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Hydrogen mediates suppression of colon inflammation induced by dextran sodium sulfate

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ABSTRACT

By its antioxidant effect, molecular hydrogen gas (H_2) was reported to protect organs from tissue damage induced by ischemia reperfusion. To evaluate its anti-inflammatory effects, we established a mouse model of human inflammatory bowel disease (IBD) by supplying mice with water containing (1) dextran sodium sulfate (DSS) (5%), (2) DSS (5%) and H_2, or (3) H_2 only *ad libitum* up to 7 days. At day-7, DSS-induced pathogenic outcomes including, loss of body weight, increase of colitis score, pathogenic shortening of colon length, elevated level of IL-12, TNF-α and IL-1β in colon lesion, were significantly suppressed by the addition of H_2 to DSS solution. Histological analysis also revealed that the DSS-mediated colonic tissue destruction accompanied by macrophage infiltration was remarkably suppressed by H_2. Therefore, the present study indicated that H_2 can prevent the development of DSS-induced colitis in mice.

Introduction

Molecular hydrogen (H_2) is recognized as possessing antioxidative effects. Previous studies demonstrated that H_2 in the form of gas or dissolved in water can suppress tissue injuries caused in brain, liver, and heart by oxidative stress from ischemia reperfusion [1–3]. Unlike other gaseous molecules, such as NO or O_2, the small H_2 molecule can penetrate solid substances, even plastic. Supported by such rapid permeability, while precise chemical mechanism by which H_2 quenches oxidative stress is to be elucidated [4], ingestion of H_2-water which holds neutral pH is thought to function as a strong antioxidant agent because of its extremely negative redox potential value [5]. However, the effects of H_2 on inflammation induced by mechanisms other than ischemia reperfusion remain to be elucidated.

Dextran sodium sulfate (DSS)-induced rodent colitis has been reported as an animal model of human inflammatory bowel disease (IBD), especially ulcerative colitis [6,7]. DSS is a sulfated polysaccharide that interferes with epithelial cell barrier function. This exposes the lamina propria to luminal bacterial antigens, which, in turn, elicits the activation of innate immunity [8,9]. When applied to mice in drinking water, DSS induces colitis, which is characterized by weight loss, diarrhea and/or grossly bloody stool, and the histopathological features of intestinal inflammation, i.e., erosion of crypt [10].

Impaired antioxidant mechanism is implicated as one of pathogenic causes of DSS-induced colitis [11]. Since both inflammation and oxidation processes are reciprocally related [12–14], as such, antioxidant effects of H_2 should prevent its development by suppressing proinflammatory cytokines, e.g., IL-1β, IL-12, and TNF-α, expressed in the colitis lesion [15–17]. Especially, these proinflammatory cytokines are considered to be responsible for the tissue destructions occurring in the DSS-induced mouse colitis as well as human IBD [6,18,19]. However, since it is unclear if ingestion of H_2-water can efficiently prevent or suppress the inflammatory outcome of DSS-induced colitis, we examined the effects of molecular H_2 dissolved in water on DSS-induced mouse colitis.

Materials and methods

Animals. BALB/c mice (8–10 w old males, $n = 6$/group) were caged in specific pathogen-free (SPF) conditions. The animals were kept in a conventional room with a 12-h light-dark cycle at constant temperature. The experimental procedures employed in this study were approved by the Forsyth IACUC.

Measurement of molecular hydrogen. Molecular hydrogen (H_2) present in water or organs of mice was measured using a needle-type

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Hydrogen Sensor (Unisense A/S, Aarhus, Denmark) following the method published by Hayashida et al. [2].

Generation of H_2 dissolved water. High purity H_2 gas (Airgas East, Salem, NH) was injected into water, Ringer's solution, or 5% DSS in water until H_2 concentration reached saturation (0.78 mM, at 25 °C).

