

VEGF and VEGF-C: Specific Induction of Angiogenesis and Lymphangiogenesis in the Differentiated Avian Chorioallantoic Membrane

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The lymphangiogenic potency of endothelial growth factors has not been studied to date. This is partially due to the lack of *in vivo* lymphangiogenesis assays. We have studied the lymphatics of differentiated avian chorioallantoic membrane (CAM) using microinjection of Mercor resin, semi- and ultrathin sectioning, immunohistochemical detection of fibronectin and α -smooth muscle actin, and *in situ* hybridization with VEGFR-2 and VEGFR-3 probes. CAM is drained by lymphatic vessels which are arranged in a regular pattern. Arterioles and arteries are accompanied by a pair of interconnected lymphatics and form a plexus around bigger arteries. Veins are also associated with lymphatics, particularly larger veins, which are surrounded by a lymphatic plexus. The lymphatics are characterized by an extremely thin endothelial lining, pores, and the absence of a basal lamina. Patches of the extracellular matrix can be stained with an antibody against fibronectin. Lymphatic endothelial cells of differentiated CAM show ultrastructural features of this cell type. CAM lymphatics do not possess mediae. In contrast, the lymphatic trunks of the umbilical stalk are invested by a single but discontinuous layer of smooth muscle cells. CAM lymphatics express VEGFR-2 and VEGFR-3. Both the regular pattern and the typical structure of these lymphatics suggest that CAM is a suitable site to study the *in vivo* effects of potential lymphangiogenic factors. We have studied the effects of VEGF homo- and heterodimers, VEGF/PlGF heterodimers, and PlGF and VEGF-C homodimers on Day 13 CAM. All the growth factors containing at least one VEGF chain are angiogenic but do not induce lymphangiogenesis. PlGF-1 and PlGF-2 are neither angiogenic nor lymphangiogenic. VEGF-C is the first lymphangiogenic factor and seems to be highly chemoattractive for lymphatic endothelial cells. It induces proliferation of lymphatic endothelial cells and development of new lymphatic sinuses which are directed immediately beneath the chorionic epithelium. Our studies show that VEGF and VEGF-C are specific angiogenic and lymphangiogenic growth factors, respectively. © 1997 Academic Press

INTRODUCTION

The development of blood vessels (angiogenesis) has been studied extensively during the past years, whereas the development of lymphatic vessels (lymphangiogenesis) has gained relatively little attention, despite the clinical relevance of lymphangiogenesis. It has been suggested to be a key process linking lymphological syndromes such as lymphedema, lymphangiectasia, lymphangioma, and lymphangiosarcoma (Witte and Witte, 1986).

Since the introduction of the chorioallantoic membrane (CAM) assay (Folkman, 1974), this method has often been used to study effects of potential angiogenic factors *in vivo*.

In a modified version of the CAM assay employing differentiated CAM (Wilting *et al.*, 1991, 1992), the vascular endothelial growth factor (VEGF) (Senger *et al.*, 1983; Connolly *et al.*, 1989; Leung *et al.*, 1989) has been shown to be a highly specific angiogenic factor. However, it has rarely been noted that CAM is drained by a dense network of lymphatic vessels, although the existence of these vessels has already been described by Budge (1887). CAM therefore appears to be a suitable site to study the effects of both potential angiogenic and lymphangiogenic factors.

Secreted proteins highly homologous to VEGF have recently been found. Among these, placenta growth factor (PlGF) shares 40% identity with VEGF (Maglione *et al.*,

1991). The function of PlGF is unknown. VEGF-B, which is highly expressed in skeletal muscle and heart, possesses 43% identity with VEGF (Olofsson *et al.*, 1996). Its function has yet to be identified. Furthermore, a growth factor with about 30% identity with VEGF has been named VEGF-C (Joukov *et al.*, 1996). VEGF-C appears to be a potential lymphangiogenic factor, as both VEGF-C receptors, VEGFR-2 and VEGFR-3 (Joukov *et al.*, 1996), are expressed in lymphatic endothelial cells (Kaipainen *et al.*, 1995; Wilting *et al.*, 1996, 1997a).

As a prerequisite for studying the effect of potential lymphangiogenic factors, the morphology and pattern of the lymphatics of normal CAM must be described. We have therefore studied the lymphatics of differentiated CAM of chick and quail embryos using semi- and ultrathin sectioning, immunohistochemical staining with anti- α -smooth muscle actin and fibronectin antibodies, *in situ* hybridization with VEGFR-2 and VEGFR-3 probes, and Mercox injection. We aimed to determine whether VEGF and PlGF homo- and heterodimers and VEGF-C homodimers affect both blood vascular and lymphatic endothelial cells.

MATERIAL AND METHODS

Embryos

Fertilized eggs of the White Leghorn chick (*Gallus gallus*) and the Japanese quail (*Coturnix coturnix*) were incubated at 37.8°C and 80% humidity. Differentiated CAM of 16-day-old chick and 13-day-old quail embryos was studied with the following methods.

Histology

Specimens were fixed in 3% glutaraldehyde and 2% formaldehyde in 0.12 M sodium cacodylate buffer, postfixed with 1% osmium solution, immersed with uranyl acetate, and embedded in Epon resin (Serva, Heidelberg, Germany). Semithin (0.75 μ m) and ultrathin (70 nm) sections were cut with an Ultracut S (Leica, Bensheim, Germany). Ultrathin sections were studied with an EM 10 (Zeiss, Stuttgart, Germany).

Immunohistochemistry

Specimens were fixed in Serra's solution (Serra, 1946), dehydrated, and embedded in Durcupan resin (Fluka, Buchs, Switzerland). Polymerization was performed at 40°C. Semithin sections (1.5 μ m) were collected on silan-coated slides. The resin was dissolved with 20% sodium ethoxide (Merck, Munich, Germany) and the sections were rinsed with ethanol and bleached with methanol/H₂O₂. Anti- α -smooth muscle actin antibody (Sigma, Deisenhofen, Germany), diluted 1:5000, was used as first antibody. Peroxidase-conjugated goat anti-mouse IgG (Sigma; 1:200) was used as second antibody and DAB as chromogen. The B3/D6 fibronectin antibody (Gardner and Fambrough, 1983) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA) and diluted 1:500. Second antibody and DAB staining were described above.

Injection Method

The lymphatics of 16-day-old chick embryos were perfused with glutaraldehyde/formaldehyde fixative. Two milliliters of Mercox-blue (Norwald, Hamburg, Germany) was mixed with 25 μ l of accelerator and injected into the lymphatics of CAM using fine glass needles and a micromanipulator.

In Situ Hybridization

In situ hybridization was performed on quail tissue with *Quek1*/VEGFR-2 and *Quek2*/VEGFR-3 probes (Eichmann *et al.*, 1993, 1996). The probes were cloned into pcDNA/Amp (Invitrogen, San Diego). The linearized antisense probes were 3.0 (*Quek1*) and 1.5 kb (*Quek2*) in length. Specimens were fixed in Serra's solution, dehydrated, and embedded in paraffin. Eight-micrometer sections were mounted on silan-coated slides, dewaxed, and hybridized at 65°C overnight. After washing, the sections were incubated with an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (1:4000) at 4°C overnight. After washing, nitroblue tetrazolium and X-phosphate (Boehringer, Mannheim, Germany) were used as chromogens to reveal a blue signal. The sense probes were used as controls and did not show a signal (Wilting *et al.*, 1997a).

CAM Assay

On Day 4 of development, a window was made into the egg shell of chick eggs. The embryos were checked for normal development and the eggs sealed with cellotape. They were further incubated until Day 13. Thermanox coverslips (Nunc, Naperville, IL) were cut into disks of about 5 mm in diameter. Sterile and salt-free growth factors were dissolved in distilled water and about 3.3 μ g/5 μ l was pipetted on the disks. After air-drying, the inverted disks were applied on CAM. After 3 days, the specimens were fixed in 3% glutaraldehyde and 2% formaldehyde and rinsed in 0.12 M sodium cacodylate buffer. They were photographed with a stereomicroscope (Wild M8) and embedded for semi- and ultrathin sectioning as described above. Controls were performed with carrier disks alone.

