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Involvement of TNF-Like Weak Inducer of Apoptosis in the Pathogenesis of Collagen-Induced Arthritis

Koichi Kamata,*† Seiji Kamijo,* Atsuo Nakajima,*§ Akemi Koyanagi, † Hisashi Kurosawa, † Hideo Yagita,2*† and Ko Okumura*

TNF-like weak inducer of apoptosis (TWEAK) is a type II membrane protein belonging to the TNF family that regulates apoptotic cell death, cellular proliferation, angiogenesis, and inflammation. However, the role of TWEAK in the pathogenesis of rheumatoid arthritis (RA) remains unclear. In this study, we have investigated the effect of neutralizing anti-TWEAK mAb on the development of collagen-induced arthritis (CIA), a well-established murine model of RA. Administration of anti-TWEAK mAb significantly ameliorated paw swelling, synovial hyperplasia, and infiltration of inflammatory cells. The levels of proinflammatory chemokines such as MCP-1 and MIP-2 in serum and knee joints were reduced by this treatment. Consistently, recombinant TWEAK enhanced the proliferation of MCP-1 and MIP-2 production by synovial cells from CIA mice in vitro. Histological examination also revealed that the treatment with anti-TWEAK mAb suppressed the development of small vessels in synovial tissues. These results indicated anti-inflammatory and angiogenic effects of the TWEAK blockade in CIA, which may be also beneficial for the treatment of RA. The Journal of Immunology, 2006, 177: 6433–6439.

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the synovial tissues in multiple joints that leads to bone atrophy and joint destruction. Although the etiology of RA has not been elucidated yet, cumulative evidence suggests that proinflammatory cytokines and chemokines play critical roles in the pathogenesis of RA (10). Especially, TNF-α plays a central role in regulating the expression and pathophysiological function of these molecules. Recent clinical investigations in which the activity of TNF-α in RA patients was blocked with a chimeric anti-TNF-α Ab have provided evidence that TNF regulates IL-6, IL-8, MCP-1, and vascular endothelial growth factor production (11–13), recruitment of immune and inflammatory cells into joints (14, 15), angiogenesis (16), and increased blood levels of matrix metalloproteinase-1 and -3 (17). However, the contribution of TWEAK to the pathogenesis of RA has not been determined yet.

Various disease models for RA have been developed to elucidate the pathogenesis of RA. Among those, collagen-induced arthritis (CIA) is a well-established murine model of RA, which can be induced by intradermal injection of type II collagen (CII) emulsified with adjuvant in DBA/1 mice (18). CIA model has also been commonly used to explore novel therapeutic strategies against RA (19, 20). In this study, we have examined the effect of a neutralizing mAb against murine TWEAK on the development of CIA to explore the involvement of TWEAK in the pathogenesis of RA.

Materials and Methods

Mice

Male DBA/1 mice were purchased from the Charles River Breeding Laboratories and were used at 7–9 wk of age. All mice were housed in the animal care facilities and were used in accordance with the guidelines of Committee on Animals of Juntendo University School of Medicine.

Reagents

The neutralizing anti-mouse TWEAK mAb (MTW-1, rat IgG1) was generated in our laboratory as previously described (9). MTW-1 did not react with transfecteds expressing related TNF family members such as TNF-α, FasL, and TRAIL (9). Control rat IgG was purchased from Sigma-Aldrich. Recombinant soluble mouse TWEAK was purchased from R&D Systems.
Induction of CIA, Ab treatment, and clinical assessment of arthritis

Male DBA/1 mice were immunized intradermally at the tail base with 200 μg of bovine type II collagen (Collagen Research Center, Tokyo, Japan) in 0.5% H37Ra Mycobacterium tuberculosis (Difco). Twenty-one days after the first immunization mice were boosted by intradermal injection with 200 μg of bovine CII with IFA. From day 21, two groups of DBA/1 mice were administered i.p. with 250 μg/mouse of either anti-TWEAK mAb (MTW-1) or control rat IgG everyday for 2 wk. We measured the levels of anti-TWEAK mAb in the serum on days 24, 27, 30, and 33 by ELISA. Recombinant TWEAK (200 ng/ml) was coated onto microtiter plates (Immunon 2; Dynatech) overnight at 4°C. After blocking with 1% BSA, serum samples were added and incubated for 1.5 h at 37°C. After washing, biotin-conjugated anti-rat IgG1 was added and incubated for 1 h at 37°C. After washing, Ab binding was visualized using Vectastain ABC kit (Vector Laboratories) and o-phenylenediamine (Sigma-Aldrich). MTW-1 was added to each plate in serial dilution and a standard curve was constructed. The titers of standard serum from untreated CIA mice (standard serum) was added to each plate in serial dilution and a standard curve was constructed. The titer of standard serum from untreated CIA mice (standard serum) was added to each plate in serial dilution and a standard curve was constructed. The titer of standard serum was defined as 1 U and the Ab titers of serum samples were determined by the standard curve.

