Mice overexpressing placenta growth factor exhibit increased vascularization and vessel permeability

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Summary

Placenta growth factor (PlGF) is a member of the vascular endothelial growth factor (VEGF) family, comprising at least five cytokines specifically involved in the regulation of vascular and/or lymphatic endothelium differentiation. Several lines of evidence indicate a role for PlGF in monocyte chemotaxis and in potentiating the activity of VEGF, but the exact function of this cytokine is not fully understood. To define the biological role of PlGF in vivo, we have produced a transgenic mouse model overexpressing this factor in the skin by using a keratin 14 promoter cassette. Our data indicate that PlGF has strong angiogenic properties in both fetal and adult life. PlGF overexpression results in a substantial increase in the number, branching and size of dermal blood vessels as well as in enhanced vascular permeability. Indeed, intradermally injected recombinant PlGF was able to induce vessel permeability in wild-type mice. The analysis of vascular endothelial growth factor receptor 1/flt-1 and vascular endothelial growth factor receptor 2/flk-1 indicates that the two receptors are induced in the skin endothelium of transgenic mice suggesting that both are involved in mediating the effect of overexpressed PlGF.

Key words: PlGF, Transgenic mice, Angiogenesis, Vessel permeability, VEGF

Introduction

Vasculogenesis is the embryonic process by which a primary capillary network is formed from differentiating endothelial cells. Angiogenesis, on the other hand, is the process responsible for the remodeling of this primary network into a mature vasculature during fetal life, and for tissue neovascularization in the adult (Carmeliet, 2000; Risau, 1997). Apart from wound healing and the reproductive and hair growth cycles, endothelium in the adult is in a resting status and neoangiogenesis is strictly associated with pathological events (Carmeliet and Jain, 2000).

Vascular endothelial growth factor (VEGF) is a mitogen for endothelial cells, at first characterized for its effect in inducing vessel permeability (Ferrara and Davis-Smith, 1997). VEGF is a key regulator of vasculogenesis and angiogenesis, as inactivation of even a single allele results in embryonic lethality (Carmeliet et al., 1996; Ferrara et al., 1996); it is also essential for growth and survival in early postnatal life when the endothelium is still proliferating (Gerber et al., 1999).

A family of growth factors structurally related to VEGF has been identified and includes placenta growth factor (PlGF), VEGF-B, VEGF-C and VEGF-D, all contributing to the regulation of the vascular and/or lymphatic endothelium development (Karkkainen and Petrova, 2000; Yancopoulos et al., 2000). PlGF is a dimeric glycoprotein that, like VEGF, can bind with high affinity to the tyrosine kinase receptor vascular endothelial growth factor receptor 1 (VEGFR-1/Flt-1) (Park et al., 1994; Persico et al., 1999). PlGF/VEGF heterodimers have been isolated and shown to bind also to the VEGFR-2/KDR/Flk-1 receptor (Cao et al., 1996; DiSalvo et al., 1995), the major mediator of VEGF activities.

With regard to the biological role of PlGF, homodimers are chemotactic for cultured endothelial cells and monocytes with an efficacy equivalent to that of VEGF (Clauss et al., 1996). PlGF has been reported to be ineffective in directly inducing endothelial cell proliferation or vascular permeability, but to act indirectly by potentiating the activity of nanomolar amounts of VEGF (Park et al., 1994). In vivo, recombinant PlGF can induce rabbit corneal vascularization (Ziche et al., 1997). Moreover, PlGF is upregulated during the active angiogenic phase of wound healing (Failla et al., 2000) and its expression has been linked to tumor angiogenesis (Donnini et al., 1999; Hatva et al., 1996; Lacal et al., 2000; Nomura et al., 1998; Takahashi et al., 1999).

To define the angiogenic role of PlGF in vivo, we have generated a transgenic mouse model constitutively overexpressing this factor in the skin under the human keratin 14 promoter (K14-PlGF-transgenic mice). Analysis of transgenic mice shows that overexpressed PlGF strongly induces vascularization, starting from fetal development and continuing during adult life. Our data also indicate that PlGF greatly enhances vessel permeability.

Materials and Methods

Mice

For generating transgenic mice, fertilized oocytes were obtained from

a (C57/Bl6×DBA/2) mouse cross. Transgenic lines were established on a BDF1 background.

All mice used for the present study were treated in accordance with the institutional guidelines for the care of experimental animals. For surgical procedures, mice were anesthetized by intraperitoneal injection of 15 μl/g of 2.5% 2,2,2-tribromoethil alcohol (Sigma-Aldrich, Milwaukee, WI).

Generation of K14-PlGF-transgenic mice

The full coding sequence of the cDNA for mouse PlGF (nucleotides 119-595, GenBank X80171) was ligated into a keratin 14 (K14) expression cassette (Vassar et al., 1989) (kindly provided by Elaine Fuchs, University of Chicago). The linearized transgene was purified and injected into fertilized oocytes. Mice were screened for the presence of the transgene by polymerase chain reaction (PCR) on genomic DNA using the following primers: forward 5′-GTGTCTCACATATTCAGTCC-3′, reverse 5′-CTCCTAGGGACTC-TAGAGG-3′ (amplification product, 315 bp). To confirm the presence of the integrated transgene and to quantify the number of inserted copies, mouse genomic DNA was *Bam*HI digested and analyzed by Southern blotting using the PlGF full length coding sequence as a probe.

