Role of Junctional Adhesion Molecule-Like Protein in Mediating Monocyte Transendothelial Migration

Ya-Lan Guo, Rui Bai, Celia X-J Chen, Dan-Qing Liu, Yuan Liu, Chen-Yu Zhang, Ke Zen

Objective—Monocyte migration across the vascular endothelium of blood vessels is a key early event in atherosclerosis. The mechanisms underlying monocyte transendothelial migration (TEM), however, are still not completely understood. Here we studied the role of junctional adhesion molecule-like protein (JAML) in regulating monocyte TEM.

Methods and Results—Firstly, by Western blot and flow cytometry, we showed that JAML was strongly expressed in monocytes and monocyte surface expression of JAML was upregulated by monocyte chemotaxis protein-1 stimulation. Both monocyte adhesion to and migration across tumor necrosis factor-α (TNFα) preactivated human microvascular endothelial cell (HMEC-1) monolayers were dose-dependently reduced by anti-JAML antisemur or soluble extracellular JAML recombinant. Secondly, short-term exposure of human monocytes and THP-1 cells to advanced glycation end products increased cell surface JAML expression, which was correlated with enhanced cell adhesion and TEM. In contrast, knockdown of JAML in THP-1 monocytes decreased both adhesion and transmigration of THP-1 monocytes. Finally, direct binding assay of the soluble JAML to HMEC-1 monolayers suggested that endothelial coxsackie and adenovirus receptor (CAR) may serve as one of the ligands for JAML.

Conclusions—Monocytic JAML plays a critical role in regulating monocyte TEM probably via binding to the endothelial CAR and other tight junction–associated adhesive molecules. (Arterioscler Thromb Vasc Biol. 2009;29:75-83.)

Key Words: monocytes ■ JAML ■ AGEs ■ transmigration ■ endothelial
more, binding assays suggested that endothelial CAR may serve as one of the counter-receptors for JAML during monocyte TEM.

Materials and Methods

Reagents and Antibodies

Recombinant human monocyte chemotaxis protein-1 (MCP-1) and TNFα were purchased from PeproTech (Rocky Hill, NJ) and Genetech Inc (San Francisco, Calif), respectively. AGEs-BSA was prepared according to a method previously described.20 Mouse anti-JAML antiserum was generated by immunizing mice with soluble JAML extracellular domain-GST chimera.19 The concentration of total IgG in anti-JAML serum and control serum was 3.7% quantitated by ELISA. Monoclonal anti-mouse JAML antibody (clone 4E10) was purchased from eBiosciences. Monoclonal anti-CAR (Rm6B, IgG1) and anti-CD11b (OKM-1) antibodies were prepared from hydridoma culture supernatant (ATCC).

Cells

Human monocytes were isolated from the peripheral blood of healthy donors. Briefly, PBMCs were isolated using Ficoll and anti-CD14 magnetic beads were used according to the manufacturer’s instruction to isolate monocytes from PBMCs (Miltenyi Biotec). Monocytes isolated with this method were more than 90% pure with no PMN contamination. All procedures related to handling human blood were approved by Nanjing University (Nanjing, China). For comparison of JAML expression in various leukocytes, whole leukocytes were also directly harvested from the “buffy coat.”21 THP-1 monocytes (China Cell Culture Center, Shanghai, China) were cultured and maintained as described.22 Immortalized HMEC-1 was kindly provided by Dr E.W. Ades (Centers for Disease Control and Prevention, Atlanta, Ga). HMEC-1 cells were grown in MCDB-131 (Invitrogen) supplemented with 10 ng/mL epidermal growth factor (Becton-Dickinson), 1 μg/mL hydrocortisone (Sigma), and 10% fetal bovine serum (HyClone). HMEC-1 cells were seeded on gelatin (Difco)-coated tissue culture plates or permeable Transwell filters (5.0 μm pore size, Corning Costar).

