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Inhibition of Inflammatory Lymphangiogenesis by Integrin α5 Blockade

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The interaction between endothelial cells and extracellular matrix proteins plays an important role in (hem)angiogenesis. Integrins are able to mediate the outgrowth of newly formed blood vessels. In contrast, the role of integrins in lymphangiogenesis, ie, the outgrowth of new from pre-existing lymphatic vessels, has so far been unclear. Here, expression and functional relevance of integrins on lymphatic endothelium *in vivo* was investigated using the mouse model of combined inflammatory corneal hemangiogenesis and lymphangiogenesis. Immunohistochemistry revealed novel expression of both integrin α5 and αv on both resting and activated lymphatic vessels *in vivo*. Integrin α5-inhibiting small molecules significantly blocked the outgrowth of new lymphatic vessels into the cornea in a dose-dependent manner. The outgrowth of blood vessels was less significantly affected by this treatment, thus allowing for selective inhibition of lymphangiogenesis at lower dosages. Combined inhibition of integrin α5 and αv using inhibiting molecules did not significantly increase the anti-lymphangiogenic effect *in vivo*, thus suggesting an important functional role of integrin α5 in lymphangiogenesis. In summary, our findings demonstrate novel expression of specific integrins on growing lymphatic endothelial cells *in vivo* and reveal their functional role during lymphangiogenesis. This opens new treatment options for selective inhibition of lymphangiogenesis, eg, in oncology and transplant immunology. ***(Am J Pathol 2007, 171:361–372; DOI: 10.2353/ajpath.2007.060896)***

Angiogenesis, ie, the outgrowth of novel blood vessels, plays an important role in tumor growth, metastasis, and other human diseases.[1] Recently, in addition to (hem)angiogenesis, lymphangiogenesis, ie, the outgrowth of novel lymphatic vessels, has gained wide attention for its essential roles in inducing immune responses after organ transplantation as well as mediating tumor growth and metastasis.[2] Angiogenesis not only depends on the expression of specific growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor, but also on cell adhesion to the extracellular matrix (ECM). During growth of new blood vessels, adhesion to the ECM, eg, via integrins αvβ3 and αvβ5, regulates proliferation, survival, and motility of endothelial cells.[3] Consequently, integrin antagonists have been shown to be able to block hemangiogenesis *in vitro* and *in vivo*.[4,5]

Integrins, as ubiquitous heterodimeric proteins, are important for cell-cell and cell-ECM connections and are composed of α and β subunits.[6,7] Because integrins serve as transmembrane linkers between their extracellular ligands and the cytoskeleton, they have the capacity to influence cell migration during embryogenesis, angiogenesis, wound healing, immune and nonimmune defense mechanisms, hemostasis, and oncogenic transformation.[6,8] During angiogenesis, a significant up-regulation of αvβ3 and α5β1 on activated vascular endothelium has been identified.[5,9]

α5 integrins play a key role during development of the vascular system. Genetic ablation of integrin α5 leads to severe vascular abnormalities.[10] In combination with its extracellular ligand fibronectin, which is able to provide proliferative signals to vascular cells, α5β1 integrin is also up-regulated in tumor blood vessels and plays a role in tumor angiogenesis and growth.[5] Very recent data have shown that the integrin α5β1 is also involved in the choroidal neovascularization in a laser injury model in mice:

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$\alpha5\beta1$ inhibition caused prevention and regression of choroidal neovascularization.[11]

In contrast to $\alpha5\beta1$, αv integrins, which are known to also participate in hemangiogenesis by providing survival signals for activated endothelial cells,[4,12] are not essential for vascular development as genetic ablation models have shown: deletion of αv, $\beta3$, and/or $\beta5$ fails to block developmental angiogenesis and in some cases may enhance vasculogenesis and angiogenesis.[13,14] Nevertheless, using integrin $\alpha v\beta3$ and $\alpha v\beta5$ antagonists, several groups were able to inhibit hemangiogenesis *in vitro* and *in vivo*.[4,12,15]

In contrast to the good knowledge on the role of integrins in hemangiogenesis, to date nothing is known about the expression of integrins on lymphatic endothelial cells (LECs) *in vivo* and their functional relevance in lymphangiogenesis. Preliminary *in vitro* and *in vivo* knockout data suggest that lymphatic vessels express integrins such as $\alpha1\beta1$ and $\alpha2\beta1$,[16] $\alpha5$, αv, and also $\alpha9$.[17] Integrin $\alpha9\beta1$ seems to play a critical role during the development of the lymphatic system as shown in integrin $\alpha9$-deficient mice.[18] Further knowledge about the role of integrins in lymphangiogenesis may lead to the development of novel specific anti-(lymph)angiogenic therapies, eg, in oncology.

Here, we used the murine model of combined inflammatory hemangiogenesis and lymphangiogenesis in the normally avascular cornea to examine expression of integrins $\alpha5$ and αv and their extracellular ligands fibronectin and vitronectin on lymphatic vascular endothelium *in vivo*. Because the normal cornea is devoid of both blood and lymphatic vessels but can secondarily be invaded by both vessel types, the cornea is a very useful model system to study to functional role of integrins in lymphangiogenesis *in vivo*.[19] Therefore, integrin-blocking small molecules were used to characterize their functional role in lymphangiogenesis and explore novel strategies to inhibit lymphatic vessel outgrowth by specifically targeting integrins.

Materials and Methods

Animals

We used the mouse model of suture-induced inflammatory corneal neovascularization.[20] Six-week-old BALB/c mice (Charles River, Sulzfeld, Germany) were put under general anesthesia with an intramuscular injection of Ketanest S (8 mg/kg) and Rompun (0.1 ml/kg), and three intrastromal 11-0 nylon sutures (Serag-Wiesner, Naila, Germany) were placed in the cornea in a standardized manner. As described before, this procedure induces combined outgrowth of blood and lymphatic vessels from the limbal arcade (border between physiologically vascularized conjunctiva and normally avascular cornea) into the normally avascular cornea.[21,22] Animals were treated in accordance with the local animal care committee and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals.

Antibodies

For immunofluorescence, podoplanin served as a specific marker for lymphatic vascular endothelium[23]: we used the monoclonal Syrian-hamster anti-mouse antibody (Acris Antibodies GmbH, Hiddenhausen, Germany), diluted 1:200. As control staining, we used Syrian-hamster IgG (Santa Cruz Biotechnology, Santa Cruz, CA); all control IgGs were used in a dilution that provided the same quantity of protein as the antibody. Secondary antibody was goat anti-Syrian hamster fluorescein isothiocyanate (FITC) antibody (Dianova, Hamburg, Germany), diluted 1:100. For whole mount preparations, we used LYVE-1 antibody rabbit anti-mouse (a kind gift of D.G. Jackson, Oxford University, Oxford, UK) as specific lymphatic endothelial marker.[24] CY3 antibody served as secondary antibody (Dianova). Detection of blood vessels was done using the panendothelial marker CD-31. We used a monoclonal FITC-conjugated rat anti-mouse antibody, diluted 1:50 (Acris Antibodies GmbH). As control staining, we used FITC-conjugated normal rat IgG2A (Santa Cruz Biotechnology). As specific antibody for integrin $\alpha5$, we used a polyclonal rabbit anti-mouse antibody (Chemicon International, Planegg-München, Germany), diluted 1:50. Integrin αv antibody was polyclonal rabbit anti-mouse antibody (Chemicon International), which was diluted 1:50; control staining was done by normal rabbit IgG (Santa Cruz Biotechnology).

A polyclonal rabbit anti-mouse vitronectin antibody (Acris Antibodies GmbH), diluted 1:200, and a polyclonal rabbit anti-mouse fibronectin antibody (Dianova), diluted 1:500, were used. As a control, we used normal rabbit control IgG, diluted 1:100 (Santa Cruz Biotechnology). The secondary antibody for integrin $\alpha5/\alpha v$ and fibronectin/vitronectin was goat anti-rabbit CY3 antibody (Dianova), diluted 1:500.

