# LABORATORY SCIENCE

# Pericyte recruitment in human corneal angiogenesis: an ultrastructural study with clinicopathological correlation

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**Background/aim:** During angiogenesis—that is, the outgrowth of new from pre-existing blood vessels, new capillaries undergo a period of "fine tuning" when vascular endothelial cells become apoptotic if sufficient supply of angiogenic factors is lacking. Morphologically, this period correlates with the absence of pericyte coverage of new vessels. Mature, pericyte covered vessels, in contrast, do not depend on elevated levels of angiogenic factors for survival. This study analyses whether, and if so when, pathological vessels in human corneal neovascularisation (CN) acquire pericyte coverage. This can be of importance for future angioregressive therapeutic strategies.
**Methods:** Vascularised human corneas obtained by keratoplasty were evaluated by electron micros-copy for pericyte coverage of new vessels. These data were correlated with the duration of CN (mean 73 (SD 95) (range 0.5–360) months; n = 15). CN was secondary to keratitis, transplant rejection, ani-ridia, or trauma.
**Results:** Overall, 196 blood vessels were analysed ultrastructurally (72 (37%) capillaries, 122 (62%) venules, and two (1%) arterioles). Electron microscopically, 170 (87%) vessels were covered by peri-cytes and two (1%) in addition by smooth muscle cells. Pericyte recruitment increased with time, evolv-ing between clinically noted onset of CN and keratoplasty. Already 2 weeks after onset of CN, more than 80% of new vessels were covered by pericytes.
**Conclusion:** Pathological new vessels in human corneal angiogenesis are rapidly covered by pericytes. Therapeutic strategies aimed at regression of immature, not yet pericyte covered vessels by antagonising angiogenic factors should thus be most effective if applied very early in the course of cor-neal neovascularisation.

T he term "pericyte" was introduced by Zimmermann in 1923 to describe blood vessel wall encircling cells consist-ing of thin processes and a prominent nucleus embedded in the basement membrane surrounding capillaries.[1–3] Peri-cytes are associated with the abluminal side of blood vascular capillaries, precapillary arterioles, and postcapillary venules, the latter two displaying gradual transition to smooth muscle cells.[1–3]

Pericyte coverage is an essential step in the maturation of new blood vessels. Mature capillaries, arterioles, and venules are surrounded by pericytes and the direct endothelial cell pericyte contact stabilises the vessel and suppresses endothe-lial proliferation—for example, by TGFβ₁.[1][2 4] Occurrence of pericyte coverage also marks the end of the transient "pruning" phase of newly formed vascular endothelial networks, which means regression of surplus vessels. This has mainly been studied in the model of retinal vessel maturation in retinopathy of prematurity (Fig 1).[5–7] In this model, the pericyte free endothelial network temporally and spatially coincides with the "fine tuning" according to the level of available oxygen.[6 7] Withdrawal of hypoxia induced ang-iogenic factors (for example, VEGF) in this phase induces selective apoptosis of new capillary endothelial cells devoid of periendothelial support cells.[5–9] With pericyte coverage occur-ring, this sensitive period ends (Fig 1).[6–8] Angiogenic factors such as VEGF therefore seem to be required to sustain newly formed vessels beyond the period of "pruning," whereas ang-iogenic factors seem to be dispensable for the mature vascular network.[6–8 10] Since the recruitment of pericytes lags behind the formation of endothelial networks, the initial pericyte free period of "pruning" constitutes a period especially suitable for an angioregressive therapy aimed at regression of newly formed immature vessels by blockade of angiogenic factors.

