Antibody against Junctional Adhesion Molecule-C Inhibits Angiogenesis and Tumor Growth

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Abstract

The junctional adhesion molecule-C (JAM-C) was recently described as an adhesion molecule localized at interendothelial contacts and involved in leukocyte transendothelial migration. The protein JAM-C interacts with polarity complex molecules and regulates the activity of the small GTPase Cdc42. The angiogenesis process involves rearrangement of endothelial junctions and implicates modulation of cell polarity. We tested whether JAM-C plays a role in angiogenesis using tumor grafts and hypoxia-induced retinal neovascularization. Treatment with a monoclonal antibody directed against JAM-C reduces tumor growth and infiltration of macrophages into tumors. The antibody decreases angiogenesis in the model of hypoxia-induced retinal neovascularization in vivo and vessel outgrowth from aortic rings in vitro. Importantly, the antibody does not induce pathologic side effects in vivo. These findings show for the first time a role for JAM-C in angiogenesis and define JAM-C as a valuable target for antitumor therapies. (Cancer Res 2005; 65(13): 5703-10)

Introduction

Angiogenesis is the formation of new blood vessels from the preexisting vasculature (1) and occurs in a multistep process involving migration, proliferation, and differentiation of endothelial cells leading to the formation of vascular loops (2). Angiogenesis occurs during embryonic development and adulthood during a variety of physiologic processes, including wound healing and the menstrual cycle of the endometrium (3, 4). Angiogenesis is also a key event in pathologic processes, such as tumor development (1) and diabetic retinopathy (5). The formation of immature endothelial sprouts is promoted by angiogenic factors, including vascular endothelial growth factor (VEGF) and angiopoietins. Maturation into functional vessels is then accomplished by the establishment of new interendothelial junctions, the organization of a new basement membrane, and the recruitment of pericytes (2). Altogether, these mechanisms result in the stabilization of a novel vascular network.

Macrophages play an important role in regulating blood vessel formation by secreting angiogenic factors during tumor development as well as during physiologic angiogenesis (6, 7). Indeed, monocytes extravasate and migrate toward hypoxic or inflammatory regions created by a growing tumor (8). Although angiogenesis is induced by angiogenic factors, additional molecules contribute to proliferation and remodeling of the vessel wall. As an example, VEGF leads to loosening of the pericyte-endothelial contacts, allowing proliferation and interaction of endothelial adhesion molecules with extracellular matrix (9). The integrins αvβ3 and αvβ5 participate in blood vessel development via a signaling cross-talk with receptors of angiogenic factors (10, 11).

Other adhesion molecules implicated in the organization of interendothelial junctions are essential in maintaining integrity of the endothelium. For example, the targeted disruption in mice of the adherens junction molecule vascular endothelial-cadherin ( VE-cadherin; 12, 13). More recently, it has been shown that the targeted disruption of the tight junction molecule, endothelial cell–selective adhesion molecule, inhibits angiogenesis in vitro and in vivo (14). In addition, in vitro experiments have shown that signaling through junctional adhesion molecule (JAM)-A and αvβ3 integrin is required for the angiogenic action of basic fibroblast growth factor (bFGF; refs. 15, 16).

We recently described JAM-C and found it expressed in vascular cell–cell contacts (17, 18). When JAM-C is transfected into epithelial cells, it localizes in tight junctions, whereas it has been found in desmosomes of enterocytes (18, 19). We and others have shown that JAM-C is involved in leukocyte transendothelial migration (17, 18, 20, 21). Furthermore, JAM-C coimmunoprecipitates with polarity complex molecules, such as PAR-3, PAR-6, or PATJ, and regulates the activity of the small GTPase Cdc42 (22, 23). These results show that JAM-C plays a role in the formation and maintenance of intercellular contacts and suggest that it may contribute to the remodeling of endothelial junctions.

We therefore investigated whether JAM-C participates in angiogenesis, a mechanism involving rearrangement of endothelial junctions. Here, we show that a monoclonal antibody directed against JAM-C totally abolishes outgrowth of microvessels in vivo aortic ring assays. When injected in vivo, the antibody reduces hypoxia-induced angiogenesis in the retina and the growth of experimental tumors. These results show a role for JAM-C in angiogenesis and underline the importance of endothelial cell adhesion molecules in the formation of new blood vessels.