RNase protection assay (RPA)

Total RNA was isolated from 6 mm back skin punch biopsies of adult mice or from the skin of 15.5 days post coitum (d.p.c.) embryos dissected under the stereomicroscope using the RNeasy kit (Qiagen, Chatsworth, CA). 3 μg of RNA were hybridized overnight with radiolabeled riboprobes, RNase- and proteinase K-treated and analyzed on a polyacrylamide gel as described (Odorisio et al., 1996). The PlGF probe was a 315 bp fragment of the transgene used to generate mice, comprising part of the PlGF cDNA and of the human K14 polyadenilation site, and was generated by PCR using the forward and reverse primers used to screen transgenic mice (see above). Labeled riboprobe was produced by in vitro transcription in the presence of [^{32}P]UTP (Amersham/Pharmacia Biotech, Uppsala, Sweden) as described (Krieg and Melton, 1987), and gel purified. The protected RNA fragments were 315 bp for the transgene and 285 bp for endogenous PlGF mRNA. The other probes used for RPA were cDNA fragments obtained by RT-PCR from mouse skin and cloned into a pCRII plasmid (Invitrogen, Leek, The Netherlands). More precisely, β-actin probe was a 250 bp fragment (bp 344-593; GenBank X03672), flt-1 was a 397 bp fragment (bp 2745-3141; GenBank D88689) and flk-1 was a 393 bp fragment (bp 268-660; GenBank X59397). Relative intensity of bands on autoradiograms was quantified using a GS-670 densitometer (Bio-Rad Laboratories, Hercules, CA).

Northern blot

Total RNA was isolated with Trizol (Invitrogen) following the manufacturer's instructions. 20 μg of RNA were electrophoresed through a 1.4% agarose gel containing 1.9 M formaldehyde. RNA was transferred to Hybond N$^+$ membrane (Amersham/Pharmacia) using 20× SSC and the membrane was hybridized to ^{32}P-labeled VEGF and β-actin cDNA probes using Quickhyb hybridization mix (Stratagene, Amsterdam, The Netherlands) as recommended by the manufacturer. Mouse VEGF and β-actin probes were the same as those used for RPA. Relative intensity of bands on autoradiograms was quantified using a GS-670 densitometer (Bio-Rad Laboratories).

In situ hybridization

In situ hybridization was performed on 4 μm thick paraformaldehyde-fixed, paraffin-embedded skin punch biopsies as previously described (Failla et al., 2000). PlGF sense and antisense riboprobes comprise the full coding sequence of mouse PlGF cDNA. Flt-1 and flk-1 probes were those described previously (Detmar et al., 1998). Vectors were linearized with appropriate enzymes and single-stranded sense and antisense RNA probes were transcribed using T3, T7 or SP6 RNA polymerases (Promega, Madison, WI) in the presence of [^{35}S]UTP (Amersham/Pharmacia Biotech), as described for RPA.

Immunohistochemical analysis

Flt-4 immunohistochemistry was performed on 8 μm thick cryosections. For all the other antibodies, 4 μm thick paraformaldehyde-fixed, paraffin-embedded sections were used and specimens were deparaffinized, rehydrated and processed as described (Failla et al., 2000). The antibodies used were: PECAM/CD31, an anti-mouse polyclonal antibody (M-20, Santa Cruz Biotechnology, Santa Cruz, CA) employed at a concentration of 2 μg/ml for 2 hours at 37°C; flt-4, a polyclonal antibody (sc-637, Santa Cruz Biotechnology) at a concentration of 1 μg/ml for 1 hour at room temperature (RT); Mac 3, a rat monoclonal antibody (clone M3/84; PharMingen Europe, Heidelberg, Germany) at a concentration of 5 μg/ml for 2 hours at RT; α-smooth muscle actin, a mouse monoclonal antibody (clone 144, Sigma-Aldrich) diluted 1:30, for 1 hour at RT. Negative controls were done by omitting the primary antibody.

Primary keratinocyte cultures

Keratinocytes were obtained from the skin of newborn (2 days post-partum) transgenic mice and wild-type littermates as described (Hager et al., 1999) except that after trypsin incubation the separated epidermis was placed in 0.05% DNase (Sigma-Aldrich). Keratinocytes were grown in N-Medium in which the fibroblast-conditioned medium was obtained from mouse 3T3-J2 (a gift from H. Green, Harvard Medical School, Boston, MA) cultures.

ELISA assay

Ninety-six-well polystyrene plates were coated with an affinity-purified polyclonal anti-mouse PlGF antibody (R&D Systems, Minneapolis, MN) as capture antibody. Triplicate 50 μl samples of keratinocyte conditioned medium or mouse blood were assayed as described (Harlow and Lane, 1988). A biotinylated polyclonal antibody against mouse PlGF-2 (R&D Systems) was used as secondary antibody and detection was performed with streptavidin-alkaline phosphatase and a colorimetric assay. Recombinant mouse PlGF-2 homodimer (R&D Systems) was used as standard. For VEGF the Quantikine Kit (R&D Systems) was employed. Statistical analysis was performed using the two-tailed *t*-test.

Western blotting analysis

Two 6 mm diameter punch biopsies were homogenized in RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 50 mM Tris-HCl pH 7.5) and immunoprecipitated using an anti-mouse PlGF polyclonal antibody (AF 465, R&D Systems) as previously described (Failla et al., 2000). 100 ng of mouse recombinant PlGF-2 (R&D Systems) were also immunoprecipitated as a positive control. Proteins were separated by 12% gel electrophoresis and transferred to a nitrocellulose filter (Amersham/Pharmacia). Protein detection was performed using a biotinylated polyclonal antibody against mouse PlGF (BAF 465, R&D Systems) and a chemioluminescence detection system (Amersham/Pharmacia).

Whole mount lectin binding analyses

Anesthetized mice were perfused with 20 ml of 5 μg/ml biotinylated-

Lycopersicon esculentum (tomato) lectin (Vector Laboratories, Burlingame, CA), 10 μg/ml of biotinylated-*Ricinus communis* (castor bean) lectin (Vector Laboratories) or 10 μg/ml of biotinylated-Wheat Germ Agglutinin lectin (Vector Laboratories), as described (Thurston et al., 1996; Thurston et al., 1998). All the analyses were carried out on groups of at least four mice.

