Junctional Adhesion Molecule-C Is a Soluble Mediator of Angiogenesis

Bradley J. Rabquer, Mohammad A. Amin, Nanditha Teegala, Matthew K. Shaheen, Pei-Suen Tsou, Jeffrey H. Ruth, Charles A. Lesch, Beat A. Imhof and Alisa E. Koch

*J Immunol* published online 30 June 2010
http://www.jimmunol.org/content/early/2010/06/30/jimmunol.1000556

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Junctional Adhesion Molecule-C Is a Soluble Mediator of Angiogenesis

Bradley J. Rabquer,* Mohammad A. Amin,* Nanditha Teegala,* Matthew K. Shaheen,* Pei-Suen Tsou,* Jeffrey H. Ruth,* Charles A. Lesch,* Beat A. Imhof,† and Alisa E. Koch*‡

Junctional adhesion molecule-C (JAM-C) is an adhesion molecule expressed by endothelial cells (ECs) that plays a role in tight junction formation, leukocyte adhesion, and transendothelial migration. In the current study, we investigated whether JAM-C is found in soluble form and whether soluble JAM-C (sJAM-C) mediates angiogenesis. We found that JAM-C is present in soluble form in normal serum and elevated in rheumatoid arthritis (RA) serum. The concentration of sJAM-C is also elevated locally in RA synovial fluid compared with RA serum or osteoarthritis synovial fluid. sJAM-C was also present in the culture supernatant of human microvascular ECs (HMVECs) and immortalized human dermal microvascular ECs, and its concentration was increased following cytokine stimulation. In addition, sJAM-C cleavage from the cell surface was mediated in part by a disintegrin and metalloproteinases 10 and 17. In functional assays, sJAM-C was both chemotactic and chemokinetic for HMVECs and induced HMVEC tube formation on Matrigel. Neutralizing anti–JAM-C Abs inhibited RA synovial fluid–induced HMVEC chemotaxis and sJAM-C–induced HMVEC tube formation on Matrigel. sJAM-C also induced angiogenesis in vivo in the Matrigel plug and sponge granuloma models. Moreover, sJAM-C–mediated HMVEC chemotaxis was dependent on Src, p38, and PI3K. Our results show that JAM-C exists in soluble form and suggest that modulation of sJAM-C may provide a novel route for controlling pathological angiogenesis.

Angiogenesis is a highly regulated process of new blood vessel formation from pre-existing vessels. It is important in a number of physiological processes, including reproduction, development, and wound healing, and is dysregulated in disease states, such as cardiovascular disease, rheumatoid arthritis (RA), and tumor growth (1). The initiation of angiogenesis depends on the release of proangiogenic mediators that activate endothelial cells (ECs) and initiate their proliferation and migration (2). Several types of proangiogenic mediators have been identified, including growth factors, cytokines, chemokines, and cellular adhesion molecules (1).

Adhesion molecules play a central role in angiogenesis. ECs use adhesion molecules for homophilic and heterophilic adhesion and adhesion to and migration through the extracellular matrix, a key step in the progression of angiogenesis (3). In addition, stimulated increase of adhesion molecule expression results in their shedding or release from ECs (4). Several EC adhesion molecules have been found in soluble form, including ICAM-1, VCAM-1, and E-selectin (5). Previously our laboratory has shown that the soluble forms of E-selectin and VCAM-1 are angiogenic (6). Both adhesion molecules induce EC chemotaxis as well as angiogenic responses in vivo (6).

Junctional adhesion molecules (JAMs) are a recently described subfamily of the Ig supergene family that localize to tight junctions between epithelial cells and between ECs (7). To date, five members of the JAM family have been identified: JAM-A (8), JAM-B (9, 10), JAM-C (11, 12), JAM4 (13), and JAML (14). On the surface of ECs, JAMs control tight junction maintenance by engaging in homophilic and heterophilic interactions with neighboring JAM molecules (11, 15, 16). In addition to binding interactions between family members, JAMs can be redistributed to the apical surface of ECs and bind specific leukocyte integrins (17–20). By undergoing an upregulation and redistribution to the cell surface from the junctional interface, JAMs mediate the influx of leukocytes during inflammation and injury. We have previously shown that JAM-C is overexpressed on RA synovial fibroblasts and mediates myeloid cell adhesion and retention in the RA synovium (21).

