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ADAM-10 Is Overexpressed in Rheumatoid Arthritis Synovial Tissue and Mediates Angiogenesis

Takeo Isozaki,[1] Bradley J. Rabquer,[2] Jeffrey H. Ruth,[1]
G. Kenneth Haines III,[3] and Alisa E. Koch[4]

Objective. **To examine the expression of ADAM-10 in rheumatoid arthritis (RA) synovial tissue (ST) and the role it plays in angiogenesis.**

Methods. **ADAM-10 expression was determined using immunohistology, Western blotting, and quantitative polymerase chain reaction. In order to examine the role of ADAM-10 in angiogenesis, we performed in vitro Matrigel tube formation and chemotaxis assays using human microvascular endothelial cells (HMVECs) transfected with control or ADAM-10 small interfering RNA (siRNA). To determine whether ADAM-10 plays a role in angiogenesis in the context of RA, we performed Matrigel assays using a coculture system of HMVECs and RA synovial fibroblasts.**

Results. **Endothelial cells and lining cells within RA ST expressed high levels of ADAM-10 compared with cells within osteoarthritis ST and normal ST. ADAM-10 expression was significantly elevated at the protein and messenger RNA levels in HMVECs and RA synovial fibroblasts stimulated with proinflammatory mediators compared with unstimulated cells. ADAM-10 siRNA–treated HMVECs had decreased endothelial cell tube formation and migration compared with control siRNA–treated HMVECs. In addition, ADAM-10 siRNA–treated HMVECs from the RA synovial fibroblast coculture system had decreased endothelial cell tube formation compared with control siRNA–treated HMVECs.**

Conclusion. **These data show that ADAM-10 is overexpressed in RA and suggest that ADAM-10 may play a role in RA angiogenesis. ADAM-10 may be a potential therapeutic target in inflammatory angiogenic diseases such as RA.**

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by inflammation and joint destruction (1). RA synovium contains high levels of inflammatory cytokines and abundant inflammatory cells, including infiltrating lymphocytes and monocytes (2). Synovial tissue (ST) macrophages represent one of the inflammatory cell types involved in RA pathogenesis. ST macrophages produce several cytokines, such as tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) (3,4). Extensive studies have identified TNFα as a key inflammatory mediator in RA, and treatment of RA has been revolutionized by the introduction of therapies blocking this cytokine. However, despite suppression of this cytokine with anti-TNFα therapy, not all patients with RA experience improvement with such treatment (5).

Angiogenesis is important in a variety of vasculoproliferative states, such as wound repair and RA (6,7). Many inflammatory mediators are also angiogenic mediators in RA ST and RA synovial fluid, including IL-8/CXCL8 (7). The process of angiogenesis is often of pivotal importance in disease progression and thus is an interesting and promising therapeutic target (8).

ADAMs are a family of proteases that are responsible for the liberation of a variety of cell surface–expressed proteins. ADAMs have been implicated in several inflammatory and degenerative pathologic conditions (9). ADAM-10 is involved in the shedding of many substrates that play roles in cancer progression,

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[1]Takeo Isozaki, MD, PhD, Jeffrey H. Ruth, PhD: University of Michigan Medical School, Ann Arbor; [2]Bradley J. Rabquer, PhD: University of Michigan Medical School, Ann Arbor, and Albion College, Albion, Michigan; [3]G. Kenneth Haines III, MD: Yale University, New Haven, Connecticut; [4]Alisa E. Koch, MD: VA Medical Services and University of Michigan Medical School, Ann Arbor, Michigan.

Address correspondence to Alisa E. Koch, MD, University of Michigan Medical School, 4045 BSRB, 109 Zina Pitcher Drive, Ann Arbor, MI 48109-2200. E-mail: aekoch@med.umich.edu.

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allergic responses, and inflammatory disease (10–13). In particular, ADAM-10 has been shown to cleave various inflammatory and angiogenic mediators from the cell surface, including fractalkine/CX3CL1 and CXCL16 (14, 15). Previous studies have shown that ADAM-15 and ADAM-17 are active in RA. ADAM-15 messenger RNA (mRNA) expression was higher in RA ST than in osteoarthritis (OA) ST (16). Expression of ADAM-17 at the protein level in RA ST was significantly higher than that in OA ST (17). However, a direct role of ADAM-10 in RA has not been demonstrated. In this study, we sought to define the function of ADAM-10 in RA angiogenesis.

MATERIALS AND METHODS

Cell culture. Dermal human microvascular endothelial cells (HMVECs) were purchased from Lonza and were maintained in EBM Endothelial Basal Medium (Lonza). Fibroblasts were isolated from synovium obtained from patients with RA who had undergone total joint replacement surgery or synovectomy (18). The study was approved by the University of Michigan Institutional Review Board. Fresh ST specimens were minced and digested in a solution of Dispase, collagenase, and DNase (19). RA synovial fibroblasts were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS).

K/BxN mouse serum transfer–induced arthritis. K/BxN mouse serum was collected at 7–8 weeks of age and pooled (20). K/BxN mouse serum (150 μl) was injected into 6–8-week-old mice on day 0 and day 2. All mice were killed on day 12, the ankles were harvested for further examination, and the joints were snap-frozen. Cryosections (10 μm) of snap-frozen joints from arthritic mice were used for immunohistologic assays. All experiments on mice were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Immunohistologic analysis. We performed immunohistologic staining on cryosections from RA, OA, normal, and psoriatic arthritis (PsA) ST, as described previously (21). Rabbit anti-human ADAM-10 (25 μg/ml; Abcam), ADAM-8 (10 μg/ml; Novus Biologicals), ADAM-9 (5 μg/ml; Novus Biologicals), and ADAM-12 (5 μg/ml; Novus Biologicals) were used as a primary antibodies. We also used joints from mice with K/BxN serum transfer–induced arthritis. Rabbit anti-mouse ADAM-10 antibody (25 μg/ml; Novus Biologicals) was used as a primary antibody. Staining was evaluated by a pathologist (GKH) who was blinded with regard to the sample group. Slides were examined for cellular immunoreactivity, and cell types were distinguished based on their characteristic morphology. ST inflammation was assigned a score of 0 (least) to 4 (most), depending on the degree of inflammatory cell invasion. ST vascularity was assigned a score of 1 (least) to 4 (most), depending on the vessel density in the ST (21). The percentage of cells expressing ADAM-8, ADAM-9, ADAM-10, and ADAM-12 was analyzed on endothelial cells and lining cells.

