Efficacy of Dibenzoylmethane Derivatives in Protecting against Endoplasmic Reticulum Stress and Inhibiting Nuclear Factor Kappa B on Dextran Sulfate Sodium Induced Colitis in Mice

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We recently reported that some dibenzoylmethane (DBM) derivatives have a protective effect against endoplasmic reticulum (ER) stress and inhibit nuclear factor kappa B (NF-κB). The aim of this study was to evaluate the effect of DBM derivatives against dextran sulfate sodium (DSS)-induced colitis in mice. The DBM derivatives used in this study were 4,4′-dibromodibenzoylmethane that protects against ER stress, and, 4,4′-dichlorodibenzoylmethane that protects against ER stress and inhibits NF-κB. In each group, the presence of faecal occult blood, the disease activity index score (DAI score) and intestinal length were examined. Both of the DBM derivatives with protective effects against ER stress significantly improved occult bleeding of the colitis induced by DSS. The 4,4′-dichlorodibenzoylmethane significantly reduced the DAI score and inhibited the shortening of colon length, but the 4,4′-dibromodibenzoylmethane did not. These findings suggest that both the protective effect against ER stress and inhibitory effect on NF-κB are needed in the treatment of DSS-induced colitis. Therefore, the effect of 4,4′-dichlorodibenzoylmethane maybe beneficial in the therapeutic regulation of ulcerative colitis.

Key words ulcerative colitis; dibenzoylmethane derivative; endoplasmic reticulum stress; nuclear factor kappa B; dextran sulfate sodium-induced colitis

Inflammatory bowel disease (IBD), which includes ulcerative colitis and Crohn’s disease, comprises a group of multifactorial disorders of unknown etiology but has a high incidence that is widely distributed throughout the human population. Although the etiology of IBD is not clear at present, recent studies suggest that IBD is a disorder involving activation of leukocytes (macrophages, lymphocytes, and neutrophils) and their infiltration into the inflamed intestine. Enhanced endoplasmic reticulum (ER) stress has been implicated in various pathological situations including inflammation. Several reports have shown that the ER stress response is induced in association with the development of IBD. Bertolli et al. reports that when dextran sulfate sodium (DSS) was administered to mice deficient in the eukaryotic inositol-requiring transmembrane kinase-endoribonuclease-1β (IRE1β) gene to induce colitis, the time to onset was shorter (early onset) than that in the wild species. The IRE1β gene is known to be involved in signal transmission in ER stress. In addition, Kaser et al. reported that the transcription factor X-box binding protein 1 (XBP1), which is an important key to ER stress response, is involved in the genetic risk of onset of IBD in humans. These findings suggest a possible correlation between IBD and ER stress.

We reported that dantrolene, an antagonist of ryanodine receptors in the ER, and some antioxidants such as α-tocopherol, partly prevented tunicamycin-induced cell death in F9 homocysteine-induced ER protein null cells. Using this cell line, our group and others have reported that 103 plant-derived compounds and 200 synthetic compounds including dibenzoylmethane (DBM) derivatives, carbazole derivatives, and pyrimidine derivatives were screened previously, and some DBM derivatives improved cell viability after tunicamycin treatment at a level similar to that achieved by dantrolene in F9 Herp null cells. However, it remains unclear whether DBM derivatives that have protective effects against ER stress are effective against colitis.

Nuclear factor κB (NF-κB) is a dimeric transcription factor that induces the expression of genes involved in the inflammatory process. Activation of NF-κB has been observed in the mucosa of patients with ulcerative colitis, and butyrate has been shown to inhibit NF-κB activation in lamina propria macrophages of patients with ulcerative colitis. Previously, our group reported that HeLaNF-κB-3 cells, which are stable transformants with a NF-κB response element, obtained a reporter gene. These cells showed an approximately 6-fold higher activation of NF-κB with tumor necrosis factor alpha (TNF-α) stimulation. Moreover, some DBM derivatives inhibited NF-κB activity. However, it remains unclear whether DBM derivatives that have NF-κB inhibitory activity are effective against colitis.