The normal human cornea is free of blood and lymphatic vessels. Secondary to different underlying diseases such as keratitis and graft rejection, pathological corneal haemangio-genesis and lymphangiogenesis can occur.[11 12] Histopathologi-cally, about 20% of corneas having undergone corneal trans-plantation display corneal neovascularisation (CN).[11] This CN not only interferes with corneal transparency, but also renders a cornea a high risk bed if subsequently corneal transplantation becomes necessary.[12] Therefore, pharmacological strategies interfering with new vessel growth and/or inducing regression of old or newly formed pathological corneal vessels are under investigation. Recently, angiogenic factors such as VEGF were identified immunohistochemically in newly formed vessels in vascularised human corneas.[13] The endothelial cells of these vessels express VEGF receptors 1, 2, and partly 3.[12 14] A corneal angioregressive therapy (inducing regression of newly formed immature vessels still depending on angiogenic growth factors) acting by blockade of angiogenic factors such as VEGF, accord-ingly should be most effective in the period of pericyte free cap-illaries, since only then can blood vessel regression be induced. Whether and if so how fast pericyte coverage of pathological blood vessels in the human cornea, a normally avascular tissue, occurs is yet unknown.

Therefore, the aims of this study were (1) to identify ultrastructurally the presence of pericytes in vascularised human corneas with angiogenesis secondary to different underlying diseases, and (2) to determine the relation between degree of pericyte coverage of new corneal blood ves-sels and duration of human CN.

## MATERIAL AND METHODS
### Vascularised human corneas
The corneas of 15 patients with corneal neovascularisation (CN), obtained by penetrating keratoplasty (8/1996–11/2000),
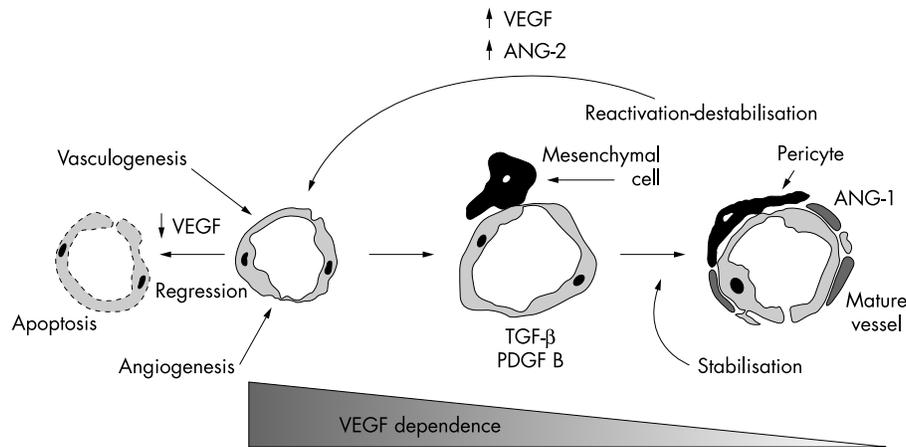
**Figure 1** Blood vessel maturation and dependence on periendothelial support cells. Blood vessel development proceeds from a stage of growth factor dependence where loss of angiogenic survival factors leads to apoptosis of vascular endothelial cells to independence of angiogenic growth factors. Vessel stabilisation marked by investment with periendothelial support cells (pericytes/smooth muscle cells) is paralleled by independence from angiogenic growth factors (with permission of the American Society for Clinical Investigation reproduced from Darland and D'Amore[8]; endothelial cell = shaded; extracellular matrix = solid; VEGF = vascular endothelial growth factor; ANG-1/2 = angiopoietin 1/2; TGF-β = transforming growth factor β; PDGF B = platelet derived growth factor B.

were retrospectively analysed in this study. Four μm paraffin embedded sections of the corneas had previously been examined histopathologically in our ophthalmic pathology laboratory using haematoxylin and eosin and periodic acid Schiff stain light microscopy.[11] Diagnoses leading to CN were herpetic keratitis (n = 6), transplant rejection (one) and transplant insufficiency after perforating keratoplasty (three), corneal trauma (one), limbal insufficiency in aniridia (one), ocular pemphigoid (one), superinfected peripheral ulcer secondary to rheumatoid arthritis (one), and pseudophakic bullous keratopathy (one). Duration of CN was defined as time between first clinical notions of corneal neovascularisation in patients' files to time of keratoplasty. In nine patients the analysed cornea was the original host cornea, in three patients the first donor cornea (regraft), and in one patient each the second, third, and fourth donor cornea. The mean time interval between onset of CN and keratoplasty was 73 (SD 95) (range 0.5–360) months. In one patient no unequivocal information about duration of CN before keratoplasty could be obtained; for analysis of time CN correlation, this patient was excluded. Patients' mean age at surgery was 47 (SD 20, range 22–82) years. Nine patients were male and six female.