Growth Factors

The following human recombinant growth factors were applied on CAM of 13-day-old chick embryos: VEGF₁₂₁ (46 experiments), VEGF₁₆₅ (29), VEGF_{121/165} heterodimer (27), PlGF-1 (11), PlGF-2 (14), VEGF₁₂₁/PlGF-1 heterodimer (33), and VEGF₁₆₅/PlGF-2 heterodimer (6). Production of the growth factors in *Escherichia coli* and subsequent purification and dimerization have been described recently (Birkenhager *et al.*, 1996). VEGF-C (25) was produced in insect cells. The endogenous signal sequence for secretion was exchanged by that of melittin. The N- and C-termini that are proteolytically processed *in vivo* to obtain biological activity were omitted (Kukk *et al.*, 1996; Joukov *et al.*, 1996, 1997). All growth factors were desalted with a PD-10 column (Pharmacia, Uppsala, Sweden) and lyophilized.

Proliferation Studies

Proliferation of blood vascular and lymphatic endothelial cells was monitored with the BrdU/anti-BrdU method (Gratzner, 1982) after application of VEGF₁₂₁ and VEGF-C on CAM of 13-day-old chick embryos. One and two days later, the embryos were incu-

bated with 100 μ l of a 40 mM BrdU solution (Sigma) for 45 min. The specimens were then fixed with ethanol containing 3% acetic acid. Paraffin sections were stained as previously described by Wilting *et al.* (1994).

RESULTS

Pattern of CAM Lymphatics

Mercox injections into the lymphatics were performed in the region of the allantoic stalk. From here it was possible to fill both the intraembryonic lymphatics and those of CAM. A pair of lymphatics was found along the arteries and arterioles of CAM (Fig. 1a). Along the arteries, the pair of lymphatics was regularly interconnected by lymphatic capillaries, and blind-ending extensions were also visible (Fig. 1b). Furthermore, lymphatics were regularly detected along veins. A dense network of lymphatics was observed surrounding the largest veins (Fig. 1c). Where the arteries and veins were running in parallel, lymphatics were usually located in the angle between them. The CAM lymphatics were continued to the embryo by collecting ducts along the chorioallantoic artery and vein in the allantoic stalk (Fig. 1d). In the embryo, the lymphatic ducts were connected with the paired thoracoabdominal trunks (thoracic ducts) reaching right and left venous angles.

Structure of CAM Lymphatics

In semithin sections, the lymphatics could be distinguished from blood vessels because of their extremely thin endothelial lining (Fig. 2a). Pores were regularly present between lymphatic endothelial cells. Formation of a media was not observed. This was confirmed in ultrathin sections (Fig. 2b). Here it was noted that lymphatic endothelial cells possessed lamellipodia in both luminal and abluminal directions (Figs. 2b and 2c). Focal contacts were observed connecting the cells. In the cytoplasm smooth and rough endoplasmic reticulum, free ribosomes, Golgi apparatus, and numerous mitochondria were observed (Fig. 2d). Furthermore, coated pits and vesicles with different inclusions were present. Bundles of filaments were found in some areas (Fig. 2d). A basal lamina was not present, but patches of extracellular matrix were visible. These patches could be stained with an antibody against fibronectin. Small amounts of fibronectin were present throughout the stroma of CAM. Higher amounts were detected in the media of veins (Fig. 2e).

The presence of smooth muscle cells of CAM lymphatics was also studied in semithin sections using an antibody against α -smooth muscle actin. Smooth muscle cells were found in all parts of the vascular tree except for the capillaries. The lymphatics of CAM did not possess a media (Fig. 3a). In contrast, media formation was observed in the region of the allantoic stalk. Here the collecting lymphatic ducts possessed a single but discontinuous layer of smooth muscle cells (Figs. 3b–3d).

Receptors of CAM Lymphatics

The expression of VEGFR-2 and VEGFR-3 in differentiated CAM of 13-day-old quail embryos was studied with digoxigenin-labeled RNA probes. In a previous study (Wilting *et al.*, 1996) it was shown that VEGFR-2 was expressed in a subpopulation of arterial endothelial cells and in accompanying vessels, which appeared to be lymphatics. This result could be confirmed (Fig. 4a). The vascular endothelial cells of the intraepithelial capillaries were negative. Furthermore, we observed expression of VEGFR-2 in a subpopulation of venous endothelial cells. Endothelial cells of the accompanying lymphatic plexus were also positive (Fig. 4b). Therefore, in differentiated CAM, a subpopulation of arterial and venous endothelial cells and those of the lymphatics expressed VEGFR-2.

In accordance with previous studies (Wilting *et al.*, 1996) we did not observe expression of VEGFR-3 in endothelial cells of arteries and capillaries (Fig. 4c). Our additional studies on CAM veins showed that these were also negative (Fig. 4d). However, a signal was detected in endothelial cells of vessels which were located adjacent to arteries and veins, indicating that these were lymphatics (Figs. 4c and 4d). Therefore, the combination of both VEGFR-2 and VEGFR-3 was only observed in lymphatic endothelium of differentiated CAM.

Effects of Endothelial Growth Factors

The angiogenic potential of different VEGF splice forms on differentiated CAM has been documented (Wilting *et al.*, 1992, 1993, 1996). The effects of PlGF homodimers, VEGF/PlGF heterodimers, and VEGF-C homodimers have not been studied in detail, and, except for VEGF₁₂₁, the potential lymphangiogenic potency of growth factors has not been studied (Wilting *et al.*, 1996).

VEGF and PlGF homo- and heterodimers were produced in *E. coli* (Birkenhäger *et al.*, 1996) and about 3.3 μ g of each factor was applied on CAM of 13-day-old chick embryos. The macroscopically visible effects are shown in Fig. 5; the corresponding semithin sections are shown in Fig. 6. In the controls, no alterations of the vascular pattern were visible (Fig. 5a). The capillary plexus was located in the chorionic epithelium, and all larger conducting and draining vessels with the accompanying lymphatics were found in the stroma of CAM (Fig. 6a). VEGF₁₂₁ induced angiogenesis. Formation of new capillaries was seen in the precapillary area of the vasculature (Fig. 5b). A great number of capillaries penetrated the stroma of CAM, which normally does not contain capillaries (Fig. 6b). The lymphatics appeared not to be affected. We did not observe sprouting or dilatation of the lymphatics in the application area (Fig. 6b). The same results were obtained with VEGF₁₆₅ and the heterodimer VEGF_{121/165}. Both forms of the growth factor induced new capillaries, whereas the lymphatics were not affected (Figs. 5c, 5d, 6c and 6d). A different result was obtained with PlGF-1 and PlGF-2. Angiogenesis and lymphangiogenesis were not detected either macroscopically (Figs. 5e and 5f)