Materials and Methods

Antibodies. Rat monoclonal antibodies (CRAM) against human and mouse JAM-C (H33 for functional assays and H36 for immunocytochemistry) and rat monoclonal antibodies against mouse platelet/endothelial cell adhesion molecule-1 (PECAM-1)/CD31 (GC51) were described previously (18, 24). Anti-human CD44 (Hermes, 9B5) used as irrelevant antibody control rat IgG2a was kindly provided by Dr. B. Engelhardt (Theodor-Kocher-Institute, Bern, Switzerland) (25, 26). Monoclonal rat anti-mouse intercellular adhesion molecule-2 (ICAM-2; 3C4) and monoclonal rat anti-mouse...
Fcy II/III receptor (2.4G2) were from BD Pharmingen (Franklin Lakes, NJ). Polyclonal rabbit antibody against PECAM-1/CD31 was described previously (27). A polyclonal rabbit serum against murine JAM-C was generated using recombinant soluble molecule as immunogen (Covalab, Lyon, France). The specificity of polyclonal antibody was confirmed by immunohistochemistry on tissue sections, the Western blot, and flow cytometry using transfected cells. Anti-JAM-C antibody H33 and isotype-matched control antibody 9B5 used in functional assays were tested for endotoxin using the Pyrogent Plus Limulus Amebocyte Lysate kit (BioWhittaker, Walkersville, MD).

Isolation and culture of primary endothelial cells. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described previously in ref. 28. Cells were used between passages 3 and 5.

Lung murine endothelial cells were isolated from wild-type C56BL/J mice (Charles River Laboratories, L'Arbresle, France) as described in ref. 29. Briefly, lungs were harvested from two mice, carefully dissected, and washed twice in 20 mL Ham's F-12 medium supplemented with antibiotics. Then, lungs were finely minced with scissors and digested in 10 mL collagenase (180–200 units/mL) at 37°C for 1 hour. The digested tissue was then mechanically dissociated by triturating, filtered through a 70 μm disposable cell strainer (Becton Dickinson Labware, Bedford, MA), and centrifuged at 1,200 × g for 5 minutes. The cell pellet was resuspended in 50:50 mix of Ham's F-12 medium/DMEM (Life Technologies/Invitrogen Corporation, Basel, Switzerland) supplemented with 20% FCS, 20 μg/mL vitronectin (Sigma-Aldrich Corp., St. Louis, MO), and antibiotics (complete medium). The cell suspension was plated on tissues precoated with 0.1% gelatin, 10 μg/mL fibronectin (Sigma-Aldrich), and 30 μg/mL Vitrogen (Nutacon B.V., Leimuiden, the Netherlands) in PBS. Endothelial cells were purified by magnetic immunosorting with a single negative sort for Fcy III/II receptor-positive macrophages and at least two positive sorts for ICAM-2-positive endothelial cells. Immunosorting was done using sheep anti-rat IgG Dynal beads (Dynal Biotech, Oslo, Norway). Cells were cultured routinely in the complete medium described above.

Vascular endothelial growth factor stimulation and immunocytochemistry. HUVECs (1 × 10⁵) were plated on 22 mm² glass slides coated with growth factor–reduced Matrigel (Becton Dickinson, Bedford, MA) and after 24 hours starved for endothelial cell growth factors. Twenty-four hours later, cells were incubated with 100 ng/mL recombinant human VEGF-165 (PeproTech House, London, United Kingdom) for 15 minutes.

In immunocytochemistry, cells were fixed with 4% paraformaldehyde in PBS for 15 minutes before permeabilization with 0.01% Triton X-100 in PBS for 15 minutes. Cells were washed with PBS/0.2% bovine serum albumin (BSA), incubated with primary antibody H36 for 1 hour, and washed before further incubation with secondary antibody coupled to FITC (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Pictures were acquired using a Zeiss LSM510 confocal microscope (Zeiss, Oberkochen, Germany).

Flow cytometry. HUVEC and LLC1 were incubated on ice with H36 and H33 anti-JAM-C monoclonal antibodies, respectively. After washing with PBS/0.2% BSA, binding of antibody was detected using a phycoerythrin-coupled anti-rat antibody (Jackson Immunoresearch Laboratories). As control, the primary antibody was omitted. The surface content of proteins was analyzed using FACS-Calibur and CellQuest software (Becton Dickinson, Mountain View, CA).

