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Prophylactic Oral Administration of Magnesium Ameliorates Dextran Sulfate Sodium-Induced Colitis in Mice through a Decrease of Colonic Accumulation of P2X7 Receptor-Expressing Mast Cells

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The number of patients with colitis has been increasing year by year. Recently, intestinal inflammation, as one of the factors for its onset, has been demonstrated to be induced by P2X7 receptor-mediated activation of colonic immune cells such as mast cells. Activation of P2X7 receptor (P2X7R) is known to be inhibited by divalent metal cations such as magnesium, but whether or not magnesium administration prevents/relieves colitis is unknown so far. Here, we report that oral (*per os* (p.o.)) administration of MgCl₂ and ingestion of commercially available magnesium-rich mineral hard water relieves dextran sulfate sodium (DSS)-induced colitis in mice. Colitis was induced through ingestion of a 3% (w/v) DSS solution *ad libitum* for 10 d. Brilliant blue G (BBG, a P2X7R antagonist), MgCl₂ or magnesium-rich mineral hard water was administered p.o. to mice *via* gastric intubation once a day or *ad libitum* from a day before DSS administration for 11 times or 11 d, respectively. DSS-treated mice exhibited a low disease activity index, a short colon and a high histological score compared to in control mice. As BBG (250 mg/kg, p.o.), administration of a MgCl₂ solution (100 or 500 mg/kg, p.o.) and *ad libitum* ingestion of the magnesium-rich mineral hard water (212 ppm as magnesium) partially, but significantly, attenuated the severity of colitis by decreasing the accumulation of P2X7-immunopositive mast cells in the colon. Therefore, prophylactic p.o. administration/ingestion of magnesium is considered to be partially effective to protect mice against DSS-induced colitis by inhibiting P2X7R-mediated activation/accumulation of colonic mast cells.

Key words magnesium; colitis; inflammatory bowel disease; mast cell; P2X7 receptor

Crohn's disease and ulcerative colitis are the two major clinical forms of inflammatory bowel disease (IBD), and are characterized by chronic and relapsing inflammation of the gastrointestinal tract.^{1,2} Although the pathogenesis of IBD is rather complex, evidence indicates the concomitant involvement of a genetic predisposition, environmental triggers, microbial agents, and immune dysfunction as essential factors for disease development.^{3,4} Recent studies have demonstrated that excessive activation of immune cells such as macrophages and helper T cells in the intestine and colon is a determinant of the course of IBD progress, because these activated immune cells release large amounts of inflammatory cytokines and bioactive molecules.^{5–7} Thus, these immune cells are accepted to be a potential target for the treatment of IBD.

Purinergic signaling is an important component of host defense against pathogens, mainly through activation of P2 receptors.⁸ Of them, P2X7 receptor (P2X7R), an ionotropic receptor, is expressed on epithelial cells and immune effector cells such as macrophages and mast cells, and is involved in the regulation of pro-inflammatory cytokines.^{9–12} Under inflammatory conditions, activation of immune cells and injury of cells induces excessive release of ATP, by which the local concentration of ATP in a tissue is drastically increased up to mM level,^{13,14} resulting in exacerbation of inflammation *via* activation of P2X7R.^{14,15} In fact, systemic blockade of P2X7R is reported to be effective for prevention of experimental colitis.^{16–18}

Kurashima *et al.* demonstrated that intraperitoneal (i.p.)

prophylactic administration of P2X7R-specific monoclonal antibodies apparently prevented the development of experimental colitis in mice by decreasing the accumulation of P2X7R-expressing mast cells in the colon.¹⁶ In addition, Marques *et al.* reported that i.p. administration of P2X7R antagonists exhibited partial, but significant, prophylactic effects on experimental colitis in rats,¹⁷ and almost the same finding was obtained in mice by Wan *et al.*¹⁸ Interestingly, in the study of Kurashima *et al.*, it was clearly indicated that ATP-P2X7R-mediated activation of colonic mast cells followed by their accumulation in the colons is the initiation phase of colitis development by induction of not only inflammatory cytokines, but also chemokines and leukotrienes for infiltration of neutrophils into the colon, leading to subsequent exacerbation of intestinal inflammation.¹⁶ Based on this, it is reasonable to consider that prophylactic oral *per os* (p.o.) administration of P2X7R blockers can prevent the development of experimental colitis by inhibiting the initiation of inflammation cascade induced by the P2X7R-mediated activation of colonic immune cells such as mast cells. However, there has been no report on the effect of p.o. administration of P2X7R blockers on experimental colitis.