Measurement of proinflammatory chemokines

Serum samples were collected at 7 days after the second immunization, and the levels of MCP-1 and MIP-2 were measured by using specific ELISA kits for MCP-1 and MIP-2 according to the protocols recommended by the manufacturer (Opt-EIA for MCP-1; BD Pharmingen; and Quantikine for MIP-2; R&D Systems).

Seven days after the second immunization, the knee joints were opened from the patellar tendon and washed in 500 μl of complete medium, and incubated for 1 h at room temperature to allow the elution of chemokines as described previously (24). Supernatants were then removed and stored at −20°C until the assay. MCP-1 and MIP-2 levels were measured using the ELISA kits as described above.

To examine the ability of synovial cells to secrete MCP-1 and MIP-2, the synovial cells were prepared as described above. An equivalent number of cells (2.5 × 10^6/well) in 500 μl of αMEM containing 10% FCS was plated onto each well of a flat-bottom 24-well microtiter plate and allowed to adhere for 24 h at 37°C. Serially diluted recombinant TWEAK containing 20 μg/ml anti-TWEAK mAb or control IgG were prepared in the medium, and 500 μl each was added to the wells, resulting in 1 ml of culture. The plate was incubated for 72 h at 37°C. Then, MCP-1 and MIP-2 levels in the supernatants were measured by ELISA as described above.

Proliferation of synovial cells

Synovial cells fed 1 day before use were trypsinized and resuspended at 1 × 10^6 cells/ml in α-MEM with 10% FCS. An equivalent number of cells (5 × 10^5/well) in 50 μl were plated onto each well of a flat-bottom 96-well microtiter plate and allowed to adhere for 24 h at 37°C. Serially diluted recombinant TWEAK containing 20 μg/ml anti-TWEAK mAb or control IgG were prepared in the medium, and 50 μl each was added to the wells, resulting in 100 μl of culture. The plate was incubated for 72 h at 37°C. 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium salt (WST-8; Dijindo) was added (10 μl/well) to each well. The plate was further incubated at 37°C for 3–5 h until color development. Absorbance was measured at 450 nm on a Microtiter manager (Bio-Rad).

Histological and immunohistochemical analyses

For histological analysis, hind limbs were removed at 14 days after the second immunization, fixed in 10% formalin neutral buffer solution, decalcified in 10% EDTA, embedded in paraffin, sectioned, and stained with H&E.

Synovial vessels were identified by immunohistochemical staining of the paraffin sections with anti-CD31 mAb (BD Pharmingen) as described (25). Endogenous peroxidase was blocked for 10 min with 3% H2O2, followed by digestion with trypsin (Sigma-Aldrich) for 30 min at room temperature. Blocking was performed for 30 min with 10% normal goat serum and 1% BSA in PBS at room temperature. The sections were incubated with anti-CD31 mAb or isotype-matched control IgG1/20 in 1% BSA/PBS overnight at 4°C in humidified chamber. After three 5-min washings with PBS, the slides were incubated with peroxidase-conjugated goat anti-rat IgG Ab (4 μg/ml; Cedarlane) for 30 min at room temperature. After washing with PBS, the slides were incubated with 0.2 mg/ml diaminobenzidine and 0.004% H2O2 for 10 min, and then counterstained with methyl green. Vessel density was determined by counting the number of CD31-expressing vessel-like structures in the synovial tissue under microscopy as previously described (26). Vessel-like structures were classified by size into small vessels (<10-μm diameter), medium vessels (10- to 50-μm diameter), and large vessels (>50-μm diameter).

Statistical analysis

All comparisons between two groups were analyzed using Student’s unpaired t test. Values of p < 0.05 were considered significant.

