

The Progression of Inflammation Parallels the Dermal Angiogenesis in a Keratin 14 IL-4-Transgenic Model of Atopic Dermatitis

LIN CHEN,* DEBORAH J. MARBLE,* RANIA AGHA,*
JENNIFER D. PETERSON,* ROBERT P. BECKER,# TIANQUAN JIN,§ JIANXUN LI,§
AND LAWRENCE S. CHAN*†,¶

Departments of *Dermatology and †Microbiology/Immunology, #Anatomy and Cell Biology, and
§Oral Biology, University of Illinois at Chicago, Chicago, Illinois, USA; and
Department of ¶Medicine Service, VA Jesse Brown Medical Center, Chicago, Illinois, USA

ABSTRACT

The role angiogenesis plays in atopic dermatitis is not well understood. The authors previously demonstrated ultrastructurally dermal microvascular angiogenesis in the IL-4-transgenic mouse model of atopic dermatitis. Here, they determine the angiogenic factors involved in dermal microvascular angiogenesis, regulatory function of inflammatory cytokines on the VEGF-A production, and microvascular permeability in this model. Computer-assisted photometric analyses for immunofluorescence-labeled CD31 demonstrated a progressive increase in blood vessel number, diameter, and percent dermal areas occupied by CD31⁺ vessels as the disease evolves in transgenic mice from before disease onset through early and late skin lesions. Similar findings were documented for VEGFR2⁺ vessels. Quantification of skin angiogenic factor mRNAs showed progressive increase of transcripts of VEGF-A, but not VEGF-B, VEGF-C, or VEGF-D. ELISA showed a similar increase of VEGF-A in the serum and skin of transgenic mice. IL-6 and IFN- γ stimulated VEGF-A mRNA production in the skin and in primary keratinocytes of transgenic mice. Other skin angiogenic factors that increased included Ang-1, Ang-2, GBP-1, and VE-cadherin. Microvascular leakage began in the transgenic mouse skin before disease onset and peaked in the late stage. In conclusion, IL-6 and IFN- γ may play important roles in upregulation of VEGF-A, along with other pro-angiogenic factors, to induce dermal microvascular angiogenesis. *Microcirculation* (2008) **15**, 49–64. doi:10.1080/10739680701418416

KEY WORDS: angiogenesis, animal model, atopic dermatitis, inflammation, VEGF

Angiogenesis has been substantially studied in cancer, as it is necessary for invasive tumor proliferation and metastasis (1). The anti-VEGF monoclonal antibody Bevacizumab, when combined with standard chemotherapy regimens, has been associated with significant improvements in patients with metastatic colorectal cancer, compared with chemotherapy alone. Bevacizumab also has promising effects as a single agent or as part of combina-

tion regimens for breast cancer, non-small-cell lung cancer, renal cell carcinoma, and other solid malignancies (2). Multitargeted kinase inhibitors sorafenib and sunitinib, which inhibit VEGF receptors 1, 2, and 3 and platelet-derived growth factor receptor, have shown promising clinical effect in treating renal cell carcinoma (3–5). Recently, angiogenesis was found to be essential in the pathophysiology of many inflammatory diseases, such as psoriasis, asthma, inflammatory bowel disease, and rheumatoid arthritis (6). In fact, anti-angiogenic treatments by various reagents, including anti-vascular endothelial growth factor (VEGF) agents, cyclooxygenase (COX-2) inhibitors, thalidomide, and anti-TNF- α (infliximab) have resulted in outstanding clinical improvement of rheumatoid arthritis, psoriasis, and inflammatory bowel disease (7–14).

One of the most common inflammatory skin diseases, atopic dermatitis (AD), is characterized by chronic itchy skin rashes and dermal and epidermal

This work is supported in part by NIH grants R01 AR47667, R03 AR47634, and R21 AR48438 to L.S.C., and Albert H. and Mary Jane Slepian Fellowship Fund to R.A.M. and L.C. We thank Kathleen Green, Lisa M. Godsel, and Kaihua Wang (Department of Pathology, Northwestern University Feinberg Medical School) for their help with primary keratinocyte culture.

Address correspondence to Lawrence S. Chan, MD, UIC-Dermatology, MC624, 808 S. Wood Street, Room 376, Chicago, IL 60612, USA. E-mail: larrycha@uic.edu

Received 25 April 2007; accepted 25 April 2007.

infiltration of inflammatory cells, including T cells, mast cells, eosinophils, and macrophages (15). Current treatments for AD focus on anti-inflammatory methodology, with topical steroids and other anti-inflammatory drugs being the mainstay of therapy (16). The role of dermal blood vessels, which are the conduit in which the inflammatory cells migrate from peripheral blood to the sites of inflammation in the skin, has not been explored. The epidermal interleukin-4-transgenic (IL-4-Tg) mouse model of AD has been a valuable model to investigate how inflammation and angiogenesis are intertwined (17). We have previously established this animal model, which closely resembles human AD clinically, histopathologically, serologically, bacteriologically, and immunologically and fulfills the diagnostic criteria for human AD (18). Further studies on this model documented the progressive increase of T-cell infiltration to the skin, T-cell surface activation molecules, T-cell proliferation in response to mitogens or superantigens (19), B-cell surface activation molecules, B-cell proliferation in response to LPS, B-cell surface IgE, total serum IgE (20), and early upregulation of Th2 type skin cytokines followed the late surge of Th1 type skin cytokines (21). Interestingly the cytokine mRNAs including IL-4, IL-5, IL-6, IL-13, IFN- γ , IL-1 β , and TNF- α were significantly upregulated in the Tg mice before disease onset (21).

The ultrastructure of the dermal blood vessels in the IL-4-Tg mouse model of AD showed changes consistent with angiogenesis. Using transmission electron microscopy, we showed that the tight junctions in the newly formed (angiogenic) blood vessels were significantly elongated, a characteristic resulting from the way that endothelial cells adhere to each other in newly formed blood vessels. In addition, transmission electron microscopy showed that newly formed blood vessels were characterized by thick endothelial cells with prominent nuclei and nucleoli, as well as abundant free ribosomes and rough endoplasmic reticulum, a finding consistent with a high metabolic state, which is consistent with angiogenic activity. Scanning electron microscopy also confirmed our findings of angiogenesis. Furthermore, sprouting and intussusception, two well-recognized surface morphological changes observed in angiogenic blood vessels, were visualized throughout the microscopic field. (17). Thus, this study strongly suggests that angiogenesis is a major change in the dermal vasculature in the inflammatory skin of IL-4 Tg mice. In addition, one study in human AD patients showed that VEGF-A protein was significantly increased in the stratum

corneum of the lesional skin (22), which further supports that angiogenesis may play an important role in the pathophysiology of AD.

In this paper, we extend our study on angiogenesis in our IL-4-Tg mouse model of AD by conducting a quantitative analysis of dermal blood vessels and by investigating possible molecular mechanisms underlying the angiogenesis process. We performed quantitative analysis of angiogenic factor mRNAs to gain insight into the underlying molecular mechanism, specifically to evaluate for the presence or upregulation of certain angiogenic factors such as VEGF, its receptor VEGFR2, angiopoietin-1 and -2 (Ang 1 and 2), guanylate-binding protein (GBP-1), and endothelial cell adhesion molecules vascular/endothelial-cadherin (VE-cad) and platelet/endothelial cell adhesion molecule (PECAM-1, CD31). Results showed that there was a significant and progressive increase of dermal vasculature (CD31⁺) and angiogenic factors as the disease evolved. Inflammatory cytokines, including IL-6 and IFN- γ that were upregulated in the Tg mouse skin before disease onset significantly increased the production of VEGF-A by primary cultured keratinocytes, the major skin cell type producing VEGF-A. In addition, in vivo study by Evans blue injection demonstrated a microvascular leakage in the skin lesions of Tg mice, a hallmark of angiogenesis. Therefore, the data from the present study indicate that there is a strong correlation of the inflammatory skin disease progression with dermal microvascular angiogenesis and upregulation of pro-angiogenic factors and suggest an initiating role of IL-6 and IFN- γ in the dermal angiogenesis.

MATERIALS AND METHODS

The Animals

The animals used in this study are keratin-14 IL-4 Tg mice and their nontransgenic littermates, as reported in previous publications (17–21). The study complied with the Animal Care Policies and Procedures of the University of Illinois at Chicago.

Genotyping

Mice born from heterozygous IL-4-Tg mouse parents were genotyped for confirmation of their positive transgenic status as described previously (17–21). We have previously determined that clinical disease developed in all (100%) Tg mice (21).

Disease Phenotype Classification

Skin lesions from IL-4 Tg mice developed for the duration of 1 week or shorter are defined as early lesions (Tg-EL). Skin lesions developed for the duration of 3 weeks or longer are defined as late lesions (Tg-LL). IL-4-Tg mice from birth until any sign of clinical disease were defined as before disease onset (Tg-BO) (17, 19–21).

Immunofluorescence Microscopy and Computer-Assisted Photometric Analysis

Tissue samples were collected exclusively from the mouse ears to reduce regional blood vessel variability. Samples collected were immediately fixed in 0.2% paraformaldehyde, followed by incubation in a 30% sucrose solution in PBS, and then embedded at -20°C in Tissue-Tek OCT compound (Fintek, Torrance, CA) and $8\text{-}\mu\text{m}$ sections of the tissue were prepared in a Leica CM1850 cryostat (Bannockburn, IL). Each slide was sequentially incubated with monoclonal rat anti-mouse CD31 (BD Biosciences,) or rat anti-mouse VEGFR2 (eBiosciences, San Diego, CA). Slides were incubated with Alexa Fluor488 goat anti-rat IgG (Invitrogen, Carlsbad, CA) for 30 min. Slides were then washed and sections were mounted with glycerol mounting medium (glycerol:PBS; 1:1; vol:vol). All procedures were performed at room temperature.

