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TRAIL-R2 (DR5) Mediates Apoptosis of Synovial Fibroblasts in Rheumatoid Arthritis

Kimihisa Ichikawa,2* Weimin Liu,2† Martin Fleck,‡ Huangge Zhang,† Limin Zhao,‡ Toshiaki Ohtsuka,* Zheng Wang,† Di Liu,† John D. Mountz,† Masahiko Ohtsuki,* William J. Koopman,† Robert Kimberly,3† and Tong Zhou3†

TRAIL has been proposed as an anti-inflammatory cytokine in animal models of rheumatoid arthritis (RA). Using two agonistic mAbs specific for TRAIL-R1 (DR4) and TRAIL-R2 (DR5), we examined the expression and function of these death receptors in RA synovial fibroblast cells. The synovial tissues and primary synovial fibroblast cells isolated from patients with RA, but not those isolated from patients with osteoarthritis, selectively expressed high levels of cell surface DR5 and were highly susceptible to anti-DR5 Ab (TRA-8)-mediated apoptosis. In contrast, RA synoviocytes did not show increased expression of TRAIL-R1 (DR4), nor was there any difference in expression of Fas between RA and osteoarthritis synovial cells. In vitro TRA-8 induced apoptosis of RA synovial cells and inhibited production of matrix metalloproteinases induced by pro-inflammatory cytokines. In vivo TRA-8 effectively inhibited hypercellularity of a SV40-transformed RA synovial cell line and completely prevented bone erosion and cartilage destruction induced by these cells. These results indicate that increased DR5 expression and susceptibility to DR5-mediated apoptosis are characteristic of the proliferating synovial cells in RA. As highly proliferative transformed-appearing RA synovial cells play a crucial role in bone erosion and cartilage destruction in RA, the specific targeting of DR5 on RA synovial cells with an agonistic anti-DR5 Ab may be a potential therapy for RA. The Journal of Immunology, 2003, 171: 1061–1069.

Rheumatoid arthritis (RA)4 is a chronic inflammatory disease for which the etiology is unknown. Although autoimmune T cells and B cells appear to play a critical role in initiation of the inflammatory response in the joints of patients with RA, highly proliferative synoviocytes that are characteristic of synovial hyperplasia erode bone and destroy cartilage, in part through release of matrix metalloproteinases (MMPs) (1). Inhibition of hyperproliferative synovial cells by intra-articular administration of agonistic anti-Fas Ab or Fas ligand is effective in abrogating disease in animal models of RA (2, 3). However, the therapeutic application of Fas-mediated apoptosis in humans has been limited severely by lethal liver toxicity (4).

A second TNF superfamily member, TRAIL (5), can also play an anti-inflammatory role in the development of RA. Local administration of an adenoviral vector encoding TRAIL inhibits the development of arthritis, and blockade of TRAIL-mediated apoptosis increases susceptibility to collagen-induced arthritis (6). However, TRAIL has several receptors, including the agonistic receptors DR4 and DR5 (7–9), the decoy receptors TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), which antagonize the death signal (10, 11), and osteoprotegerin (OPG) (12). TRAIL binding to OPG might inhibit osteoclast maturation and activation. Thus, the net effect of TRAIL administration is difficult to predict, and its potential impact on bone metabolism and integrity is unclear. Furthermore, concern about TRAIL-induced hepatotoxicity in humans has been raised (13).

Recent experience has demonstrated increased DR5 expression on most malignantly transformed cells, which can be killed by a novel agonistic anti-human DR5 mAb, TRA-8. TRA-8 induces a strong apoptosis response without hepatocellular toxicity (14). These results prompted us to consider the possibility of increased DR5 expression on semitransformed synovial cells. To our surprise, DR5, but not DR4, expression was enhanced on both RA primary and transformed synovial cells. This increased expression and corresponding sensitivity to TRA-8-mediated apoptosis was not seen in osteoarthritis (OA) synovial cell lines and primary OA synovial tissues. Furthermore, TRA-8 was effective in blocking joint destruction in an animal model of arthritis induced by human RA synovial cells. Our results indicate that DR5 may be a unique target on semitransformed synovial cells and that a selective agonistic anti-DR5 Ab may be an effective therapeutic strategy for RA.

