Increased Microvascular Density and Enhanced Leukocyte Rolling and Adhesion in the Skin of VEGF Transgenic Mice

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Vascular endothelial growth factor (VEGF) has been implicated in the pathologic angiogenesis observed in psoriasis and other chronic inflammatory skin diseases that are characterized by enhanced expression of VEGF by epidermal keratinocytes and of VEGF receptors by tortuous microvessels in the upper dermis. To investigate the functional importance of chronic VEGF overexpression *in vivo*, we used a keratin 14 promoter expression cassette containing the gene for murine $VEGF_{164}$ to selectively target VEGF expression to basal epidermal keratinocytes in transgenic mice. These mice demonstrated an increased density of tortuous cutaneous blood capillaries with elevated expression levels of the high affinity VEGF receptors, VEGFR-1 and VEGFR-2, most prominently during the neonatal period. In contrast, no abnormalities of lymphatic vessels were detected. In addition, the number of mast cells in the upper dermis was significantly increased in transgenic skin. Intravital fluorescence microscopy revealed highly increased leukocyte rolling and adhesion in postcapillary skin venules that were both inhibited after injection of blocking antibodies against E- and P-selectin. Combined blocking antibodies against intercellular adhesion molecule-1 and lymphocyte function-associated antigen-1 were without effect, whereas an anti-vascular cell adhesion molecule-1/VLA-4 antibody combination almost completely normalized the enhanced leukocyte adhesion in transgenic mice. This study reveals VEGF as a growth factor specific for blood vessels, but not lymphatic vessels, and demonstrates that chronic orthotopic overexpression of VEGF in the epidermis is sufficient to induce cardinal features of chronic skin inflammation, providing a molecular link between angiogenesis, mast cell accumulation, and leukocyte recruitment to sites of inflammation. ***Key words: angiogenesis/endothelium/VPF/mast cells. J Invest Dermatol 111:1–6, 1998***

Common chronic inflammatory diseases such as psoriasis and rheumatoid arthritis are characterized by leukocyte infiltration, angiogenesis, and vascular remodelling leading to enhanced tortuosity of blood microvessels (Ryan, 1980; Braverman and Sibley, 1982; Braverman and Keh-Yen, 1986; Bull *et al*, 1992; Fava *et al*, 1994; Koch *et al*, 1994). Previously, we have demonstrated increased expression of vascular endothelial growth factor (VEGF; also known as vascular permeability factor) by epidermal keratinocytes, and of the two high-affinity VEGF receptors, VEGFR-1 (Flt-1) (deVries *et al*, 1992) and VEGFR-2 (KDR) (Terman *et al*, 1992) by dermal microvessels in psoriasis, contact dermatitis, and several chronic inflammatory bullous skin diseases with enhanced angiogenesis (Detmar *et al*, 1994; Brown *et al*, 1995a, b). Moreover, enhanced VEGF and VEGF receptor expression was a characteristic feature of lesional skin in a recently described mouse model for chronic, psoriasiform skin inflammation (Schön *et al*, 1997); however, it has remained unclear whether keratinocyte-secreted VEGF, predominantly the $VEGF_{165}$ and $VEGF_{121}$ splice variants (Ballaun *et al*, 1995; Detmar *et al*, 1995, 1997), can penetrate the epidermal–dermal basement membrane to reach its target cells on dermal microvessels, and whether VEGF itself might be involved in the observed upregulation of its receptors.

Previously, we have shown that keratinocyte-derived VEGF is a potent mitogen for human dermal microvascular endothelial cells that express both VEGFR-1 and VEGFR-2 *in vitro* (Detmar *et al*, 1995). VEGF also enhanced endothelial cell migration through upregulation of the $\alpha_v\beta_3$ integrin (Senger *et al*, 1996) that has been found upregulated on skin microvessels in psoriasis (Creamer and Barker, 1995), and of the $\alpha_1\beta_1$, and $\alpha_2\beta_1$ integrins (Senger *et al*, 1997). These findings suggested a major role of VEGF in the mediation of the vascular remodelling characteristic of chronic skin inflammation; however, the precise biologic importance of VEGF for this process is still unknown because VEGF deficiency is lethal during early embryonic development (Carmeliet *et al*, 1996; Ferrara *et al*, 1996), preventing evaluation of skin angiogenesis in VEGF deficient mice. Moreover, whereas injection of VEGF into the skin leads to acutely increased microvascular permeability to plasma macromolecules (Senger *et al*, 1990), chronic topical delivery of VEGF to the skin could not be achieved.

To study the specific biologic consequences of chronic VEGF overexpression in the skin, we generated transgenic mice, using a transgene vector in which the coding sequence of murine $VEGF_{164}$ was cloned into a human keratin 14 promoter expression cassette. The human keratin 14 expression cassette has previously been shown to selectively target transgene expression to basal keratinocytes of the skin

Manuscript received January 30, 1998; revised March 25, 1998; accepted for publication April 14, 1998.

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Abbreviations: VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial cell growth factor; VEGFR-1, VEGF receptor-1 (Flt-1); VEGFR-2, VEGF receptor-2 (Flk-1).