Recent studies have begun to demonstrate the role that JAMs play in angiogenesis. JAM-A has been shown to interact with integrin αvβ3 to mediate basic fibroblast growth factor (bFGF)-induced angiogenesis (22–24). In addition, Lamagna et al. (25) have suggested an indirect role for JAM-C in angiogenesis. In this study, a neutralizing anti–JAM-C Ab abolished angiogenesis ex vivo and in vivo and reduced tumor growth and vascularization (25). Currently, we hypothesized that JAM-C may be a soluble mediator of angiogenesis. We report that JAM-C is present in soluble form and elevated in RA serum and synovial fluid. Soluble JAM-C (sJAM-C) was also found to be present in the culture supernatant of cytokine-stimulated ECs. Moreover, we demonstrate that sJAM-C stimulates human dermal microvascular EC (HMVEC) migration in vitro.
a process that requires Src, p38, and PI3K. In addition, using the Matrigel plug and sponge granuloma models, we found that sJAM-C–stimulated angiogenesis in vivo. Our results show that JAM-C exists in soluble form and that sJAM-C is a potent proangiogenic mediator.

Materials and Methods

Patients

Synovial fluids were isolated from patients meeting the American College of Rheumatology criteria for psoriatic arthritis (PsA), RA, and osteoarthritis (OA) (25). OA and RA were confirmed by arthrocentesis. RA and normal peripheral blood were obtained by venipuncture. All specimens were obtained following approval from the University of Michigan Institutional Review Board.

Animals

All experiments performed with animals were done with approval from the University of Michigan Committee on Use and Care of Animals. C57BL/6 mice (National Cancer Institute, Bethesda, MD) were used for both the Matrigel plug and sponge granuloma experiments.

Cell culture

HMVECs (2 × 10^4/well) were grown in complete EC basal medium (EBM)-2 medium with EC growth medium-2 SingleQuots (Lonza, Basel, Switzerland). Twenty-four hours prior to the experiment, the medium was changed to serum-free EBM-2 with or without TNF-α (25 ng/ml; R&D Systems, Minneapolis, MN). Cell culture supernatants were collected 48 h after the stimulation, cells and cellular debris were removed by centrifugation, and the supernatants were concentrated using Amicon Ultra centrifugal filters (3000 m cutoff; Millipore, Billerica, MA) following the manufacturer’s instructions.

In addition, SV-40 immortalized human microvascular endothelial ECs (HMECs) (HMEC-1; 2 × 10^4/well) were grown in complete EBM-2 medium with EC growth medium-2 SingleQuots (Lonza, Switzerland). In one series of experiments, the medium was changed to serum-free EBM-2 24 h prior to used for experiments and then the HMECs were treated with the following mediators (all from R&D Systems) at concentrations previously established (26, 27): TNF-α (25 ng/ml), INF-γ (25 ng/ml), IFN-γ (25 ng/ml), IL-1β (25 ng/ml), IL-1β (25 ng/ml), IL-1β (25 ng/ml), LPS (50 μg/ml), macrophage inhibitory factor (MIF, 300 ng/ml), bFGF (50 ng/ml), acidic FGF (aFGF, 50 ng/ml), or PMA (50 ng/ml). Cell culture supernatants were collected at 48 h and treated as above.

In a second series of experiments, HMEC-1s were cultured as above and stimulated with TNF-α (25 ng/ml) in the presence of DMSO (solvent control), TAPI-2 (a broad spectrum inhibitor of matrix metalloproteinases [MMPs], TNF-α converting enzyme [TACE], and a disintegrin and metalloproteinases [ADAMs]) [20 μM]; Calbiochem, San Diego, CA), GM 1489 (an inhibitor of MMPs 1, 2, 3, 8, and 9 [20 μM]; Calbiochem), aprotinin (a serine protease inhibitor; Sigma-Aldrich, St. Louis, MO), leupeptin (an inhibitor of serine and cysteine proteases [5 mM]; Sigma-Aldrich), pepstatin A (an aspartic protease inhibitor [3 μM]; Sigma-Aldrich), and PMSF (an inhibitor of serine proteases [575 μM]; Sigma-Aldrich). Cell culture supernatants were collected at 48 h and treated as above.