Evans blue permeability assay and Miles assay

100 μl of a 30 mg/kg Evans blue dye (Sigma-Aldrich) solution in saline was injected into the tail vein of anesthetized transgenic and wild-type control mice. One hour after injection, mice were perfused as described (Thurston et al., 1998). Ears were removed, dried, weighted and left for 48 hours in 1 ml of formamide at 60°C to extract the Evans blue from the tissue. Dye concentration was measured at 595 nm and the value obtained reported as ng/mg of ear tissue. For Miles assay, after Evans blue dye injection, 100 μl of PBS alone or different concentrations of human recombinant PlGF (R&D Systems) were injected intradermally in 8-week-old CD1 female mice and the appearance of a blue spot monitored.

Ultrastructural analysis

Back skin biopsies were fixed in Karnovsky solution, postfixed in OsO_4 and embedded in Epon resin. Ultrathin sections were counterstained and observed in a Philips CM100 transmission electron microscope (Eindoven, The Netherlands).

Microlymphangiography

Microlymphangiography was performed by injecting in the tail dermis 10 μl of a 25% solution of FITC-dextran (M_r 2×10^6, Sigma-Aldrich) as described (Leu et al., 1994).

Results

Characterization of PlGF expression in transgenic mice

Four transgenic lines were established and two of them, containing approximately 12 and 10 transgene copies inserted in their genome and showing the highest levels of transgene transcription, were used for the phenotypic analysis. In situ hybridization on dorsal skin sections confirmed the targeted expression of the transgene to the epidermal basal layer and to the outer root sheet keratinocytes of hair follicles (Fig. 1C,D), while no specific hybridization could be detected in wild-type controls (Fig. 1A,B). RNase protection assay (RPA) analysis, using a riboprobe that discriminates the transgenic transcript from the endogenous one, showed a strong hybridization signal corresponding in length to the transgenic mRNA in K14-PlGF mouse skin (Fig. 1E). A faint signal from the endogenous mRNA could be detected in the skin of both transgenic and wild-type mice only after a long exposure time. By western blotting on skin lysates a band corresponding in molecular weight to mouse PlGF could be visualized only in transgenic mice, indicating that, as expected, PlGF is expressed below

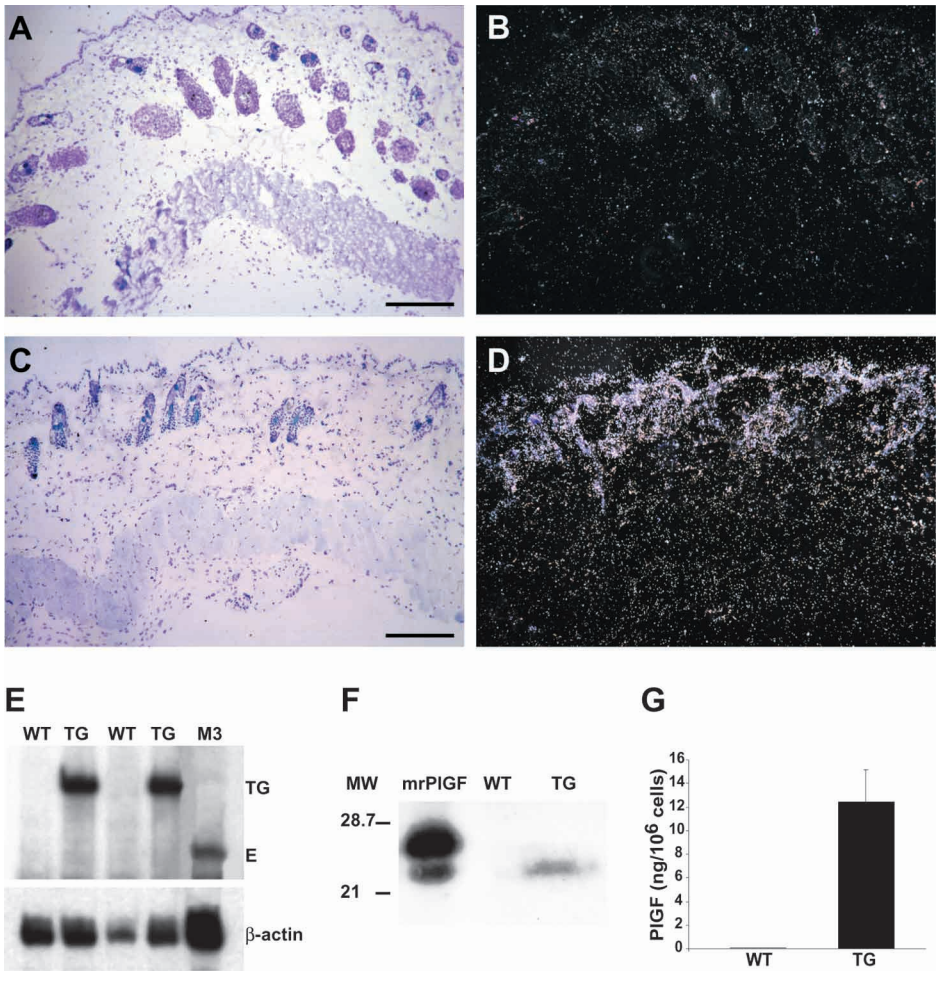

Fig. 1. PlGF overexpression in the skin of transgenic mice. In situ hybridization analysis with an antisense PlGF riboprobe on the back skin of wild-type (A,B) and transgenic (C,D) mice. (A,C) Bright field; (B,D) dark field. Bars, 100 μm. (E) RNase protection assay for PlGF on total RNA from back skin biopsies of wild-type (WT) and transgenic (TG) littermates. The murine melanoma cell line M3 was used as a positive control for the endogenous PlGF transcript (E). (F) Western blot for PlGF on skin lysates. As a control, mouse recombinant PlGF (mrPlGF) was included. (G) ELISA for PlGF on 24-hour-conditioned medium of cultured keratinocytes from newborn transgenic and wild-type mice.

detectable levels in wild-type animals (Fig. 1F). Moreover, high levels of PlGF protein (12.46±2.67 ng/10^6 cells, mean±s.d.) were measured by enzyme linked immunoadsorbent assay (ELISA) in the 24-hour-conditioned medium of cultured keratinocytes isolated from newborn transgenic mice, while PlGF was barely detectable in the supernatant of keratinocytes from wild-type littermates (52.40±19.50 pg/10^6 cells, mean±s.d.) (Fig. 1G). Significantly increased PlGF levels (P=0.0214) were also detected in the blood of transgenic mice (463±168 pg/ml, mean±s.d.) compared with wild-type littermates (159±57 pg/ml, mean±s.d.), suggesting that overexpressed PlGF could reach and possibly act on other tissues in addition to skin.

