

# Histological findings of surgically excised choroidal neovascular membranes after photodynamic therapy

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## Abstract

**Aim**—To investigate effects of photodynamic therapy (PDT) on human choroidal neovascularisation (CNV).

**Methods**—Two patients with recurrences after PDT with verteporfin underwent surgical extraction of the CNV. Immediately after surgical excision the subfoveal neovascular membranes were divided for light microscopic and for electron microscopic processing. For light microscopy tissues were embedded in paraffin. Sections were stained with haematoxylin and eosin, and the periodic acid Schiff (PAS) reaction was performed to determine histological diagnosis and to ensure tissue quality. For electron microscopy the specimens were fixed in glutaraldehyde and embedded in epoxy resin. Semithin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope.

**Results**—Light microscopy showed thick fibrovascular membranes in both cases. On the outer surface remnants of retinal pigment epithelial cells resting on thickened inner aspect of Bruch's membrane were found. On the retinal side some outer segments were found. The membrane showed areas with irregularly shaped vessels. Electron photomicrographs showed occluded vessels within the CNV containing thrombotic masses and/or ultrastructural damage of the neovascular endothelium. Most of the vessels presented regressive changes with vacuolisation and fragmentation of the neovascular endothelium accompanied by disintegration of the endothelial cell layer. Extravasation of red blood cells was observed. Occasionally, vessels with normal endothelium containing intact red blood cells were observed. Some vessels contained immature endothelial cells. At some locations the retinal pigment epithelium cells (RPE) were metaplastic showing highly vacuolised cytoplasm.

**Conclusions**—These findings suggest that the evidence of fluorescein leakage from the CNV and enlargement of the neovascular complex following PDT could be related to new vessel growth and recanalisation of occluded vessels. Additionally, RPE disturbances were observed in the specimens. This finding may be related to the original pathology or could indicate that PDT treatment may result in RPE atrophy.

(*Br J Ophthalmol* 2001;85:1086–1091)

Age related macular degeneration (AMD) is the leading cause of new blindness in the elderly, and choroidal neovascularisation (CNV) is responsible for 80% of severe visual loss in patients with AMD.<sup>1,2</sup> Subfoveal occurrence of CNV leads to progressive loss of visual acuity due to subretinal fluid accumulation and fibrosis. It has been demonstrated that laser photocoagulation of subfoveal CNV has a long term beneficial effect on visual acuity, although patients experience an immediate decline in vision after laser photocoagulation.<sup>3–5</sup> In view of the poor visual results following laser photocoagulation in subfoveal CNV photodynamic therapy (PDT) has been developed for the management of this condition.<sup>6–8</sup> Recently, the beneficial effect of PDT using benzoporphyrin derivative, or verteporfin has been demonstrated by the TAP study group.<sup>9,10</sup> Previous experimental studies using a rabbit model have shown that subretinal vessels could be occluded by PDT without alteration of the adjacent retina.<sup>6,11</sup> However, the exact mechanism of PDT induced CNV occlusion is unclear. In particular, the high number of CNV with evidence of fluorescence leakage after PDT suggests reopening or regrowth of the pre-existing neovascular proliferations.<sup>12</sup>

To characterise the cellular and extracellular constituents of CNV after PDT, we have studied morphological features of surgically excised subfoveal CNV that had been treated with PDT.

## Material and methods

Two patients with predominantly classic CNV underwent PDT with verteporfin under an expanded access programme. Clinically, these two patients had experienced a significant deterioration of visual acuity and considerable subretinal fibrosis within 3 months after initial PDT therapy. The fluorescein angiograms at this time demonstrated evidence of leakage from the CNV with increase in size of the lesion. We suggested repeated treatment with PDT to the patients. Both patients refused repeated PDT. As alternative treatment we offered surgical extraction of the CNV to the patients. After full explanation of the experimental nature of this treatment the patients gave written informed consent and underwent surgical membrane extraction.

Immediately after surgical excision the subfoveal neovascular membranes were divided for light microscopic and for electron microscopic processing. For light microscopy tissues were fixed for at least 4 hours in a solution of 2% paraformaldehyde. Tissues were dehydrated