Cell lysis and Western blotting. HMVECs and RA synovial fibroblasts were incubated in 6-well plates in 0.1% FBS medium overnight, before stimulation with phorbol myristate acetate (PMA; Sigma-Aldrich), lipopolysaccharide (LPS) derived from *Escherichia coli* O111 (Sigma-Aldrich), recombinant human interleukin-17 (rhIL-17), rhIL-1β, or human rhTNFα (all from R&D Systems). The concentrations of the mediators that were used have been previously described (22). Cell lysis and Western blotting were also performed as previously described (21). Membranes were probed with rabbit anti-human ADAM-10 antibody (Abcam). Densitometric analysis of the bands was performed using Un-Scan-It software, version 5.1 (Silk Scientific).

RNA extraction and quantitative reverse transcription–polymerase chain reaction (RT-PCR). RNA extraction and quantitative RT-PCR were performed as previously described (23). The ADAM-10 and actin primer pairs were purchased from Invitrogen. All samples were run in duplicate, and Eppendorf software was used to analyze the read-out data.

RNA silencing. HMVECs were seeded in 6-well plates at a density of 1×10^5 cells/well. The cells were maintained in growth factor complete endothelial cell basal medium, at which time they were 60–70% confluent. Small interfering RNA (siRNA; 100 n*M*) against ADAM-10 or nonsilencing siRNA and TransIT-TKO (Mirus) in 250 μl serum-free endothelial basal medium were mixed gently at room temperature for 15 minutes to allow complexes to form. Thereafter, 1,250 μl of complete medium without antibiotics was added, and the resultant solution was mixed and overlaid on the cells. Cells were incubated with the siRNA and TransIT-TKO for 24 hours at 37°C. Control and ADAM-10 siRNA were purchased from Invitrogen. The target sequences of siRNA specific for ADAM-10 or control were as follows: for ADAM-10-1, 5′-GAGGAAAUACCAGAUGACUGGUGUA-3′; for ADAM-10-2, 5′-UACACCAGUCAUCUGGUAUUUCCUC-3′; for control, 5′-GACCGAAUGGGUUCUAAGAAACACA-3′. The siRNA sequences for both ADAM-10-1 and ADAM-10-2 are provided in the same master mixture (Invitrogen). To investigate the transfection efficiency of cells, fluorescein-conjugated nonsilencing siRNA (Santa Cruz Biotechnology) was transfected into cells with TransIT-TKO. The percent knockdown of ADAM-10 expression was determined using quantitative RT-PCR and Western blotting. In order to investigate whether knockdown was specific, we used quantitative RT-PCR to assess the expression of ADAM-8, ADAM-9, and ADAM-12.

Enzyme-linked immunosorbent assay (ELISA). Ninety-six–well plates were coated with synovial fluid from patients with RA, synovial fluid from patients with PsA, or recombinant ADAM-10 (R&D Systems), which was used as a standard. Goat anti-human ADAM-10 antibody (R&D Systems) was added as a primary antibody. The plates were then washed, and biotinylated horse anti-goat antibody (Vector) was added, followed by streptavidin–horseradish peroxidase (streptavidin–HRP; BD Biosciences). The plates were developed using tetramethylbenzidine substrate (TMB; Sigma-Aldrich) and were read on a microplate reader.

Ninety-six–well plates were coated with rabbit anti-human CXCL16 antibody (PeproTech) or mouse anti-human CX3CL1 antibody (R&D Systems). ADAM-10 siRNA– or control siRNA–treated HMVEC–conditioned medium, recombinant CXCL16 (PeproTech), or CX3CL1 (R&D Systems), used as a standard, was added. Biotinylated rabbit anti-human CXCL16 antibody or mouse anti-human CX3CL1 antibody

was used to detect CXCL16, using a streptavidin–peroxidase method (BD Biosciences) with TMB. The concentration in each sample was measured at 450 nm.

A direct ELISA was also developed using recombinant soluble junctional adhesion molecule A (sJAM-A; R&D Systems) or sJAM-C (R&D Systems) as a standard, and 96-well plates were coated with standard or samples overnight at 4°C. The conditioned medium was added directly to the plate. Goat anti-human JAM-A antibody (R&D Systems) or goat anti-human JAM-C antibody (R&D Systems) was added as a primary antibody. The plates were then washed, and biotinylated horse anti-goat antibody (Vector) was added, followed by streptavidin–HRP (BD Biosciences). The plates were developed using TMB and were read on a microplate reader.

In vitro HMVEC chemotaxis assay. Chemotaxis assays were performed using a 48-well modified Boyden chamber system, as previously described (24,25). In order to confirm the role of ADAM-10, we performed HMVEC chemotaxis assays using recombinant ADAM-10 (100 n*M*; R&D Systems), control siRNA–transfected HMVEC–conditioned medium, and ADAM-10 siRNA–transfected HMVEC conditioned medium.

HMVECs were transfected with control siRNA or ADAM-10 siRNA, using TransIT-TKO. HMVECs treated with control siRNA (100 n*M*) or ADAM-10 siRNA (100 n*M*) were placed in the bottom wells of the chambers, and diluted RA synovial fluid (1:10 in phosphate buffered saline [PBS]) was added to the top wells of the chambers. In addition, basic fibroblast growth factor (bFGF; 60 n*M*) or vascular endothelial growth factor (VEGF; 10 n*M*) (R&D Systems) was used as a positive control. Readings represent the number of cells migrating through the membrane (the sum of 3 high-power fields/well, averaged for each quadruplicate well).

In vitro Matrigel tube formation assay. Matrigel tube formation assays using growth factor–reduced Matrigel (BD Biosciences) were performed (26,27). HMVECs were transfected with control or ADAM-10 siRNA, using TransIT-TKO. Basic FGF (10 n*M*) or VEGF (1 n*M*) was used as a positive control, and PBS was used as a negative control. The treated HMVECs (1.8×10^4 cells/400 μl) were plated on Matrigel in the presence of bFGF, VEGF, or PBS for 6 hours at 37°C. Photographs (100× magnification) were obtained, and an observer counted the tubes in a blinded manner. Tubes were defined as elongated connecting branches between 2 identifiable HMVECs.

Endothelial cell tube formation assay using cocultured HMVECs and RA synovial fibroblasts. In order to confirm the influence of ADAM-10 on tube formation by HMVECs, we cocultured HMVECs and RA synovial fibroblasts using a Transwell system (28). HMVECs were first transfected, as described above, with either control siRNA or ADAM-10 siRNA and were plated in the bottom of the Transwell system. RA synovial fibroblasts were concurrently grown on the top inserts. HMVECs and RA synovial fibroblasts were cocultured with serum-free medium for 24 hours. HMVECs were collected from the coculture plates and subsequently plated on Matrigel in serum-free medium for 6 hours at 37°C. Tubes formed by HMVECs were counted by an observer in a blinded manner.

Conditioned medium from the coculture system was then used in an in vitro Matrigel assay in which the medium was added to HMVECs to determine whether tube formation could be altered. To do this, HMVECs from the coculture system were plated on Matrigel with the cocultured conditioned medium for 6 hours at 37°C, and an observer counted the number of tubes in a blinded manner.