VEGFR-2 antibody, a rabbit anti-mouse antibody, diluted 1:50 (Santa Cruz Biotechnologies), and VEGFR-3 antibody, a goat anti-mouse antibody, diluted 1:100 (R&D Systems, Minneapolis, MN) were used as primary antibodies. For co-localization studies, we used integrin $\alpha5\beta1$ rat anti-mouse antibody (diluted 1:25; Chemicon International) as secondary antibody. As a proliferation marker, we used Ki67 antibody (Biozol, Eching, Germany), diluted 1:100. After rinsing, we incubated with the secondary antibodies CY3 (goat anti-rabbit, diluted 1:500; Dianova) and FITC (donkey anti-rat, diluted 1:100; Chemicon International). Normal rabbit IgG and normal goat IgG (Santa Cruz Biotechnologies) were used as controls. For immunofluorescence on LECs in chamber slides, we used integrin $\alpha5\beta1$ antibody (mouse anti-human; Chemicon International), VEGFR-3 antibody (rabbit anti-human; Acris Antibodies GmbH), and the proliferation marker Ki67 (Biozol), diluted 1:50.

Integrin Inhibitors

JSM6427 is a $\alpha5\beta1$-specific integrin-inhibiting small molecule further described in Umeda and colleagues.[11]

JSM6424 is a derivative of JSM6427 recognizing $\alpha5\beta1$ as well as $\alpha v\beta3$ integrin. The inhibitory activity of these small molecules was determined using a solid-phase binding assay as described in Umeda and colleagues.[11] The integrin inhibitor JSM6427 is an antagonist of binding to integrin $\alpha5\beta1$ with an IC_{50} of 0.55 nmol/L and is at least 1500-fold less potent in the inhibition of binding to $\alpha v\beta3$ and other integrins such as $\alpha v\beta5$ and $\alpha IIb\beta3$. JSM6424 is an antagonist of binding to integrin $\alpha5\beta1$ with an IC_{50} of 0.9 nmol/L and to $\alpha v\beta3$ with an IC_{50} of 4 nmol/L. For testing of selectivity compared with other nonintegrin receptors, the integrin $\alpha5\beta1$ inhibitor was tested in a standard screening assay for activity on 50 nonrelated pharmacological relevant receptors, ion channels, and transporters. The inhibitor showed for all of them no activity or at least 1000-fold less activity than for the target integrin $\alpha5\beta1$ and no permeability into cells (Cerep, Paris, France; data not shown). These small molecules are characterized by good tissue penetration because of their low molecular weight and stability in physiological buffers (data not shown). Both small molecules were purchased from Jerini AG, Berlin, Germany.

Immunofluorescence

Staining protocols were standardized for whole mount preparations and cryosections as described previously.[22,25] We used indirect immunofluorescence to localize integrin $\alpha5$ and αv, fibronectin, and vitronectin on blood and lymphatic vessels in the pathologically vascularized, as well as in normal, nonvascularized mouse corneas and at the limbus. Blood and lymphatic vessels are physiologically present at the limbal border between cornea and conjunctiva. For immunofluorescence, murine eyes were cryopreserved in OCT embedding medium, and 5- to 7-μm cryosections were obtained. Sections were dried (15 minutes, 37°C) and fixed in acetone for 15 minutes on Superfrost slides (Menzel GmbH & Co. KG, Braunschweig, Germany). After rinsing with phosphate-buffered saline (PBS) (3×5 minutes on a shaker) and 1 hour incubation in 2% bovine serum albumin (BSA) at room temperature, incubation of primary antibodies followed overnight at 4°C. On the 2nd day, the antibodies were rinsed with PBS (5×5 minutes on a shaker) and blocked with 2% BSA (1 hour) and incubated with CD-31 or podoplanin antibody overnight at 4°C in the dark. On the 3rd day, after rinsing with PBS (5×5 minutes) and blocking with BSA, secondary antibodies were incubated for 45 minutes at room temperature in the dark. All dilutions were done with 2% BSA in PBS, and all incubations were performed in a humid chamber. After a final rinsing step (5×5 minutes PBS), sections were covered with DAKO (Glostrup, Denmark) fluorescent mounting medium and stored at 4°C in the dark. Fluorescence microscopy and photography was done using the Olympus BX51 fluorescence microscope (Olympus Optical Co., Hamburg, Germany) and the F-View II monochrome charge-coupled device camera (Soft Imaging System, Münster, Germany) software provided by analySISB (Soft Imaging Systems).

Electron Microscopy

Immunoelectron microscopy was performed on vascularized murine cornea segments as described previously.[25] Specimens were embedded in resin (LR-White; London Resin Co., Berkshire, UK) and ultrathin sections were used. Sections were incubated successively in drops of Tris-buffered saline (TBS), 0.05 mol/L glycine in TBS, and TBS-ovalbumin-gelatin. For immunogold labeling, we used a polyclonal rabbit anti-mouse antibody against integrin αv and a polyclonal rabbit anti-mouse antibody against integrin $\alpha5$ (Chemicon International) diluted in TBS and ovalbumin overnight at 4°C and a 10-nm gold-conjugated goat anti-rabbit secondary antibody (BioCell, Cardiff, UK) diluted in 1:30 TBS-ovalbumin-gelatin for 1 hour at room temperature. After rinsing, sections were stained with uranyl acetate and examined with an electron microscope (LEO 906 E; LEO, Zeiss, Oberkochen, Germany).

Systemic Application of Inhibiting Small Molecule Solutions

Inhibition of integrin $\alpha5\beta1$ function was achieved by a small molecule antagonist, JSM6427 (Jerini AG). We used the following doses of integrin $\alpha5\beta1$ inhibitor: 10, 23, and 46 mg/kg/day. For combined inhibition of integrin $\alpha5\beta1$ and integrin $\alpha v\beta3$, a combined small molecule antagonist (JSM6424; Jerini AG) was used at a dose of 23 mg/kg/day.

Because of rapid elimination of the small molecule inhibitors, systemic application was provided by osmotic pumps (Alzet; Durect Corp., Cupertino, CA). After filling with the inhibiting molecule solution or vehicle solution (control), pumps were incubated in 0.9% NaCl overnight at 37°C to induce pumping function. Osmotic pumps were then placed subcutaneously at the back of the mice after skin incision, simultaneously with the corneal suturing under general anesthesia (see above). The incision was closed by nonresolvable 6-0 silk sutures (Serag-Wiesner). After 1 week, osmotic pumps were changed to new ones that were filled before with solution (inhibiting molecule or vehicle) and again preincubated overnight at 37°C. After a total of 2 weeks of continuous systemic application, mice were sacrificed. The eyes were enucleated, the pumps removed and blood samples taken. Pumps and plasma were analyzed to control sufficient pumping rate and plasma levels. Experiments were repeated three times, with four animals per group. For analysis of macrophage recruitment into the cornea, mice were treated with integrin $\alpha5\beta1$ inhibitor JSM6427 (Jerini AG) at a dosage of 46 mg/kg/day ($n = 5$) or vehicle solution as control ($n = 5$) for 1 week after corneal suturing as described above.

Whole Mount Preparations and Immunostaining

Preparation was done as previously described.[22] In brief, mice were sacrificed, eyes were enucleated and the cornea dissected from the eye behind the corneal limbus.