To confirm the above results, HMVECs were cultured as described above. After reaching ~70% confluence, the cells were transfected with small interfering RNAs (siRNAs) using TransIT-TKO (Mirus, Madison, WI) following the manufacturer’s instructions. Control, ADAM10, and ADAM17 siRNAs were purchased from Invitrogen (Carlsbad, CA). The transfection was allowed to proceed for 72 h, at which point cell culture supernatants were collected and treated as described above. Transfection efficiency was monitored with the use of fluorescence-labeled control siRNA and was found to be >90%. Specific knockdown of ADAM10 and ADAM17 was confirmed by Western blotting using Abs specific for either ADAM10 or ADAM17 (both from Abcam, Cambridge, MA).

ELISA

An ELISA was designed to determine the concentration of sJAM-C in biological fluids and cell culture supernatants. Ninety-six–well plates (Nalge Nunc International, Rochester, NY) were coated overnight at 4°C with goat anti-human JAM-C Ab (R&D Systems). The plates were then washed and blocked with Starting Block blocking buffer (Thermo Scientific, Waltham, MA), and incubated overnight with the following media: sera, synovial fluids, or supernatants at 4°C. Prior to use, serum and synovial fluids were immunodepleted of rheumatoid factor to avoid interference with the assay (28). The plates were then washed, and biotinylated goat anti-human JAM-C Ab (R&D Systems) was added, followed by streptavidin-HRP (BD Biosciences, San Jose, CA). The plates were developed using tetramethylbenzidine (TMB) and were read on a microplate reader. A standard curve was prepared using sJAM-C/Fc (R&D Systems). PBS served as the negative control.

Expression and purification of sJAM-C

sJAM-C was produced as described previously (12). Briefly, a pcDNA vector containing murine JAM-C in frame with a Flag tag sequence was expanded in chemically competent DH5α cells (Invitrogen) following the manufacturer’s protocol. BOSC23 cells were then transfected with the vector using a calcium-phosphate technique (12). Flag-tagged sJAM-C was purified using an anti-Flag M2 affinity gel column (Sigma-Aldrich). The purity and specificity of sJAM-C were confirmed by silver staining, Western blot analysis, and ELISA. The amount of endotoxin in the purified sJAM-C was determined using the QCL-1000 Endotoxin Chromogenic Limulus amebocyte lysate assay following the manufacturer’s instructions (Lonza).

Western blot analysis

Western blotting was performed as described previously (21). To determine the presence of sJAM-C in HMVEC supernatants, a mouse monoclonal anti-JAM-C Ab was used (R&D Systems). For cell signaling experiments, Abs against phosphorylated and total Src family kinases, p38, and PI3K (Cell Signaling Technology, Danvers, MA) were used. As a loading control, the blots were stripped and probed with an anti-actin Ab (Sigma-Aldrich).

In vitro HMVEC chemotaxis assays

HMVEC chemotaxis assays were performed as described previously (26). sJAM-C was diluted in PBS and used as a test substance at concentrations ranging from 1 μM to 10 μM. bFGF (60 nM) was used as a positive control, and PBS was used as the negative control.

To determine whether the sJAM-C present in RA synovial fluid contributes to RA synovial fluid-mediated HMVEC chemotaxis, we neutralized sJAM-C and performed HMVEC chemotaxis. RA synovial fluids were first depleted of rheumatoid factor and then incubated with neutralizing anti-JAM-C Abs F26 and H33 (each at 25 μg/ml) or rat IgG (50 μg/ml, negative control) for 15 min prior to the assay. The depleted RA synovial fluids were then used as test substances in the assay.

Checkerboard analysis was performed to determine whether sJAM-C was chemotactic or chemokinetic for HMVECs. HMVEC chemotaxis was performed with concentrations of sJAM-C in the upper chamber ranging from 0 to 100 nM and concentrations of sJAM-C in the lower chamber ranging from 0 to 100 nM. PBS was used as a negative control, and bFGF (60 nM) was used as a positive control.

To determine which kinases were required for sJAM-C–mediated HMVEC chemotaxis, cells were incubated with chemical signaling inhibitors. HMVECs were preincubated with chemical signaling inhibitors for 2 h prior to the assay, and the inhibitors were present in the lower chamber with the HMVECs during the assay. The following inhibitors were purchased and used at concentrations recommended by Calbiochem (La Jolla, CA): PD98059 (10 μM Erk1/2 inhibitor), LY294002 (10 μM PI3K inhibitor), PP2 (1 μM Src inhibitor), SB203580 (10 μM p38 MAPK inhibitor), and suramin (40 μM protein inhibitor).