Slides were examined with a $20\times$ objective lens using a Zeiss Axiovert 100 fluorescence microscope and 5 areas in each slide were identified and photographed with a SensiCam CCD camera (Cooke Corporation, Orlando, FL). Samples were oriented in a consistent fashion, horizontally across field, capturing dermis and epidermis of one side of the mouse ear. Slides were analyzed using a computer-assisted photometric analysis program written in house by Tiaquin Jin, which used an objective micrometer as the scale standard. For measuring the diameter of blood vessels, the shorter of the two outside dimensions of the center of stained vessels (usually elongated) were determined manually by two point clicking, followed by computerized calculation. Vessel number was recorded simultaneously and later reported as number per linear width of the field. The areas of blood vessels were automatically determined by the program after recognition of fluorescence. Fluorescent contaminants were removed manually before final area was calculated. The program then gave total area of the positive staining vessels per photo, which was later divided by the total area of the skin

sample in the photo to a percent area occupied by vessels.

Primary Keratinocyte Cell Culture

For primary keratinocyte cell culture, neonatal mouse pups of both IL-4-Tg mice and non-Tg mice between 3 and 4 days postpartum were used. After the mice were sacrificed, the pelts from the pups were placed dermis-side down into a 60-mm tissue culture dish containing 3 mL of dispase (Roche, Indianapolis, IN, 10 mg/mL in keratinocyte culture medium) and incubated overnight at 4°C . The epidermis was separated from the dermis and transferred to another tissue culture dish containing trypsin versene and incubated for 15 min at 37°C . The trypsinized epidermal sheets were then transferred to a pre-scratched dish (to provide rougher surface optimal for cell release) containing 2 mL FBS and rubbed back and forth for 30 s. The released keratinocytes were then collected and washed with PBS. Finally, the cells were resuspended in KGM-2 keratinocyte media (Cambrex Bio Science, Walkersville, MD) with 50 mM CaCl_2 and placed onto rat tail collagen I-coated 75 cm^2 flasks. When cells were 70–80% confluent they were subcultured into 24-well collagen I-coated plates. When cells had again reached 70–80% confluence, cytokines, including IL-1 β (20 ng/mL), IL-6 (20 ng/mL), IFN- γ (30 ng/mL), and TNF- α (20 ng/mL), were added. Then cells were collected 8 h after culture for total RNA extraction and culture media were collected at 48 and 72 h for VEGF ELISA. The tails from pups were genotyped as described above.

Western Blot Analysis

Mouse ears were collected and ear samples from 5 mice in each group (NT, BO, EL, and LL) were pooled together for protein extraction. The ears were then cut into small pieces in tissue lysis buffer containing 20 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 0.1% SDS (pH 7.5) with a protease inhibitor cocktail for mammalian cells (Sigma, St. Louis, MO). Tissues were then homogenized and centrifuged for 20 min at 13,000 rpm at 4°C . Supernatants were collected and concentrations of proteins were quantified using protein microassay (Bio-Rad, Hercules, CA). The protein concentrations in all samples were diluted to 5 mg/mL. Skin protein extracts were mixed with the same volume of $2\times$ SDS loading buffer (Invitrogen), then 2-mercaptoethanol (5% of final concentration)

was added. The samples were boiled for 5 min before loading. Then 125- μ g proteins from each sample group and molecular weight marker (Invitrogen) were loaded in a 4–20% Tris–glycine acrylamide gel (Invitrogen). Separated proteins by SDS-PAGE were then transferred to nitrocellulose membrane. Membrane was immunoreacted using Odyssey (Li-Cor Biosciences, Lincoln, NE) Western blot detection protocol. Briefly, the membrane was first blocked with a blocking buffer (Li-Cor). A polyclonal goat anti-mouse GBP-1 (2 μ g/mL, Santa Cruz, CA) or a polyclonal rabbit anti-mouse Ang-1 (170 μ g/mL, Abcam, Cambridge, UK) was applied to the membrane for 1 h at room temperature followed by incubation of a 1:5000 dilution of Alexa Fluor 680-labeled goat anti-rabbit IgG, or rabbit anti-goat IgG secondary antibodies (Invitrogen). Images were acquired and analyzed using an Odyssey infrared scanner and its software (Li-Cor). The band intensities (pixels/mm²) in the NT group were used as background. The intensities of other groups (BO, EL, and LL) were obtained by subtracting the background pixel from the pixel values of these groups.

ELISA

The concentrations of VEGF protein in undiluted primary IL-4 Tg mice keratinocyte culture medium, 10-fold diluted mouse sera, and skin protein extracts (200 μ g/sample) from different groups of mice prepared above were determined using Quantikine mouse VEGF kit (R&D Systems, Minneapolis, MN), which detects mouse VEGF-A isoforms 120 and 164. All standards, controls, and samples were done in duplicate and the assay was performed according to the

manufacturer's directions. The plates were read by a Bio-Tek μ Quant ELISA reader (Winooski, VT). The lower detection limit was 6.3 pg/mL. IL-6 and IFN- γ concentrations in the skin protein extracts prepared in the section of Western blot analysis were determined by commercially available ELISA kits (eBioscience) according to manufacturer's instructions and 100 μ L (500- μ g proteins) of each diluted sample was used in the assay. These ELISA kits have a lowest detection limit of 4 pg/mL (for both IL-6 and IFN- γ).

Quantification of Angiogenic Factor mRNAs by Real-Time PCR

Skin samples taken from mouse ears were immediately placed in a RNA-preserving medium *RNAlater* (Ambien, Austin, TX) and kept at -20°C before they were used to analyze the mRNA levels of VEGF-A120,164, and 188, VEGF-B, VEGF-C, VEGF-D, VEGFR1, VEGFR2, VEGFR3, CD31, VE-cadherin, angiopoietin-1, angiopoietin-2, Tie-2, and GBP-1. Skin samples for total RNA extraction were obtained from NT, Tg-BO, Tg-EL, and Tg-LL mice ($n = 8$ in each group). Total RNA was extracted using *TRIzol* (Invitrogen) and treated with DNase (Invitrogen). One microgram total RNA of each sample was reverse transcribed to cDNA using a Retroscript Kit (Ambion). The sequences of primers for amplifying these factors are listed in Table 1. Specifically, the sequences for CD31, VE-Cad, angiopoietin-1, angiopoietin-2, and Tie-2 were obtained from a previous report (23). Sequences for VEGF-A (including isoforms 120, 164, and 188) were obtained from another reference (24). Sequences for VEGFR1 (flt-1) and VEGFR2 (flk-1) were obtained from

Table 1. Primer sequences and references

	Forward (5'–3')	Reverse (5'–3')	Reference
VEGF-A121	TGCAGGCTGCTGTAACGATG	CCTCGGCTTGTCACATTTTCT	24
VEGF-A165	TGCAGGCTGCTGTAACGATG	GAACAAGGCTCACAGTGATTTTCT	24
VEGF-A189	TGCAGGCTGCTGTAACGATG	CTCCAGGATTTAAACCGGGATT	24
VEGF-B	TGCCATGGATAGACGTTTATGC	TGCTCAGAGGCACCACCAC	24
VEGF-C	AAGACCGTGTGCGAATCGA	ACACAGCGGCATACTTCTTCAC	24
VEGF-D	GCTGCTGAATGAGATCTCCC	GCAAGACGAGACTCCACTGC	26
VEGF-R1	GAGGAGGATGAGGGTGTCTATAGGT	GTGATCAGCTCCAGGTTTGACTT	25
VEGF-R2	GCCCTGCCTGTGGTCTCACTAC	CAAAGCATTGCCCATTCGAT	25
VEGF-R3	GTTTCCTGATGGGCAAAGG	TCAGTGGGCTCAGCCATAGG	24
PECAM (CD31)	GAGCCCAATCACGTTTCAGTTT	TCCTTCCTGCTTCTTGCTAGCT	23
Angiopoietin-1	CATTCTTCGCTGCCATTCTG	GCACATTGCCCATGTTGAATC	23
Angiopoietin-2	TTATGCACAAAGGATTCGGACAAT	TTTTGTGGCTAGTACTGTCCATTG	23
Tie-2	ATGTGGAAGTCGAGAGGCGAT	CGAATAGCCATCCACTATTGTCC	23
VE-cadherin	TCCTCTGCATCCTCACTATCACA	GTAAGTGACCAACTGCTCGTGAAT	23
GBP-1	ATATGCCTCGCCTGTGTA	TCAGCTGGTCCCTCCTGTA	
GAPDH	TCACCACCATGGAGAAGGC	GCTAAGCAGTTGGTGCTGCA	27

reference (25). Sequences for VEGF-B, VEGF-C, and VEGFR3 (flt-4) were obtained from reference (24), and those for VEGF-D were cited from (26), GAPDH primers were published in reference (27). For real-time PCR, we used SYBR green supermix (Bio-Rad) with target gene specific primers and 25-ng cDNA templates of individual sample. Total reaction volume was 25 μ L. For all targets, identical thermal cycling conditions were used: 15 s at 95°C and 30 s at 60°C with a total of 40 cycles followed by a melting curve data collection set up to identify the nonspecific products or primer-dimers. Real-time PCR was performed in an MX3000P real-time machine (Stratagene, La Jolla, CA) in duplicate wells. Relative quantification of these molecules was obtained by the PCR values of the targets calculated by a factor normalized to the values of the respective NT mice GAPDH using MX3000p software, expressing in folds of increase over that of the NT mouse samples. VEGF-A120 mRNA expression in keratinocytes isolated from NT and IL-4 Tg mice 8 h after cytokine stimulation as described above was also examined by real-time PCR.