Statistical analysis. Data were analyzed using Student's *t*-test, assuming equal variances. Data are reported as the mean ± SEM. P values less than 0.05 were considered significant.

RESULTS

Expression of ADAM-10 on endothelial cells and ST lining cells. We hypothesized that ADAM-10 is overexpressed in RA ST and plays a role in angiogenesis in RA. To test this hypothesis, we first determined whether endothelial cells and RA synovial fibroblasts could be induced to express ADAMs. ADAM expression was determined in RA, OA, normal, and PsA ST samples and in the joints of mice with K/BxN serum–transfer arthritis, using immunohistologic staining. Both synovial inflammation and vascularity were significantly increased in RA ST samples compared with OA and normal ST samples ($P < 0.05$; results not shown).

Figures 1A–D show representative photomicrographs of sections stained for ADAM-10 or control IgG antibody. Endothelial cells within RA ST expressed high levels of ADAM-10 (mean ± SEM 46 ± 7%), while endothelial cells within OA ST and normal ST expressed significantly less ADAM-10 (9 ± 3% and 1 ± 1%, respectively) (Figure 1I). Expression of ADAM-10 on endothelial cells within PsA ST (n = 1) was 2%. However, there was no correlation between ADAM-10 expression and vascularity. ADAM-10 was highly expressed on lining cells in RA ST (72 ± 6%), while lining cells within OA ST and normal ST expressed significantly less ADAM-10 (47 ± 11% and 9 ± 3%, respectively) (Figure 1J). Expression of ADAM-10 on lining cells within PsA ST was 4%. When experiments examining ADAM-8, ADAM-9, and ADAM-12 expression were performed, no differences were observed between RA ST and OA ST for any cell type (results not shown).

Figures 1E–H show the joints of mice with K/BxN serum–induced arthritis and nonarthritic controls immunostained with ADAM-10 or control IgG antibody. ADAM-10 was overexpressed on lining cells in mice with K/BxN serum–induced arthritis (day 12) compared with mice without arthritis (day 0) (mean ± SEM 64 ± 6% versus 21 ± 11%) (Figure 1K). However, ADAM-10 expression on endothelial cells in the joints was not different between the mice with K/BxN serum–induced arthritis and the normal control group (0.5 ± 0.2% versus 0.1 ± 0.1%).

Elevated ADAM-10 concentration in RA synovial fluid compared with PsA synovial fluid. In order to examine ADAM-10 secretion, we performed an

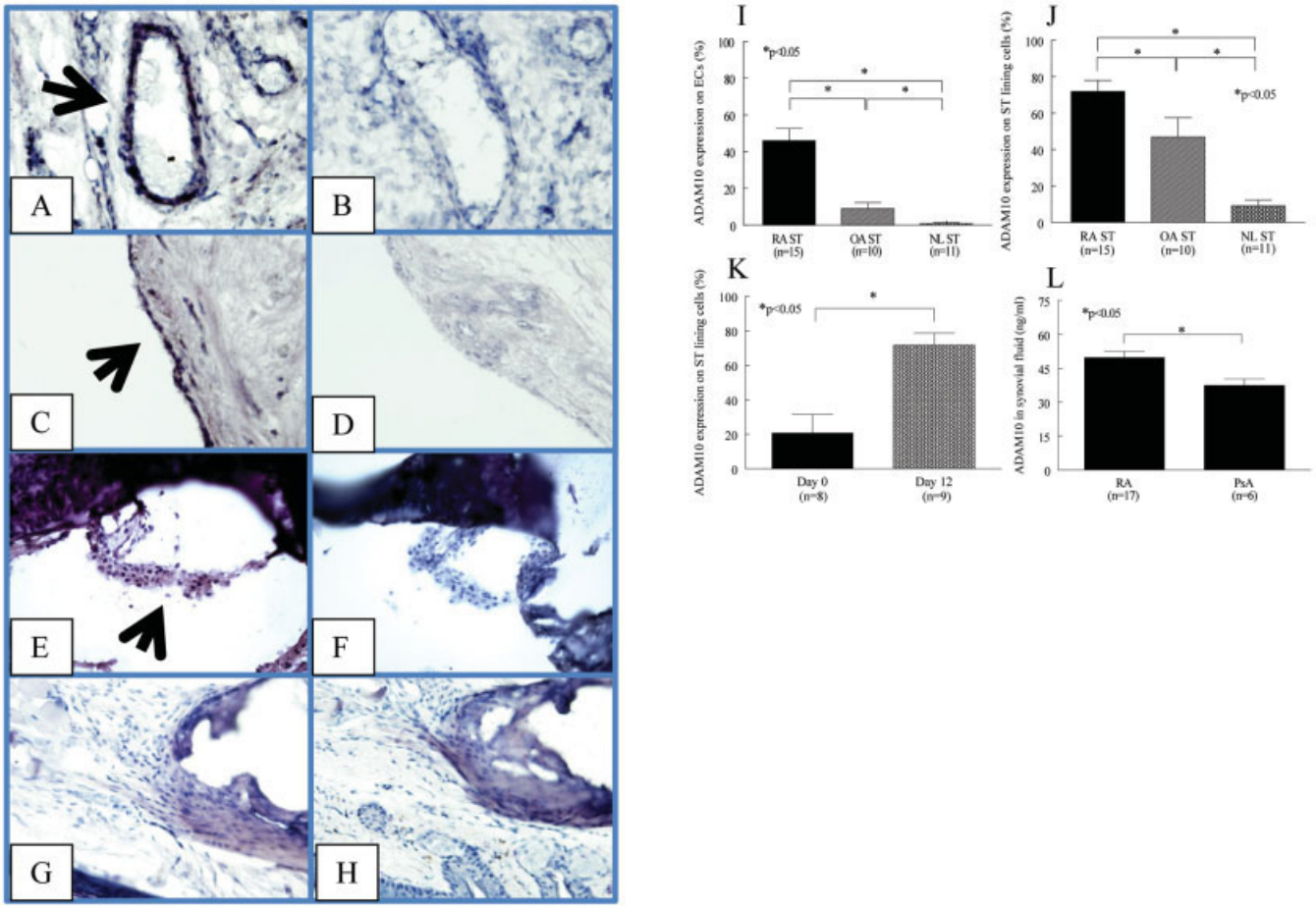

Figure 1. Immunohistologic analysis of ADAM-10. Endothelial cell (EC) and lining cell staining was graded from 0% to 100%, where 0% indicated no staining and 100% showed that all the cells were immunopositive. **A–D,** Representative photomicrographs of synovial tissue (ST) samples from patients with rheumatoid arthritis (RA). **E** and **F,** Representative photomicrographs of joints from mice with K/BxN serum–induced arthritis (day 12). **G** and **H,** Representative photomicrographs of nonarthritic mouse joints (day 0). Cryosections were stained for ADAM-10 (**A, C, E,** and **G**) or control IgG (**B, D, F,** and **H**). **Arrows** show ADAM-10 expression on endothelial cells (**A**) and ST lining cells (**C** and **E**). Original magnification × 400. **I,** ADAM-10 expression on RA ST, osteoarthritis (OA) ST, and normal (NL) ST endothelial cells. **J,** ADAM-10 expression on RA, OA, and NL ST lining cells. **K,** ADAM-10 expression on ST lining cells from arthritic mouse joints (day 12) and nonarthritic mouse joints (day 0). **L,** ADAM-10 concentration in RA synovial fluid and psoriatic arthritis (PsA) synovial fluid. Values in **I–L** are the mean ± SEM.

