Fibroblast Growth Factor-2 Failed to Induce Angiogenesis in Junctional Adhesion Molecule–Deficient Mice

Vesselina G. Cooke, Meghna U. Naik, Ulhas P. Naik

Objective—We have previously shown that JAM-A regulates fibroblast growth factor-2 (FGF-2)–induced endothelial cell morphology, proliferation, and migration. Whether JAM-A is involved in FGF-2–induced angiogenesis in vivo is not known. We used JAM-A null mice to conclusively determine the role of JAM-A in FGF-2–induced neovascularization.

Methods and Results—We generated JAM-A null (JAM-A<sup>−/−</sup>) mice using gene trap technology. These mice, although viable and fertile, exhibited distorted Mendelian and sex ratios, suggesting partial embryonic lethality. Retinal fluorescein angiogram did not reveal any significant morphological differences in the vasculature of JAM-A<sup>−/−</sup> mice compared with wild-type (JAMA-A<sup>+/+</sup>) litters. To evaluate the role of JAM-A in angiogenesis, we performed an aortic ring assay. FGF-2–induced microvessel growth was evident in aortic rings from JAM-A<sup>−/−</sup> mice, but FGF-2 failed to induce microvessel sproutings in aortic rings from JAM-A<sup>+/−</sup> mice. In a Matrigel plug assay, a known in vivo model for angiogenesis, we found that FGF-2 induced a robust vessel growth in JAM-A<sup>+/−</sup> mice, whereas FGF-2 failed to induce blood vessel formation in plugs from JAM-A<sup>−/−</sup> mice.


Key Words: angiogenesis ■ aortic ring assay ■ bFGF ■ FGF-2 ■ integrin α<sub>4</sub>β<sub>3</sub> ■ JAM-A ■ JAM-1 ■ matrigel plug assay

Blood vessel formation occurs through two distinct processes, vasculogenesis and angiogenesis. During vasculogenesis, the precursor stem cells differentiate into endothelial cells which later accumulate into blood vessels, whereas during angiogenesis endothelial cells from the preexisting capillaries or individual angioblasts grow into new blood vessels. Blood vessel growth is a tightly regulated process, and its disruption can lead to the pathogenesis of many diseases, such as cancer, psoriasis, arthritis, blindness, heart and brain ischemia, neurodegeneration, hypertension, and respiratory distress. These molecules are also involved in transmitting signals for endothelial cell survival.

We have recently shown that in quiescent endothelial cells JAM-A is located at the cell–cell junctions, where it forms a complex with integrin α<sub>4</sub>β<sub>3</sub>. On treatment of the cells with growth factors such as FGF-2, JAM-A dissociates from its complex with α<sub>4</sub>β<sub>3</sub> and redistributes to the cell surface. Function blocking anti-JAM-A antibody inhibits FGF-2–induced endothelial cell morphology, proliferation, and migration.

Additionally, knockdown of JAM-A using RNAi technology in endothelial cells showed decreased adhesion and migration of these cells on vitronectin in response to FGF-2 indicating a possible role for JAM-A in FGF-2–induced angiogenesis. Consistent with previous studies, our results presented here demonstrate that JAM-A has a crucial role in FGF-2–induced angiogenesis. We have generated JAM-A<sup>−/−</sup> mice by the gene trap strategy and these mice exhibit partial lethality and have a distorted sex ratio. We show that FGF-2 induces abundant vessel growth in control JAM-A<sup>+/−</sup> mice, whereas this growth is completely absent in JAM-A<sup>−/−</sup> mice as determined by both ex vivo and in vivo assays. These results conclusively determine an essential role of JAM-A in FGF-2–induced angiogenesis and may contribute to further understand the complex regulation of blood vessel formation.
Methods

Generation and Genotyping of JAM-A−/− Mice
JAM-A−/− mice were generated using the gene trap approach20–22 by disrupting the endogenous JAM-A (F11r) gene. This resulted in the production of a JAM-A−/−β-galactosidase chimeric protein containing the N-terminal Ig domain of JAM-A. The JAM-A−/− mice are from a mixed C57Bl6 and Ola129s background and were backcrossed once to C57Bl6 genetic background. Three- to four-week-old mice were used for all experiments. All experimental protocols were approved by the University of Delaware Institutional Animal Care and Use Committee. Genotyping was performed using Southern blot analysis as described previously.23 Mice were screened by hybridization of KpnI/HindIII-digested genomic DNA with a 580 bp probe derived from the genomic sequence between exons 2 and 4 upstream of the trap vector. The probe was generated by polymerase chain reaction (PCR) of genomic DNA using primers: 2F, 5′-ttgtaacaagccaggggtcc-3′, and 4R, 5′-aaggggaagagcagagacagcagc-3′.