In Vivo Pro-Angiogenic Function of IL-6 and IFN- γ

To examine if administration of IL-6 and IFN- γ to the skin can induce VEGF-A production, we intradermally injected recombinant mouse IL-6 (150 ng) and IFN- γ (150 ng) in PBS on the shaved abdomen of 4- to 5-week-old IL-4 Tg-BO mice. Control mice received PBS in the same volume. Twelve hours later, the skin of the injected site was collected and the total RNA was extracted. After reverse transcription, semi-quantitative real-time PCR was performed to detect mRNA of VEGF-A 120 as described above.

In Vivo Blood Vessel Leakage Study

To study the permeability of dermavascultures to Evans blue, we used a protocol published by Hulthen et al. (28) with some modifications. Briefly, NT, Tg-BO, Tg-EL, and Tg-LL mice ($N = 5$ in each group) were anesthetized by intraperitoneal injection of ketamine 100 mg/kg. After the mice were fully anesthetized, an incision over the left thigh was made to expose the left saphenous vein. Then 0.3-mL 1% Evans blue (Sigma, St. Louis, MO) in normal saline was injected slowly in the saphenous vein. Five minutes postinjection, images of ears were taken. The animals were then sacrificed and the ears were excised. Identical weights (51 mg) of lesional or nonlesional ears from each mouse were placed in pure formamide,

dissected, and homogenized. The supernatants were collected after a 5-min centrifugation at 5000 rpm. The ODs at 620 nm were read using the same plate reader mentioned above.

Statistical Analysis

Statistical analyses were performed using the Student *t* test with GraphPad InStat (GraphPad software, San Diego, CA). All experimental data were expressed as means $\pm$ SD. *p* value $\leq .05$ was considered to be significantly different.

RESULTS

The Number of Vessels, Average Vessel Diameter, and Percent Dermis Occupied by Vessels Increased in Tg Mice Dermis with Skin Disease

To investigate whether there is an increase of microvasculature in this AD model, ear skin samples were taken from IL-4 Tg mice, which were subsequently immunostained with anti-CD31 to identify endothelial cells of blood vessels. Microvascular architecture and number differed significantly among the four groups. The vessels in the Tg mouse groups (BO, before disease onset; EL, early lesion; and LL, late lesion) appeared to be larger, more numerous, and more dilated, when compared to those of the NT (non-Tg) group (Figure 1a). Statistical analyses of data obtained from computer-assisted photometric analysis showed significant increases in vessel number per mm skin length (Figure 1b.1), average diameter of blood vessels (Figure 1b.2), and total percentage of dermal areas occupied by blood vessels in both the EL and LL groups (Figure 1b.3) compared to the NT group. In addition, the average vessel diameter in the BO group is also significantly larger than that of the NT group (Figure 1b.2). The percentage of dermal areas occupied by vessels in the LL group was significantly higher than that of the EL group (Figure 1b.3), while the average diameter of vessels in the LL group showed significantly greater reduction than that of the EL group (Figure 1b.2). Consistent with the above result, the mRNA expression of CD31 in the skin examined by RT real-time PCR (Figure 1c) increased as the disease progressed.

Endothelial cells possess VEGF receptors on their surfaces and they function to receive signals from VEGF and through signal transduction initiate cell migration, specialization, and proliferation of endothelial cells (29). The influence of VEGF on endothelial cells via VEGF receptors has been shown

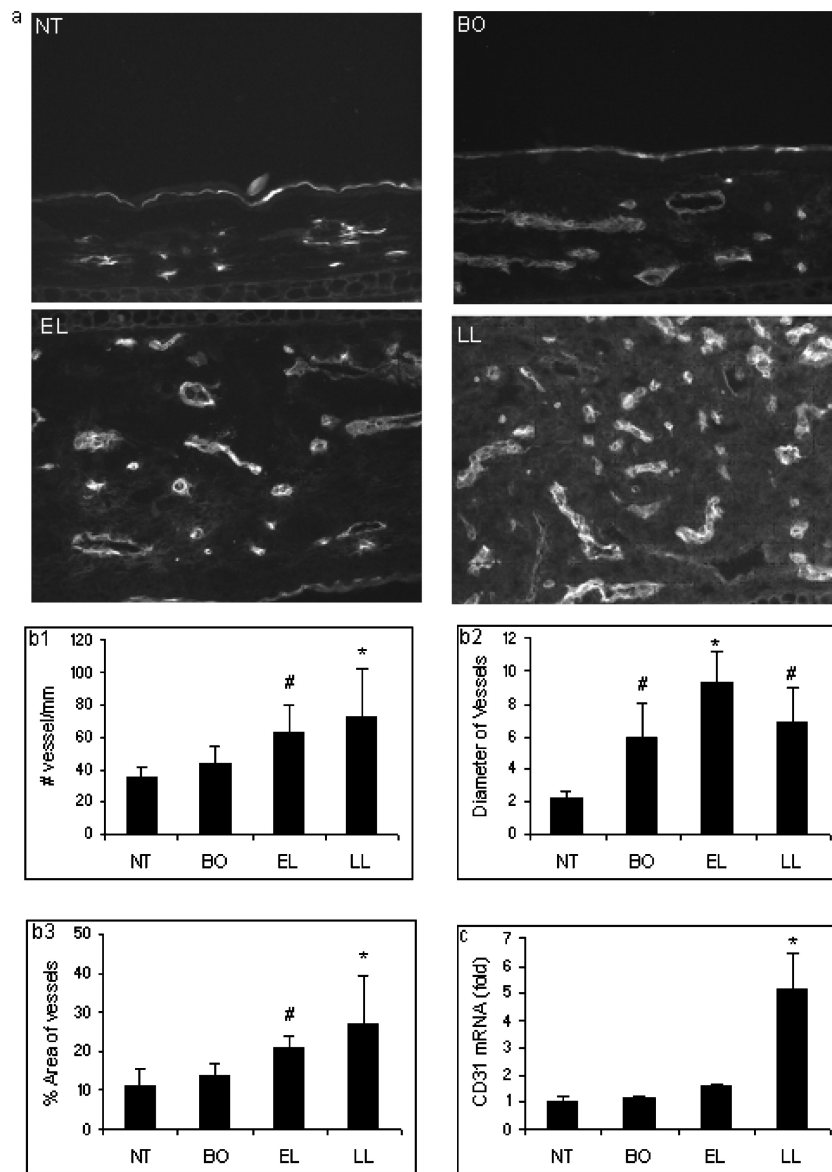


Figure 1. The increases of CD31 and VEGFR2 expressions parallel the progression of skin inflammation. (a) The dermis of Tg mouse skin showed increased microvasculatures. Frozen sections stained with rat anti-mouse CD31, followed by Alexa Fluor 488-labeled goat anti-rat IgG. (b) Quantification of vessel parameters using immunofluorescence microscopy and computer-assisted photometric analysis reveal a progressively increased number of vessels/mm skin length (b.1), increased average diameter of vessels (b.2), and percentage of skin area occupied by vessels (b.3) as the disease evolves. [$*p < .001$, statistical significance vs. NT, $\#p < .05$, statistical significance vs. NT, other statistical significances include increase in number of vessels (b.1): LL vs. BO $p < .001$, EL vs. BO $p < .05$; increase in diameter of vessels (b.2): EL vs. BO $p < .001$, LL vs. EL $p < .01$; increase in area of vessels (b.3): EL vs. BO $p < .05$, LL vs. EL $p < .05$, LL vs. BO $p < .001$.] (c) CD31 mRNA expressed as fold of increase compared to NT group (with value of 1) examined by a semi-quantitative RT real-time PCR in the skin. $N = 8$ in each group. [$*p < .001$, statistical significance vs. NT]. (d) Increased dermal blood vessel expression of VEGFR2 in dermis of Tg mouse skin. Frozen sections of mouse ear samples were stained with rat anti-mouse VEGFR2, followed by Alexa Fluor 488-labeled goat anti-rat IgG. (e) Quantification of VEGFR2 expression using immunofluorescence microscopy and computer-assisted photometric analysis reveals increased number of VEGFR2 positive vessels/mm skin (e.1) and increased percent area of VEGFR2 expression (e.2). [$*p < .001$, $\#p < .05$, statistical significance vs. NT; other statistical significances include increase in VEGFR2 positive vessels (e.1): LL vs. BO $p < .001$, LL vs. EL $p < .05$, EL vs. BO $p < .05$; increase in percent area of VEGFR2 (e.2): EL vs. BO $p < .01$, LL vs. EL $p < .01$]. (f) VEGFR2 mRNA expressed as fold of increase compared to NT group (with value of 1) examined by a semi-quantitative RT real-time PCR in the skin. $N = 8$ in each group. [$*p < .001$, $\#p < .05$, statistical significance vs. NT.] (Continued)