ADAM-10 ELISA with synovial fluid. The ADAM-10 concentration in RA synovial fluid (n = 17) was significantly elevated compared with that in PsA synovial fluid (n = 6) (mean ± SEM 50 ± 3 ng/ml and 37 ± 3 ng/ml, respectively) (Figure 1L).

Expression of ADAM-10 on HMVECs and RA synovial fibroblasts. To determine whether ADAM-10 was expressed by endothelial cells and RA synovial cells and whether its expression was regulated by proinflammatory cytokines or proangiogenic mediators, Western blotting and quantitative PCR were performed. ADAM-10 expression at the protein level was significantly elevated on PMA-, LPS-, IL-17–, and TNFα-stimulated HMVECs compared with unstimulated endothelial cells (Figure 2A). In addition, treatment with LPS induced a 1.6-fold increase in the expression of ADAM-10 mRNA in HMVECs within 1 hour (Figure 2B). ADAM-10 expression on RA synovial fibroblasts was inducible by IL-1β (Figure 2C). The expression of ADAM-10 mRNA was also significantly elevated in IL-1β–, IL-17–, PMA-, and LPS-stimulated RA synovial fibroblasts compared with unstimulated cells (Figure 2D).

ADAM-10–mediated release of potent angiogenic factors. In order to confirm the function of ADAM-10, we used siRNA directed against ADAM-10. The mean ± SEM transfection efficiency in HMVECs was 88 ± 2% (n = 3 replicates), and the percent knockdown

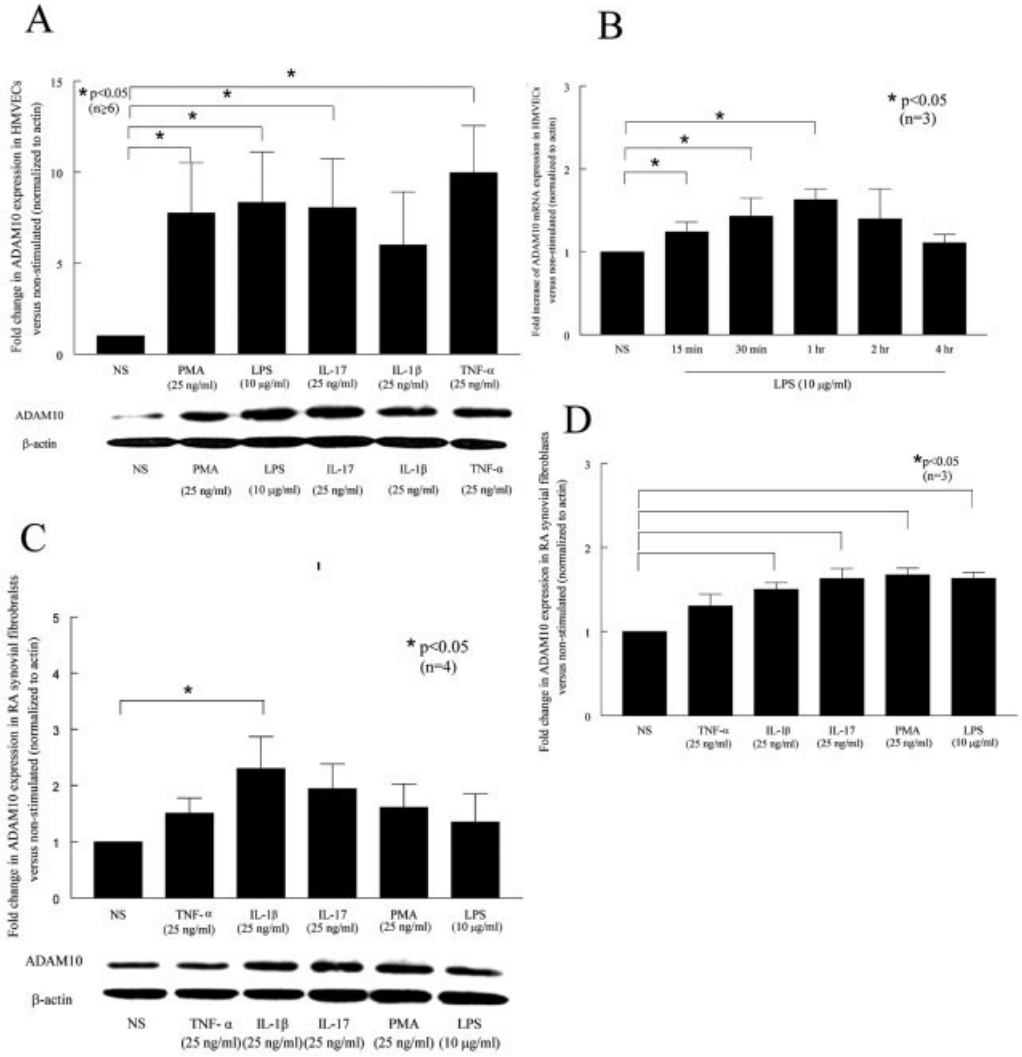

Figure 2. ADAM-10 expression on human microvascular endothelial cells (HMVECs) and rheumatoid arthritis synovial fibroblasts. **A,** ADAM-10 expression in HMVECs, as determined by Western blotting. HMVECs were incubated for 24 hours with phorbol myristate acetate (PMA), lipopolysaccharide (LPS), interleukin-17 (IL-17), IL-1β, or tumor necrosis factor α (TNFα), and the band intensities were quantified. A representative blot is shown. **B,** ADAM-10 mRNA expression on HMVECs following stimulation with LPS, as determined by quantitative polymerase chain reaction (PCR). **C,** ADAM-10 expression on RA synovial fibroblasts, as determined by Western blotting. RA synovial fibroblasts were incubated for 72 hours with PMA, LPS, IL-17, IL-1β, or TNFα, and the band intensities were quantified. A representative blot is shown. **D,** ADAM-10 mRNA expression on RA synovial fibroblasts following stimulation with TNFα, IL-1β, IL-17, PMA, and LPS, as determined by quantitative PCR. Bars show the mean ± SEM. n = number of experiments using cells from a single donor; NS = not stimulated.

