Ameliorating effect of anti-Fas ligand MAb on wasting disease in murine model of chronic colitis


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—Fas/Fas ligand (FasL) interaction has been implicated in the pathogenesis of various diseases. To clarify the involvement of Fas/FasL in the pathogenesis of intestinal inflammation, we investigated the preventive and therapeutic effects of neutralizing anti-FasL monoclonal antibody (MAb) on the development of chronic colitis induced by adoptive transfer of CD4+CD45RB-high T cells to SCID mice. Administration of anti-FasL MAb from 1 day after T cell transfer (prevention study) resulted in a significant improvement of clinical manifestations such as wasting and diarrhea. However, histological examination showed that mucosal inflammation in the colon, such as infiltration of T cells and macrophages, was not improved by the anti-FasL MAb treatment. In vitro studies showed that anti-FasL MAb did not inhibit IFN-γ production by anti-CD3/CD28-stimulated lamina propria CD4+ T cells but suppressed TNF-α and IL-1β production by lamina propria mononuclear cells. Therapeutic administration of anti-FasL MAb from 3 wk after T cell transfer also improved ongoing wasting disease but not intestinal inflammation. These results suggest that the Fas/FasL interaction plays a critical role in regulating systemic wasting disease but not local intestinal inflammation.

Fas/FasL; murine model; Crohn’s disease; therapy

INFLAMMATORY BOWEL diseases, including Crohn’s disease and ulcerative colitis, are chronic and wasting diseases whose etiology and pathogenesis are poorly understood. However, increasing evidence indicated an important role of immunological mechanisms (16). These diseases are characterized by a massive infiltration of T cells and macrophages in the inflamed mucosa and the production of proinflammatory cytokines such as IFN-γ, TNF-α, and IL-1β by these cells (5). In fact, TNF-α has been implicated in the pathogenesis of chronic colitis models (17), and recent clinical application of anti-TNF-α MAb (Infliximab) has shown a dramatic improvement of clinical symptoms in patients with Crohn’s disease (18, 24), although the precise mechanism of its action has not been fully understood.

Fas and its ligand (FasL) are members of the TNF receptor and ligand families (14, 20). Fas is constitutively expressed on various cells and in tissues, including lamina propria (LP) lymphocytes, macrophages, and intestinal epithelial cells (IEC), and is dramatically upregulated at the site of inflammation (1, 15). FasL is expressed on activated T cells and may be involved in T cell-mediated cytotoxicity against IEC (2, 15). In addition, nonlymphoid cells, such as IEC, can also express FasL, which may induce apoptosis in neighboring IEC and lead to breakdown of the epithelial barrier function (23). On the other hand, the Fas/FasL system also plays a critical role in downregulation of excessive immune responses (20, 21) and has been implicated in regulation of T cell apoptosis in the mucosa (3, 12). Therefore, it remains still controversial whether the Fas/FasL interaction plays an aggressive or a regulatory role in the pathogenesis of inflammatory bowel disease. In the present study, to clarify the role of Fas/FasL interaction, we evaluated the effect of a neutralizing anti-FasL MAb (MFL) on the development of chronic colitis by utilizing a murine model induced by adoptive transfer of CD4+CD45RB-high T cells to SCID mice.

MATERIALS AND METHODS

Mice. Eight-to-ten-week-old female BALB/c SCID mice and normal BALB/c mice were purchased from Japan Clea (Tokyo, Japan) and maintained under specific pathogen-free conditions in our animal facilities. Mice were treated according to the ethical guidelines of our institution.

Induction of colitis and MAb treatment. Colitis was induced by adoptive transfer of CD4+CD45RB-high T cells from normal BALB/c mice to BALB/c SCID mice, essentially as described previously (25). CD4+ T cells were isolated from the spleen of BALB/c mice by positive selection using an anti-CD4 (L3T4) MACS magnetic separation system (Miltenyi Biotec, Auburn, CA). The isolated CD4+ T cells were labeled with FITC-conjugated anti-CD45RB MAb (model

Address for reprint requests and other correspondence: T. Kanai, Dept. of Gastroenterology and Hepatology, Graduate School of Medicine, Tokyo Medical and Dental University, Tokyo 113-8519, Japan (E-mail address: taka.gast@tmd.ac.jp).

