Spontaneous Corneal Hem- and Lymphangiogenesis in Mice with Destrin-Mutation Depend on VEGFR3 Signaling

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Lymphangiogenesis, the formation of new lymphatic vessels, is important for tumor metastasis and induction of immunity to peripheral antigens including organ transplants. We herein describe a novel mouse model of spontaneous, secondary lymphangiogenesis in the normally avascular cornea. *corn1* mice, which suffer from a deletion in the gene encoding the cytoskeletal protein destrin, develop hemangiogenesis as well as spontaneous outgrowth of LYVE-1⁺⁺⁺/CD31⁺ lymphatic vessels into the cornea starting at age 4 weeks. Corneal lymphangiogenesis is delayed in onset, is less intense, and regresses earlier compared with hemangiogenesis. Moreover, the lymphangiogenesis is preceded only by a mild recruitment of CD45⁺ inflammatory cells into the cornea. In contrast to mice with inflammation-induced hem- and lymphangiogenesis, *corn1* mice do not develop breakdown of the blood-aqueous barrier. Finally, in this novel mouse model, a blocking anti-VEGFR3 antibody significantly inhibited not only lymph- but also hemangiogenesis. In summary, destrin deletion has differential effects on spontaneous hem- and lymphangiogenesis in the normally avascular cornea and represents a novel mouse model to study the mechanisms of lymphangiogenesis and to test the antihem- and antilymphangiogenic properties of known or new antiangiogenic agents. (*Am J Pathol* 2005, 166:1367–1377)

Lymphangiogenesis, the development of new lymph vessels, has recently gained wide interest for its important role in tumor metastasis and induction of alloimmunity after organ transplantation (for review, see Refs. 1, 2). Antilymphangiogenic strategies improved survival in animal tumor models by reducing tumor metastasis.^{3,4} Furthermore, antihem- and antilymphangiogenic strategies improve graft survival after organ transplantation in the mouse model of (corneal) transplantation.⁵ On the other hand, pro-lymphangiogenic treatment is desirable for patients with congenital or acquired (eg, postmastectomy) lymphedema.⁶ Therefore, a broad spectrum of potential applications for novel pro- and antilymphangiogenic treatments exists, making it essential to test novel agents for their potential pro- or antilymphangiogenic effect *in vivo*. Surprisingly, however, there is little knowledge about potential antilymphangiogenic properties of the plethora of antihemangiogenic agents that have already entered clinical trials in tumor patients⁷ and about the mechanisms involved in maintenance of (pathological) lymphatic vessels (for review, see Refs 1 and 6).

Several types of animal models have been used to study lymphangiogenesis *in vivo*. The first type monitors lymphangiogenesis during wound healing in the mouse tail,^{8–10} the rabbit ear,⁹ the rat tail,¹⁰ the lizard tail,¹¹ or the rat limb.¹² The second type of animal model involves transgenic or viral overexpression of ligands or antagonist molecules usually in the skin.^{13,14} In addition, the CAM assay in chicken eggs has been adopted for use in lymphangiogenic assays.¹⁵ Finally, the (mouse) cornea has been used for inflammatory models of hem- and lymphangiogenesis, either by implantation of micropel-

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lets releasing lymphangiogenic growth factors^{16–18} or by microsurgical manipulations of the cornea.¹⁷ Knockout mice are of limited value because VEGF-C-, VEGF-D-, and VEGFR3-deficient mice do not survive into adulthood.¹ Transgenic mice targeting the primarily hemangiogenic growth factor VEGF-A (such as VEGF-A isoform transgenic mice) allow insights into at least some aspects of lymphangiogenesis.¹⁷ In summary, so far, no simple, reliable, and easy quantifiable mouse model enabling the study of hem- and lymphangiogenesis without external (usually inflammatory) intervention is available.¹⁹