Induction of DSS-induced colitis. On Day-0, control regular distilled water, DSS (5% [wt/vol], 30–40 kDa; Acros Organics, Morris Plains, NJ) dissolved in distilled water with or without saturated H_2 or distilled water with saturated H_2 only were applied to mice *ad libitum* as drinking water in a feeding glass bottle (Schott Duran, Mainz, Germany) with rubber top and metal tube. The biophysical property of fresh water containing saturated H_2 showed (1) H_2 concentration, 0.78 mM, (2) pH 7.43 ~ 7.76, and (3) ORP −462 to −511 mV. After 24 h, these values were measured as (1) H_2 concentration, 0.39 ~ 0.42 mM, (2) pH 7.34 ~ 7.63, and (3) ORP −388~−420 mV. Fresh DSS solution, with or without hydrogen, and hydrogen-only water was prepared daily. Mice were monitored on a daily basis for the next 7 days to measure body weight and colitis score. After 7 days, mice were sacrificed, and colonic tissues were collected. The colon was removed at the two positions closest to the ileocecal valve and the rectum, and the length was measured. Sections (1 cm) of the distal and proximal colon were fixed and embedded in OCT compound for histological analysis. The remaining part of the colon was weighed and frozen in liquid nitrogen for detection of cytokines.

Colitis score. The colitis score was assessed daily during DSS induction by trained individuals blinded to the treatment groups [20]. The baseline colitis score was determined on day 0. Briefly, no weight loss was scored as 0; weight loss of 1–5% from baseline as 1; 5% to 10% as 2; 10% to 20% as 3; and more than 20% as 4. For stool consistency, a score of 0 was assigned for well-formed pellets, 2 points for pastey and semi-formed stools that did not adhere to the anus, and 4 points for liquid stools that did adhere to the anus. For bleeding, a score of 0 was assigned for no blood, 2 points for positive hemoccult, and 4 points for gross bleeding. These scores were added together and divided by three, resulting in a total clinical score ranging from 0 (healthy) to 4 (maximal activity of colitis).

Histological evaluation. Tissue sections (8 μm) of distal portion of transversal colon were stained with hematoxylin and eosin (H & E). Histological scoring was performed based on amount and depth of inflammation and amount of crypt regeneration or damage [21] Briefly, scores were graded as follows: (1) Inflammation amount: none, 0; slight, 1; moderate, 2; severe, 3 (2) Inflammation depth: none, 0; mucosa, 1; mucosa and submucosa, 2; transmural, 3 (3) Crypt damage: none, 0; basal 1/3 damaged, 1; basal 2/3 damaged, 2; only surface epithelium intact, 3; entire crypt and epithelium lost, 4. Sections were scored for each feature separately, and the scores were added to reach the final histological scoring for individual colon specimens.

Immunofluorescent identification of F4/80-positive macrophages. Sectioned colon tissues were fixed with a mixture of acetone (50%) and methanol (50%). Macrophages present in the colon were stained with anti-F4/80 MAb conjugated with biotin (Rat IgG2b, AbD Serotec, Oxford, UK) followed by FITC-Avidin (BD Pharmingen, San Diego, CA). Irrelevant Rat MAb conjugated with biotin (BD Pharmingen) was used as control. The staining pattern was analyzed at ×400 magnification using a Leica TCS/SP-2 laser scan confocal microscope.

ELISA. Dissected transversal colonic tissues were homogenized with a Dounce glass homogenizer in PBS supplemented with 0.05% Tween 20, phenylmethyl sulfonyl fluoride (1 mM; Sigma, St. Louis, MO) and protease inhibitor cocktail (Sigma), followed by centrifugation for 10 min at 18,000 rpm. The resulting supernatant was subjected to ELISA for the measurement of TNF-α (Mouse TNF-α ELISA MAX™ Set, Biolegend, San Diego, CA), and IL-1β or IL-12p40 (Murine ELISA Development kit, Peprotech, Rocky Hill, NJ, respectively).

Results

Inhalation of H_2 gas can suppress oxidation-dependent tissue injury of the small intestine caused by ischemia reperfusion which was mediated by syngeneic small intestinal transplantation [22]. While this indicates that administration by gas can reach the small intestine, it is unclear whether H_2-dissolved water applied orally can affect the concentration of H_2 in the colon. Efficacy would depend on the interplay between H_2 produced or absorbed by intestinal bacteria and exogenously supplied H_2. Therefore, temporal change of H_2 concentration in stomach (Fig. 1A) and in colon (Fig. 1B) after oral administration of H_2-saturated water to mice was monitored. Measured at 5 min, the concentration of H_2 present in the stomach showed 503.8 ± 160.3 μM, rapidly decreasing to 200.8 ± 106.6 μM at 10 min, and returning to baseline level at 60 min. In contrast, the concentration of H_2 in the colon gradually increased to its highest level, 88.0 ± 26.5 μM, at 30 min and then returned to the baseline level of 58.0 ± 15.1 μM at 60 min. As shown in Fig. 1, the orally administered H_2 seemed to increase the H_2 already present in the colon, presumably by a combination of cross-epithelial diffusion [23] and vascular-based transport processes [24].