Materials and Methods

Synovial tissues and primary synovial cells

Thirteen RA patients (age range 40–75; 11 females and 2 males) meeting the American College of Rheumatology criteria for RA (15) and 10 OA patients provided synovial tissues that were obtained at the time of surgery for synovectomy or joint replacement. All synovial tissues were collected from patients provided synovial tissues that were obtained at the time of surgery for synovectomy or joint replacement. All synovial tissues were collected from patients with RA (15) and 10 OA patients provided synovial tissues that were obtained at the time of surgery for synovectomy or joint replacement. All synovial tissues were collected from patients with RA (15) and 10 OA patients provided synovial tissues that were obtained at the time of surgery for synovectomy or joint replacement. All synovial tissues were collected from patients.
Germany). Eight lines of human primary RA synovial cells (RA-1014, RA-1016, RA-1021, RA-512, RA-707, RA-811, RA-716, and RA-929) were kindly provided by Dr. H. Matsuno (Department of Orthopedic Surgery, Toyama Medical and Pharmaceutical University, Toyama, Japan) and cultured in DMEM supplemented with 10% FCS, penicillin, streptomycin, and glutamine. Seven OA synovial cell lines were isolated from the synovial tissues of OA patients by a standard collagenase method (16) and cultured under the same conditions. A SV40-transformed RA synovial fibroblast cell line was prepared as previously described (16). All primary cells used in this study were studied within five passages of the initial culture. A normal human skin fibroblast cell line, clone Malme-3, and a cancer fibroblast cell line, clone Hs 911T, were purchased from American Type Culture Collection (Manassas, VA).

Generation of anti-human DR4 mAb
A fusion protein containing the extracellular domain of human DR4 (aa 1–236) and the FC portion of human IgG1 was expressed in Cos-7 cells transfected with a recombinant adeno viral vector. The fusion protein was purified by protein A affinity column. BALB/c mice were immunized with the purified fusion protein as previously described (14). One hybridoma clone, 2E12 (IgG1, κ), with specific binding to DR4 and the capability to induce apoptosis of human Ramos B lymphoma cells was subcloned three times. The binding specificity of 2E12 was determined by ELISA and Western blot analysis using human DR5, DrR1, DrR2, and IgG1 fusion protein as control Abs. The binding of 2E12 to cell surface DR4 was determined by flow cytometry analysis of Cos-7 cells transfected with the full-length cDNA encoding human DR4. Apoptosis-inducing activity was determined by incubating Ramos cells with 1 μg/ml 2E12 in the presence of goat anti-mouse IgG1. Cell viability was determined by ATPLite assay as previously described (14).

In situ immunohistochemical analysis of synovial tissues
Paraffin sections of formalin-fixed RA and OA synovial tissues were stained with H&E. The infiltrating T cells in RA synovial tissues were stained with a HRP-conjugated polyclonal anti-human CD3 Ab (DAKO, Carpinteria, CA) according to the manufacturer’s instructions. For TRA-8 staining, frozen sections of RA or OA synovial tissues were fixed in 70% ethanol for 30 min and blocked with 10% calf serum in PBS for another 30 min. The sections were stained with 20 μg/ml affinity-purified anti-human DR5 mAb, TRA-8 (14), or isotype control murine IgG1 followed by anti-mouse IgG ABC kit (Vector Laboratories, Burlingame, CA). The color reaction was developed by diaminobenzidine substrate and counterstained with methyl green. Three to five sections of each tissue were examined by at least two independent observers. Each observer graded the intensity of immunostaining based on a visual assessment of the intensity of brown reaction product within the cell cytoplasm or on the cell membrane on a scale of 0 (no staining) to 4+ (intense staining). The final score reported is the average of replicated sections assessed by these observers.

Flow cytometry analysis
The cultured synovial cells and control fibroblast cells were detached from the culture plate with 1 mM EDTA and suspended in FACS buffer (PBS supplemented with 5% FBS and 0.1% NaN₃). A total of 5 × 10⁶ cells were first incubated with 10 μg/ml affinity purified anti-human DR5 (TRA-8, (14)) or anti-DR4 (2E12) or anti-Fas (DX2; BD PharMingen, San Diego, CA) Ab, or the equal amount of murine IgG1 as isotype control at room temperature for 30 min as previously described. After the wash, cells were further incubated with PE-conjugated goat anti-mouse IgG1 for another 30 min at room temperature. The stained cells were fixed, and 5,000–10,000 viable cells were analyzed by FACSVantage flow cytometer (BD Biosciences, San Jose, CA).