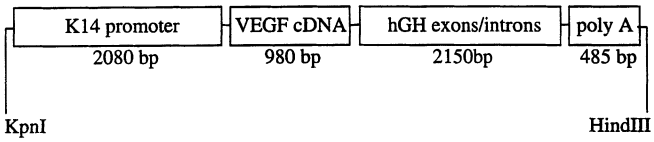

Figure 1. Schematic representation of the K14-VEGF transgene construct. A 980 bp murine $VEGF_{164}$ BamHI cDNA fragment, coding for the 164-amino acid VEGF splice variant, was ligated to the BamHI restriction site of the keratin 14 promoter expression cassette.

(Vassar *et al*, 1989; Vassar and Fuchs, 1991; Turksen *et al*, 1992; Guo *et al*, 1993), providing thereby an orthotopical model to assess the effects of epidermis-derived VEGF on skin angiogenesis *in vivo*. In this study, we found that VEGF transgenic mice were characterized by an increased density of tortuous cutaneous blood capillaries with elevated expression levels of VEGFR-1 and VEGFR-2, most prominently during the neonatal period, whereas no abnormalities of lymphatic vessels were detected, establishing VEGF as a blood vessel-specific skin angiogenesis factor. Using intravital fluorescence microscopy, we detected dramatically increased leukocyte rolling and adhesion in postcapillary skin venules that were both completely inhibited after injection of blocking antibodies against E- and P-selectin. In addition, an anti-vascular cell adhesion molecule-1 (VCAM-1)/VLA-4 antibody combination almost completely normalized the enhanced leukocyte adhesion in transgenic mice. Thus, chronic orthotopic overexpression of VEGF in the epidermis was sufficient to induce cardinal pathologic features of psoriasis, providing further evidence for the role of VEGF as a major skin angiogenesis factor in chronic inflammation and as a novel molecular link between angiogenesis and leukocyte recruitment to sites of inflammation.

MATERIALS AND METHODS

Generation of mice A 980 bp murine $VEGF_{164}$ cDNA (GenBank accession number M95200) was ligated into the BamHI restriction site of the keratin 14 expression cassette (Vassar *et al*, 1989; kindly provided by Dr. Elaine Fuchs, University of Chicago; **Fig 1**), and a KpnI-HindIII fragment was purified and injected into FVB/N mouse zygotes. The injected embryos were transplanted into the uterus of pseudo-pregnant C21 mice. Transgenic founders were detected by Southern blot analysis of BamHI digested genomic DNA using a ^{32}P-labeled 980 bp murine VEGF cDNA as the probe. Genomic tail DNA also was subjected to polymerase chain reaction using two primers specific for human growth hormone sequences contained in the expression vector; 5′-CTCACCTAGCTGCAATGG-3′ and 5′-AAGGCACTGCCCTCTTGAA-GC-3′. Initial denaturation at 94°C for 4 min was followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by final extension at 72°C for 5 min. Transgenic lines were established on the FVB genetic background.

***In situ* hybridization** *In situ* hybridization of paraffin and frozen sections, obtained from four wild-type and four transgenic mice for each time point, was performed as described earlier (Detmar *et al*, 1994) using pGEM or pBluescript II plasmids containing mouse VEGF, VEGFR-1, or VEGFR-2 cDNA fragments. *In situ* hybridizations were performed on abdominal, dorsal, and tail skin with comparable results. VEGFR-1 and −2 clones were a kind gift from Clive Wood (Genetics Institute, Cambridge, MA). The flk-1 and flt-1 sequences were isolated by polymerase chain reaction from a mouse fetal thymus cDNA library (Finnerty *et al*, 1993). The murine flk-1 transcription template was a 392 bp fragment encompassing amino acids 1–130 (nucleotides 268–660 of the flk-1 sequence described previously; Matthews *et al*, 1991), cloned into pGEM-T (Promega, Madison, WI). The sequence for murine flt-1 was obtained by degenerate polymerase chain reaction cloning of kinase domains that resulted in a 640 bp cDNA fragment encoding the insert region from amino acid 832–1045 of a sequence described previously (Finnerty *et al*, 1993). The VEGFR-3 probe was kindly provided by Dr. Kari Alitalo and has been described elsewhere (Jeltsch *et al*, 1997). Transcription reactions were carried out using a Riboprobe Gemini II kit (Promega) in the presence of (α-^{35}S) UTP. Anti-sense (and control sense) probes were evaluated on alternate sections.

Immunohistochemistry For immunohistochemistry, 6 μm cryostat sections of ear, abdominal, or dorsal skin obtained from four wild-type and four transgenic mice were stained with anti-mouse platelet-endothelial cell adhesion molecule-1 (PECAM-1; CD31), anti-mouse intercellular adhesion molecule-1 (ICAM-1; CD54), anti-mouse E-selectin, P-selectin, L-selectin, anti-mouse CD3, CD4, CD8, CD 11a, CD11b, CD45, CD 49d (all from Pharmingen, San Diego, CA), anti-mouse collagen type IV, and anti-desmoplakin I/II (Biodesign International, Kennebunk, ME), and affinity purified rabbit anti-mouse collagen XVIII (Rehn and Pihlajaniemi, 1994; kindly provided by Dr. Marko Rehn), using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Rat IgG1 (R59–40), rat IgG2a (R35–95), or hamster IgG (Ha4/8; all purchased from Pharmingen) were used as isotype matched negative controls in monoclonal antibody staining, and normal rabbit serum was used as control for polyclonal rabbit sera. For quantitation of microvascular densities in the skin, abdominal skin samples were obtained after CO_2 euthanasia from six transgenic and six wild-type control mice each at 3 d, 11 d, 3 wk, and 6 wk after birth. Five micrometer paraffin sections were stained for CD31, and the number of vascular profiles per high-power field (×100 objective) was determined in the areas immediately below the epidermal–dermal basement membrane zone. At least five random fields per section were evaluated, and data were evaluated using the paired Student's t test. Three micrometer plastic sections were stained for chloroacetate-esterase reactivity (n = 6) using naphthol-AS-D-chloroacetate as substrate and 1% methyl green as counterstain. Mast cell staining was confirmed by methylene blue and Giemsa staining.