of ADAM-10 mRNA was 92 ± 1% (n = 3). The specific knockdown of ADAM-10 was confirmed by Western blotting, and ADAM-10 protein levels were shown to be decreased (Figure 3A). To examine whether knockdown was specific, we assayed for ADAM-8, ADAM-9, and ADAM-12 mRNA and observed 7 ± 9% knockdown of ADAM-8 mRNA, 3 ± 1% knockdown of ADAM-9 mRNA, and 0 ± 0% knockdown of ADAM-12 mRNA (n = 3). We then tested whether ADAM-10 cleaves potent angiogenic factors such as JAM-A, JAM-C, CXCL16, and CX3CL1 from the cell surface. When ADAM-10 was inhibited using siRNA, sJAM-A expression was decreased 17 ± 2%, sJAM-C expression was decreased 78 ± 19%, CXCL16 expression was decreased 71 ± 1%, and CX3CL1 expression was decreased 65 ± 9% ($P < 0.05$; n = 4 replicates) (Figures 3B–E).

Effect of ADAM-10 on angiogenesis. To determine the role of ADAM-10 in angiogenesis, we per-

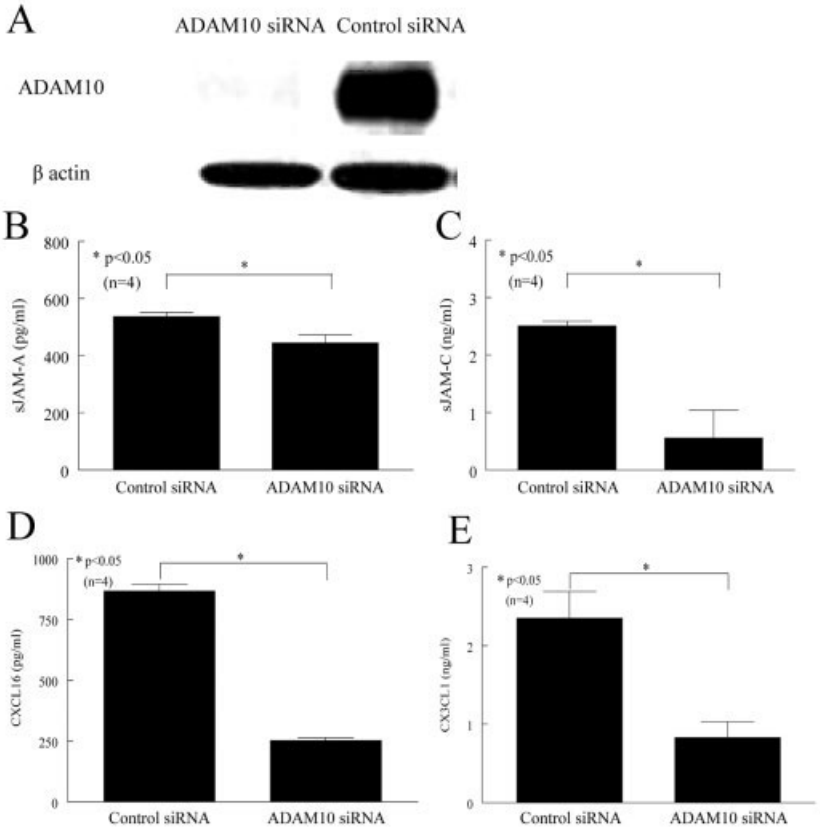

Figure 3. Decreased ADAM-10 expression on HMVECS, using small interfering RNS (siRNA) directed against ADAM-10. Cells were stimulated with TNFα (25 ng/ml) for 24 hours. **A,** Representative Western blot showing ADAM-10 expression on ADAM-10 siRNA–treated HMVECs and control siRNA–treated HMVECs. **B–E,** Expression of soluble junctional adhesion molecule A (sJAM-A) (**B**), sJAM-C (**C**), soluble CXCL16 (**D**), and soluble CX3CL1 (**E**) on ADAM-10 siRNA– or control siRNA–treated HMVECs. Bars show the mean ± SEM. n = number of replicates (see Figure 2 for other definitions).

formed in vitro chemotaxis assays and Matrigel tube formation assays. Endothelial cell chemotaxis is an initial step in the angiogenic process. HMVECs were assayed for their chemotactic response to ADAM-10, using a modified Boyden chamber. In order to determine whether ADAM-10 mediates angiogenesis directly or indirectly, we performed HMVEC chemotaxis toward recombinant human ADAM-10, control siRNA–transfected HMVEC–conditioned medium, and ADAM-10 siRNA–transfected HMVEC–conditioned medium. HMVECs did not migrate toward recombinant human ADAM-10. In contrast, ADAM-10 siRNA–transfected HMVEC–conditioned medium caused a significant decrease in the chemotactic potential for HMVECs compared with control siRNA–transfected HMVEC–conditioned medium (mean ± SEM number of HMVECs migrated 26 ± 2 and 47 ± 3, respectively; $P < 0.05$) (Figure 4A). ADAM-10 siRNA–treated HMVECs showed decreased migration toward RA synovial fluid compared with control siRNA (mean ± SEM number of HMVECs migrated 19 ± 2 and 35 ± 4, respectively; $P < 0.05$) (Figure 4B).

To confirm which mediators are important in ADAM-10–induced angiogenesis, we used bFGF and VEGF as chemotactic stimuli. ADAM-10 siRNA–treated HMVECs showed decreased migration toward bFGF compared with control siRNA–treated HMVECs and untreated HMVECs (mean ± SEM number of HMVECs migrated 3 ± 0, 8 ± 1, and 12 ± 1, respectively; $P < 0.05$) (Figure 4C). ADAM-10 siRNA–treated HMVECs also had decreased migration toward VEGF compared with control siRNA–treated HMVECs and untreated HMVECs (mean ± SEM number of HMVECs migrated 5 ± 1, 10 ± 1, and 12 ± 2, respectively; $P < 0.05$) (Figure 4D), indicating that both bFGF and VEGF have important roles in ADAM-10–induced angiogenesis.