Preparation of Recombinant Proteins

Soluble recombinant proteins consisting of the extracellular domain of JAML and CAR were prepared using methods previously described.19 To produce JAML-Fc, cDNA encoding the extracellular domain of human JAML was amplified by polymerase chain reaction (PCR) from a human leukocyte cDNA library (Clontech) using primers: 5′-GACAAGAGCTTTCGAGTTCTGGAAGACTCT-3′ and 5′-GAGCTTTCTCAGCTTCGTCG-3′. The amplified cDNA product of the JAML extracellular domain was then fused to a cDNA encoding a modified region of rabbit IgG1 Fc. cDNA for JAML-Fc fusion protein was then cloned into pcDNA3.1 (Invitrogen) followed by transfections in COS-7 cells. Secreted JAML-Fc was affinity-purified by Protein A-Sepharose resin (Sigma). To produce CAR-GST chimera, cDNA encoding the extracellular domain of CAR was amplified by PCR and the amplified cDNA products were fused to a GST fusion protein-encoding region in pSj26(mod),23 which was designed for eukaryotic expression and secretion of recombinant GST fusion proteins. Fc-only (the same Fc fragment of rabbit IgG derived from the same expression vector) and GST-only were also prepared as previously described.19 Chimeric proteins were dialyzed against PBS and filter sterilized. All chimeric protein reagents used in these experiments were free of detectable endotoxin (<0.1 endotoxin units) by limulus amoebocyte lysate assay.24

Immunofluorescence, Flow Cytometry, and Confocal Microscope

For surface JAML labeling, cells were incubated with blocking solution for 30 minutes and then incubated with anti-JAML antiserum for 1 hour on ice. After washing, cells were fixed with 3.7% paraformaldehyde (PF) for 5 minutes and then incubated with Alexa Fluor 488-conjugated goat antimouse antibody. Flow cytometry was performed on a FACSscan instrument, and data were analyzed using CELLQUEST software (BD Biosciences). In morphological study, fluorescently labeled cells were visualized using fluorescence microscope and confocal microscope (Olympus). Images shown are representative of at least three separated experiments.

Binding of JAML-Fc to Nonfixed HMEC-1 Monolayers

HMEC-1 monolayers cultured to confluence were gently permeabilized with Triton X-100 in the presence of protease inhibitors.19 After blocking, HMEC-1 monolayers were incubated with 10 μg/mL JAML-Fc for 1 hour on ice. Monolayers were then washed 3 times, fixed with 3.7% PF, and incubated with Alexa Fluor 488-conjugated goat antirabbit IgG for 45 minutes. As a control for JAML-Fc binding, HMEC-1 monolayers were incubated with 10 μg/mL Fc-only.

siRNA Treatment

Chemically synthesized duplex siRNA against JAML and nontargeting control siRNA (Scramble Duplex) were obtained from Sigma. All of the siRNA transfections were performed using Lipofectamine 2000 (Invitrogen) in Opti-MEM I medium with a final siRNA concentration of 100 nM.

Cell Adhesion Assay

To assess cell adhesion to HMEC-1 monolayers, confluent HMEC-1 cell monolayers cultured on gelatin-coated Transwell filters were treated with 25 ng/mL TNFα for 6 hours. Monocytes were prelabeled with 2′,7′-bis-(2-carboxyethyl)-6-carboxyfluorescin acetoxymethyl ester (BCECF-AM) (Molecular Probes). Fluorescently labeled monocytes (∼105 cells per well) were then added and incubated for 30 minutes at 37°C in the presence or absence of JAML antibody or JAML recombinant proteins. Nonadherent monocytes were removed by 3 washes with HBSS. Adherent monocytes were harvested by treating HMEC-1 monolayers with trypsin. For cell adhesion to immobilized GST recombinants, 96-well plates were incubated with CAR-GST or GST-only (20 μg/mL each) overnight at 4°C. After removal of nonbound recombinants, plates were blocked with 1% BSA. Fluorescently labeled monocytes (5×104 cells per well) were added and incubated for 1 hour at 37°C. Cell fluorescence intensity was measured by a fluorescence plate reader at excitation/emission wavelengths of 485/535 nm (Millipore),19 and cell adhesion was presented as percentage of total applied cells according to the equation: adhesion rate=fluorescence intensity (adherent monocytes)/fluorescence intensity (total applied monocytes)×100%. Adhesion was measured with triplicate replicates.