To investigate the possible preventive efficacy of H_2 on the development of IBD, the effect of H_2 on DSS-induced colitis was tested in BALB/c mice. Significant body weight loss in mice was observed after 5 days of consecutive administration with DSS, persisting until Day-7 when animals were sacrificed (Fig. 1C). In contrast, BALB/c mice receiving DSS solution containing H_2-water or H_2-water alone showed no loss in body weight (Fig. 1C). The colitis scores of mice receiving DSS alone increased in a time-dependent manner until Day-7. Especially, significant difference of colitis scores between mice supplied with DSS alone and those receiving DSS solution containing H_2 was detected from Day-4 to Day-7 (Fig. 1D). By contrast, there was no significant difference between the mice supplied with control regular water and those receiving H_2 dissolved in water (Fig. 1B). These data indicated that H_2 administered orally can suppress the onset of DDS-induced colitis.

According to the macroscopic examination of the colons sampled at Day-7, administration of DSS alone induced remarkable colon contraction (Fig. 2A). In contrast, DSS with H_2 in solution prevented the contraction. It is noteworthy that H_2-water alone did not show any effect on colon length (Fig. 2A). As shown in Fig. 2B, the decreased colon length caused by DSS was abrogated by supplementing DSS solution with H_2, and both changes were statistically significant.

Based on the results of histochemical analyses (Fig. 3), colonic tissue sections sampled from the mice treated with DSS alone were characterized by inflammatory cell infiltration into mucosa and extensive damage of epithelium along with crypt destruction (Fig. 3B and E). Remarkably, tissue sections of mice receiving DSS solution containing H_2 showed attenuation of inflammation, as demonstrated by the reduced infiltration of inflammatory cells into the mucosa and protection of epithelium and crypt structures (Fig. 3C and F). In addition, tissue sections from mice treated with control regular water or H_2-water alone indicated no signs of inflammation (control water, Fig. 3A and D; H_2-water, data not shown). Accordingly, the histological score of mice treated with DSS and H_2 in water was significantly lower than that of mice treated with DSS only (Fig. 3G). Therefore, it seems that H_2 did affect the susceptibility of mice to DSS-induced colitis by modulating inflammatory responses.

