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Evidence for intussusceptive capillary growth in the chicken chorio-allantoic membrane (CAM)*

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Summary. The aim of our investigations was to test whether the chicken chorio-allantoic membrane (CAM) could be an adequate in vivo model for a new mode of capillary growth, originally described in the rat lung and termed intussusceptive microvascular growth. According to that concept the capillary system does not grow by sprouting of vessels, but expands by insertion of transcapillary tissue pillars or posts which form new intercapillary meshes. In the present study, we observed slender transcapillary tissue pillars with diameters around 1 μm in the CAM by in vivo microscopy, and analyzed their ultrastructure by transmission electron microscopic investigation of serial sections. The pillars corresponded in size to those previously described in rat lung microvasculature. On day 7, the pillar core contained endothelial-, endothelial-like cells and collagen fibers, and on day 12 additionally chorionic epithelial cells. As a hypothesis we propose that slender cytoplasmic extensions of endothelial cells, heavily interdigitated in the post area and often projecting into the vascular lumen, could initiate the first step of pillar formation, i.e., interconnect opposite capillary walls. During both stages of development endothelial-like cells were observed in close relationship with the pillars. These cells seem to be relevant for tissue post completion and growth, as they were found to invade the core of the pillars. From the localization of the interendothelial junctions in the post region, a certain similarity to the concept proposed for the lung can be found. The observations confirm that the CAM is a very suitable material for the in vivo investigation of intussusceptive capillary growth.

Key words: Chorio-allantoic membrane – Capillary growth – Development – Endothelial cell – Microcirculation

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Introduction

A new growth process of microvasculature called intussusceptional or intussusceptive capillary growth has been described in the lungs of rats (Caduff et al. 1986; Burri and Tarek 1990). Instead of growing by vascular sprouting, the capillary network expands by insertion of slender transcapillary tissue pillars (Fig. 1). Pillars or posts are defined as capillary meshes with diameters below 2.5 μm, but mostly in the range of 1 to 1.5 μm. By electron microscopic (EM) investigation of serial sections of interalveolar septa, four consecutive stages in pillar formation could be assessed (Burri and Tarek 1990). In phase I, an area of contact between opposite endothelial walls is formed (Fig. 1a). During phase II, the endothelial junctions are reorganized (Fig. 1b) and the endothelial leaflets are broken up centrally (Fig. 1c). By these means, an interstitial cylindrical core arises in

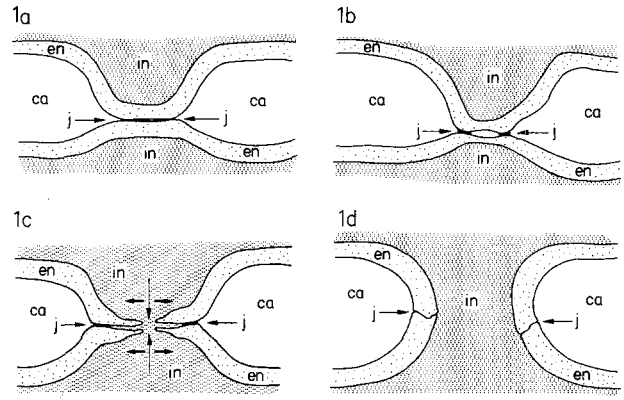

Fig. 1a–d. Formation of transcapillary tissue pillar in intussusceptive capillary growth in the lung. A disk-like zone of contact between opposite endothelial cells is established across the capillary lumen (**a**), this zone being then demarcated by the formation of intercellular junctions (**b**). The endothelial cell leaflets become perforated centrally (**c**). This region is then invaded by the cellular and acellular elements of the interstitial tissue (**d**). *ca*, capillary; *en*, endothelial cell; *in*, interstitial tissue; *j*, interendothelial junction (reproduced with permission from Patan et al. 1992)