Here, we describe a novel mouse model of spontaneous lymphangiogenesis. Mice homozygous for the autosomal recessive mutation, cornea1 (*corn1*) have a mutation in the destrin gene, an actin-depolymerizing cytoskeletal protein.^{20–22} That defect leads to focal hyperplasia and increased turnover of the corneal epithelium and to spontaneous ingrowth of blood vessels into the normally avascular cornea by day 21.^{20–22} The normal cornea is devoid of blood and lymphatic vessels and maintains its avascularity unless severe inflammatory or other strains cause a disruption of the antiangiogenic privilege of the cornea with subsequent ingrowth of blood and lymphatic vessels from the adjacent (physiologically vascularized) conjunctiva into the corneal stroma.² The results presented herein demonstrate that 1) deletion of the cytoskeletal destrin gene in *corn1* mice indirectly causes spontaneous lymphangiogenesis in addition to clinically obvious hemangiogenesis; that 2) importantly, the two processes are differentially affected by the destrin mutation; and that 3) lymphangiogenesis in *corn1* mice is low-inflammatory in nature. The cornea of *corn1* mice therefore is a reliable and easy quantifiable model system to study the mechanisms of hem- and lymphangiogenesis and to test the antihem- and antilymphangiogenic properties of known or new antiangiogenic agents. Finally, we use this new model system to demonstrate a novel, combined antihem- and antilymphangiogenic effect of blocking VEGFR3 signaling *in vivo*.

Materials and Methods

corn1 Mice and Wild-Type Controls

corn1 mice (A.BY H2bH2-T18f/SnJ-Dstncorn1/J) and age-matched control mice from the parent strain (A.BY/SnJ) were obtained from The Jackson Laboratory (Bar Harbor, ME; gift of S. Ikeda). Mice aged 1, 2, and 4 weeks and 2, 3, 5, 8, and 12 months from both groups were analyzed biomicroscopically using slit-lamp examinations and immunohistochemically (see below). Mice were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals. For slit-lamp examinations, mice were anesthetized using a mixture of ketamine and xylazine (120 and 20 mg/kg body weight, respectively).

Immunohistochemistry and Morphometry of Corneal Hem- and Lymphangiogenesis

Briefly, corneal flat mounts were rinsed several times in phosphate-buffered saline (PBS), fixed in acetone, rinsed in PBS, blocked with 2% bovine serum albumin, stained with a FITC-conjugated CD31/PECAM-1 antibody overnight (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), washed, blocked, and stained with anti-LYVE-1 antibody (1:500; a lymphatic endothelium specific hyaluronic acid receptor; D. Jackson, Oxford),²³ which was visualized with a Cy3-conjugated secondary antibody (1:100; Jackson ImmunoResearch Laboratories, Westgrove, PA). Double-stained sections were analyzed using a Zeiss Axiophot microscope (Zeiss, Jena, Germany). Digital pictures of the flat mounts were taken using the Spot Image Analysis system, and the area covered by CD31⁺⁺/LYVE-1⁻ blood vessels and CD31⁺/LYVE-1⁺⁺ lymphatic vessels^{17,24,25} was measured using NIH Image software. The total corneal area was outlined using the innermost vessel of the limbal arcade as the border, and the area of hem- versus lymphangiogenesis within the cornea was then calculated and normalized to the total corneal area (expressed as a percentage of the cornea covered by blood versus lymphatic vessels).¹⁷

Immunohistochemistry for Destrin, VEGFR3, VEGF-A, VEGF-C, and VEGF-D

Indirect immunohistochemistry for CD31/PECAM1 (1:100; Santa Cruz Biotechnology) and destrin/cofilin (1:100; gift of Dr. J.R. Bamberg) was performed on frozen sections of wild-type controls as described previously.^{20,24,25} Indirect immunohistochemistry for VEGFR3 (polyclonal goat-anti-mouse antibody; 1:200; R&D Systems, Inc., Minneapolis, MN), VEGF-A, VEGF-C, and VEGF-D (polyclonal goat-anti-mouse antibody; Santa Cruz Biotechnology; 1:100) was performed on frozen sections of wild-type controls and same-aged *corn1* mice as previously described.¹⁷