Corneas were washed 3 × 5 minutes in PBS at room temperature. Fixation was done by acetone for 30 minutes, followed by washing 3 × 5 minutes in PBS. Afterward, we incubated with 2% BSA in PBS for 2 hours at room temperature. As primary antibody, we used LYVE-1 antibody rabbit anti-mouse 1:500 with 2% BSA in PBS, incubated overnight at 4°C. On the 2nd day, after washing 5 × 5 minutes in PBS, the antibody was blocked with 2% BSA in PBS for 2 hours. The secondary antibody CD31-FITC rat anti-mouse (Acris Antibodies GmbH), diluted 1:50 with 2% BSA in PBS, was added for incubation overnight at 4°C in the dark. On day 3, after washing 5 × 5 minutes in PBS, the antibody was blocked with 2% BSA in PBS for 2 hours. The third antibody, CY3 goat anti-rabbit (Dianova), diluted 1:100 with 2% BSA in PBS, was incubated for 45 minutes at room temperature in the dark. For detection of macrophages in the cornea, we used FITC-conjugated rat anti-mouse CD11b antibody (diluted 1:100; Serotec, Oxford, UK) instead of CD31-FITC antibody. As a final step, antibody was washed 5 × 5 minutes in PBS. Corneas were moved to Superfrost slides and covered with DAKO fluorescent mounting medium and stored at 4°C in the dark. Fluorescence microscopy and photography was done using the BX51 camera (Olympus Optical Co., Hamburg, Germany). For analysis, digital photographs were taken as multialignment pictures using ×100 magnification (analySISB; Soft Imaging System).

Functional and Statistical Analysis

Quantitative analysis of blood and lymphatic vessels was performed in a standardized procedure using analySISB (Soft Imaging System) software by means of threshold analysis. For measurements, we used rectangles of a standardized size (1.11 mm^2), which were aligned along the limbus. The corneal area filled with blood or lymphatic vessels (hemvascularized or lymphvascularized area) was measured in each rectangle. The vascularized areas of the control groups were defined as being 100%. For macrophage analysis, a standardized rectangle in the central cornea was analyzed by threshold analysis as described above. The area filled with macrophages was measured. Subsequent statistical analysis was done using InStat 3 Version 3.06 (GraphPad Software Inc., San Diego, CA). Graphs were drawn using Prism4, version 4.03 (GraphPad Software Inc.).

Lymphatic and Blood Endothelial Cells

For *in vitro* studies, we used human lymphatic microvascular endothelial cells (HMVEC-dLyAd-Der Lym Endo cells; Cambrex Bio Science, Walkersville, MD), which were identified as lymphatic cells via fluorescence-activated cell sorting analysis, revealing more than 95% positivity for podoplanin (as a specific marker for LECs) and for CD31 (as a panendothelial marker) expression. For blood endothelial cells (BECs), we used human umbilical vein endothelial cells (HUVECs; PromoCell, Heidelberg, Germany).

Western Blot Analysis

HUVECs (PromoCell) and human lymphatic microvascular endothelial cells (HMVEC-dLyAd-Der Lym Endo cells; Cambrex Bio Science) were cultured in endothelial growth medium (EGM) supplemented with a EGM-2-MV bullet kit (Cambrex Bio Science, Nottingham, UK) in a humidified atmosphere at 37°C, 5% CO_2. Cells were grown to 80% confluence. Cells (2×10^6) were lysed in 200 μl of lysis buffer [1% Triton X-100, 150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5, protease-inhibitor-cocktail (Sigma-Aldrich, Taufkirchen, Germany), 25 mmol/L glycerine phosphate, 50 mmol/L NaF, 10 mmol/L $Na_4P_2O_7$, and 1 mmol/L orthovanadate] and centrifuged for 5 minutes at 4°C at 12,000 rpm. Cells (7.5×10^4) (corresponding to 18.9 or 9 μg of total protein for HMVECs or HUVECs, respectively) per lane were separated on a 4 to 15% Tris-HCl gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions and transferred to polyvinylidene difluoride membranes by semidry Western blotting. Twenty ng of human integrin α5 (Chemicon Europe, Chandlers Ford, UK) was run as a positive control. Membranes were blocked with 3% BSA. $\alpha5\beta1$ integrin was visualized by rabbit anti-integrin α5 polyclonal antibody (Chemicon Europe) and actin, using a rabbit anti-actin polyclonal antibody (Sigma-Aldrich), respectively, followed by detection with anti-rabbit POD-conjugated antibody (Sigma-Aldrich) and BM chemiluminescence Western blotting POD substrate (Roche, Penzberg, Germany). The luminescence signal was measured using the Lumi imager (Boehringer-Mannheim GmbH, Mannheim, Germany) and corresponding software.

Immunofluorescence on LECs in Vitro

LECs (see above) were cultivated in chamber slides (Lab-Tek; Nalge Nunc International, Rochester, NY) in 500 μl of EGM medium for 24 hours. After removal of the medium, attached cells were fixed with 500 μl of ice-cold methanol for 10 minutes at room temperature. After three rinsing steps with PBS for 5 minutes, we added heat-inactivated fetal bovine serum for 30 minutes at 37°C. As primary antibody, we used rabbit anti-human Ki67 antibody (diluted 1:50 in 0.2% BSA in PBS; Biozol) overnight at 4°C. CY3 goat anti-rabbit antibody (diluted 1:250, for 1 hour at room temperature in the dark; Dianova) served as secondary antibody. For double staining, podoplanin mouse anti-human antibody (Acris Antibodies GmbH), diluted 1:100, was used, with FITC goat anti-mouse (Acris Antibodies GmbH; diluted 1:100) as secondary antibody. Mouse anti-human integrin α5 antibody (Chemicon International) was incubated overnight at 4°C (diluted 1:50 in 0.2% BSA in PBS), with secondary antibody FITC goat anti-mouse (diluted 1:100; Acris Antibodies GmbH). After each step, we rinsed three times with PBS. Following the same protocol, we used the VEGFR-3 rabbit anti-human antibody from Acris Antibodies GmbH, diluted 1:50 in 0.2% BSA (incubation overnight at 4°C) as primary antibody. Fluorescence microscopy and pho-

tography was done using the Olympus BX51 fluorescence microscope (Olympus Optical Co.) and the F-View II monochrome charge-coupled device camera (Soft Imaging System) software provided by analySISB (Soft Imaging Systems).

Light Microscopy of LECs in Vitro

To analyze the impact of integrin antagonists on the morphology of LECs *in vitro*, glass coverslides were precoated with 10 μg/ml fibronectin and blocked with 1% BSA. Cells (1.5×10^5) were plated on coverslips in the presence of 50 μmol/L of small molecule antagonists JSM6427 or JSM6424. Cells were allowed to attach and spread for 3 hours in growth medium as described for the proliferation assay. Photographs were taken with a Nikon Eclipse TS100 microscope (Tokyo, Japan) at ×100 magnification.

LEC and BEC Culture Proliferation Enzyme-Linked Immunosorbent Assay (ELISA)

Human lymphatic microvascular endothelial cells (HMVEC-dLy-Ad-der; Cambrex Bio Science) and HUVECs (PromoCell) were cultured in EGM-2-MV medium (Cambrex Bio Science) and endothelial cell growth medium (PromoCell), respectively, according to the instructions of the manufacturer. Ninety-six-well plates were precoated with 10 μg/ml fibronectin (Chemicon Europe) and blocked with 1% BSA. HMVECs were seeded at a density of 4×10^3 cells and HUVECs at 8×10^3 cells per well in the presence of indicated concentrations of integrin α5 small molecule antagonist (JSM 6427) or combined inhibitor of α5 and αv (JSM6424) as well as 5-bromo-2′-deoxyuridine (BrdU) (10 μl/ml, Cell Proliferation ELISA BrdU; Roche, Penzberg, Germany). Dimethyl sulfoxide concentrations were adjusted. Cells were grown for 3 days in growth medium containing serum and supplements. Cells were fixed and stained after 3 days following the manufacturer's instructions for BrdU [Cell Proliferation ELISA 5-bromo-2′-deoxyuridine (BrdU) Roche]. Colorimetric analysis was performed with a Spectra Max M5 microplate reader (Molecular Devices, Sunnyvale, CA). Data were analyzed with GraphPad Prism software.

Results

Expression of Integrins α5 and αv on Physiological Lymphatic Vessels in Vivo

We used untreated murine eyes to investigate whether physiological lymphatic vessels at the limbus (junction of vascularized conjunctiva and nonvascularized cornea) express integrins α5 and αv. By immunofluorescence, we were able to co-localize integrin α5 and αv on resting podoplanin-positive lymphatic vessels at the limbal arcade and adjacent physiologically vascularized conjunctiva in normal, nontreated mice. Some resting lymphatic vessels showed no staining for integrin α5. Only few lymphatic vessels at the limbus were integrin αv-positive. Staining for fibronectin and vitronectin as extracellular ligands of integrin α5 and αv was positive around most of the resting limbal lymphatic vessels.