In vitro Matrigel tube formation assays

Matrigel tube formation assays using growth factor-reduced Matrigel (BD Biosciences) were performed (26). Test substances used were sJAM-C (10 nM), bFGF (60 nM, R&D Systems, positive control), and PBS (negative control). After an overnight incubation at 37°C, the cells were fixed and counterstained. Photographs (×100) were taken, and tubes were counted by a blinded observer. Tubes were defined as elongated connecting branches between two identifiable HMVECs.

A second series of experiments was performed to determine whether depletion of sJAM-C from the sJAM-C–mediated HMVEC chemotaxis cells was incubated with chemical signaling inhibitors. HMVECs were preincubated with chemical signaling inhibitors for 2 h prior to the assay, and the inhibitors were present in the lower chamber with the HMVECs during the assay. The following inhibitors were purchased and used at concentrations recommended by Calbiochem (La Jolla, CA): PD98059 (10 μM Erk1/2 inhibitor), LY294002 (10 μM PI3K inhibitor), PP2 (1 μM Src inhibitor), SB203580 (10 μM p38 MAPK inhibitor), and suramin (40 μM protein inhibitor).

In vivo Matrigel plug angiogenesis assays

Matrigel plug assays were performed as described previously (26). C57BL/6 mice were anesthetized and injected s.c. with 500 μl growth factor-reduced Matrigel containing either sJAM-C (100 nM), aFGF (62.5
pM, positive control; R&D Systems, or PBS (negative control). Mice were euthanized after day 7, plugs were dissected, and angiogenesis was analyzed by hemoglobin measurement using the TMB method (26). Hemoglobin measurements were normalized to plug weight. Alternatively, some of the plugs removed were frozen in optimal cutting temperature medium for immunohistological examination. Sections were cut, and immunohistochemistry was performed as described previously (21). Polyclonal rabbit anti-von Willebrand factor (vWF; DakoCytomation, Glostrup, Denmark) was used as a primary Ab, with rabbit IgG serving as a negative control. FITC-labeled anti-rabbit IgG (Invitrogen) was used as a secondary Ab, and DAPI (Invitrogen) was used to observe nuclear staining. Photographs were taken with a fluorescence microscope (Olympus, Melville, NY).

In vivo sponge granuloma angiogenesis assays

The in vivo sponge granuloma assay is a model of inflammatory angiogenesis and was performed as described previously (29). Briefly, 1-cm² sponge discs were cut from 2-mm-thick polyvinyl alcohol foam sponges (M-pact, Eudora, KS). A 2-mm pellet was cut into the disc center to serve as a depot for sJAM-C (250 nM), sFGF (62.5 pM, positive control), or PBS (negative control). After adding the test substances to the center pellet, the sponges were sealed with Millipore filters (0.45 μm) using Millipore glue number 1 (Millipore, Bedford, MA). C57BL/6 mice were then anesthetized, and the sponges were inserted s.c. into the back of each mouse. After 7 d, the animals were sacrificed, and the sponge discs were harvested. The sponges were then cut, minced, homogenized, and analyzed for the amount of hemoglobin per sponge using the TMB method. Hemoglobin values were normalized to sponge weight.

Statistical analysis

Data were analyzed using Student t test assuming equal variances. The p values < 0.05 were considered statistically significant. Data are represented as the mean ± SEM.

Results

JAM-C is present in soluble form

To determine whether JAM-C was present in soluble form, we designed an ELISA. We found that JAM-C is soluble and detectable in normal serum (mean, 0.7 ± 0.1 ng/ml) (Fig. 1) and that it is significantly elevated in RA serum (1.2 ± 0.1 ng/ml; p < 0.05). Moreover, we found that sJAM-C is upregulated locally in RA synovial fluid (2.4 ± 0.3 ng/ml; p < 0.05) and PsA synovial fluid (2.3 ± 0.5 ng/ml; p < 0.05) and that both are significantly elevated compared with noninflammatory OA synovial fluid (1.3 ± 0.1 ng/ml; p < 0.05). This is the first demonstration of JAM-C in soluble form in biological fluids.

sJAM-C is present in EC supernatants

To determine the source and m.w. of sJAM-C, HMVECs and HMEC-1s were cultured, the cell culture supernatants were collected and concentrated, and Western blotting and ELISAs were performed. Our results indicate that sJAM-C is present in the culture supernatant of HMVECs (mean, 15.5 ± 8.4 pg/ml) and HMEC-1s (80.0 ± 50.0 pg/ml) (Fig. 2). In addition, sJAM-C was elevated in the culture supernatant of TNF-α–stimulated HMVECs (43.4 ± 13.9 pg/ml) compared with nonstimulated (Fig. 2A). The sJAM-C in HMVEC supernatants had an approximate molecular mass of 40 kDa, whereas the anti–JAM-C Ab identified two proteins of 40 and 50 kDa, suggesting the presence of both soluble and full-length JAM-C in HMVEC protein lysates (Fig. 2B).