Zhang and Kaufman suggest that an enhanced ER stress response contributed to the progression of inflammation through the activation of NF-κB. Therefore, the aim of this study was to evaluate the effect of DBM, which has protective effects against ER stress and inhibits NF-κB, on dextran sulfate sodium (DSS)-induced colitis in mice.
MATERIALS AND METHODS

Materials  The DBM derivatives used in this study were 4,4′-dibromodibenzoylmethane, and 4,4′-dichlorodibenzoylmethane. These compounds were synthesized as described previously.11,17  

Analysis of the Protective Effects against ER Stress  
The DBM derivatives were analyzed for their protective effects against ER stress in F9 homocysteine-induced endoplasmic reticulum protein (Herp) null cells, as described previously.11 In brief, F9 Herp null cells were developed as described previously and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% fetal bovine serum (FBS).8 ER stress was induced by treating the cells with tunicamycin (0.8 μg/ml; Sigma, St. Louis, MO, U.S.A.). Each DBM derivative was added (20 μM) to the cell together with tunicamycin. The cells were incubated for 48 h. Dantrolene (20 μM) and α-tocopherol (120 μM) were used as positive controls. Cell viability under ER stress was measured by 3-(4,5-dimethyl-2-thiazoyl)-2,4-diphenyl-2H tetrazolium bromide (MTT) assay (Nacalai Tesque, Kyoto) as described previously.19 Treatment with tunicamycin decreased viability to 40% of the non-stressed cells. Addition of α-tocopherol improved the viability to 50—70% in tunicamycin-treated cells. Furthermore, addition of dantrolene improved the viability to 70—80% in tunicamycin-treated cells. The evaluation was as follows: (−): if the activity was lower than that of α-tocopherol (cell viability; <50%); ( ): if the activity was equal to that of α-tocopherol (cell viability; 50—70%); (+): if the activity was equal to that of Dantrolene (cell viability; 70—80%); and (++) if the activity was more than that of dantrolene (cell viability; 80%).

Analysis of NF-κB Inhibition Activity  The DBM derivatives were also evaluated for NF-κB inhibition using HeLa(NF-κB)-3 cells, as described previously.15 In brief, 0.5×10⁵ cells of HeLa(NF-κB)-3 were seeded on a 96-well tissue culture plate. After 24 h, aliquots of test sample solution (20, 200 μM) were added into each well. After 1 h, 10 μl of 800 ng/ml tumor necrosis factor-α (TNF-α) was also added into each well. After 24 h, the medium of each well was recovered and assayed for secreted alkaline phosphatase (SEAP) activity according to the manufacturer’s protocol. The value of NF-κB inhibitory activity in this state was 0%. Before TNF-α was added, the value of NF-κB inhibitory activity was 100%. Partenolide (1 μM), a known NF-κB inhibitor,19 was used as a positive control.

Preparation of a Mouse Model of DSS-Induced Colitis  
The experimental protocols were approved by the Animal Care Committee of the Division of Research and Development, Meiji Dairies Corporation. Six-week-old female BALB/c mice (SLC, Shizuoka, Japan) were used for this study. The mice were housed in a room with a 12/12 h light/dark cycle at a constant temperature (22±3 °C), and acclimatized to the colony room for 1 week with free access to standard feed and water before the experiments were performed. They were then divided into four groups of eight mice (group 1: DSS-untreated group, group 2: DSS-induced colitis group, group 3: DSS+4,4′-dibromodibenzoylmethane treated group, and group 4: DSS+4,4′-dichlorodibenzoylmethane treated group). DSS (5000 molecular weight (MW), Nacalai Tesque, Kyoto, Japan) was dissolved in tap water to prepare a 5% DSS solution. Mice in groups 2 to 4 were allowed free access to this solution as drinking water for 7 d according to the method reported by Hirata et al.19 Group 1 was kept as the negative control and received drinking water without DSS throughout the experimental period. Each DBM derivative suspended in 1% Arabic gum (Wako, Osaka, Japan), was administered intraperitoneally (i.p.) to mice at a dose level of 100 mg/kg once daily on days 1 to 7. In the in vitro examination above, NF-κB inhibitory activity was measured as 20 μM and 200 μM, confirming NF-κB inhibitory activity. The calculation was done such the blood concentration of one mouse was 200 μM, and the dose was set at 100 mg/kg. In groups 1 and 2, 1% arabic gum was administered intraperitoneally to mice instead of DBM.

Evaluation of General Signals, Stool Consistency, Occult Blood and DAI Score of Colitis  On day 8 of the experiment, each animal was weighed to check the body weight, fecal appearance and the presence of fecal occult blood using a commercial kit (Shionogi, Osaka, Japan), followed by calculation of the DAI score according to the method reported by Murthy et al.20 (Table 1). The disease activity index of each mouse was equal to the average of the combined score for weight loss, stool consistency and occult blood.

Evaluation of Intestinal Shorting  On day 8 of the experiment, each mouse was sacrificed and the large intestines removed immediately to measure intestinal length and evaluate intestinal shortening.