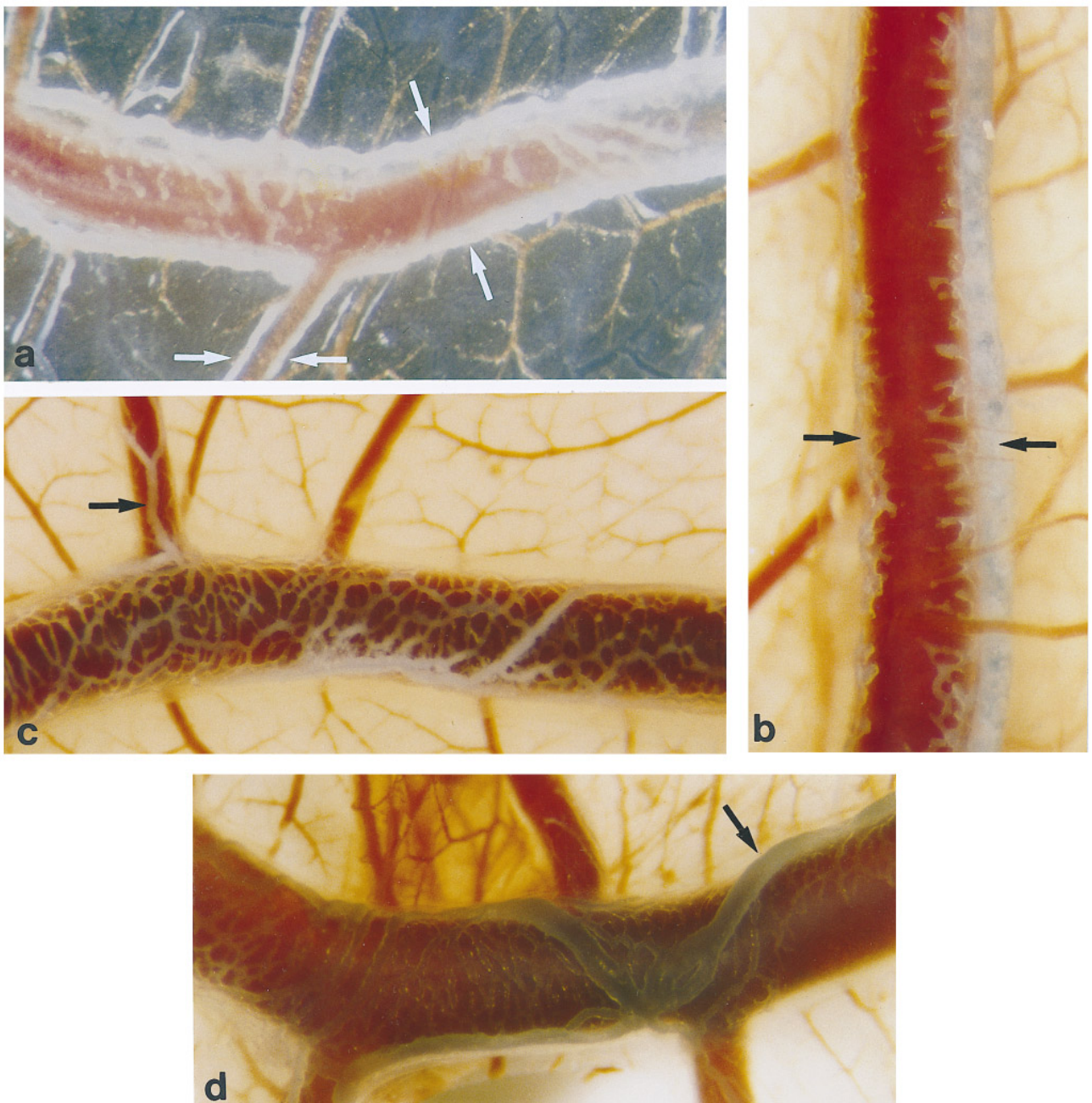
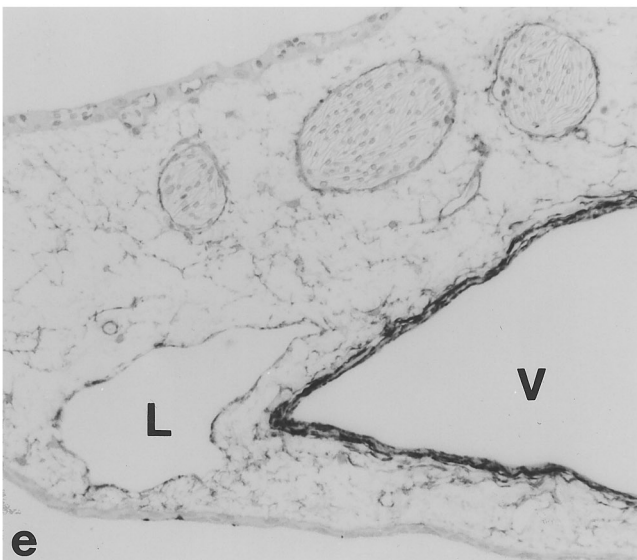
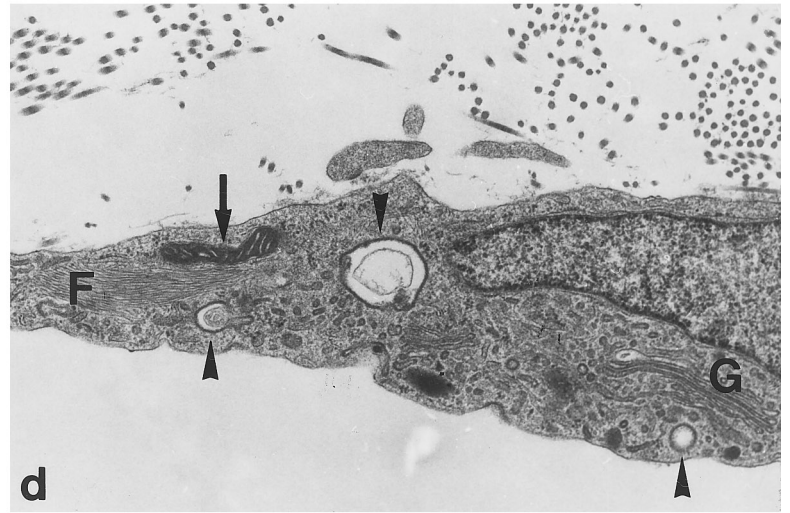
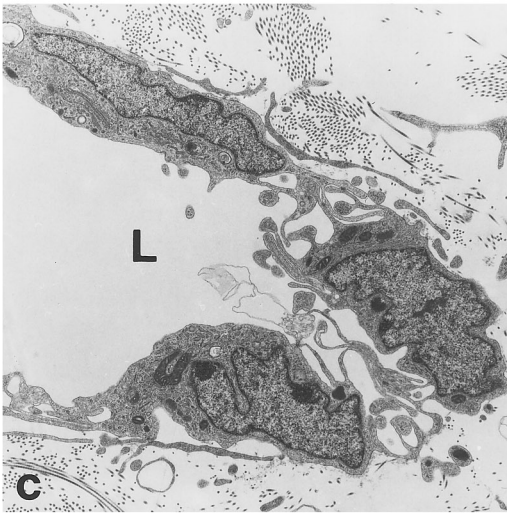
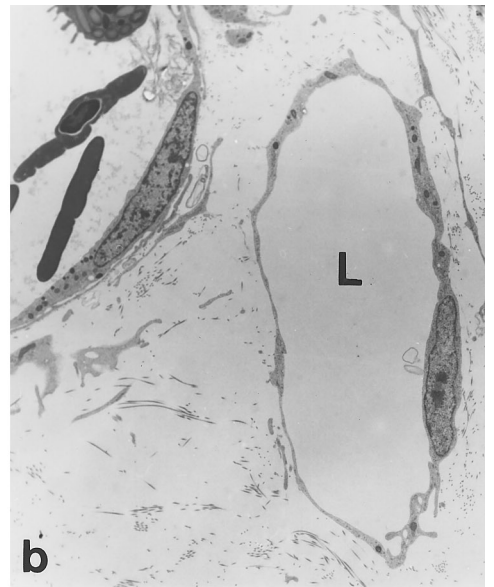
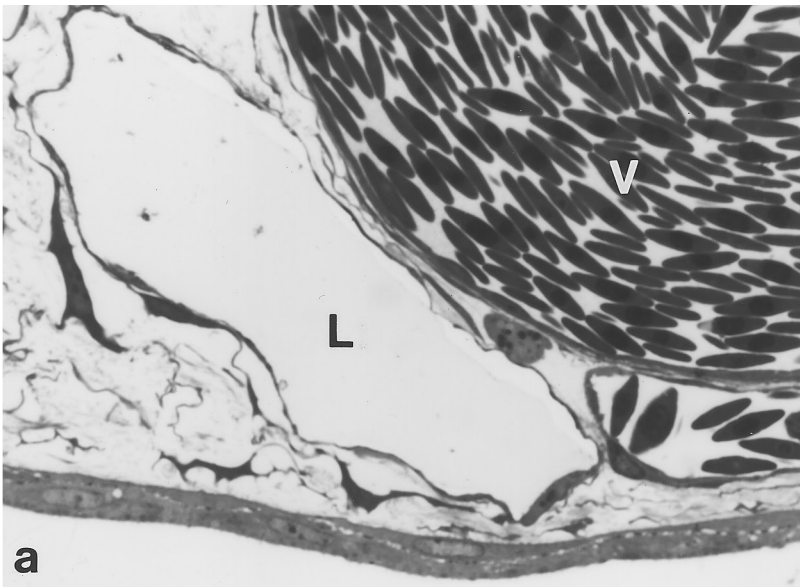