Induction of apoptosis
A total of 500-1000 cells per well were cultured in flat-bottom 96-well plate in triplicates in 10% FCS DMEM medium with the indicated concentration of the recombinant FLAG-tagged soluble TRAIL. Cells were cultured in the presence of anti-FLAG Ab as a cross-linker and enhancer for induction of apoptosis (Alexis, San Diego, CA) according to the manufacturer’s instructions, affinity purified TRA-8, or 2E12 with anti-mouse IgG1, or CH11 overnight at 37°C. For caspase inhibition assays, cells were incubated with a fixed concentration of TRAIL or TRA-8 in the presence of variable concentrations of the tetra-peptide caspase inhibitors (R&D Systems, Minneapolis, MN). Apoptosis of synovial cells and control cells was determined by cell viability using an ATPLite kit (Packard Instrument, Meriden, CT) according to the manufacturer’s instructions.

Gal shift assay and Western blot analysis for NF-κB activation
Cells were stimulated with the recombinant soluble TRAIL, plus enhancer, TRA-8, or with TNF-α. The nuclear and cytosolic extracts were prepared as described previously (17) with slight modifications. Double-stranded oligonucleotide containing a consensus NF-κB recognition sequence (Promega, Madison, WI) was end-labeled with T4 polynucleotide kinase in the presence of [γ-32P]ATP. Gel shift assay was performed using the Gel Shift Assay System (Promega) according to the supplier’s instructions. After 20 min of the DNA binding reaction at room temperature, the samples were subjected to electrophoresis on 4% non-denaturing polyacrylamide gels in 0.1 M Tris-borate EDTA buffer. Gels were dried, and protein-DNA complexes were visualized by the Cyclone phosphor imager. For Western blot analysis, cytosolic proteins were separated by SDS-PAGE and transferred to the polyvinylidene difluoride membrane (Millipore, Bedford, MA). Blots were saturated with 5% skim milk and 0.5% Tween 20 in TBS (pH 7.4) and probed using anti-Ieα-α Ab (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with peroxidase-conjugated secondary Ab. Bound Abs were detected using the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions.

Measurement of MMP production
Synovial cells were cultured in medium alone or with 50 ng/ml IL-1β, 10 ng/ml TNF-α, 50 ng/ml TRAIL plus enhancer, or 50 ng/ml TRA-8 overnight. The levels of the MMP-1 and MMP-3 in the culture supernatants were determined by ELISA (R&D Systems).

Human RA synovial cell xenograft model
The 6- to 8-wk-old female BALB/c nude mice were injected i.p. with 10 μg of anti-asialo-GM1 rabbit Ab (WAKO, Richmond, VA) to deplete NK cells and a subarthritogenic dose (50 μg) of mixed monoclonal anti-collagen Abs (Stratagene, La Jolla, CA) to induce mild inflammation in the joints (18, 19). Twenty-four hours later the knee joints of pretreated mice were injected with 1 × 10⁶ DR5-positive SV40-transformed RA synovial cells. Starting with day 1 after implantation of RA synovial cells, mice were injected i.p. daily with 250 μg of recombinant human IL-1β (R&D Systems) for a total of three doses. Mice were randomly divided into two groups: one group received 100 μg of TRA-8 i.p. injection, and another received the equal amount of murine IgG1 as control. The treatment started at day 1 after implantation of RA synovial cells and was repeated every day for 3 days. Seven days after the last treatment dose, the knee joints and other control joints were fixed in formalin and decalcified. The paraffin sections were stained with H&E. The histological lesions were evaluated for synovial cell proliferation, cartilage erosion, and bone destruction with modification (20). For synovium hyperplasia, a score (0–5) was given with the following values: 0 representing normal single layer of synovial cells, 1 representing mild proliferation with 2–5 layers of synovial cells, 2 representing intermediate proliferation with 5–10 layers of synovial cells, 3 representing severe proliferation with >10 layers of synovial cells, and 4 representing massive synovial cell proliferation. For cartilage erosion and bone destruction, a score (0–4) was given with the following values: 0 representing normal cartilage and bone structure; 1 representing minor cartilage erosion without bone destruction, and the erosion was limited on the surface; 2 representing severe cartilage erosion, but without bone destruction; 3 representing severe cartilage erosion with minor bone destruction; and 4 representing severe cartilage erosion and bone destruction. At least three representative cutting levels of each involved joint were examined, and the severity scores at each level were averaged.