ADAM-10 siRNA–treated HMVECs showed de-

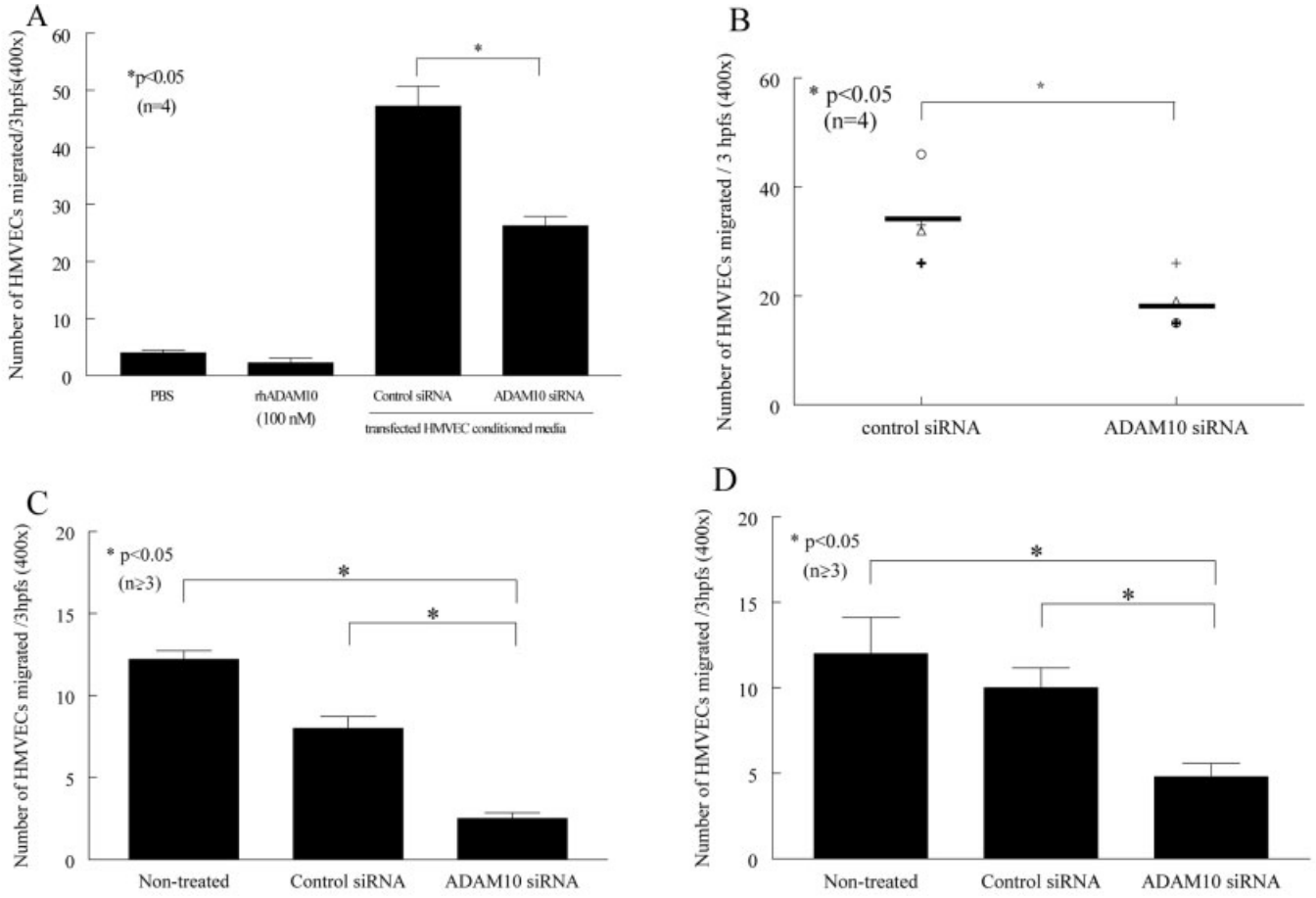

Figure 4. Human microvascular endothelial cell (HMVEC) chemotaxis induced by ADAM-10. **A,** HMVEC chemotaxis toward recombinant human ADAM-10 (rhADAM-10), control siRNA–transfected HMVEC–conditioned medium, and ADAM-10 siRNA–transfected HMVEC–conditioned medium. HMVECs did not migrate toward recombinant ADAM-10. **B,** Contribution of ADAM-10 to rheumatoid arthritis (RA) synovial fluid–mediated HMVEC chemotaxis. RA synovial fluid was depleted of rheumatoid factor, and HMVECs were treated with control small interfering RNA (siRNA) or ADAM-10 siRNA. **C,** Contribution of ADAM-10 to basic fibroblast growth factor (bFGF)–mediated HMVEC chemotaxis. Basic FGF (60 n*M*) was used as a stimulus for HMVEC chemotaxis. **D,** Contribution of ADAM-10 to vascular endothelial growth factor (VEGF)–mediated HMVEC chemotaxis. VEGF (10 n*M*) was used as a stimulus for HMVEC chemotaxis. In **A, C,** and **D,** values are the mean ± SEM, and n = number of replicates. In **B,** bars show the mean of 4 separate adjacent wells, and n = number of RA synovial fluid samples used. hpf = high-power field; PBS = phosphate buffered saline.

creased endothelial cell tube formation in response to bFGF compared with control siRNA–treated HMVECs or untreated HMVECs (mean ± SEM number of endothelial cell tubes formed 2 ± 1, 32 ± 5, and 35 ± 3, respectively; $P < 0.05$) (Figures 5A and B). In addition, ADAM-10 siRNA–treated HMVECs showed decreased endothelial cell tube formation in Matrigel in response to VEGF compared with control siRNA–treated HMVECs and untreated HMVECs (mean ± SEM number of endothelial cell tubes formed 4 ± 1, 30 ± 5, and 27 ± 3, respectively; $P < 0.05$) (Figures 5C and D).

In order to examine the role of ADAM-10 in angiogenesis with respect to RA, we used untreated HMVECs, ADAM-10 siRNA–treated HMVECs, and control siRNA–treated HMVECs and cocultured these endothelial cells with RASFs in an in vitro chamber system. ADAM-10 siRNA–treated HMVECs from the coculture system showed decreased endothelial cell tube formation compared with control siRNA–treated and untreated HMVECs (mean ± SEM number of endothelial cell tubes formed 4 ± 2, 23 ± 1, and 27 ± 1, respectively; $P < 0.05$) (Figures 6A and B).

We also examined the possibility that fibroblasts may affect HMVEC tube formation, potentially confounding our findings. To control for this possibility, we added HMVECs obtained from the coculture system