**Electron microscopy**
Non-paraffin embedded, fixed corneal tissue was available from each cornea for electron microscopy (n = 15). Semithin sections were stained with toluidine blue, ultrathin sections were stained with uranyl acetate-lead citrate and were examined with an electron microscope EM 906 (Leo, Oberkochen, Germany). Areas with pathological corneal vessels were selected from semithin sections. From each cornea, one representative complete supporting grid, covering the entire corneal diameter and 2 mm in length, was analysed ultrastructurally (in all sections the area with the highest vessel density was chosen). This area was always located in the peripheral third of the cornea. For each vessel included in the section, morphology of the vessel wall and contents of the vessel lumen were assessed. Morphological parameters included presence of erythrocytes and/or leucocytes in the lumen, presence of tight junctions, presence and continuity of basement membrane, presence and location of pericytes, and presence of smooth muscle cells in the vessel wall. In addition, thickness of endothelial basement membrane, number of basement membrane layers, and diameter of the vessel lumen in two directions were measured.

Definitions used for the classification of pathological CN and vessel wall components were as follows.[15–17] A blood capillary is defined as an endothelial lined vessel just sufficient for the passage of a red blood cell—that is, up to maximal diameters of 8 μm in both directions being occasionally filled with erythrocytes and displaying single layered endothelial lining, partly with tight junctions. Blood capillaries have a continuous basement membrane and are partly covered by pericytes, whereas smooth muscle cells are absent. A postcapillary venule is defined as a blood vessel with similar anatomy as a blood capillary but with minimal diameters of more than 8 μm in both directions. Smooth muscle cells are usually not present. A precapillary arteriole is defined as a blood vessel with size and anatomical features of a postcapillary venule, but in addition it has to be covered by at least a single layer of smooth muscle cells. A pericyte is defined as an adventitial cell characterised ultrastructurally by long cytoplasmic processes, cytoplasmic filaments, pinocytotic vesicles, and a prominent basement membrane merging into the endothelial basement membrane—that is, an "intramural pericyte."[18] A smooth muscle cell ultrastructurally displays myofilament bundles with characteristic subplasmalemmal densities, pinocytotic vesicles, and usually is surrounded by a separate basement membrane. Assessment was masked as to the duration of CN in an individual specimen.

**Indirect immunohistochemistry**
Indirect immunohistochemistry for α-smooth muscle actin was performed as described previously.[12 13] One corneal section from each of the 15 vascularised human corneas was stained with a monoclonal antibody against human α-smooth muscle actin recognising perivascular smooth muscle cells and pericytes (clone 1A4, 1:100, Sigma, St Louis, MO, USA) using the streptavidin biotin method. Briefly, the corneas were fixed in neutral buffered formalin, embedded in paraffin, and cut to into 4 μm sections. After deparaffinisation and rehydration, a proteolytic digestion using proteinase K— prediluted in TRIS buffer—was performed (Dako, Germany). Peroxidase blocking was done for 10 minutes followed by incubation with the primary antibody (30 minutes) and the staining procedure according to the manufacturer's instructions. AEC (3-amino 9-ethyl carbazol; Dako, Germany) was used as a chromogenic substrate, Mayer's haemalum as counterstain (Chroma, Germany). Negative controls using isotypic antibody were done as well as positive controls using a normal human tissue checkerboard (Dako, Germany).