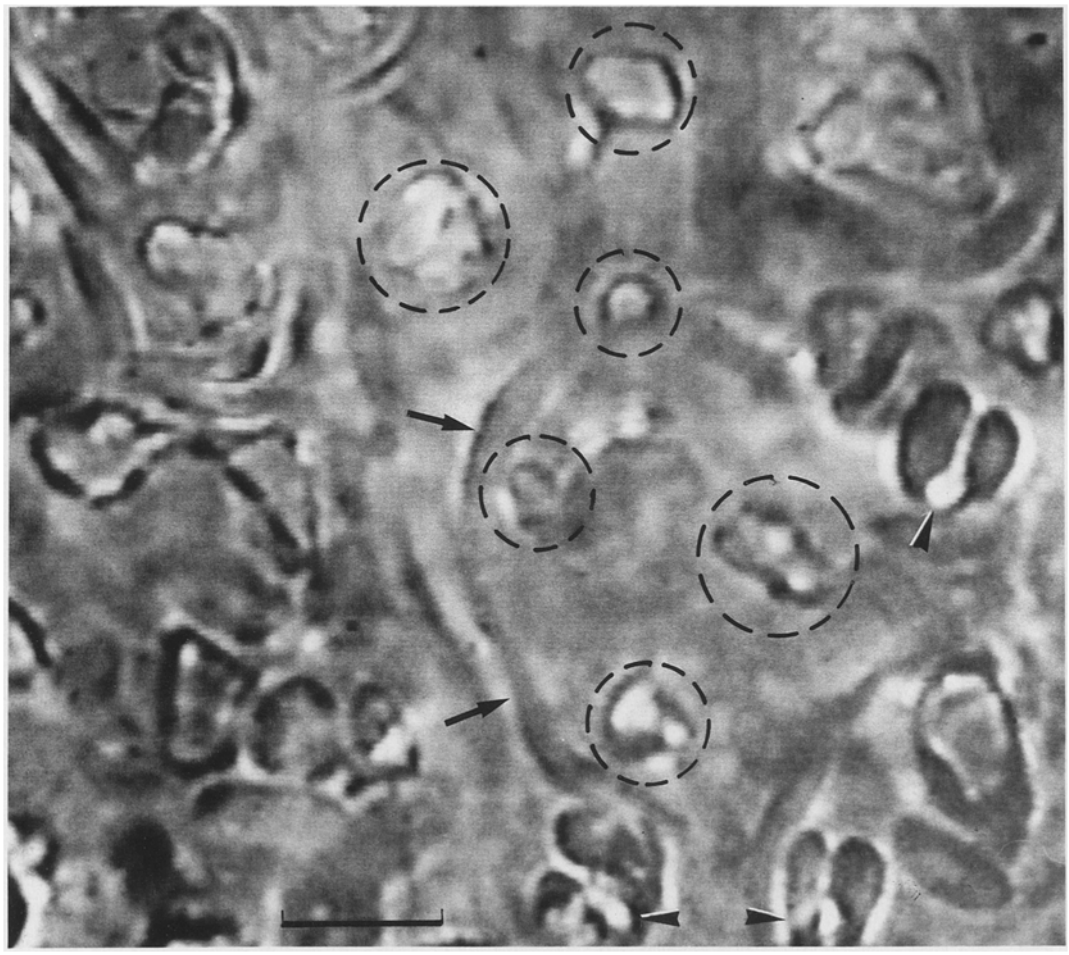

Fig. 2. Still video image of CAM capillary network obtained during live microscopy. Image contrast was enhanced by means of the Visilog Software. The intercapillary meshes represent "islands" (*encircled by dotted lines*) of varying dimensions within the blood circulation, the pillars appear as tiny refracting spots with diameters around 1.5 μm. Note an erythrocyte stuck to a pillar (*arrowhead*). *Arrows* point to regions of floating erythrocytes. Bar 8.0 μm

the post centre (phase III; Fig. 1c). The post core is successively invaded by cytoplasmic processes of myofibroblasts and pericytes and finally definitively stabilized by deposition of connective tissue fibers (Fig. 1d).

Despite the highly suggestive sequence of structural alterations in post morphology, static EM pictures can hardly ever be a proof for a dynamic process. Only the in vivo observation of pillar formation could definitely demonstrate the existence of growth by intussusception.

In microvascular research, the capillary system of the chick chorio-allantoic membrane (CAM) represents a well-known material for in vivo microscopy of blood circulation. In this study, we combined live video microscopy with the analysis of perpendicular serial EM sections of the CAM microvascular layer in an attempt to document, as a first step, the existence and the morphology of transcapillary tissue pillars.

Materials and methods

White Leghorn eggs were incubated for 4 days. After the shell had been cleaned with a solution of Betadine, the eggs were cracked open with a scalpel and their contents carefully poured into a plastic Petri dish of 80 mm diameter. The dishes were then incubated for several days at 37° C in a humid atmosphere of air (Auerbach et al. 1974; Narbaitz and Jande 1978; Dunn and Fitzharris 1979). In vivo light microscopy of CAM capillaries was performed with a Zeiss microscope (Standard RA) equipped with a thermostatically controlled specimen stage of our own design. Flow in the capillary network was monitored over periods of up to 12 h by means of a black and white video camera attached to a time-lapse video cassette recorder. Still images were digitized by means of a frame grabber board (Advanced Frame Grabber from Imaging Technologies) installed in a 486-DOS-Personal Computer. Using Visilog[1], contours and contrast of the digital images were enhanced and output to the video-screen. Screen photographs were taken from a Sony monitor (type PVM-1444 QM) with a Canon F1 camera fitted with a Canon 50 mm/3.5 macrolens on Agfapan 100 material.

For EM-investigations, CAMs aged 7 and 12 days were fixed by submerging them in a solution of 2.5% glutaraldehyde in 0.03 M K-phosphate buffer (350 mOsm, pH 7.4) for about 24 h. The membranes were then dissected and cut into several pieces and postfixed in Na-cacodylate buffered 1% OsO_4 (osmolarity 340 mOsm). After dehydration in ascending concentrations of ethanol, the tissue was embedded in Epon 812. From selected blocks, several batches of either vertical or horizontal serial sections were obtained. A batch

[1] Visilog is the trade name of an image analysis software produced by Noësis Corporation

usually consisted of 120–180 ultrathin sections of 70–90 nm thickness. Every single section was picked up on a Formvar-coated one-hole grid, double stained with lead citrate (Reynolds 1963) and uranylacetate (Frasca and Parks 1965) and viewed in a Philips EM 300 electron microscope.

Results

In vivo microscopy

Single CAM specimens could be investigated for periods of up to 12 h while blood flow in the capillary network was continuously monitored. In vivo microscopy was, however, limited to developmental stages from day 10 onward, because only by then had the capillary network grown beyond the egg yolk. Before day 10, light absorption in the yolk was so heavy, that the video recordings were too noisy. On day 12 of development, the CAM capillary system represents a flat network extending practically in two dimensions only with meshes of varying sizes. Viewed from on top, the meshes appear as round or polygonal tissue pillars of various diameters. Live observation reveals great local and temporal variations of blood flow. High and low velocities, or even a stop and go of erythrocytes, are found side by side in directly connected vessels. Flow characteristics in a given capillary can also change markedly with time: periods of rapid flow sometimes alternate with clogging of the capillary lumen by blood cells.