Histological Quantification of Inflammatory Cells

The presence of inflammatory cells in wild-type and *corn1* mice corneas was analyzed in hematoxylin & eosin (H&E)-stained serial sections of plastic-embedded corneas fixed in 10% paraformaldehyde after enucleation. In addition, acetone-fixed corneal frozen sections were stained for the panleucocyte marker CD45 (clone 30-F11; Pharmingen, San Diego, CA). Isotype controls were used as negative controls. As a positive control, frozen sections of vascularized corneas of mice 2 weeks after corneal transplantation were used.⁵ Always, three separate sections per eye were analyzed, and CD45⁺ cells were counted using an image analysis software (NIH Image) on digital pictures taken using the Spot Image Analysis system. Ten eyes were examined per group.

Neutralizing Anti-VEGFR3 Antibody

A neutralizing anti-VEGFR antibody (mF4-31C1; ImClone Systems, Inc., New York)¹⁹ was used to test the importance of VEGFR3 signaling for hem- and lymphangiogenesis in the *corn1* model system. We compared the effect of systemic application of a blocking anti-VEGFR3 antibody (mF4-31C1, 0.7 mg/mouse intraperitoneally (i.p.), at 14 and 21 days old) with the effect of control IgG solution (Jackson Immunoresearch, Inc., West Grove, CA) at the same concentration on hem- and lymphangiogenesis measured at age 5 weeks.

Quantification of Protein and Leukocytes in Aqueous Humor

Mice were euthanized by cervical dislocation. A puncture was made at the center of the cornea with tip of a 30-gauge needle, and the aqueous humor (AqH) was expressed. The AqH was collected into 10- μ l glass micropipettes (Fisher Scientific, Pittsburgh, PA) by capillary attraction. AqH from two eyes of a mouse was pooled as a sample. AqH was centrifuged. One microliter of the supernatant was used for total protein analysis using a protein assay kit (BCA; Pierce, Rockford, IL). The rest of the supernatant was removed, and the pellets were resuspended in 10 μ l of PBS. Presence of cells in the suspension was analyzed and counted using a hemocytometer.

Reverse Transcription-Polymerase Chain Reaction for VEGF-A, VEGF-C, VEGF-D, and VEGFR3

Reverse transcription-polymerase chain reaction (PCR) was carried out as previously described.^{17,24} Briefly, total RNA was extracted from central corneas immediately after euthanasia of *corn1* and wild-type mice by using RNeasy-60 (Tel-Test, Inc., Friendswood, TX). For this, the corneas of 10 mice (20 corneas) were pooled. From 1 μ g of mRNA, cDNA was synthesized with M-MLV reverse transcriptase (Promega, Madison, WI) according to manufacturer's instructions. The following primers were used for PCR from 5' to 3': GAPDH sense, GGTGAAGGTCGGTGTGAACGGA; GAPDH antisense, TGTTAGTGGGGTCTCGCTCTG; VEGFR3 sense, GCGACAGGGTTCTCATAA; VEGFR3 antisense, CGTTGCCTCATTGTGATTAG; VEGF-A sense, GTACATCTTCAAGCCGTCCT; VEGF-A antisense, TTACACGTCT GCGGATCTT; VEGF-C sense, GTCTGTGTC-CAGCGTAGATG; VEGF-C antisense, GCTGGCAGAGAACGTCTAAT; VEGF-D sense, GCGGCAACTTCTATGACA; VEGF-D antisense, AGCACTTACAACCCGTATGG. All primers were designed by Sigma Genosys (Woodlands, TX). PCR was carried out under the following conditions: denaturation at 94°C; annealing at 55°C; and extension at 72°C. After 40 cycles of amplification (AmpliTaQ DNA Polymerase; Applied Biosystems, Foster City, CA), PCR products were electrophoresed in 2% agarose gel and visualized by ethidium bromide staining (0.5 μ g/ml ethidium bromide) for 40 minutes. Photo-

graphs of the gel were taken with a high-resolution camera, and the density of the bands was analyzed on the gel using UV illumination and Image One image analysis software (Bio-Rad, Hercules, CA). The expression level of mRNA was standardized by the expression of GAPDH as an internal control. The predicted sizes of PCR products are 245 bp for GAPDH, 276 bp for VEGF-A, 531 bp for VEGF-C, 307 bp for VEGF-D, and 290 bp for VEGFR3.