Reverse-Transcriptase PCR Analysis
One-step reverse-transcriptase PCR (RT-PCR) was performed using total mouse heart RNA. The forward and reverse primers used and the expected product size are given in supplemental Table I (available online at http://atvb.ahajournals.org). RT-PCR conditions were 20 minutes at 50°C, 5 minutes at 94°C, followed by 28 to 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 1 minute at 72°C, and final extension of 5 minutes at 72°C. Densitometric analysis was used to determine the relative expression of specific genes.

Western Blot Analysis and Immunofluorescence
Western blot analysis and immunofluorescence were performed as previously described.24,25 For Western blot analysis, rat anti-mouse JAM-A monoclonal antibody (1:500) (R&D Systems) was used as a primary antibody, followed by a peroxidase-conjugated secondary donkey anti-rat antibody (1:10 000) (Jackson ImmunoResearch Laboratories). For immunofluorescence, goat anti-mouse JAM-A polyclonal antibody (1:50), and rat anti-mouse CD31 antibody (1:50) (MEC13.3; BD Biosciences) were used as primary antibodies. Donkey anti-goat and rabbit anti-rat (Molecular Probes) were used as secondary antibodies (1:200). Sections were views using Zeiss LSM510 laser-scanning confocal microscope.

X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) Staining
Sections (16 μm) of Tissue-Tek Optical Cutting Temperature (Sakura Fintek) embedded tissues were air-dried at room temperature, and fixed in a solution containing 1% paraformaldehyde/0.2% (wt/vol) glutaraldehyde/0.02% (wt/vol) NP-40/0.01% sodium deoxycholate for 1 to 6 hours at 4°C. After fixation, slides were stained as described previously.26

Retinal Fluorescein Angiogram
Mice were anesthetized with ketamine/xylazine (3 to 5 mg/kg), and fluorescein isothiocyanate (FITC)-dextran (FITC-dextran) (Sigma-Aldrich) was injected into the portal vein as previously described.16 Briefly, 1 mL of phosphate-buffered saline containing 25 mg of 2×1010 molecular weight FITC-dextran was injected into the portal vein. Thirty minutes later, mice were euthanized by CO2 asphyxiation; the eyes were removed and fixed in 4% paraformaldehyde in phosphate-buffered saline for 3 hours. The retina was dissected and mounted on a slide using GelMount medium (Biomed). Fluorescence micrographs were taken using Zeiss LSM510 laser-scanning confocal microscope.

Aortic Ring Assay
Aortic ring assay was performed as previously described.18,27 Briefly, 1 mm mouse aortic rings were embedded in 3-dimensional growth factor reduced Matrigel (BD Biosciences), treated with or without FGF-2 (50 ng/mL) and heparin (60 U/mL), and incubated at 37°C. Vascular sproutings were counted every day for a period of 7 days, at which time they were photographed using a Nikon Eclipse TS 100 (Nikon) phase-contrast microscope.

Matrigel Plug Assay
Matrigel plug assay was performed as described previously.28 Briefly, mice were subcutaneously injected with 200 μL of growth factor reduced Matrigel supplemented with or without FGF-2 (80 ng/mL) and heparin (60U/mL). Fourteen days later, the Matrigel plugs were excised, and H&E stained paraffin sections were analyzed for the presence of blood vessels.

Statistical Analysis
Standard statistical tests (mean value, SEM, Student t test) were performed for data analysis. Results are expressed as mean±SEM. P<0.05 was regarded as statistically significant.