To establish the presence of intussusceptive capillary growth in the CAM, it was important, as a first step, to find extremely small meshes, i.e., tissue pillars with diameters less than 1.5–2 μm: Such structures could easily be made out. They appeared as tiny refractile spots within the capillary lumina (Fig. 2). The proof that these spots were not artefacts produced by the optical projection of structures located underneath, was clearly established by the observation that red blood cells bounced against these pillars, wrapped around them and hung there sometimes for minutes (Fig. 2). The erythrocytes underwent dramatic shape distortions due to the surrounding rapid flow, but seemed not to be destroyed, despite the extreme mechanical stress they were subjected to.

Electron microscopy

Day 12. The previously video-taped CAM specimens presented the following structural features in the vertical EM sections. On top, the chorionic ectoderm consists of two to three layers of epithelial cells closely juxtaposed to each other, with multiple interdigitations and desmosomes. The microvascular network, located immediately underneath the epithelium, mostly appears to be embedded half-way into the epithelial sheet. At some places, the epithelium extends deeply in between the capillaries and reaches the mesenchymal side of the capillary layer. According to this "embedded" position of the capillaries in the chorionic epithelium, the intercapillary meshes often contain solid masses of epithelial cells

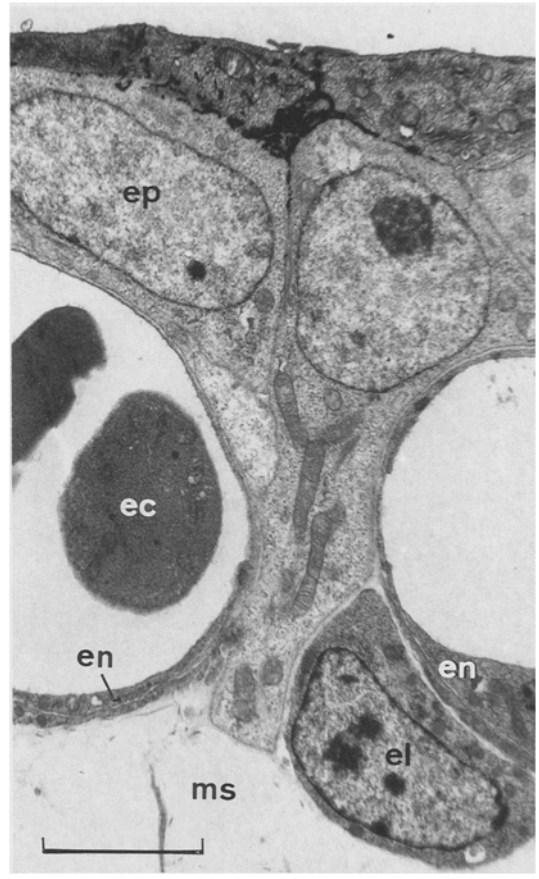

Fig. 3. Chorionic epithelial layer containing the capillary plexus and positioned on top of the mesenchyme at day 12 of CAM development. Bar 3.0 μm. *ec*, erythrocyte; *el*, endothelial-like cell; *en*, endothelial cell; *ep*, chorionic epithelial cell; *ms*, mesenchymal layer

that rest on the ground substance of the mesenchymal layer. They are laterally bounded by the endothelial leaflets of the adjacent capillaries, and frequently also by processes of what we call endothelial-like cells (Fig. 3).

The endothelial-like cells have morphological features similar to those of endothelial cells. They are, however, located outside the capillary wall proper. They form intercellular junctions with each other and with endothelial cells. In some cases, a single extension of an endothelial-like cell is intercalated between the capillary endothelium and reaches the capillary lumen.

A few larger vessels run underneath the capillary layer in the deeper zones of the mesenchyme. At this age their walls are still composed of a simple endothelial coat surrounded in many places by endothelial-like cells. The mesenchymal sheet is lined below by the allantoic epithelium which consists of one to three layers of flat cells.

In the vertical serial sections of the capillary layer, very slender transcapillary tissue pillars with maximal widths of 1–2 μm, corresponding to the pillars found in the video frames, could frequently be observed. Such a tissue post is illustrated in Fig. 4a–d. Since it extends from section 94 to section 116, it measures about 1.8 μm in depth. Its width amounts to 2.0 μm. Its outer lining is composed of the cytoplasmic processes of interdigitat-