Results
RA synovial tissues and primary synovial fibroblast cells express high levels of DR5
Six RA and five OA synovial tissues were stained with TRA-8 and isotype control murine IgG1 to determine DR5 expression at the protein level. All six RA synovial tissues were positively stained with TRA-8 with a range of the intensity score from 2 to 4. In contrast, the OA synovial tissues did not exhibit significant staining with TRA-8 with a range of the intensity score from 0 to 0.5. Fig. 1a illustrates representative staining of one OA and three RA synovial tissues. The RA synovial tissues exhibited typical pathological features of RA, including hyperplasia of synovium, infiltration of inflammatory cells, and increased vascularity as shown.
stromal cells of RA tissues were positively stained with TRA-8. In contrast, the OA synovial tissue did not exhibit significant staining with TRA-8.

To confirm that RA synovial cells express cell surface DR5, two panels of primary cultured synovial fibroblast-like cells comprised of eight samples from patients with RA and seven from patients with OA, respectively, were examined by flow cytometry. The primary synovial cells derived from all eight RA patients expressed significant levels of cell surface DR5 (Fig. 1b, upper graphs (RA)), and the expression levels of DR5 were very similar among the different patients. In contrast, there was no significant cell surface expression of DR5 in any OA synovial cells (Fig. 1b, lower graphs (OA)). These results indicate that DR5 expression is increased in the synovial tissues and synovial fibroblast cells of RA patients.

**DR5 expression is selectively increased on RA synovial cells**

Both DR4 and DR5 are the death receptors for TRAIL and are increased in expression on malignantly transformed cells. To compare the expression and function of DR4 in RA and OA synovial cells, we generated an agonistic anti-human DR4 mAb (2E12). 2E12 is specific for human DR4, as it did not bind to other TRAIL receptors such as DR5, DcR1, and DcR2 in ELISA (Fig. 2a). 2E12 recognized cell surface DR4 as demonstrated by flow cytometry analysis of DR4 transfected Cos-7 cells (Fig. 2b). 2E12 was able to induce apoptosis of human Ramos lymphoma cells in the presence of second Ab cross-linking in a dose-dependent fashion (Fig. 2c). In vitro treatment of Ramos cells with 2E12 resulted in a time-dependent activation of caspases 8, 9, and 3, and cleavage of poly(ADP-ribose) polymerase (PARP) (Fig. 2d). These results indicate that 2E12 is an agonistic anti-DR4 Ab that induces apoptosis in a caspase-dependent fashion.

To determine whether DR5 is uniquely expressed by RA synovial cells, we compared the expression of other death receptors, DR4 and Fas, using panels of primary synovial fibroblast cells from RA and OA patients, the SV40-transformed RA synovial fibroblast cells, a normal human skin fibroblast cell line (Malme3), and a fibrosarcoma cell line (Hs 913T). Like many other types of cancer cells, fibrosarcoma cells expressed high levels of cell surface DR5 similar to that of both RA primary and SV40-transformed synovial fibroblast cells (Fig. 3, top), whereas neither OA synovial cells nor normal skin fibroblast cells expressed significant levels of DR5. Both RA (primary and SV40-transformed) and OA synovial cells as well as normal skin fibroblast cells had low levels of cell surface DR4 (Fig. 3, middle). Fibrosarcoma cells exhibited higher levels of DR4, and all five fibroblast cell types expressed high levels of Fas (Fig. 3, bottom). The mean fluorescence intensity of DR4 reactivity was 10.34 ± 2.80 and 9.58 ± 2.10 (mean ± SD) in five RA and five OA synovial cell lines, respectively. DR5 expression levels in RA synovial cells were ~10-fold higher than DR4, with a mean fluorescence intensity ratio of 9.36 ± 2.7 (mean ± SD). In contrast, DR5 expression levels in OA synovial cells were lower than that of DR4, with a ratio of 0.74 ± 0.27 (mean ± SD). These results further demonstrate that RA synovial fibroblast cells differentially express high levels of DR5.