Intravital fluorescence microscopy Twenty-four day old wild-type or transgenic mice (n = 6 for each group) were injected intravenously with 50 μl fluoroscein isothiocyanate-labeled dextran (MW, 2000000; Sigma; 10 mg per ml), and video recording of ear skin perfusion was performed as described previously (Fukumura *et al*, 1995). Mice were also injected with 20 μl Rhodamin-6G (Molecular Probes, Eugene, OR; 50 mg per ml) to visualize peripheral blood leukocytes (Fukumura *et al*, 1995). In additional experiments, 24 d old mice (n = 3 for each group) were injected intraperitoneously with the following antibodies (150 μg each), either alone or in different combinations (**Fig 6**): blocking antibodies against E-selectin (clone 10E9.6), P-selectin (clone RB.40.34), ICAM-1 (clone 3E2), VCAM-1 (clone 429), L-selectin (clone MEL-14), lymphocyte function-associated antigen-1 (CD11a; clone M17/4), or VLA-4 (CD49d; clones R1–1 and 9C10; all purchased from Pharmingen) 3 h prior to examination. For fluorescence microlymphangiography, 5–10 μl of fluoroscein isothiocyanate-dextran (25 mg per ml) were injected intradermally into the tail skin (n = 3 for each group), and fluorescence micrographs were recorded 40–60 min after injection (Leu *et al*, 1994). The capillary tortuosity index was calculated by dividing 100 by the average length (μm) of capillaries without branching; a minimum of 500 branches was evaluated in each animal. The rolling count was calculated as 100 × number of rolling leukocytes/total leukocyte flux and adhesion density was calculated as the number of adherent leukocytes per mm^2 vascular surface of postcapillary venules as described (Fukumura *et al*, 1995).

RESULTS

The aim of this study was to characterize the biologic consequences of chronic VEGF overexpression in the epidermis of transgenic mice, using a keratin 14 promoter expression cassette containing the mouse $VEGF_{164}$ gene to target VEGF expression selectively to basal epidermal keratinocytes. Southern blot analysis of genomic DNA revealed transgene incorporation in nine of 30 mice with copy numbers between three and ≈20 (data not shown). Whereas one of these transgenic mice died and four did not transmit the transgene, four founders with 8–10 transgene copies in their genome transmitted the transgene to their offspring with a Mendelian inheritance pattern.

Increased expression of VEGF and its receptors in transgenic mice Targeted expression of the K14-VEGF transgene was confirmed by *in situ* hybridization, demonstrating selectively increased VEGF expression in the basal keratinocyte layer of the epidermis and in the outer root sheath keratinocytes of hair follicles that also express keratin 14 (**Fig 2*A–F***). Within the upper dermis and surrounding hair follicles, microvessels expressed highly increased levels of VEGFR-2 mRNA (**Fig 2*G–J***) and moderately increased levels of VEGFR-1 mRNA (**Fig 2*K*, *L***). No VEGFR-1 mRNA expression was detectable in wild-type skin (not shown). In contrast, expression of VEGFR-3 (Flt-4) appeared unchanged (data not shown). These findings suggest a positive feedback mechanism, resulting in selective induction of the two high-affinity, endothelial cell VEGF receptors by keratinocyte-derived VEGF *in vivo*.

Increased density of blood microvessels in the dermis of transgenic mice VEGF transgenic mice were characterized by visibly increased skin vascularization (**Fig 3*A***). Immunohistochemical staining for PECAM-1, an endothelial junction molecule (**Fig 3*B*, *C***) (Dejana