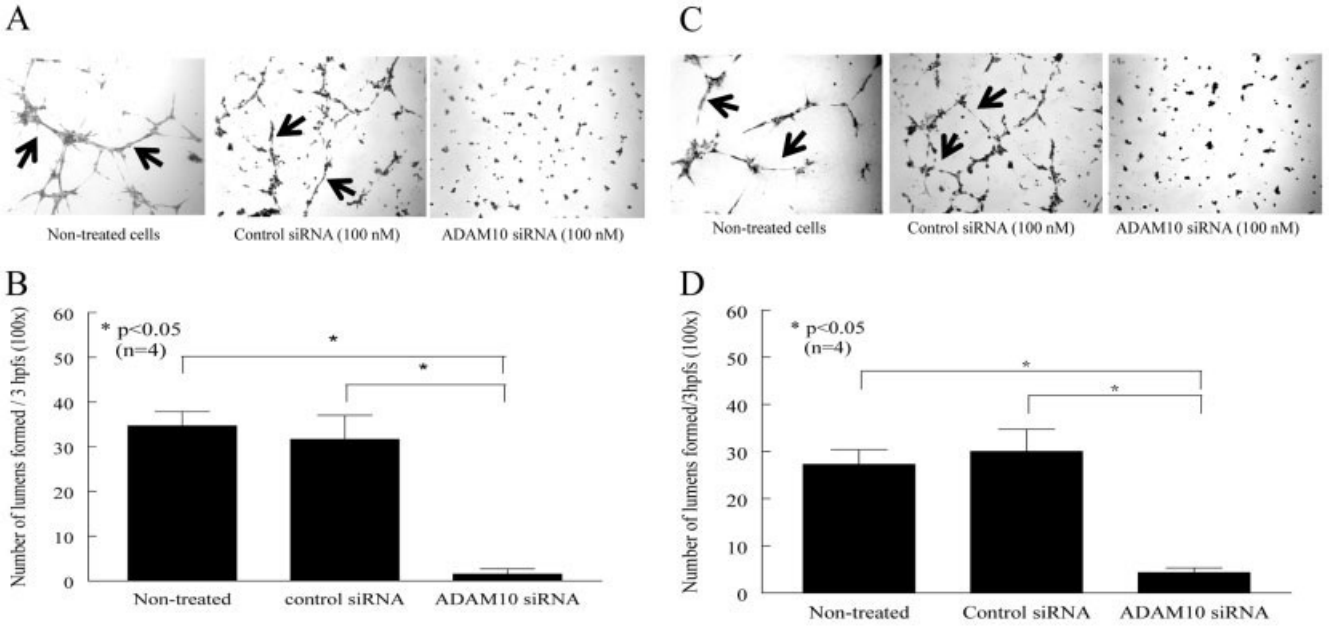

Figure 5. Tube formation by untreated HMVECs and HMVECs treated with ADAM-10 siRNA or control siRNA. **A** and **C,** Representative images showing HMVEC tube formation on Matrigel following stimulation with bFGF (**A**) and VEGF (**C**). **Arrows** indicate tube formation. Original magnification × 100. **B** and **D,** Quantification of HMVEC tube formation on Matrigel following stimulation with bFGF (**B**) and VEGF (**D**). Bars show the mean ± SEM. n = number of individual experiments (see Figure 4 for other definitions).

directly to Matrigel containing RA synovial fibroblast–conditioned medium and cultured them for 6 hours. ADAM-10 siRNA–treated HMVECs from RA synovial fibroblast–conditioned medium showed decreased endothelial cell tube formation compared with control siRNA–treated and untreated HMVECs (mean ± SEM

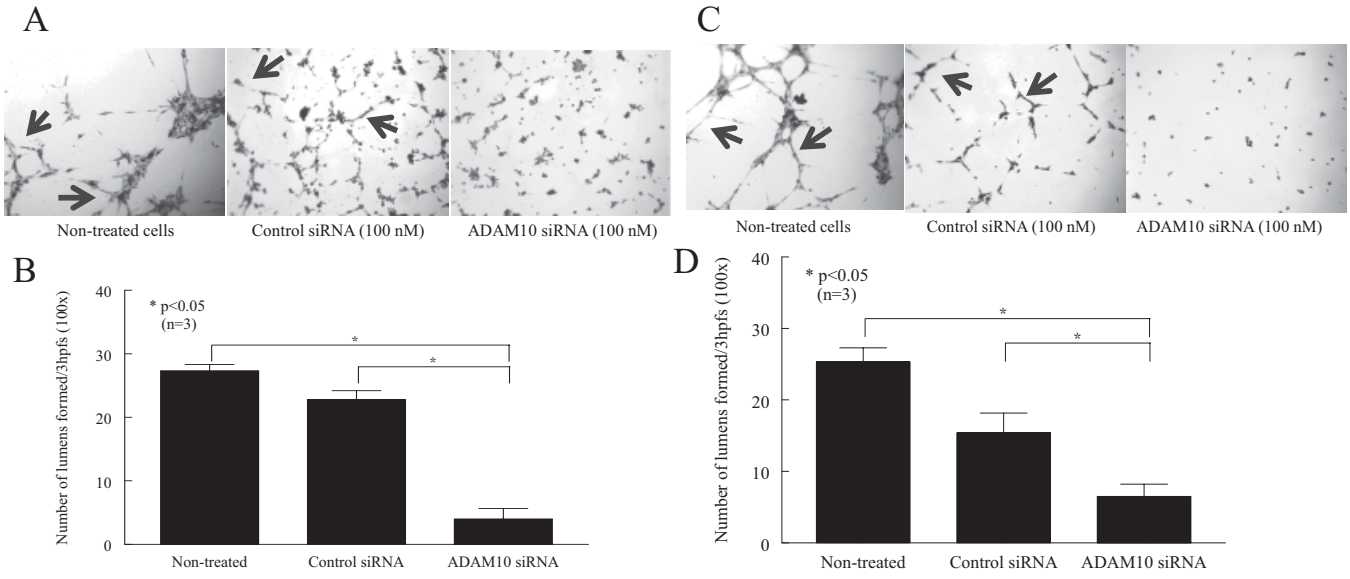

Figure 6. Tube formation by human microvascular endothelial cells (HMVECs) cocultured with rheumatoid arthritis synovial fibroblasts. HMVECs were first transfected with control or ADAM-10 small interfering RNA (siRNA) and plated in the bottom wells of the Transwell system wells. RA synovial fibroblasts were grown on the top inserts of the Transwell system. The treated HMVECs were plated on Matrigel. **A** and **C,** Representative images showing tube formation by untreated HMVECs and HMVECs treated with control siRNA or ADAM-10 siRNA (**A**) and HMVECs from the RA synovial fibroblast coculture medium (**C**). **Arrows** indicate tube formation. Original magnification × 100. **B** and **D,** Quantification of tube formation by HMVECs from the coculture system (**B**) and HMVECs from the RA synovial fibroblast coculture medium (**D**). Bars show the mean ± SEM. hpf = high-power field; n = number of RA synovial fibroblasts.

number of endothelial cell tubes formed 7 ± 2, 15 ± 3, and 25 ± 2, respectively; $P < 0.05$) (Figures 6C and D). These results showed that RA synovial fibroblasts were not involved in HMVEC tube formation, and that transfected HMVECs that were unable to express ADAM-10 had a reduced capacity to form tubes in vitro.

