Essential Role of Junctional Adhesion Molecule-1 in Basic Fibroblast Growth Factor–Induced Endothelial Cell Migration

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Objectives—Recently, we have shown that blocking of junctional adhesion molecule-1/A (JAM-1/A) inhibits basic fibroblast growth factor (bFGF)-induced angiogenesis. Because the process of endothelial cell proliferation is a key initial step of neovascularization, we studied the effect of functional knockdown of JAM-1 on human umbilical vein endothelial cell (HUVEC) adhesion and migration induced by bFGF.

Methods and Results—We introduced small interfering RNAs specific to JAM-1 in HUVECs, stimulated them with bFGF, and studied the resultant adhesion and migration of these cells on vitronectin and fibronectin. We show that depletion of JAM-1 inhibits bFGF-induced HUVEC migration specifically on vitronectin. This inhibition is not attributable to the failure of junctional organization, because expression and distribution of other junctional proteins remained unaffected. This inhibition was in fact attributed to an inability of JAM-1–depleted HUVECs to adhere and spread on vitronectin. Furthermore, we find that JAM-1–depleted HUVECs failed to activate extracellular signal–related kinase (ERK) in response to bFGF treatment.

Conclusions—Our results show that JAM-1 is required for the bFGF-induced ERK activation that leads to endothelial cell migration on vitronectin. These data thus implicate JAM-1 as an integral part of both bFGF and ERK signaling pathways in endothelial cells. (Arterioscler Thromb Vasc Biol. 2003;23:GGG–GGG.)

Key Words: junctional adhesion molecule-1 ■ basic fibroblast growth factor ■ extracellular signal–related kinase ■ endothelial cell migration ■ small interfering RNA

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JAM-1 may regulate the bFGF and ERK signaling pathways that are involved in endothelial cell migration, leading to wound repair.

**Methods**

**siRNA Synthesis**

Double-stranded RNAs of 19 nucleotides were synthesized by Dharmacon Research. The targeting sequence of human JAM-1 mRNA (5’-GGA GAC ACC AGA CUC G-3’) corresponds to the region 199-217, relative to the first nucleotide of the start codon. Firefly (Photinus pyralis) luciferase siRNA served as control. The targeting sequence of luciferase mRNA (accession No. X65324) (5’-CGU CCG AAT ACC AUC UCG A-3’) corresponds to the region 153-171, relative to the first nucleotide of the start codon.

**Cell Culture and siRNA Transfection**

HUVECs and appropriate growth media were purchased from Clonetics. Transfection of HUVECs with duplex synthetic siRNA was performed using Lipofectamine reagents (Invitrogen) as described previously.14 Cells were assayed after 48 hours of transfection. For mock transfection, all procedures listed above were performed in the absence of siRNA duplex. To routinely obtain greater than 70% to 80% transfection efficiency, transfection conditions were standardized by cotransfecting siRNA oligos with Green Lantern expression vector (Life Technologies).

**Reverse Transcriptase–Polymerase Chain Reaction**

Reverse transcriptase (RT) reactions were performed by using the cells to cDNA II kit (Ambion) as per the manufacturer’s protocol. This kit allows for the generation of cDNA without the need to perform a separate RNA isolation protocol. The polymerase chain reaction (PCR) amplification of JAM-1 cDNA was performed using the following gene-specific primers: sense, (5’- ATG GGG ACA AAG GCC CAA GTC GAG AGG AAA 3’); antisense, (5’-TCA CAC CAG GAA TGA CTG TTT GAA 3’). During PCR, a pcDNA construct of JAM-1 was used as a positive control, and a RT reaction sample without RT was used as a negative control.