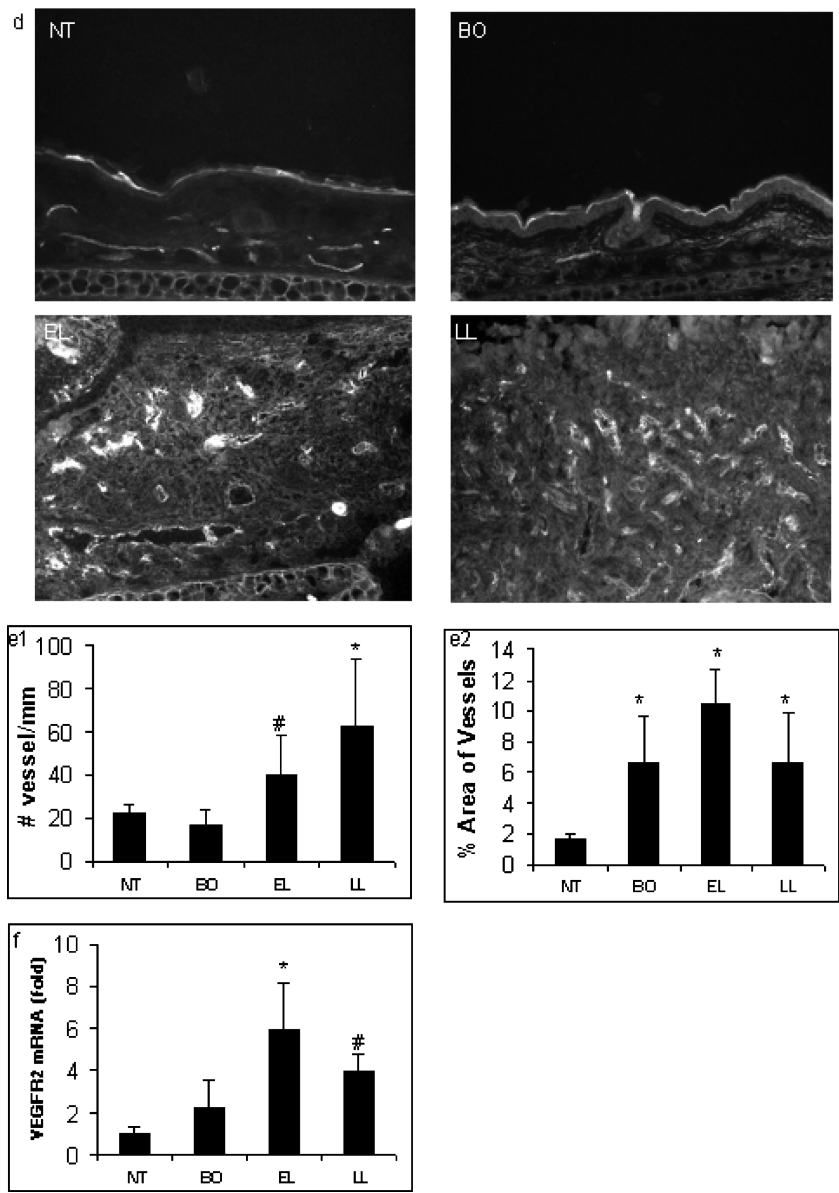


Figure 1. Continued

to cause blood vessel leakiness, enlargement, and sprouting (30). We therefore next asked whether the expression of such receptor was also upregulated in the inflamed skin. As depicted in Figure 1d, the alteration of microvascular architecture as identified by anti-VEGFR2 antibodies and the increase of VEGFR2⁺ blood vessels appear to be similar to that identified by anti-CD31 antibodies (Figure 1d). Analyses of data obtained from computer-assisted photometric analysis revealed that the number of VEGFR2⁺ blood vessels per millimeter skin (Figure 1e.1) and the total percentage of dermal area occu-

pied by VEGFR2 expression (Figure 1e.2) were significantly increased in the EL and LL groups, when compared to that of the NT group. Furthermore, the percent dermis occupied by VEGFR2 expression was also significantly increased in the BO group compared to the NT group (Figure 1e.2). Correlated with the blood vessel VEGFR2 expression, the mRNA expression of VEGFR2 in the skin determined by RT real-time PCR began to increase in BO stage and continued to increase and peak at EL, and then slightly decreased at LL stage compared to EL stage (Figure 1f).

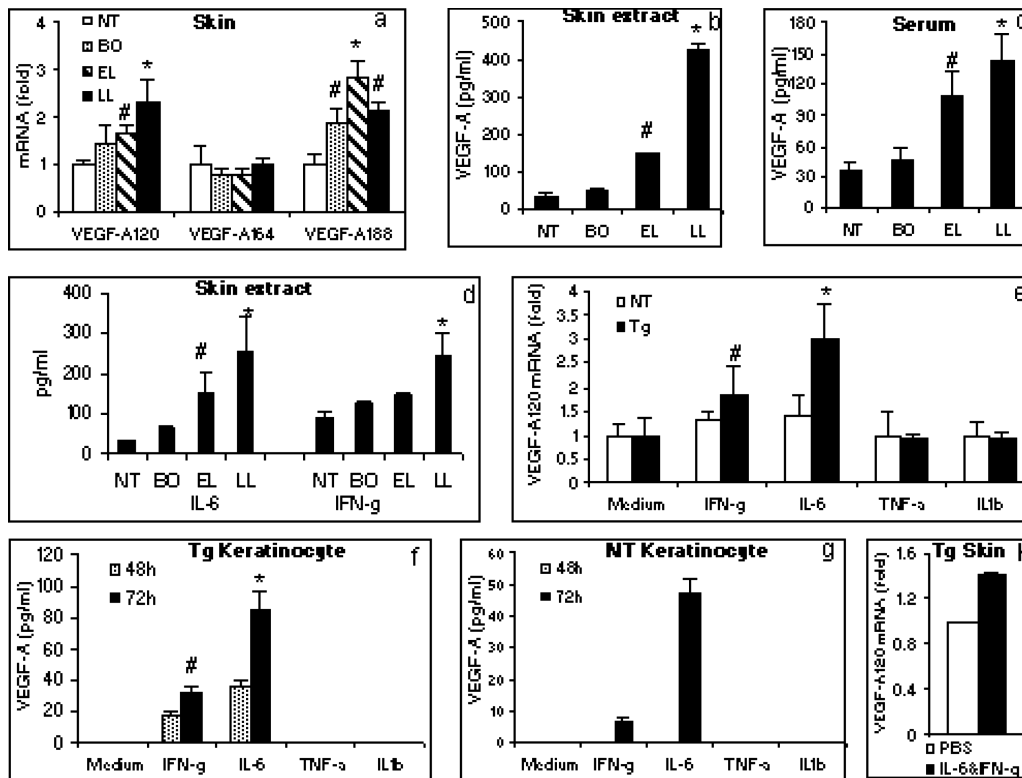


Figure 2. Progressive elevation of VEGF-A as the disease evolves in IL-4 Tg mice and the upregulation of VEGF-A in keratinocytes by cytokine stimulation. (a) Skin mRNA levels of VEGF-A isoforms 120, 164, and 188 analyzed by semi-quantitative RT real-time PCR ($N = 8$ in each group). (b) VEGF-A protein in the skin protein extracts pooled from 5 mice for each group analyzed by ELISA. $*p < .001$, $\#p < 0.05$, statistical significance vs. NT. (c) Serum VEGF-A protein levels quantified by ELISA. $N = 15$ in each group. $*p < .001$, $\#p < .05$, statistical significance vs. NT. (d) IFN- γ and IL-6 in the skin protein extracts pooled from 5 mice for each group. The concentrations of IFN- γ and IL-6 in the skin extracts of NT, BO, EL, and LL mice were determined by ELISA. $*p < .01$, $\#p < .05$, statistical significance vs. NT. (e) NT and IL-4-Tg mouse cultured primary keratinocyte VEGF-A 120 isoform mRNA expression examined by semi-quantitative RT real-time PCR 8 h after IFN- γ , IL-6, TNF- α , and IL-1 β stimulation. $*p < .01$, $\#p < .05$ compared to keratinocytes of Tg mice treated with medium only. IL-4-Tg mouse (f) and NT mouse (g) primary cultured keratinocyte VEGF-A protein secretion in the culture media examined by ELISA 48 and 72 h after IFN- γ , IL-6, TNF- α , and IL-1 β stimulation. (h) Intradermal administration of IL-4 Tg mice with IL-6 and IFN- γ induces upregulation of VEGF-A 120 mRNA. The values for Figure 2e–h were the averages of duplicate wells. Results with similar trends were obtained in another experiment.

VEGF-A in Tg Mice with Skin Disease Increases as the Disease Progresses and Is Upregulated by Inflammatory Cytokines

VEGF-A is a major pro-angiogenic factor that stimulates proliferation and migration of endothelial cells (31) and serum VEGF-A elevation has been associated with angiogenesis (32, 33). In addition, it is now known that skin epidermal cells are the major producers of VEGF-A (34, 35). Therefore, it would be logical to examine its presence in our model system. Using RT real-time PCR, we first examined mRNA levels of three isoforms of VEGF-A (VEGF-A120, VEGF-A164, and VEGF-A188) in the skin as a result

of alternative splicing (24). As shown in Figure 2a, mRNAs of VEGF-A120 and 188 in the IL-4 Tg mice especially in EL and LL stages significantly increased in the skin as compared with NT mice. However, there was no increase of VEGF-A164 isoform mRNA expression. Then we further asked whether VEGF-A protein level was also upregulated in the skin. ELISA analysis on whole skin protein extracts demonstrated that the concentrations of VEGF-A in the lesional skin (EL and LL stages) significantly increased compared to those in NT and BO skin. Such increase was not observed in the BO and NT groups (Figure 2b). Similar results were obtained in the serum levels of

VEGF-A (Figure 2c). Therefore, the levels of VEGF-A in the skin and serum were correlated as the skin inflammation progressed.