Results
JAM-A is expressed at endothelial cell TJs and is shown to be involved in leukocyte transmigration.29 We have previously shown that blockage of the extracellular domain of JAM-A inhibits FGF-2–induced endothelial cell morphology, proliferation, and migration.19 This effect of JAM-A is through its interaction with integrin αβ1, an essential component of the FGF-2–induced angiogenic signaling pathway.30,31 We found that blockage of integrin αβ1, a component of the vascular endothelial growth factor-induced angiogenic pathway, did not affect JAM-A–induced human umbilical vein endothelial cells migration, suggesting a specific role of JAM-A in FGF-2–induced pathway. This was further confirmed by the finding that anti-JAM-A function blocking antibody had little effect on vascular endothelial growth factor–induced human umbilical vein endothelial cell migration (data not shown).

Generation of JAM-A−/− Mice
To conclusively determine the role of JAM-A in FGF-2–induced angiogenesis, we sought to generate JAM-A−/− mice. The mouse JAM-A gene is located on chromosome 1 and it contains 10 exons, where exons 2 to 4 encode the first Ig-like domain, while exons 5 and 6 encode the second Ig-like domain. Using a gene trap approach,20–22 the JAM-A−/− mice were generated using the gene trap approach20–22 by random insertion of a gene trap vector (supplemental Figure I, available online at http://atvb.ahajournals.org). The secretory trap vector (11.98-kb) includes the mouse En-2 splice acceptor (gene) together with a downstream internal ribosome entry site, the placental alkaline phosphatase gene, and the simian virus 40 polyadenylation signal. The use of 5′ rapid amplification of cDNA ends and sequencing analysis revealed that gene splicing occurred between the splice donor site in exon 4 of the JAM-A gene and the splice acceptor site of the trap vector22 (supplemental Figure I). This insertion resulted in the production of a chimeric protein corresponding to the first Ig-loop domain of JAM-A fused to the transmembrane domain inserted in frame with β-geo (LacZ-neomycin fusion gene) together with a down stream internal ribosome entry site, the placental alkaline phosphatase gene, and the simian virus 40 polyadenylation signal. The use of 5′ rapid amplification of cDNA ends and sequencing analysis revealed that gene splicing occurred between the splice donor site in exon 4 of the JAM-A gene and the splice acceptor site of the trap vector22 (supplemental Figure I). This insertion resulted in the production of a chimeric protein corresponding to the first Ig-loop domain of JAM-A fused to the transmembrane region of CD4 and β-geo.

We determined the generation of the JAM-A−/− mice by Southern blot (Figure 1A), RT-PCR (Figure 1B), Western blot (Figure 1C), and X-gal staining (Figure 1D). The RT-PCR results also demonstrated that the gene trap did not cause a splice around for the JAM-A gene, which is documented to occur in some instances.32 Additionally, X-gal staining of JAM-A+/+ and JAM-A−/− embryos revealed ex-
pression of JAM-A starting from the blastula stage until 18.5 dpc as reported previously. JAM-A was also expressed in the developing kidneys, lungs, and olfactory system during branching morphogenesis and developing epithelia.

The disruption of the JAM-A gene was further confirmed by immunohistochemistry of kidney (supplemental Figure IIA), heart (supplemental Figure IIB), lung (supplemental Figure IIC), and aorta (Figure 2). Double staining of tissues with antibodies against JAM-A (red) and PECAM-1 (green, used as an endothelial cell marker) not only confirmed that JAM-A was absent in the endothelium but also confirmed that the random insertion of the gene trap in the JAM-A gene was efficient in all investigated organs. Together, these results confirm the generation of JAM-A\(^{-/-}\) mice, which were used to study the in vivo role of JAM-A in angiogenesis.