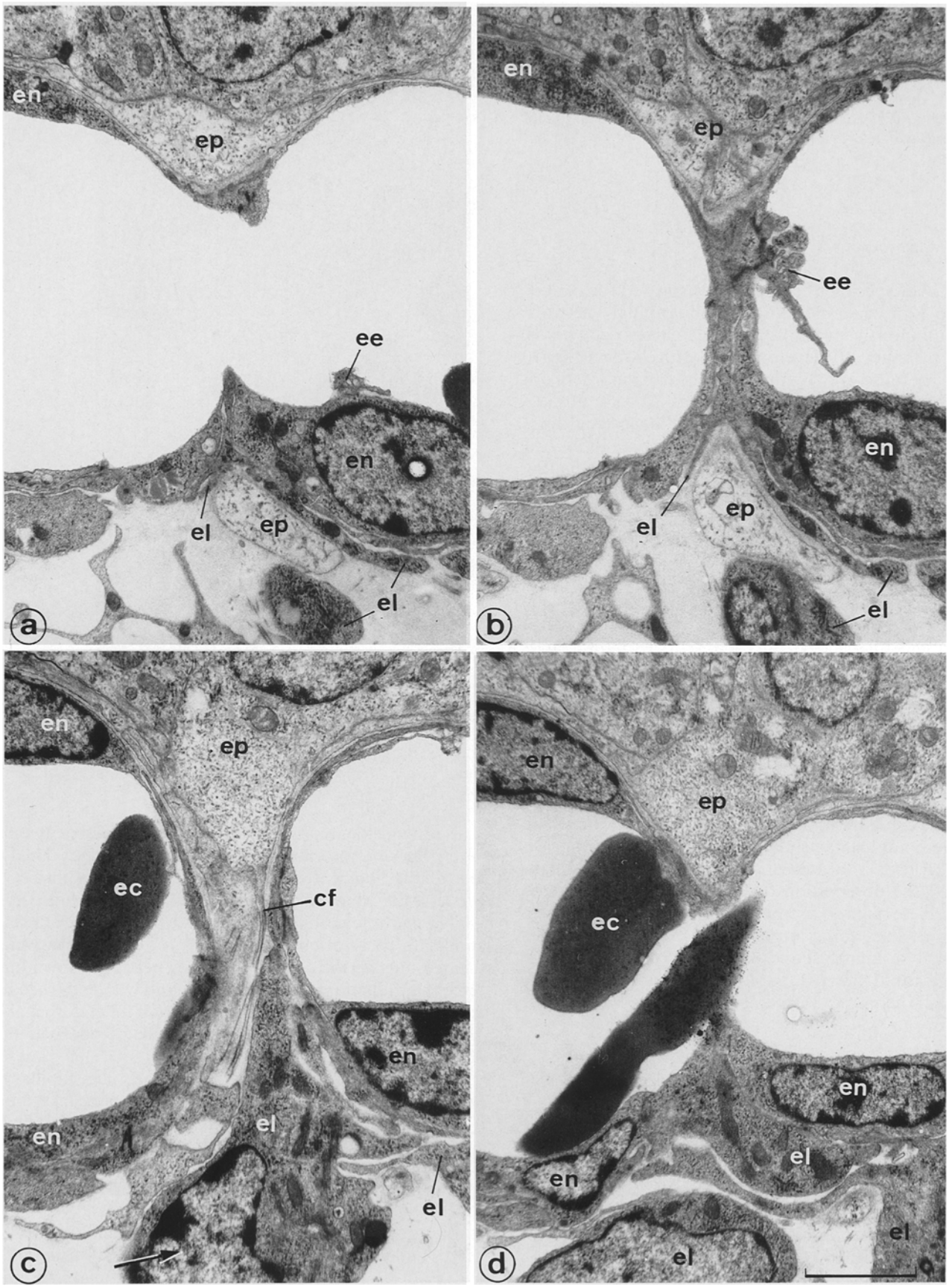

Fig. 4a–d. Electron micrographs of tissue pillar in perpendicular serial sections at day 12 of CAM development. The pillar extended from section 94 to 116 (mean diameter ≈1.8 μm). **a** Section 91, capillary lumen is open; **b** Section 96, lumen is closed by the pillar; **c** Section 110, core of the pillar; note a chorionic epithelial cell projecting from above and an endothelial-like cell from below; they form a cushion on the mesenchymal side of the capillary layer (*arrow*); collagen fibrils are found in the pillar core. **d** Section 118, lumen is open. Bar 2.0 μm. *cf*, collagen fibrils; *ec*, erythrocyte; *ee*, endothelial extension; *el*, endothelial-like cell; *en*, endothelial cell, *ep*, chorionic epithelial cell