**Table 1** Pericyte coverage index (PCI; %) of pathological corneal blood vessels in vascularised human corneas with different duration of corneal neovascularisation (CN) as detected using electron microscopy (n=15)

| Corneal disease | Duration of CN (months)* | Electron microscopy | | |
| --- | --- | --- | --- | --- |
| | | PCI of capillaries (n)† | PCI of precapillary and postcapillary vessels (n)† | PCI of all vessels (n)† |
| Bullous keratopathy | –‡ | 50 (8) | 82 (11) | 68 (19) |
| Trauma | 0.5 | 88 (8) | 81 (21) | 83 (29) |
| Ocular pemphigoid | 3 | – | 100 (2) | 100 (2) |
| Peripheral ulcer in PCP | 3 | 27 (11) | 82 (17) | 61 (28) |
| Transplant insufficiency | 3 | 100 (3) | 100 (7) | 100 (10) |
| Transplant insufficiency | 11 | 100 (2) | 100 (3) | 100 (5) |
| Transplant insufficiency§ | 36 | 100 (2) | 95 (19) | 95 (21) |
| Transplant insufficiency§ | 60 | 50 (2) | 100 (9) | 91 (11) |
| Herpetic keratitis | 60 | 100 (2) | 100 (9) | 100 (11) |
| Herpetic keratitis§ | 60 | 100 (5) | 83 (6) | 91 (11) |
| Herpetic keratitis§ | 72 | 83 (6) | 100 (1) | 86 (7) |
| Herpetic keratitis | 72 | 100 (8) | 100 (5) | 100 (13) |
| Herpetic keratitis | 96 | – | – | – |
| Aniridia | 204 | 100 (14) | 100 (8) | 100 (22) |
| Herpetic keratitis | 360 | 100 (2) | 100 (5) | 100 (7) |
| Mean (SD) | 73 (95) | | | 91 (13) (196) |

*CN = pathological corneal neovascularisation; †percentage of vessels with pericyte coverage (in parentheses the number of vessels analysed for this cornea is given); ‡not unequivocally detectable; §corneas with clinically ongoing angiogenesis.

## Statistics

Correlation analysis between pericyte coverage index of blood vessels and duration of CN (Spearman) and group comparison using Mann-Whitney test were performed using SPSS (version 10.0).

## RESULTS

### Electron microscopy

Overall, 196 blood vessels were detectable, out of which 72 (37%) were capillaries, 122 venules (62%), and two (1%) arterioles displaying smooth muscle cells in their wall. One hundred and seventy vessels (87%) had pericytes within their vessel wall, whereas 26 (13%) did not. Smooth muscle cells were electron microscopically found in only two blood vessel walls (1%; duration of CN: 11 and 72 months). Blood vessel lumina varied between 2 and 313 µm (mean 21.7 (SD 25.7) µm), single basement membrane layer thickness between 0.01 and 0.66 µm (0.086 (0.078) µm), and total basement membrane thickness between 0.02 and 1.98 µm (0.16 (0.2)). Blood vessels often displayed reduplicated basement membranes with one layer in 64 vessels (33%), two layers in 61 vessels (32%), three in 46 (24%), four in 15 (8%), five in two (1%), six in one (0.05%), and seven layers in four vessels (2.1%). Tight junctions were present between endothelial cells in 144 blood vessels (74%) and adherent junctions in 128 blood vessels (65%).

After 3 months, more than 80% of all vessels (capillaries, precapillary and postcapillary vessels, all vessels) were at least partly covered by pericytes (Table 1, Fig 2). In corneas without clinically ongoing angiogenesis, after 3 years all vessels were partly covered by pericytes (pericyte coverage index (PCI; see Benjamin et al[6 7]): 100%). In the corneas with ongoing angiogenesis (herpetic keratitis, two; transplant insufficiency, two), a mean PCI of 91% was found (Table 1, Fig 2).

In the only cornea with a history of CN under 3 months available (penetrating keratoplasty 2 weeks after trauma), already 80% of capillaries and of venules were electron microscopically covered by pericytes.