It is well known that divalent metal cations (DMCs) have inhibitory effects on activation of P2X7R.^{19–22} Recently, we revealed that the inhibitory effects of DMCs were greater in the order of copper > zinc ≈ nickel ≈ magnesium > calcium for both human and mouse P2X7Rs, and that there was species differences in the effects of nickel and calcium, but not of

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the others, between humans and mice.²³⁾ Of these DMCs, we have focused on magnesium as a blocker for P2X7R activation because magnesium oxide, which gives magnesium chloride by reacting with gastric acid in the stomach, is used as a medicine for gastritis/gastric ulcers and constipation, guaranteeing safety and colonic delivery of magnesium *via* p.o. administration, and has a relatively greater inhibitory ability as to P2X7R activation (the IC_{50} =0.3–0.4 mM as $MgCl_2$), compared to the case of calcium (IC_{50} value=2 or more mM as $CaCl_2$).²³⁾ In addition, magnesium at concentrations found *in vivo* inhibited agonist-induced P2X7R activation *in vitro* experiments,^{19,21)} while the other P2XRs, except for P2X1R, are known to be potentiated or unaffected by DMCs.^{24–30)} Therefore, there is the possibility that DMCs might exhibit specific antagonism as to P2X7R without any effect on other P2XRs. Thus, we postulated that p.o. administration of magnesium might prevent P2X7R-mediated experimental colitis in mice.

On the market, there are a lot of mineral hard waters, the majority of which contains higher concentrations of calcium (180–530 ppm) than magnesium (50–100 ppm). In a commercially available refined deep-seawater (RDSW), in contrast, the concentration of magnesium is 212 ppm (8.72 mM), which is higher than that of calcium (73.8 ppm (1.85 mM)),³¹⁾ and is approximately 40-fold greater than the IC_{50} value for P2X7R, there being the possibility to prevent P2X7R activation expressed by colonic immune cells through its ingestion.

Therefore, we investigated whether or not p.o. administration of a $MgCl_2$ solution, and *ad libitum* ingestion of a $MgCl_2$ solution and RDSW had prophylactic effects on the development of dextran sulfate sodium (DSS)-induced experimental colitis in mice.

MATERIALS AND METHODS

Chemicals In this study, to induce experimental colitis, we used dextran sulfate sodium salt (colitis grade) (Cat. and Lot Nos.: 160110 and Q3526, respectively, molecular weight (MW)=36000–50000, MP Biomedicals, LLC, OH, U.S.A.). As commercially available magnesium-rich mineral hard water, RDSW (Amami's Water®/Water Hardness 1000, Ako Kasei Co., Ltd., Ako, Japan) was used, its ingredients being as follows: magnesium (212 ppm), calcium (73.8 ppm), sodium (60.1 ppm) and potassium (57.5 ppm).³¹⁾ Brilliant blue G (BBG) and $MgCl_2 \cdot 6H_2O$ were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.), and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. All other chemicals and reagents were purchased commercially and were of analytical grade requiring no further purification, except where otherwise noted.

Animals All experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (Permit No.: 16-13-033), and were performed according to the Guidelines for Animal Experimentation of Kyoto Pharmaceutical University. Female C57BL6/Ncr1 mice (7 or 8 weeks-old, Charles River, Yokohama, Japan) housed with food and water available *ad libitum* in a controlled environment with a 12h/12h light/dark cycle were used.

Induction of Colitis and Experimental Protocol Following a generally accepted protocol,³²⁾ to induce the experimental colitis in mice, they were allowed to ingest a 3% (w/v) DSS solution in distilled water *ad libitum* for 10 d (from days 0 to 10).