**JAM-A\(^{-/-}\) Mice Exhibit Partial Lethality, Distorted Sex Ratio, and Reduced Litter Size**

A genotype analysis of offspring from 40 JAM-A\(^{+/+}\) crosses revealed a deviation from the Mendelian ratio of expected JAM-A\(^{-/-}\) mice. Viable homozygous JAM-A\(^{-/-}\) mice were produced, but at a frequency 40% lower than the expected, JAM-A\(^{+/+}\) mice, n=97, JAM-A\(^{-/-}\) mice, n=163, and JAM-A\(^{+/-}\) mice, n=57, generating a Mendelian ratio of 1:1:7:0.6 (Figure 3A). Breeding data from 43 JAM-A\(^{-/-}\) crosses revealed that the percentage of JAM-A\(^{-/-}\) males is significantly less as compared with the percentage of JAM-A\(^{-/-}\) females: 43% males, n=122 versus 56%, n=158 females. The percentage of males to females in JAM-A\(^{+/-}\) crosses was as expected: 50% males, n=82 versus 50% females, n=82 (Figure 3B). In addition, the average litter size of the JAM-A\(^{-/-}\) crosses, 6.56 pups per litter was smaller as compared with the average litter size of the JAM-A\(^{+/-}\) crosses, 7.13 pups/litter, but these numbers were not significant (Figure 3C). These findings suggest that deletion of the JAM-A gene causes partial embryonic lethality and a preferential female survival. Liveborn JAM-A\(^{-/-}\) mice appeared healthy with normal postnatal survival into adulthood. Histological analysis of tissues (lung, liver, spleen, kidney, and heart) obtained from 3-month-old mice did not reveal any significant difference as compared with the control mice (data not shown).

**Embryonic and Retinal Vasculature Appears Normal in JAM-A\(^{-/-}\) Mice**

During the onset of angiogenesis, vessels become permeable and dilated in response to growth factors as a result of the disruption of the junctional proteins that tighten the vessels. The mice in which the JAM-A gene is disrupted exhibited partial embryonic lethality prompting us to investigate whether vessels in the JAM-A\(^{-/-}\) mice are prone to leakage, dilation, and abnormal organization. Embryonic vasculature of 13.5 dpc embryos with their attached yolk sacs was examined and no visible differences were observed between the JAM-A\(^{+/-}\) and JAM-A\(^{-/-}\) embryos (Figure 4A). To study the vascular network in adult JAM-A\(^{+/-}\) and JAM-A\(^{-/-}\) mice, we performed a fluorescein retinal angiography by injecting FITC-dextran in vivo. Similar blood vessel density was observed between JAM-A\(^{+/-}\) and JAM-A\(^{-/-}\) mice. There was no visible difference in the number of large vessels, and small branching vessels were also similar between JAM-A\(^{+/-}\) and \(^{-/-}\) mice. Vessels in JAM-A\(^{-/-}\) retinas did not appear tortuous or dilated, and no leakage was detected (Figure 4B). Additionally, H&E staining of JAM-A\(^{+/-}\) and JAM-A\(^{-/-}\) aortas revealed no major differences in the morphology of these vessels (Figure 4C). These findings demonstrated that the disruption of the JAM-A gene has no apparent visible effect on the vasculature, suggesting that JAM-A is probably not involved in vasculogenesis. The reasons for the observed partial embryonic lethality therefore cannot be attributed to vascular defect and needs further investigation.

**JAM-A Inhibits FGF-2–Induced Vessel Sprouting in an Ex Vivo Angiogenesis Assay**

Normal vessel growth in the adult involves the recruitment of bone marrow-derived endothelial progenitors, which become...
incorporated into the nascent vessels or stimulate new vessel growth by releasing angiogenic factors. Angiogenesis then commences and sprouting of new endothelial cell-lined vessels from the preexisting ones occurs. To investigate if JAM-A has a role in this initial sprouting of endothelial cells, we performed an ex vivo aortic ring assay in which we cross-sectioned mouse thoracic aortas into 1-mm rings and cultured them in triplicate in 3-dimensional growth factor-reduced Matrigel. Because endothelial cell sprouting is typically dependant on growth factor signaling, we characterized sproutings from JAM-A+/+ and JAM-A−−/− aortic rings in the presence or absence of FGF-2. The sprouting phenotype was not seen in the JAM-A+/+ or JAM-A−−/− aortic rings that were not supplemented with FGF-2 (Figure 5A, upper panels). However, when supplemented with FGF-2, aortic rings from JAM-A+/+ mice showed robust sproutings (Figure 5A, lower left panel). Interestingly, FGF-2 failed to induce sproutings in aortic rings from JAM-A−−/− mice (Figure 5A, lower right panel). This phenomenon was highly reproducible (n=19) and deemed significant (Figure 5B). This data indicated that JAM-A is needed for the FGF-2-induced initial sprouting of endothelial cells.