FIG. 1. Intralymphatic injection of Mercox-blue into a 16-day-old chick embryo. (a) A pair of lymphatics (arrows) is present beside arteries and arterioles of CAM. (b) Higher magnification showing an artery accompanied by a pair of lymphatics (arrows). (c) CAM veins accompanied by lymphatics (arrow) and surrounded by a lymphatic plexus. (d) Vein leading to the allantoic stalk, surrounded by a lymphatic plexus. Note formation of lymphatic trunk (arrow). Magnification $\times 20$.

or in semithin sections (Figs. 6e and 6f). However, the heterodimers VEGF₁₂₁/PlGF-1 and VEGF₁₆₅/PlGF-2 were angiogenic (Figs. 5g, 5h, and 6g). An effect of the smallest, non-heparin-binding form could be detected even at some distance from the application area. As before, the lymphatics remained unaffected (Fig. 6g).

The mitogenic effect of VEGF₁₂₁ was studied by monitoring the incorporation of BrdU into the DNA. In previous studies we have found that VEGF induces a three- to four-fold increase of the proliferation rate of endothelial cells of CAM (Wilting *et al.*, 1993; Kurz *et al.*, 1995). We now distinguished between blood vascular and lymphatic endo-



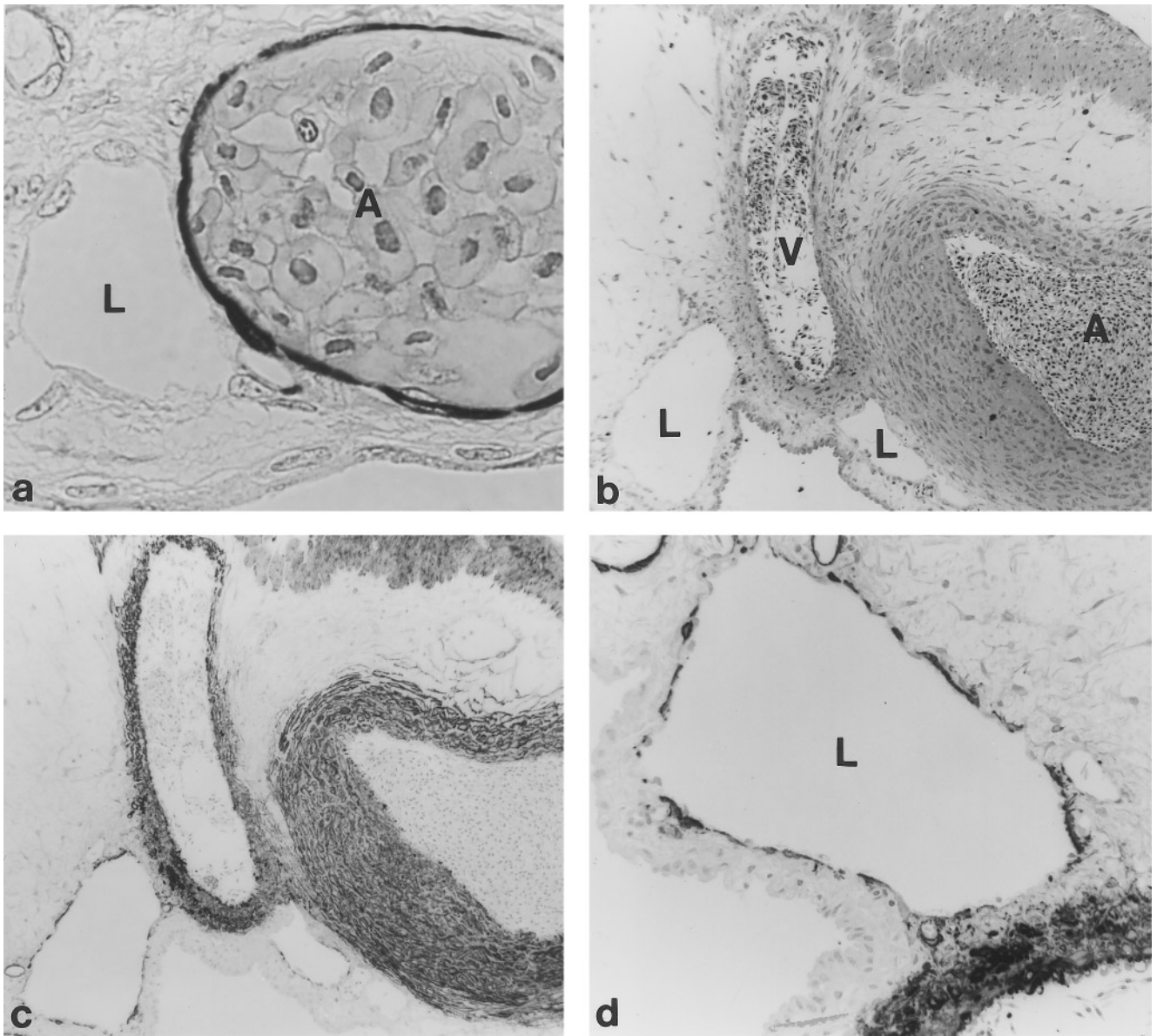


FIG. 3. Semithin sections of CAM and the allantoic stalk of 16-day-old chick embryos. (a, c, d) Anti- α -smooth muscle actin staining. (a) CAM artery (A) invested by smooth muscle cells. The lymphatics (L) do not possess a media. Magnification $\times 1100$. (b) Section showing umbilical artery (A), vein (V), and lymphatic trunks (L). Magnification $\times 120$. (c) Parallel section showing the presence of a media of lymphatic trunks. Magnification $\times 120$. (d) Higher magnification of c. The lymphatic trunk (L) is invested by a single layer of smooth muscle cells. Magnification $\times 300$.

FIG. 2. Structure of CAM lymphatics of 16-day-old chick embryos. (a) Semithin section showing a vein (V) and a lymphatic capillary (L) with its thin endothelial lining. Magnification $\times 1100$. (b) Ultrathin section of a lymphatic capillary. There is no investment by any other cell type. Magnification $\times 2500$. (c) Lymphatic endothelial cells (L) with numerous lamellinodia. Magnification $\times 3500$. (d) Higher magnification of c. Lymphatic endothelial cell with Golgi apparatus (G), microfilaments (F), mitochondria (arrow), and several vesicles (arrowheads). There is no continuous basal lamina. Magnification $\times 15,500$. (e) Semithin section stained for fibronectin. There is weak staining in the stroma and around lymphatic capillaries (L) and strong staining in the media of veins (V). Magnification $\times 300$.