Administration Protocol BBG, a specific antagonist for P2X7R,³³⁾ and $MgCl_2$ were dissolved in distilled water at the concentrations of 10 mg/mL and 5 or 25 mg/mL, respectively, and then administered to mice p.o. at the doses of 250 mg/kg and 100 or 500 mg/kg, respectively, once a day from a day before initiation of DSS treatment (day –1) to day 9, *i.e.*, for 11 times. With the BBG group acting as a positive control, we preliminarily evaluated its inhibitory effect on DSS-induced colitis at 100, 250 and 500 mg/kg by p.o. and i.p. administration following the reports of Marques *et al.*¹⁷⁾ and Apolloni *et al.*,³⁴⁾ and its optimum dose was judged to be 250 mg/kg (p.o.) (data not shown). As for the doses of $MgCl_2$, we also confirmed in preliminary experiments that p.o. administration of a $MgCl_2$ solution at the dose of 500 or less mg/kg did not induce diarrhea in mice (data not shown), and thus its doses were determined to be 100 or 500 mg/kg.

In the cases of *ad libitum* ingestion, mice had free access to a $MgCl_2$ solution or RDSW as drinking water from day –1 to day 10, *i.e.*, for 11 d. The concentration of $MgCl_2$ in the drinking water was set at 800 ppm, which corresponded to the magnesium concentration in RDSW (212 ppm), as stated above, and the mice that ingested the $MgCl_2$ solution and RDSW were confirmed to exhibit no diarrhea (data not shown). For DSS-treated mice, $MgCl_2$ dissolved in 3% DSS solution and 3% DSS in RDSW were used. In the preliminary experiments, the daily drinking volume for these mice was approximately 4 mL/mouse/d (data not shown), and was almost comparable to in the control and DSS groups.

Assessment of Disease Activity Index The disease activity index (DAI) was expressed as the sum of the scores assigned to reflect the following: body weight loss (0=<5, 1=5–10, 2=11–15, and 3=16–20%), stool consistency (0=normal, 1=soft but still formed, 2=very soft, and 3=diarrhea), and rectal bleeding (0=no blood, 1=occult blood in stool, 2=trace of blood visible in stool, and 3=rectal bleeding).³²⁾ The minimum DAI score was 0, and the maximum one was 9. The DAI scoring was carried out by two independent investigators who were blind as to the experimental groups.

Macroscopic and Histological Evaluation At the end of DSS treatment (day 10), mice were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) containing 0.2% picric acid under deep anesthesia (pentobarbital sodium, 25 mg/kg, i.p.), and then the entire colon (from the caecum to the anus) was removed, and the colon length was determined. Thereafter, following additional overnight immersion in 4% paraformaldehyde at 4°C, the tissues were immersed in a 30% sucrose solution at 4°C overnight, and then cut into 10 μ m frozen sections and stained with haematoxylin and eosin (HE), photomicrographs being obtained under a light microscope (BX40; Olympus, Tokyo, Japan) equipped with a digital camera (moticam 2000; Shimadzu, Kyoto, Japan).

Histological scoring was performed on the basis of 3 parameters: the severity of inflammation, crypt damage and ulceration.³⁵⁾ Each parameter was scored as follows: inflammation: rare inflammatory cells in the lamina propria (score 0), increased number of granulocytes in the lamina propria (score 1), confluence of inflammatory cells extending into the submucosa (score 2), and transmural extension of the inflammatory infiltrate (score 3); crypt damage: intact crypts (score 0), loss of the basal one-third (score 1), loss of the basal two-

thirds (score 2), entire crypt loss (score 3), a change of the epithelial surface with erosion (score 4), and confluent erosion (score 5); ulceration: an absence of ulcer (score 0), 1 or 2 foci of ulcerations (score 1), 3 or 4 foci of ulcerations (score 2), and confluent or extensive ulceration (score 3). These values were added to give a maximal histological score of 11. A minimum of 2 sections of different parts of the distal colon per animal were scored. This scoring was carried out by two independent investigators who were blind as to the experimental groups.