Because FGF-2–induced vessel sprouting was affected in the JAM-A−−/− mice, we tested whether this was due to the downregulation of the major FGF-2 receptors fibroblast growth factor receptor 1 (FGFR-1) and 2 (FGFR-2) in the JAM-A−−/− mice using RT-PCR (supplemental Figure IIIA). β-actin and FGF-2 were used as controls (supplemental Figure IIIA and IIIB, respectively). Quantitation of these data revealed that there is no significant decrease in the level of expression of these genes in the JAM-A−−/− mice (supplemental Figure IIIC and IID), suggesting that the decreased vessel...
sprouting in the JAM-A−/− mice is specific to the ablation of the JAM-A gene. Integrin αvβ3 is known to participate in FGF-2–induced signaling leading to angiogenesis.30 Further, we have shown that JAM-A forms a functional complex with αvβ3,18,34 therefore, we determined its level of expression in the JAM-A+/+ and JAM-A−/− mice as well. The level of expression of the β3 subunit of the integrin remained unaffected in JAM-A−/− mice (supplemental Figure IIIA and IIIE), ruling out the possibility that the observed angiogenic effect is caused by downregulation of integrin αvβ3 expression in the JAM-A−/− mice supporting the contention that defect in FGF-2 signaling is the cause.

**JAM-A−/− Mice Show a Decrease in FGF-2–Induced Neovascularization in Matrigel Plugs**

To confirm our ex vivo results, we performed an in vivo assay for angiogenesis. We implanted Matrigel, supplemented with or without FGF-2, in the flank region of JAM-A+/+ and JAM-A−/− mice. In this model, the host endothelial cells migrate and form a capillary network in the Matrigel implants, which are normally avascular. Fourteen days after implantation, mice were euthanized by CO2 asphyxiation. The plugs were excised, photographed, and sections were stained with H&E. The plugs were quantified and deemed significant (Figure 6C) and the level of expression in JAM-A+/+ mice (supplemental Figure IIIA and IIIE), ruling out the possibility that the observed angiogenic effect is caused by downregulation of integrin αvβ3 expression in the JAM-A−/− mice supporting the contention that defect in FGF-2 signaling is the cause.

**Discussion**

JAM-A is a multifunctional protein that is involved in platelet activation,25 TJ integrity,34 and leukocyte transmigration.29 We have previously shown that JAM-A is involved in FGF-2–induced endothelial cell proliferation, and migration.18 This effect is believed to be specific to FGF-2 and not vascular endothelial growth factor. In this study, we demonstrate the generation of JAM-A−/− mice by the gene trap strategy. Our results show that JAM-A−/− mice exhibit partial embryonic lethality, and have a distorted sex ratio. Using these mice, we found that deletion of JAM-A results in a complete blockage of FGF-2–induced angiogenesis. Thus, we provide conclusive evidence that JAM-A is crucial for FGF-2–induced neovascularization.

One of the roles of the TJs is to seal the endothelial cell layer and maintain the resting state of the endothelial cells.35 During the outgrowth of a new blood vessel, the existing contacts between the cells are broken and new ones are made. These processes are linked to cell proliferation, migration, and survival in a tightly controlled manner.35 The role of cell–cell junctions in angiogenesis has been shown with the use of transgenic animals. Inactivation of several genes in the AJs such as VE-cadherin or β-catenin lead to inhibition of the vessel growth in the embryo.17,36 Although inactivation of claudin-5 leads to permeability defects in the blood–brain barrier, the absence of some TJ proteins, such as occludin or claudin-5, did not cause any defects in the vascular growth.37 A recently identified TJ protein, ESAM, however, has been found to promote in vitro tube formation in endothelial-like yolk sac cells and the genetic ablation of ESAM leads to decreased tumor size and vascularization.16 These findings indicate that TJs might offer a new frontier for the regulation of angiogenesis. Our finding of the role of JAM-A in FGF-2–induced neovascularization is the second record to demonstrate an involvement of a TJ protein in angiogenesis. This novel finding marks JAM-A as a potential regulator of angiogenesis and highlights its significance as a physiologi-
cally important molecule for neovascularization. The fact that aortic rings from JAM-A−/− mice failed to respond FGF-2–induced sprouting as compared with JAM-A+/+ controls reveals that JAM-A is important for the initial budding of the endothelial cells during the early stages of angiogenesis. The failure to detect any apparent vascular abnormalities in 13.5-dpc embryos and the surviving JAM-A−/− mice as determined by fluorescent retinal angiography, suggests that JAM-A may not be important for vasculogenesis. However, we cannot exclude the possibility that differences in vasculature can exist depending on the organ it supplies. In tumor angiogenesis, for example, vascular permeability and angiogenesis can depend on the type of tumor and the tissue where the tumor is growing, because each organ has different stromal cells and produces different pro and anti-angiogenic molecules.33 It has been shown that mice injected with a monoclonal antibody directed against VE-cadherin show a concentration- and time-dependant increase in vascular permeability in heart and lung without a change in other organs.38