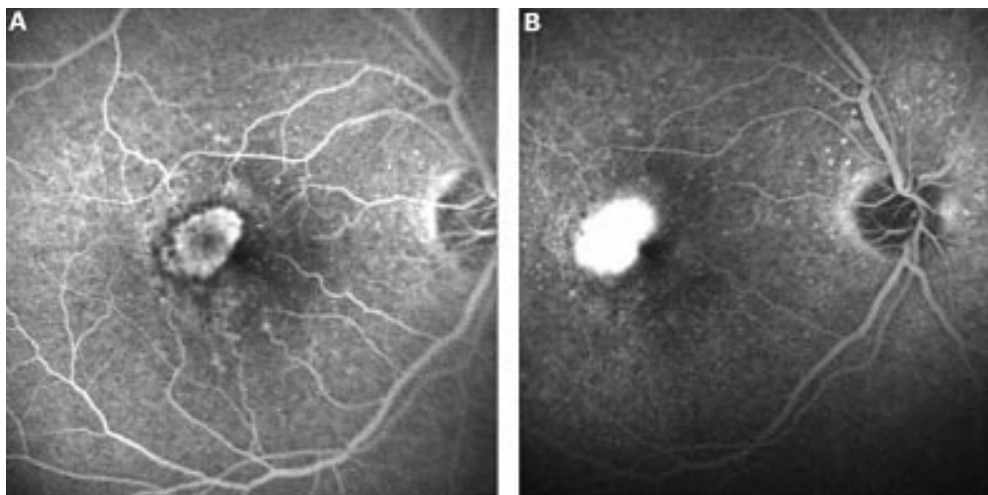
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Accepted for publication  
3 April 2001



*Figure 1* Angiographic studies before PDT treatment (patient 1) He had stable fixation within the area of new vessels visible in the early angiographic study. (A) Early phase angiogram. (B) Late phase angiogram.

with declining alcoholic dilutions, embedded in paraffin, and sectioned at 1.5  $\mu\text{m}$ . Sections were stained with haematoxylin and eosin and the periodic acid Schiff (PAS) reaction was performed to determine histological diagnosis and to ensure tissue quality. Five to eight paraffin sections from each CNV were used for immunostaining. Deparaffined sections were hydrated in a descending alcohol cascade, treated for 5 minutes with 3%  $\text{H}_2\text{O}_2$  solution, rinsed in distilled water, treated for 5 minutes in TBS using Triton x-100 at pH 7.6, and stored in serum protein block serum free (Dako $\times$ 909) for 10 minutes. Overnight the sections were stored with the primary antibody (rabbit anti-human collagen type III, polyclonal antibody (Chemicon); rabbit anti-human collagen type IV, polyclonal antibody (Chemicon); rabbit anti-human collagen type VI, polyclonal antisera Chemicon) at different dilutions ranging from 100:1 to 200:1 at 4°C, in a moist chamber and rinsed in TBS with Tritonx-100, stored for 1 hour with the diluted secondary antibody at room temperature, rinsed in TBS with Triton x-100 for 3  $\times$  10 minutes, stored with the PAP complex (rabbit PAP Dako Z 113), diluted in TRIS buffer pH 7.6 for 1 hour, and rinsed three

times in TBS-Triton x-100. After reaction with the DAB-Set the sections were developed for 1–5 minutes, rinsed in distilled water and dehydrated in ascending alcohol, and embedded in Canada balm.<sup>13</sup>

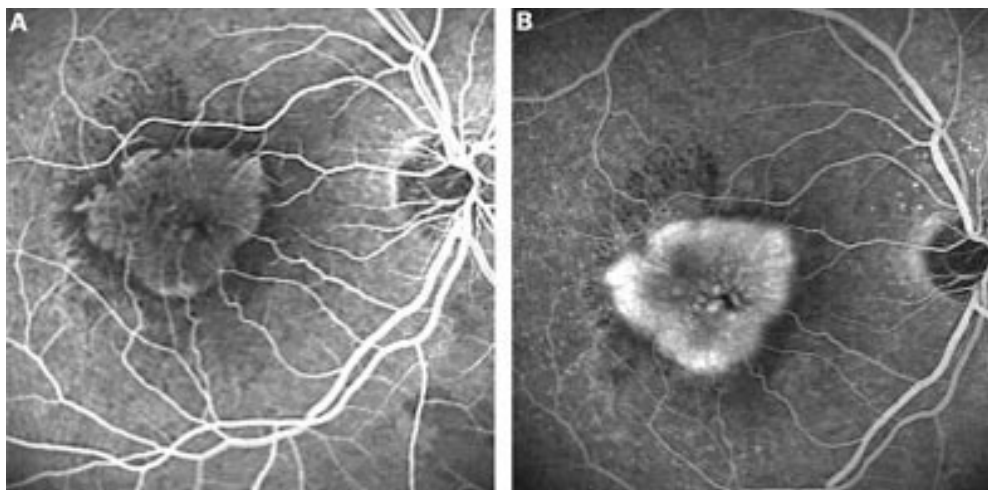
For electron microscopy the specimens were fixed in buffered 2.5% glutaraldehyde for 2 hours, rinsed in buffer, postfixed in 1% osmium tetroxide for 2 hours, dehydrated in an acetone cascade, and embedded in epoxy resin.