Results

Anti-TWEAK mAb prevents the development of CIA

To investigate the functional contribution of TWEAK to the development of CIA, we first examined the effect of a neutralizing anti-TWEAK mAb in vivo. DBA/1 mice were immunized twice with bovine CII emulsified with an equal volume of adjuvant to elicit CIA. Two groups of mice were i.p. administrated with either 250 μg of anti-TWEAK mAb or 250 μg of control IgG every day from the day of second immunization for 2 wk. The mice treated with control IgG developed severe arthritis manifested by swelling of paws and joint ankylosis (Fig. 1A, Table I). In contrast, the
administration of anti-TWEAK mAb significantly inhibited these clinical manifestations (Fig. 1A, Table I). We also compared the effects of an irrelevant monoclonal rat IgG1 and the polyclonal control rat IgG on the clinical course of CIA, but there was no significant difference between these two preparations (Fig. 1B). Histological examination showed significantly reduced levels of synovial hyperplasia and infiltration of inflammatory cells in the anti-TWEAK mAb-treated mice compared with the control IgG-treated mice (Fig. 1C). When CIA mice were treated with anti-TWEAK mAb from day 7 after second immunization, it was not effective (data not shown). This result suggested that TWEAK/Fn14 interaction contributed to the initiation but not progression of arthritis. These results indicated a substantial role for TWEAK in the pathogenesis of CIA.

Anti-TWEAK mAb does not affect secondary B cell and T cell responses

CIA is a model of autoimmune arthritis in which both T cell-mediated autoimmune response against CII and B cell-mediated production of CII-specific autoantibodies play an essential role (18–20). To investigate whether the treatment with anti-TWEAK mAb had any effect on these responses, we first examined serum levels of CII-specific autoantibodies. As shown in Fig. 2A, the levels of CII-specific Abs (IgG1, IgG2a, and total IgG) in the sera of anti-TWEAK mAb-treated mice were not significantly different from those of control IgG-treated mice. These results indicated that the anti-TWEAK mAb treatment did not affect the production of CII-specific autoantibodies in response to the secondary immunization.

Next, we examined the effect of anti-TWEAK mAb on the T cell-mediated immune response. We isolated DLN cells from the anti-TWEAK mAb- or control IgG-treated mice at 7 days after the second immunization and restimulated the DLN cells with CII in vitro. As shown in Fig. 2B, no significant difference in the proliferative response was observed between the DLN cells from anti-

**Table I.** Effect of anti-TWEAK mAb treatment on CIA

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Treatment</th>
<th>Incidence</th>
<th>Arthritis Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control IgG</td>
<td>10/10</td>
<td>6.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Anti-TWEAK mAb</td>
<td>10/10</td>
<td>2.8 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Control IgG</td>
<td>10/10</td>
<td>6.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Anti-TWEAK mAb</td>
<td>10/10</td>
<td>3.5 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>Control IgG</td>
<td>10/10</td>
<td>7.1 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Anti-TWEAK mAb</td>
<td>10/10</td>
<td>4.0 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> CIA was induced as described in Fig. 1 and treated with either 250 μg of control IgG or anti-TWEAK mAb everyday for 2 wk. Values represent the mean ± SEM from each group of 10 mice.

<sup>b</sup> Statistically different from the control IgG-treated group (p < 0.05).
TWEAK mAb-treated mice and those from control IgG-treated mice. We also examined the levels of IFN-γ in the culture supernatants, but no significant difference was observed between the two groups (data not shown). Furthermore, in vitro treatment with anti-TWEAK mAb also did not significantly affect the CII-specific proliferative response of DLN cells from CII-immunized mice, whereas anti-B7.1 and anti-B7.2 mAbs efficiently inhibited it (Fig. 2C). These results indicated that the anti-TWEAK mAb treatment did not affect the secondary CII-specific T cell response. Because the anti-CII serum Abs or the CII-specific proliferative response of DLN cells was not detectable after primary immunization, the effect of anti-TWEAK mAb on primary immune responses could not be examined in the present model.

Anti-TWEAK mAb inhibits the production of proinflammatory chemokines in serum and joint