**TRA-8 induces apoptosis of RA synovial cells**

In general, all of the primary cultures of synovial cells isolated from patients with RA were susceptible to both TRAIL- and TRA-8-induced apoptosis (Fig. 4a), and all OA cells were resistant to...
TRAIL- and TRA-8-induced apoptosis (Fig. 4b). Two of the primary RA synovial cell cultures (RA-512 and RA-707) were extremely susceptible, with 80% of the cells being killed upon treatment with TRAIL or TRA-8 at concentrations below 10 ng/ml. Five of the RA primary cultures (RA-716, RA-811, RA-929, RA1021, and RA1014) exhibited intermediate susceptibility to TRAIL or TRA-8, with nearly 100% cell death occurring in the presence of higher concentrations (>50 ng/ml) of TRAIL or TRA-8. In one of the primary RA cultures (RA-1016), although the majority (>60%) of cells were killed by low doses of TRAIL or TRA-8, a portion of the cells survived in the presence of high concentrations of TRAIL or TRA-8. The susceptibility to apoptosis induced by TRAIL and TRA-8 varied among the RA synovial cells, and this susceptibility was well correlated between TRAIL and TRA-8 in these RA synovial cells. These results indicate that DR5 plays a major role in TRAIL-mediated apoptosis of RA synovial cells. Consistent with their surface expression of DR5, all OA cells were much less susceptible to apoptosis induced by TRAIL or TRA-8. No more than 30% of OA81F, OA58, and OA69F cells were killed even in the presence of the highest concentrations of TRAIL or TRA-8.

As previously shown in cancer cells, TRA-8-induced apoptosis of RA synovial cells is caspase dependent. The peptide inhibitors for caspases 8, 9, and 3 partially inhibited TRA-8-mediated apoptosis, whereas a general caspase inhibitor completely blocked apoptosis (Fig. 4c). Interestingly, methotrexate, a commonly used anti-arthritis drug, significantly increased susceptibility of RA, but not OA, synovial cells to TRA-8-mediated apoptosis (Fig. 4d). These results indicate that RA synovial fibroblast cells not only express elevated levels of cell surface DR5, but are also more susceptible than OA synovial cells to DR5-mediated apoptosis.

The susceptibility of the fibroblast cells to 2E12-mediated apoptosis appeared to be associated with the expression levels of DR4. Only the fibrosarcoma cells, which have the highest levels of DR4, were relatively susceptible to 2E12-induced apoptosis (Fig. 4e). An anti-Fas Ab (CH-11) was able to induce apoptosis of all the fibroblast cells, although the degree was variable (Fig. 4e). Importantly, the susceptibility of RA and OA synovial cells to anti-Fas-mediated apoptosis was not different. Thus, increased expression and function of DR5, but not DR4, is associated with RA and not OA synovial cells. These results suggest that DR5 might be a more selective and effective target on RA synovial cells than either DR4 or Fas.

TRA-8 does not activate NF-κB and inhibits production of MMPs

Similar to other members of the TNFR superfamily, DR5 can activate a NF-κB pathway in addition to transducing apoptosis signals (7). NF-κB activation transduces an anti-apoptosis signal (21)
and can promote proinflammatory actions, in part through enhanced production of MMPs (22). Thus, on balance, TRAIL might play either an anti-inflammatory role or a pro-inflammatory role in RA. To determine the effect of TRA-8 on the activation of NF-κB and production of MMPs in RA synovial cells, we examined the NF-κB activity by gel-shift and Western blot analysis of IκB-α degradation. Although TNF-α strongly induced activation of NF-κB and production of MMPs in RA synovial cells, as demonstrated by both increased DNA binding and decreased content of IκB-α, untreated RA synovial cells did not exhibit detectable activation of NF-κB (Fig. 5a). TRAIL was able to activate NF-κB in Jurkat cells (Fig. 5b) and induced a weak activation of NF-κB in RA synovial cells at 60 min after the treatment (Fig. 5a). In contrast, TRA-8 did not induce any significant activation of NF-κB in either RA synovial cells or Jurkat cells (Fig. 5, a and b). The failure of TRA-8 to activate NF-κB was not associated with its apoptosis-inducing activity, as NF-κB activation was not detected in either TRA-8-sensitive or -resistant cells. These results suggest that TRA-8 might selectively activate apoptosis signaling, but not the NF-κB pathway in RA synovial cells. In contrast to TNF-α, which induced a dose-dependent production of both MMP1 (Fig. 5c) and MMP3 (Fig. 5d) in two RA synovial cell lines, neither TRAIL nor TRA-8 increased production of these MMPs. More importantly, TRA-8 was also able to completely inhibit production of MMPs induced by IL-1β in RA synovial cells (Fig. 5e). These results suggest that the primary function of DR5 in RA synovial cells is anti-inflammatory through induction of apoptosis.