Semithin sections for orientation were stained with paraphenylenediamine and 1% toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (EM 900 Zeiss, Germany).

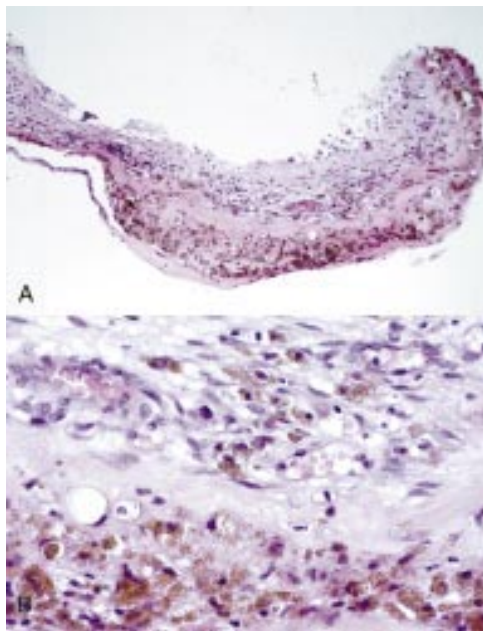
## Results

### CASE 1

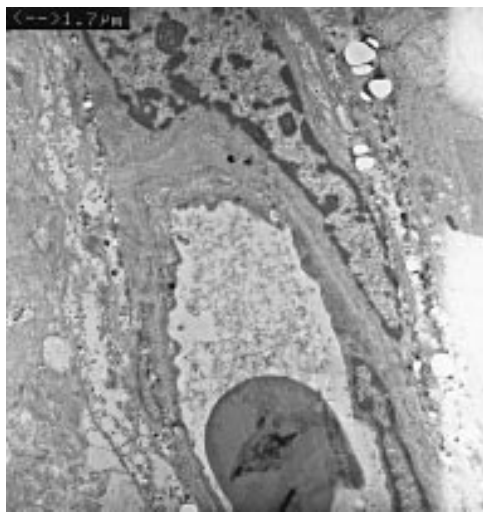
A 74 year old man was referred for visual loss of recent onset in his right eye. Visual acuity was 20/50 in the right eye. The left eye had a long standing exudative AMD with severe visual loss and a fibrovascular scar. Ophthalmoscopy and fluorescein angiography revealed a classic subfoveal CNV in the right eye (Fig 1). The patient underwent PDT with verteporfin for treatment



*Figure 2* Angiographic studies after PDT (patient 1) (A) Early phase angiogram demonstrating a marked enlargement of the neovascular complex. (B) Mid-phase angiogram.



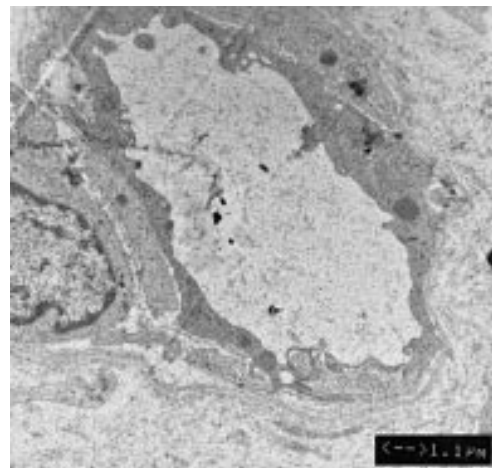
**Figure 3** (A) General view of CNV after PDT represents hyaline masses between retinal pigment epithelium and fibrovascular tissue with severe oedema (haematoxylin and eosin staining,  $\times 100$ ). (B) The hyalinised masses in the CNV were partly surrounded by small red cell containing vessels partly invading the CNV (haematoxylin and eosin  $\times 400$ ).



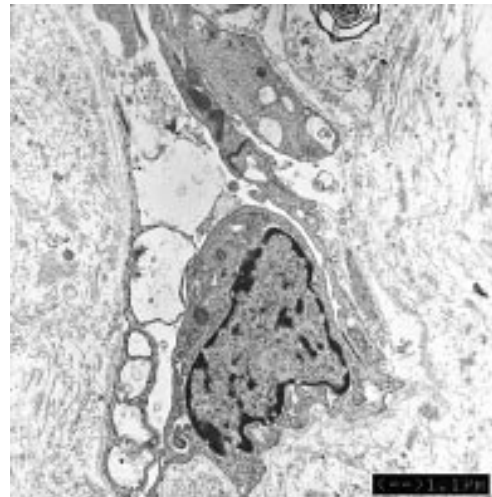
**Figure 4** Capillary with structurally damaged endothelium and part of an pericyte. Note the fibrin, debris, and the erythrocyte in the lumen of the vessel.