We then determined whether other proinflammatory or proangiogenic mediators were able to increase EC expression of sJAM-C. We found that sJAM-C was significantly elevated in the culture supernatant of IL-1β–, IL-17–, LPS–, MIF–, TNF-α–, or PMA–stimulated HMEC-1s compared with nonstimulated cells (all p < 0.05) (Fig. 2C). As with the HMVEC supernatants, we again observed that the form of JAM-C present in HMEC-1 supernatants had an apparent molecular mass of 40 kDa, with no presence of the full-length m.w. protein (Fig. 2D). These results suggest that ECs release sJAM-C following stimulation with proinflammatory mediators.

ADAM10 and ADAM17 mediate release of sJAM-C

After finding that sJAM-C was present in the cell culture supernatant of HMEC-1s and upregulated in the presence of proinflammatory mediators, we sought to determine the mechanism for its release. First, we performed quantitative PCR to determine whether the increase of sJAM-C following TNF-α stimulation was the result of increased JAM-C production. However, we found that TNF-α stimulation had no effect on JAM-C mRNA expression in HMEC-1s (data not shown). Therefore, we determined whether the increase in sJAM-C in TNF-α–stimulated EC supernatants was a result of membrane-bound JAM-C being shed from the cell surface. HMVEC-1s were stimulated with TNF-α in the presence of various sheddase inhibitors (Fig. 3A). We found that the concentration of sJAM-C was significantly reduced in the cell culture supernatants of HMVEC-1s stimulated with TNF-α in the presence of TAPI-2 (p < 0.05), an inhibitor of MMPs, TACE, and ADAMs. Specific inhibitors of MMPs, serine, cysteine, or aspartic proteases had no effect on the release of JAM-C from the surface of these cells (Fig. 3A).

We then performed siRNA experiments to further elucidate which type of protease inhibited by TAPI-2 is responsible for the release of JAM-C from the cell surface. We found that the amount of sJAM-C in the culture supernatants of HMVECs transfected with specific siRNA against either ADAM10 or ADAM17 was decreased compared with those transfected with control siRNA (Fig. 3B). Collectively, these results suggest that JAM-C is cleaved from the surface of ECs by ADAM10 and ADAM17.

Characteristics of purified sJAM-C

To determine the role of sJAM-C in angiogenesis, we purified sJAM-C from the culture supernatant of BOSC23 cells transfected with a vector containing the extracellular domain of JAM-C as described previously (12). We found that our purified sJAM-C was >95% pure by Western blot analysis and silver staining with a molecular mass of ~35 kDa (Fig. 4A). In addition, we found that 50 nM sJAM-C contains 0.1 EU/ml endotoxin, which equates to 10 pg/ml or <0.01 ng endotoxin/1 μg total protein (data not shown). Collectively, these data indicate that our purified sJAM-C is free of contaminating proteins and contains only trace amounts of endotoxin.
sJAM-C mediates facets of angiogenesis in vitro and contributes the angiogenic potential of RA synovial fluid

We wondered whether sJAM-C may stimulate angiogenesis. Therefore, we performed in vitro HMVEC chemotaxis assays in the presence of sJAM-C, because EC chemotaxis is an initial step in the angiogenic process. Our results indicate that sJAM-C stimulates HMVEC chemotaxis in a dose-dependent manner, with migration significantly greater than PBS, occurring from 10 to 500 nM ($p < 0.05$) (Fig. 4A). In addition, a checkerboard analysis was performed to determine whether sJAM-C was chemotactic and/or chemokinetic for HMVECs. Our findings suggest that sJAM-C is both chemotactic and chemokinetic for HMVECs (Table I).