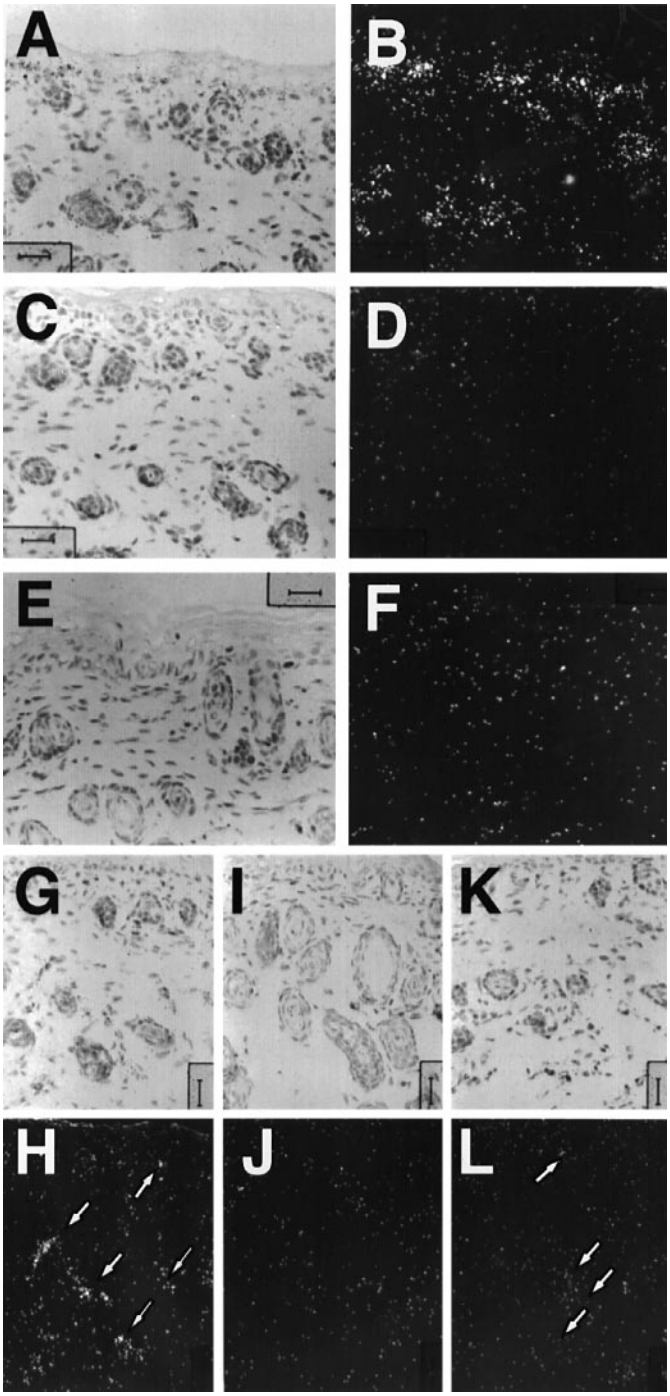

Figure 2. Induced overexpression of VEGF and VEGFR-2 in the abdominal skin of K14-VEGF transgenic mice. *In situ* hybridization of transgenic skin with mouse VEGF anti-sense (*A*, *B*) and sense (*C*, *D*) probes demonstrates strong VEGF mRNA expression in basal epidermal keratinocytes and in follicular keratinocytes. Parts (*E*) and (*F*) show only weak VEGF mRNA expression in the skin of a wild-type littermate control. Hybridization with mouse VEGFR-2 anti-sense probe demonstrates increased VEGFR-2 mRNA expression in dermal microvessels (*arrows*) in transgenic skin (*G*, *H*), as compared with wild-type skin (*I*, *J*). Weak VEGFR-1 mRNA expression in dermal microvessels (*arrows*) in transgenic skin (*K*, *L*). No VEGFR-1 expression was detected in wild-type skin (not shown). Bright-field (*A*, *C*, *E*, *G*, *I*, *K*) and dark-field (*B*, *D*, *F*, *H*, *J*, *L*) microscopy. *Scale bar*: 25 μm.

et al, 1995), and for collagen types IV and XVIII revealed an increased number of dermal microvessels within transgenic skin (**Fig 3*F*, *G*** and data not shown). Morphometric analysis of microvascular profiles per area unit, using paraffin sections stained for CD31, showed that microvascular density in wild-type controls was highest during the neonatal period (**Fig 4**), and was substantially lower after 3 and 6 wk. These findings corresponded to the degree of visible skin vascularization. VEGF transgenic mice were characterized by highly increased numbers of microvessels per high power microscope field in the abdominal skin of 3 d old mice (+58.3% *versus* control; $p < 0.01$; $n = 6$) (**Fig 4**). In 11 d old, 3 wk old, and 6 wk old mice, the increase was less dramatic (+30.2–31.3% *versus* control; $p < 0.05$; $n = 6$), due to the diminished expression of the transgene construct in the epidermis of older animals (Vassar *et al*, 1989; data not shown). Comparable results were obtained in dorsal and ear skin. The induced dermal microvessels in VEGF transgenic mice were not stained by monoclonal antibodies against desmoplakin I and II (data not shown) expressed by lymphatic, but not by blood vascular endothelial cells (Schmelz *et al*, 1994). This indicated that the induced dermal microvasculature was of blood vessel rather than lymphatic origin.

Increased density of mast cells in the dermis of transgenic mice Angiogenesis and increased mast cell localization to sites of newly forming microvasculature are often associated (Meininger and Zetter, 1992). When Epon sections of skin of VEGF transgenic mice were stained for chloroacetate esterase activity (**Fig 3*D*, *E***) or were Giemsa stained, a significantly increased density of dermal mast cells was detected (+35.2% *versus* wild-type littermates, $p < 0.05$, $n = 6$). These results suggest a role of VEGF in mast cell recruitment to sites of angiogenesis *in vivo*. In contrast, skin-infiltrating leukocytes were not increased in VEGF transgenic mice, as assessed by immunohistochemical staining for CD3, CD4, CD8, and CD45 expressed by lymphocytes and CD11b (α_M-integrin) expressed by granulocytes and macrophages.