Transendothelial Migration

Migration of human monocytes or THP-1 cells across HMEC-1 monolayers was performed as previously described.25 Briefly, HMEC-1 cells cultured on gelatin-coated transwell filters were pretreated with 25 ng/mL TNFα for 6 hours. Monocytes (∼105 cells per well) or THP-1 cells (∼105 cells per well) were added to the upper chamber of Transwells inserts containing 150 μL HBSS with or without various antibodies and soluble chimeric proteins. 10 nmol/L MCP-1 was placed in the bottom chamber to initiate transmigration. After 2-hour migration, cells that had transmigrated to the lower chamber were harvested, labeled with phycocyanin-conjugated anti-CD14 antibody, and counted by flow cytometry. Migration was measured with triplicate replicates.26 The transmigration of monocytes was presented as percentage of total applied cells.

Statistical Analysis

Data are presented as the mean±SD. Paired Student t test was used for analysis of statistical significance. Values of P<0.05 were considered statistically significant.
Results

JAML Expression on Monocyte Cell Surface Is Upregulated by Chemoattractant Stimulus

A previous study demonstrated that JAML is expressed in PMNs and can mediate human PMN migration across cultured colonic T84 epithelial monolayers via interaction with epithelial CAR. Here, we investigated the potential role of monocytic JAML in regulating monocyte-endothelial interactions during monocyte TEM. Whole human leukocytes were directly obtained from the “buffy coat.” Cells were washed once with HBSS and then directly labeled with mouse antihuman JAML antiserum. As shown in Figure 1A, JAML was expressed in both monocytes and PMNs. The majority of lymphocytes were JAML-negative though a small population of lymphocytes was labeled by anti-JAML antibody. A 2-color labeling of cell fractions of gate R2 and R3 was performed to further define the distribution of JAML. As can be seen, anti-JAML strongly labeled CD14-positive monocytes. CD11b was used as a marker to separate T or B lymphocytes from natural killer lymphocytes. However, no significant anti-JAML labeling was observed in either CD11b-negative or CD11b-positive lymphocytes. At this stage, the nature of small lymphocyte fraction that expresses JAML remains unclear. Immunoblot further confirmed that JAML was strongly expressed in monocytes and PMN, as well as human monocytic THP-1 cells, but not or only weakly expressed in Jurkat (human T lymphoblast-like cell line), nondifferentiated HL-60 cells, and HMEC-1 cells (Figure 1B).

Immunofluorescence labeling further showed that JAML expression on the surface of monocytes was upregulated by MCP-1 stimulation (Figure 2A). However, total amount of JAML in monocytes was not affected by stimulation with either MCP-1 or TNFα (Figure 2B), suggesting that surface upregulation of JAML is likely attributable to JAML translocation from intracellular domain to cell surface. Figure 2C showed the localization of JAML on monocyte cell surfaces before and after a short exposure to MCP-1. Compared to nonstimulated cells, monocytes stimulated by MCP-1 had a brighter surface staining by anti-JAML antibody (arrows).

JAML Mediates Monocyte Migration Across TNFα-Preactivated HMEC-1 Monolayer

Next, we examined the role of JAML in monocyte migration across TNFα-prestimulated endothelial monolayers. For these experiments, HMEC-1 cells were cultured to confluence on gelatin-coated permeable transwell filters. Monolayers were stimulated with 25 ng/mL TNFα to upregulate the expression of surface adhesive molecules before migration assay. In the absence of JAML antibody or soluble JAML recombinant, a significant amount of monocytes migrated across TNFα-preactivated HMEC-1 monolayers in response to MCP-1 (Figure 3). As can be seen, more than 30% of total applied monocytes migrated through after 2-hour incubation. Transmigration of monocytes, however, was reduced by anti-JAML antibody (A) or soluble recombinant JAML extracellular domain (C) in a dose-dependent fashion. As controls, normal mouse serum or Fc-only had no effects on monocyte TEM. At concentration of 5.5 μg/mL, anti-JAML antibody inhibited monocyte TEM by nearly 40% percent. However, at the same concentration, no effect of anti-JAML antibody on the adhesion of monocyte to TNFα prestimulated endothelial monolayers was observed (Figure 3B). Although anti-JAML antibody did show a significant inhibition to monocyte...
adhesion at higher concentration, its effect on monocyte adhesion was less than on monocyte TEM. The results imply that JAML may be mainly involved in modulating the postadhesion process of monocyte TEM.