RA synovial fluid is rich in angiogenic mediators. Therefore, we sought to determine whether sJAM-C is a significant contributor to the chemotactic potential of RA synovial fluid for ECs in vitro. We found that depleting JAM-C from RA synovial fluids resulted in a significant decrease in the chemotactic potential of RA synovial fluid for HMVECs compared with RA synovial fluid sham depleted with an isotype-matched IgG control Ab (18% decrease; $p < 0.05$) (Fig. 4B). This result further demonstrates that sJAM-C is an angiogenic mediator and that sJAM-C is a significant angiogenic component in RA synovial fluid.

Because HMVEC chemotaxis is only one facet of angiogenesis, we determined whether sJAM-C can induce other aspects of new
blood vessel development and thus performed in vitro HMVEC tube formation assays. We found that sJAM-C induced a significantly greater number of HMVEC tubes on Matrigel compared with PBS ($p < 0.05$) (Fig. 5A–D). Collectively, these results indicate that sJAM-C mediates angiogenesis in vitro.

To confirm that the observed tube formation on Matrigel was directly attributable to sJAM-C and not other contaminating proteins or endotoxin, we performed Matrigel in vitro assays with neutralizing anti-JAM-C Abs F26 and H33 (25 μg/ml each) or IgG control Ab (50 μg/ml) and used as a stimulus for HMVEC chemotaxis. The percentage of maximal migration was calculated by dividing the number of cells migrating in the IgG control group by the number migrating in the JAM-C–depleted group. $n$, the number of different RA synovial fluid samples used. For both chemotaxis assays, means are presented with SEM, and differences were determined using the Student $t$ test. *$p < 0.05$ was significant. HPF, high-power field.

**sJAM-C induces angiogenesis in vivo**

To determine whether sJAM-C has angiogenic properties both in vitro and in vivo, we initially performed in vivo Matrigel plug angiogenesis assays. Matrigel was mixed with PBS, aFGF, or sJAM-C and injected s.c. into C57BL/6 mice. Blood vessel formation was assessed by immunohistology and hemoglobin content, a measure of plug vascularity. We found that mixing Matrigel with either angiogenic aFGF or sJAM-C resulted in a greater number of blood vessels than PBS alone (Fig. 6A–C). Moreover, when plugs were removed and the amount of hemoglobin was determined, we observed that Matrigel plugs containing sJAM-C had a significantly greater amount of hemoglobin compared with plugs containing PBS ($p < 0.05$) (Fig. 6D).

To further examine the role of sJAM-C in angiogenesis in vivo, we performed an in vivo sponge granuloma angiogenesis assay, which is a model of inflammatory angiogenesis. In this study, we found that sponges containing sJAM-C induced a significantly greater angiogenic response compared with those with PBS ($p < 0.05$) (Fig. 6E). Collectively, these findings suggest that sJAM-C is angiogenic in vivo.

**Src, p38, and PI3K are required for sJAM-C–mediated angiogenesis**

To determine the EC signaling mechanism involved in sJAM-C–mediated angiogenesis, we performed HMVEC chemotaxis assays in the presence of inhibitors to known signaling intermediates previously shown to be important in angiogenesis. Inhibitors targeting Src, p38, or PI3Ks significantly reduced the ability of sJAM-C to induce HMVEC chemotaxis compared with sJAM-C with DMSO-treated cells (all $p < 0.05$) (Fig. 7A). In contrast, Erk 1/2 and G protein inhibitors had no effect on sJAM-C–mediated HMVEC chemotaxis.

After observing that Src, p38, and PI3K are required for sJAM-C–induced HMVEC chemotaxis, Western blots were performed to determine whether stimulation with sJAM-C results in the phosphorylation of these mediators. We found that sJAM-C stimulated the phosphorylation of each of these mediators, with PI3K phos-
phorylation occurring first, followed by the phosphorylation of p38, and finally Src family kinases (Fig. 7B). Taken together, these results suggest that sJAM-C stimulates the phosphorylation of Src, p38, and PI3K and that these pathways are required for sJAM-C–mediated angiogenesis.

Discussion

Angiogenesis is a critical process in both physiological and pathological conditions, of which adhesion molecules are known to play key roles. Adhesion molecules regulate angiogenesis both indirectly and directly. They regulate angiogenesis by promoting the influx and retention of leukocytes capable of secreting proangiogenic factors or by being cleaved or alternatively spliced into soluble form and directly stimulating ECs.

