学 位 論 文 の 要 旨

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学 位 論 文 名　MFG-E8 Attenuates Intestinal Inflammation in Murine Experimental Colitis by Modulating Osteopontin-dependent $\alpha_v\beta_3$-integrin Signaling

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論 文 内 容 の 要 旨

INTRODUCTION

Milk fat globule-epidermal growth factor 8 (MFG-E8) is a secreted glycoprotein that participates in phagocytosis of apoptotic cells by macrophages. MFG-E8 deficiency causes immune-mediated disorders due to abnormal tissue homeostasis. Although MFG-E8 is involved in several cell surface-mediated regulatory events and modulates immune responses in a variety of conditions, comparatively little is known regarding its functions in gastrointestinal tract disorders. We investigated whether this glycoprotein has a protective role in colitis by use of a recombinant MFG-E8 protein in an *in vivo* model of acute colitis and then elucidated its effects by evaluating colitis parameters. In addition, we conducted an *in vitro* investigation with macrophages. Our findings indicate that novel MFG-E8-mediated anti-inflammatory effects are generated via NF-κB inhibition through modulation of $\alpha_v\beta_3$-integrin signaling.

MATERIALS AND METHODS

Mouse wild-type and RGD mutant MFG-E8 proteins were prepared using a mammalian expression system. MFG-E8 coding regions without the signal peptide sequence were cloned

into the *EcoRI* and *XhoI* sites of a 6x HispTriEx-3hygro vector, then transfected into HEK293 cells using Lipofectamine 2000. At 48 hours after transfection, the cells were lysed and MFG-E8 protein was purified using Ni-NTA columns. Protein purity was checked by SDS-PAGE and western blotting assays, and functional activity was evaluated by examining the phagocytic potential of apoptotic cells with fluorescent microscopy. Next, 7-week-old male specific pathogen-free BALB/c mice were fed 2.5% DSS in drinking water for 9 days to create an experimental colitis model, while the control group received only normal drinking water.

Purified recombinant wild-type or mutant MFG-E8 (30 μg/kg) was then intravenously injected through the tail vein of mice in both groups, and colitis parameters, e.g., body weight, colon length, rectal bleeding, histology, and pro-inflammatory cytokine contents, were determined. To investigate the *in vitro* effects, recombinant MFG-E8 was added to LPS- or flagellin-treated macrophages, after which the levels of pro-inflammatory cytokines, NF-κB, and $\alpha_v\beta_3$-integrin associated protein-tyrosine-kinase phosphorylation were determined by EIA, western blotting, and immunoprecipitation methods, respectively. Furthermore, to examine binding of MFG-E8 to $\alpha_v\beta_3$-integrin, recombinant MFG-E8 was added to $\alpha_v\beta_3$-integrin coated plates and they were incubated for 2 hours at room temperature. After subsequent washing, the wells were treated with HRP-labeled anti-mouse MFG-E8 antibody and the resulting signals were measured using a plate reader. Since osteopontin (OPN) also binds to $\alpha_v\beta_3$-integrin, to assess the competitive binding of OPN and MFG-E8 to $\alpha_v\beta_3$, a fixed amount of exogenous OPN was allowed to bind with $\alpha_v\beta_3$-integrin in coated wells in the presence of various concentrations of recombinant MFG-E8. After incubation with HRP-labeled anti-mouse OPN antibody, the resulting signals were measured at a wavelength of 450 nm and analyzed using the Curve Expert 1.3 software package. All quantitative data are expressed as the mean ± SE. Student's t-test was used for statistical determinations. P values of <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

MFG-E8, which is expressed in normal mice in a tissue-specific manner, was significantly down-regulated in inflamed colons during the acute phase (days 1 to 9) of colitis. As for therapeutic potential, body weight loss of about 23% commenced on day 4 and continued to day 9 in mice who received DSS with PBS, while mice injected with the wild-type, but not those with mutant MFG-E8, showed significantly lower rates of body weight loss from day 4, which reached only 14% on day 9. Histological scores, and pro-inflammatory cytokine and myeloperoxidase (MPO) contents were also significantly decreased in MFG-E8-treated DSS-colitis mice. To elucidate the MFG-E8-dependent anti-inflammatory mechanism, we utilized an *in vitro* system using a lipopolysaccharide (LPS)-based inflammatory model with cultured murine peritoneal macrophages. Our results showed that LPS-induced pro-inflammatory cytokines as well as NF-κB activity in cells pre-treated with recombinant MFG-E8 were both significantly down-regulated. Using $\alpha_v\beta_3$-integrin si-RNA, we also confirmed that the exogenous effects of MFG-E8 were mediated by $\alpha_v\beta_3$-integrin. Moreover, we found that OPN, an extracellular-matrix protein with pro-inflammatory cytokine-like properties, was elevated in DSS-colitis mice *in vivo* as well as LPS-treated peritoneal macrophages *in vitro*. Since OPN shares the same integrin ($\alpha_v\beta_3$) with MFG-E8, we investigated the relative binding affinities of these two ligands for $\alpha_v\beta_3$-integrin and observed a higher level for MFG-E8. In addition, treatment with recombinant MFG-E8 significantly decreased OPN-induced cytokine production and NF-κB activation in the cells in a dose-dependent manner. Finally, our results showed that MFG-E8 inhibits NF-κB activity by modulating $\alpha_v\beta_3$-integrin-mediated focal adhesion kinase (FAK) phosphorylation.

CONCLUSION

Our results indicate that MFG-E8 has a novel therapeutic potential for treatment of colitis by modulating $\alpha_v\beta_3$-integrin signaling.