Histology and quantification of vascular volume fraction, apoptotic cells, and macrophage contents into the tumors. For immunochemistry on tumor cryosections with monoclonal antibody anti-PECAM-1 (GC31), sections were fixed with acetone/methanol (1:1) for 5 minutes at −20°C, dried, and hydrated in PBS/0.2% gelatin/0.05% Tween 20. Sections were incubated with primary antibody for 1 hour at room temperature and after three washes in PBS incubated with a secondary antibody coupled to peroxidase (Jackson Immunoresearch Laboratories).

For immunochemistry on paraffin-embedded eye sections with polyclonal antibodies against PECAM-1 and JAM-C, sections were dewaxed following the classic procedure. Tissue sections were then treated with H₂O₂, 0.3% in methanol for 10 minutes, washed in PBS, and blocked with PBS/3% BSA/0.1% Tween 20 for 30 minutes. Sections were incubated with polyclonal antibodies for 1 hour at room temperature and after washes in PBS incubated with EnVision system for 30 minutes (DakoCytoquantation AG, Baar, Switzerland). Peroxidase activity was detected using 3-amin-9-ethylcarbazol (Sigma-Aldrich) as substrate and sections were counterstained for 1 minute with hemalum before mounting in Aquatex (Merck, Darmstadt, Germany).

Acid phosphatase activity was detected on tumor cryosections using the method described previously in ref. 30. Detection of apoptotic cells on tumor cryosections was based on labeling of DNA strand breaks [terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL–fluorescence method) and done using terminal transferase and biotin-16-dUTP according to the manufacturer's instructions (Roche Diagnostics AG, Rotkreuz, Switzerland). Bound biotin-16-dUTP was detected with streptavidin coupled to Texas red dye (Jackson Immunoresearch Laboratories).

Pictures were acquired using a Zeiss LSM510 confocal microscope or a Zeiss Axioskop 1 microscope equipped with an Axiocam color CCD camera. Images were recorded and treated using the AxiosVision software (Zeiss).

To quantify vascular volume fraction, the number of macrophages, and the number of apoptotic cells into tumors, pictures of the entire cryosection (four cryosections per tumor) were analyzed using Zeiss KS400 or Openlab software. The vascular volume fraction was quantified by determining the total area of PECAM-1-positive staining across whole sections of tumors. Results are expressed as percentage of PECAM-1 staining by applying the formula: % PECAM-1 staining = [Total area of PECAM-1 staining (mm²)] / [Total area of tumor section (mm²)] × 100. The number of acid phosphatase–positive cells and the number of TUNEL-positive cells present across the entire area of each tumor section were counted and divided by the area of the whole section. This determined the number of macrophages per square millimeter and the number of apoptotic cells per square millimeter, respectively.

Ex vivo aortic ring assay. Mouse aortic ring assay was adapted from ref. 31. Briefly, 1 mm thoracic aortic rings were placed between two layers of 50 μL growth factor–reduced Matrigel and overlaid with 100 μL DMEM supplemented with 20% FCS, 20 units/mL heparin, and endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY) in the presence or absence of 50 μg/mL 9B5 or H33 antibodies. Microvessel outgrowth was visualized by phase microscopy using a Zeiss Axioskop microscope.

Tumor graft. Eight- to 10-week-old female C56BL/J mice were inoculated s.c. with 1 × 10⁶ murine Lewis lung carcinoma cells (LLC1; obtained from the European Collection of Cell Cultures, Salisbury, United Kingdom). Mice were then injected i.p. every second day with 150 μg monoclonal antibody H33, isotype-matched control antibody 9B5, or PBS. When the control tumors (PBS-injected mice) had reached 1 cm, animals were sacrificed and tumors were excised and analyzed. Tumor volume was measured by using a caliper, applying the following formula for approximating the volume of an ellipsoid: Volume (mm³) = 4/3π × (Length / 2) × (Width / 2) × (Height / 2).

Hypoxia-induced retinal angiogenesis. Postnatal day 7 (P7) mice were placed in 75% oxygen in air for 5 days causing central avascularity of retinas followed by housing the mice for 5 additional days (until P17) under normoxic conditions. Mice were injected i.p. with 50 μg monoclonal antibodies at P12, P14, and P16. After anesthesia with 150 mg/kg ketamine plus 12.5 mg/kg xylazine, 17-day-old mice were perfused with a nondiffusible fluorescein–dextran solution (Sigma-Aldrich). Neovascularization was visualized and quantified on flat-mounted retinas by counting the number of vascular glomeruli under the microscope. Pictures were acquired using a Zeiss LSM510 confocal microscope.