**Correlation Between Upregulation of JAML and Enhancement of Monocyte Chemotaxis**

It has been reported that advanced glycation end products (AGEs) are involved in tissue damage associated with diabetic complications and aging. Although the mechanism is still not clear, monocytes tend to be activated by AGEs and show a higher chemotaxis under such inflammatory conditions. Because our results from Figure 3 suggest that JAML facilitates monocyte TEM, next we examined whether AGEs treatment would increase JAML expression level in monocytes, which in turn, increased monocyte TEM. As shown in Figure 4A, treatment with AGEs-BSA for 3 hours dose-dependently increased the level of surface JAML expression in human monocytes. As a control, BSA had no effect on monocyte JAML expression. Increased JAML expression level on monocyte cell surfaces by AGEs-BSA treatment was positively correlated with an enhanced monocyte adhesion to (Figure 4B) and migration across (Figure 4C) the TNFα-preactivated HMEC-1 monolayers. The enhancement of monocyte TEM by AGEs-BSA, however, was largely abolished by treatment with anti-JAML antibody (Figure 4D).
Given that monocyte surface JAML is upregulated by MCP-1 stimulation (Figure 2) and JAML plays a critical role in regulating monocyte TEM (Figure 3), next we used THP-1 cells as a model cell line to further analyze a potential correlation between JAML expression and monocyte TEM. In a similar fashion, we treated THP-1 cells with AGEs-BSA or BSA and then tested the expression of JAML and other essential molecules for THP-1 cell adhesion and transmigration. As shown in supplemental Figure I (available online at http://atvb.ahajournals.org), the expression of total cellular JAML and the receptor for AGEs (RAGE) in THP-1 cells was increased by AGEs-BSA treatment in a dose-dependent fashion (A). In contrast, expression of JAM-A and β-actin was not altered by AGEs-BSA treatment. Compared to upregulation of total JAML expression, the upregulation of THP-1 cell surface expression of JAML by AGEs-BSA treatment was more dramatic. After overnight treatment with 0.5 mg/mL AGEs-BSA, the level of THP-1 monocyte surface JAML was more than 2.5-fold higher than the level in BSA-treated cells (B). The correlation between JAML expression levels and monocyte adhesion or transmigration was further confirmed by JAML knockdown in THP-1 monocytes using JAML-specific siRNA. As shown in Figure 5, JAML siRNA-transfected THP-1 monocytes showed a significantly lower expression level of JAML than mock-transfected THP-1 monocytes (A). Cell surface expression of JAML in JAML siRNA-transfected THP-1 cells was also significantly decreased though a fraction of cells still maintained a normal JAML expression (B). As can be seen, both THP-1 monocyte adhesion to (C) and migration across (D) TNFα-preactivated HMEC-1 monolayers were significantly reduced after JAML siRNA transfection.

**Endothelial CAR May Be One of Counter-Receptors for JAML During Monocyte TEM**

Previously we reported that epithelial expression of coxsackie and adenovirus receptor (CAR) served as a counter-receptor for JAML in regulating PMN migration across colonic epithelial monolayers. As CAR is also expressed at endothelial cell tight junction (TJ) complexes, we next determined whether CAR-JAML interactions have a similar role in regulating monocytic cell migration across endothelial monolayers. The binding of soluble JAML extracellular domain-Fc fusion protein to nonfixed HMEC-1 monolayers was performed. As shown in Figure 6A, soluble JAML-Fc specifically bound to the cellular borders of HMEC-1 monolayers (arrows) whereas no binding of Fc-only was observed. Binding of JAML-Fc to the borders of HMEC-1 monolayers was specifically abolished by anti-JAML antiserum at a concentration of 25 μg/mL. Functional anti-CAR antibody, at a concentration of 50 μg/mL, also partially blocked the binding of soluble recombinant JAML to HMEC-1 monolayers.