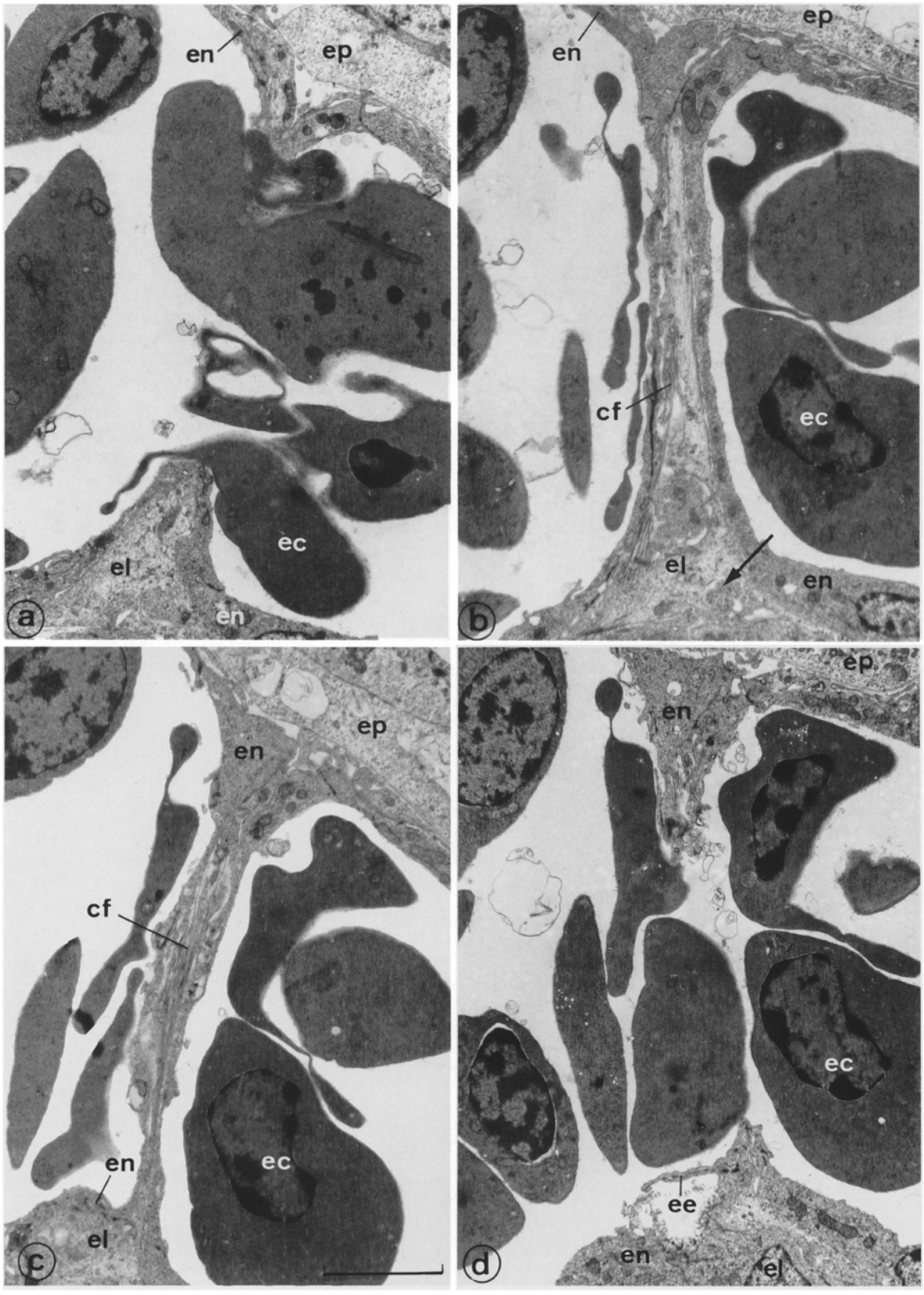

Fig. 5a–d. Example of tissue pillar as observed in electron microscopic perpendicular serial sections at day 7 of CAM development. The pillar extended from section 80 to 95 (mean diameter ≈1.4 μm). **A** Section 76, capillary lumen is open; **b** Section 85, lumen is closed by the pillar (pillar core); **c** Section 90, mantle region of the pillar; note the complex arrangement of interendothelial junctions running laterally around the pillar; **d** Section 96, lumen is open again. Endothelial-like cells form a cushion on the mesenchymal side of the capillary walls at the base of the pillar (*arrow*); collagen fibrils are found in the pillar core. Bar 3.0 μm. *cf*, collagen fibrils; *ec*, erythrocyte; *ee*, endothelial extension; *el* endothelial-like cell; *en*, endothelial cell; *ep*, chorionic epithelial cell

ing endothelial cells. Hence, the interendothelial junctions run circumferentially around the pillar (Fig. 4b). The pillar core is formed by solid buds of epithelial cells projecting from the apical chorionic layer and by processes of endothelial-like cells entering from the mesenchymal side and interposed between epi- and endothelium (Fig. 4c). Where the epithelial core reaches the mesenchyme, the epithelial cells can form, together with the endothelial-like cells, a cushion-like structure, located on the mesenchymal side of the capillary meshes. In the vicinity of the posts, endothelial cell processes often protrude into the capillary lumen (Fig. 4b). The long process shown in Fig. 4b could be traced intraluminally over a distance of 5.4 µm (68 sections); it originated at the nuclear region of an endothelial cell.

Day 7. Although it was not possible, because of the limited light sensitivity of our videocamera, to videotape the CAM-capillaries live on day 7, we were able to observe posts directly by live microscopy, so we searched for them at this earlier developmental stage at the EM level.

On day 7, the chorionic ectoderm consists of two to three layers of loosely interconnected cells with broad intercellular spaces. The capillary network is located in the mesenchyme underneath the epithelium. Occasionally, the epithelial cells just begin to reach into the upper parts of the intercapillary mesh spaces. The larger vessels of the mesenchymal zone and the epithelium of the allantoic layer already exhibit the features described for day 12.

The intercapillary meshes of the chorionic microvascular plexus are of variable dimensions and morphology. The capillaries are often wrapped by endothelial-like cells lying outside the capillary walls. These cells can build a compact column of tissue completely filling some of the capillary meshes, and form cytoplasmic lamellae on the mesenchymal side of the capillaries.

Again we found very slender tissue pillars with diameters in the range of 1–2 µm.Their morphology resembled closely the one described for day 12.

Figure 5a–d shows successive transverse step sections of a single pillar at this age. It extends from section 80 to section 95; its depth hence measures about 1.2 µm; its width amounts to 1.6 µm. In Fig. 5a, the capillary lumen is open, in Fig. 5b, c, it is closed by the pillar, and in Fig. 5d, it is open again. The mantle of the pillar is formed by the walls of the adjacent capillaries, composed of slender cytoplasmic processes of several endothelial cells. The latter are interconnected with each other by cytoplasmic interdigitations and tight junctions (Fig. 5c). Figure 5b shows the pillar at its largest diameter. Collagen fibrils and processes of endothelial-like cells are found side by side entering the pillar from below.