Statistical Analysis

Statistical significance was analyzed by Mann-Whitney's test. Differences were considered significant at $P < 0.05$. Each experiment was performed at least twice with similar results. Graphs were drawn using Graph Pad Prism, Version 3.02.

Results

Spontaneous Lymphangiogenesis in Corneas of Mice with a Destrin Mutation (*corn1*)

To investigate whether spontaneous hemangiogenesis occurring in destrin-mutant *corn1* mice is associated with spontaneous lymphangiogenesis, *corn1* mice and wild-type controls of different ages (1, 2, and 4 weeks and 2, 3, 5, 8, and 12 months) were analyzed biomicroscopically and using double immunohistochemistry (with LYVE-1 as lymphatic vascular endothelial and CD31 as blood vascular endothelial marker).¹⁷ Slit-lamp examination (Figure 1A) confirmed the finding of spontaneous outgrowth of limbal blood vessels into the normally avascular cornea starting around week 3 (ie, 1 week after lid opening). These blood vessels covered the whole corneal surface at about week 6. There was no sign of blood vessel regression over the 1st year of life. LYVE-1 immunohistochemistry at 2 months of age demonstrated that in addition to LYVE-1⁻/CD31⁺⁺ blood vessels, biomicroscopically invisible LYVE-1⁺⁺/CD31⁺ lymphatic vessels were present in the cornea of *corn1* mice (Figure 1, B and C).

Spontaneous Lymphangiogenesis in *corn1* Mice Is Delayed in Onset Compared with Hemangiogenesis

Having shown the existence of spontaneous lymphangiogenesis in *corn1* mice, we next analyzed the time course of hem- versus lymphangiogenesis in *corn1* mice (Figure 2). Double immunohistochemistry confirmed the slit-lamp finding that hemangiogenesis started with single sprouts around week 2 and was biomicroscopically visible at week 3 postpartum. In contrast, there was no sign of lymphangiogenesis until week 3. Starting with single small spouts around week 3, outgrowth of lymphatic vessels was clearly visible around weeks 4 to 6. Lymphangiogenesis thereby followed spontaneous hemangiogenesis with a delay of 1 to 2 weeks (Figure 2).

Figure 1. Spontaneous hem- and lymphangiogenesis in mice with a destrin gene deletion (*corn1* mice). **A:** Representative slit-lamp aspect of the cornea of an 8-week-old *corn1* mouse with blood vessels growing into the normally avascular cornea (hemangiogenesis). **B:** Double immunostaining of the cornea using LYVE-1 (red; **arrow:** L) as a specific lymphatic endothelial marker and CD31 (green; **arrowhead:** B) as a blood vascular marker demonstrates both hem- and lymphangiogenesis into the cornea (magnification, $\times 40$). **C:** Detail form B with abundant blood (B) and some lymphatic (L) vessels (magnification, $\times 100$; limbus at the left: transition zone from physiologically vascularized conjunctiva to normally avascular cornea).

Secondary Lymphangiogenesis in *corn1* Mice Is Less Intense and Less Persistent Than Hemangiogenesis

Because spontaneous corneal lymphangiogenesis was delayed compared with hemangiogenesis in *corn1* mice, we next asked whether lymphangiogenesis in *corn1* mice is less intense and less robust than hemangiogenesis, suggesting a “secondary” hemangiogenesis-triggered lymphangiogenesis. Morphometry of a time course of double-immunolabeled corneal flat mounts (at age 1, 2, and 4 weeks and 3, 5, 8, and 12 months; $n = 4$ per time-point; Figure 3) demonstrated that lymphangiogenesis in *corn1* was not only delayed but also significantly less extensive than hemangiogenesis (10 to 60% of hem-vascularized area; $P < 0.001$ at all time points) and that lymphatic vessels regressed much earlier than blood vessels (which did not show signs of regression within the

1st year). Lymphatic vessel fragmentation was observable starting already at 2 months of age (Figure 1C).