By contrast, homodimeric VEGF secreted by keratinocytes of newborn transgenic mice (259.18±63.86 pg/10^6 cells, mean±s.d.) was significantly reduced (P=0.00062) compared with control littermates (713.25±101.88 pg/10^6 cells, mean±s.d.), suggesting a possible negative regulation on VEGF by overexpressed PlGF. In addition, the evaluation of homodimeric VEGF on cultured keratinocyte lysates showed a significant decrease (P=0.0291) in transgenic mice (24.12±3.95 pg/10^6 cells, mean±s.d.) compared with wild-type littermates (49.59±17.43 pg/10^6 cells, mean±s.d.). However, northern blot analysis on total RNA extracted from cultured keratinocytes indicated that the mRNA levels for VEGF were comparable in transgenic and wild-type mice (data not shown).

Increased dermal vascularization in K14-PlGF mice

Transgenic mice from the two lines analyzed looked healthy and bred normally, despite being somewhat smaller than their wild-type littermates. K14-PlGF skin appeared swollen and redder than that of control mice at birth (Fig. 2A). Since K14 starts to be expressed at 14.5 days post coitum (d.p.c), we checked whether the reddish color of the skin was evident in transgenic fetuses. A redder color of the skin, suggesting increased vascularization, was visible starting from day 16.5 of fetal development in K14-PlGF mice compared with wild-type controls (data not shown). The erythematous color of the skin not only persisted in adult transgenic mice but became even more intense with age (Fig. 2B).

To visualize the dermal vasculature in transgenic mice, a whole mount analysis of the ears after vascular perfusion with *Lycopersicon esculentum* biotin-labeled lectin was performed. This analysis revealed that dermal vessels, of both the venular and the arteriolar component, were more numerous and branched, as well as strongly enlarged in K14-PlGF mice (Fig. 2C,D). Moreover, ear microvasculature of transgenic mice was characterized by neoangiogenesis-associated phenomena, such as collateral vessel emission, intussusception (Fig. 2E) and numerous varicosities (Fig. 2F) resembling the glomeruloid bodies described in VEGF-induced and tumor-associated angiogenesis (Sundberg et al., 2001). When mice at different ages (8 weeks, 12 weeks and 18 weeks) were analyzed with the same technique, an age-related increase in the dermal vascularization could be appreciated (data not shown).

To quantify the extent of dermal vascularization induction in K14-PlGF mice, immunohistochemical staining was performed on dorsal skin sections using an antibody against the endothelial cell-specific marker PECAM/CD-31 (Dejana et al., 1995). Computer-assisted morphometric analysis of stained sections confirmed that the number of small blood vessels was significantly increased in the upper dermis of transgenic mice compared with wild-type littermates (Table 1). Moreover, the diameters of small blood vessels in the dermis of transgenic mice were strongly enlarged compared with those of control animals (Table 1). By staining serial skin sections with an antibody directed against the α-smooth muscle actin, which detects both smooth muscle cells and pericytes surrounding endothelial cells, a significant increase in smooth-muscle-coated vessels was detected in transgenic mice compared with those of wild-type littermates; such an increase was comparable with that observed for blood vessel density by PECAM staining (Table 1).

Vessel branching was quantified by measuring the distance between branches in the small vessels from *L. esculentum*