We hypothesized that JAM-C is present in soluble form and mediates angiogenesis. We found that JAM-C is present in soluble form and is detectable in normal serum. To date, the only other JAM family member to be found in soluble form is JAM-A, which is elevated in the blood of patients with cardiovascular diseases (30, 31). In addition, we found that sJAM-C is more highly expressed in RA serum and synovial fluid compared with normal serum and OA synovial fluid, respectively. sJAM-C was also elevated in PsA synovial fluid, whereas OA synovial fluid was used as a noninflammatory synovial fluid control, because normal synovial fluid is present in small amounts and not readily obtainable. Previous studies have shown that the RA synovial fluid is rich in both proinflammatory and proangiogenic mediators (4). Moreover, soluble adhesion molecules, such as E-selectin, P-selectin, VCAM-1, ICAM-1, and ICAM-3, have been shown to be upregulated in RA synovial fluid (4). However, of these soluble adhesion molecules, only soluble E-selectin, ICAM-1, and VCAM-1 have been shown to be directly angiogenic (6, 32).

We and others have previously shown JAM-C to be present on the cell surface of several cell types, including fibroblasts, epithelial cells, and ECs (11, 12, 21). To determine the cellular source of sJAM-C, we cultured ECs and found sJAM-C in their unstimulated cell culture supernatants. Moreover, we found that the concentration of sJAM-C was increased in EC supernatants following stimulation with the proinflammatory mediators IL-1β, IL-17, LPS, MIF, TNF-α, IL-18, or PMA. Koenen et al. (33) found similar results for JAM-A, as stimulation of HUVECs with PMA, a combination of IFN-γ and TNF-α, or platelet-activating factor resulted in
the release of sJAM-A. In addition, previous studies have shown similar findings with members of the selectin and Ig adhesion molecule families (4). Pigott et al. (5) found that cytokine-stimulated ECs released soluble forms of E-selectin, ICAM-1, and VCAM-1. Importantly, these adhesion molecules, aside from E-selectin, are also secreted or released from a variety of other cell types in response to various conditions. Therefore, although we have established that ECs are a source of sJAM-C, they are likely not the only source, and further studies will be needed to determine which other cell types secrete or release sJAM-C.

After finding that ECs are a source of sJAM-C, we sought to determine how sJAM-C is produced by these cells. Adhesion molecules that are found in soluble form have previously been shown to be the result of cleavage from the cell surface or from alternative splicing events (4). Membrane-bound E-selectin, ICAM-1, and VCAM-1 are proteolytically cleaved from the surface of ECs (5, 34). In addition, however, splice variants of ICAM-1 and VCAM-1 without their transmembrane domains have also been observed (35–37). Moreover, Koenen et al. (33) demonstrated that JAM-A is cleaved from the surface of ECs by ADAM17 and, to...
a lesser extent, ADAM10. We found that JAM-C is proteolytically cleaved from the cell surface of ECs, because treatment with the inhibitor TAPI-2 decreased the amount of sJAM-C in the cell culture supernatant. In contrast, inhibitors of MMPs, serine, cysteine, or aspartic proteases had no effect on the release of JAM-C from the surface of these cells. Because TAPI-2 is a broad spectrum inhibitor of MMPs, TACE, and ADAMs, these results suggested the release of JAM-C from the surface of ECs is dependent on TACE and ADAMs. To confirm these findings, we performed siRNA experiments and found that ADAM10 and ADAM17 (TACE) are able to cleave JAM-C from the cell surface. However, as we also found lesser amounts of sJAM-C in HMVEC and HMEC-1 protein lysates, it is likely that JAM-C is also alternatively spliced into soluble form.

Previous studies have shown that adhesion molecules, both membrane bound and soluble, play key roles in mediating angiogenesis. We have shown that the soluble forms of E-selectin and VCAM-1 mediate angiogenesis directly (6). An indirect role for JAM-C in angiogenesis was established by Lamagna et al. (25). They found that an anti–JAM-C Ab abolished neovascularization of aortic rings in vitro and reduced tumor volume and vascularization in vivo (25). On the basis of this study and our finding that JAM-C is present in soluble form following cleavage from the surface of ECs, we postulated that sJAM-C is a proangiogenic factor. Our results show that sJAM-C is angiogenic in vitro and able to promote two facets of angiogenesis. We found that sJAM-C stimulates HMVEC chemotaxis, an initial event in angiogenesis, in the nanomolar range. Our previous studies have shown that soluble E-selectin and soluble VCAM-1 are chemotactic for HMVECs at a similar concentration range (6). Using a checkerboard analysis of sJAM-C–induced HMVEC migration, we found that sJAM-C is both chemotactic and chemokinetic for HMVECs. Moreover, we found that sJAM-C is a significant contributor to the chemotactic potential of RA synovial fluid for ECs. This result demonstrated that sJAM-C is a significant angiogenic factor present in RA synovial fluid.