of the subfoveal CNV in the right eye. At 12 weeks after PDT the fluorescein angiogram showed significant leakage in the area of the CNV and an enlargement of the neovascular complex (Fig 2). Visual acuity had dropped to 20/400. We suggested repeated treatment with PDT to the patient, but he refused. As an alternative treatment we offered macular translocation surgery to the patient. Surgery was performed 2 weeks later with surgical extraction of the CNV. Six months after surgery visual acuity was 20/200.

After excision, the general macroscopic configuration of the membranes was that of fibrovascular tissue adjacent to the retinal pigment epithelium (RPE). Light microscopy showed a thick fibrovascular membrane with a major



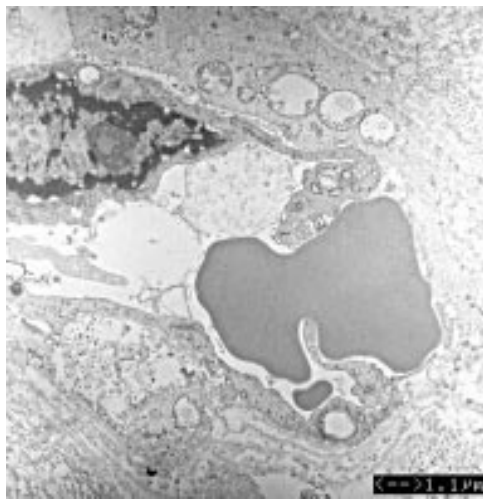
**Figure 5** Capillary with structurally damaged endothelium and part of a pericyte. The lumen contains lots of fibrin and debris. Additionally, cellular debris can be seen in the matrix around the vessel.



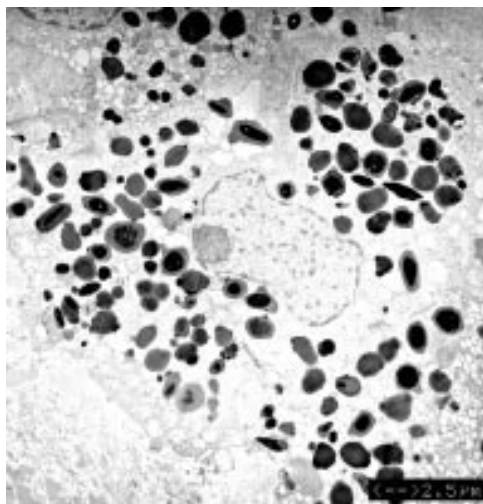
**Figure 6** Part of a CNV membrane after PDT containing a damaged microvessel. The endothelium shows excessive bubbling and partly total loss of continuity.

subretinal component (Fig 3). On the outer surface remnants of retinal pigment epithelial cells resting on the thickened inner aspect of Bruch's membrane were found. Additionally, some minor parts of the membrane were detected under the retinal pigment epithelium. On its retinal aspect amorphous tissue with striation perpendicular to the plane of the membrane was found. This tissue represents degenerated outer segment material indicating the neuroretinal side of the specimen. The membrane contained areas with irregularly shaped vessels. Extracellular constituents consisted of homogeneous matrix, which showed positive PAS reaction, and small fibres of connective tissue. Scattered fibroblasts, macrophages, extravasated red blood cells, and non-pigmented stromal cells were present (Fig 3). Immunohistochemical staining gave evidence of type III collagen and type VI fibres within some CNV membrane in places.

Electron photomicrographs showed occluded vessels within the CNV containing thrombotic masses and/or ultrastructural damage of the neovascular endothelium (Figs 4 and



*Figure 7* The endothelium with split basal lamina, structural disintegration, and loss of continuity; further margination of condensed chromatin of the nucleus. Note the extravasated red blood cells. An erythrocyte just penetrates the endothelium.

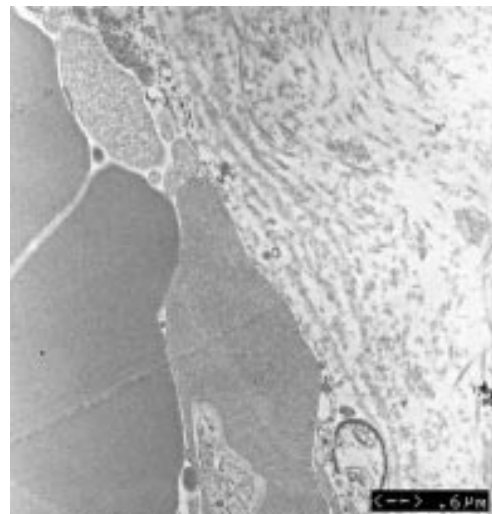


*Figure 8* Retinal pigment epithelial cells characterised by numerous lipofuscin and melanolipofuscin, and lancet-shaped melanin granules. Note the vacuolated cytoplasm.