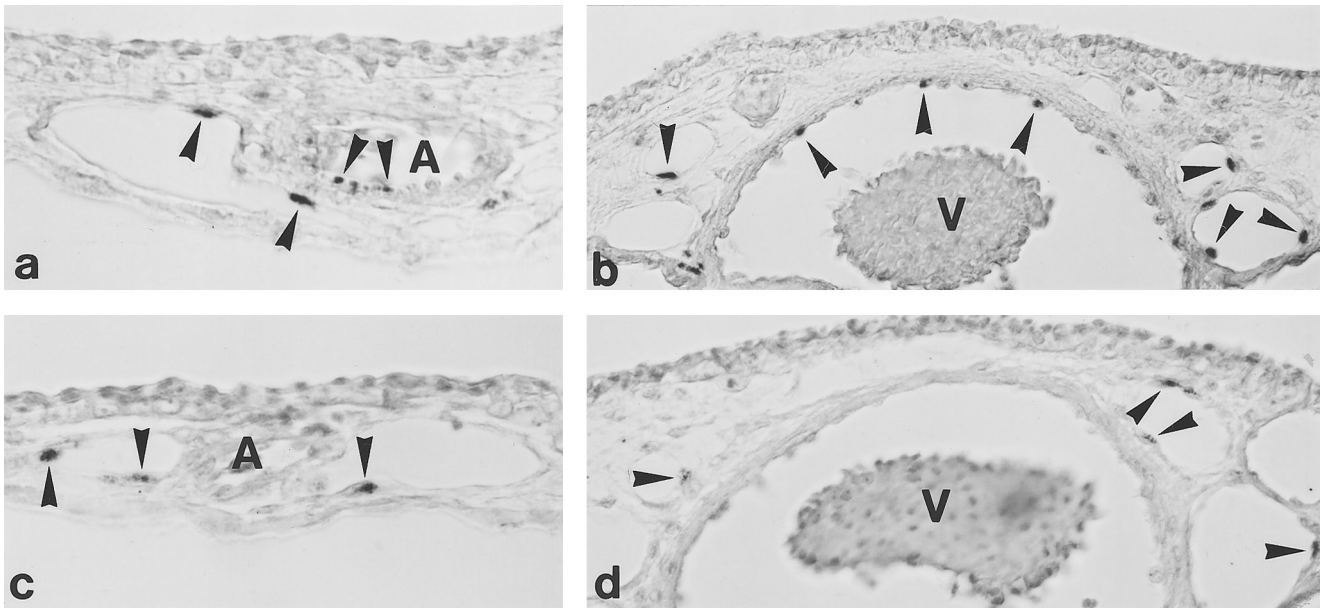


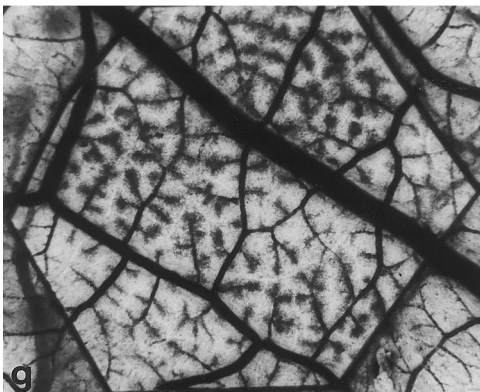
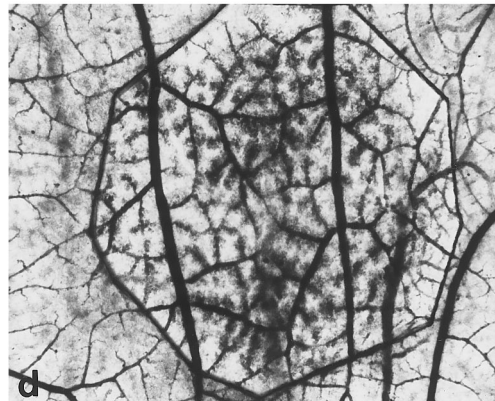
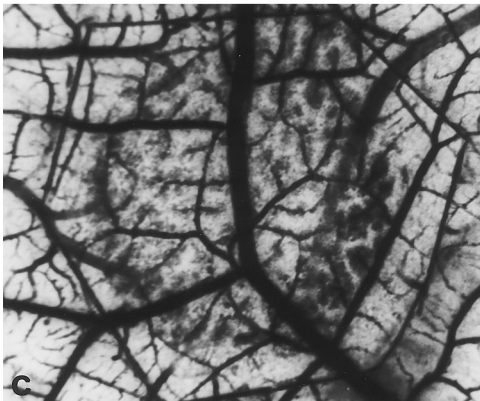
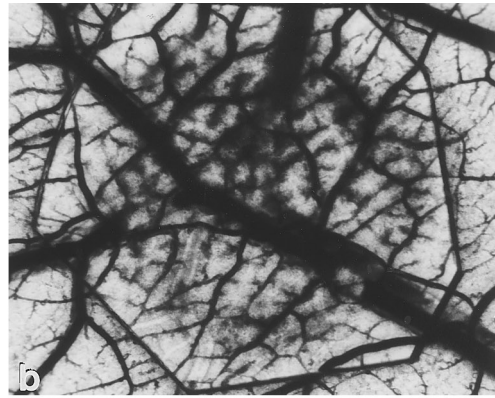
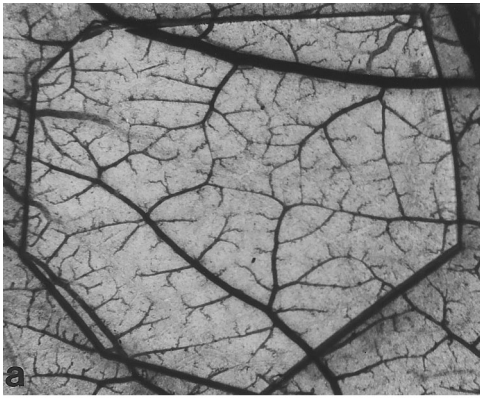
FIG. 4. *In situ* hybridization on paraffin sections of CAM of 13-day-old quail embryos. (a) VEGFR-2/*Quek1* expression (arrowheads) is detectable in endothelial cells of arteries (A) and accompanying lymphatics. Magnification $\times 600$. (b) VEGFR-2/*Quek1* is expressed in endothelial cells (arrowheads) of veins (V) and accompanying lymphatics. Magnification $\times 400$. (c) VEGFR-3/*Quek2* expression (arrowheads) is detectable in endothelial cells of lymphatics. A, artery. Magnification $\times 600$. (d) VEGFR-3/*Quek2* expression (arrowheads) in a lymphatic plexus surrounding a vein (V). Magnification $\times 400$.

thelial cells. A great number of BrdU-labeled blood vascular endothelial cells were found after application of VEGF₁₂₁, whereas those of the lymphatics were negative (Fig. 7).

VEGF-C was produced in insect cells (Kukuk *et al.*, 1996; Joukov *et al.*, 1996, 1997) and about 3.3 μg was applied on differentiated CAM. When a 5- μl droplet of VEGF-C-containing solution was dried on the carrier disk, most of the protein was found at the border of the droplet. Therefore, the highest amount of growth factor was arranged in a circle, as has also been demonstrated with other proteins (Wilting *et al.*, 1991). According to the distribution of the growth factor, a circular effect of VEGF-C was visible (Fig. 8a). Within this circle, a weak alteration of the vascular system was observed. However, this was not the main effect. By injecting Mercox-blue into the lymphatics it was possible to demonstrate that in the circular VEGF-C-affected area numerous lymphatics were present (Fig. 8b). High density of lymphatics was observed in the whole application site. A huge lymphatic sinus was located in the circle of highest growth factor concentration. Whereas the normal lymphatics of differentiated CAM were accompanying blood vessels, the newly

formed lymphatics could as well be found as isolated vessels. In semithin sections, the lymphatic nature of the VEGF-C induced vessels was also visible. They were huge sinuses with an extremely thin endothelial lining (Fig. 8c). Many folds and endothelial-lined islands were present within the sinuses, indicative of plexus formation. As there were no obvious signs of sprouting, the new lymphatics seemed to grow by intercalation. They were located immediately beneath the chorionic epithelium, where they are never found in normal CAM (Figs. 8c and 8d). In the circular region of highest growth factor concentration newly formed vascular capillaries surrounded the lymphatics (Fig. 8d). The extremely thin endothelial lining of the lymphatics and the absence of a basal lamina were also visible in ultrathin sections (Fig. 8e). Here it was also observed that there was no formation of a media, which could be confirmed in semithin sections stained with an antibody against α -smooth muscle actin (not shown). Proliferation studies revealed a great amount of BrdU-labeled nuclei of lymphatic endothelial cells after 1 and 2 days of VEGF-C application (Fig. 8f), the effect being greater after 1 day.

FIG. 5. Effects of endothelial growth factors applied on differentiated CAM of chick embryos. (a) Control. The carrier does not induce vascular alterations. (b) VEGF₁₂₁ induces angiogenesis. (c) VEGF₁₆₅ induces angiogenesis. (d) VEGF_{121/165} heterodimer induces angiogenesis. (e) PlGF-1 does not induce angiogenesis. (f) PlGF-2 does not induce angiogenesis. (g) VEGF₁₂₁/PlGF-1 heterodimer induces angiogenesis. (h) VEGF₁₆₅/PlGF-2 heterodimer induces angiogenesis. Magnification $\times 12$.