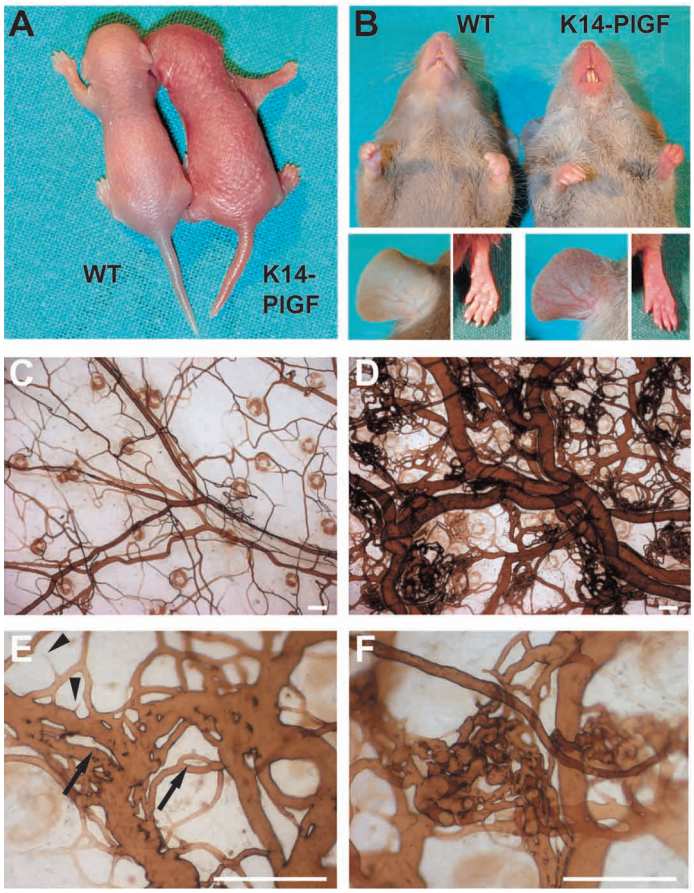

Fig. 2. Hypervascularized skin phenotype of PlGF-transgenic mice. (A) Newborn mice are visibly redder than wild-type controls. (B) The redder color of the skin in transgenic mice persists in the adult (10-week-old). Enlarged vessels are evident in the ear of transgenic mice compared with controls. (C,D) Whole mount ear preparations from control (C) and transgenic (D) 10-week-old mice perfused with biotinylated-*L. esculentum* lectin showing the increase in vessel number, tortuosity and size in the transgenic ears. (E,F) Signs of active angiogenesis in transgenic mice: vessel spike emission (E, arrowheads), intussusception (E, arrows) and glomeruloid bodies (F). Bars, 100 μm.

Table 1. Comparison of different vessel parameters in transgenic and wild-type mice

	Wild-type	Transgenic	*P* value
Blood vessel density (vessels/mm^2)	159.03±3.44	199.39±3.37	0.034
Smooth muscle actin-stained vessel density (vessels/mm^2)	150.58±2.33	192.11±3.88	0.049
Average blood vessel area (μm^2)	26.73±3.27	110.42±17.12	0.00029
Average blood vessel length without branching (μm)	36.11±1.36	11.57±0.45	<0.00001
Lymphatic vessel density (vessels/mm^2)	123.09±1.04	126.53±1.17	0.725

Ten independent fields/animal were analyzed for each parameter, corresponding to a total of 67 and 107 blood vessel areas and 296 and 312 blood vessel tracts without branching for wild-type and transgenic mice, respectively. The fields analyzed were localized in the upper dermis. Areas and distances were calculated by computer-assisted image analysis, using a Zeiss KS300, Version 3.0 program. Results are expressed as mean±s.e.m. The two-tailed *t*-test was employed to determine the probability value (*P*).

whole-mount ear preparations. A strong increase in vessel branching was seen in transgenic mice compared with that in wild-type controls (Table 1).

When immunohistochemistry was performed using an antibody directed against the VEGFR-3/Flt-4 tyrosine kinase receptor, which is specifically expressed in the lymphatic endothelium in the adult (Skobe and Detmar, 2000), no significant difference in the number of dermal lymphatic vessels in transgenic and wild-type mice could be detected (Table 1). Moreover, whole mount analysis by fluorescent microlymphoangiography indicated that the diameter of lymphatic vessels was not increased in transgenic mice compared with that in wild-type littermates (Fig. 3).

PlGF is chemotactic for monocytes (Clauss et al., 1996). Therefore, we monitored whether an increased number of monocytes/macrophages was recruited in the skin of K14-PlGF mice by staining skin sections with an antibody directed against the Mac-3 antigen expressed on mouse monocytes and activated macrophages. As shown in Table 2, no significant difference in the number of macrophages/monocytes in wild-type and transgenic mice could be detected.

Increased vascular permeability in K14-PlGF mice

One of the features of neoangiogenesis is represented by vascular hyperpermeability to plasma proteins, leading to deposition of an extravascular fibrin gel, which acts as a substrate for endothelial cell growth and migration. PlGF has been reported to be ineffective in inducing vessel permeability per se, but to strongly potentiate VEGF activity (Park et al., 1994). To analyze the permeability of the vasculature in K14-PlGF mice we injected the Evans blue dye into the tail vein of transgenic mice and wild-type controls and monitored the extravasation of the dye. After buffer perfusion and fixation, the skin of transgenic mice appeared visibly bluer than that of wild-type littermates indicating a possible increase in vascular leakage in K14-PlGF mice (Fig. 4A,B). Spectrophotometric quantitation of the extravasated blue confirmed the augmented leakage, with a fourfold increase in the dye concentration in transgenic mice (65.81±14.05 ng/mg, mean±s.d.) compared with that in wild-type controls (17.79±2.81 ng/mg, mean±s.d.). To assess whether the increased amount of extravasated dye was due only to enhanced vessel density in transgenic mice or also to increased leakage sites in the endothelium, mice were perfused with biotin-labeled *Ricinus communis* I lectin, which binds strongly to the basement membrane (Thurston et al., 1996). In wild-type mice lectin bound uniformly and faintly to the luminal surface of blood vessels (Fig. 4C), while in transgenic animals numerous dark staining patches, corresponding to sites of binding to exposed basement membrane, were distributed in the endothelium (Fig. 4D). Perfusion with the wheat germ agglutinin biotin-labeled lectin, which allows discrimination of veins from

Table 2. Monocyte/macrophage density in the upper dermis of transgenic and wild-type mice

	Wild-type	Transgenic	*P* value
Monocyte/macrophage density (cells/mm^2)	288.21±9.69	269.83±6.56	0.633

Ten independent fields/animal were analyzed. Results are expressed as mean±s.e.m. The two-tailed *t*-test was employed to determine the probability value (*P*).

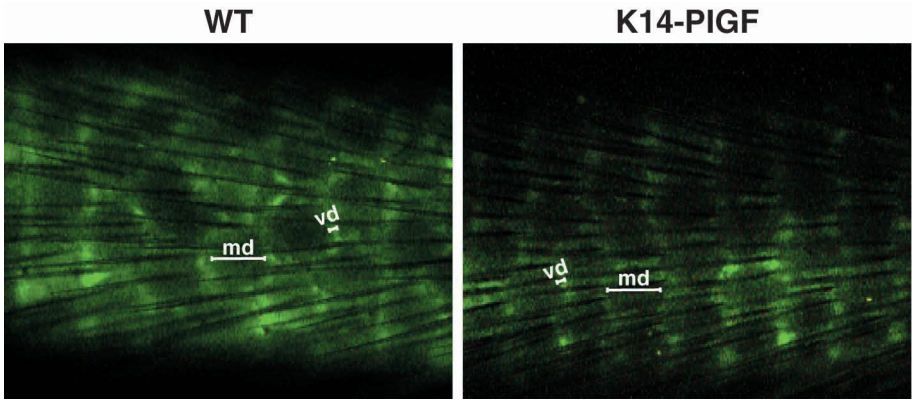

Fig. 3. Microlymphoangiography of the tail skin showing that in K14-PlGF-transgenic mice both lymphatic vessel diameters (vd) and mesh diameters (md) are comparable in size with those of wild-type mice.