**Immunofluorescence**

Immunofluorescence studies were performed as described previously. HUVECs transiently transfected with JAM-1 or luciferase siRNA constructs were incubated with indicated primary antibodies (1:100) at 4°C overnight. The next day, slides were incubated with rhodamine-X-conjugated donkey anti-mouse IgG secondary antibody (1:300) (Jackson ImmunoResearch Laboratories) for 1 hour at room temperature. For visualization of F-actin, FITC-conjugated phalloidin (1:100) (Molecular Probes) was included during incubation along with the secondary antibody. SlowFade (Molecular Probes) was added to minimize fading of the fluorescence intensity. Optical sections were taken using Zeiss LSM510 laser-scanning confocal microscope to visualize the cell-cell junctions.

**Migration Assays**

The capacity for migration of HUVECs transiently transfected with JAM-1 or control siRNA constructs was monitored by 2 different assays. A wound-induced migration assay was performed as described previously. Wounds were monitored for 24 hours under serum-free conditions. In some experiments, after 24 hours of incubation, serum-free media was replaced with growth medium containing 10% FBS or containing 10 ng/mL bFGF (Sigma) and wounds were monitored for an additional 4 hours. Additionally, a more quantitative haptotactic migration assay was performed essentially as described previously. Transwell inserts (8-µm pore size) were coated on the underside with vitronectin (10 µg/mL) or fibronectin (10 µg/mL) as indicated. Mock-transfected, luciferase siRNA-transfected, or JAM-1 siRNA-transfected HUVECs were serum starved overnight, harvested (5×10⁵ cells/mL), and treated with 10 ng/mL bFGF where indicated, and then 100 µL of the cell suspension was added to the inserts and allowed to migrate for 5 hours at 37°C. The average number of migrated cells in 10 randomly chosen fields of view was taken to quantify the extent of migration. In addition, each set of experiments was performed in triplicate. In some experiments, mock-transfected or JAM-1–transfected cells were preincubated with DMSO or PD98059 (50 µmol/L) for 20 minutes and migration assay was performed as above.

**Cell Adhesion and Morphology**

Luciferase or JAM-1 siRNA-transfected HUVECs were serum starved overnight and then harvested. Cells (5×10⁵) were then plated on a vitronectin or fibronectin precoated 24-well dish and allowed to spread under serum-free conditions for 4 hours at 37°C. Wells were rinsed with PBS to remove the nonadherent cells and then fixed and stained using Diff-Quik (Dade Behring). Attached cells were counted, and morphological changes were visualized using Nikon phase-contrast microscope under ×400 magnification.

**ERK Activation**

ERK activation was assessed essentially as described previously.17 In brief, HUVECs (1.5×10⁵ cells/mL) mock-transfected or transfected with indicated siRNA constructs were plated on vitronectin, serum starved overnight, and then treated with 10 ng/mL bFGF for 10 minutes where indicated. Lysates were then added immediately after bFGF treatment. Samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then immunoblotted as described previously. Phosphorylation of ERK1/2 was detected by probing with anti-phospho-ERK1/2. Blots were then stripped and reprobed with anti-ERK1/2 antibodies to show the total amount of ERK1/2.

**Statistical Analysis**

Standard statistical tests (mean value, SEM, paired Student’s t test) were performed for data analysis. Results are expressed as mean±SEM. P<0.05 was regarded as statistically significant.