Previous reports have revealed that TWEAK receptor is expressed on endothelial cells and RA synoviocytes, and that production of MCP-1 and IL-8 were induced upon TWEAK stimulation (5, 7). In light of these previous findings, we investigated the levels of proinflammatory chemokines in the sera and swollen joints. We measured MCP-1 and MIP-2 in the sera collected from anti-TWEAK mAb- or control IgG-treated mice at day 7 after the booster immunization. The serum levels of MCP-1 and MIP-2 in the anti-TWEAK mAb-treated mice were significantly lower than those of the control IgG-treated mice. (Fig. 3). We also measured the MCP-1 and MIP-2 levels in the joints collected from anti-TWEAK mAb- or control IgG-treated mice at day 7 after the booster immunization. Although MIP-2 was undetectable, the level of MCP-1 in the anti-TWEAK mAb-treated mice was significantly lower than that in the control IgG-treated mice (Fig. 3). These results suggested that the reduced production of proinflammatory chemokines was at least partly responsible for the ameliorating effect of TWEAK mAb on MCP-1 production by synovial cells in vitro. Synovial cells were prepared from CIA mice and cultured with the indicated concentrations of recombinant mouse TWEAK in the presence or absence of anti-TWEAK mAb (■) or control IgG (□) (10 µg/ml each) for 24 h. The levels of MCP-1 (A) and MIP-2 (B) in the supernatants were measured by ELISA. Data are represented as the mean ± SD of triplicate samples. Similar results were obtained in three independent experiments. *, Significantly different (p < 0.05).
effect of anti-TWEAK mAb on CIA such as the reduced infiltration of inflammatory cells and edema.

**TWEAK induces MCP-1 and MIP-2 secretion by synovial cells**

To further substantiate the contribution of TWEAK to the production of proinflammatory chemokines in arthritic lesions, we next examined whether TWEAK could induce the secretion of MCP-1 by CIA mouse-derived synovial cells in vitro. As shown in Fig. 4A, recombinant TWEAK induced the secretion of MCP-1 in a dose-dependent manner, which was abrogated by anti-TWEAK mAb. Furthermore, recombinant TWEAK could also induce secretion of MIP-2 (Fig. 4B). These results suggested that TWEAK was involved in the secretion of proinflammatory chemokines by synovial cells in the joints of CIA mice.

**TWEAK enhances proliferation of synovial cells**

The histological examination (Fig. 1C) showed that the synovial hyperplasia in knee joint was significantly ameliorated by anti-TWEAK mAb. We have detected the expression of TWEAK on synovial cells from CIA mice by FACS analysis. In contrast, CD45-negative synovial cells from normal mice did not express TWEAK (data not shown). Because we previously observed that TWEAK enhanced the proliferation of endothelial cells (5), we next examined whether TWEAK could induce the proliferation of synovial cells in vitro. As shown in Fig. 5, recombinant TWEAK enhanced the proliferation of synovial cells in a dose-dependent manner, which was abrogated by anti-TWEAK mAb. These results suggested that TWEAK might be also involved in the proliferation of synovial cells, leading to synovial hyperplasia in the joints of CIA mice.

**Involvement of TWEAK in synovial angiogenesis**

It has been demonstrated that TWEAK induces angiogenesis in vivo (3, 6). It is also well accepted that angiogenesis plays an important role in the pathogenesis of RA (10). Therefore, we also examined the effect of anti-TWEAK mAb on the angiogenesis in inflamed synovial tissue of CIA mice by immunohistochemical staining of vessels with anti-CD31 mAb. As shown in Fig. 6, the density of small vessels was significantly decreased in the anti-TWEAK mAb-treated mice compared with the control IgG-treated mice. These results suggested that TWEAK was also involved in the angiogenesis in synovial tissue of CIA mice.

**Discussion**

In this study, we demonstrated the involvement of TWEAK in the pathogenesis of CIA. The administration of anti-TWEAK mAb ameliorated the clinical and histological manifestations of inflammatory arthritis (Fig. 1). Elevation of proinflammatory chemokines in the serum and joints were significantly inhibited by the anti-TWEAK mAb treatment (Fig. 3), which appeared to result in the beneficial effect. It has been demonstrated that TWEAK induced the secretion of chemokines such as IL-8 and MCP-1 by HUVEC (5), and that TWEAK induced the mRNA for IL-8 and MCP-1 in RA synovial cells (6). It has also been reported that the reduction in synovial lining-layer macrophages and sublining inflammatory cell infiltrates was accompanied by significantly diminished expression of MCP-1 in synovial tissue (27), and IL-8 is known to induce transendothelial migration of neutrophils to sites of inflammation (28, 29). In light of these previous findings, we speculated that not only endothelial cells but also synovial cells might be
responsible for the TWEAK-mediated chemokine production observed in this arthritis model. Consistent with this notion, our present results have shown that TWEAK induces secretion of MCP-1 and MIP-2 by synovial cells from CIA mice in a dose-dependent manner (Fig. 4).