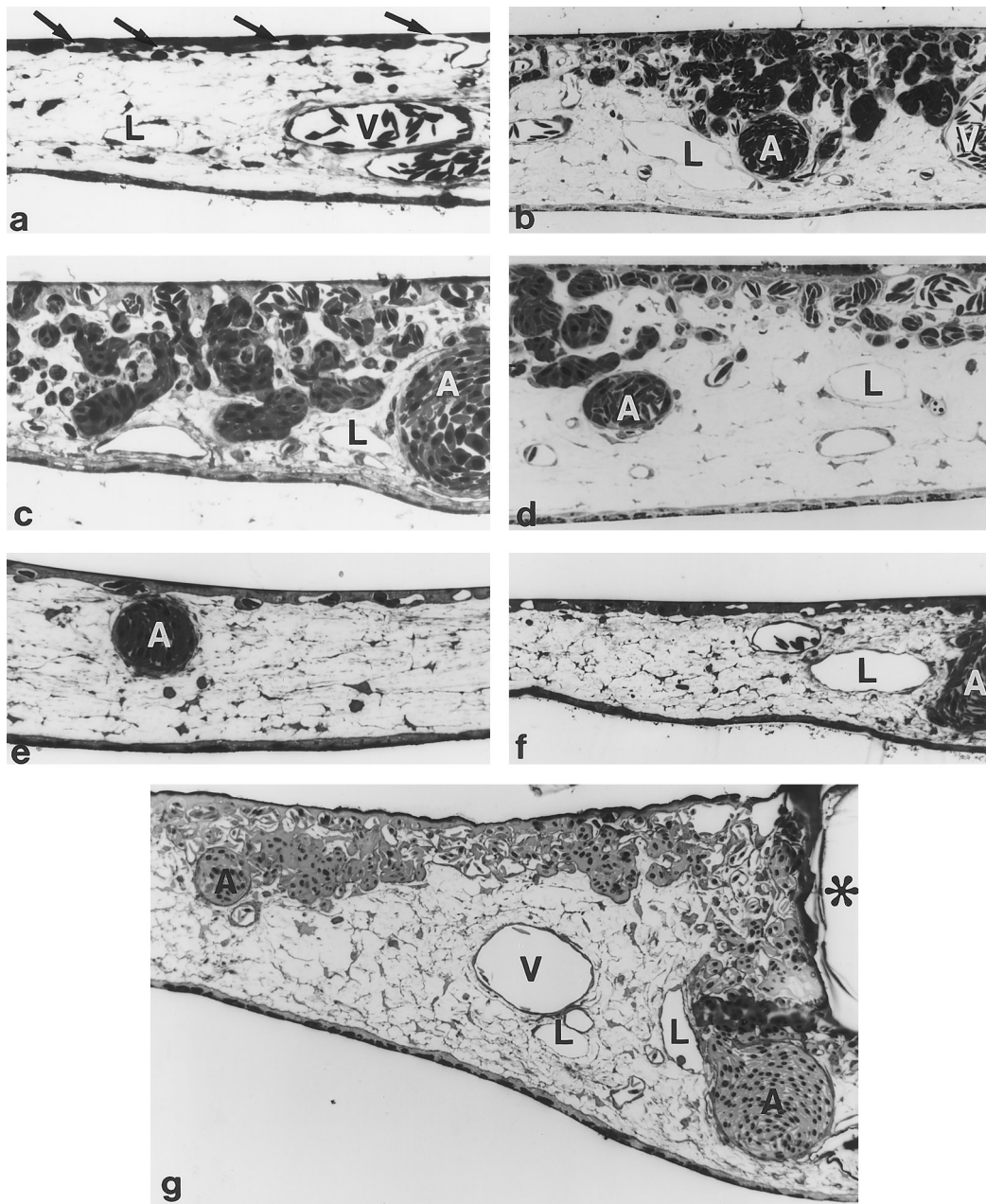


FIG. 6. Semithin sections of growth-factor-treated CAMs corresponding to those in Fig. 5. (a) Control. The capillaries (arrows) are located in the chorionic epithelium. Arteries, veins, and lymphatics are found in the stroma of CAM. Magnification $\times 450$. (b) Effect of VEGF₁₂₁. Numerous capillaries are found in the stroma of CAM. Lymphatics seem to be unaffected. Magnification $\times 350$. (c) Effect of VEGF₁₆₅. Note large amount of blood-filled capillaries. Lymphatics are not affected. Magnification $\times 450$. (d) Effect of VEGF_{121/165} heterodimer. New capillaries are present in the stroma of CAM. The lymphatics are not affected. Magnification $\times 450$. (e) PlGF-1. Neither an angiogenic nor a lymphangiogenic effect is present. Magnification $\times 450$. (f) PlGF-2. There are no signs of angiogenesis or lymphangiogenesis. Magnification $\times 350$. (g) VEGF₁₂₁/PlGF-1 heterodimer. The border of the carrier disk (star) is shown. Due to diffusion, formation of new capillaries in the stroma of CAM is found even at a distance of 0.6 mm from the application site. Lymphatics are not affected. Magnification $\times 350$. A, artery; L, lymphatics; V, vein.

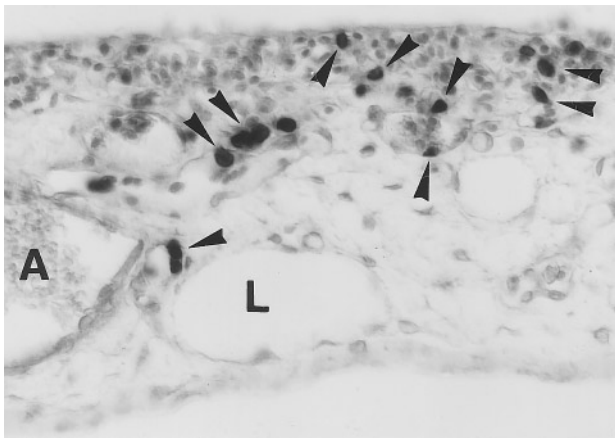


FIG. 7. Proliferation studies with the BrdU/anti-BrdU method after application of VEGF₁₂₁. Numerous BrdU-positive vascular endothelial cells (black nuclei) are present. Lymphatic (L) endothelial cells are negative. A, artery. Original magnification $\times 400$.

DISCUSSION

Lymphatics of CAM Possess Typical Morphology

The lymphatics of the chick embryo were first studied by Albrecht Budge during the seventh and eighth decades of the 19th century. After his death, a survey of these studies was published by Wilhelm His (Budge, 1887). As early as 1887, Budge had already noted that the arteries of the CAM are accompanied by a pair of lymphatics which are interconnected. The lymphatics are drained into two lymph-hearts located in the angle between the pelvic and the sacral bones. Furthermore, they are connected to the paired thoracic ducts. These are located adjacent to the aorta and lead to the venous angles. Since the original description of CAM lymphatics by Budge (1887), they have only been briefly mentioned in the textbooks by Hamilton (1965) and Romanoff (1960).