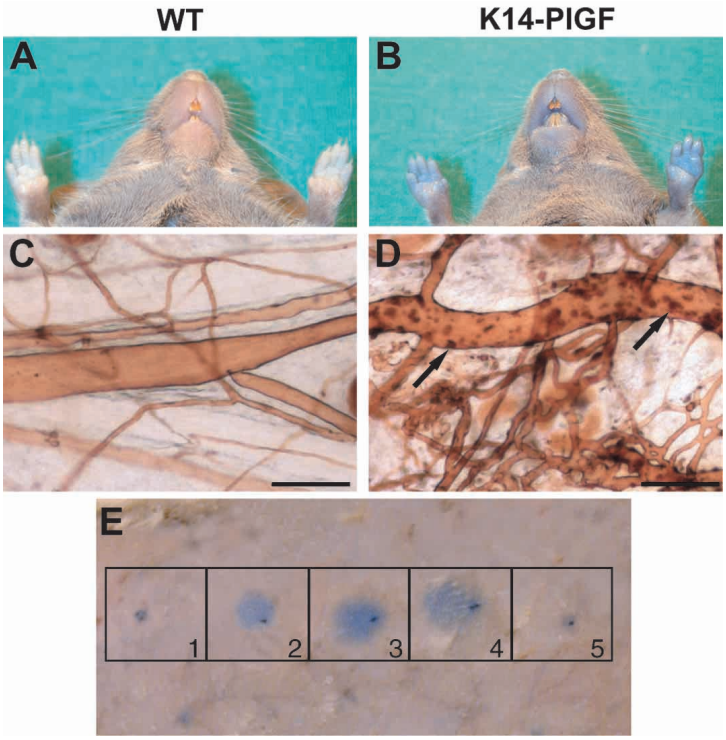

Fig. 4. Enhanced vascular permeability in K14-PlGF mice. (A,B) Evans blue dye-perfused transgenic mice show increased extravasated blue tracer in the skin compared with perfused control mice. (C,D) Whole mount ear preparations from 10-week-old mice perfused with biotinylated *R. communis* lectin. Numerous leakage sites are present in the ear vessels of transgenic mice (D, arrows). Bars, 100 μm. (E) Miles vascular permeability assay. 1-4: 25, 50, 125, 250 ng of injected PlGF, respectively; 5: site of PBS injection.

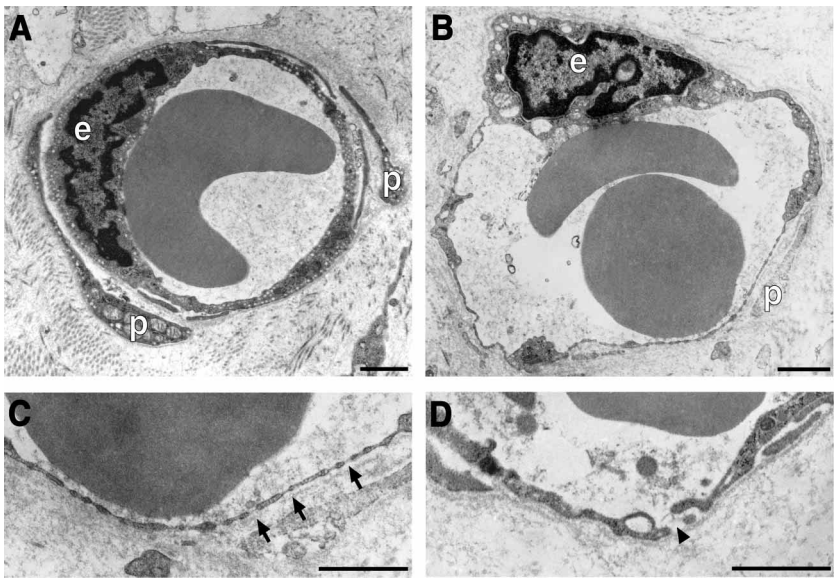

Fig. 5. Ultrastructure of dermal capillaries in the skin of transgenic (B) and control (A) mice. The plasma membrane of endothelial cells (e) appears thinner and more irregular in transgenic mice compared with wild-type littermates. Several fenestrae (C, arrows) and gaps (D, arrowhead) are visible in the plasma membrane of transgenic mouse endothelial cells. p, pericyte. Bars, 1 μm.

arteries (Thurston et al., 1996), demonstrated that the vast majority of leakage sites were localized in venules and capillaries (data not shown), as described in models of acute inflammation (McDonald, 1994). Ultrastructural analysis further confirmed the increased vessel permeability in transgenic mice, revealing the presence of multiple fenestrations and gaps in the skin endothelium (Fig. 5).

To verify the ability of PlGF to induce vessel permeability in vivo, human recombinant PlGF was injected in the skin of wild-type mice in a Miles assay. Administrated PlGF resulted in Evans blue extravasation in a dose-dependent manner (Fig. 4E).