On day 7, (as was manifested on day 12, but in conjunction with epithelial cells), we observed at the base of the pillar the presence of a conglomerate of endotheli-

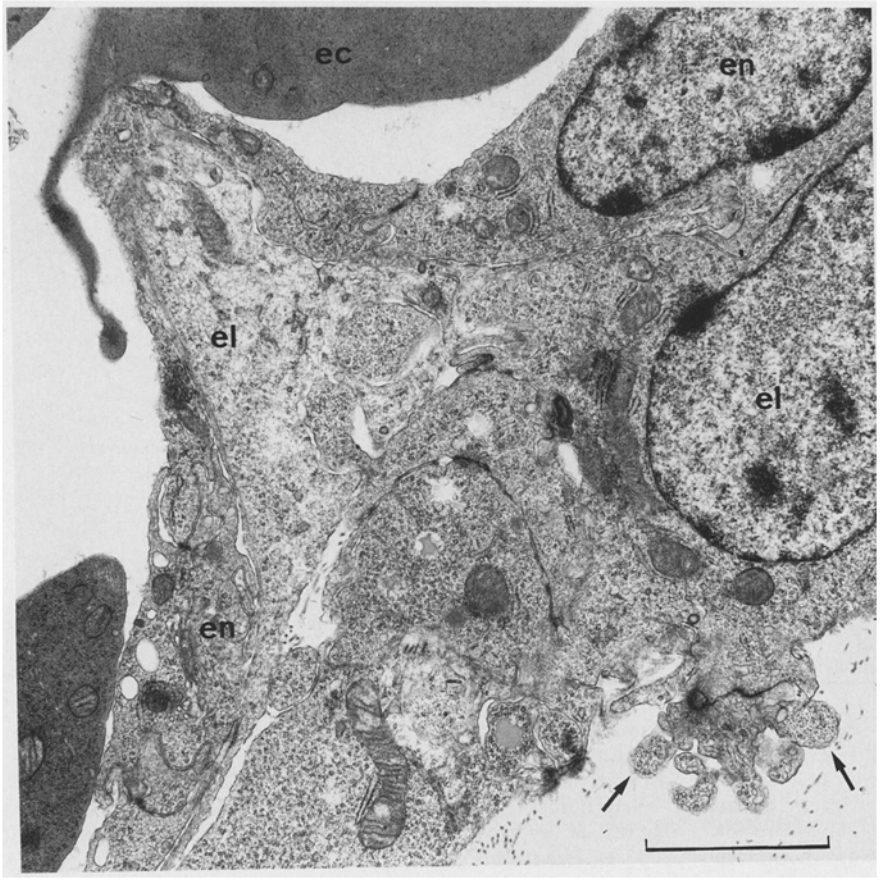

Fig. 6. Detail of tissue pillar of day 7 of CAM development. A conglomerate of endothelial-like cells is bulging towards the mesenchyme; the endothelial-like cells are interconnected with each other in a complex way by junctions and interdigitations. Note tiny cytoplasmic buds (*arrows*) projecting into the mesenchyme. Bar 2.0 µm. *ec*, erythrocyte; *el*, endothelial-like cell; *en*, endothelial cell