Intravital characterization of skin microvessels To functionally characterize the induced dermal blood vessels in VEGF transgenic mice, intravital microscopy of ear skin was performed after intravenous injection of fluorescently labeled dextran (Fukumura *et al*, 1995). Diameter, red blood cell velocity, and shear rate of postcapillary venules were similar in VEGF transgenic and wild-type mice (**Fig 5*G*, *H***); however, in accordance with the data obtained by morphometric analysis of CD31 stained skin sections, the microvascular density was significantly increased in the skin of 3 wk old transgenic mice (+20.1% *versus* control) (**Fig 5*A*, *B*, *I***). These capillaries had a highly increased tortuosity index (+69.0%), corresponding to a decreased average capillary length without branching (**Fig 5*J***). In addition, capillaries in VEGF transgenic ear skin were hyperpermeable, as evidenced by early leakage of fluoroscein isothiocyanate-dextran into the perivascular space (**Fig 5*B*** and data not shown). In contrast, microlymphangiography demonstrated normal numbers and diameters of lymphatic vessels in transgenic skin (**Fig 5*E*, *F***).

Enhanced leukocyte rolling and adhesion in postcapillary venules in transgenic skin Postcapillary venules in VEGF transgenic ear skin showed a significantly increased proportion of rolling leukocytes (47.9% *versus* 15.6% in wild-type skin, $p < 0.005$) (**Fig 5*K***) and, even more prominent, an enhanced density of adherent leukocytes (251.9 *versus* 61.3 cells per mm^2, $p < 0.005$) (**Fig 5*C*, *D*, *L***). The enhanced leukocyte rolling and adhesion was not due to increased numbers of circulating leukocytes, as white blood cells counts were comparable in wild-type and transgenic mice. Blocking antibodies against E- and P-selectin brought the proportion of rolling and adherent leukocytes back down to levels seen in wild-type mice (**Fig 6*A***). In contrast, an anti-ICAM-1/lymphocyte function-associated antigen-1 antibody combination had no effect on leukocyte adhesion; however, no pronounced alterations of endothelial cell expression levels of E- or P-selectin were detected within the skin of VEGF transgenic mice by immunohistochemical staining (data not shown). Leukocyte adhesion in transgenic mouse skin was potently inhibited by antibody combinations of anti-VCAM-1/VLA-4 and of anti-VCAM-1/ICAM-1 (**Fig 6**). This suggested that the increased rolling and adhesion of peripheral blood leukocytes in postcapillary venules of VEGF transgenic mice resulted from specific cell adhesion molecule interactions.

DISCUSSION

Previously, we identified VEGF as a major keratinocyte-derived mitogen for dermal microvascular endothelial cells (Detmar *et al*, 1995)

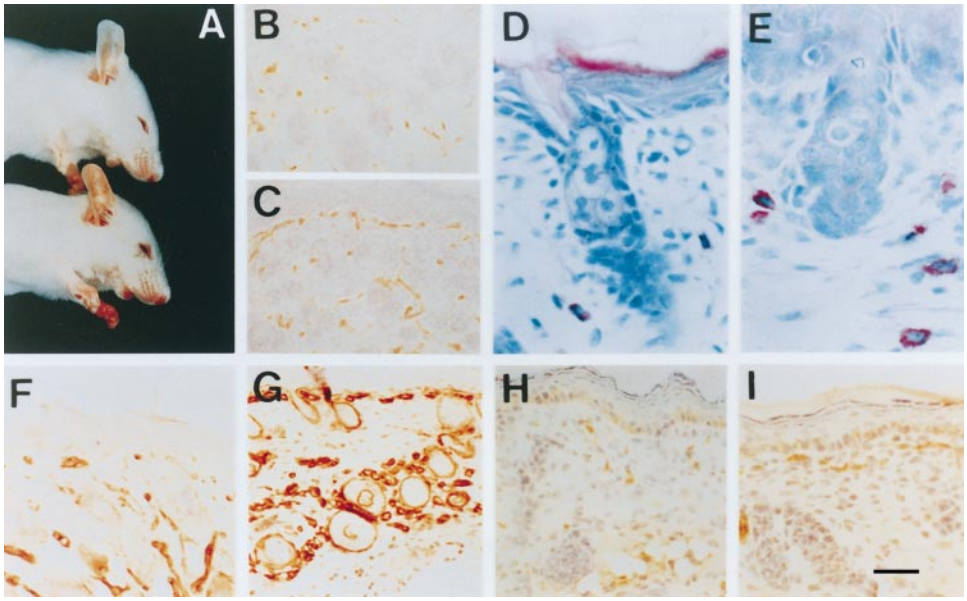

Figure 3. Increased vascularization in the skin of K14-VEGF transgenic mice. (*A*) Three week old male transgenic mouse and wild-type littermate. Note the increased vascularization of transgenic skin, most visible in the area of the inner surface of the ear. (*B, C*) Staining of frozen sections of wild-type and transgenic abdominal skin, respectively, with anti-mouse PECAM-1 (CD 31). Chloroacetate-esterase staining of 3 μm plastic sections of wild-type (*D*) and transgenic (*E*) skin. Mast cells are visualized by purple granules. (*F, G*) Anti-mouse collagen type IV; (*H, I*) anti-mouse ICAM-1 (CD 54) in wild-type (*F, H*) and transgenic (*G, I*) skin. *Scale bars*: (*B, C*) 25 μm; (*D, E*) 100 μm; (*F–I*) 50 μm.