Generally, older vessels (duration of CN >3 months) displayed multilayered basement membranes, more often had pericyte coverage and more often tight junctions between endothelial cells compared to vessels in corneas with recent onset of CN (Figs 3 and 4). Vessels in corneas with a short history of CN (≤3 months: trauma and rekeratoplasty) had a sig-

nificantly lower number of basement membrane layers (1.14 (0.7) compared to 2.2 (1.1); p<0.001; all vessels), the degree of pericyte coverage was significantly lower (85% (19%) compared to 97% (5%); p = 0.04; only capillaries) and a significantly lower percentage of tight junctions between endothelial cells was found (39% compared to 88%; p<0.001; all vessels). No significant difference in pericyte coverage index was found between younger and older precapillary and postcapillary vessels. The number of basement membrane layers in all vessels was correlated with duration of CN (p<0.001; r 0.63) as was the basement membrane thickness (p<0.001; r 0.49). A scatter plot of PCI and duration of CN is shown in Figure 2.

### Immunohistochemistry (α-smooth muscle actin; clone 1A4, Sigma)

Out of the 15 vascularised corneas analysed, in nine corneas *all* vessels (defined as lumina within the stroma or subepithelially filled with erythrocytes and/or leucocytes) had at least
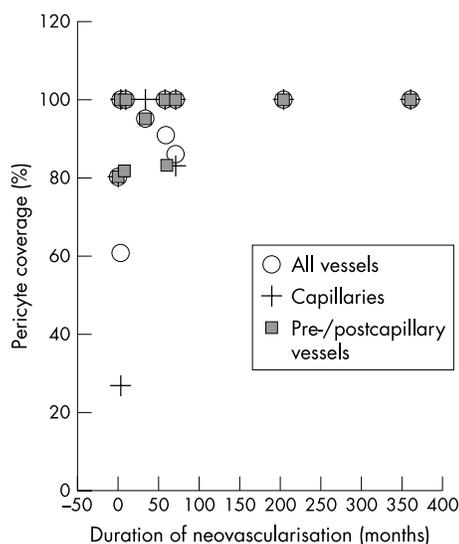


**Figure 2** Correlation between PCI (pericyte coverage index) as detected ultrastructurally and duration of corneal neovascularisation noted clinically (months). Note that high PCIs are established early and increase with time.
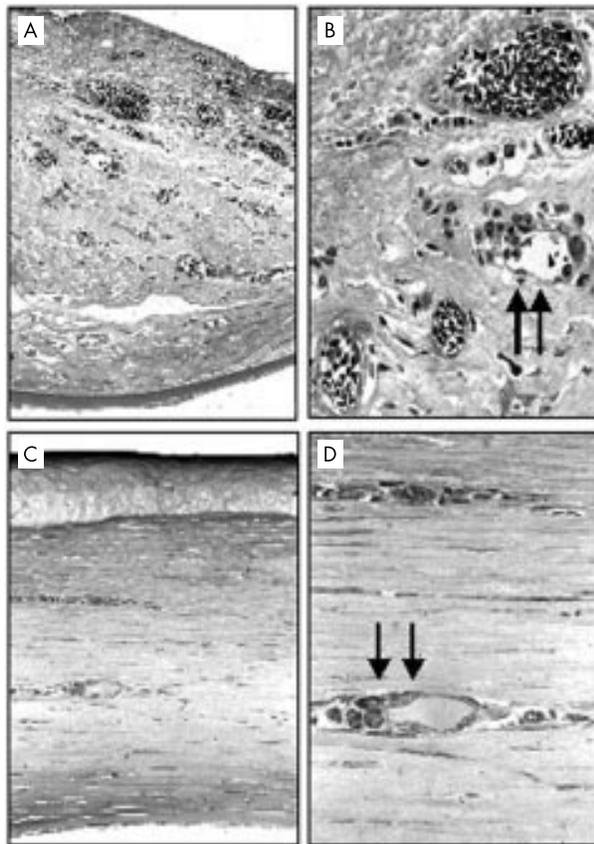
**Figure 3** Semithin cross sections of human corneas with recent onset (A, B) and longstanding corneal neovascularisation (C, D). (A) Immature, recently ingrown pathological vessels in a cornea 2 weeks after a perforating corneal injury (toluidine blue; ×150). Note extravasated erythrocytes within disorganised corneal stroma. (B) Detail from (A) showing new blood vessels partly lacking periendothelial support cells (×400; arrows). (C) Older vessels after longstanding corneal neovascularisation from a patient with 5 year history of recurrent herpetic keratitis (toluidine blue; ×150). Note organised, non-oedematous corneal stroma. (D) Detail from (C) showing periendothelial support cells around blood vessel (×800; arrows).