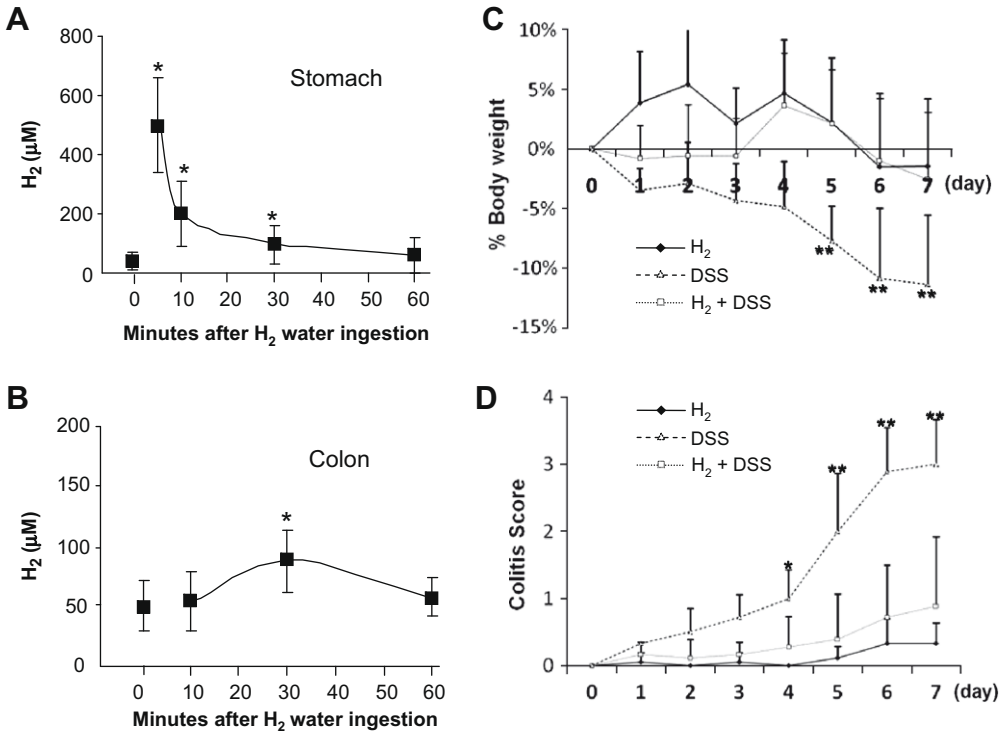

Fig. 1. Effects of H_2 on the clinical features of DSS-induced colitis in BALB/c mice. Temporal change of H_2 concentration in stomach (A) and in colon (B) after the oral administration of H_2-saturated water to mice (n = 5/each time point) was monitored. Distilled water saturated with H_2 (0.78 mM, ORP = −511 mV, pH 7.67) was applied to mice using a Popper® feeding needle (1 ml/mouse). Immediately after the sacrifice of animals in each group, H_2 concentrations of liquid substance present in stomach and colon were measured. To examine the effects of H_2 on DSS-induced colitis, H_2 (0.78 mM) water alone or 5% DSS with or without H_2-water were administered to mice (8 w males, n = 6/group) *ad libitum* for 7 days. The percent of body weight change (C) and colitis score (D) were measured on a daily basis. Data points and bars of H_2-water alone (♦), 5% DSS water alone (□), and 5% DSS including H_2-water (△) indicate means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$: Value is significantly different from the control group receiving DSS alone in the same measurement day (t-test).

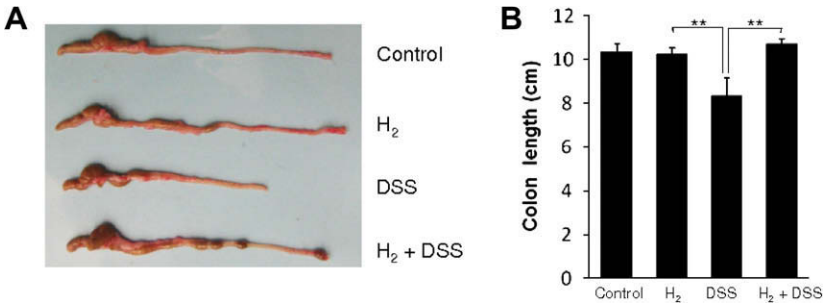

Fig. 2. H_2-mediated alleviation of colon contraction induced by DSS. To assess the severity of colitis, regular water, H_2 (0.78 mM) water alone, or 5% DSS with or without H_2-water were administered to mice for 7 days after which the colon length was measured. (A) Representative macroscopic features of the colons isolated from mice treated with regular water (Control), H_2-containing water (H_2), 5% DSS water (DSS) and 5% DSS containing H_2-water (H_2 + DSS) are shown. (B) The measurement of mean ± SD of colon length in each group at Day-7 is depicted. $^{**}p < 0.01$: Value differs significantly between the groups indicated by brackets (t-test).

It is plausible that H_2 dissolved in the DSS solution directly affected the biochemical activity of DSS which resulted in the attenuated pathophysiological outcomes of DSS-induced colitis. To rule this out, H_2 was administered in Ringer's solution (H_2, 0.78 mM; 1 ml/day/mouse) by peritoneal injection (i.p.) to the mice receiving DSS solution orally *ad libitum* (H_2-ip + DSS-po group). As a negative control, H_2-free Ringer's solution (1 ml/day/mouse, [i.p.]) was applied to the mice receiving DSS solution orally *ad libitum* (control DSS-po group). At the Day-8, the clinical readouts of these two groups were compared as follows: body weight (−3.40 ± 3.02% and −9.42 ± 6.74%; t-test; $P < 0.05$); colitis score (0.944 ± 1.04; DSS-po and 2.77 ± 0.86%; t-test; $P < 0.01$); and colon length (10.35 ± 0.59 and 7.13 ± 2.08 cm; t-test; $P < 0.01$), respectively. Therefore, even though H_2 was administered via a route (i.p.) different from that used for DDS administration (p.o.), H_2 still significantly prevented the onset of DSS-induced colitis, suggesting that H_2-mediated attenuation of pathophysiological outcomes of DSS-induced colitis is related to the action of H_2 on tissues and cells present in the colon.