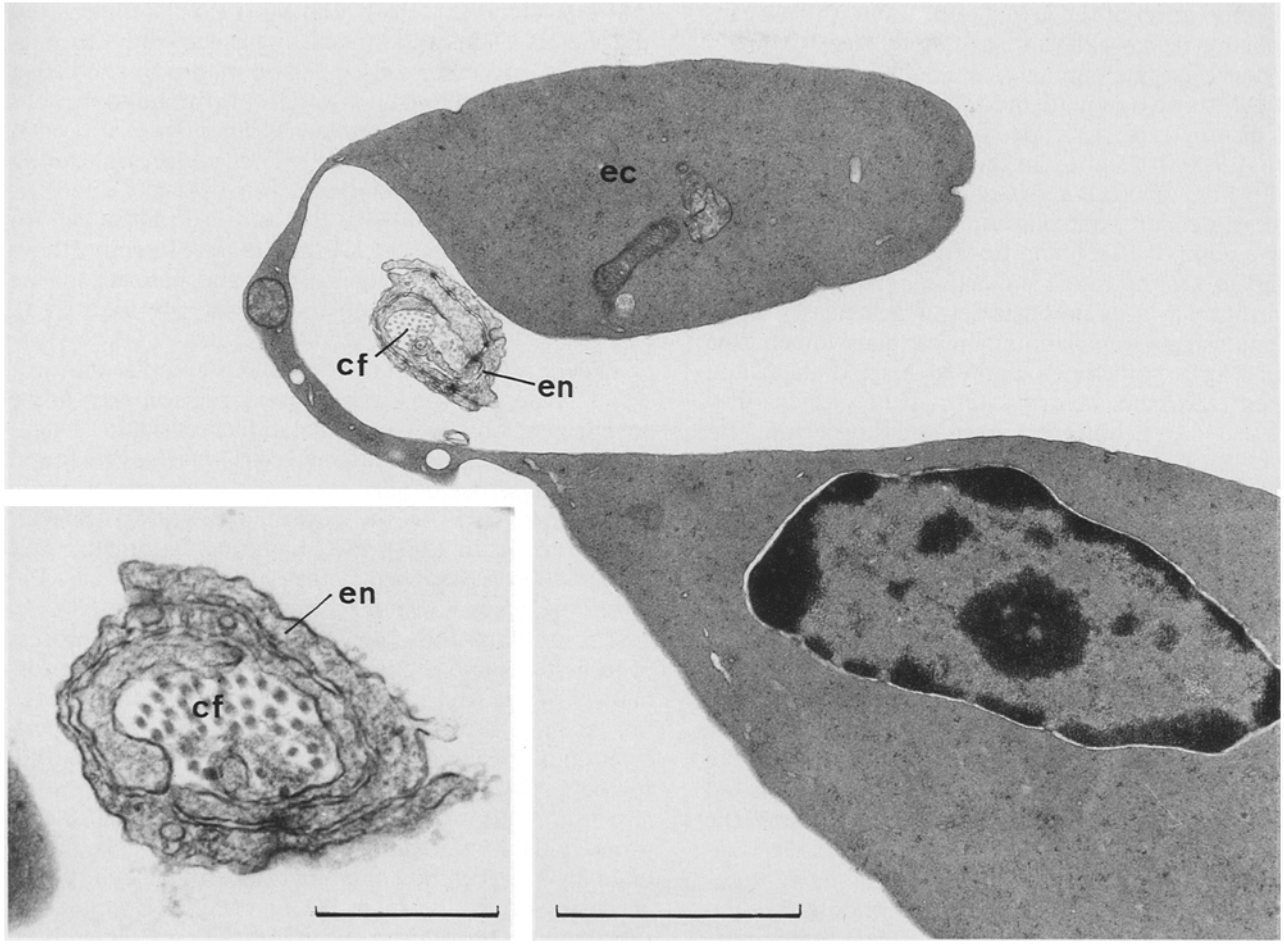

Fig. 7. Tissue pillar as observed in electron microscopic horizontal serial sections at day 7 of CAM development (section 50, diameter ≈1.0 μm, length ≈4 μm). The picture corresponds to the top view of the CAM as seen in live microscopy (see Fig. 2). Note the huge dimensions of the erythrocyte stuck to the tiny pillar. Bar 2.0 μm. *Inset* (section 70): endothelial cells and eventually endothelial-type cells form cytoplasmic lamellae running around the pillar's core that contains collagen fibrils. Bar 0.5 μm. *cf*, collagen fibrils; *ec*, erythrocyte; *en*, endothelial cell

al-like cells bulging towards the mesenchyme. These cells are interconnected with each other in a complex way by junctions and interdigitations. At their base, they are irregularly surrounded by small cytoplasmic buds projecting into the mesenchyme (Fig. 6). A basement membrane is usually missing at this age.

As described for the specimens aged 12 days (Fig. 4a, b), multiple intraluminal cytoplasmic projections of endothelial cells were also present at this age (Fig. 5d). They were more frequently observed in the vicinity of tissue posts.

Figure 7 illustrates a cross section of a tissue pillar at day 7 observed in a series of horizontal sections. The pillar's cross-section measures 1.2 by 0.8 μm. Its core consists of collagen fibrils and is enclosed by processes of endothelial cells, and probably also by processes of endothelial-like cells.

In many places in the capillary network we found very short and slender capillaries which correspond in morphology to the tubular sprouts described in the nineteenth century (Thoma 1893) and many times since then (Clark and Clark 1939; Wolff 1964; Ausprunk and Folkman 1977; Nicosia et al. 1982; Furusato et al. 1984). They are located within the intercapillary meshes or towards their mesenchymal basis.

Discussion

In rat lung development, the existence of slender transcapillary tissue pillars could be demonstrated while the capillary volume was markedly growing (Burri and Tarek 1990). Assuming that capillary growth should match increasing oxygen requirements, we decided to investigate the CAM during the second week of development, when the oxygen consumption of the chick embryo was reported to increase rapidly from 1.4 to 10.0 ml/h (Needham 1932). Retrospectively, our observations of days 7 and 12 confirm that the CAM microvascular network was indeed expanding very rapidly during this interval. Finally, day 12 was primarily selected because by then, the developing capillary system had regularly expanded over the egg white, thus facilitating microscopic observation by the transillumination method.

The final aim of the in vivo study of the CAM being

the documentation of the actual appearance of new capillary pillars, it was relevant, as a first step, to prove the existence of intercapillary meshes of varying sizes. If the CAM is an adequate model, its capillary network should display typical slender meshes with dimensions between 1.0 and 1.5 μm like those occurring in the rat lungs. In vivo microscopy had already confirmed the presence of slender tissue pillars in the critical size range. When we observed the blood flow in the CAM, we were surprised to see the rough impactions of the red blood cells onto tiny cylindrical obstacles, which clearly represented solid structures spanning the capillary lumen. The red blood cells sometimes wrapped around them and underwent extreme deformations, from which they seemed to recover, however, when stress was over. Tazawa (1978), noted that red blood cells often suffered various deformations due to the narrowness of the vessels. In the present situation, blood cell deformation was more often due to obstacles and angular pathways than to vessel diameter.

EM investigation of serial sections confirmed the existence of tissue pillars and enabled us to analyze their morphology. We should expect a certain structural congruence between chick CAM and rat lung pillars, if both are involved in the same or a similar process of capillary growth. Our findings show that in both organs, the elements that contribute to the structure of normal-sized intercapillary meshes are also found in or around the slender pillars. These are pericytes, myofibroblasts and collagen fibrils in the lung, and, judging from topographical relationships, endothelial, endothelial-like cells and collagen fibrils in the CAM. The undifferentiated mesenchymal cells of the chorionic plate are usually too far away to play a role in post formation. In the CAM aged 12 days, the capillary network is in intimate contact with the chorionic epithelium. These latter cells therefore also appear as elements of the mesh and pillar morphology. This does, however, not necessarily imply that they are actively involved in post initiation. Because the chorionic epithelium forms a much thicker layer on day 12 than on day 7, and the capillaries are almost embedded in it, it seems plausible that the proliferating epithelial cells are more or less pushed into the core of the post after its formation.