Absence of Massive Inflammation Preceding Spontaneous Hem- and Lymphangiogenesis in *corn1* Mice

To elucidate the mechanism governing the spontaneous lymphangiogenesis in *corn1* mice (given that recently inflammatory cells and especially macrophages have been identified as prime sources of lymphangiogenic growth factors VEGF-C and VEGF-D),^{17,26} we analyzed whether *corn1* mice display increased recruitment of bone-marrow-derived cells into the corneal stroma preceding spontaneous lymphangiogenesis.²⁷ To test this, corneal sections of *corn1* mice aged 4 weeks and age-matched wild-type controls were analyzed for CD45⁺

Figure 3. Spontaneous, secondary lymphangiogenesis in *corn1* mice is less extensive and regresses earlier compared with hemangiogenesis. Depicted are the relative proportions of the normally avascular cornea covered by blood (black bars) versus lymphatic vessels (white bars; mo., months; $P < 0.001$ at all time points).

cells. Hem- and lymphvascularized corneas after mouse corneal transplantation⁵ served as positive controls. A quantitative analysis demonstrated that there were slightly, but significantly, more CD45⁺ inflammatory cells in *corn1* mice (132.9 ± 75) compared with WT controls (51.6 ± 21 ; $P < 0.01$). But the level of stromal CD45⁺ cells in wild-type and in *corn1* mice was still significantly lower compared with vascularized and inflamed (positive control) corneas (322.5 ± 121.6 cells/section; $P < 0.01$; Figure 4). These findings demonstrate that the spontaneous corneal lymphangiogenesis in *corn1* mice occurs in a low-inflammatory context.

Normal Protein Content and Leukocyte Count in Aqueous Humor of *corn1* Mice

To further support the notion that induction of hem- and lymphangiogenesis in *corn1* is not mediated primarily by inflammation, we quantified protein and leukocyte content in the aqueous humor, the clear fluid filling the anterior chamber. Normally, inflammation-induced corneal neovascularization is associated with breakdown of the blood-aqueous barrier and subsequent increases in leukocytes and protein content of the aqueous humor.^{27–29} In contrast, no protein (less than 0.3 mg/ml) and no leukocytes were detectable in the aqueous from the ABY wild-type mice and from the aqueous humor of eyes of *corn1* mice with intense corneal neovascularization.

Absent Dextrin Immunoreactivity on Wild-Type Limbal Blood and Lymphatic Vessels

To evaluate whether angiogenesis and lymphangiogenesis associated with absence of dextrin in the corneas of *corn1* mice are due to a direct effect of absence of the

Figure 2. Delayed onset of spontaneous lymphangiogenesis compared with hemangiogenesis in *corn1* mice (representative segments from corneal flat mounts stained with LYVE-1 [red] and CD31 [green]; magnification, $\times 100$): **A:** At postnatal day 7, neither blood nor lymphatic vessels grow out from the limbal arcade (Li, limbus). **B:** At 2 weeks of age, very small sprouts of blood vessels (arrowhead: B) grow into the cornea. **C:** At 3 weeks, a robust hemangiogenesis (green) can be observed with lymphangiogenesis showing initial minor outgrowth. **D:** At 6 weeks, the whole cornea is occupied by blood vessels and partly covered by lymphatic vessels (arrow: L).

protein on limbal blood and lymphatic vessels, we evaluated the expression of destrin on the normal limbal vasculature. As depicted in Figure 5, there is strong expression of destrin in the corneal epithelium of wild-type mice but no expression on the limbal blood or lymphatic vasculature of wild-type mice. This suggests an indirect angiogenic and lymphangiogenic effect of absent destrin.