Determination of Colonic Magnesium Contents According to the previous report,³⁶⁾ we measured the magnesium concentrations in mouse colonic feces obtained on day 10. Feces were subjected to a wet-ashing process involving nitric acid, perchloric acid and hydrogen peroxide at 200–250°C, and the ashed samples were dissolved in 5 mL of 5% nitric acid followed by the addition of 6 µL of a 40 ppm indium solution as an internal standard. The magnesium concentrations were measured using an inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7700X ICP-MS; Agilent Technologies, CA, U.S.A.). Standard curves for determination of magnesium concentrations were prepared by dilution of multi-element standard solution BM (Wako).

Immunohistochemistry As reported previously,³⁷⁾ after washing with ice-cold Sørensen's phosphate-buffered saline (Sørensen's PBS; 19 mM NaH₂PO₄ and 81 mM Na₂HPO₄, pH 7.4), the colonic free-floating sections were treated with a blocking buffer (1% donkey serum, 0.3% triton X-100, 0.3% bovine serum albumin and 0.05% sodium azide in PBS) at 4°C. Then, the sections were immunoreacted with primary antibodies in the blocking buffer for 3 d at 4°C, followed by incubation for a day at 4°C with secondary antibodies in the blocking buffer. The primary antibodies used were mouse anti-mast cell tryptase antibodies (1:50; Cat. No. sc-59585, Santa Cruz, CA, U.S.A.) and rabbit anti-P2X7 receptor antibodies (1:100, Cat. No. APR004, Alomone Labs., Jerusalem, Israel), and the secondary ones were donkey anti-mouse immunoglobulin G (IgG) antibodies conjugated with Alexa Fluor® 594 (1:1000; Cat. No. 21203, Life Technologies, CA, U.S.A.) and donkey anti-rabbit IgG antibodies conjugated with Alexa Fluor® 488 (1:1000; Cat. No. 21206, Life Technologies). For all immunostaining, a negative control, which was prepared by omitting the primary antibodies, was prepared, and the reproducibility of immunostaining was confirmed by assessing sections from 3 or 4 rats per immunostaining. The sections were mounted on glass slides and then enclosed using a Prolong® antifade kit (Life Technologies). Photomicrographs were obtained under a confocal laser microscope (LSM510META; Carl Zeiss, Jena, Germany). Fluorescence intensity was measured using the histogram program of the Photoshop software (Adobe Systems, CA, U.S.A.).

Statistical Analysis All data were expressed as means ± standard deviation (S.D.). Comparisons among groups, of which the populations were considered to have a normal distribution, were performed by means of the Tukey–Kramer test, and differences with a *p*-value of 0.05 or less were considered statistically significant.

RESULTS

Colitis Symptoms In DSS-treated mice, the DAI score increased day by day up to day 10, and significant differences

between the control and DSS groups were detected from day 3 (Fig. 1a). The mice administered BBG (250 mg/kg, p.o.) also showed a time-dependent increase of the DAI score, but the score was partially, but significantly, less than that in the DSS group, and the same profiles were found in the mice p.o. administered with MgCl₂ at the doses of 100 and 500 mg/kg, the values on days 9 and 10 in the 100 mg/kg group being significantly lower. With *ad libitum* ingestion of MgCl₂ (800 ppm) and RDSW, the DAI scores tended to be lower than those in the control group, but significant differences were not detected. Figure 1b shows the colon length in these mice. The mice treated with DSS had a significantly shorter colon length compared to the control group. As the BBG group, in contrast, the colon in the MgCl₂ p.o. administration groups, 100 and 500 mg/kg doses, was significantly greater than that in the DSS group. The *ad libitum* ingestion of RDSW significantly prevented shortening of the colon length, while there was no alteration in the colon length in the MgCl₂ *ad libitum* ingestion group. These findings indicated that p.o. administration of MgCl₂ prevented the progress of DSS-induced colitis partially as in the case of BBG administration, and almost the same prophylactic effect was found on *ad libitum* ingestion of RDSW, but it seemed to be less than in the case of MgCl₂ p.o. administration.