In addition, we found that sJAM-C induces HMVEC tube formation on growth factor-reduced Matrigel. When ECs are grown on Matrigel in the presence of an angiogenic substance, robust tube formation occurs (38). This assay is a reliable in vitro model of angiogenesis, as we and others have previously shown a strong correlation between angiogenic factors that stimulate EC tube formation on Matrigel and those with the ability to promote in vivo angiogenesis (6, 39, 40). In addition, we used this model to demonstrate that neutralizing anti–JAM-C Abs block the angiogenic effect of our sJAM-C protein preparation. Collectively, these studies indicate that sJAM-C stimulates two different facets of angiogenesis in vitro.

After finding that sJAM-C induces HMVEC chemotaxis and tube formation in vitro, we assessed the effect of sJAM-C on angiogenesis in vivo using two different models. We first used an in vivo Matrigel angiogenesis assay in which liquid Matrigel is mixed with a potential angiogenic substance and injected s.c. into mice. The resulting solid plug, when supplemented with angiogenic factors, supports an intense vascular response (41). We found that sJAM-C induces the growth of blood vessels into the Matrigel plug and increases the amount of hemoglobin per plug. Previously, we have shown that the adhesion molecule soluble E-selectin elicits a robust angiogenic response in vivo using this method (39). In addition, we found that JAM-C induced in vivo angiogenesis in a sponge granuloma assay. This model represents inflammatory angiogenesis, because fibroblasts, lymphocytes, mast cells, and macrophages have been observed to be present at sites of neovascularization (42). Thus, it is possible that sJAM-C also mediates inflammatory cell migration, an avenue that warrants further investigation. In all, these results suggest that sJAM-C is a proangiogenic mediator in vivo.

To date, little is known regarding signaling cascades initiated by membrane-bound JAM-C, and this is the first study to show the presence of sJAM-C and to begin to examine the signaling pathways initiated by sJAM-C. Previous reports have demonstrated that JAMs can engage in homophilic and heterophilic interactions with neighboring JAM molecules (11, 15, 16). Therefore, it is possible that sJAM-C may bind membrane-bound JAM-B or JAM-C on the surface of ECs to initiate angiogenesis. It is also possible that as-yet unidentified additional receptors for sJAM-C are present on ECs. We observed that sJAM-C initiates the phosphorylation of Src, p38, and PI3K in HMVECs. In addition, we found that these kinases are required for sJAM-C–mediated HMVEC chemotaxis. Other studies have shown that vascular endothelial growth factor also uses Src and PI3K pathways to mediate angiogenesis (43, 44). In addition, we have previously shown that Src and Erk 1/2 are required for soluble E-selectin–mediated angiogenesis (39). Our results further demonstrate that Src and PI3K play key roles in mediating angiogenesis through a variety of proangiogenic mediators.

We have previously shown that JAM-C is overexpressed on ECs in the RA synovium (21). In this study, we report the novel finding of sJAM-C and demonstrate that sJAM-C is elevated in the serum and synovial fluid of patients with RA. In addition, we have shown that ECs are a source of sJAM-C and that proinflammatory cytokines, which are elevated in RA synovial fluid, upregulate the expression of sJAM-C. Moreover, it is now evident that sJAM-C mediates facets of angiogenesis in vitro and in vivo angiogenesis. Thus, in the case of RA, it is likely that the local overexpression of JAM-C combined with the presence of inflammatory cytokines results in the production of sJAM-C, which acts to promote angiogenesis in the synovium. Collectively, these findings suggest that therapies aimed at modulating the release or function of sJAM-C may be beneficial to combating angiogenic diseases, such as RA, cardiovascular disease, and tumor growth.

Acknowledgments
We thank Drs. Edwin Ades of the U.S. Centers for Disease Control (Atlanta, GA) and Thomas Lawley of Emory University (Atlanta, GA) for providing the HMEC-1 cells.

Disclosures
The authors have no financial conflicts of interest.

References