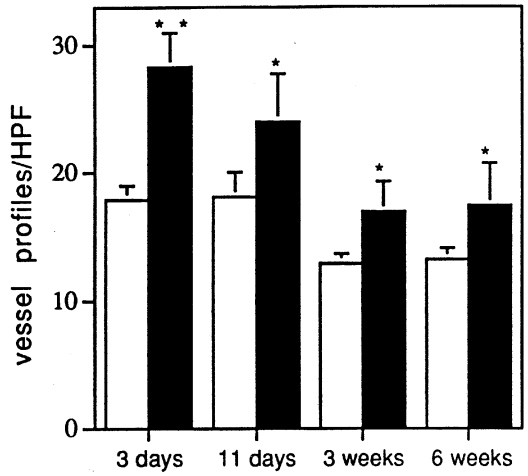

Figure 4. Increased density of microvessels underlying the epidermal–dermal basement membrane in the abdominal skin of VEGF transgenic mice (*black bars*), as compared with wild-type controls (*white bars*). Microvascular density was measured by the average number of microvascular profiles per high-power field. Mean ± SD (n = 6 for each group). *$p < 0.05$; **$p < 0.01$.

with increased expression in psoriasis and other inflammatory diseases characterized by tortuous, hyperpermeable microvessels and increased inflammatory infiltration (Detmar *et al*, 1994; Brown *et al*, 1995a, b). To characterize the biologic importance of VEGF for skin angiogenesis *in vivo*, we generated a transgene construct to overexpress VEGF selectively in the epidermis of transgenic mice, using a keratin 14 promoter expression cassette that reliably led to transgene expression in basal epidermal keratinocytes. Results of this study provide evidence that selective overexpression of VEGF in mouse epidermis was sufficient to induce an increased number of dermal blood microvessels, as demonstrated by morphometric analysis of skin samples stained for the endothelial cell junction molecule PECAM-1 (Dejana *et al*, 1995). In accordance with our findings, increased new blood vessel formation was recently reported when VEGF was expressed under control of a bovine rhodopsin promoter, targeting transgene expression to the retina (Okamoto *et al*, 1997). The increase in microvascular density was age dependent and was most prominent in the early postnatal period with a 58.3% increase of microvascular density in the upper dermis of K14/VEGF transgenic mice. At later time points, the increase in microvascular density was less pronounced, though still statistically significant, caused by a diminished expression level of the K14/VEGF construct with increasing age of the animals. *In vivo* fluorescence angiography of ear skin after intravenous injection of fluoresceinated high-molecular weight dextran confirmed an increased number of skin capillaries in transgenic mice. These vessels were tortuous and hyperpermeable, characteristic features of abnormal microvessels in psoriasis (Braverman and Sibley, 1982). Thus, these findings support our hypothesis that, in psoriatic lesions, keratinocyte-derived VEGF is able to penetrate the epidermal–dermal basement membrane barrier to exert its biologic effects on target endothelial cells in the upper dermis (Detmar, 1996).

In K14/VEGF transgenic mice, the expression of the VEGF receptor flk-1 (VEGFR-2) was highly increased in dermal microvessels in the vicinity of VEGF overexpressing keratinocytes, whereas the expression of flt-1 (VEGFR-1) was only moderately induced. These findings are comparable with the results of our previous *in situ* hybridization studies in psoriasis and other inflammatory skin diseases (Detmar *et al*, 1994; Brown *et al*, 1995a, b) that are characterized by epidermal VEGF overexpression, strong endothelial cell expression of VEGFR-2, and moderate expression of VEGFR-1. They also suggest a positive feedback mechanism by which VEGF may upregulate its receptors on endothelial cells *in vivo*. This concept is supported by a recent developmental study using a retroviral vector to overexpress VEGF in the avian limb, resulting in increased endothelial expression of VEGF receptors during early embryogenesis (Flamme *et al*, 1995).

The induced dermal microvessels in VEGF transgenic mice were stained by an antibody against collagen XVIII that has been suggested as a specific marker for blood vessel endothelial cells (Rehn and Pihlajaniemi, 1994). In contrast, no staining was observed with monoclonal antibodies against desmoplakin I and II that have been reported to be selectively expressed by lymphatic endothelial cells (Schmelz *et al*, 1994). Moreover, *in vivo* microlymphangiography after intradermal injection of fluoroscein isothiocyanate-labeled high molecular weight dextran, demonstrated normal numbers and diameters of lymphatic vessels in transgenic skin. Taken together, these findings indicate that the VEGF-induced dermal microvasculature is of blood vessel rather than lymphatic origin, and that VEGF acts as a selective angiogenesis