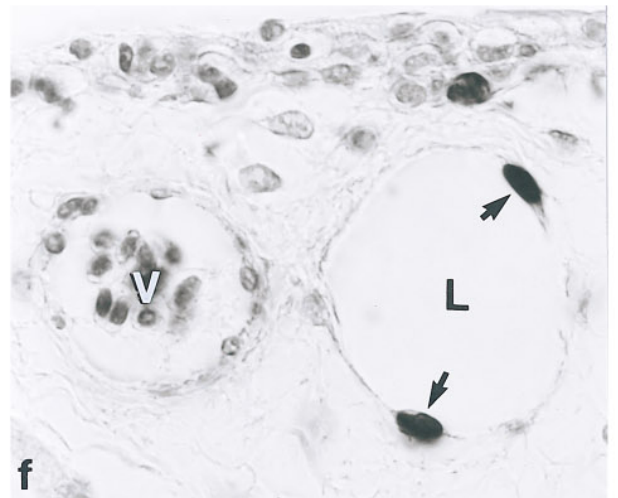
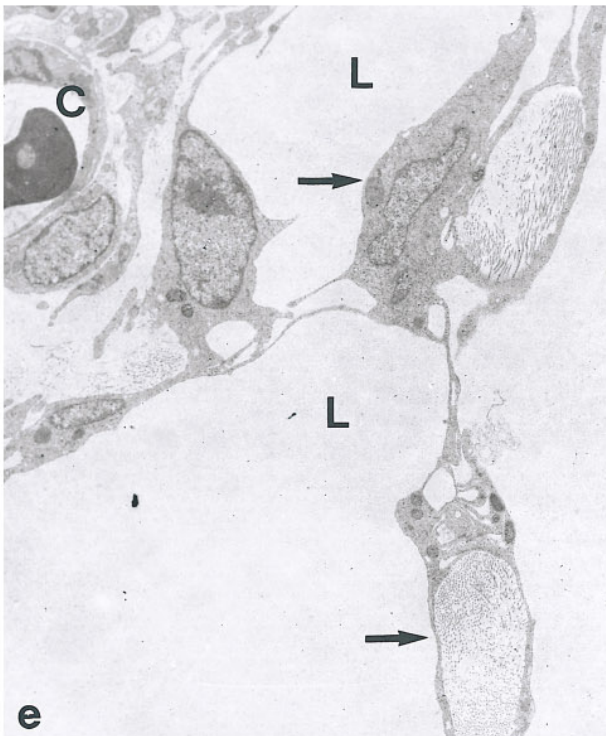
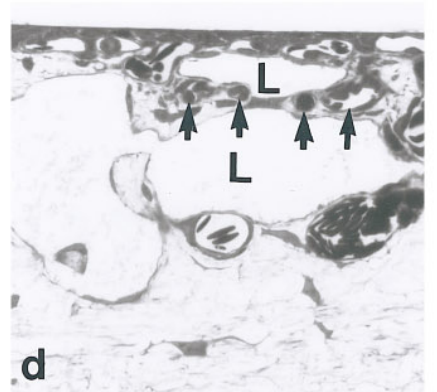
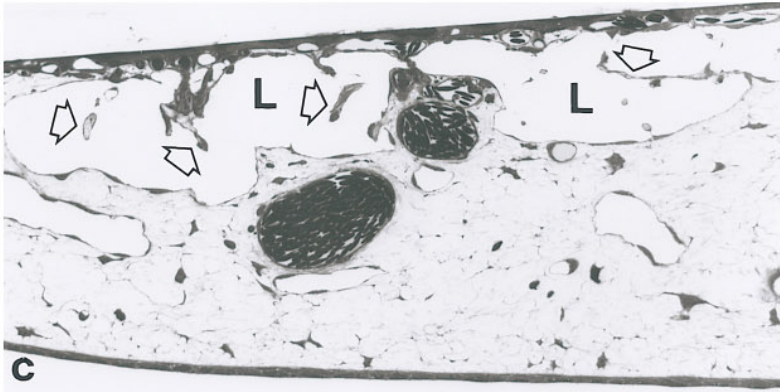
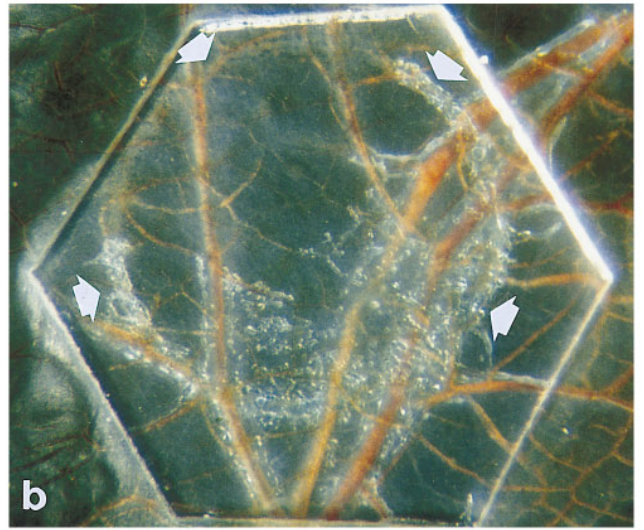
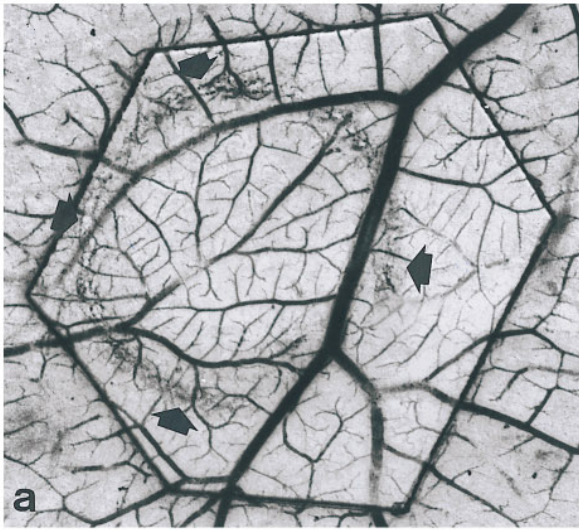
In contrast to lymphatics, the blood vessels of avian CAM have gained considerable attention. In the search for angiogenic factors that may promote tumor vascularization, the CAM assay has been used to assess effects of growth factors. The assay was originally performed on Day 10 of development (Folkman, 1974). In a modified version, differentiated CAM of 13-day-old embryos has been shown to respond selectively to different growth factors (Wilting *et al.*, 1991, 1992). VEGF was observed to induce endothelial cell proliferation, upregulation of VEGFR-2, and angiogenesis, without affecting other cells of CAM (Wilting *et al.*, 1993, 1996; Kurz *et al.*, 1995). Recently, three more proteins sharing approximately 30–40% identity with VEGF have been described and named VEGF-B, VEGF-C, and PlGF (Maglione *et al.*, 1991; Olofsson *et al.*, 1996; Joukov *et al.*, 1996). VEGF-C binds with high affinity to VEGFR-2 (*flk-1*, KDR) and VEGFR-3 (*flt-4*) (Joukov *et al.*, 1996). During development VEGFR-2 is expressed in endothelial cells of blood vessels

and lymphatics (Wilting *et al.*, 1997a), and VEGFR-3 becomes restricted to those of lymphatics (Kaipainen *et al.*, 1995; Wilting *et al.*, 1997a). It has therefore been suggested that VEGF-C may exert its activities on lymphatics (Kukk *et al.*, 1996; Wilting *et al.*, 1997b). Lymphatic endothelial cells have been studied *in vitro* (Gumkowski *et al.*, 1987; Witte and Witte, 1987); however, an *in vivo* assay for potential lymphangiogenic factors has not previously been described.

We have studied the lymphatics of avian CAM. Our results support the findings of Budge (1887). A pair of lymphatics is present along all arteries and arterioles of CAM. With increasing size of the arteries, an increasing number of lymphatic capillaries interconnects the pair of lymphatics. Blind-ending extensions are also visible. Veins are also accompanied by lymphatics. Again, the number of lymphatic capillaries and the complexity of the lymphatic plexus increase with increasing size of the veins. Lymph of CAM flows into the embryo. Huge lymphatics are located in the angle between the umbilical vessels. These lymphatics are easily visible with a stereomicroscope. They have to be regarded as lymphatic trunks since they possess a single layer of media smooth muscle cells. In avian CAM, lymphatics do not possess a media and they are made up solely of endothelium.