No Difference in the mRNA Levels of Angiogenic Growth Factors of the VEGF Family between *corn1* Mice and Wild-Type Controls

To test whether the spontaneous lymphangiogenesis in *corn1* mice is related to changes in the expression of the major known lymphangiogenic growth factors (VEGF-A, VEGF-C, VEGF-D, or their VEGF receptor 3), we compared the mRNA/GAPDH-ratios of VEGF-A (0.19 [*corn1*] versus 0.18 [*wildtype*]), VEGF-C (0.1 versus 0.13), VEGF-D (0.5 versus 0.51), and VEGF receptor 3 (0.56 versus 0.5) between *corn1* mice and wild-type controls at 4 weeks of age. There was no obvious, significant difference in the expression levels of these growth factors (Figure 6). Furthermore, immunohistochemistry for the lymphangiogenic growth factors VEGF-C, VEGF-D, and VEGF-A as well as the main lymphangiogenic receptor VEGFR3 in the cornea did not reveal significant differences in expression. Figure 6 exemplarily demonstrates that the strong immunoreactivity of the lymphangiogenic VEGFR3 is primarily located on corneal epithelial cells (and in vascularized *corn1* corneas in addition to new stromal vessels).

A Blocking Anti-VEGFR3 Antibody Inhibits Spontaneous Hem- and Lymphangiogenesis in *corn1* Mice

To test the usefulness of spontaneous corneal hem- and lymphangiogenesis in *corn1* mice as a model system to study the molecular mechanisms of hem- and lymphangiogenesis, we asked the question whether in addition to lymphangiogenesis,¹⁸ hemangiogenesis also depends on VEGFR3 signaling and whether a novel blocking anti-VEGFR3 antibody (mF4-31C1)¹⁹ can inhibit hem- and lymphangiogenesis. This question has significant relevance for antiangiogenic cancer treatments.^{3,4,7} *corn1* mice received i.p. injections of a blocking anti-VEGFR3 antibody at days 14 and 21, before onset of spontaneous hem- and lymphangiogenesis (control mice received i.p. injections of the control IgG). When the extent of hem- and lymphangiogenesis was evaluated at

5 weeks of age (Figure 7), anti-VEGFR-treated mice displayed nearly complete inhibition of lymphangiogenesis ($1.1 \pm 0.8\%$) compared with IgG controls ($12.7 \pm 15.8\%$; $P < 0.01$). In addition, they had a significant inhibition in hemangiogenesis ($32.5 \pm 7.3\%$ versus 99.8% in the controls; $P < 0.0001$), providing significant support for the principle that anti-VEGFR3-blocking antibodies can inhibit hem- and lymphangiogenesis *in vivo*.

Discussion

The results presented in this study allow two important conclusions. First, we conclude that there is a low-inflammatory form of (corneal) lymphangiogenesis that is not dependent on massive recruitment of bone-marrow-derived cells and may primarily be a sequel of initial hemangiogenesis rather than a parallel companion of it, as is the case in situations of intense inflammation-induced corneal hem- and lymphangiogenesis.¹⁷ Deletion of the gene encoding the cytoskeletal protein destrin causes spontaneous hem- and lymphangiogenesis into the normally avascular cornea, but with hemangiogenesis starting earlier, being more intense, and lasting longer than the lymphangiogenic response. The destrin-mutant *corn1* mouse therefore constitutes a novel mouse model to study the mechanisms of (especially this secondary, low-inflammatory form of) hemangiogenesis and especially lymphangiogenesis. Second, we provide novel evidence that anti-VEGFR3-blocking antibodies not only inhibit lymphangiogenesis but also hemangiogenesis. At least two implications may be drawn from this. First, that the apparent partial functional redundancy between proangiogenic and lymphangiogenic receptors (VEGFR1/-2 and VEGFR3, respectively) may in some cases limit total abrogation of the angiogenic response if only hemangiogenic receptors are targeted. Second, pharmacological anti-VEGFR3 strategies may prove important in a combined antihemangiogenic and antilymphangiogenic approach in which such a "dual track" may be highly desirable, as in cancer treatment.