Expression of Integrins α5 and αv on Proliferating Lymphatic Vessels in Vivo

In pathologically vascularized murine corneas, proliferating pathological corneal lymphatic vessels were characterized by integrin α5 and integrin αv positivity (Figure 1). Nevertheless, some podoplanin-positive pathological lymphatic vessels showed no staining for these integrins. Fibronectin and vitronectin as extracellular ligands of integrin α5 and αv were identified around most of the activated, proliferating corneal lymphatic vessels (Figure 2).

Co-Localization of VEGF Receptors 2 and 3 with Integrin α5 on LECs in Vivo

To analyze a potential co-localization of integrin α5 with the VEGF receptors 2 and 3 on lymphatic vessels *in vivo*, we performed staining for integrin α5 on VEGFR-3- and VEGFR-2-positive lymphatic vessels in the stroma of pathologically vascularized cornea. This demonstrated a co-localization of integrin α5 with both VEGF receptors (Figure 3). Because the normal cornea is devoid of lymphatic vessels, all lymphatic vessels observed within the corneal stroma early after an inflammatory stimulus are by definition proliferative. Furthermore, these lymphatic vessels stain positive for proliferation marker Ki67 (Figure 3A).

Ultrastructural Localization of Integrins α5 and αv on LECs in Vivo

As described before, lymphatic vessels are characterized by specific ultrastructural features in electron microscopy: a thin vessel wall; an absent continuous basement membrane; erythrocyte-free lumen; partly overlapping, thin endothelial cells; and lack of pericytes or tight junctions.[25,26] We used these criteria to distinguish lymphatic vessels from blood vessels in electron microscopy of vascularized corneas. By immunogold labeling, we were able to localize integrin αv and α5 to the endothelium of proliferating lymphatic as well as blood vessels in vascularized corneas. In LECs, immunogold labeling for both integrins was mainly localized apical at the luminal side (Figure 4), whereas its localization in blood vessel endothelium was more often basal (data not shown). Nevertheless, there was also immunogold labeling for integrins αv and α5 at the basal side of LECs.

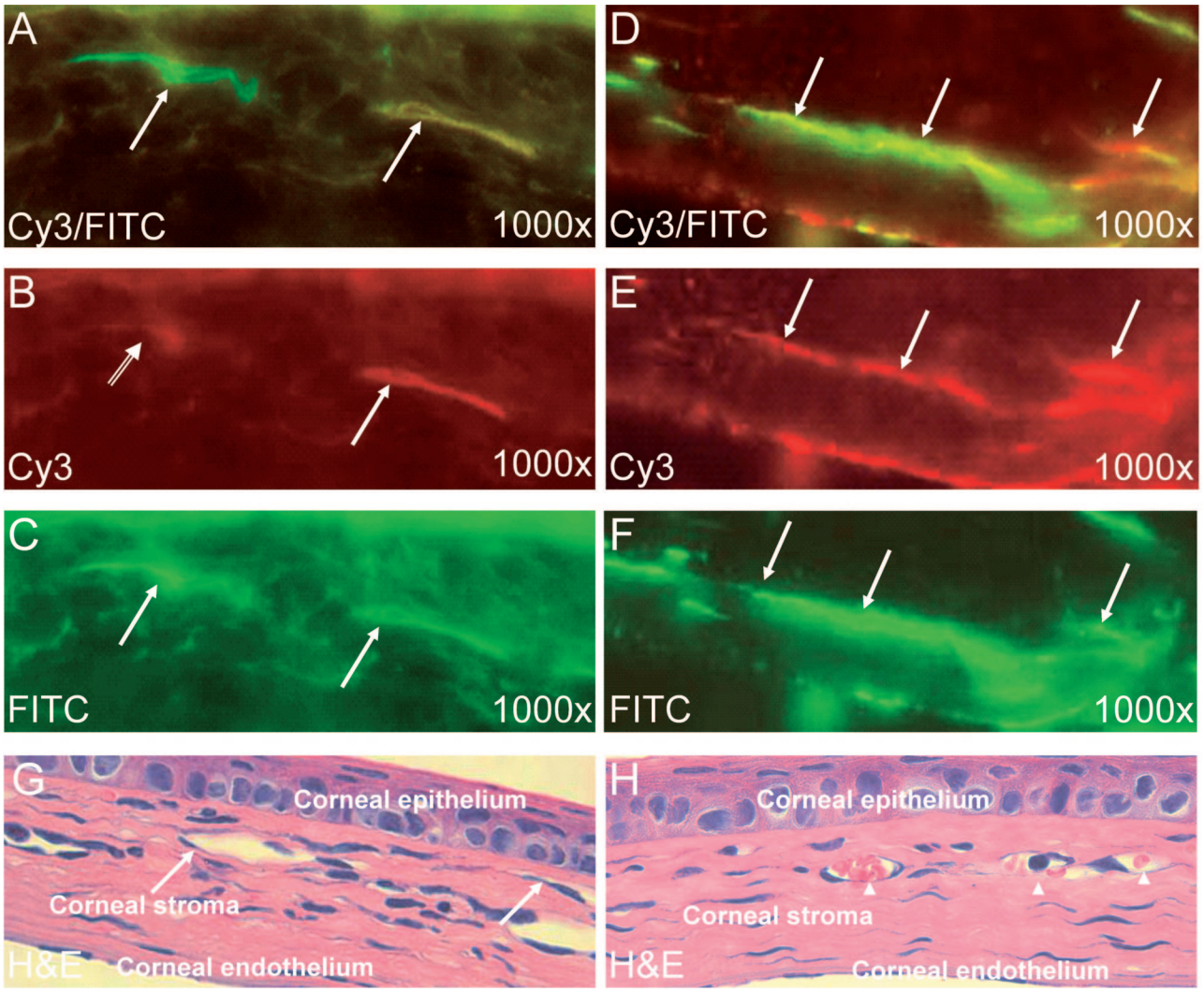

Figure 1. Expression of integrins $\alpha 5$ and αv on lymphatic vascular endothelium (**arrows**) *in vivo*. **A–C:** Integrin $\alpha 5$ (red, CY3; **B**) is co-localized with podoplanin-positive activated lymphatic vessels (green, FITC; **C**) in a representative segment of a pathologically vascularized murine cornea (**A**, merged). Note that not all podoplanin-positive vessels are integrin $\alpha 5$-positive (**open arrow**). **D–F:** Integrin αv (red, CY3; **E**) is co-localized with podoplanin-positive activated lymphatic vessels (green, FITC; **F**) in a representative segment of a pathologically vascularized murine cornea (**D**, merged). **G** and **H:** Light microscopy of representative sections of pathologically vascularized murine corneas (H&E) with the superficially located epithelial layer, the corneal stroma in the middle, and the basal endothelial layer, oriented to the anterior chamber of the eye. Pathological, erythrocyte-free, putative lymphatic vessels (**arrows** in **G**) and erythrocyte-filled blood vessels (**arrowheads** in **H**) are located in the stromal portion of the cornea. All immunostainings in this and the following figures are taken from the stromal portion of the cornea. Original magnifications, ×1000.

Expression of Integrin $\alpha 5$ on LECs in Vitro *(Western Blotting)*

$\alpha 5$ integrin expression on proliferating LECs (HMVEC-dLy-Ad-der; Cambrex Bio Science) and BECs (HUVECs) was analyzed by Western blotting. Both cell lines highly expressed integrin $\alpha 5$ in the same order of magnitude (Figure 5).

Expression of VEGFR-3 and Integrin $\alpha 5$ on Proliferating LECs in Vitro

The proliferation status of LECs was identified by positive staining with proliferation marker Ki67. LECs *in vitro* were positive for the lymphatic endothelial markers VEGFR-3 and podoplanin as well as for integrin $\alpha 5$ (Figure 6).