An extremely thin endothelial lining is one of the characteristics of lymphatic capillaries. Furthermore, their diameter is usually much bigger than that of blood capillaries. They also possess pores, and a basal lamina is missing (Casley-Smith, 1980; Witte and Witte, 1987; Berens von Rautenfeld and Drenckhahn, 1994). Therefore, staining with antibodies against type IV collagen and laminin is negative around lymphatic capillaries and in lymphangiomas (Barsky *et al.*, 1983; Ezaki *et al.*, 1990). The lymphatics of CAM show all characteristics that have been described for normal lymphatic capillaries: huge diameter, porous and very thin endothelial lining, and absence of a basal lamina. From these characteristics, it is possible to recognize lymphatics in semithin sections although, in routine paraffin histology, one cannot be sure to recognize them. However, in CAM, their regular pattern and location beside arteries and veins is a good help for the recognition of lymphatics, even in paraffin sections. Our findings therefore indicate that the regular pattern and the normal structure of CAM lymphatics make this organ highly suitable for the study of lymphangiogenic factors.

Our studies also show that the lymphatics of CAM express VEGFR-2 and VEGFR-3. This expression pattern has also been found in intraembryonic sites during late embryogenesis (Kaipainen *et al.*, 1995; Wilting *et al.*, 1997a). VEGFR-2 is expressed in blood vascular and lymphatic endothelial cells during all stages examined (Wilting *et al.*, 1997a). This receptor has only been studied in context of angiogenesis, and not lymphangiogenesis (Millauer *et al.*, 1993; Shalaby *et al.*, 1995). In early stages of development, VEGFR-3 is expressed by all endothelial cells. Later it becomes restricted to a subpopulation of endothelial cells, disappearing first from the arteries. Expression persists only



in lymphatic endothelial cells, with very few exceptions (Kaipainen *et al.*, 1995; Wilting *et al.*, 1997a). The newly identified growth factor, VEGF-C, binds with high affinity to VEGFR-2 and VEGFR-3 (Joukov *et al.*, 1996; Kukk *et al.*, 1996).

VEGF and VEGF-C Induce Angiogenesis and Lymphangiogenesis, Respectively

The angiogenic potential of VEGF is well established. This growth factor binds to VEGFR-1 (*flt-1*) and VEGFR-2 (Terman *et al.*, 1992; Millauer *et al.*, 1993). Targeted mutations of either the growth factor or its receptors result in early embryonic lethality (Shalaby *et al.*, 1995; Fong *et al.*, 1995; Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Both VEGF₁₆₅ and VEGF₁₂₁ induce angiogenesis in differentiated CAM (Wilting *et al.*, 1992, 1996). No effect of VEGF₁₂₁ on the lymphatic system was observed in a previous study (Wilting *et al.*, 1996) or in this study. The factor induces expression of VEGFR-2 in endothelial cells, but it does not influence expression of VEGFR-3 (Wilting *et al.*, 1996), to which it does not bind. Here, we have compared the activities of different homo- and heterodimeric forms of VEGF. All forms that contained at least one VEGF chain were capable of inducing angiogenesis. Histologically we have never observed effects in the lymphatics. Since VEGFR-2 is expressed in both types of vessels, differences may reside in the expression of VEGFR-1. It is probable that a combined activation of VEGFR-1 and VEGFR-2 is necessary to induce angiogenesis. However, differential expression of VEGFR-1 in blood vascular and lymphatic endothelium has not been studied to date. VEGF/PlGF heterodimers induce angiogenesis in differentiated CAM. Such heterodimers have been isolated from GS-9L glioma cells (DiSalvo *et al.*, 1995) and have been found in conditioned media of human tumor cell lines (Cao *et al.*, 1996). Therefore, in some tumors this heterodimer may be an important angiogenic factor.

The function of PlGF is much less established. PlGF shares 40% amino acid sequence identity with VEGF, and it binds only to VEGFR-1 (Maglione *et al.*, 1991; Hauser and Weich, 1993; Park *et al.*, 1994). In differentiated CAM, PlGF-1 and PlGF-2 induce neither angiogenesis nor lymphangiogenesis. Activation of VEGFR-1 does not seem to be sufficient to induce angiogenesis. In the avian, however, we

have to be aware of the fact that the VEGFR-1 has not been cloned to date. Since PlGF is almost exclusively expressed in the placenta (Park *et al.*, 1994), it may induce placenta-specific characteristics of the vasculature.

Our studies show that VEGF-C is a highly specific lympho-angiogenic factor. This is consistent with the expression of its receptors in lymphatic endothelial cells of differentiated tissues (Kaipainen *et al.*, 1995; Wilting *et al.*, 1997a). Whereas VEGFR-2 is expressed in vascular and lymphatic endothelial cells throughout development (Wilting *et al.*, 1997a), expression of VEGFR-3 becomes restricted to those of lymphatics (Kaipainen *et al.*, 1995; Wilting *et al.*, 1997a). The early lymphatics are therefore indistinguishable from blood vessels, which supports the assumption that they are derived from blood vessels (Sabin, 1909; Clark and Clark, 1920). We suggest that the activity of VEGF-C in embryonic tissues is different from that in differentiated tissues. In differentiated CAM, VEGF-C induces a very mild angiogenic response in regions of high growth factor concentration. Preliminary studies in our lab indicate that the angiogenic activity of VEGF is enhanced by VEGF-C. However, the main effect of VEGF-C is induction of new lymphatics. These are located immediately beneath the chorionic epithelium, which indicates that VEGF-C is a chemoattractant for lymphatic endothelial cells. Whereas VEGF induces development of vascular capillaries with relatively small diameter, VEGF-C induces huge lymphatic sinuses, obviously due to proliferation and intercalated growth. By morphological criteria, the lymphatic nature of these vessels can clearly be demonstrated. The great amount of lymphatics in the application area and their unusual location immediately beneath the chorionic epithelium show that they are newly formed.

No growth factors specific for the lymphatic vascular system have yet been described. VEGF regulates vascular permeability and angiogenesis, but it does not promote lympho-angiogenesis. Overexpression of VEGF-C, a ligand of VEGFR-3 and VEGFR-2, in the skin of transgenic mice resulted in lymphatic, but not vascular, endothelial proliferation and vessel enlargement (Jeltsch *et al.*, 1997). Thus, VEGF-C appears to induce selective hyperplasia of the lymphatic vasculature, which is involved in the draining and filtering of interstitial fluid, in immune function, in inflammation, and in tumor metastasis. VEGF-C may have a

FIG. 8. Effect of VEGF-C. (a) VEGF-C does not induce gross alteration of the vascular system of CAM. A circular effect (arrows) is only visible in the area of highest growth factor concentration. Original magnification $\times 12$. (b) Intralymphatic injection of Mercox-blue outside the application area. The solution has filled most, but, for technical reasons, not all of the lymphatics in the circular application area. Note the high density of lymphatics in the application area. A huge lymphatic plexus is located in the circle of highest growth factor concentration (arrows). Original magnification $\times 12$. (c, d) Semithin sections of the application area. (c) Newly developed lymphatics (L) located immediately beneath the chorionic epithelium. Note many intraluminally located endothelial cells (arrows), indicative of plexus formation. No other cell types are affected. Original magnification $\times 450$. (d) In the region of highest VEGF-C concentration, some newly formed lymphatics (L) are surrounded by vascular capillaries (arrows). Original magnification $\times 450$. (e) Ultrathin section of the application area. Newly formed lymphatics (L) located adjacent to an intrachorionic capillary (C). Note intraluminally projecting lymphatic endothelial cells (arrows). Original magnification $\times 3500$. (f) Proliferation studies with the BrdU/anti-BrdU method 1 day after application of VEGF-C. Lymphatic (L) endothelial cells (arrows) are BrdU positive. V, Vein. Original magnification $\times 1000$.

role in various disorders involving the lymphatic system and it may prove to be useful for therapeutic lymphangiogenesis.

In summary, our studies show that differentiated CAM comprises a regular pattern of lymphatics. The lymphatic endothelial cells possess ultrastructural characteristics usually described in this cell type. They express VEGFR-2 and VEGFR-3. CAM is therefore a suitable site to study lymphangiogenic factors. We have shown that VEGF homo- and heterodimers induce angiogenesis, but not lymphangiogenesis; that PlGF is neither angiogenic nor lymphangiogenic; and that VEGF-C is the first member of the VEGF family that specifically induces lymphangiogenesis.

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