some positive reaction in the vessel wall (60%; mean number of vessels per corneal section: 9.5 (11.7); range: 1–35). In six of the vascularised corneas, *some* corneal vessels (16.1% (13.7%); range 6.7–42.9%) did not display positive reaction with the monoclonal anti-α-sm actin antibody in the vessel wall (six of 15 corneas: 40%). The mean number of analysed vessels in these six corneas was 26.3 (10.6) (range: 14–39). The overall α-smooth muscle actin antibody positivity of all analysed vessels (immunohistochemical PCI) was 91% (212 of 234 vessels).

The PCI of vessels with duration of CN ≤3 months was 94% (6%), whereas PCI was 98% (4%) for vessels with duration of CN greater than 3 months (except for two corneas with ongoing herpetic keratitis: PCI 67% and 82%; Table 1; not significant).

## DISCUSSION

Pericytes can cover pathological new blood vessels in the human cornea, a primarily avascular tissue. Pericyte recruitment in human CN occurs early. Already 2 weeks after onset of CN, 80% of vessels were coated by pericytes. As shown by ultrastructural and immunohistochemical analysis, pericyte coverage then increases with time and after 36 months all pathological corneal vessels are pericyte covered (except for corneas with ongoing angiogenic disease). This most probably

reflects regression of vessels lacking pericyte coating when angiogenic factors are downregulated, a phenomenon also occurring in developing retinal vasculature, in corpus luteum and in human tumours.[5–7 9] From animal CN the existence of pericytes and smooth muscle cells around experimentally induced new vessels is known.[16–24] Limbal capillaries and venules were the predominant source of CN in the rat, but after 3 weeks some new vessels demonstrated smooth muscle cell coverage thus assuming morphological features of arterioles.[21 22] It was yet unknown whether this arteriolar differentiation could also be found in human CN. Here, only 1% of vessels demonstrated smooth muscle coverage, whereas most retained capillary (37%) or venous characteristics (62%). At least some of the morphological venules must functionally act as afferent "arteriolar" vessels bringing blood into the cornea. The low rate of smooth muscle coverage may be related to low rates of fluid shear stress imposed on new corneal vessels thus preventing induction of an arteriolar phenotype. The presence of pericytes in vascularised human corneas, to our knowledge, has not been systematically analysed, but pericytes have been shown in experimental CN.[20–23] In this study, 91% of corneal vessels had a positive immunoreaction against α-smooth muscle actin detecting mAb clone 1A4, reacting with both pericyte and smooth muscle α-smooth muscle actin, which is commonly used to quantify vascular periendothelial coverage indices (PCI).[6 7 25] The immunohistochemical results are in good agreement with the ultrastructural findings, even more taking into account the fact that immunohistochemical analysis, in contrast with electron microscopy, evaluates the whole cornea. Thus, pathological human corneal vessels are commonly covered by pericytes, but only rarely by smooth muscle cells. A wide variability in PCI of pathological new vessels is known from tumour angiogenesis (13% in glioblastomas to 67% in mammary carcinomas) implying different degrees of vessel maturation and different amenability to therapies aiming at blood vessel regression.[25]