**TRA-8 prevents erosive arthritis induced by RA synovial cells**

To determine the therapeutic efficacy of a selective anti-DR5 strategy in the treatment of arthritis, we developed a novel murine xenograft model for human RA in which an SV40-transformed RA synovial cell line (16) was injected into the knee joint of NK cell-depleted BALB/c nude mice. The cell surface expression of DR5 and susceptibility of SV40-transformed RA synovial cells to TRA-8-mediated apoptosis was very similar to that of primary RA synovial cells (Figs. 3 and 4e). The SV40-transformed RA synovial cells alone were not sufficient to cause erosive arthritis, although they could grow in nude mice. Similarly, treatment with low, sub-arthritisic doses of anti-collagen Abs and IL-1β did not induce significant lesions in the joints in the absence of implanted RA synovial cells (data not shown). However, with the combination of anti-collagen Abs, IL-1β, and implanted RA synovial cells, 100% of the mice developed severe erosive arthritis within 7 days (Fig. 6a). These results suggest that anti-collagen Abs might cause sub-clinical damage and create a pro-inflammatory environment in which IL-1β may help to activate implanted synovial cells, thereby leading to the development of arthritis that is dependent on human RA synovial cells. Except for the absence of lymphocyte and monocyte infiltration in the nude mouse, the pathological features in this model are similar to those seen in human RA, that is synovial cell hypercellularity, pannus formation, cartilage destruction, and bone erosion (Fig. 6a). The majority of proliferating cells in the joints are fibroblast-like cells of human origin, that is MHC class I Ag positive, DR5 positive, CD3 and F4/80 negative, and CD11b low (data not shown). These results indicate that the human RA synovial cells play an essential role in the development of destructive arthritis in this model, thereby providing an excellent model to examine the in vivo efficacy of an agent that targets RA synovial cells.

To determine the efficacy of TRA-8, the recipient mice were treated with three doses of 100 μg TRA-8 after implantation of RA synovial cells. The development of erosive arthritis was examined at day 7 after the last treatment dose. In contrast to control mice (Fig. 6a), none of TRA-8-treated mice developed cartilage destruction and bone erosion (Fig. 6b). The severity of arthritis was graded according to each histological lesion, synovial cell hypercellularity (Fig. 6c), cartilage erosion, and bone destruction (Fig. 6d). Whereas 100% (6 of 6) of control mice had severe synovial cell hypercellularity (4+), 50% (3 of 6) of TRA-8-treated mice did not have any increased synovial cells in the joints, and 50% of the mice exhibited only minor (1+) synovial hypercellularity. One hundred percent of control mice exhibited severe (3+ to ~4+) erosion of cartilage and bone destruction. In contrast, none (0 of 6) of TRA-8-treated mice exhibited cartilage erosion or bone destruction. Thus, the treatment with TRA-8 completely eliminated the invasive RA synovial cells and effectively inhibited the development of destructive arthritis. These results indicate that induction of apoptosis of RA synovial cells by TRA-8 is effective in suppressing proliferation of human RA synovial cells and in the prevention of joint destruction caused by RA synovial cells in this model.