Besides interendothelial cell contacts, adhesion to the extracellular matrix is very important for angiogenesis.33 We have previously shown that JAM-A–induced cell migration on vitronectin is αβ3-dependent.18 In inactivated human umbilical vein endothelial cells, most integrin αβ3 localizes at the cell–cell junctions concurrent with JAM-A localization. Furthermore, immunoprecipitation of JAM-A followed by Western blotting using anti-β3 showed that the two proteins physically associate with each other. On FGF-2–stimulated signaling, however, the complex between JAM-A and FGF-2–induced signaling pathway, because levels of FGF-2, its receptors (FGFR-1 and FGFR-2), and integrin αβ3 remained unaffected in JAM-A−/− mice.

The genetic ablation of the JAM-A gene causes only a partial lethality, raising the possibility that a compensatory mechanism may be rescuing the mice thus resulting in their survival. Similarly, mice, in which the β3 subunit of the αβ3 integrin has been genetically ablated, also survive, even though those mice incur a bleeding disorder caused by the loss of the integrin α3β3 on platelets.39 Further, it is interesting to note that FGF-2−/− mice are also viable and fertile despite observed neuronal defects.40 Whether occurrence of functional compensation by other JAM family members or other TJ and AJ proteins remains unclear, although a recent finding that an antibody against JAM-C inhibits angiogenesis and tumor growth in vivo supports this possibility.41 Functional knock-down of JAM-A by siRNA technology, however, did not cause any upregulation of ZO-1, VE-cadherin and β-catenin19 suggesting that if a functional compensation by junctional proteins occurs, it may be a result of a developmental adaptation.

Overall, this report demonstrates that JAM-A has a significant role in FGF-2–induced angiogenesis in vivo thus marking JAM-A as a novel key molecule in the FGF-2–induced pathway. We show that a TJ protein has the ability to regulate blood vessel formation, which furthers our knowledge of how this complicated and extremely well-regulated process works. Additional investigations, however, are needed to study the potential use of this molecule as a therapeutic target in pathological angiogenesis.

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Disclosures

None.

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Table I
RT-PCR primers used and the expected size of the products

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**Figure SI.** Gene targeting vector used to generate \textit{JAM-A-/-} mice. Genomic structure of the \textit{JAM-A} gene and gene-trap strategy. The secretory trap vector was inserted between exons 4 and 5 of the \textit{JAM-A} gene.

**Figure SII.** Histological analysis of \textit{JAM-A-/-} mice. A, Immunofluorescence staining of cryosections of \textit{JAM-A}+/+ (upper panel) and \textit{JAM-A-/-} (lower panel) kidney; B, heart; and C, lung. All sections were triple stained for PECAM-1 (green), JAM-A (red), and ToPro-3 (blue). Scale bar, 50 µm.
**Figure SIII.** Gene expression analysis of *JAM-A* -/- mice. Expression of FGF-2, FGFR-1, FGFR-2, integrin β3, and β-actin was determined by RT-PCR analysis (A). B-E, Relative band density of FGF-2 (B), FGFR-1 (C), FGFR-2 (D), and integrin β3 (E) is shown (n=9). Band densities were normalized to the levels of β-actin expression.