Proliferation assays. Primary mouse lung endothelial cells or LLC1 tumor cells were plated at a density of 2,500 cells/cm² in 24- or 6-well plates, respectively. Twenty-four hours later, the medium was replaced by medium supplemented with control or H33 antibodies at 50 μg/mL. Cells were trypsinized from wells daily for 4 days and counted using a Casy-1 Coulter counter (Schärfe System GmbH, Reutlingen, Germany).

Real-time quantitative PCR. Retinal mRNA from newborn mice undergone hypoxia-induced angiogenesis were extracted by Trizol.
according to the manufacturer's instructions (Life Technologies/Invitrogen). Reverse transcription was done by using 1 μg total RNA, random hexanucleotide primers, and SuperScript II reverse transcriptase (Invitrogen). 1:25 Dilution of the resulting cDNA was used for real-time quantitative PCR using the SYBR Green PCR Master Mix kit as recommended by the provider and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The following primers were used: JAM-C forward 5'-GCTGGGAGACGACATGCAA and reverse 5'-CAGGAGCTCTGGGCTCACA, RPS-9 forward 5'-GACCAGGAGC-TAAAGGTGATTGGA and reverse 5'-TCTTGGCCAGGGTAAACTTGA, and TBP forward 5'-TTGACCTAAAGACCATGACTTC and reverse 5'-TTCTC-ATGATGACTGCAGCAAA. JAM-C relative mRNA content was normalized by geometric averaging of internal control genes RPS-9 and TBP according to ref. 32.

Statistical analysis. Each column in graphs represents the mean ± SE. All experiments were evaluated with the Mann-Whitney's t test using the statistical software StatView (Abacus Concepts, Inc., Berkeley, CA). P < 0.05 was considered as statistically significant.

Animal regulation. All animal experiments were done under the ethical approval and the recommendations of the Veterinary Office of Geneva state according to the Swiss federal laws.

Results

Vascular endothelial cells express junctional adhesion molecule-C and vascular endothelial growth factor changes its localization. The adhesion molecule JAM-C is constitutively expressed by endothelial cells and partially localized in cell-cell contacts (21). We investigated by fluorescence-activated cell sorting analysis whether the level of JAM-C proteins at the surface of endothelial cells can be regulated by angiogenic stimuli. Treatment with the angiogenic factor VEGF did not change JAM-C surface expression levels (Fig. 1A). However, analysis of the distribution of the molecule in VEGF-stimulated endothelial monolayer by immunocytochemistry revealed a redistribution of the existing JAM-C in cell-cell contacts within minutes (Fig. 1B and C). Other endothelial adhesion molecules, such as JAM-A or PECAM-1, were not redistributed on VEGF stimulation (data not shown). Because VEGF is a promoter of angiogenesis, this result prompted us to investigate whether JAM-C may play a role in the reorganization of endothelial cells junctions during angiogenesis.

In vitro vessel outgrowth is inhibited by anti-junctional adhesion molecule-C monoclonal antibody. We first tested whether anti-JAM-C antibody would interfere with angiogenesis using ex vivo aortic ring assays. This consisted of embedding freshly dissected mouse aortic rings into Matrigel in the presence of endothelial growth factors. Outgrowths of vascular sprouts from aortic rings were followed over a period of 12 days of culture (Fig. 2). The anti-JAM-C antibody H33 blocked vessel outgrowth, whereas control antibody had no effect (Fig. 2). These in vitro experiments suggested a role for JAM-C in angiogenesis and showed that anti-JAM-C antibodies might be valuable tools preventing angiogenesis in vivo.

Anti-junctional adhesion molecule-C monoclonal antibody reduces tumor growth and angiogenesis in vivo. Given the finding that in vitro vessel outgrowth can be blocked with the H33 anti-JAM-C antibody, we investigated whether it affected tumor angiogenesis and tumor growth. Mice were s.c. injected with Lewis lung carcinoma cells (LLC1) and the antibodies were given i.p. every second day. Tumor size, volume, and weight were significantly decreased when mice were treated with H33 anti-JAM-C antibody compared with isotype-matched control antibody or PBS (Fig. 3A-C). Because LLC1 tumor cells did not express JAM-C (Fig. 4A), we suggested that the reduction in tumor growth was due to an effect of the H33 antibody on endothelial cells.