The presence and the morphology of the endothelial-like cells are highly suggestive of an active role. The origin and the prospective fate of these cells are not yet known. It remains speculation, whether the endothelial-like cells constitute an endothelial cell population outside the capillary wall, and originate from the endothelium, or whether they are directly derived from the underlying mesenchyme, and may join the endothelium. In growing capillaries of the cornea and muscle, cells that first appeared to be peri-endothelial could be traced to the inner vascular lining (Schoefl 1963). We made the same observation for some of our endothelial-like cells, which were found to form part of the endothelial lining of the capillary wall. The relationship between endothelial-like cells and pericytes is also a matter of debate. Pericytes, which are located on the mesenchymal side of the capillaries on the 14th–21st day of chick CAM development (Hoshi and Mori 1971; Wangensteen and Weibel 1982) cannot be distinguished from their mesenchymal precursor cells at earlier stages. Our so-called endothelial-like cells could as well correspond to the cells characterized as 'pericyte-like' by Shumko et al. (1988) and Sweeny and Bather (1968). They are present during all stages of CAM development. Cytoplasmic interdigitations between endothelial-like and endothelial cells in the CAM at days 7 and 12 (Fig. 6) closely resemble those described between endothelial cells and pericytes in the capillaries of human granulation tissue (Furusato et al. 1990).

During wound healing "bubbling cytoplasmic processes" reaching into the perivascular space, such as we have seen belonging to endothelial-like cells, and "extensively interdigitating endothelial cells", such as we found in our pillars, have been observed frequently in newly formed capillaries in the cornea and muscle (Schoefl 1963; Yamagami 1967). Interdigitating endothelial cells have also been described in the chick CAM at days 14–18 of development (Shumko et al. 1988).

The primary and crucial event in the whole process of intussusceptive capillary growth is obviously the formation of the first transcapillary endothelial bridge. In the rat lung, it was suggested that contractility of pericytes could be involved, by forming an endothelial fold. In the CAM aged 7 and 12 days no such observation has been made so far. We can only suggest that a first transcapillary interendothelial contact could be facilitated by the presence of numerous slender endothelial processes projecting into the lumen of the capillaries. Intraluminal projections of endothelial cells have been observed in a variety of organs, in many species, under normal and pathological conditions and during development (Clark and Clark 1939; Tanaka 1960; Fawcett and Wittenberg 1962; Fawcett 1963; Warren 1966; Sethi and Brooks 1971; Smith et al. 1971; Wagner 1980; Pexieder 1981). Their function is still unknown, but they have been found to bridge the lumen by joining processes originating from the opposite endothelial wall (Warren 1966). Clark and Clark (1939) also made a similar observation in their in vivo studies of capillary growth in tadpole tails and in adult rabbit ears. They found endothelial processes originating from the perikaryon of endothelial cells projecting along the wall, across the lumen, and also contacting each other. Long and slender endothelial cell processes along the luminal surface of the capillary wall have also recently been described by Wilms et al. (1991) in the chick embryo. They have been interpreted by these authors as originating from endothelial cells in the process of intraluminal migration. Although intraluminal projections are also present in adult endothelium, they are claimed to be particularly prominent in developing vessels (Fujimoto et al. 1975; Wagner 1980). Whether such intraluminal endothelial cell processes do really initiate pillar formation remains an open question. It is clear, however, that, by whatever means, the first phase of pillar formation resides in establishing a transcapillary interendothelial contact. The second step consists in the formation of a transcapillary endothelial tube, the core of which is then invaded by the

interstitium, (i.e., by endothelial-like or epithelial cells and collagen fibrils). In the lung, it is assumed that after the formation of junctional complexes, the endothelial leaflets are centrally broken up to allow for the interstitial invasion (Fig. 1c, see Burri and Tarek 1990). In the CAM, no precise description of these steps can be proposed yet, but the presence (as in the lung) of interendothelial junctions on the pillar itself (or in its immediate vicinity) is in favour of a process of junctional formation and reorganization. Furthermore, the reports that the basement membrane is usually very thin and frequently discontinuous in 4–10 days old CAMs on the mesenchymal side of the capillaries (Ausprunk et al. 1974; Shumko et al. 1988), and our observations that some of the thinner posts found on day 7 showed incomplete endothelial basement membranes in the central region of the pillar, favour the idea of an endothelial remodeling.

According to the concept of intussusceptive capillary growth, the proliferation of endothelial and endothelial-like cells is not *a priori* a necessary step in early pillar formation, because of the plasticity and the motility of the cellular elements involved. With a certain delay, however, mitoses of endothelial cells related to growth and expansion of the capillary network are expected to be distributed throughout the network, with no need for mitotic foci as found in sprouting. This is in agreement with the observation that, following administration of tritiated thymidine, LM autoradiography revealed an even distribution of labeled endothelial cells throughout the capillary bed (Ausprunk et al. 1974). These authors concluded that the extension of the capillary network in the CAM prior to day 11 might be accomplished by a general lengthening of the existing vascular system rather than by the process of endothelial sprouting. It is obvious, however, that a mere lengthening of capillary segments would result in a coarser capillary network, which would be of little functional benefit regarding gas diffusion and/or substrate transport. De Fouw and coworkers (1989) showed that intercapillary distances were substantially shorter at days 10 and 14 when compared with day 6.

In conclusion, the existence of tissue pillars with dimensions corresponding to those found in the rat lungs was confirmed by in vivo microscopy and EM in the chick CAM. The analysis of their ultrastructure suggested some possible, but still hypothetical mechanisms of their formation. We propose that slender intraluminal cytoplasmic extensions of endothelial cells (described for many years, with no clues as to their function) may be involved in initiating tissue post formation in the CAM. In view of the similarity in the positioning of the interendothelial junctions, we suggest, that as in the lung, the transcapillary interendothelial bridge is remodeled to a slender cylindrical endothelial tube invaded by the interstitium. It is evident that further studies are needed to clarify the mode of implementation of pillar formation.

This study yielded good evidence that intussusceptive capillary growth occurs in the CAM, and that the CAM represents a very suitable model for investigating the concept of intussusceptive capillary growth by in vivo microscopic observations.

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