Effect of Integrin Antagonists on LEC Proliferation in Vitro *(ELISA)*

Small molecule inhibitors of integrin $\alpha 5$ (JSM6427) as well as the combined inhibitor of integrins $\alpha 5$ and αv (JSM6424) reduced proliferation of LEC ($P = 0.0086$ and $P = 0.0003$) as well as BEC ($P = 0.0392$ and $P = 0.0013$) *in vitro* significantly (Figure 7). For both types of endothelial cells the combined inhibitor of integrins $\alpha 5$ and αv (JSM6424) *in vitro* is significantly more effective than

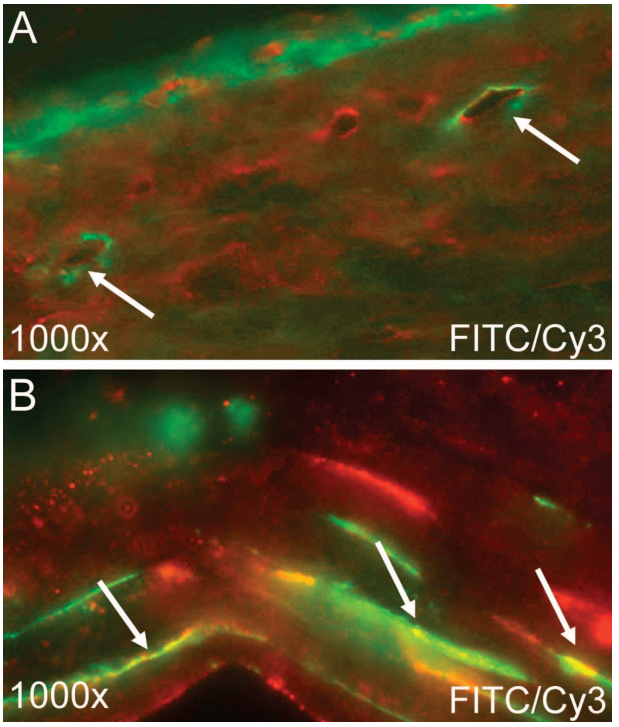

Figure 2. Expression of extracellular ligands of integrins α5 and αv (fibronectin and vitronectin) around pathological lymphatic vessels (**arrows**) *in vivo*. Positive reaction against fibronectin (red, CY3; **A**) and vitronectin (red, CY3; **B**) around activated, podoplanin-positive lymphatic vessels (green, FITC) in the stroma of a pathologically vascularized cornea. The anatomical layers of the cornea are depicted in Figure 1, G and H. Original magnifications, ×1000.

the selective small molecule inhibitor of integrin α5 (JSM6427) ($P = 0.002$ for LECs and $P = 0.0392$ for BECs).

Effect of Integrin Antagonists on Morphology of LECs in Vitro

To evaluate the effect of integrin antagonist on the morphology of lymphatic microvascular endothelial cells (HMVECs) under treatment with different integrin antagonists, LECs were grown on fibronectin with small molecule integrin antagonist JSM6424 and JSM6427 for 3 hours. Small molecule integrin antagonists caused reduced adhesion and attenuated spreading of cells. Compared with control (dimethyl sulfoxide, 0.5%), we found a twofold (JSM6427, 12.0%) and threefold increase (JSM6424, 18.8%) in the amount of rounded, nonadherent cells (Figure 8).

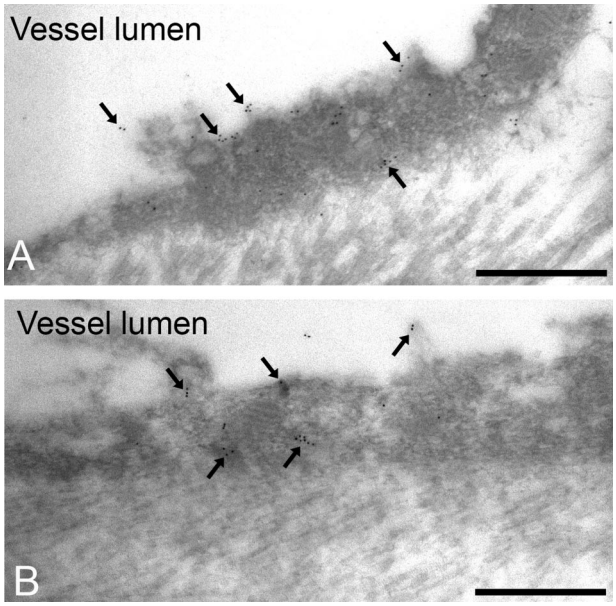

Figure 4. Ultrastructural localization of integrins α5 and αv in pathological lymphatic vessels *in vivo*. Clusters of 10-nm immunogold labeling (**arrows**) against integrin α5 (**A**) and integrin αv (**B**) are localized mostly apically but also basally on lymphatic vascular endothelium of pathological lymphatic vessels in vascularized murine corneas (electron microscopy). Scale bars = 555 nm.

Blockade of Integrin α5 Inhibits Lymphangiogenesis in Vivo

Systemically applied integrin α5-inhibiting small molecules (JSM6427) significantly inhibited lymphatic vessel outgrowth *in vivo* in the murine model of inflammatory corneal hemangiogenesis and lymphangiogenesis. After 2 weeks of application, a dose of 10 mg/kg/day did not significantly inhibit lymphangiogenesis compared with controls (20% increase of the lymphvascularized area

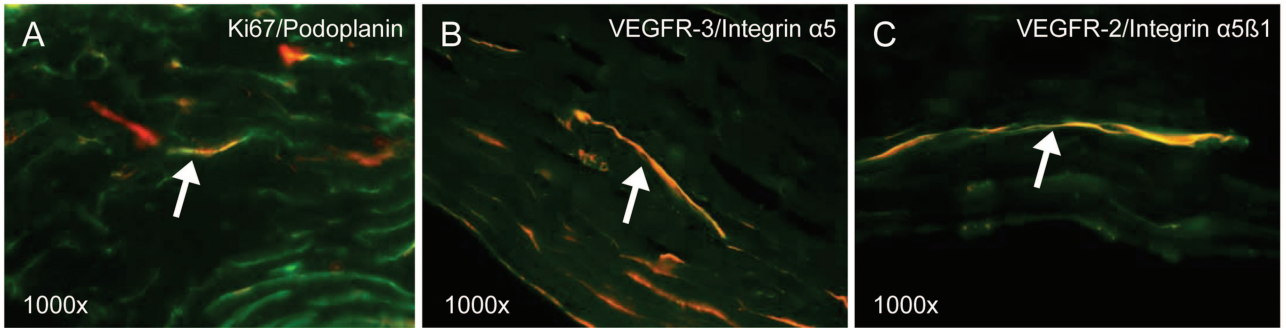

Figure 3. Co-localization of integrin α5 with VEGFR-2 and VEGFR-3 on lymphatic vessels *in vivo*. **A:** Podoplanin-positive (green, FITC) lymphatic vessel (**arrow**) shows staining for proliferation marker Ki67 (red, CY3). Co-localization of integrin α5 [red, CY3 (**B**); green, FITC (**C**)] on VEGFR-3-positive (green, FITC) and VEGFR-2-positive (red, CY3; **C**) lymphatic vessels (**arrows**) in a representative aspect of the stroma of a pathologically vascularized murine cornea. The anatomical layers of the cornea are depicted in Figure 1, G and H. Original magnifications, ×1000.