corn1 mice are characterized by early, irregular thickening of the corneal epithelium, by development of stromal hemangiogenesis around week 3, by cataract formation around week 7,²² and—as shown in this study—by spontaneous lymphangiogenesis. A deletion in the gene encoding the cytoskeletal protein destrin²⁰ has been found to be causative for the *corn1* phenotype.²⁰ Destrin is an essential actin-regulatory protein belonging to the ADF/cofilin family.²⁰ The phenotype seems to be specific for the cornea, although destrin is widely expressed, eg, in epithelial cells.²⁰ The molecular mechanism(s) causing spontaneous hemangiogenesis and secondary lym-

Figure 4. Absence of the massive corneal inflammation (normally associated with inflammatory corneal neovascularization) in *corn1* mice developing spontaneous hem- and lymphangiogenesis compared with wild-type controls (and "positive" inflamed controls). **A:** Numbers of CD45⁺ cells per corneal section comparing wild-type controls (age, 4 weeks), *corn1* mice (age, 4 weeks), and experimentally vascularized corneas (positive control; inflammation-associated vascularization in corneas 14 days after mouse corneal transplantation).⁵ **B through D:** Representative segments of central cornea from inflamed vascularized corneas (**B**; positive control with numerous inflammatory cells; $\times 100$), 4-week-old *corn1* mice (**C**) and same-aged wild-type control (**D**) both showing absence of significant stromal inflammatory cell influx (H&E; magnification, $\times 100$). **E and F:** CD45 immunohistochemistry (red, with DAPI blue counterstaining of nuclei) demonstrates very few CD45⁺ cells in a wild-type cornea (**F**) compared with significantly more, yet still few, CD45⁺ cells in a representative *corn1* mouse cornea (**E**; magnification, $\times 100$).

Figure 5. Absent destrin immunoreactivity of limbal blood and lymphatic vessels in wild-type control mice. **A:** Positive immunoreactivity of limbal blood and lymphatic vessels with CD31/PECAM1 (magnification, $\times 100$; green). **B:** Absent immunoreactivity with destrin antibody at the same location (magnification, $\times 100$; red). **C:** Positive immunoreactivity of central corneal epithelium with the destrin antibody (magnification, $\times 100$; red; DSTN/CFN).

phangiogenesis in *corn1* mice remains unknown, but the absence of a massive inflammatory response in the cornea before either hem- or lymphangiogenesis and absent signs of blood-aqueous barrier breakdown are compatible with a noninflammatory cause for the spontaneous angiogenic response in *corn1* mice.^{27–30} Moreover, the fact that spontaneous lymphangiogenesis in *corn1* mice followed hemangiogenesis with a delay of 1 to 2 weeks argues against an inflammation-induced induction of both vascular processes, because in pathologies leading to recruitment of bone-marrow derived cells and especially macrophages that release both hem- and lymphangiogenic growth factors,^{17,26} a parallel outgrowth of both vessel types into the cornea is observed, with lymphatic vessels sometimes even preceding the outgrowth of blood vessels.¹⁷ The delayed onset of corneal lymphangiogenesis in *corn1* mice rather suggests a “secondary” form of lymphangiogenesis, potentially triggered by release of lymphangiogenic factors (eg, VEGF-C) by the advancing vascular endothelium.³¹ In that respect, spontaneous lymphangiogenesis in *corn1* mice parallels the “secondary” lymphangiogenesis following hemangiogenesis in wound healing scenarios.³² Nevertheless, it cannot be ruled out with certainty that the small but significant increase in primarily macrophage-type inflammatory cells in *corn1* mice accounts for the spontaneous angiogenesis and lymphangiogenesis in these mice. The delayed onset of lymphangiogenesis could also be caused by delayed build-up or altered composition of lymphangiogenic versus angiogenic growth factors released by inflammatory cells in this low-inflammatory context or due to different sensitivity thresholds of different growth factors that become apparent in this model because of the low number of inflammatory cells present potentially secreting growth factors. Because lymph- and hemangiogenesis into the normally alymphatic and avascular cornea can easily and reliably be quantified using flat-mount double-immunohistochemistry and image analysis software,¹⁷ we suggest the destrin-deleted *corn1* mouse as a novel model system to study the mechanisms of induction, regulation, maintenance, and regression of hemangiogenesis and especially lymphangiogenesis. This is especially relevant because the theoretically more specific knockout mice for the lymph-

phangiogenic growth factors VEGF-C and VEGF-D as well as their receptor VEGFR3 are not viable. Our finding that the normal (wild-type) limbal vasculature does not express destrin suggests that the angiogenic and lymphangiogenic effect of absence of destrin in *corn1* mice is an indirect one, not directly related to an effect of destrin on limbal blood or lymphatic vessels.