**Discussion**

Although mRNA levels of DR5 are detectable in normal tissues, our recent data using TRA-8 demonstrate that most normal tissues and cells do not express detectable DR5 at the protein level, and normal cells from different tissue origins are completely resistant to DR5-mediated apoptosis. In contrast, most cancerous tissues and cells express high levels of DR5 and are susceptible to DR5-mediated apoptosis. These results suggest that increased DR5 expression and susceptibility to DR5-mediated apoptosis might be a characteristic of transformed cells (14). Interestingly, in the present study we demonstrate that RA synovial cells, like malignant transformed cells, express high levels of DR5 and are highly susceptible to DR5-mediated apoptosis. This property appears to be selective for RA synovial cells, because OA synovial cells and...
FIGURE 4. TRA-8 induced caspase-dependent apoptosis of RA synovial cells. (a, b) TRA-8 induced apoptosis of RA and OA synovial cells. One thousand cells per well of primary culture RA (a) or OA (b) synovial cells were cultured overnight in triplicates in flat-bottom 96-well plates in the presence of the indicated concentrations of recombinant soluble TRAIL (○) or affinity-purified TRA-8 (●). The results are presented as an average of triplicate cultures and as a percentage of viable cells compared with 100% viable cells in control medium. The representative results from three repeated experiments are presented. c, Caspase-dependent apoptosis by TRA-8. RA synovial cells (RA512) were incubated overnight with 1 μg/ml TRA-8 in the presence of the indicated caspase inhibitors at the indicated concentrations. The results are presented as an average of the triplicates. d, Methotrexate enhances TRA-8 mediated apoptosis of RA synovial cells. RA synovial cells from three patients (RA1021, RA1014, and RA 1016) were incubated overnight with indicated concentrations of TRA-8 in the absence or presence of 20 μM methotrexate. The results are presented as the mean ± SEM of RA synovial cells of three patients. e, TRA-8, but not Fas, selectively induces apoptosis of RA synovial and sarcoma cells. One thousand cells per well of each type of fibroblast cell were cultured overnight in flat-bottom 96-well plates in the presence of the indicated concentrations of TRA-8 (●), 2E12 with anti-mouse IgG1 (■) or CH-11 anti-Fas Ab (○). The results are presented as the mean ± SEM of RA synovial cells from five patients and as an average of the triplicates of the fibroblast cell lines. Cell viability was determined by the ATPLite assay and is presented as the percentage survival of untreated control cells.
normal skin fibroblast cells did not express DR5 and were resistant to DR5-mediated apoptosis. Moreover, the expression and function of the other two death receptors were no different among different types of fibroblast cells, further supporting the notion that increased expression and function of DR5 in synovial cells is selective for RA.

Hyperproliferation of the synovial fibroblast cells is a characteristic feature of RA. Although the hyperproliferative synovial cells are nonmalignant, they have been described as “transformed-appearing synoviocytes” because they share some common features of transformed cells, including a dense rough endoplasmic reticulum, numerous irregular nuclei, and changes in the normally spindle-shaped cell skeleton (23). In addition, RA synovial cells exhibit a more invasive and erosive behavior than the synovial cells in other diseases. It would be interesting to compare DR5 expression and function of RA synovial cells with other types of inflammatory arthritis.

Although the molecular mechanisms leading to proliferation of synovial cells in RA are not fully understood, some have proposed that the expression of oncogenes and virus-derived genes might be the primary triggers for the transformed appearance of RA synovial cells (23–29). Pronounced proliferation, despite high levels of DR5 expression and susceptibility to DR5-mediated apoptosis, suggests that inflamed RA synovial cells may escape TRAIL-mediated apoptosis during the development of RA. Whereas it is still unclear whether there is defective TRAIL-mediated apoptosis in the joints of RA patients, one possible explanation is that the synovial environment in RA might lack sufficient expression of TRAIL or might contain blocking factors such as soluble TRAIL receptors for TRAIL-mediated apoptosis. Decoy receptors for TRAIL have been implicated as crucial anti-apoptosis mechanisms for TRAIL, although the parallel susceptibility of RA synovial cells to both TRAIL and anti-DR5 Ab suggests that this might not apply to RA synovial cells. Alternatively, the synovial levels of endogenous TRAIL apoptosis blockers, such as soluble DR4, DR5, or OPG, might be increased in RA patients (30, 31). Blockade of TRAIL-mediated apoptosis with the soluble DR5 is known to increase susceptibility to the development of arthritis in mice (6). In any case, TRAIL can play an anti-inflammatory role in immune privileged tissues (32), and it is likely that TRAIL might function as a critical negative regulator for inflammatory synovial cells.