*Figure 4.* Upregulation of monocytic JAML expression, monocyte adhesion, and TEM by AGEs. Isolated human monocytes from periphery blood stream were treated with AGEs-BSA in culture medium for 3 hours. Cells treated with BSA served as a negative control. Treated monocytes were then used in the assays of monocyte adhesion to and migration across TNFα-preactivated HMEC-1 monolayers. A, Upregulation of monocyte cell surface JAML by AGEs-BSA. B, Inhibition of monocytes adhesion by AGEs. C, Inhibition of monocyte TEM by AGEs-BSA. D, TEM of AGEs-treated monocytes or BSA-treated monocytes in the presence of various antibodies at concentration of 20 μg/mL. All data are mean±SD of three independent experiments. *P*<0.05.
ers, suggesting a potential role of endothelial CAR as JAML counter-receptor. However, only partial inhibition of JAML-Fc binding by anti-CAR antibody, even at relative high concentration, also imply the existence of other ligand(s) for JAML at the border regions of HMEC-1 monolayers. The role of binding interactions between monocytic JAML and endothelial CAR was further characterized by assessing the adhesion of monocytes to immobilized CAR-GST recombinant. As shown in Figure 6B, freshly isolated human monocytes specifically adhered to the 96-well plate coated with CAR-GST, and the adhesion was strongly blocked by anti-JAML antiserum and JAML-Fc recombinant. The adhesion was not affected by anti-CD11b antibody a44 and by mAb J10.4, an monoclonal antibody against another tight junction molecule JAM-A.19 Taken together, these results suggest that endothelial monolayer CAR is one of the adhesive molecules that serve as the binding partners for JAML during JAML-mediated monocyte TEM.

**Discussion**

In the present study, we investigated the role of JAML in modulating monocyte migration across TNFα-preactivated HMEC-1 monolayers. The results show that JAML expression in monocytes is upregulated by stimulation of chemoattractant or proinflammatory reagents, and through interaction with endothelial CAR or other tight junction-associated adhesive molecule(s), JAML facilitates monocyte migration across endothelial monolayers.

Unlike other junctional adhesion molecules, JAML is not expressed in the cell lines that have a well-defined tight junction complex such as epithelial or endothelial cells. In contrast, it is expressed preferentially in granulocytes and monocytes. The function of JAML in those cells is not clear, though there is evidence suggesting that it may be involved in leukocyte differentiation.18 Here, we present evidence that expression of JAML in monocytes can be upregulated by various factors related to inflammation such as MCP-1 and AGEs. Interestingly, these factors all directly or indirectly promote monocyte adhesion and transmigration. Therefore, upregulation of monocytic JAML expression under these proinflammatory conditions suggests that JAML may be involved in monocyte recruitment. The positive correlation between JAML expression and monocyte influx was also suggested by an in vivo study using streptozotocin (STZ) treatment-induced hyperglycemia mice. Under certain conditions, STZ-treated animals showed an elevated level of proinflammatory cytokines such as TNFα and a decreased level of antiinflammatory interleukin (IL)-10, therefore they have been used as a model of chronic inflammation.33 In STZ-treated mice, monocytes were more activated and had a higher capacity of adhesion and chemotaxis. In agreement with this, we found that, after 10 to 12 days of STZ treatment, JAML expression levels in mouse monocytes were nearly 2-fold higher than those of mice treated with vehicle only (data not shown). The mechanism underlying the upregulation of JAML expression by proinflammatory factors such as

![Figure 5.](https://example.com/figure5.png)
MCP-1 (Figure 2) and AGEs-BSA (Figure 4) is unknown, although it may be related to AGEs-activated transcription factor nuclear factor-kappaB (NF-κB). It would be interesting to test whether JAML upregulation can be blocked by terminating the NF-κB signaling pathway. Because monocyte adhesion to endothelial monocytes is mediated by multiple cell surface adhesion molecules including JAML, upregulation of JAML onto the cell surface of monocytes by MCP-1 or other inflammatory factors such as AGEs may be essential for successful adhesion of monocytes during the TEM process. There are 2 likely pathways that may contribute to the upregulation of JAML onto the cell surface of monocytes: (1) JAML translocation from intracellular vesicles or granules onto the cell surface of monocytes, possibly in the case of MCP-1 stimulation, and (2) de novo synthesis of JAML in monocytes under chronic inflammation as shown in AGEs-treated THP-1 monocytes (supplemental Figure 1).