Methodical drawbacks of this retrospective study in human corneas include the lack of more corneas with very early CN, a potential overlap between more and less mature vessels in corneas with ongoing CN, and the not unequivocally exact clinical timing of CN. A true time course of pericyte recruitment can only be established using animal models. However, to our knowledge, this is the first study systematically analysing pericyte recruitment in human CN. Whether the process of "pruning" really takes place in human CN cannot be proved by this study. However, coverage of all older vessels in quiescent corneas by pericytes and blood vessel regression occurring in animal CN[10 21 22] support this assumption. The origin of corneal pericytes remains unknown. They might either migrate into the cornea from pre-existing limbal vessels or differentiate from corneal keratocytes. In fact, fibroblasts can transform into pericytes and further into smooth muscle cells.[26 27] As in experimental CN,[10 16] the presence of a thickened basal lamina consisting of multiple concentric lamellae, in addition to the presence of pericytes, was a conspicuous feature of persisting mature blood capillaries in human CN. New blood vessels (<3 months CN) significantly more often lacked pericyte coverage (capillaries), had fewer layers of basement membranes (all vessels), and fewer tight junctions (all vessels) compared to older vessels.

Regarding the time course of pericyte coverage, in experimental and developmental angiogenesis, pericyte recruitment lags behind establishment of new capillaries by days to up to weeks.[5–7 16 28 29] In chemically burned mice corneas, pericyte coverage was recently observed to occur within 2 weeks.[24] In this study, already 2 weeks after CN, 80% of new vessels were covered by pericytes.

Pathological corneal blood vessels can be approached therapeutically in two ways: (1) stop further outgrowth of new from pre-existing blood vessels in cornea or limbus in an angiogenically stimulated cornea (*antiangiogenic* therapy), or (2)
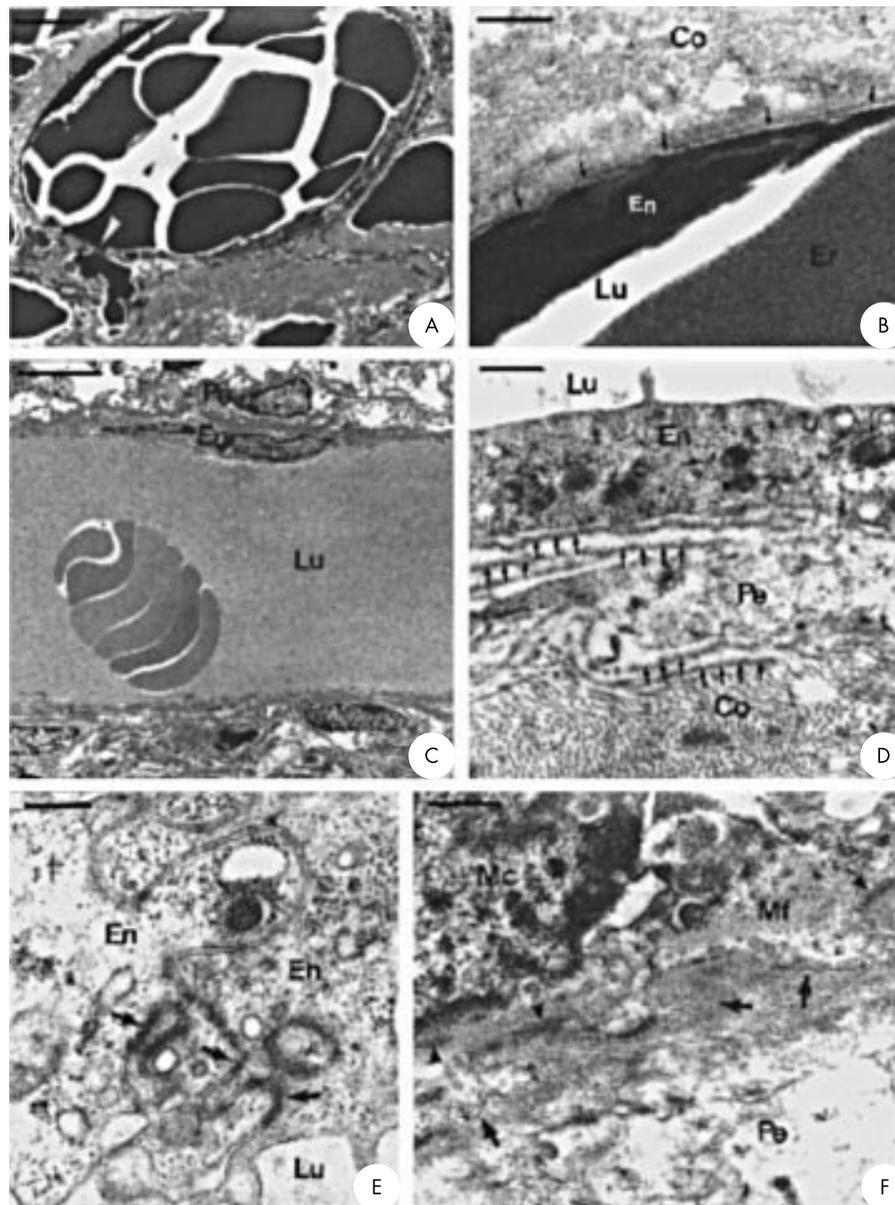
**Figure 4** Transmission electron micrographs of new (A, B, 2 weeks after perforating injury) and old (C–F, duration of corneal neovascularisation between 11 and 60 months) pathological human corneal blood vessels displaying typical ultrastructural features of vessel maturation. (A) New blood vessel lacking pericytes with erythrocyte extravasations (white arrowhead) from Figure 3A (bar = 3 µm). Boxed area indicates detail shown in (B). (B) Note thin, single layered basement membrane (arrows) between endothelial cells (En) and perivascular stromal collagen fibres (Co). No tight junctions were present between endothelial cells (Lu = lumen; Er = erythrocyte; bar: 0.3 µm). (C) Mature corneal stromal blood vessel with erythrocytes in the lumen (Lu) and pericytes (Pe) covering the endothelial cells (bar = 5 µm; recurrent herpetic keratitis with 5 years' duration of CN). (D) Mature blood vessel displaying a thick multilayered basement membrane (arrows) surrounding a pericyte (Pe) and endothelial cell (En; Lu = lumen; Co = collagen fibres; bar: 0.5 µm; longstanding bullous keratopathy with 5 years' duration of CN). (E) Arrows indicate intercellular junctions including tight junctions between endothelial cells (En) of a mature stromal blood vessel (Lu = lumen; bar: 0.3 µm; transplant insufficiency with 11 months CN). (F) Mature blood vessel with smooth muscles cell (Mc) in vessel wall adjacent to a pericyte (Pe). The smooth muscle cell is characterised by myofilaments (Mf) with focal subplasmalemmal densities (arrowheads) and clusters of pinocytotic vesicles (arrows; bar: 0.5 µm; transplant insufficiency with 11 months duration of CN).