Figure 2 shows the magnesium contents in the colons of the control, DSS MgCl₂ and RDSW groups. Compared to the control group, the DSS group showed lower magnesium contents in the feces, this being considered to be the result of diarrhea. The p.o. administration MgCl₂ significantly increased the magnesium contents in the feces and there was no difference in them between the doses of 100 and 500 mg/kg. The *ad libitum* ingestion of RDSW increased the magnesium contents in mouse colonic feces, while there was tendency of an increase of them on *ad libitum* ingestion of MgCl₂ (800 ppm). Therefore, it is considered that the prophylactic effects of MgCl₂ p.o. administration and RDSW *ad libitum* ingestion on DSS-induced colitis are due, at least in part, to the increase in the magnesium content in mouse colons.

Histological Alterations To determine whether or not the p.o. administration of a MgCl₂ solution and *ad libitum* ingestion of RDSW prevent histological and immunohistochemical alterations induced by DSS treatment, we performed a second set of experiments. In this set, we confirmed that the DSS treatment induced the elevation of the DAI scores and the shortening of the colon, and these alterations were partially prevented by MgCl₂ p.o. administration (100 mg/kg) and *ad libitum* ingestion of RDSW (data not shown), as in the first set of experiments shown in Figs. 1 and 2.

As shown in Fig. 3, histological examination revealed that DSS treatment induced severe colitis, which was characterized by extensive disruption of the epithelium, massive loss of goblet cells and diffuse loss of crypts, and these changes partially disappeared on BBG administration. Similarly, the p.o. administration of MgCl₂ and the *ad libitum* ingestion of RDSW reduced the DSS-induced increase of histological scores with decreases of the inflammatory infiltration and crypt damage.

Colonic Accumulation of Mast Cells Finally, we evaluated the accumulation of mast cells in the colons of mice (Fig. 4). Treatment of mice with DSS increased significantly the number of mast cells in the colon with increase of P2X7R-immunopositive mast cells. As BBG, both the MgCl₂ p.o.

Fig. 1. Effect of MgCl₂ or RDSW Administration on the DAI Score (a) and Colon Length (b) in DSS-Treated Mice

To induce IBD, mice were treated with 3% DSS *ad libitum* for 10 d. A MgCl₂ or BBG solution was administered p.o. at the dose of 100 or 500 mg/kg, or 250 mg/kg, respectively, once a day from a day before initiation of the DSS administration for 11 times. The MgCl₂ solution (800 ppm) or RDSW was administered to mice *ad libitum* from a day before initiation of the DSS administration for 11 d. Evaluation of DAI was performed every day before the administration, and the colon length was measured on day 10. In panel b, a representative photograph of colons of three to four mice in each group is shown. Each point/bar represents the mean $\pm$ S.D. ($N=3-4$). Scale bar=1 cm. * $p<0.05$ (vs. control). † $p<0.05$ (vs. DSS).

Fig. 2. Effect of MgCl₂ or RDSW Administration on the Colonic Magnesium Content in DSS-Treated Mice

To induce IBD, mice were treated with 3% DSS *ad libitum* for 10 d. A MgCl₂ solution was administered p.o. at the dose of 100 or 500 mg/kg once a day from a day before initiation of the DSS administration for 11 times. The MgCl₂ solution (800 ppm) or RDSW was administered to mice *ad libitum* from a day before initiation of the DSS administration for 11 d. Colonic magnesium contents were measured on day 10 with an ICP-MS. Each bar represents the mean $\pm$ S.D. ($N=3-4$). * $p<0.05$ (vs. control). † $p<0.05$ (vs. DSS).

administration and *ad libitum* ingestion of RDSW decreased the colonic accumulation of mast cells and the proportions of P2X7R-immunopositive cells in them.

DISCUSSION

In this study, we demonstrated that in addition to p.o. administration of MgCl₂, *ad libitum* ingestion of RDSW, which contains a high concentration of magnesium, increased the colonic magnesium content and prevented partially, but significantly, the development of DSS-induced experimental colitis in mice, and that these prophylactic effects were due, in part, to inhibition of the activation/accumulation of colonic mast cells including P2X7R-immunopositive cells as the initiation phase of colitis development. Together with the finding that an increase in the numbers of P2X7R-expressing mast cells is found in the colons of not only mice with colitis but also patients with Crohn's disease,¹⁶⁾ we believe that these findings should provide a novel strategy for the development of a prophylactic approach for IBD symptoms such as Crohn's disease.