Destruction of mucosal barrier caused by DSS is thought expose the lamina propria to luminal bacterial antigens, which, in turn, recruits and activates innate immune cells, including neutrophils and macrophages [8,9]. It is reported that elevated infiltration of F4/80-positive macrophages in the colonic mucosa is associated with severity of DSS-induced colitis in mice [25]. Therefore, we monitored the presence of F4/80-positive macrophage infiltration by immunofluorescent microscopy to determine the effect of H_2 administration. A remarkable increase in F4/80-positive macro-

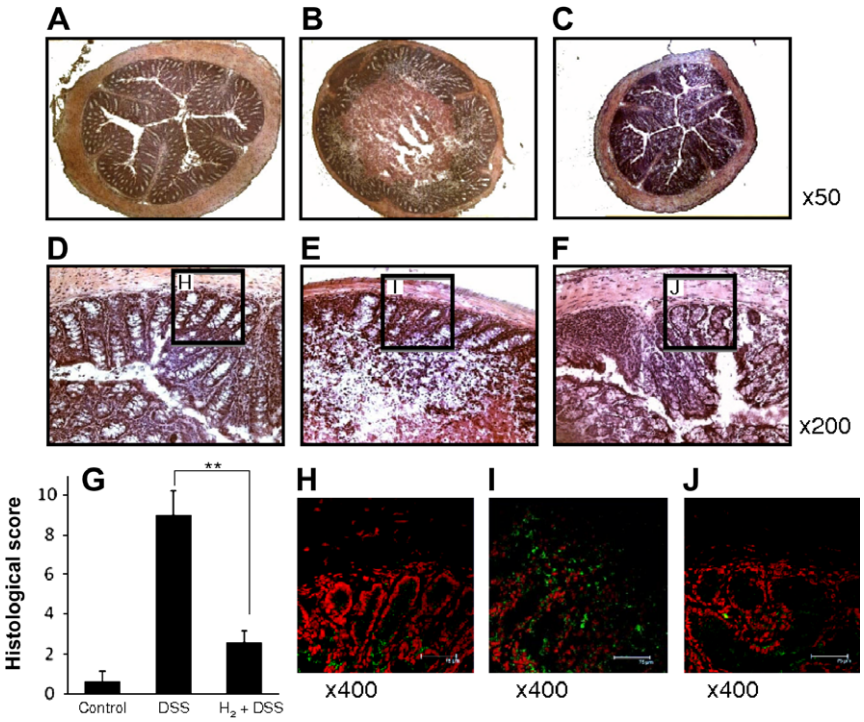

Fig. 3. Administration of H_2 attenuates the pathophysiological outcomes of DSS-induced colitis. To assess histological damage of the crypt of colon, control regular water or 5% DSS water solution with or without H_2 (0.78 μM) was administered to mice for 7 days. The section of colon sampled from sacrificed mice on Day-7 was stained with H & E. (A–F) Representative tissue section of mice treated with control regular water (A and D), 5% DSS water alone (B and E) and 5% DSS containing H_2-water (C and F) are shown at the magnification indicated in the figure. (A–C) Original magnification × 50; (D–E) Original magnification × 200. (G) Histological score was graded as described in Materials and methods. Data are the mean ± SD of five mice per group. $^{**}p < 0.01$: Value differs significantly between the groups indicated by brackets (*t*-test). (H–J) The macrophages infiltrating in colon epithelium and mucosa were stained with anti-F4/80 MAb conjugated with biotin, followed by FITC-avidin, followed by nuclear staining with propidium iodide. The colon sections sampled from mice receiving control regular water (H), 5% DSS water alone (I) and 5% DSS containing H_2-water (J) are shown. The fluorescent staining was carried out on the consecutive sections of D–F. The corresponding sites selected for fluorescent imaging shown in H–J are indicated by a square box shown in HE staining image of D–F, respectively.

phage infiltration was observed in the colon of mice receiving DSS alone (Fig. 3I) compared to control mice (Fig. 3H). However, the addition of H_2 in the DSS solution had a suppressive effect (Fig. 3J), indicating that H_2 reduced the migration of innate immune macrophages into the colonic mucosa.

To examine the effect of H_2-water on the colonic expression of proinflammatory cytokines induced by the administration of DSS, IL-1β (Fig. 4A), IL-12 (Fig. 4B) and TNF-α (Fig. 4C) present in the homogenates of colonic tissue were evaluated using ELISA. The increased the levels of these proinflammatory cytokines in colonic tissue of mice receiving DSS alone was significantly suppressed by the addition H_2, while H_2-water alone did not affect the levels of any cytokine tested, indicating that the H_2-mediated suppression of pathophysiological outcomes of DSS-induced colitis appeared to be derived from the down-regulation of inflammatory responses by H_2.