**Results**

**Knockdown of JAM-1 Using RNA Interference Technology**

To precisely determine the involvement of JAM-1 in bFGF-induced endothelial cell physiology, we used RNA interference (RNAi) technology to knock down JAM-1 expression. Small interfering RNAs (siRNAs) specific to JAM-1 and to a control luciferase gene not expressed in HUVECs were transfected into these cells. RT-PCR was performed on isolated HUVEC RNA to determine the extent of JAM-1 mRNA knockdown by siRNA transfection (Figure 1A). Mock-transfected HUVECs (lane 1) show a considerable amount of endogenous JAM-1 expression, which is comparable to control JAM-1 amplification from 1 ng of plasmid vector (lane 4). Luciferase siRNA-transfected cells also express a considerable amount of JAM-1 mRNA (lane 2), indicating that transfection of siRNA against an unrelated protein does not affect JAM-1 mRNA expression. However, JAM-1 siRNA transfection drastically reduced endogenous JAM-1 mRNA levels (lane 3), indicating that the JAM-1 message can in fact be knocked down in these cells using this technology. To determine whether this drastic decrease of JAM-1 mRNA would result in reduction in JAM-1 protein expression, mock-transfected and siRNA-transfected HUVEC lysates were subjected to Western blot analysis. As expected, JAM-1 protein expression was considerably knocked down in JAM-1 siRNA-transfected cells compared with mock-
transfected or luciferase siRNA-transfected cells (Figure 1B). Although not 100% knocked down because of the limitation of transient transfection, the profound reduction of JAM-1 protein that did occur was indeed attributable to the effectiveness of JAM-1 siRNA and not to the overall inhibition of protein synthesis, because $\beta$-tubulin expression among samples remained unchanged (Figure 1B). To additionally demonstrate the effectiveness of JAM-1 siRNA, transfected cells were observed for JAM-1 expression under confocal microscopy. Mock-transfected and luciferase siRNA-transfected HUVECs exhibited typical JAM-1 staining at the cell-cell junctions (Figure 1C). However, in JAM-1 siRNA-transfected cells, JAM-1 staining is barely visible, indicative of the extent of depletion of JAM-1 expression (Figure 1C). Interestingly, in JAM-1 siRNA-transfected cells, we consistently observed a decrease in membrane-associated actin (Figure 1C), suggesting that JAM-1 may be required for the stability of the membrane-associated cytoskeleton. Together, these data confirm the generation of JAM-1–depleted cells, which can now be used to study the effect of JAM-1 functional knockdown in bFGF-induced events.

Reduction of JAM-1 Expression Does Not Affect the Localization of Other Tight Junctional Components

Before testing the functional relevance of the lack of JAM-1 expression, we first sought to confirm that JAM-1 knockdown did not affect the expression or localization of other proteins that are known to be functionally associated with JAM-1. To do so, we analyzed luciferase siRNA-transfected and JAM-1 siRNA-transfected HUVECs for expression of integrin $\alpha$,$\beta_3$ and the tight junction protein ZO-1, which are known to associate with JAM-1,13,19,20 and 2 other cell-cell junction–associated proteins, VE-cadherin and $\beta$-catenin. Western blot analysis indicated that although JAM-1 expression is reduced (Figure 1B), the expression levels of all of these proteins remained unaffected in cells transfected with JAM-1 siRNA oligos (Figure 2A). Additionally, immunofluorescence analysis of these proteins indicated that neither was their localization affected in the absence of JAM-1. VE-cadherin and $\beta$-catenin are adherens junction proteins, and optical sections taken at such junctions in both luciferase siRNA-transfected and JAM-1 siRNA-transfected cells exhibited similar staining patterns (Figure 2B). JAM-1 siRNA-transfected cells showed no detectable JAM-1 staining, indi-
cating that these cells were indeed JAM-1 deficient (Figure 1C). ZO-1 is a tight junction protein that binds JAM-1 through its PDZ domain.\textsuperscript{19,20} At optical sections taken through areas of tight junctions, ZO-1 localization is also similar in both luciferase siRNA-transfected and JAM-1 siRNA-transfected HUVECs. B, Confocal images of VE-cadherin, ZO-1, \(\beta\)-catenin localizations in luciferase siRNA-transfected and JAM-1 siRNA-transfected HUVECs. Scale bar=10 \(\mu\)m.

Reduction of JAM-1 Blocks bFGF-Induced HUVEC Migration on Vitronectin

We have recently shown that bFGF-induced neovascularization requires JAM-1.\textsuperscript{13} Because the angiogenic process requires endothelial cell migration, we sought to investigate the role of JAM-1 in this process and thus performed a wounding assay using luciferase and JAM-1 siRNA-transfected HUVECs. A wounded monolayer of cells was monitored in the absence of serum or bFGF for 24 hours, during which time no visible migration occurred in either luciferase or JAM-1 siRNA-transfected cells (data not shown). After this time, cells were additionally incubated for 4 hours in the presence of bFGF and, as expected, luciferase siRNA-transfected cells migrated to fill the wounded area (Figure 3A). However, even in the presence of bFGF, migration of JAM-1 siRNA-transfected cells into the wound was greatly reduced (Figure 3A).