DISCUSSION

Angiogenesis is initiated by proangiogenic mediators that promote the release of proteolytic enzymes, including matrix metalloproteinases, resulting in the degradation of endothelial cell basement membrane and the perivascular extracellular matrix. In addition, specific angiogenic mediators activate endothelial cells and induce their proliferation and migration. The activated endothelial cells proliferate and migrate into the surrounding area, thus forming primary sprouts. Our group and others have previously shown that TNFα and other key mediators of RA pathogenesis, including fractalkine/CX3CL1, CXCL16, JAM-A, and JAM-C, exist as cell surface–expressed and soluble proteins (22,24,29). As soluble factors, these mediators promote inflammation and angiogenesis and thus drive the progression of RA.

ADAM-10 is a protease with a range of substrates that play roles in cancer proliferation, inflammation, and angiogenesis (30,31). These substrates include proinflammatory chemokines such as fractalkine/CX3CL1 and CXCL16 (14,15). We previously demonstrated that several of these ADAM-10 substrates are up-regulated in RA ST or RA synovial fluid (24,32). However, the expression and regulation of ADAM-10 in RA have not been completely described. In the only previous study of ADAM-10 in RA, Komiya et al showed that ADAM-10 mRNA was found in RA ST at levels similar to those in OA ST, but information regarding localization of ADAM-10 expression in the different ST cell types was not provided (16). Komiya and colleagues showed that the mRNA levels of other ADAMs, such as ADAM-8, ADAM-9, and ADAM-12, are similar between RA and OA. The present study clearly demonstrated that ADAM-10 is expressed on both endothelial cells and lining cells in normal, OA, and RA ST. In addition, the results showed that ADAM-10 was more highly expressed on endothelial cells and lining cells in RA ST than on endothelial cells and lining cells in OA and normal ST. We also observed that ADAM-8, ADAM-9, and ADAM-12 expression was not different between RA ST and OA ST.

Because of the more selective expression of ADAM-10 in RA ST than in OA ST, we focused on ADAM-10. In mice, ADAM-10 expression was significantly increased on the lining cells of arthritic joints compared with noninflamed control joints. In contrast, ADAM-10 expression on endothelial cells was low in the joints of both arthritic and nonarthritic mice. In addition, we observed that ADAM-10 expression was elevated in RA synovial fluid compared with PsA synovial fluid.

After observing ADAM-10 expression in RA ST endothelial cells and RA synovial fibroblasts, we determined which mediators control the expression of ADAM-10 in key cell types in RA ST. Western blotting and quantitative PCR revealed increased amounts of ADAM-10 expression on HMVECs and RA synovial fibroblasts upon stimulation with proinflammatory mediators such as TNFα, IL-1β, IL-17, PMA, or LPS. These data demonstrate the importance of both endothelial cell– and fibroblast-expressed ADAM-10 expression in RA.

We observed that ADAM-10 is also important in angiogenesis. Schulz et al showed that ADAM-10 actively plays a role in thrombin-induced decreases in endothelial cell–endothelial cell interactions (10). Those investigators reported that knockdown of ADAM-10 in human umbilical vein endothelial cells (HUVECs) by siRNA impaired T cell transmigration. Glomski et al studied ADAM-10–deficient mice and showed that these mice had vascular abnormalities such as aberrant subcapsular hepatic veins, enlarged glomeruli, intestinal polyps containing endothelial cell masses, and abnormal endochondral ossification (31). Donners et al showed that VEGF induced ADAM-10–mediated cleavage of VE-cadherin, which increases endothelial cell migration (33). They also demonstrated that VEGF increased vascular permeability in an ADAM-10–dependent manner, and that inhibition of ADAM-10 decreased endothelial cell migration.

After defining the activity of ADAM-10 using HMVEC chemotaxis and tube formation assays, we next assessed the role of ADAM-10 in angiogenesis with respect to RA. Specifically, we assessed the ability of ADAM-10 siRNA–transfected HMVECs cocultured with RA synovial fibroblasts to form tube structures. In general, coculture systems of normal dermal fibroblasts and HUVECs are used for tube formation assays (34). However, the RA joint is rich in angiogenic mediators. A number of infiltrating cells contribute to the accumulation of angiogenic factors, including T cells and monocytes. In addition to these cells, resident ST lining fibroblasts account for many of the pathologic and clinical manifestations of RA (35). Moreover, fibroblasts

produce many inflammatory and angiogenic mediators, including cytokines and chemokines (36). Therefore, we used RASFs to replicate the environment of the RA joint.

We observed that ADAM-10 siRNA–treated HMVECs had decreased endothelial cell tube formation activity compared with control siRNA–treated HMVECs in coculture with RA synovial fibroblasts or conditioned medium from the coculture system. ADAM-10 siRNA–treated HMVECs stimulated with bFGF or VEGF exhibited decreased migration compared with control siRNA–treated HMVECs stimulated with either bFGF or VEGF. We also controlled for the possibility that soluble factors such as bFGF or VEGF may be produced by RA synovial fibroblasts in the coculture system. This was done by isolating the treated HMVECs from the coculture system, washing them, and incubating these cells on Matrigel. We observed a decrease in tube-forming activity from the ADAM-10 siRNA–treated endothelial cells similar to that observed using the RA synovial fibroblast–conditioned medium, confirming that the inhibitory effect on tube formation is endothelial cell specific.

RA synovial fibroblasts secrete angiogenic factors capable of inducing endothelial cell tube formation. However, the reduction in tube formation could be attributed to the lack of ADAM-10 expression in vitro. Because ADAM-10 cleaves potent angiogenic factors such as JAM-A, JAM-C, CXCL16, and CX3CL1 from the cell surface (25,37–39), limiting cleavage of these molecules via ADAM-10 inhibition may impair the ability of endothelial cells to initiate angiogenesis. We observed no HMVEC chemotaxis toward recombinant ADAM-10. However, we observed that ADAM-10 siRNA–transfected HMVEC–conditioned medium induced less HMVEC chemotaxis compared with control siRNA–transfected HMVEC–conditioned medium. This finding suggests that ADAM-10 mediates angiogenesis indirectly. In addition, decreased ADAM-10–mediated cleavage of adhesion molecules may result in increased endothelial cell–endothelial cell interactions mediated by JAMs, thus preventing migration of endothelial cells. It would be interesting to examine these mechanisms in an in vivo environment such as a murine model of collagen-induced arthritis. Indeed, the finding that ADAM-10 inhibits joint inflammation by reducing vasculature would be novel and exciting. The in vitro results presented here indicate that such a study would be relevant and would validate the use of ADAM-10–null mice to confirm our current findings.

We report here that ADAM-10 was overexpressed in RA ST. In addition, we demonstrated that endothelial cells and ST lining cells are a source of ADAM-10, and that proinflammatory mediators upregulate the expression of ADAM-10. Moreover, we showed that ADAM-10 plays a very important role in angiogenesis in RA.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Isozaki had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Isozaki, Rabquer, Ruth, Koch.
Acquisition of data. Isozaki, Rabquer, Haines, Ruth.
Analysis and interpretation of data. Isozaki, Rabquer, Ruth, Koch.

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