A better way to understand the possible initiation role of inflammatory cytokines in upregulation of VEGF in keratinocytes in our mouse model would be to examine VEGF production by primary keratinocytes of NT and IL-4 Tg mice co-cultured in the presence of these inflammatory cytokines. Therefore, the primary candidate cytokines would include IL-1 β , IL-6, TNF- α , and IFN- γ , since the mRNAs of these four cytokines were significantly increased in the BO skin documented by our previous studies (21). Furthermore, we demonstrated that the proteins of IL-1 β and TNF- α were upregulated in the BO skin (36). To further confirm the elevation of IL-6 and IFN- γ in the skin of IL-4 Tg mice at protein level, their concentrations in the skin protein extracts were determined by ELISA. Results showed that the protein levels of IL-6 in BO, EL, and LL skin extracts were elevated, especially in EL and LL skin lesions, which had significantly higher IL-6 concentrations than that of NT skin (Figure 2d). Similar results were obtained in the IFN- γ experiment (Figure 2d). When primary keratinocytes were co-cultured with these cytokines, we found that IL-6 and IFN- γ , but not IL-1 β or TNF- α , induced the increase of mRNA expression of VEGF-A120 after 8 h culture in Tg mice, but not in NT mice (Figure 2e). The protein secretion of VEGF-A in the culture media was also increased after 48 h culture and further increased at 72 h (Figure 2f) after IL-6 and IFN- γ stimulation in Tg mice. Keratinocytes from NT mice produced a smaller amount of VEGF-A after 72 h stimulation by IFN- γ and IL-6 than that of Tg mice (Figure 2g). The VEGF-A production by NT keratinocytes at 48 h was not detectable (Figure 2g). To further confirm that IL-6 and IFN- γ indeed induce VEGF-A in IL-4 Tg mice in vivo, we administered recombinant murine IL-6 and IFN- γ to the Tg mice intradermally for 12 h and found that VEGF-A 120 mRNA was upregulated by the treatment, compared to the PBS control group (Figure 2h).

Marked Elevation of Angiogenic Factors VE-Cadherin, Ang-1, Ang-2, and GBP-1 in Lesional Skin

Besides VEGF, other pro-angiogenic factors, including VE-Cad, Ang-1, and Tie-2, have been recently defined and their functional roles identified (23). Using the RT real-time PCR semi-quantitative method,

we found that there were significantly increased mRNA expressions of VE-Cad and Ang-1 in diseased mice especially in LL stage (Figure 3a). The significantly increased levels of Ang-1 were confirmed by Western blot (Figure 4b). There was a slight increase of VEGFR1 mRNA in Tg-EL and Tg-LL mice, but statistical significance as compared to NT was not observed (Figure 3a). Similarly, there was a small but not significant increase of Tie-2 in Tg-BO and Tg-EL mice as compared to NT mice (Figure 3a). No increase of mRNA expression was noted in VEGF-B, VEGF-D, and VEGFR3 in IL-4 Tg mice, as compared to NT mice (Figure 3a). VEGF-C mRNA was not detected in NT, BO, EL, or LL mice skin (data not shown).

We further investigated the presence of possible anti-angiogenic molecules including Ang-2 (37) and GBP-1 (38, 39) in our mouse model system to see if there was a negative feedback against the strong pro-angiogenic factors in the inflammatory skin. RT real-time PCR demonstrated that the GBP-1 mRNA expression in the skin was slightly increased in the EL group and highly elevated in the LL group when compared to NT mice (Figure 3a). Increased protein levels of GBP-1 detected by Western blot in the skin extracts of EL and LL stages of AD in IL-4 Tg mice confirmed the result of PCR (Figure 3c). Ang-2 mRNA was also found significantly increased in LL skin lesions (Figure 3a).

Dermal Vascular Permeability Increased in IL-4 Tg Mice

To examine if there was an alteration of dermavascular permeability, a marker of angiogenesis, in the diseased IL-4 Tg mice, Evans blue was intravenously injected into mice. As shown in Figure 4a, blue was only seen in the blood vessels of ears of NT mice 5 min after injection. However, there was a striking increase in the intensity of blue color in the ears of Tg-BO mice, as compared to NT mice, even though visible inflammatory lesions were not yet observed in the BO mice. As the disease progressed in the EL and LL stage blue color in the ears became darker (Figure 4a). The relative concentrations of Evans blue in lesional skin tissues (Tg-BO, EL, and LL) all had a significantly higher amount of Evans blue than those of NT mice ($p < .001$ to $p < .05$, Figure 4b), strongly suggesting that dermal vasculatures have dramatically increased permeability in Tg mice particularly in diseased mice.

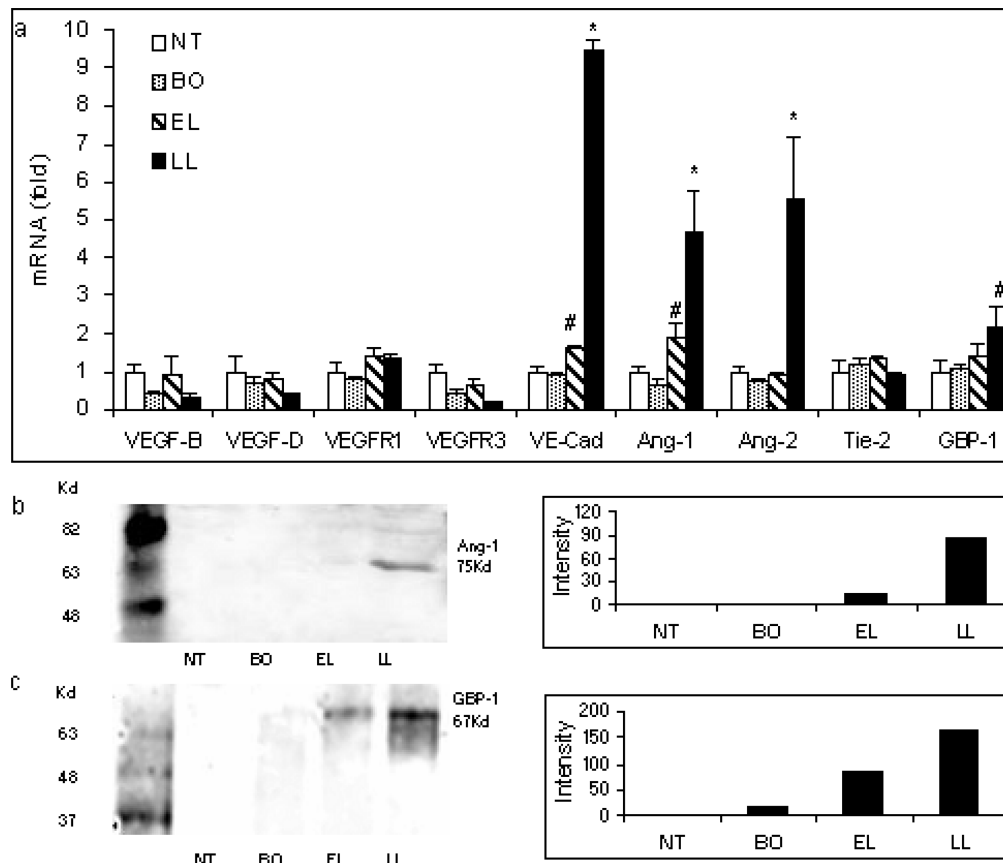


Figure 3. Upregulation of mRNA expressions of other angiogenic factors in inflamed mouse skin. (a) Total RNA extracted from skin was reverse transcribed followed by a semi-quantitative real-time RT-PCR analysis for VEGF-B, VEGF-D, VEGFR1, VEGFR3, VE-cadherin, Ang-1, Ang-2, Tie-2, and GBP-1. The increased factors in diseased mice represented here as fold increases compared to NT (with value of 1). $N = 8$ in each group. Dotted line indicates the level of target mRNA in NT mice. (b) Western blot analysis of Ang-1 in the skin extracts (left) and band intensities (pixels/mm²) of Ang-1 (right). (c) Western blot analysis of GBP-1 in the skin extracts (left) and band intensities (pixels/mm²) of GBP-1 (right). Similar results were obtained in a second experiment.

DISCUSSION

Our study demonstrates a link between angiogenesis and AD, and the molecular mechanism leading to the angiogenesis that has not been previously described in AD. Our study demonstrates an increase in vessel number, vessel diameter, and percent area occupied by vessels, as disease progresses from no visible lesions to late lesions. Interestingly, the diameter of vessels decreases in late lesions, while total number and area occupied increases. On examination of the vessel architecture it is noted that the early lesions demonstrate larger but fewer vessels as compared to late lesions. These findings lend strong support for a microvascular angiogenesis process taking place in this inflammatory skin disease. We next sought to uncover the factors that might have contributed to

this angiogenesis process in this model, among them VEGF-A120, VEGF-A164, VEGF-A188, VEGF-B, VEGF-C, VEGF-D, VEGFR1, VEGFR2, VEGFR3, VE-cadherin, Ang-1, and Tie-2 (23, 30, 40).