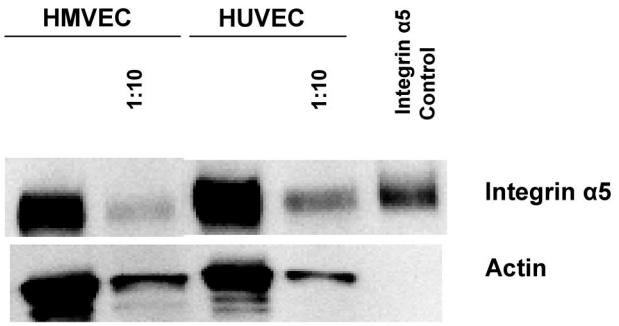

Figure 5. Expression of integrin α5 on proliferating lymphatic (HMVECs) and blood vascular endothelial cells (HUVECs) *in vitro*. Cells were grown to 80% confluence and lysed. Equal amounts of cells and dilutions of 1:10 were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein levels of integrin α5 and actin were visualized by Western blotting and chemiluminescence. Integrin α5 control: 20 ng of purified integrin α5 (CC1026; Chemicon Europe).

versus control). Higher doses of 23 and 46 mg/kg/day caused a significant inhibition of lymphangiogenesis ($P < 0.001$, lymphvascularized area 52.36% versus control set as 100%; $P < 0.001$, lymphvascularized area 50.19% versus control set as 100%, respectively) (Figure 9). Interestingly, inhibition of integrin α5 did inhibit hemangiogenesis less effectively. Doses of 10 and 23 mg/kg/day could not prevent sprouting of blood vessels significantly ($P = 0.1046$, hemvascularized area 103.4% versus control set as 100%, and $P = 0.5261$, hemvascularized area 96.76% versus control set as 100%, respectively), although the latter already inhibited lymphangiogenesis significantly. Only higher doses (46 mg/kg/day) were able to reduce both lymphangiogenesis and hemangiogenesis ($P < 0.01$, hemvascularized area 79.63% versus control set as 100%).

Combined Blockade of Integrins α5 and αv Inhibits Lymphangiogenesis in Vivo

A simultaneous inhibition of integrin α5 and integrin αv by means of systemic application of inhibitory small molecules (JSM6424) at a dose of 23 mg/kg/day resulted in a highly significant inhibition of lymphangiogenesis ($P <$

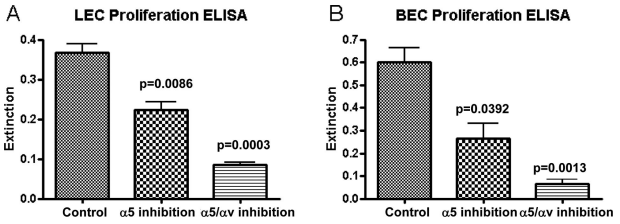

Figure 7. Blockade of integrin α5 and combined blockade of integrin α5 and αv inhibits proliferation of LECs (HMVECs) and BECs (HUVECs) *in vitro*. Diagrams of statistical analysis of cell proliferation ELISA. Cell proliferation of LECs (HMVECs) (**A**) and BECs (HUVECs) (**B**) was significantly inhibited by 50 μmol/L of small molecule integrin α5 ($P = 0.0086$ and $P = 0.0392$) or combined small molecule integrin α5 and αv antagonists ($P = 0.0003$ and $P = 0.0013$).

0.0001, lymphvascularized area 40.83% versus control set as 100%), whereas hemangiogenesis again was less affected ($P < 0.05$, hemvascularized area 87.15% versus control set as 100%) (Figure 10).

Blockade of Integrin α5 Does Not Significantly Affect Recruitment of CD 11b-Positive Macrophages in Vivo

To investigate whether the anti-lymphangiogenic effect of systemically applied integrin α5-inhibiting small molecules (JSM6427) is secondary to effects on recruitment of CD11b-positive macrophages, we analyzed this in the corneal suture model. There was no significant difference in CD11b-positive macrophage recruitment into the central cornea between the treated group and the control group ($P = 0.5476$) when mice were treated for 7 days.

Discussion

Our study elucidates two novel aspects of the mechanisms of lymphangiogenesis. First, we show expression of specific integrins (α5 and αv) on resting and proliferating lymphatic vessels *in vivo*. Expression of integrins α5 was also detected on proliferating LECs (HMVECs) *in vitro*. Second, we identify a novel and significant func-

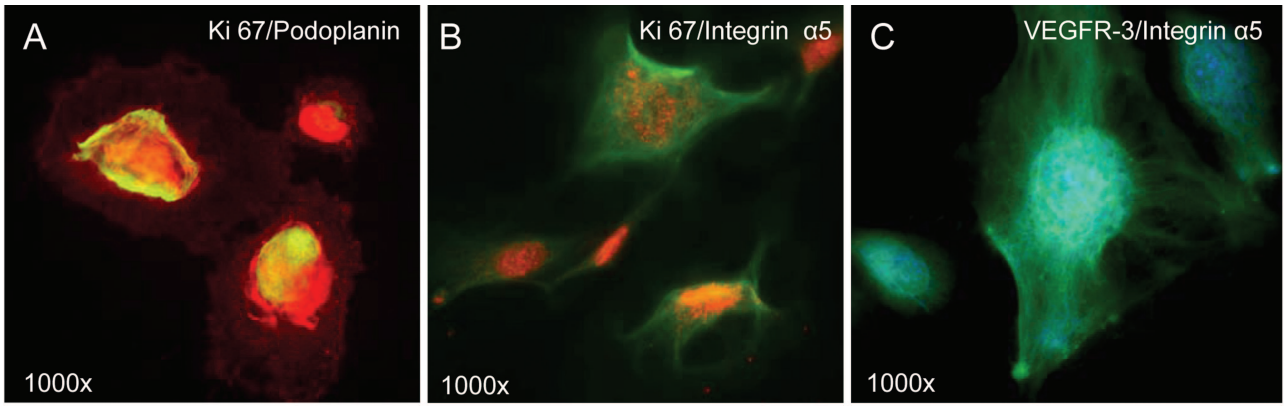

Figure 6. Expression of VEGFR-3 and integrin α5 on proliferating, Ki67-positive and podoplanin-positive LECs *in vitro*. Cultivated LECs (HMVECs) show positivity in chamber slide stainings for lymphatic endothelial marker podoplanin (**A**, green, FITC), for proliferation marker Ki67 (**A**, **B**; red, CY3), integrin α5β1 (**B**, **C**; green, FITC), and VEGFR-3 (**C**; FITC, miscolored in blue). Original magnifications, ×1000.

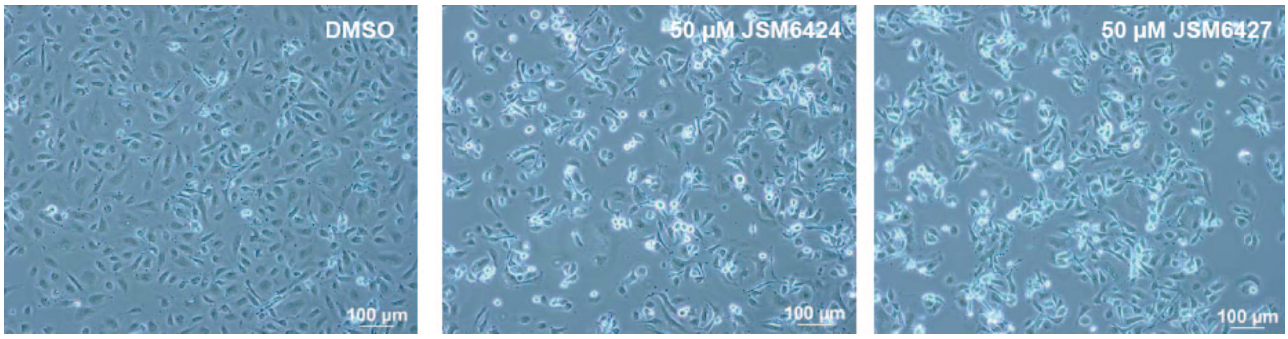

Figure 8. Cell morphology of LECs (HMVECs) grown on fibronectin in the presence of small molecule integrin antagonists. Cells were grown on fibronectin with small molecule integrin antagonists JSM6424 and JSM6427 (blocking integrins α5 and α5/αv, respectively) for 3 hours and photographed. After incubation with the integrin inhibitors, the LECs show diminished attachment, rounded detachment of cells, and reduced spreading. DMSO, dimethyl sulfoxide.

tional role of these integrins for inflammatory lymphangiogenesis *in vivo*: outgrowth of novel lymphatic vessels depends on normal function of specific integrins such as integrin α5 and, possibly to a lesser extent, αv.