To visualize the tumor vasculature, tumor vessels were labeled with the endothelial marker PECAM-1 (Fig. 4). The vascular volume fraction was then quantified by calculating the surface of PECAM-1 staining across tumor sections. Tumors excised from H33-treated animals exhibited a reduction in the percentage of PECAM-1 staining when compared with control animals (Fig. 4B).

To deliver a conclusive proof of JAM-C implication in angiogenesis, we then used the antibody in hypoxia-induced retinopathy in neonatal mice, a model of tumor-independent neovascularization. Central avascularization of the retina of P7 mice was caused by exposure of the animals to 75% oxygen in air during 5 days. Then, by housing the mice until P17 under normoxia, retinal neovascularization was induced. During this period, mice were injected with anti-JAM-C or control monoclonal antibodies. Neovascularization was detected by fluorescence microscopy after perfusion of

![Figure 1.](https://www.aacrjournals.org/cancerres.35.22.4089/Figure1.jpg)
the entire vasculature with a nondiffusible fluorescein-dextran. Vascular glomeruli, corresponding to highly proliferating clusters of vessels, were counted as a measure of neovascularization (10). As shown in Fig. 5A and B, the number of glomeruli was significantly reduced when mice were treated with the H33 anti-JAM-C antibody. It is important to note that de novo formed vessels and vascular glomeruli expressed JAM-C, as do retinal vessels of control mice that have not been exposed to hypoxia (Fig. 5C). Taken together, these in vivo experiments showed that anti-JAM-C antibody reduced pathologic angiogenesis.

Junctional adhesion molecule-C expression is not regulated during retinal angiogenesis. In Fig. 1, we described that JAM-C surface expression level was not modified on VEGF stimulation of endothelial cells in vitro. We explored whether this absence of regulation would also be found in vivo. To this end, retinal mRNA extracts from mice that have undergone hypoxia-induced retinal angiogenesis were isolated at different time points of the experiment and subjected to real-time quantitative PCR. As a control, retinal mRNA from nonexposed newborn and adult mice were processed in parallel. In agreement with in vitro observations, JAM-C mRNA contents were not regulated during the time course of the angiogenic process (Fig. 5D). These results support the hypothesis that the redistribution of JAM-C might play an integral part in the angiogenic function as already found in vitro (Fig. 1B and C).

H33 anti–junctional adhesion molecule-C antibody has no effect on endothelial cell proliferation or apoptosis. Angiogenesis is a complex process orchestrated by the proliferation and architectural reorganization of endothelial cells on angiogenic stimuli. We first tested whether the H33 antibody blocks angiogenesis by inhibiting proliferation of lung primary endothelial cells in vitro and found no effect (Fig. 6A). To avoid any direct consequence of H33 treatment on tumor cell proliferation, we also did the experiment with tumor cells in vitro and no effect was detectable (Fig. 6B). These results indicated that the reduction of angiogenesis observed after H33 administration...
was not caused by prevention of vascular or tumor cell growth. Stimulation of apoptosis would be another explanation for the reduction of angiogenesis induced by H33 treatment. We tested this hypothesis on tumor sections by identifying endothelial cells with PECAM-1 staining and apoptosis by a standard labeling protocol of apoptotic cells (TUNEL). Results revealed that H33 antibody had no consequence on endothelial cell apoptosis in vivo and in vitro (Fig. 6C; data not shown). However, tumor cells showed increased TUNEL labeling in vivo, suggesting that apoptosis occurred as a consequence of antibody-mediated reduced vascularization (Fig. 6C).

**H33 anti-junctional adhesion molecule-C antibody has no pathologic side effect in vivo.** It has been shown that antibodies can be toxic when injected in vivo by increasing vascular permeability or inducing organ failure (33, 34). We injected animals with the H33 anti-JAM-C antibody and tested whether it induced pathologic side effects in vivo. Because JAM-C is expressed by endothelium in the kidney, we first analyzed whether the antibody would affect the normal function of the kidney. Using histochecmistry and periodic acid-Schiff staining, we did not observe accumulation of proteins in tubules and the morphology of glomeruli appeared normal (Supplementary Fig. S1A). Because JAM-C has been involved in controlling paracellular permeability of monolayers (18), we tested whether the antibody would increase permeability of blood vessels in vivo. No change in permeability was detectable in the organs analyzed, such as heart, kidney, and brain (Supplementary Fig. S1B).