We have indeed found an increase of VEGF-A in the skin of Tg mice, with the most prominent increase in mice with late skin lesions. The correlation of serum and skin VEGF-A concentrations with the degrees of angiogenesis and the progression of skin inflammation in our mouse model strongly suggest a positive influence of VEGF-A in angiogenesis and inflammation. This notion is further supported by the finding that VEGF-A itself has a pro-inflammatory property (41). Another support for the influence of VEGF-A on skin angiogenesis comes from the generation of an animal model of psoriasis,

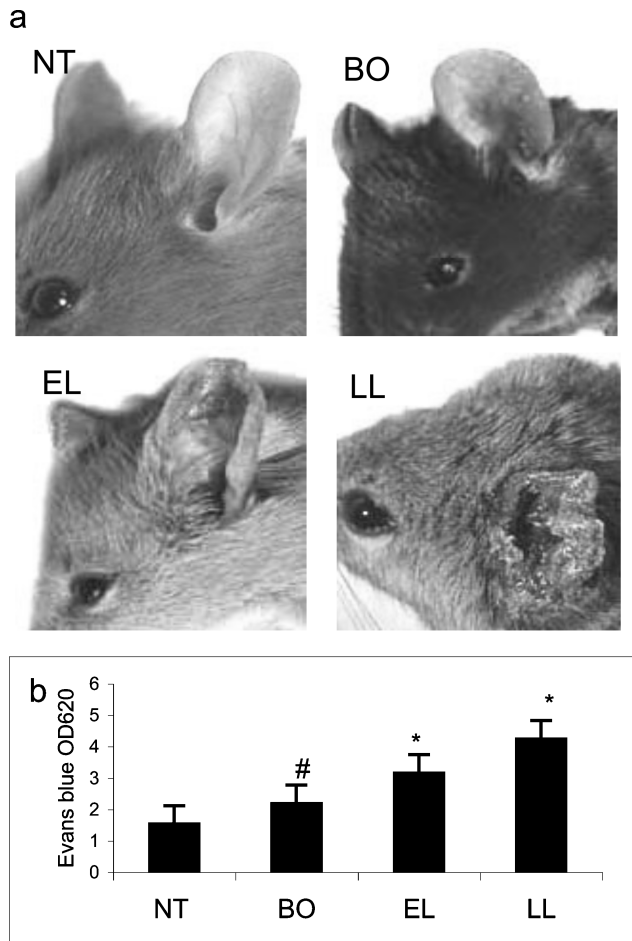


Figure 4. Dermal vasculature leakage increases as the disease progresses. Evans blue was intravenously injected into NT, Tg-BO, Tg-EL, and Tg-LL mice ($N = 5$ in each group). (a) Photographs of ears taken 5 min later after injection. (b) Relative concentrations (OD at 620) of Evans blue by unit weight of lesional or non-lesional skin were determined. * $p < .01$, # $p < .05$ compared to NT.

a typical inflammatory skin disease characterized by prominent angiogenesis, by transgenic expression of VEGF-A into the mouse epidermis (42). By contrast, we did not detect any mRNA upregulation of VEGF-B or VEGF-D in Tg mouse skin and were not able to detect any mRNAs for VEGF-C in NT or Tg mouse skin. Thus, VEGF-A seems to be the major factor, among the VEGF family proteins, that plays a significant role in the angiogenesis of our model. VEGF-A120 and -A164 are two predominant forms of VEGF-A. They induce proliferation of endothelial cells and in vivo angiogenesis (43). However, the significance of upregulation of VEGF-A120 but not VEGF-A164 in our model is not clear. Cur-

rent literature has not yet reported on functional differentials between these two isoforms of VEGF-A, with respect to angiogenesis. This point needs further investigation.

Having demonstrated the upregulation of VEGF-A in our mouse model of AD, the natural question that follows is what induced this upregulation? It has been determined that IL-4 and IFN- γ differentially regulate the VEGF expressions in normal skin epidermal cells and fibroblasts, with the epidermal cells being the major producers (35). While IL-4 upregulates and downregulates VEGF-A in human fibroblasts and keratinocytes, respectively, IFN- γ has the opposite effect (35). We have previously reported that both IL-4 and IFN- γ mRNAs were significantly increased in Tg-BO mouse skin as compared to NT mouse skin (21). Put all these data together and it is possible to reason that upregulation of IFN- γ might have been responsible for the initial upregulation of skin VEGF-A, due to its ability to enhance VEGF-A production by keratinocytes, a major VEGF producer in the skin. However, the effects of different cytokines on the induction/production of VEGF-A are still controversial. One previous study reported that inflammatory cytokine TNF- α and IL-1 β induced VEGF-A mRNA expression in the human keratinocyte cell line HaCat. But IL-6 had no effect on VEGF-A expression in these cells (44). Another report showed that IL-1 β , IL-6, IL-8, TNF- α , IFN- γ and TGF- β had no effect on VEGF secretion in human primary keratinocytes (34). A third study demonstrated that IFN- γ but not IL-4, enhanced the secretion of VEGF-A by human primary keratinocytes (35). Our investigation demonstrated that IL-6 and IFN- γ both increased the expression and production of VEGF-A by IL-4 Tg mouse keratinocytes at both mRNA (8 h) and protein levels (48 and 72 h) (Figure 2e, f). These two cytokines also induced VEGF-A protein production by keratinocytes from NT mice (72 h) (Figure 2g).

These results may indicate an initiating role of these two cytokines in angiogenesis by upregulating VEGF-A in our animal model and IL-4 transgene in Tg keratinocytes may play some roles in inducing VEGF-A expression. The reasons for why IL-6 and IFN- γ upregulated VEGF-A and other inflammatory cytokines active in this model did not yet delineated by current literature, and therefore this mechanism needs further investigation. It is worth noting that the controversial data in the literature with regard to the activities of IL-6 and IFN- γ on keratinocyte-derived

VEGF-A are derived from human keratinocytes (34, 35, 44), not from the mouse keratinocytes that we used. The lesson we learned from this skin inflammation model is that pro-inflammatory cytokines of both Th1 (IFN- γ) and Th2 (IL-6) types have the potential to upregulate pro-angiogenesis factor VEGF-A.

VEGF-A has been shown to support cell growth and is known to form chemotactic gradients and release proteases, which facilitate migration of certain inflammatory cell types into tissues (45). Our AD model, as well as human AD, is known to be an inflammatory disease characterized by prominent inflammatory cell infiltration in the dermis of the skin (19). Recently VEGF-A has been shown to be released by T cells in response to specific antigen, IL-2, or hypoxia. These activated T cells, themselves expressed VEGFR2, in turn could respond to VEGF by reverting to the Th1 phenotype (46). However, in the present study, we focused on the role of VEGF-A in neovascularization, not its role in facilitating the transport of inflammatory cells to the tissue. Therefore, the involvement of VEGF-A in inflammatory cell recruitment and VEGF-A production by inflammatory cells in this model remains unclear.

Microvascular leakage is a hallmark of angiogenesis. The increased permeability of microvasculature is closely related to VEGF-A (47, 48). The functional integrity of the dermal blood vessels *in vivo* in our animal model was assessed by intravenous injection of Evans blue dye. We found that there is a strong correlation of the inflammatory skin disease progression with dermal microvascular permeability (Figure 4). The increased permeability could in turn further enhance the angiogenesis process by allowing more inflammatory cells and serum pro-angiogenic factors to reach the inflamed site.

Two additional factors, Ang-1 and Ang-2, are known to be increased in conjunction with VEGF-A during angiogenesis. (30, 49–51). Both of these ligands have been shown to bind to Tie-2, a receptor tyrosine kinase, expressed almost exclusively on endothelial cells (50, 52). Deletion of Tie-2 causes early death from immature blood vessels (52). However, the binding of Tie-2 by Ang-1, but not Ang-2, causes phosphorylation of Tie-2 receptor (37). Since Ang-2 does not actually transduce a signal, it is proposed that it acts as an antagonist to Ang-1 (37). However, recent findings showed Ang-2 can act as Tie-2 agonist or antagonist (53–55). At high concentration, Ang-2 activated the Tie-2 receptor (54). Ang-1 ap-

pears to be involved in vessel remodeling and maturation (30, 56), while Ang-2 is able to promote new vessel sprouting by inhibition of Ang-1 (37). The skin of K-14 VEGF-A-transgenic mice had numerous twisted capillary-sized vessels with higher permeability. In contrast, the skin of K-14 Ang-1-transgenic mice had enlarged dermal vessels without a significant increase in vessel number and leakage. However, in the K-14 VEGF and Ang-1 double transgenic mice, both the number and size of the skin vessels were increased (57), indicating that the combined functions of VEGF-A and Ang-1 are responsible for the increases of vessel number and size. The findings in our model that both vessel size and number are increased in EL disease stage, corresponding to combined VEGF-A and Ang-1 upregulation, are consistent with the implicated combined functions of VEGF-A and Ang-1 as illustrated by the double transgenic model (57). Similarly, in our model, vessel number was increased, but vessel size was decreased in LL disease stage as compared to that of EL stage. This could reflect the effect of VEGF-A, while Ang-1 is inhibited by Ang-2, which showed increase only at the LL disease stage. However, the actual roles of Ang-1, Ang-2, and Tie-2 in the dermal angiogenesis of this animal model are in need of further investigation.

VE-Cad, an adhesion molecule of intracellular junctions, is required for angiogenesis. Monoclonal antibody directly against VE-Cad inhibited the formation of capillary tubes and disrupted the capillary network when added to form capillary tubes (58). VE-Cad-deficient embryos and yolk sacs showed arrested vascularization process at a very primitive stage with no sign of angiogenic sprouting (59). Recently VE-Cad has been shown to be associated with VEGFR2 during signal transduction from VEGF-A binding (60). VE-Cad phosphorylation in response to VEGF binding has been proposed as a mechanism to loosen cell-cell contacts, allowing for vessel sprouting and cell migration associated with VEGF-induced angiogenesis (61). The increased transcription of VE-Cad in early and late atopic lesions in our model may indicate a positive role of VE-Cad in angiogenesis by way of VEGF. However, the elevation of mRNA expression of VE-Cad most likely resulted from the increase in numbers of endothelial cells in the skin lesions as they became more vascularized as established in Figure 1, not due to a change in level of expression by individual endothelial cells.