Overexpressed PlGF upregulates both flt-1 and flk-1 in transgenic mice

To evaluate whether the angiogenic phenotype we observed in K14-PlGF mice could be mediated by PlGF activation of VEGFR-1/flt-1 in endothelial cells, we analyzed the transcription of this receptor and of VEGFR-2/flk-1 by in situ hybridization on embryos at 15.5 d.p.c., the stage just before the appearance of a visible hypervascularized phenotype in the transgenic fetuses. The probe used for the analysis of flt-1 recognizes the intracytoplasmic domain of the receptor (Detmar et al., 1998), thus discriminating the active form from the soluble one, which comprises only the extracellular domain (Kendall and Thomas, 1993). In the skin of wild-type fetuses, a hybridization signal for flt-1 mRNA was not detectable (Fig. 6A,B) and the expression of this mRNA was induced in the dermal endothelium of transgenic embryos (Fig. 6C,D). Flk-1 was transcribed in the skin endothelium of wild-type animals (Fig. 6E,F) and significantly upregulated in transgenic embryos (Fig. 6G,H) compared with wild-type littermates.

To quantify flt-1 and flk-1 transcription in transgenic and wild-type mice an RNase protection assay experiment was performed on total RNA extracted from the skin of 15.5 d.p.c. embryos. The results obtained confirmed that both flt-1 and flk-1 transcripts are increased in transgenic embryos (Fig. 6I,J). Normalization of the hybridization signals with β-actin revealed that the increase in the transcription of the two receptors was similar, being 1.9-fold for flk-1 and 2.3-fold for flt-1 (n=3).

Discussion

The role of PlGF as an angiogenic factor has been debated for a long time. In this study we investigated the in vivo angiogenic activity of PlGF by overexpressing this factor in the skin under the control of a keratin-14 promoter. Our results show that PlGF has strong angiogenic properties in vivo, its overexpression resulting in an increase in the number and a striking induction in the

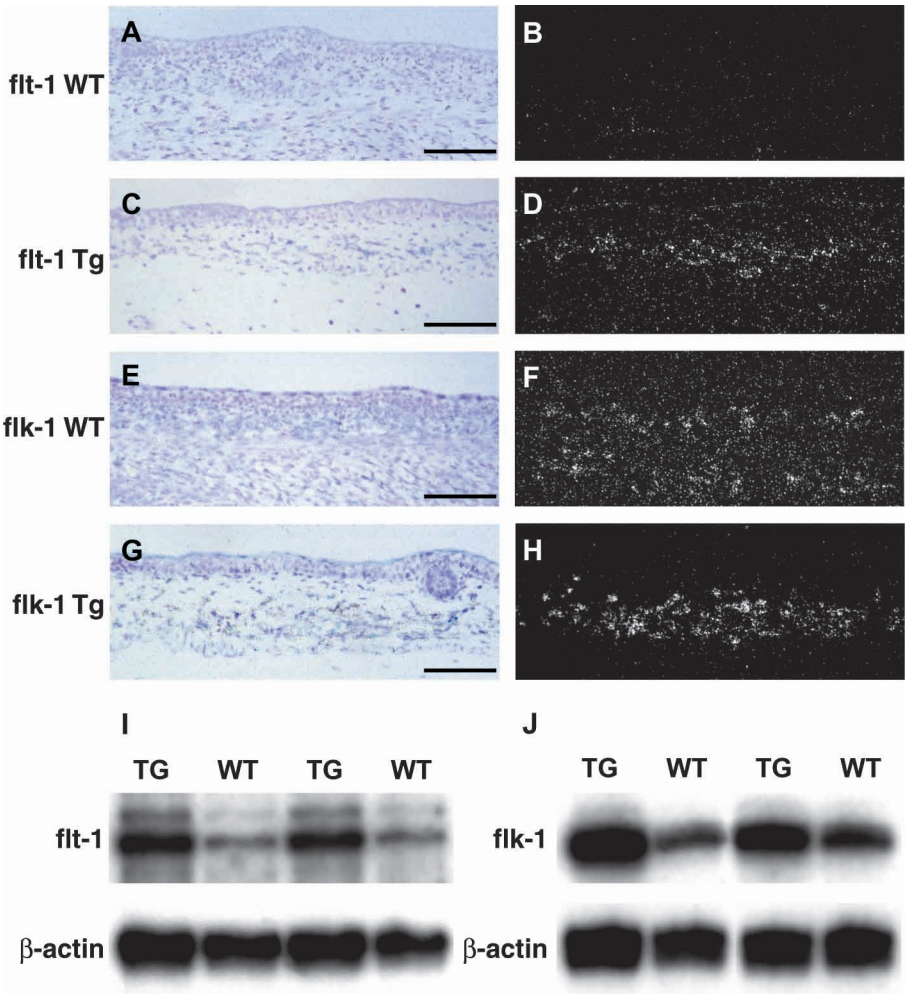

Fig. 6. Flt-1 and flk-1induction in the skin of transgenic mice. In situ hybridization analysis for flt-1 and flk-1 in the skin of 15.5 d.p.c. embryos. The mRNA for flt-1 transmembrane receptor is undetectable in the skin of wild-type embryos (A,B) and is induced in the dermal endothelium of transgenic embryos (C,D). Flk-1 is transcribed in the skin endothelium of wild-type mice (E,F) and consistently upregulated in the dermal endothelium of transgenic embryos (G,H). (A,C,E,G) Bright field; (B,D,F,H) dark field. Bars, 40 μm. RNase protection assay for flt-1 (I) and flk-1 (J) confirmed that mRNA levels for both receptor are increased in the skin of transgenic 15.5 d.p.c. embryos compared with wild-type controls.

branching and size of blood vessels as well as in enhanced vascular leakiness. PlGF overexpression effects are specific for blood vessels, as transgenic mouse lymphatic vessels appear neither more numerous nor hypertrophic.

K14-PlGF transgenic mice show a clear hypervascularized phenotype starting from fetal development and continuing throughout the adult life. Recently published observations from PlGF knockout mice indicate that this factor is redundant for vasculogenesis, as well as for fetal angiogenesis, and define a role for PlGF in supporting VEGF activity in adult pathological angiogenesis (Carmeliet et al., 2001). By contrast, our data indicate that, despite not being essential for correct fetal angiogenesis, PlGF is able to potently stimulate it. The lack of fetal angiogenesis impairment observed in PlGF knockout mice may be due to the low levels of PlGF physiologically expressed in the embryo (Persico et al., 1999).