**H33 anti–junctional adhesion molecule-C antibody reduces the recruitment of macrophages into the tumors.** Tumor angiogenesis is often accompanied by inflammation and macrophages represent prominent tumor-associated inflammatory cells (6). Indeed, macrophages participate in angiogenesis by secreting angiogenic factors, such as VEGF, mostly under hypoxic conditions (8). JAM-C is implicated in leukocyte adhesion and transmigration through endothelial and epithelial cells (19, 20, 28, 35). We thus investigated whether H33 antibody might affect recruitment of macrophages into tumors. As shown in Fig. 6D, mice treated with H33 antibody showed reduced macrophage content in tumors compared with control mice. This indicates that the H33 effect on angiogenesis is mediated in part via its action on recruitment of macrophages.

**Discussion**
In the present study, we show that the administration of a monoclonal antibody directed against JAM-C reduces angiogenesis in two different in vivo models: hypoxia-induced neovascularization of the retina and angiogenic vascularization of tumors. We show for the first time an important role for a member of the JAM family in angiogenesis in vivo. More importantly, JAM-C is now validated as a potential target for antiangiogenic treatment.

We reported previously that the molecule JAM-C is present on resting endothelium in different organs (18). Thus, the angiogenesis-blocking antibody H33 does not exclusively bind to angiogenic zones of the vasculature. However, the H33 antibody has no effect on in vivo vascular permeability, suggesting that the antibody does not interfere with established interendothelial junctions. In addition, the antibody does not induce other detrimental effects on the resting endothelium, such as kidney endothelial cells, which express large amounts of JAM-C. These observations suggest that H33 antibody neither interferes with the integrity of established interendothelial junctions nor creates vascular immunopathologies but rather interferes with a specific function of angiogenic endothelium.

In the exploration of anticancer treatments, antibody-based therapies have been shown to be good strategies to reduce vascularization of tumors and to limit their growth (36). Several growth factor receptors and adhesion molecules, such as VEGF-receptor-2 (VEGFR-2), VE-cadherin, or α5β3 integrin, have been successfully exploited as targets for antibody-based therapies (33, 37, 38). Although the mechanisms of action and the doses of antibodies required to get antiangiogenic effect differ from one target to the other, these strategies always consist in perturbing the mechanisms involved in migration, proliferation, or apoptosis of endothelial cells (33, 37, 38).
Based on the comparison of results obtained with antibodies and knockout mice on angiogenesis, it is possible to classify the targets into two categories: (a) targets antagonized in a fashion mimicking their absence and (b) targets used to transduce a specific signal in angiogenic endothelial cells. The endothelial molecules VE-cadherin and VEGFR-2 belong to the first category. Indeed, the targeted inactivation of these genes lead to embryonic lethality due to vasculogenesis defects (12, 39). In contrast, mice lacking αv or αvβ3 integrin subunits survive and maintain developmental blood vessel formation as well as angiogenesis in adult mice (10, 40). The integrin αvβ3 is known to mediate cell adhesion to extracellular matrix. This receptor-ligand interaction leads to outside-in signaling, which delivers intracellular survival signals. The contradiction between the antangiogenic effect of αvβ3 integrin inhibitors and enhanced angiogenesis in αvβ3-deficient animals has been explained as follows: when antagonists block engagement of αvβ3 with its ligands, the survival signal is switched into a death signal. In contrast, when the αvβ3 integrin is not expressed, the induction of apoptotic signals is absent (9).

Likewise to αvβ3 integrin, we show that antibody against JAM-C reduces angiogenesis, although preliminary data suggest that the genetic inactivation of JAM-C may not lead to apparent vascular defects (23). This indicates that the antibody H33 targets a specific function of JAM-C on angiogenic endothelial cells. However, we can exclude an effect on apoptosis or proliferation of endothelial cells, indicating that the antibody acts by a different mechanism.