The important role of integrins in lymphangiogenesis may be attributable to several factors. First is their function in substrate binding of endothelial cells. Without interactions between endothelial cells and the ECM, LECs, as BECs, are not able to sprout and form new vessels. Integrins mediate this cell-ECM interaction, eg, adhesion and de-adhesion of endothelial cells. Morphological analysis of LECs *in vitro* showed that the inhibition of cell adhesion to fibronectin via inhibition of α5β1 integrin causes diminished or attenuated spreading of cells. Detached cells lose their survival signal, attenuate proliferation, and most likely undergo apoptosis as described for BECs.[11] Loss of integrin-mediated attachment to the ECM triggers apoptosis through activation of caspases but also via caspase-independent effectors of apoptosis.[27,28]

Cell death does not result from the loss of adhesion alone (anoikis) but from the selective blockade of a single integrin and its signal transduction on adherent, tissue-associated cells.[29] Immunogold localization of integrins α5 and αv not only to the apical but also to the basal side of LEC, and inhibition of LEC proliferation by blocking molecules *in vitro*, support this concept of integrins being essential in lymphangiogenesis because of mediating LEC adhesion to ECM. As our immunohistochemistry data suggest, the expression of integrins on LECs might depend on their grade of activation and their particular microenvironment.

Second, integrins are involved in mediating growth factor signaling to (lymphatic) endothelial cells via various signaling pathways.[30] Integrin α5β1 has been shown to play a functional role in LEC survival and

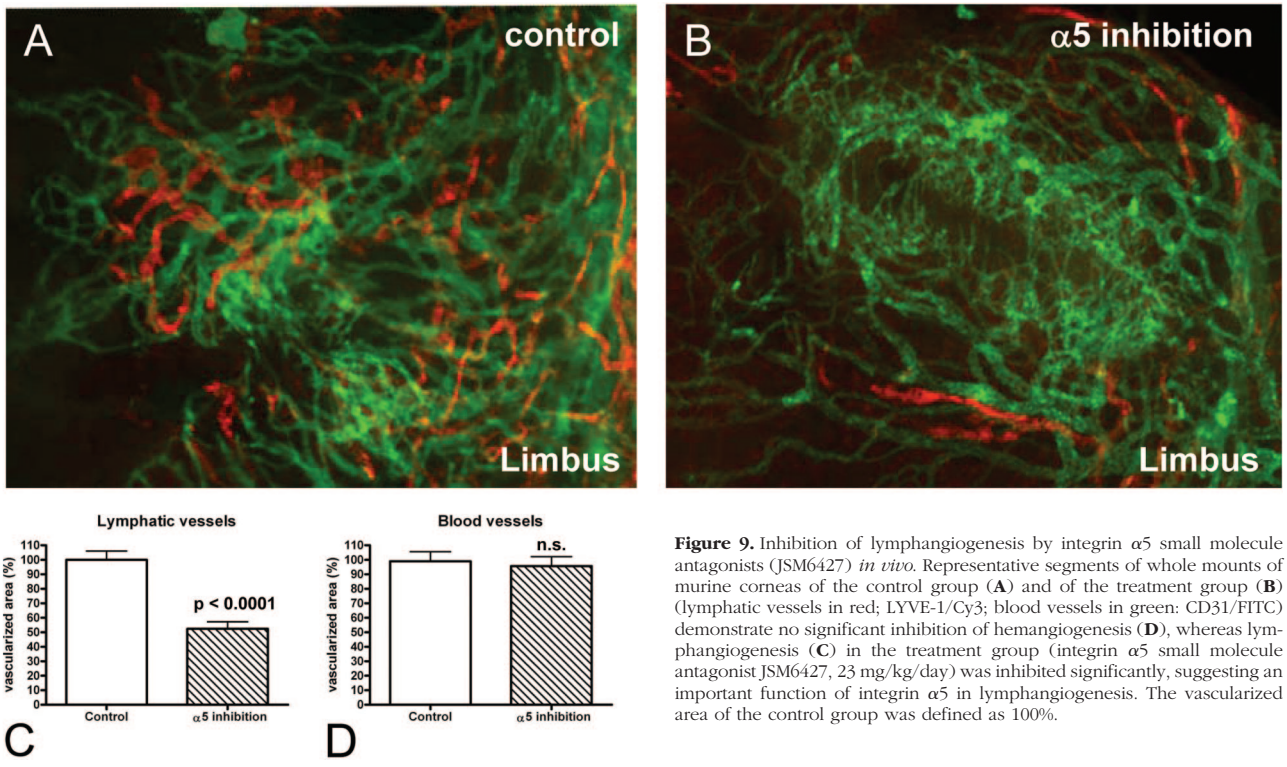

Figure 9. Inhibition of lymphangiogenesis by integrin α5 small molecule antagonists (JSM6427) *in vivo*. Representative segments of whole mounts of murine corneas of the control group (**A**) and of the treatment group (**B**) (lymphatic vessels in red; LYVE-1/Cy3; blood vessels in green: CD31/FITC) demonstrate no significant inhibition of hemangiogenesis (**D**), whereas lymphangiogenesis (**C**) in the treatment group (integrin α5 small molecule antagonist JSM6427, 23 mg/kg/day) was inhibited significantly, suggesting an important function of integrin α5 in lymphangiogenesis. The vascularized area of the control group was defined as 100%.