To more quantitatively investigate these data, we performed a haptotactic transwell motility assay. In the absence of bFGF, mock-transfected, luciferase siRNA-transfected, and JAM-1 siRNA-transfected HUVECs all migrated minimally on a vitronectin matrix (Figure 3B). However, on treatment with bFGF, only mock-transfected and luciferase siRNA-transfected HUVECs showed enhanced migration (Figure 3B). Consistent with the observed wound-induced migration, JAM-1 siRNA-transfected HUVECs showed sig-
Significantly reduced bFGF-induced migration on vitronectin compared with control cells (Figure 3B, *P < 0.005). It has been well established that bFGF-induced signaling is specific to vitronectin, because bFGF signals through the vitronectin receptor, α,β. Interestingly, this JAM-1–dependent migration also seemed to be specific to vitronectin, because bFGF marginally induced HUVEC migration on fibronectin and basal migration was unaffected by JAM-1 depletion (Figure 3C). Taken together, these data suggest that JAM-1 is required for bFGF-induced HUVEC migration on vitronectin.

Knockdown of JAM-1 Inhibits HUVEC Adhesion on Vitronectin

It is well understood that a symbiotic relationship must exist between adhesive and motile properties for efficient cell migration. A nonadhesive cell cannot migrate any better than would one that was overly adherent; consequently, a progression of rapid adhesion and deadhesion events promote the most effective cellular motility. Our data to this point suggest that JAM-1 is required to promote endothelial cell migration on vitronectin, so we next sought to investigate the role of JAM-1 in HUVEC adhesion. Consistent with our migration data above, luciferase siRNA-transfected cell adhesion on vitronectin was only slightly enhanced by treatment with bFGF (Figure 4A). JAM-1 depletion, however, significantly reduced HUVEC adhesion to vitronectin, even in the presence of bFGF (Figure 4A, *P < 0.005). Concomitantly, adhesion to fibronectin was also slightly increased on bFGF treatment, and this adhesion was unaffected by JAM-1 depletion (Figure 4B). Thus, it seems as though the promotion of bFGF-induced endothelial migration on vitronectin is attributable to the cell’s ability to adhere, and this process is dependent on the level of JAM-1 expression.

Reduction of JAM-1 Affects HUVEC Spreading on Vitronectin

The above adhesion data suggest that in the absence of JAM-1, HUVECs fail to adhere as well to vitronectin as control cells, even in the presence of bFGF stimulation. To more precisely determine the cause, we stained adherent luciferase siRNA-transfected and JAM-1 siRNA-transfected HUVECs to observe their cellular morphology. Luciferase siRNA-transfected HUVECs spread well on vitronectin, taking on a very flat morphology (Figure 5A, top left). The presence of wide and well-spread lamellipodia (arrow) was also apparent (Figure 5A, top left). However, JAM-1 siRNA-transfected cells seemed only partially spread (Figure 5A, top right). Narrow filopodia-like structures were evident (arrows) but not spread into the wide lamellipodia, as observed in control cells. Additionally, JAM-1–depleted cells were much

**Figure 3.** bFGF-induced HUVEC migration is inhibited on JAM-1 depletion. A, Wound-induced migration assay using luciferase siRNA-transfected and JAM-1 siRNA-transfected cells in the presence of bFGF. Arrows indicate the original wound edge. Magnification ×200. B, Haptotactic transwell migration assay of mock-transfected, luciferase siRNA-transfected, or JAM-1 siRNA-transfected HUVECs on vitronectin. Depletion of JAM-1 significantly (*P < 0.005) inhibits bFGF-induced migration on vitronectin. C, bFGF treatment only slightly increases HUVEC migration on fibronectin, and this migration is unaffected by JAM-1 depletion.