Previously, several approaches for prevention of development of experimental colitis focusing on P2X7R blockade were successful, but the antagonists/antibodies for P2X7R were administered *via* parenteral routes such as i.p. and intrarectal (i.r.) administration.¹⁶⁻¹⁸⁾ In addition, Marques *et al.* reported that twice intra-colonic administration of BBG at 40 mg/kg on days -1 and 3 could not prevent the development of DSS-induced colitis in rats, suggesting systemic P2X7R

Fig. 3. Effect of MgCl₂ or RDSW Administration on the Colonic Histology in DSS-Treated Mice

To induce IBD, mice were treated with 3% DSS *ad libitum* for 10 d. A MgCl₂ or BBG solution was administered *p.o.* at the dose of 100 mg/kg or 250 mg/kg, respectively, once a day from a day before initiation of the DSS administration for 11 times. RDSW was administered to mice *ad libitum* from a day before initiation of the DSS administration for 11 d. Colonic histology was evaluated on day 10 with HE staining. Representative photomicrographs of three mice in each group (a) and the quantitative results (b) are shown. Each bar represents the mean ± S.D. (*N*=3). Scale bar=50 μm. **p*<0.05 (vs. control). †*p*<0.05 (vs. DSS).

blockade was needed.¹⁷⁾ In our preliminary experiments, in contrast, we found that the *i.p.* administration of BBG at 50 and 100 mg/kg based on the dose of Marques *et al.* had no apparent effects on the DSS-induced IBD symptoms in mice (data not shown). Although differences in experimental conditions including species difference might explain this discrepancy, the details are not clear. However, as aforementioned, when the colonic concentrations of P2X7R blockers reach effective ones such as IC₅₀ values on their *p.o.* administration, it is reasonable to expect that they should show preventive effects on colitis by inhibition of ATP-P2X7R-mediated activation/accumulation of colonic mast cells, followed by induction of not only inflammatory cytokines, but also chemokines and leukotrienes for infiltration of neutrophils into the colon, leading to subsequent exacerbation of intestinal inflammation.¹⁶⁾ In fact, we found that as the *p.o.* administration of BBG, the *p.o.* administration of MgCl₂ and *ad libitum* ingestion of RDSW is effective to prevent the DSS-induced colitis. These prophylactic effects on the DSS-induced colitis in mice is considered to be due, at least in part, to inhibition of the colonic accumulation of P2X7R-expressing mast cells, because the accumulation is primed by activation of P2X7R expressed by them,¹⁶⁾ although there is a possibility that

mechanisms other than P2X7R inhibition might be involved in the BBG- and magnesium-mediated prevention of the DSS-induced colitis. Recently, we reported that there is no species difference in the sensitivity of P2X7R to magnesium between humans and mice.²³⁾ Overall, it is suggested that daily ingestion of magnesium might have a prophylactic effect in IBD patients through inhibition of inflammatory conditions including P2X7R-induced mast cell activation.

Administration of magnesium and RDSW prevented the development of the DSS-induced colitis symptoms and histological changes, but it was only partial, although its efficacy was statistically significant. In the studies of Marques *et al.*¹⁷⁾ and Wan *et al.*,¹⁸⁾ the *i.p.* administration of P2X7R antagonists had significant, but partial, preventive effects on experimental colitis, while on *i.r.* administration of the P2X7R antibodies, the colitis symptoms in mice recovered to those in the control group,¹⁶⁾ suggesting that activation of colonic mast cells *via* P2X7R activation has to be almost completely inhibited for clear prevention of colitis onset. In addition, colitis symptoms have been shown to be exacerbated by neutrophil infiltration induced by chemokines released from activated colonic mast cells.¹⁶⁾ Thus, it is considered that with our experimental protocols, *p.o.* administration of P2X7R blockers had partial

Fig. 4. Effect of MgCl₂ or RDSW Administration on Colonic Mast Cell Accumulation in DSS-Treated Mice

To induce IBD, mice were treated with 3% DSS *ad libitum* for 10 d. A MgCl₂ or BBG solution was administered p.o. at the dose of 100 mg/kg or 250 mg/kg, respectively, once a day from a day before initiation of the DSS administration for 11 times. RDSW was administered to mice *ad libitum* a day before initiation of the DSS administration for 11 d. The numbers of total and P2X7R-immunopositive mast cells were determined on day 10 by immunohistochemistry with counterstaining nuclei with Hoechst33258 (10 µg/mL). Representative photomicrographs of three mice in each group (a) and the quantitative results (b, c) are shown. Each bar represents the mean ± S.D. (N=3). Scale bar=50 µm. **p*<0.05 (vs. control). †*p*<0.05 (vs. DSS).

preventive effects on the DSS-induced colitis.