Figure 6. Absent differences in corneal mRNA levels of angiogenic growth factors of the VEGF family and VEGF receptor 3 (VEGFR3) between wild-type controls (WT; lane 1) and *corn1* mice (lane 2; **A** through **F**). **G** and **H:** Representative sections from central cornea demonstrate equal expression of VEGFR3 protein in corneas from wild-type controls (**G**; WT) and *corn1* mice (**H**). VEGFR3 is primarily expressed on corneal epithelial cells (**arrows**) and in the vascularized *corn1* cornea in addition to new stromal blood and lymphatic vessels (**arrowhead**; magnification, $\times 400$). Immunohistochemistry for VEGF-C, VEGF-D, and VEGF-A revealed no significant difference in expression between wild-type controls and *corn1* mice.

Figure 7. Systemic application of a neutralizing anti-VEGFR3 antibody (mF4-31C1)¹⁹ significantly inhibits spontaneous hemangiogenesis as well as lymphangiogenesis in the *corn1* mouse model. Whereas *corn1* mice receiving control IgG injections intraperitoneally at day 14 and 21 of age display a robust hem- and lymphangiogenesis at age 5 weeks (**A**: whole mount of cornea stained with LYVE-1 [red] and CD31 [green], magnification, $\times 40$; **C**: detail from **A**, magnification, $\times 100$), there is significant inhibition of hemangiogenesis and nearly complete inhibition of lymphangiogenesis in *corn1* mice that received anti-VEGFR3 antibodies at the same time points (**B**, **D**, and **E**: morphometrical analysis; Li, limbal vascular arcade).

Furthermore, our finding that there is no significant difference in the expression of the major lymphangiogenic growth factors VEGF-A, VEGF-C, and VEGF-D as

well as their VEGF receptor 3 between *corn1* mice and wild-type controls suggests that the spontaneous angiogenesis and lymphangiogenesis in *corn1* mice are

not related to alterations in the expressional pattern of growth factors of the VEGF family. Therefore, either up-regulation of other (potentially yet unknown) angiogenic and lymphangiogenic growth factors or a deficient release of antiangiogenic inhibitors at the epithelial-basement junction (such as angiostatin and endostatin) may be responsible for the low-inflammatory induction of angiogenesis and lymphangiogenesis in *corn1* mice.

Because the receptor for the hem- and lymphangiogenic growth factors VEGF-C and VEGF-D (VEGFR3) in the adult becomes largely restricted to lymphatic vascular endothelium,³³ it has been shown that pharmacological strategies blocking VEGFR3 signaling are potent inhibitors of lymphangiogenesis.^{18,19} However, VEGFR3 is also up-regulated on some proliferating blood vascular endothelia at least in tumor vessels,²⁴ and VEGFR3 signaling has been implicated in early maintenance of proliferating blood vessels.³⁴ Hence the question arose of whether anti-VEGFR3 strategies may also inhibit hemangiogenesis? We show herein, using this novel model of spontaneous corneal hem- and lymphangiogenesis in *corn1* mice, that anti-VEGFR3-blocking antibodies can profoundly suppress lymph- and hemangiogenesis. We cannot definitely comment on why different anti-VEGFR3 strategies used in two previous reports could not document an effect on hemangiogenesis. This may be related to different methods of quantification of hemangiogenesis, different antibodies used, different mouse strains, and dosage regimens.^{18,35} We conclude, however, that the blocking antibody to the mouse VEGFR3 used in our study is a promising tool for investigating a combined antihem- and antilymphangiogenic approach in animal disease models. A similar antibody that blocks the human VEGFR-3 has been developed and may represent an important new therapeutic in oncology or transplantation applications.¹⁹

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