Statistical Analysis  The results are presented as means±S.E. For comparison among multiple groups, the data were analyzed for homogeneity of variance using Bartlett’s test. When the variances were homogeneous, parametric Dunnett’s multiple comparison test was performed. When the variances were not homogeneous by Bartlett’s test, metric Dunnett’s multiple comparison test was performed. In all the statistical analyses, an associated probability (p value) of <5% was considered significant.

RESULTS AND DISCUSSION  
The structures of the DBM derivatives used in this study to protect against ER stress and inhibit NF-κB are shown in Table 2. While 4,4′-dibromodibenzoylmethane has protecting effects against ER stress (+ +) but poor NF-κB inhibitory activity (8%), 4,4′-dichlorodibenzoylmethane has both protecting effects against ER stress (+ +) and NF-κB inhibiting activity (50%).

Body weight of each group on days 1 and 8, occult/gross bleeding and stool consistency in each group on day 8 are shown in Table 3. Body weight on days 1 and 8 among

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Table 1. Disease Activity Index Score

<table>
<thead>
<tr>
<th>Score</th>
<th>Weight loss (%)</th>
<th>Stool consistency</th>
<th>Occult/gross bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>—</td>
<td>Normal</td>
<td>Guia (−)</td>
</tr>
<tr>
<td>1</td>
<td>1—5</td>
<td>Normal</td>
<td>Guia (−)</td>
</tr>
<tr>
<td>2</td>
<td>6—10</td>
<td>Loose</td>
<td>Guia (+)</td>
</tr>
<tr>
<td>3</td>
<td>11—15</td>
<td>Loose</td>
<td>Diarrhea</td>
</tr>
<tr>
<td>4</td>
<td>&gt;15</td>
<td>Diarrhea</td>
<td>Gross bleeding</td>
</tr>
</tbody>
</table>

Normal stool=well formed pellets; Loose stool=pasty stool that does not stick to the anus; Diarrhea=liquid stool that sticks to the anus.
these four groups were not significantly different. Drinking volume in the entire experimental period was no significant differences in any of the groups.

Occult/gross bleeding on day 8 were more severe in group 2 than in group 1. In groups 3 and 4, there were significant improvements compared to group 2. These data seem to suggest that ER stress may affect bloody excrement due to colitis. Stool consistency was looser in group 2 than in group 1. Compared to group 2, group 4 had significantly improved stool consistency, but group 3 showed no significant difference.

Figure 1 shows the DAI score of each group on day 8. The highest DAI score was observed in group 2. Compared to group 2, the score for DAI in group 3 tended to decrease, although this difference was not statistically significant. The score for DAI in group 4 was significantly lower than that in group 2. Tang et al. reported that IBD-associated diarrhea results from NF-κB-mediated tight junction protein internalization and increased paracellular permeability.\(^{21}\) In fact, in this study, we demonstrated that 4,4′-dichlorodibenzoylmethane, which possesses NF-κB inhibitory activity, improved stool consistency (Table 3). Our result suggested that the improvement in stool consistency was at least in part, associated with the inhibition of NF-κB activity.

Figure 2 shows the length of colon in each group. The length of the colon in group 2 was shortened significantly compared to that in group 1. The length of the colon in group 3 showed no difference compared to group 2, but improved significantly in group 4.

Recently, Zhang and Kauffman suggested that enhanced ER stress response contributed to the progression of inflammation through the activation of NF-κB.\(^{16}\) However, both the DBM derivatives used in this experiment possess protective activity against ER stress, but showed different effects towards colitis. For 4,4′-dichlorodibenzoylmethane which has effects against the DSS-induced colitis, namely a protective effect against ER stress and inhibitory effect against NF-κB.

The unfolded protein reaction (UPR) initiates the mechanisms that are required to resolve ER stress. Three proximal effectors of the UPR exist in cells that sense the accumulation of misfolded proteins. These include eukaryotic inositol-requiring transmembrane kinase-endoribonuclease-1 (IRE1α/β), pancreatic ER kinase (PERK) and activated transcription factor 6 (ATF6).\(^{22,23}\) An increase in the protein-folding load in the ER can lead to the accumulation of reactive oxygen radi-
trolled cell death largely through the transcription factor has on intestinal tract epithelium cells in the future.

In summary, the DBM derivative, 4,4′-dichlorodibenzoylmethane, with both a protective effect against ER stress and inhibitory effect on NF-κB, improved DSS-induced colitis in mice. These results indicate a possible beneficial effect of 4,4′-dichlorodibenzoylmethane on the therapeutic regulation of ulcerative colitis.

REFERENCES