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To assess the production of TNF-α by CD4+ T cells and CD11b+ macrophages, we analyzed intracellular TNF-α by flow cytometry. Freshly isolated LPMCs were cultured with PMA (50 ng/ml; Sigma) and ionomycin (250 ng/ml; Sigma) to activate CD4+ T cells or LPS (1 μg/ml; Sigma) to activate macrophages in the presence of MFL (10 μg/ml) or control IgG for 6 h. Brefeldin A (10 μg/ml; Sigma) was added for the last 5 h to induce accumulation of cytokines in the Golgi body. The cells were then stained with anti-CD4-FITC (model L3T4; BD Pharmingen) or anti-CD11b-FITC (model M1/70; BD Pharmingen). After being washed, the cells were fixed and permeabilized for 15 min with 4% formaldehyde containing 0.1% saponin. For intracellular staining, the cells were incubated with anti-TNF-α-PE (model MP6-XT22; BD Pharmingen) and analyzed on a FACS Calibur.

To exclude the possibility that the MFL treatment induces nonspecific killing of FasL-expressing cells, we treated the colitic mice at 6 wk after the transfer of CD4+ CD45RBhigh cells with a single dose of MFL or control IgG (250 μg). After isolating LPMCs, we determined the number of apoptotic/dead cells by staining with annexin V-FITC/propidium (PI; MBL, Nagoya, Japan). To assess the production of TNF-α, IFN-γ, and IL-1β concentrations in the culture supernatant were determined by specific ELISA according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).

Cytokine ELISA. To measure IFN-γ production, LP CD4+ T cells (1 x 10^5) were cultured in 200 μl of complete medium supplemented with 1 μg/ml anti-CD28 MAb (model 37.51; BD Pharmingen) in 96-well flat-bottomed plates (Costar, Cambridge, MA) precoated with 10 μg/ml anti-CD3 MAb (model 145–2C11; BD Pharmingen) for 48 h. To measure TNF-α and IL-1β production, LPMCs (5 x 10^5) were cultured in 200 μl of complete medium supplemented with 10 μg/ml anti-CD3 MAb and/or 10 μg/ml MFL in 96-well flat-bottomed plates for 24 h. IFN-γ, TNF-α, and IL-1β concentrations in the culture supernatant were determined by specific ELISA.

Clinical scoring. Mice were weighed weekly and monitored for clinical appearance and signs of soft stool and/or diarrhea. Histological examination and immunohistochemistry.

Histological examination and immunohistochemistry. Tissue samples were fixed in PBS containing 6% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with hematoxylin and eosin. The degree of inflammation (histological score) was determined as previously described (6). Tissue samples for immunohistochemistry were embedded in optimal cutting temperature compound and snap frozen in liquid nitrogen. Six-micrometer sections were incubated with biotinylated anti-mouse CD4 MAb (model RM4-5), biotinylated anti-mouse F4/80 MAb (Serotec, Oxford, UK), and biotinylated isotype-matched control IgG (BD Pharmingen). Biotinylated antibodies were detected using Vectastain ABC kit (Vector, Burlingame, CA). The sections were finally counterstained with hematoxylin.

Preparation of LPMC. The entire dissected colon was opened longitudinally, washed with PBS, cut into small pieces, incubated with Ca2+ - and Mg2+ -free Hank’s balanced salt solution containing 1 mM dithiothreitol (Sigma, St. Louis, MO) for 30 min to remove mucus, and then serially incubated two times in medium containing 0.75 mM EDTA (Sigma) for 60 min. The supernatants from these incubations were collected and treated with 1 mg/ml collagenase A (Roche, Indianapolis, IN) in medium for 2 h. The cells were pelleted two times through a 40% isotonnic Percoll solution, and then mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation (40/70%) at the interface. CD4+ LP T cells were isolated from the LPMCs by positive selection using the anti-CD4 (L3T4) MACS magnetic separation system. The cells were >95% CD4+ as analyzed by flow cytometry.

Flow cytometry. Isolated LPMC were preincubated with an Fc-γ receptor-blocking MAb (model 2.4G2; BD Pharmingen) for 20 min, followed by incubation with FITC-conjugated anti-CD4 MAb for 30 min on ice. Flow cytometric analysis was performed on FACS Calibur (Becton Dickinson) equipped with CellQuest software.