Although TWEAK induces cell death in some tumor cell lines (1, 4, 30), it has been demonstrated that TWEAK induces proliferation of endothelial cells (5, 6). We now demonstrated that TWEAK induced proliferation of synovial cells in a dose-dependent manner (Fig. 5). Although some other TNF family members and receptor family such as FasL and TRAIL induces apoptosis of synovial fibroblasts (31–33), our present results indicated that TWEAK induces proliferation rather than apoptosis of synovial cells. Thus, TWEAK might be directly involved in the synovial hyperplasia in the joints of CIA mice. Consistent with this notion, anti-TWEAK mAb ameliorated the synovial hyperplasia in CIA mice (Fig. 1).

It has also been reported that TWEAK not only induces proliferation and migration of endothelial cells in vitro but also acts as a potent inducer of angiogenesis in vivo (3, 6). We now demonstrated that the density of small vessels in synovial tissue of CIA mice was significantly reduced by anti-TWEAK mAb treatment (Fig. 6). Because the disease progression of CIA is directly correlated with the level of synovial angiogenesis (34, 35), the reduction of small vessel density might be also responsible for beneficial effect of anti-TWEAK mAb treatment.

Various TNF, TNFR family molecules, including TNF-α (21, 36–41), TRAIL (42), Fas (43), CD40 (44), and OX40L (45), have treatment with anti-CD40 mAb exacerbated CIA by enhancing proliferation of endothelial cells (46). We now demonstrated that the anti-TWEAK mAb treatment did not affect the production of IFN-γ and anti-CII IgG2a production (44). In this study, the treatment with anti-OX40L mAb ameliorated CIA by suppressing IFN-γ and anti-CII IgG2a production (45). In contrast, in vivo treatment with anti-CD40 mAb exacerbated CIA by enhancing IFN-γ and anti-CII IgG2a production (44). In this study, the treatment with anti-TWEAK mAb in vivo or in vitro did not affect the proliferative response of and IFN-γ production by DLN cells upon restimulation with CII in vitro (Fig. 2, B and C, and data not shown), indicating that the activation of CII-reactive T cells was not inhibited by the anti-TWEAK mAb treatment. Furthermore, the anti-TWEAK mAb treatment did not affect the production of anti-CII IgG1 or IgG2a (Fig. 2A). These results showed that TWEAK was not involved in the activation of pathogenic T cells and B cells.

In conclusion, the administration of anti-TWEAK mAb ameliorated CIA through anti-inflammatory and antiangiogenic actions. Thus, the blockade of TWEAK may be a novel strategy for the treatment of RA and possibly other chronic inflammatory diseases.

Disclosures
The authors have no financial conflict of interest.

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9. Nakayama, M., N. Harada, E. Kominami, K. Okumura, and H. Yagita. 2003. Characterization of monoclonal antibodies and receptor family such as FasL and TRAIL induces apoptosis of synovial fibroblasts (31–33), our present results indicated that TWEAK induces proliferation rather than apoptosis of synovial cells. Thus, TWEAK might be directly involved in the synovial hyperplasia in the joints of CIA mice. Consistent with this notion, anti-TWEAK mAb ameliorated the synovial hyperplasia in CIA mice (Fig. 1).

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Various TNF, TNFR family molecules, including TNF-α (21, 36–41), TRAIL (42), Fas (43), CD40 (44), and OX40L (45), have been implicated in the pathogenesis of CIA. Accumulating evidence supports the concept that a dominance of either Th1 or Th2 is associated with distinct manifestations of autoimmune diseases, and some therapies that induce a shift from Th1 to Th2 or vice versa may result in amelioration of diseases (46–48). In vivo treatment with anti-OX40L mAb ameliorated CIA by suppressing IFN-γ and anti-CII IgG2a production (45). In contrast, in vivo treatment with anti-CD40 mAb exacerbated CIA by enhancing IFN-γ and anti-CII IgG2a production (44). In this study, the treatment with anti-TWEAK mAb in vivo or in vitro did not affect the proliferative response of and IFN-γ production by DLN cells upon restimulation with CII in vitro (Fig. 2, B and C, and data not shown), indicating that the activation of CII-reactive T cells was not inhibited by the anti-TWEAK mAb treatment. Furthermore, the anti-TWEAK mAb treatment did not affect the production of anti-CII IgG1 or IgG2a (Fig. 2A). These results showed that TWEAK was not involved in the activation of pathogenic T cells and B cells.

In conclusion, the administration of anti-TWEAK mAb ameliorated CIA through anti-inflammatory and antiangiogenic actions. Thus, the blockade of TWEAK may be a novel strategy for the treatment of RA and possibly other chronic inflammatory diseases.

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