GBP-1 is a member of the large GTPase protein family (62). It is a cytoplasmic protein expressed

in endothelial cells. It is selectively induced by inflammatory cytokines, including IFN- γ , IL-1 α , IL-1 β , and TNF- α (63). In humans, endothelial cells of normal skin do not express GBP-1. The expression of GBP-1 was highly detected in the endothelial cells in inflammatory skin diseases with angiogenesis, such as psoriasis, adverse drug reaction, and Kaposi's sarcoma (63). However, GBP-1 was found to be inversely related to endothelial cell proliferation in both in vitro and in vivo studies without affecting endothelial cell adhesiveness for monocytes (38). In addition, GBP-1 mediates the inhibition of endothelial cell invasiveness by inflammatory cytokines via downregulation of matrix metalloproteinase-1 (MMP-1) expression (39), an important proteolytic enzyme in tissue remodeling (39). The anti-angiogenic effect of GBP-1 was determined to be through its GTPase activity that inhibits of MMP-1 expression, rather than its anti-proliferative function (39). The elevated GBP-1 in the skin lesions is likely induced by increased inflammatory cytokines (IFN- γ , IL-1 β , and TNF- α) in the skin of our Tg mice (21), as in the case for VEGF-A. It is interesting that inflammatory cytokines simultaneously induce both pro-angiogenic factor VEGF-A and angiogenic inhibitor GBP-1. The upregulation of GBP-1 in skin lesions probably serves as a negative feedback signal, attempting to terminate excessive angiogenesis induced by VEGF-A.

The angiogenesis process is thought to depend on a balance between endogenous positive and nega-

tive regulatory molecules (pro-angiogenic and anti-angiogenic factors, respectively) (64). This is most likely what happens in our animal model, in light of the significantly increased expression of pro-angiogenic molecules, including VEGF-A, Ang-1, and VE-cadherin, in the IL-4 Tg mice. In the LL stage of the skin lesion, anti-angiogenic factors Ang-2 and GBP-1 are significantly augmented, suggesting that these upregulated anti-angiogenic factors attempt to control the strongly developed angiogenesis as a negative back mechanism. However, the role of these factors in the angiogenesis remains undefined and merits further investigation.

Taken together, an angiogenesis process has occurred in the atopic lesions in our mouse model as demonstrated by the increased number and density of CD31 positive vessels, the upregulation of pro-angiogenic factors VEGF-A, VEGFR2, VE-cadherin, and Ang-1, as well as significant dermal microvascular leakage. Inflammatory cytokines IL-6 and IFN- γ seem to play an initiating role of angiogenesis by upregulating epidermal VEGF-A. In addition, our results provided a strong correlation of the progression of inflammatory disease with that of dermal angiogenesis. These data together thus depict a picture of skin immunological microenvironment that promotes dermal microvascular angiogenesis in our keratin 14-IL-4-Tg mouse model of AD (Figure 5). Future studies may elucidate the detailed mechanism of angiogenesis in the pathogenesis of AD, leading possibly to development of target-specific therapies for AD.

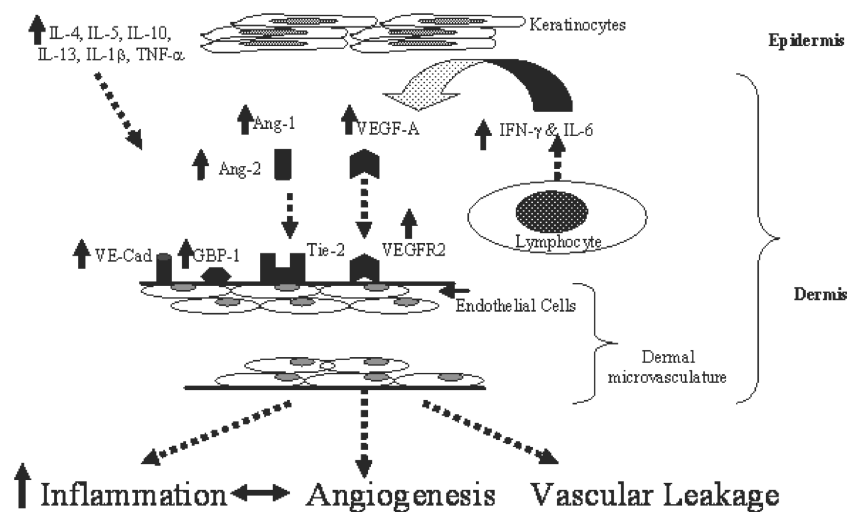


Figure 5. Skin immunological microenvironment that may promote or inhibit angiogenesis, vascular leakage, and inflammation as presented in the keratin 14-IL-4-Tg mouse model of AD. Solid one-sided arrows indicate upregulation; dotted one-sided and curve arrows indicate direction of influence or direction of process. Double-sided arrow indicates co-dependency.

REFERENCES

- Folkman J. (2002). Role of angiogenesis in tumor growth and metastasis. *Semin Oncol* 29:15–18.
- Shih T, Lindley C. (2006). Bevacizumab: an angiogenesis inhibitor for the treatment of solid malignancies. *Clin Ther* 28:1779–1802.
- Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Siebels M, Negrier S, Chevreau C, Solska E, Desai AA, Rolland F, Demkow T, Hutson TE, Gore M, Freeman S, Schwartz B, Shan M, Simantov R, Bukowski RM. (2007). Sorafenib in advanced clear-cell renal-cell carcinoma. *N Engl J Med* 356:125–134.
- Motzer RJ, Michaelson MD, Redman BG, Hudes GR, Wilding G., Figlin RA, Ginsberg MS, Kim ST, Baum CM, DePrimo SE, Li JZ, Bello CL, Theuer CP, George DJ, Rini BI. (2006). Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. *J Clin Oncol* 24:16–24.
- Feldman DR, Motzer RJ. (2006). Novel targets and therapies for metastatic renal cell carcinoma. *Oncology (Williston Park)* 20:1745–1753; discussion 1756.
- Bagli E, Xagorari A, Papetropoulos A, Murphy C, Fotis T. (2004). Angiogenesis in inflammation. *Autoimmun Rev* 3 (Suppl 1):S26.
- Brennen WN, Cooper CR, Capitosti S, Brown ML, Sikes RA. (2004). Thalidomide and analogues: current proposed mechanisms and therapeutic usage. *Clin Prostate Cancer* 3:54–61.
- Du W., Hattori Y, Hashiguchi A, Kondoh K, Hozumi N, Ikeda Y, Sakamoto M, Hata J, Yamada T. (2004). Tumor angiogenesis in the bone marrow of multiple myeloma patients and its alteration by thalidomide treatment. *Pathol Int* 54:285–294.
- Gately S, Li WW. (2004). Multiple roles of COX-2 in tumor angiogenesis: a target for antiangiogenic therapy. *Semin Oncol* 31:2–11.
- Koehne CH, Dubois RN. (2004). COX-2 inhibition and colorectal cancer. *Semin Oncol* 31:12–21.
- Streck CJ, Zhang Y, Miyamoto R, Zhou J, Ng CY, Nathwani AC, Davidoff AM. (2004). Restriction of neuroblastoma angiogenesis and growth by interferon- α /beta. *Surgery* 136:183–189.
- Shishodia S, Aggarwal BB. (2004). Cyclooxygenase (COX)-2 inhibitor celecoxib abrogates activation of cigarette smoke-induced nuclear factor (NF)- κ B by suppressing activation of IkappaB α kinase in human non-small cell lung carcinoma: correlation with suppression of cyclin D1, COX-2, and matrix metalloproteinase-9. *Cancer Res* 64:5004–5012.
- Inai T, Mancuso M, Hashizume H, Baffert F, Haskell A, Baluk P, Hu-Lowe DD, Shalinsky DR, Thurston G, Yancopoulos GD, McDonald DM. (2004). Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. *Am J Pathol* 165:35–52.
- Grosios K, Wood J, Esser R, Raychaudhuri A, Dawson J. (2004). Angiogenesis inhibition by the novel VEGF receptor tyrosine kinase inhibitor, PTK787/ZK222584, causes significant anti-arthritis effects in models of rheumatoid arthritis. *Inflamm Res* 53:133–142.
- Leung DY, Bieber T. (2003). Atopic dermatitis. *Lancet* 361:151–160.
- Leung DY, Boguniewicz M, Howell MD, Nomura I, Hamid QA. (2004). New insights into atopic dermatitis. *J Clin Invest* 113:651–657.
- Agha-Majzoub R, Becker RP, Schraufnagel DE, Chan LS. (2005). Angiogenesis: the major abnormality of the keratin-14 IL-4 transgenic mouse model of atopic dermatitis. *Microcirculation* 12:455–476.
- Chan LS, Robinson N, Xu L. (2001). Expression of interleukin-4 in the epidermis of transgenic mice results in a pruritic inflammatory skin disease: an experimental animal model to study atopic dermatitis. *J Invest Dermatol* 117:977–983.
- Chen L, Martinez O, Venkataramani P, Lin SX, Prabhakar BS, Chan LS. (2005). Correlation of disease evolution with progressive inflammatory cell activation and migration in the IL-4 transgenic mouse model of atopic dermatitis. *Clin Exp Immunol* 139:189–201.
- Chen L, Lin SX, Overbergh L, Mathieu C, Chan LS. (2005). The disease progression in the keratin 14 IL-4-transgenic mouse model of atopic dermatitis parallels the upregulations of B cell activation molecules, proliferation, and surface and serum IgE. *Clin Exp Immunol* 142:21–30.
- Chen L, Martinez O, Overbergh L, Mathieu C, Prabhakar BS, Chan LS. (2004). Early up-regulation of Th2 cytokines and late surge of Th1 cytokines in an atopic dermatitis model. *Clin Exp Immunol* 138:375–387.
- Zhang Y, Matsuo H, Morita E. (2006). Increased production of vascular endothelial growth factor in the lesions of atopic dermatitis. *Arch Dermatol Res* 297:425–429.
- Shih SC, Robinson GS, Perruzzi CA, Calvo A, Desai K, Green JE, Ali IU, Smith LE, Senger DR. (2002). Molecular profiling of angiogenesis markers. *Am J Pathol* 161:35–41.
- Voros G, Maquoi E, Demeulemeester D, Clerx N, Collen D, Lijnen HR. (2005). Modulation of angiogenesis during adipose tissue development in murine models of obesity. *Endocrinology* 146:4545–4554.
- Shih SC, Ju M, Liu N, Smith LE. (2003). Selective stimulation of VEGFR-1 prevents oxygen-induced retinal vascular degeneration in retinopathy of prematurity. *J Clin Invest* 112:50–57.
- Thijssen VL, Brandwijk RJ, Dings RP, Griffioen AW. 2004. Angiogenesis gene expression profiling in xenograft models to study cellular interactions. *Exp Cell Res* 299:286–293.

27. Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. (2001). An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* 25:386–401.
28. Hulthen UL, Cao Z, Rumble JR, Cooper ME, Johnston CI. (1996). Vascular hypertrophy and albumin permeability in a rat model combining hypertension and diabetes mellitus. Effects of calcium antagonism, angiotensin converting enzyme inhibition, and angiotensin II-AT1-receptor blockade. *Am J Hypertens* 9:895–901.
29. Ruhrberg C. (2003). Growing and shaping the vascular tree: multiple roles for VEGF. *Bioessays* 25:1052–1060.
30. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. (2000). Vascular-specific growth factors and blood vessel formation. *Nature* 407:242–248.
31. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoek A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380:435–439.
32. Lee SS, Joo YS, Kim WU, Min DJ, Min JK, Park SH, Cho CS, Kim HY. (2001). Vascular endothelial growth factor levels in the serum and synovial fluid of patients with rheumatoid arthritis. *Clin Exp Rheumatol* 19:321–324.
33. Harada M, Mitsuyama K, Yoshida H, Sakisaka S, Taniguchi E, Kawaguchi T, Ariyoshi M, Saiki T, Sakamoto M, Nagata K, Sata M, Matsuo K, Tanikawa K. 1998. Vascular endothelial growth factor in patients with rheumatoid arthritis. *Scand J Rheumatol* 27:377–380.
34. Detmar M, Yeo KT, Nagy JA, Van de Water L, Brown LF, Berse B, Elicker BM, Ledbetter S, Dvorak HF. 1995. Keratinocyte-derived vascular permeability factor (vascular endothelial growth factor) is a potent mitogen for dermal microvascular endothelial cells. *J Invest Dermatol* 105:44–50.
35. Trompezinski S, Denis A, Vinche A, Schmitt D, Viac J. 2002. IL-4 and interferon-gamma differentially modulate vascular endothelial growth factor release from normal human keratinocytes and fibroblasts. *Exp Dermatol* 11:224–231.
36. Chen L, Lin SX, Agha-Majzoub R, Overbergh L, Mathieu C, Chan LS. 2006. CCL27 is a critical factor for the development of atopic dermatitis in the keratin-14-IL-4-transgenic mouse model. *Int Immunol* 18:1233–1242.
37. Maisonnier PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD. (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277:55–60.
38. Guenzi E, Topolt K, Cornali E, Lubeseder-Martellato C, Jorg A, Matzen K, Zietz C, Kremmer E, Nappi F, Schwemmle M, Hohenadl C, Barillari G, Tschachler E, Monini P, Ensoli B, Sturzl M. (2001). The helical domain of GBP-1 mediates the inhibition of endothelial cell proliferation by inflammatory cytokines. *Embo J* 20:5568–5577.
39. Guenzi E, Topolt K, Lubeseder-Martellato C, Jorg A, Naschberger E, Benelli R, Albini A, Sturzl M. (2003). The guanylate binding protein-1 GTPase controls the invasive and angiogenic capability of endothelial cells through inhibition of MMP-1 expression. *EMBO J* 22:3772–3782.
40. Bhushan M, Young HS, Brenchley PE, Griffiths CE. (2002). Recent advances in cutaneous angiogenesis. *Br J Dermatol* 147:418–425.
41. Reinders ME, Sho M, Izawa A, Wang P, Mukhopadhyay D, Koss KE, Geehan CS, Luster AD, Sayegh MH, Briscoe DM. (2003). Proinflammatory functions of vascular endothelial growth factor in alloimmunity. *J Clin Invest* 112:1655–1665.
42. Xia YP, Li B, Hylton D, Detmar M, Yancopoulos GD, Rudge JS. (2003). Transgenic delivery of VEGF to mouse skin leads to an inflammatory condition resembling human psoriasis. *Blood* 102:161–168.
43. Nakamura M, Abe Y, Tokunaga T. (2002). Pathological significance of vascular endothelial growth factor A isoform expression in human cancer. *Pathol Int* 52:331–339.
44. Frank S, Hubner G, Breier G, Longaker MT, Greenhalgh DG, Werner S. (1995). Regulation of vascular endothelial growth factor expression in cultured keratinocytes: implications for normal and impaired wound healing. *J Biol Chem* 270:12607–12613.
45. Ancelin M, Chollet-Martin S, Herve MA, Legrand C, El Benna J, Perrot-Appianat M. (2004). Vascular endothelial growth factor VEGF189 induces human neutrophil chemotaxis in extravascular tissue via an autocrine amplification mechanism. *Lab Invest* 84:502–512.
46. Mor F, Quintana FJ, Cohen IR. (2004). Angiogenesis-inflammation cross-talk: vascular endothelial growth factor is secreted by activated T cells and induces Th1 polarization. *J Immunol* 172:4618–4623.
47. Detmar M, Brown LF, Schon MP, Elicker BM, Velasco P, Richard L, Fukumura D, Monsky W, Claffey KP, Jain RK. (1998). Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice. *J Invest Dermatol* 111:1–6.
48. Dvorak HF, Brown LF, Detmar M, Dvorak AM. (1995). Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 146:1029–1039.
49. Holash J, Wiegand SJ, Yancopoulos GD. (1999). New model of tumor angiogenesis: dynamic balance

- between vessel regression and growth mediated by angiopoietins and VEGF. *Oncogene* 18:5356–5362.
50. Asahara T, Chen D, Takahashi T, Fujikawa K, Kearney M, Magner M, Yancopoulos GD, Isner JM. (1998). Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization. *Circ Res* 83:233–240.
 51. Papapetropoulos A, Garcia-Cardena G, Dengler TJ, Maisonpierre PC, Yancopoulos GD, Sessa WC. (1999). Direct actions of angiopoietin-1 on human endothelium: evidence for network stabilization, cell survival, and interaction with other angiogenic growth factors. *Lab Invest* 79:213–223.
 52. Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W, Qin Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 376:70–74.
 53. Teichert-Kuliszewska K, Maisonpierre PC, Jones N, Campbell AI, Master Z, Bendeck MP, Alitalo K, Dumont DJ, Yancopoulos GD, Stewart DJ. (2001). Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2. *Cardiovasc Res* 49:659–670.
 54. Kim I, Kim JH, Moon SO, Kwak HJ, Kim NG, Koh GY. (2000). Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. *Oncogene* 19:4549–4552.
 55. Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, Martin C, Witte C, Witte MH, Jackson D, Suri C, Campochiaro PA, Wiegand SJ, Yancopoulos GD. (2002). Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by angiopoietin-1. *Dev Cell* 3:411–423.
 56. Hanahan D. (1997). Signaling vascular morphogenesis and maintenance. *Science* 277:48–50.
 57. Thurston G. (2002). Complementary actions of VEGF and angiopoietin-1 on blood vessel growth and leakage. *J Anat* 200:575–580.
 58. Bach TL, Barsigian C, Chalupowicz DG, Busler D, Yaen CH, Grant DS, Martinez J. (1998). VE-Cadherin mediates endothelial cell capillary tube formation in fibrin and collagen gels. *Exp Cell Res* 238:324–334.
 59. Gory-Faure S, Prandini MH, Pointu H, Roullot V, Pignot-Paintrand I, Vernet M, Huber P. (1999). Role of vascular endothelial-cadherin in vascular morphogenesis. *Development* 126:2093–2102.
 60. Zanetti A, Lampugnani MG, Balconi G, Breviario F, Corada M, Lanfrancone L, Dejana E. (2002). Vascular endothelial growth factor induces SHC association with vascular endothelial cadherin: a potential feedback mechanism to control vascular endothelial growth factor receptor-2 signaling. *Arterioscler Thromb Vasc Biol* 22:617–622.
 61. Esser S, Lampugnani MG, Corada M, Dejana E, Risau W. (1998). Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. *J Cell Sci* 111 (Pt 13):1853–1865.
 62. Naschberger E, Bauer M, Sturzl M. (2005). Human guanylate binding protein-1 (hGBP-1) characterizes and establishes a non-angiogenic endothelial cell activation phenotype in inflammatory diseases. *Adv Enzyme Regul* 45:215–227.
 63. Lubeseder-Martellato C, Guenzi E, Jorg A, Topolt K, Naschberger E, Kremmer E, Zietz C, Tschachler E, Hutzler P, Schwemmle M, Matzen K, Grimm T, Ensoli B, Sturzl M. (2002). Guanylate-binding protein-1 expression is selectively induced by inflammatory cytokines and is an activation marker of endothelial cells during inflammatory diseases. *Am J Pathol* 161:1749–1759.
 64. Hanahan D, Folkman J. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86:353–364.

Copyright of *Microcirculation* is the property of Taylor & Francis Ltd and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.