Our findings indicate that the effect of H33 antibody on tumor growth may be due to impaired recruitment of monocytes into neovascularized areas. It is well described that local hypoxia induces secretion of chemokines by tumor cells responsible for monocyte recruitment (8, 41). Hypoxia also stimulates the secretion of angiogenic factors by monocytes/macrophages (42). Hence, tumor-associated angiogenesis is enhanced by the presence of macrophages. Recently, we showed that antibody H33 affects the adhesion of monocytes to the endothelium by modulating the availability of endothelial JAM-C for the monocyte integrin α4β2.3 We propose that JAM-C is also implicated in the recruitment of macrophages into tumors. Nevertheless, we cannot distinguish whether the reduction of

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Figure 5. H33 antibody reduces hypoxia-induced retinal angiogenesis. Newborn mice (P7) were exposed for 5 days to 75% oxygen in air, producing a central avascularization of the retina. Mice were then housed from P12 to P17 under normoxia leading to a hypoxia-induced situation and to neovascularization of the retina. Mice at P12, P14, and P16 were i.p. injected with PBS (n = 9), isotype-matched control antibody (n = 9), or H33 antibody (n = 13; 50 μg/injection). Mice at P17 were perfused with a nondiffusible FITC-dextran solution to visualize vasculature and were sacrificed, and retinal angiogenesis was analyzed. A, flat mounts of retinas from mice treated with control or H33 antibody. H33-treated retinas showed a reduced number of vascular glomeruli, which corresponded to highly proliferative clusters of vessels produced in response to hypoxia (arrowheads) compared with control retinas. Bar, 200 μm. B, number of vascular glomeruli was counted under the microscope. Columns, mean; bars, SE. ***, P < 0.001. C, serial sections of retina from P17 mice nonexposed (top) or exposed to hypoxia (bottom) were immunostained with polyclonal antibodies raised against JAM-C or PECAM-1. JAM-C is exclusively detected on blood vessels (arrow) and glomeruli (arrowhead). Bar, 20 μm. D, retinal JAM-C mRNA contents were quantified by real-time quantitative PCR. JAM-C expression level is not regulated by hypoxia compared with control animals.
tumor-associated macrophages is cause or consequence of reduced tumor vascularization in H33-treated mice. Indeed, the antibody abolishes vessel outgrowth in *ex vivo* aortic ring assays done in the presence of angiogenic growth factors and in the absence of possible recruitment of macrophages in culture dishes. This indicates that H33 antibody has also a direct, monocyte-independent effect on angiogenesis.

Although we can exclude an effect of the antibody H33 on endothelial cell proliferation or apoptosis, an alternate explanation for a direct effect of H33 antibody on endothelial cells could have been the reduction of endothelial cell migration. Indeed, angiogenesis depends in part on migration of endothelial cells, mainly supported by the coordination of signals from growth factors and the extracellular matrix (10, 43, 44). Moreover, regulation of cell-cell adhesion is also critical for migration. For example, VE-cadherin supports a cross-talk with VEGFR-2 signaling, which allows sprouting and endothelial cell migration during angiogenesis (45). A cross-talk was also suggested between JAM-A and bFGF to induce endothelial cell migration (15, 16). Nevertheless, at this time, no results have confirmed a role for JAM-A in angiogenesis *in vivo*. We found that H33 antibody does not affect migration of primary lung endothelial cells in response to VEGF *in vitro* (Supplementary Fig. S2). However, we cannot exclude that JAM-C plays a role in migration of endothelial cells *in vivo*, indicating that the antiangiogenic effect of anti-JAM-C antibody is indeed more complex. Whether JAM-C interacts with vascular growth factor receptors or extracellular matrix receptors, such as integrins, awaits further investigations.

Interestingly, we observe that JAM-C is relocalized in cell-cell contacts on VEGF or tumor necrosis factor-α stimulation (Fig. 1; data not shown). Such stimuli have been involved in activation of small Rho GTPases in human endothelial cells (46–48). In addition, the small GTPases Cdc42 and Rac have been implicated in capillary lumen formation in three-dimensional extracellular matrices *in vitro* (49). We showed recently that JAM-C modulates the localization and activity of Cdc42, which is essential for the...
polarization of round spermatids in vitro (23). In addition, in vitro studies have revealed that JAM-C interacts with polarity complex molecules, such as PAR-3 and PAR-6, suggesting a role for JAM-C in polarity establishment (22). Disassembly of molecular complexes essential to maintain endothelial polarity and the establishment of new interendothelial junctions occur during angiogenesis. We propose that the antibody H33 interferes with one or both steps. Current experiments aim to determine whether JAM-C is able to interact with polarity complex proteins in endothelial cells and participates to the polarization of the endothelium.

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