While *ad libitum* ingestion of RDSW partially prevented the development of DSS-induced colitis, *ad libitum* ingestion of a MgCl₂ solution, of which the concentration of 800 ppm was the same as that of RDSW, had no effect on the colitis

symptoms. One of the possible reasons is considered to be the lower magnesium concentration in the colon on *ad libitum* ingestion of MgCl₂ solution (800 ppm) group than that in the *ad libitum* ingestion of RDSW group, although details of this difference are unknown. In addition to magnesium, RDSW contains calcium at the concentration of 78.3 ppm (1.85 mM) as another DMC. In our previous study, calcium exhibited an inhibitory effect on P2X7R activation, and its inhibitory effect was the weakest among the DMCs examined, its IC₅₀ value being 2 or more mM.²³⁾ Thus, the concentration of calcium in RDSW is low to inhibit P2X7R activation, but interaction of magnesium and calcium such as an additive/synergistic effect might explain the difference between the MgCl₂ solution and RDSW. This might be supported by the findings that nevertheless the colonic magnesium concentration in RDSW-ingested mice was approximately half of that in MgCl₂-p.o. administered ones, their preventive effects on the DSS-induced colitis symptoms were almost the same (Figs. 2, 3). Further detailed investigations are needed to clarify this.

There is the possibility that administration of high doses of magnesium may induce diarrhea and hypermagnesemia. As described in Materials and Methods, p.o. administration of MgCl₂ solution at the dose of 500 mg/kg, and *ad libitum* ingestion of RDSW and MgCl₂ solution (800 ppm) did not cause diarrhea in mice (data not shown). Furthermore, under the condition shown in Fig. 1, the scores of stool consistency of mice in control, DSS, BBG, MgCl₂ (100 mg/kg, p.o.), MgCl₂ (500 mg/kg, p.o.), MgCl₂ (800 ppm, *ad libitum*) and RDSW (*ad libitum*) groups on day 10 were 0.25±0.50, 2.33±0.58, 2.33±0.58, 2.00±0.82, 2.75±0.5, 2.50±0.58 and 2.50±0.58, respectively, and on other days, there was no apparent increase in the scores by administration/ingestion of magnesium. Thus, we think that administration/ingestion of magnesium under the condition adopted in this study does not induce diarrhea in mice. As for hypermagnesemia, we did not measure the plasma magnesium concentrations in this study, but there seems to be no or negligible possibility to induce hypermagnesemia in MgCl₂- and RDSW-administered mice, because hypermagnesemia is known to be found in magnesium-administered patients with renal dysfunction and constipation.

Very recently, colonic inflammation with colonic accumulation of macrophages induced by ingestion of a high-fat diet endowed mice with insulin resistance in an intestinal Ccl2/Ccr2-dependent manner.³⁸⁾ Since macrophages are representative cells expressing P2X7R highly,⁹⁻¹²⁾ and Kurashima *et al.*, demonstrated that mast cells treated with ATP showed the expression of chemokines such as Ccl2,¹⁶⁾ it is suggested that administration of magnesium might inhibit the development of high-fat diet-induced insulin resistance, leading to prevention of onset of lifestyle-related type II diabetes mellitus.

CONCLUSION

Here, we demonstrated that p.o. administration and *ad libitum* ingestion of magnesium had preventive effects on the development of experimental colitis and that an underlying mechanism was inhibition of colonic mast cell activation *via* P2X7R. As there is no species difference in the inhibitory effect of magnesium between humans and mice,²³⁾ this prophylactic effect of magnesium on colitis might be obtained in IBD patients.

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