrows and arrowheads indicate mucosal edema and demolition of urothelial cells, respectively.