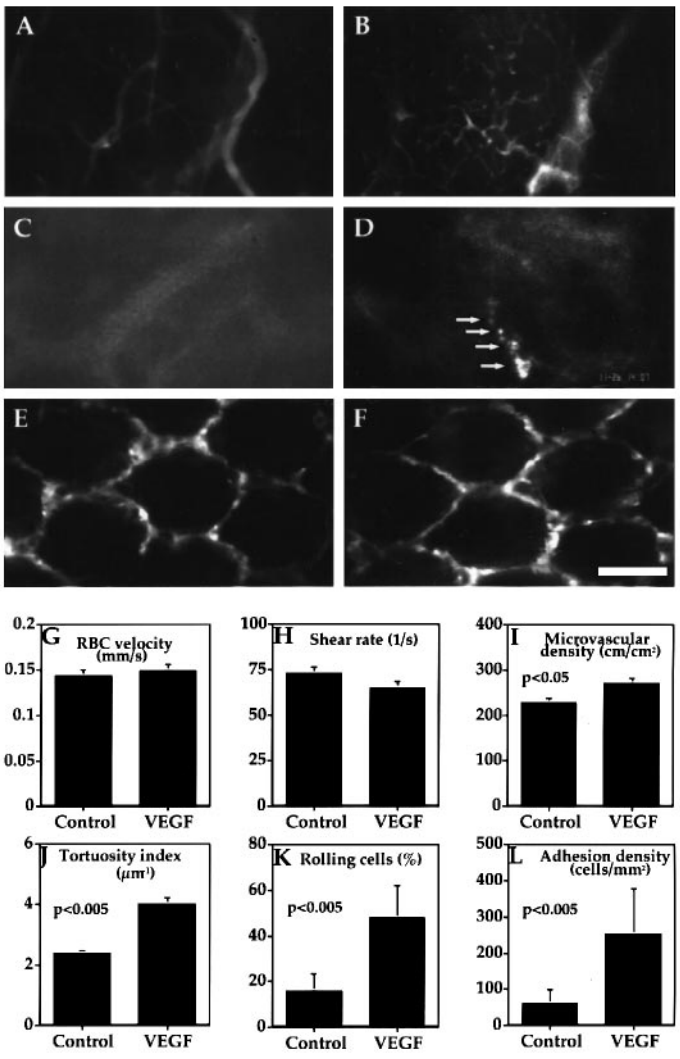

Figure 5. Intravital analysis of microcirculation, leukocyte rolling and adhesion, and lymphatic vascularization in VEGF transgenic skin. Increased numbers of tortuous and hyperpermeable microvessels in transgenic ear skin (*B*) as compared with littermate controls (*A*). After injection of Rhodamin-6G, increased numbers of leukocytes adherent to postcapillary venules were detected in transgenic mouse ear skin (*D*; *arrows*) as compared with controls (*C*). Parts (*E*) and (*F*) demonstrate similar numbers and diameters of lymphatic vessels in wild-type and VEGF transgenic tail skin. No significant differences of red blood cell velocity (*G*) and shear rate (*H*) were detected between wild-type and transgenic postcapillary venules; however, transgenic skin showed a 20.1% increase in microvascular density (*I*), a 69.0% increase in microvascular tortuosity (*J*), a 3-fold increase in leukocyte rolling (*K*), and a 4.1-fold increase in leukocyte adhesion to the wall of postcapillary venules (*L*). *Scale bars*: (*A*, *B*) 500 μm; (*C*, *D*) 250 μm; (*E*, *F*) 350 μm.

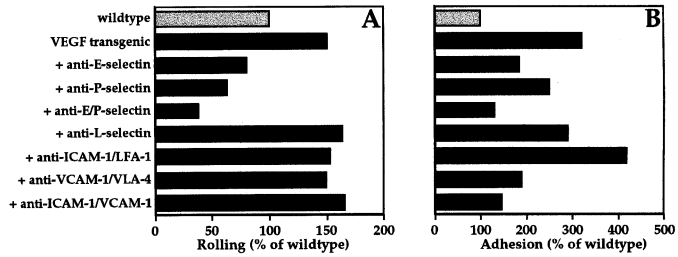

Figure 6. Specific inhibition of VEGF-induced leukocyte rolling and adhesion by blocking antibodies to adhesion molecules. Mice were injected with 150 μg of each of the respective blocking antibodies or with equivalent amounts of isotype control antibodies 2 h prior to injection of rhodamin-6G. Data for leukocyte rolling (*A*) and adhesion (*B*) are given as the percentage of wild-type control and represent means of 2–3 experiments.

factor for blood vessels in the skin. In contrast, a closely related new member of the VEGF family of growth factors, VEGF-C, did not induce blood microvessels, but caused lymphatic proliferation and vessel enlargement when selectively overexpressed in the epidermis of transgenic mice, using a keratin 14 promoter construct (Jeltsch *et al*, 1997). Thus, closely related angiogenesis factors exert clearly distinct effects on the vascular system.

Hyperpermeability of microvessels precedes and accompanies angiogenesis and vascular remodeling observed in inflammatory and neoplastic skin diseases, resulting in leakage of plasma proteins including clotting factors such as fibrinogen and prothrombin (for review, see Dvorak *et al*, 1995). Extravasated plasma proteins activate the extrinsic coagulation system, leading to the generation of thrombin and cleavage of fibrinogen to fibrin that is readily cross-linked by activated factor XIIIa. Cross-linked fibrin as well as other RGD-containing extravasated plasma proteins such as fibronectin and vitronectin provide a provisional matrix supporting endothelial cell migration. Our findings of increased microvascular permeability in the skin of VEGF transgenic mice, together with the observed increase in microvascular density, further support the hypothesis that VEGF promotes angiogenesis and vascular remodeling in skin diseases such as psoriasis and squamous cell carcinomas through both induction of vascular leakiness and direct stimulation of endothelial cell migration and proliferation (Detmar, 1996; Senger *et al*, 1996), and suggest a significant role of vascular hyperpermeability in the clinical course of these diseases.