**Figure 4.** HUVEC adhesion to vitronectin is inhibited on JAM-1 depletion. A, Luciferase siRNA-transfected and JAM-1 siRNA-transfected HUVECs were allowed to adhere to vitronectin in the absence and presence of bFGF. Adhesion of JAM-1 siRNA-transfected cells is significantly (*P < 0.005) inhibited on JAM-1 depletion, even in the presence of bFGF. B, bFGF treatment minimally increases HUVEC adhesion to fibronectin, and this adhesion is unaffected by JAM-1 depletion.
more 3D and raised, because it appeared as though a substantially lower amount of membrane surface was engaged by matrix (Figure 5A, top right). On treatment with bFGF, the morphology of luciferase siRNA-transfected cells appeared similar to that of cells in the absence of bFGF, indicating bFGF treatment had no effect on cell spreading (Figure 5A, bottom left). JAM-1 siRNA-transfected cells, however, remained only partially spread in the presence of bFGF (Figure 5A, bottom right), suggesting that bFGF treatment was not sufficient to overcome the partially spread morphology that occurred on JAM-1 depletion. Quantitation of the amount of well-spread cells among the observed cell populations indicated that on JAM-1 knockdown, the amount of fully spread cells decreased as partially spread cells increased (Figure 5B). In comparison, cells adhered to fibronectin exhibited a fully spread morphology with wide lamellipodia (arrows) independent of bFGF treatment or JAM-1 depletion (Figure 5C), suggesting that cell spreading on fibronectin is not dependent on bFGF or JAM-1 signaling. Taken together, these data indicate that the decrease in adhesion to vitronectin observed in JAM-1–depleted cells could be attributable to the fact that these cells could not spread as well as control cells. This inability to fully spread could thereby explain the inhibition of HUVEC migration on...
Reduction of JAM-1 Blocks bFGF-Induced ERK Activation

We next sought to investigate these adhesion and migration data in terms of a signaling mechanism. It has been shown previously that bFGF-induced migration requires ERK activation. Because we have now implicated JAM-1 in the bFGF-induced signaling pathway leading to HUVEC adhesion and migration, we consequently investigated the effect of JAM-1 knockdown on the bFGF-induced ERK1/2 response. We found that in the absence of bFGF, very little active ERK (P-ERK1/2) was present in mock-transfected, luciferase siRNA-transfected, or JAM-1 siRNA-transfected cells that were plated on vitronectin (Figure 6A). As expected, on bFGF treatment, ERK activity was greatly enhanced; and it was also enhanced to a similar extent in mock-transfected and luciferase siRNA-transfected cells (Figure 6A). However, ERK activity in JAM-1 siRNA-transfected cells on bFGF treatment was substantially reduced compared with control (Figure 6A). To determine if ERK activation is required for JAM-1–induced endothelial cell migration, we performed transwell migration in the presence of MEK inhibitor PD98059. We found that JAM-1–induced HUVEC migration was blocked on inhibition of ERK activation. Thus, the reduced adhesive and migratory capability of these cells must be linked to the inhibition of ERK activation on JAM-1 depletion, which is consistent with the report that bFGF-induced ERK activity is required for endothelial cell migration. Moreover, it seems that JAM-1 is required in bFGF-induced signaling that leads to ERK activation and endothelial cell migration. Thus, these data introduce JAM-1 as a key player in the bFGF-induced signaling pathway.

Discussion

In response to vascular injury, a vast number of signaling processes must take place to regulate the necessary restorative events that include endothelial cell adhesion and migration. It has been previously reported that bFGF activates ERK signaling, which is fundamental to the endothelial cell migration that seals an injured vasculature. In this study, by silencing JAM-1, we show that bFGF-induced HUVEC migration on vitronectin requires JAM-1. In JAM-1–depleted cells, adhesion to vitronectin is hindered, thus accounting for their impeded migratory ability. Furthermore, we find that in the absence of JAM-1, bFGF-induced activation of ERK is greatly inhibited. Because ERK activity is required for endothelial cell migration, we thus attribute the inability of JAM-1–depleted cells to migrate on vitronectin to an inability of bFGF to activate ERK in the absence of JAM-1. Thus, this study defines JAM-1 as a primary signaling component of the bFGF-ERK pathway.