To explore the contribution of Fas/FasL interaction to the development of chronic colitis, the recipient mice were treated with a neutralizing MFL or control hamster IgG for 8 wk from 1 day after T cell transfer. As shown in Fig. 1A, the control IgG-treated mice manifested progressive weight loss (wasting disease) during 3–8 wk after T cell transfer. In contrast, the MFL-treated mice did not show the progressive weight loss. The average body weight at 8 wk relative to the initial weight was 103.6 ± 3.5% for the MFL-treated mice and 87.5 ± 3.7% for the control IgG-treated mice (p = 0.009). The MFL-treated mice looked healthy during the observation period, and the assess-
ment of colitic symptoms by clinical scoring showed a significant difference ($P = 0.01$) between the MFL-treated mice and the control IgG-treated mice (Fig. 1B). Dissection at 8 wk showed that the colon from the control IgG-treated mice was enlarged (Fig. 2A) and had a greatly thickened wall (Fig. 2B). In contrast to the improvement of wasting and clinical symptoms by the MFL treatment (Fig. 1), these changes in the colon were not improved in the MFL-treated mice (Fig. 2, A and B). Histological examination showed massive inflammatory infiltrates composed of a large number of lymphocytes and macrophages/dendritic cells and a small number of neutrophils and eosinophils in the colon from the control IgG- or MFL-treated mice. No significant difference was observed by histological scoring of multiple colon sections, which was $6.5 \pm 0.7$ for the MFL-treated mice vs. $7.0 \pm 0.4$ for the control IgG-treated mice (Fig. 2C). Immunohistochemical staining also showed no apparent difference in the infiltration of CD4$^+$ T cells and F4/80$^+$ macrophages in the colon between the control IgG- and MFL-treated mice (Fig. 3). A further quantitative evaluation of CD4$^+$ T cell infiltration was made by isolating LPMC from the whole colon and flow cytometric analysis.
average CD4⁺ T cell number at 8 wk was 30.4 ± 5.9 × 10⁵ cells/colon for the MFL-treated mice vs. mice given 34.5 ± 7.2 × 10⁵ cells/colon for the control IgG-treated mice (P = 0.67, not significantly different). Because the numbers of CD4⁺ T cells recovered from the colon of these mice were comparably higher than the number of injected cells (3–5 × 10⁵), these results suggested that neither expansion nor deletion of CD4⁺ cells was affected by the MFL treatment. Finally, to exclude the possibility that the MFL treatment induced nonspecific killing of FasL-expressing cells, we treated the colitic mice at 6 wk after the transfer of CD4⁺ CD45RBhigh T cells with a single dose of MFL or control IgG (250 μg) and determined the percentages of apoptotic or dead cells in LP cells by the PI/annexin V staining. We confirmed that there was no significant difference between anti-FasL-treated and control IgG-treated LPMCs (anti-FasL vs. control IgG; annexin V⁺ PI cells, 9.4 ± 0.5 vs. 8.5 ± 1.0, P = 0.31; annexin V⁺ PI⁺ cells, 22.5 ± 0.9 vs. 20.9 ± 0.8, P = 0.56).

Effect of MFL on proinflammatory cytokine production. To explore the mechanism by which the MFL treatment ameliorated the wasting disease and clinical symptoms without affecting histological inflammation, we next examined the effect of MFL on the function of LPMC in vitro. As shown in Fig. 4A, LP CD4⁺ T cells from the control IgG-treated mice and those from the MFL-treated mice produced a comparable level of IFN-γ on stimulation with anti-CD3 and CD28 MAbS in vitro, indicating that the development and infiltration of IFN-γ-producing Th1 cells was not affected by the MFL treatment. Interestingly, production of TNF-α and IL-1β by LPMC from colitic mice was significantly inhibited by the addition of MFL (Fig. 4B).

We next examined the effect of MFL on TNF-α production by CD4⁺ T cells and CD11b⁺ macrophages by intracellular staining. As shown in Fig. 4C, TNF-α production by both CD4⁺ T cells and CD11b⁺ macrophages were significantly decreased by MFL to control IgG. These results suggested that the MFL treatment prevented the wasting disease possibly by inhibiting the TNF-α release from both LP CD4⁺ T cells and CD11b⁺ macrophages.

Therapeutic effect of MFL. We next evaluated whether a delayed MFL treatment could improve ongoing wasting disease. Since the wasting disease started 2–3 wk after T cell transfer and the infiltration of lymphocytes was already detectable at 2 wk in our model, we started the MFL treatment from 3 wk after T cell transfer. As shown in Fig. 5A, the delayed MFL treatment significantly improved weight loss compared with control IgG treatment at 8 wk after T cell transfer (P = 0.034). The clinical score at 8 wk was also significantly improved by the delayed MFL treatment (Fig. 5B, P = 0.015). Consistent with the prevention study, however, there was no histological difference in colonic sections between the MFL-treated mice and the control IgG-treated mice (data not shown).