The increasing vascularization with age, associated with multiple signs of active neoangiogenesis in the dermal microvasculature, indicate that also the quiescent endothelium of the adult is responsive to PlGF activation. Endothelial cell-dependence on VEGF has been reported to be lost after the fourth postnatal week (Gerber et al., 1999), and mice overexpressing VEGF in the skin manifest the strongest vascular induction in the postnatal period, the number of dermal vessels declining thereafter (Detmar et al., 1998). Our data support the concept that the role played by VEGF in adult angiogenesis could depend on the concomitant induction of PlGF. Indeed, during wound healing PlGF is expressed in endothelial cells of small blood vessels surrounding the granulation tissue prior to VEGF induction in migrating keratinocytes (Failla et al., 2000), suggesting that an autocrine loop is important for activating the endothelium and making it responsive to VEGF.

VEGFR1/Flt-1 receptor is expressed on human pericytes and smooth muscle cells (Ishida et al., 2001; Wang and Keiser, 1998) and PlGF has been shown to induce smooth muscle cell migration but not proliferation in vitro (Ishida et al., 2001). We therefore tested whether in K14-PlGF mice the increased dermal vascularization was associated with enhanced recruitment of pericytes/smooth muscle cells. By staining skin sections with an antibody recognizing the α-smooth muscle actin we could observe that the increase in vessel density, detected by PECAM/CD31 staining, was comparable with that in smooth-muscle-coated vessels. These data are in agreement with the reported observation that PlGF knockout mice contain more naked vessels than smooth-muscle-coated vessels in the wound granulation tissue compared with wild-type controls (Carmeliet et al., 2001).

Despite the chemotactic effect of PlGF on monocytes (Clauss et al., 1996), we could not detect any significant

increase in the density of these cells in transgenic mice compared with that in wild-type mice. An explanation for this finding might be that in our transgenic mice the high levels of PlGF constantly secreted, starting from fetal development, may render circulating monocytes unresponsive to its chemotactic effect. Further investigations are required to clarify this aspect.

Interestingly, we found a significant decrease in the amount of both released and intracellular homodimeric VEGF in cultured keratinocytes of transgenic mice compared with wild-type littermates. In the same cells, VEGF mRNA levels were similar, thus excluding a negative regulatory effect of overexpressed PlGF on VEGF transcription. VEGF monomers might form heterodimers with PlGF present in excess within the cell, with a reduction in the total amount of homodimeric cellular and secreted VEGF. Indeed, higher amounts of VEGF/PlGF heterodimers were released by cultured keratinocytes in transgenic mice compared with wild-type littermates (T.O., C.S., M.L.Z. et al., unpublished), even though we could not exactly quantify the amount of heterodimers because of the lack of a mouse VEGF/PlGF standard.

PlGF has been thought to act indirectly in angiogenesis by displacing VEGF from the decoy VEGFR-1/Flt-1 receptor and making it available for activating VEGFR-2/KDR/Flk-1 (Park et al., 1994). This latter receptor mediates the full spectrum of VEGF angiogenic activities (Ferrara, 1999). PlGF could also act directly in inducing angiogenesis by binding and activating the VEGFR-1/Flt-1 receptor. A question that arises from the analysis of K14-PlGF mice is whether the angiogenic effect we observe can be ascribed to the direct or indirect effect of overexpressed PlGF. The vascular phenotype of mice overexpressing PlGF has several features that differ from those of VEGF-induced angiogenesis (Detmar et al., 1998). In addition to showing the strongest vascularization in the postnatal period, mice overexpressing VEGF display a moderate increase in vessel number and no significant change in vessel size. Together with the observation that homodimeric VEGF is downregulated in cultured keratinocytes from K14-PlGF-transgenic mice, such different vascular traits suggest a direct role of overexpressed PlGF in inducing angiogenesis, independent of the hypothesized effect as a decoy agent.

By in situ hybridization on a 15.5 d.p.c. embryo, we could show an induction of both flk-1 and the transducing membrane form of flt-1 in the skin endothelial cells of transgenic mice compared with wild-type controls. The quantitation of receptor transcription by RPA confirmed such induction and indicated that the increment is comparable in the two receptors. The observation that the transcription levels of both flt-1 and flk-1 are upregulated supports the hypothesis that overexpressed PlGF, on one side, directly induces angiogenesis by binding and activating its own receptor and, on the other side, acts by potentiating the effect of VEGF and/or VEGF/PlGF heterodimers through upregulation of the flk-1 receptor.

The striking angiogenic phenotype induced by overexpressed PlGF raises the possibility that this factor could be used in the modulation of angiogenesis for therapeutic purposes. If PlGF activity is necessary for making the endothelium responsive to VEGF and amplifying its effect in adult neoangiogenesis, the coordinated administration of these two factors could be used to obtain a stronger angiogenic effect at a lower VEGF dosage. Moreover, in cases where angiogenesis is associated with disease, an antagonist to PlGF activity, acting upstream of the VEGF signal cascade, might be effective also in blocking VEGF-induced angiogenesis. Nevertheless, increased permeability could be a limit of PlGF-induced therapeutic angiogenesis. A study with double-transgenic mice overexpressing in the skin both VEGF and angiopoietin-1 (Ang-1), an angiogenic factor playing a major role in vascular remodeling during angiogenesis (Suri et al., 1996), has indicated that these two factors have an additive effect in inducing vascularization and that Ang-1 is able to oppose the effect of VEGF on vessel permeability (Thurston et al., 1999). The combined use of different factors may therefore be the way to go for a more accurate therapeutic modulation of neoangiogenesis.

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