A historic approach to study the function of a particular protein is to investigate physiological events in its absence. We have decided to take advantage of the highly specific technology of RNA interference to study the process of bFGF-induced endothelial cell migration in the absence of vitronectin observed in JAM-1 siRNA-transfected cells on treatment with bFGF.
JAM-1. RNAi technology is based on the idea of interfering with protein expression at the transcriptional level. When transfected into cells, gene-specific duplex synthetic short interfering RNAs have been shown to significantly knock down expression of the targeted gene.10,23 Although a commercially available vector-based RNAi technology allows for the production of a clonal population of gene-deficient cells, we decided to use synthetic siRNAs because of the limitation of HUVEC passage number and subsequent inability to produce a clonal population. In this manner, only transiently transfected cells could be generated for our studies. Nevertheless, we were able to achieve highly significant knockdown of JAM-1 mRNA and thus protein expression without vector-based technology.

On siRNA knockdown of JAM-1, we sought to determine whether JAM-1 depletion affected functionally related proteins. JAM-1 has been well established as a tight junctional component, interacting at the cell-cell junction in a homophilic manner.7–9 JAM-1 is known to have a PDZ domain–binding motif, through which it has been shown to bind other PDZ domain–containing tight junction proteins, such as ZO-1, PAR-3, and AF-6.19,20,24 Additional reports have suggested that because JAM-1 is expressed early in tight junction formation, it may be involved in the recruitment of such proteins.19,24 Interestingly, our results show that in the absence of JAM-1, neither ZO-1 expression nor its localization was affected (Figures 2A and 2B), suggesting that JAM-1 may not be involved in the recruitment of ZO-1 as originally thought. The possibility that JAM-1 indeed recruits ZO-1 to the tight junctions is not necessarily negated by our observations, however. Because of the limitation of transient siRNA transfection, minimal JAM-1 expression may occur in these cells. This slight expression may be sufficient for adequate ZO-1 recruitment to the tight junctions. Yet another possibility could be that previous studies concerning JAM-1 recruitment of ZO-1 were performed using epithelial cells where tight junctions are well-organized.19 However, our studies were performed using endothelial cells. Thus, this discrepancy could be attributable to cell-type specificity or a cell type–specific function of JAM-1. A more in-depth investigation of this phenomenon may be required for conclusive evidence of the recruitment abilities of JAM-1.

Other observations in this study, however, are more consistent with the published data. It is known that stimulation of endothelial cells by bFGF induces a signaling cascade that leads to the activation of ERK, which culminates in the initiation of endothelial cell proliferation and migration.3,25 These processes have been previously shown to involve signaling through integrin αvβ3.2,21 Additionally, previous findings of ours using JAM-1 function-blocking antibody and mutational studies show that JAM-1 interacts with integrin αvβ3 and this interaction is involved in bFGF-induced signaling, leading to angiogenesis.13 In this report, we show that functional ablation of JAM-1 inhibits bFGF-induced endothelial cell adhesion and migration specifically on vitronectin, a ligand of αvβ3. Thus, these knockdown data functionally link JAM-1 to the bFGF, αvβ3, and ERK signaling pathways. Future study as to the relationship of these proteins with each other in regards to their internal signaling cascade is ongoing and will be fundamental to fully understand bFGF-related events.

In conclusion, the data presented here establish a link between bFGF, JAM-1, and the activation of the ERK signaling pathway leading to cell adhesion and migration on vitronectin. Future study as to how JAM-1 regulates the bFGF-induced signaling pathway would be of broad interest, considering the wide physiological and pathological conditions in which bFGF plays a role, which include repair of vascular injury, atherosclerosis, and ischemic- or tumor-induced angiogenesis. In such a manner, JAM-1 may one day become a potential target for the development of therapeutic intervention for such disorders.

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References


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