DISCUSSION

In this study, we demonstrated that the administration of neutralizing MFL ameliorated wasting disease and clinical symptoms without affecting local inflammation in a murine model of chronic colitis. In vitro experiments suggested that the ameliorating effect of MFL might be mediated by suppression of TNF-α production by both LP CD4⁺ T cells and CD11b⁺ macrophages.

It has been suggested that the Fas/FasL interaction may regulate T cell apoptosis in the mucosa (3, 12). On the other hand, it has been reported that the Fas/FasL interaction is not only involved in T cell apoptosis but also in T cell costimulation (8, 21). Our present results
showed that the MFL treatment did not affect the number of CD4+ LP T cells collected from the inflamed colon. In addition, IFN-γ production by LP CD4+ T cells was not also affected by the MFL treatment.

Fig. 4. Effect of anti-FasL MAb on cytokine production. A: lamina propria CD4+ T cells were isolated from normal BALB/c mice and control-IgG- or anti-FasL MAb-treated mice at 8 wk after T cell transfer, cultured for 48 h in the presence of anti-CD3 and anti-CD28 MAb, and then culture supernatants were analyzed for concentration of IFN-γ by ELISA. Data are indicated as the means ± SE of 6 mice in each group. *P < 0.05 compared with control IgG. B: clinical score was determined at 8 wk after T cell transfer. Data are indicated as the means ± SE of 7 mice in each group. *P < 0.05.

Fig. 5. Therapeutic effect of anti-FasL MAb. Recipient mice were administered with anti-FasL MAb or control IgG for 5 wk starting at 3 wk after T cell transfer (arrow). A: change in body weight over time is expressed as the percent of original weight. Data are indicated as the means ± SE of 4 mice in each group. *P < 0.05 compared with control IgG. B: clinical score was determined at 8 wk after T cell transfer. Data are indicated as the means ± SE of 4 mice in each group. *P < 0.05.
These results suggested that the Fas/FasL interaction does not play a critical role in either costimulating T cell expansion and Th1 development or depleting infiltrating T cells in the mucosa.

Recent studies (9, 11, 19) have shown that FasL could induce TNF-α and IL-1β production by activated CD4+ T cells, macrophages, and dendritic cells in vitro. Therefore, the inhibitory effect of MFL on TNF-α and IL-1β production by LPMC in vitro is likely mediated by interruption of the interaction between FasL-expressing T cells and Fas-expressing macrophages and/or dendritic cells. Since both TNF-α and IL-1β have been implicated in the pathogenesis of wasting disease associated with colitis (5), the ameliorating effect of MFL on wasting disease might be mediated by suppression of TNF-α and IL-1β production in vivo. Consistent with this notion, we recently demonstrated that therapeutic administration of neutralizing anti-TNF-α MAb improved the ongoing wasting disease without improving the intestinal inflammation (26), just like MFL in the present study. Further studies are needed to determine the effect of MFL on the production of these cytokines in vivo.

It has been reported that FasL expression is massively upregulated in Fas-deficient T cells from lpr mice, and transplantation of bone marrow cells from lpr mice to syngeneic wild-type recipients induces graft-vs.-host-type disease-like wasting disease (4). In contrast, transplantation of Fas/FasL double-deficient bone marrow cells did not induce the wasting disease (27). This substantiates a critical contribution of the Fas/FasL interaction among bone marrow-derived leukocytes to the pathogenesis of wasting disease, which is consistent with our present observation in a murine colitis model.

Previous studies (2, 15, 23) have suggested that the Fas/FasL interaction may be involved in the IEC damage, since activated T cells or IEC expressed FasL and killed IEC. Although we have not directly assessed the IEC damage in this study, the ameliorating effect of MFL on some clinical symptoms, such as diarrhea, might be partly mediated by improvement of the IEC damage. Further studies using an oxazolone colitis model, which is characterized by the presence of epithelial cell loss and patchy ulceration, are needed to address this point. Alternatively, the perforin pathway should also be addressed, because it was previously reported (10, 22) to be, at least in part, involved in the T cell-mediated mucosal injury and might explain the minor therapeutic effects of MFL in this study.

In the present study, we also demonstrated that the MFL treatment could ameliorate ongoing wasting disease and improve clinical symptoms in a therapeutic protocol. This suggests that the FasL blockade with a humanized MFL (13) may be a novel strategy to improve the quality of life of the patient with chronic colitis such as Crohn’s disease.

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DISCLOSURES

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