induction of regression of already formed immature or old established corneal blood vessels (*angioregressive* therapy). An antiangiogenic therapy can be delivered by blocking angiogenic growth factors. Regarding an angioregressive therapy of human CN according to our results, two different therapeutic targets exist: (1) newly sprouted, immature vessels not yet or only partially covered by pericytes and surrounded only by a single layered basement membrane, and (2) older, established blood vessels covered by pericytes, multilayered basement membrane, and partially even by smooth muscle cells. Only in the first group should pharmacological approaches via blockage of angiogenic factors be able to

induce blood vessel regression, if applied very early. In contrast, older, established vessels do not depend any longer on angiogenic growth factors and might only disappear after vessel disruption—for example, via application of the Tie2 receptor ligand angiopoietin II, targeted toxins, or mechanical/photodynamic vessel disruption.[26][30] The time cut off between the two strategies according to our results seems to be within the first 3 months after onset of CN, probably within the first few weeks. In human corneal diseases, obviously old and new vessels can be mixed if the angiogenic stimulus is still active. In addition, antiangiogenic strategies inhibiting further CN should be possible in both settings of old and new CN.

In conclusion, in pathological human CN new vessels acquire pericyte coating. Pathological new vessels become rapidly covered by pericytes during vessel maturation. An angioregressive strategy aiming at regression of immature, pericyte free blood vessels relying on blockade of angiogenic factors should be most effective if applied very early in the course of human CN.

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