Production of high levels of metalloproteinases by inflammatory synovial cells is a leading cause for the degradation of cartilage and bone erosion in RA. Pro-inflammatory cytokines such as TNF-α and IL-1β play a pivotal role in the production of MMPs in RA synovium (22). Although previous studies demonstrate that DR5 is able to activate NF-κB translocation, our results indicate that TRA-8 does not activate NF-κB in RA synovial cells compared with TNF-α and TRAIL. More importantly, TRA-8 strongly inhibits the release of MMPs from pro-inflammatory cytokine-activated RA synovial cells in vitro and inhibits the invasive properties of RA synovial cells in vivo. The differential activation of apoptosis and the NF-κB signaling pathway in RA synovial cells by TRA-8 further supports the potential therapeutic value of TRA-8 in the treatment of RA.

Our results suggest that selective targeting of DR5 may provide a novel and effective therapeutic strategy for RA. Although induction of apoptosis in inflamed synovial tissues by Fas ligand is also effective in animal models (2), the systemic toxicity of Fas ligand

**FIGURE 5.** TRA-8 does not induce activation of NF-κB and inhibits production of MMPs. *a,* RA707 cells were incubated with 100 ng/ml TRAIL plus 1 μg/ml cross-linker, 100 ng/ml TRA-8, or 30 ng/ml TNF-α for the indicated times. Nuclear and cytosolic extracts were prepared and subjected to gel shift and Western blotting to measure NF-κB activation, respectively. *b,* Jurkat cells were incubated with 1 μg/ml TRAIL plus 2 μg/ml cross-linker, 1 μg/ml TRA-8, or 30 ng/ml TNF-α for the indicated times. Nuclear and cytosolic extracts were prepared and subjected to gel shift and Western blotting, respectively, to measure NF-κB activation. *c* and *d,* Production of MMP-1 and MMP-3. A total of 1 × 10^6 cells per milliliter of each indicated RA synovial cell line were incubated with the indicated concentrations of TRA-8, TRAIL, or TRA-8 plus 100 ng/ml TRAIL, respectively. RA synovial cells were stimulated with 50 ng/ml IL-1β in the presence of the indicated concentrations of TRA-8. After overnight culture, the levels of MMP1 and MMP3 in culture supernatants were determined by ELISA. The data, averages of duplicates, were replicated in at least two independent experiments.
has raised serious concerns regarding the application of this strategy to humans. The application of TRAIL as a therapeutic agent might also be questioned because of the ability of soluble TRAIL to induce apoptosis of normal hepatocytes (13) and the potential for TRAIL to accelerate osteoporosis in RA patients through OPG (12), although this is not proven in TRAIL-deficient mice (33).

Unlike TRAIL, TRA-8 does not induce apoptosis of normal human hepatocytes (14), and by specifically targeting DR5, TRA-8 does not react with other TRAIL receptors including OPG. Furthermore, unlike DR4 and Fas, increased expression of DR5 and susceptibility to TRA-8-mediated apoptosis is selective for RA synovial cells and is not characteristic of OA synovial cells and perhaps normal synovial cells. In addition to RA synovial fibroblast cells, T cells, B cells, and monocytes/macrophages involved in immune-mediated inflammation might also be targets for DR5-mediated apoptosis. DR5-mediated apoptosis is a critical alternative mechanism for activation-induced cell death in T and B cells, and preliminary data indicate that T cells isolated from the synovial fluid of some RA

**FIGURE 6.** TRA-8 inhibits the development of erosive arthritis induced by RA synovial cells. The representative histology of two control mice with severity scores of 4 (proliferation) and 3 (bone and cartilage erosion) (a) and two TRA-8-treated mice with severity scores of 1 and 0 (b) are shown. The severity of the joint lesion was graded according to synovial cell proliferation (c) and cartilage destruction and bone erosion (d). A score of 4 represents the most severe lesion, whereas a score of 0 indicates normal tissue. The individual severity scores of six mice in each group are presented. The photomicrographs were taken at ×40, ×100, and ×400 from left to right.
patients express high levels of DR5. Thus, a DR5-specific strategy may be effective not only as an anti-cancer agent, but also as an anti-inflammatory agent for RA.

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