There are many factors that participate in modulating the entire process of monocyte diapedesis. Our data demonstrated that JAML is one of those essential molecules that mediate the adhesion and migration events of monocytes. JAML antibody and the soluble JAML extracellular domain are both potent inhibitors of monocyte adhesion to and migration across HMEC-1 monolayers preactivated by TNFα (Figure 3). Additionally, an essential role for JAML in regulating monocyte adhesion and transmigration has been also tested in THP-1 cells by upregulation (Figure 4) or downregulation (Figure 5) of JAML expression. Previously Moog-Lutz et al reported that human HL-60 cells began to express JAML when stimulated by RA or DMSO. In a separate experiment, we also observed that JAML in differentiated HL-60 PMN is largely located at granular-like structures, and that these granular-like structures are largely associated with actin filaments (data not shown). We are therefore tempted to hypothesize that JAML may modulate the mobilization of HL-60 PMN granular structures via directly interacting with actin filaments during cell differentiation. Future study would be required to determine whether JAML can directly interact with F-actin through its intracellular domain.

Through direct labeling of nonfixed HMEC-1 monolayers with soluble JAML extracellular domain fusion protein, we demonstrated that the endothelial cell binding partners for JAML are likely endothelial cellular junction-associated proteins (Figure 6A). Given the unique localization of its counter-receptors, JAML may execute its role in modulating monocyte TEM at the level of the cellular junctions. This conclusion is supported by the differential effect of anti-JAML antibody on monocyte adhesion and TEM. As shown in Figure 3, anti-JAML antibody strongly blocks monocyte TEM but shows a less inhibitory role on monocyte adhesion. Because the counter-receptor(s) of JAML, including CAR, are located at the tight junction of endothelial monolayers, they are not accessible for migrating monocytes at the initial adhesion stage. According to the current model of monocyte diapedesis, adherent monocytes will crawl along the endothelial monolayer surface to get to transmigration site where they migrate across. These transmigration sites are generally within the tight junction region. Therefore, JAML may mainly modulate the transmigration process of monocyte after cell crawl to the tight junction complex of endothelial monolayers. The present study confirmed that endothelial tight junction-associated protein CAR may serve as one of the ligands for JAML during monocyte TEM. At this stage, however, we have no direct evidence to demonstrate where the monocytes are blocked by anti-JAML antibody. To clarify this issue, future morphological study of whole monocyte TEM process is required. Cell adhesion assays clearly showed that JAML-CAR interactions can mediate monocyte adhesion (Figure 6B). Through their heterophilic interactions, monocyctic JAML and endothelial CAR may play a critical role in regulating monocyte migration across endothelial monolayers. Because CAR has been shown to be associated with actin and microtubulin, the binding of JAML to CAR may trigger actin rearrangements that permit leukocyte movement across the tight junction of endothelial monolayers, in a mechanism similar to coxsackie viruses crossing the tight junction gate. Interestingly, although CAR was initially cloned as a receptor for both coxsackie B viruses and adenoviruses, its primary biological functions and regulatory mechanisms have been gradually realized in recent years.
It has been postulated that epithelial or endothelial monolayers would compromise their barrier functions under inflammatory stimuli to allow leukocytes to migrate across. Although normally localized at endothelial tight junctions, JAM-A has been found to be redistributed away from cell junctions to the apical pole on activation. Similar redistribution of CAR from tight junction complexes to the apical surface of cells under inflammatory condition may significantly increase JAML-mediated adhesion of monocytes to endothelial monolayers. In addition, there are several CAR-like membrane proteins, including CAR-like soluble protein (CCLSP), adipocyte adhesion molecule (ACAM), and CAR-like membrane protein (CLMP), that have been identified in epithelial cells. Additional studies are required to identify whether these CAR-like adhesive molecules are expressed at the tight junction of endothelial cell monolayers and serve as binding partners for JAML during monocyte TEM. Studies focusing on the identification of new adhesion molecules that interact with JAML and regulate monocyte TEM may provide new targets for anti-inflammatory therapies.

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Disclosures
None.

References


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**Supplemental Figure 1**  Upregulation of total (A) and cell surface (B) JAML expression in THP-1 cells by AGEs. THP-1 cells were treated with AGEs-BSA or BSA (served as a negative control) in culture medium overnight.