In addition to the observed vascular changes, VEGF transgenic mice showed an approximately 35% increase in mast cell densities in the upper dermis. Although this increase was rather moderate, these findings are in agreement with observations of increased mast cell localization to sites of angiogenesis (Meininger and Zetter, 1992). In particular, psoriasis and murine psoriasiform skin lesions (Schön *et al*, 1997) are characterized by both increased epidermal VEGF expression (Detmar *et al*, 1994) and enhanced dermal mast cell numbers (Kreuter *et al*, 1978). At present, a mechanistic explanation for the mast accumulation in VEGF transgenic mice remains to be established. Whereas we were unable to detect expression of VEGFR-1 or VEGFR-2 by mast cells in the skin, a direct chemotactic effect of VEGF on mast cells *in vitro* has been recently reported (Gruber *et al*, 1995). Alternatively, VEGF released from transgenic epidermal keratinocytes might indirectly induce mast cell accumulation through induction of increased microvascular density in the skin that may lead to increased release of mast cell recruiting factors by endothelial cells. It has been shown that injection of stem cell factor into mouse skin potently induced mast cell hyperplasia (Tsai *et al*, 1991), and that overexpression of nerve growth factor in the skin of transgenic mice led to increased dermal mast cell density (Getchell *et al*, 1995). Both stem cell factor (Aye *et al*, 1992; Meininger *et al*, 1995) and nerve growth factor (M. Detmar and C. Pincelli, unpublished data) are produced by endothelial cells, and it remains to be explored in future studies whether VEGF might regulate their expression in dermal microvascular endothelial cells. Notwithstanding our incomplete knowledge of the molecular mechanisms involved, our results for the first time demonstrate a role of VEGF in mast cell recruitment to sites of angiogenesis *in vivo*.

VEGF transgenic mice were also characterized by dramatically increased leukocyte rolling (>3-fold) and adhesion (>4-fold) in postcapillary venules in ear skin. These findings were not due to increased numbers of circulating leukocytes, as white blood cell counts were comparable in wild-type and transgenic mice. Moreover, blood flow and shear rate were identical in postcapillary venules of wild-type and transgenic skin, suggesting that specific adhesion molecule interactions were responsible for the observed increase in leukocyte adhesion and rolling in transgenic skin. Indeed, *in vivo* blocking studies using intraperitoneal application of various combinations of blocking antibodies to adhesion molecules demonstrated that the increased number of rolling and adherent leukocytes was normalized after injection of an anti-E- and P-selectin antibody combination. We were unable, however, to detect pronounced alterations of E- or P-

selectin expression within the skin of VEGF transgenic mice by immunohistochemical staining. This may reflect the low sensitivity of the immunohistochemical approach, or may reflect only subtle differences in selectin expression that are already sufficient to lead to biologic effects. This is supported by the only moderate increase of *in vitro* E-selectin expression in VEGF treated human umbilical vein endothelial cells reported recently (Melder *et al*, 1996). Combinations of anti-VCAM-1/VLA-4 and VCAM-1/ICAM-1 antibodies potently down-regulated leukocyte adhesion. These findings are in accordance with the previously reported *in vitro* effects of VEGF on the expression of cell adhesion molecules in cultured human umbilical vein endothelial cells (Melder *et al*, 1996).

It is of interest that the observed increases in leukocyte rolling and adhesion in postcapillary venules in transgenic ear skin were not associated with increased numbers of infiltrating leukocytes in the dermis or epidermis in mouse ears. Immunohistochemical staining for CD3, CD4, CD8, CD11a, or CD49d did not reveal an increased number of infiltrating leukocytes in the dermis of transgenic mice. Thus, it appears that VEGF plays an important role in early steps of leukocyte recruitment to sites of angiogenesis, but is not sufficient to induce the entire cascade of leukocyte localization seen in inflammatory conditions.

In summary, targeted overexpression of VEGF in epidermal keratinocytes led to a significantly increased density of hyperpermeable and tortuous skin microvessels. Whereas VEGF has been widely regarded as an endothelial cell specific growth factor, VEGF transgenic mice also demonstrated increased mast cell numbers and enhanced leukocyte rolling and adhesion to postcapillary venules, characteristic features of healing wounds and chronic inflammatory skin diseases such as psoriasis. Thus, our findings suggest much broader, previously unsuspected biologic functions of VEGF, providing a molecular link between angiogenesis, mast cell accumulation, and enhanced leukocyte adhesion to postcapillary venules in chronic inflammation.

We thank K. Tognazzi, K. Herzberg, and D. Brown for their excellent technical assistance, and Drs. H.F. Dvorak, and R. Melder for their helpful comments. This work was supported by NIH/NCI grant CA69184, by Deutsche Forschungsgemeinschaft grant De483/3–2, by the National Psoriasis Foundation, and by the Beth Israel Pathology Foundation, Inc.

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