Discussion

The present study demonstrated that H_2 can attenuate DSS-induced colitis by down-regulating the expression of proinflammatory cytokines, as well as suppressing the infiltration of

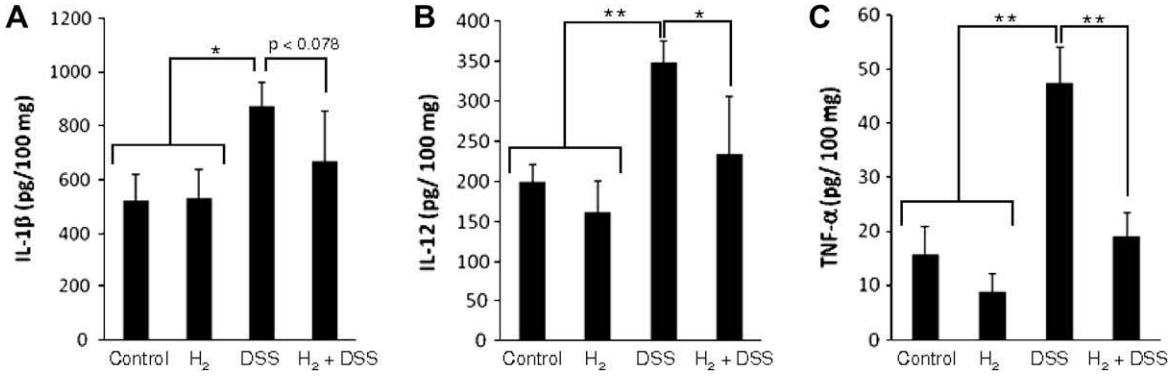

Fig. 4. Effects of H_2 on proinflammatory cytokines produced in the colon of DSS-induced colitis mice. To investigate whether H_2-water inhibits the inflammatory cytokine production caused by DSS, ELISA was performed on whole colonic tissue homogenates. (A–C) Colonic tissue homogenates were sampled from the sacrificed mice treated with control regular water (Control), H_2-water, 5% DSS water alone (DSS), or 5% DSS water containing H_2 (H_2 + DSS) for 7 days. IL1-β (A), IL-12 (B) and TNF-α (C) in colonic tissue homogenates were quantified by ELISA. Data are the mean ± SD of three mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$: Value differs significantly (*t*-test) between the groups indicated by brackets.

macrophages in the colon lesion. The administration of H_2 remarkably reduced the clinical symptoms of DSS-induced colitis, i.e., body weight loss, visible fecal blood and diarrhea, colitis score, and shortening of colon length. Histopathological evaluation further supported the effects of H_2 on the prevention of DSS-mediated destruction of epithelium crypt structure. Therefore, this is the first study to demonstrate that H_2 can suppress the production of tissue-destructive proinflammatory cytokines, including IL-1β, IL-12 and TNF-α, in colon.

However, reactive oxygen species (ROS) can activate TNF-α expression by up-regulation of the NF-κB signaling pathway [13], while, at the same time, it can activate NADPH-Oxygenase (NOX) expression that generates ROS from NADPH [14]. Therefore, both inflammation and oxidation processes are reciprocally related. Such complexity of cross reactions between ROS and proinflammatory mediators indicates that the H_2-mediated suppression of proinflammatory cytokines, as manifested in DSS-induced colitis, may also involve antioxidant effects by H_2. Nonetheless, since it has been suggested that various inflammatory mediators, especially IL-1β, IL-12, and TNF-α, are involved in the pathogenesis and exacerbation of human and mouse colitis [15–17,26], the suppression of these cytokines by H_2 appeared to play a role in the attenuation of DSS-induced colitis.

Furthermore, the administration of H_2 seemed to suppress the activation of macrophages because the migration of F4/80-positive macrophages in mice receiving DSS was remarkably suppressed by H_2 (Fig. 3). Macrophages are also one of the major inflammatory cells producing proinflammatory cytokines in human IBD [27,28]. The expression of TNF-α by RAW264.7 cells (a mouse macrophage cell line) in response to *in vitro* stimulation with LPS was significantly suppressed by the presence of H_2 dissolved in the culture medium (unpublished data). Therefore, it is assumed that the H_2-mediated attenuation of DSS-induced colitis is derived from the suppressive effects of H_2 on macrophage activation in response to luminal bacterial antigens, such as LPS.

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