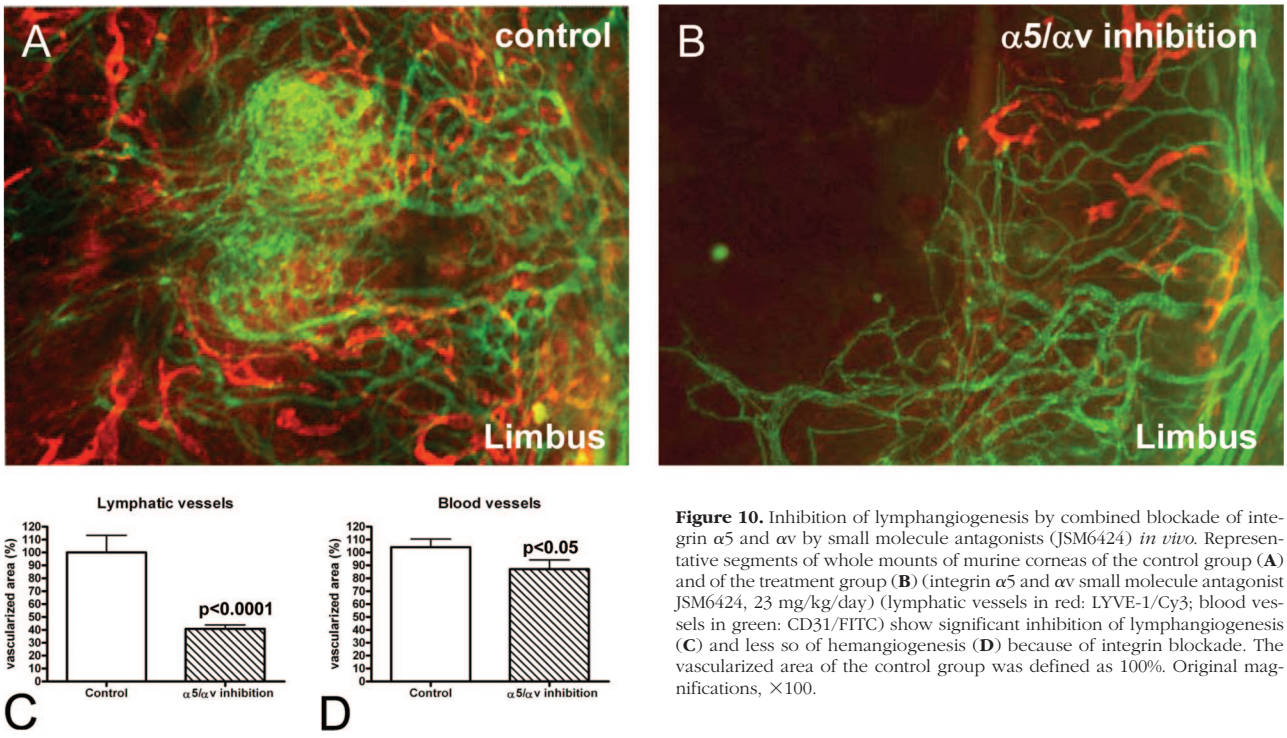

Figure 10. Inhibition of lymphangiogenesis by combined blockade of integrin α5 and αv by small molecule antagonists (JSM6424) *in vivo*. Representative segments of whole mounts of murine corneas of the control group (**A**) and of the treatment group (**B**) (integrin α5 and αv small molecule antagonist JSM6424, 23 mg/kg/day) (lymphatic vessels in red: LYVE-1/Cy3; blood vessels in green: CD31/FITC) show significant inhibition of lymphangiogenesis (**C**) and less so of hemangiogenesis (**D**) because of integrin blockade. The vascularized area of the control group was defined as 100%. Original magnifications, ×100.

proliferation *in vitro*.[31] Integrin α5β1 seems to be essential for VEGFR-3-mediated cell signaling because the tyrosine phosphorylation of VEGFR-3 can be blocked by anti-α5β1 antibody.[31] The downstream phosphatidylinositol 3 kinase/Akt signaling pathway of VEGFR-3 is the most important cell survival signaling cascade, being essential for regulation of cell proliferation and survival. VEGFR-3 selectively associates with α5β1, and furthermore, fibronectin as an extracellular ligand of integrin α5β1 selectively promotes the growth of LECs.[31] At present, little is known regarding tissue-specific signaling pathways in lymphangiogenesis, but recent data give evidence that integrins take part in these processes. VEGFR-3 and its ligands VEGF-C and VEGF-D are major regulators of lymphangiogenesis and stimulate proliferation, migration, and survival of LECs; they play an important role in physiological and tumor-related lymphangiogenesis.[32] Lymphangiogenic growth factors VEGF-C and VEGF-D have been identified as ligands for α9β1.[17] The effect of hepatocyte growth factor, which has recently been identified as lymphangiogenic growth factor, on LECs is partially mediated via α9β1.[33] Integrins α1β1, α2β1, and VEGFR-2 are involved in VEGF-A-promoted lymphangiogenesis.[16] Ultrastructural immunogold localization of α5 integrin to the apical side of LECs is compatible with a role in mediating signaling events, from lymphangiogenic growth factors in the lymphatic vessel lumen to the LEC. Furthermore, co-localization of integrin α5 with VEGFR-3 on proliferating lymphatic vessels is compatible with a role of integrin α5 in inducing VEGFR-3 phosphorylation.

Third, inflammatory and antigen-presenting cells such as macrophages play a crucial role in lymphangiogenesis. Immune cells seem to respond to lymphangiogenic signals but also induce lymphangiogenesis both by releasing lymphangiogenic growth factors as well as by integrating into new lymphatics.[22,34,35] Because integrins are also expressed on immune cells (eg, α5β1 on macrophages[36]), they seem to have complex functions in the regulation of lymphangiogenesis. Jin and colleagues[37] were able to show that monocyte trafficking in tumors is promoted by integrins such as α4β1. Because we found no significant reduction of CD11b-positive macrophages in the central cornea by systemic integrin α5 inhibition, we suppose that the inhibitory effect on lymphangiogenesis in the corneal suture model might be a direct effect on LECs rather than an indirect one via inhibition of macrophage recruitment. Nevertheless, a functional impairment of the immune cell interaction with α5 integrin cannot be excluded, and more investigations are underway.

Our data suggest α5 integrin to have a more important role in pathological lymphangiogenesis compared with αv. When mice were treated with combined α5 and αv antagonists, there was only a slightly higher inhibition of lymphangiogenesis *in vivo* compared with α5 antagonists alone. Even though we have seen *in vitro* an additive effect for inhibition of proliferation via addressing both integrins, the *in vitro* cultivation of endothelial cells is more different from the complex three-dimensional ECM environment *in vivo*. As Zhang and colleagues[31] suggest, integrin α5β1 may play a more important role in VEGFR-3-mediated lymphangiogenesis than integrin αvβ3.

Different expression patterns of the various integrins might be able to control the time course of hemangiogenesis and lymphangiogenesis.[16] Although α5β1 interactions are clearly proangiogenic in hemangiogenesis, αv

integrins have also anti-angiogenic properties.[6] The effect on angiogenesis may depend on different ligands of αv integrins, thus either enhancing or inhibiting $\alpha v\beta 3$-mediated angiogenesis.[14] Some *in vitro* studies suggest that integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ influence each other through cross-talk signaling events.[38,39] These facts might also explain the lower than expected (and not significantly different) additive effect of combined integrin α5 and αv blocking small molecules on lymphangiogenesis *in vivo*.

The organ-specific and the matrix-specific microenvironment seem to determine the process of lymphangiogenesis, and different growth factors promote lymphangiogenesis under different conditions.[16] Because the eye is an immunologically and morphologically very specific site, the observed phenomena in corneal lymphangiogenesis have to be seen in this specific ocular context. The cornea is normally not only devoid of blood and lymphatic vessels (this fact has been named "corneal angiogenic privilege") but also an immune-privileged site and tissue (termed corneal "immune privilege" by Streilein[40]). Because of its normal avascularity, in contrast to the normally vascularized skin, the cornea is an excellent *in vivo* model system to study the mechanisms of angiogenesis and lymphangiogenesis.[19,41] Because lymphatic vessels show the same morphological and functional characteristics in the eye as they do in other tissues,[25] we can assume that lymphangiogenesis in other organs might be also affected by integrin inhibition, but the exact timing and the molecular mechanisms might be other than in the corneal site because of the different microenvironment.

Our data suggest integrin inhibition to be an effective novel tool to prevent not only hemangiogenesis but also lymphangiogenesis. Furthermore, at certain doses it was possible to selectively inhibit lymphangiogenesis. Selective inhibition of lymphangiogenesis could be an important tool, especially in transplant immunology. By inhibition of lymphatic vessel outgrowth, the afferent arm of the immune reflex arc after organ transplantation (allowing for efflux of antigen-presenting cells and thereby causing host sensitization) can be (at least temporarily) interrupted without affecting hemangiogenesis (being essential for wound healing) and thereby increase graft survival after transplantation. The important role of the afferent arc of the immune reflex arc in mediating immune response after (corneal and) organ transplantation has recently been shown in several studies.[41–43] Furthermore, the novel treatment concept of anti-angiogenic therapy to improve (corneal) graft survival has recently been shown to be effective.[41,42] Thus novel strategies to selectively inhibit lymphangiogenesis are needed. In oncology, antilymphangiogenic therapy could prevent tumors from metastasizing, such as malignant melanomas, which are spread predominantly by lymphatic metastases.[44,45] Antagonists of several integrins ($\alpha 5\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$) are under clinical investigation to determine their therapeutic potential in anti-tumor treatment because of their antihemangiogenic effect. Our data suggest that at least some of them also should display anti-lymphangiogenic effects *in vivo*.

Conclusion

Lymphangiogenesis and hemangiogenesis depend on specific interactions between endothelial cells and ECM. Here, we demonstrated in a murine model of inflammatory corneal lymphangiogenesis and hemangiogenesis the novel expression of integrin α5 and αv on proliferating LECs *in vivo*. Selective inhibition of lymphangiogenesis was possible by specific blockade of integrin α5 and $\alpha 5/\alpha v$. The results indicate an important role of integrin α5 and less so αv in mediating lymphangiogenesis *in vivo* and reveal new therapeutic options for (selective) antilymphangiogenic treatment, eg, in oncology and transplant immunology.

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