5). Most of the microvessels presented regressive changes, consisting in vacuolisation and fragmentation of the neovascular endothelium accompanied by disintegration of the endothelial cell layer (Fig 6). Extravasation of red blood cells could be observed in these areas. Endothelial cells with pyknotic nuclei were also found. The majority of mitochondria appeared damaged and myelin figures were frequent. The endothelial basal lamina was partially split (Fig 7). Occasionally we observed vessels with immature endothelium containing intact red blood cells.

In some central locations of the sections the retinal pigment epithelium cells showed marked degeneration (Fig 8). In the apical part of the pigment epithelial cell, degenerated outer segments of photoreceptors were engulfed and highly vacuolated in the cytoplasm. Other areas showed relatively normal retinal pigment epithelium.

Additionally, we have seen abundant small collagen fibrils in the CNV (Fig 9). Scattered



*Figure 9* Part of a dehiscent blood vessel showing isolated remnants of endothelium. Loosely arranged collagen fibrils are found outside the basal membrane.

fibroblastic cells and parts of their processes with marked cytoplasmic organelles, especially rough endoplasmic reticulum (rER), were present. Cellular debris as well as cellular elements which could not otherwise be specified by electron microscopy were found.

#### CASE 2

A 50 year old woman consulted the clinic for acute visual loss in the right eye. Visual acuity was 20/60 in the right eye and 20/20 in the left eye. Ophthalmoscopy and fluorescein angiography revealed a predominantly classic CNV in the right eye. The left eye demonstrated soft drusen at the posterior pole. We performed PDT with verteporfin for treatment of the subfoveal CNV in the right eye. Three months later visual acuity was 20/200. Clinically, the centre of the CNV showed significant fibrosis but the fluorescein angiogram showed an active neovascular net adjacent to the fibrovascular tissue. The patient refused repeated PDT and selected surgical membrane extraction as an alternative treatment. Surgery was performed 4 months after initial PDT. Visual acuity was 20/400 at the last follow up visit 6 months after submacular surgery.

Light microscopy showed a thick fibrovascular membrane with a predominant subretinal component (Fig 10). In most parts of the specimen the outer surface showed remnants of retinal pigment epithelial cells resting on the thickened inner aspect of Bruch's membrane. At the retinal face some outer segments were found. The membrane showed areas with irregularly shaped vessels. Extracellular constituents consisted of homogeneous matrix and small fibres of connective tissue. Scattered cells, fibroblasts, pericytes and their processes, macrophages and extravasated blood cells, non-pigmented stromal cells and pigmented cells were present. Immunohistochemical staining gave evidence of type III collagen and type VI fibres within some CNV membrane in places.

Electron photomicrographs showed similar features to case 1. The vessels within the CNV were partly occluded by thrombotic material

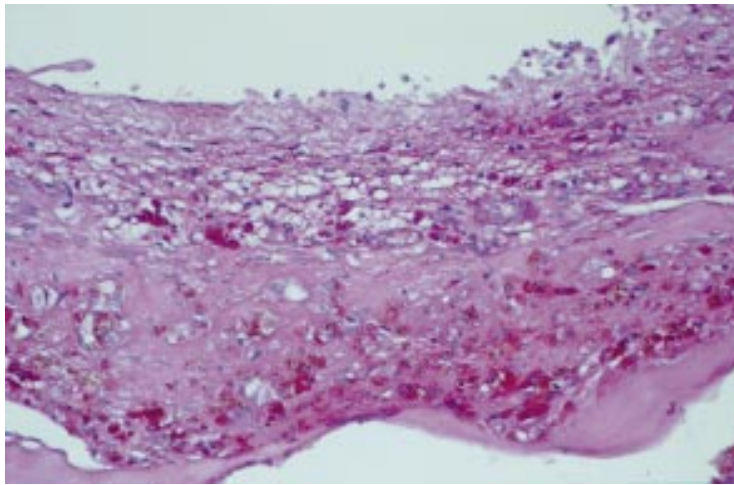


Figure 10 General view of CNV after PDT with a major subretinal fibrovascular component. Membrane contains hyaline masses between retinal pigment epithelium and fibrovascular tissue with severe oedema (PAS reaction,  $\times 200$ ).

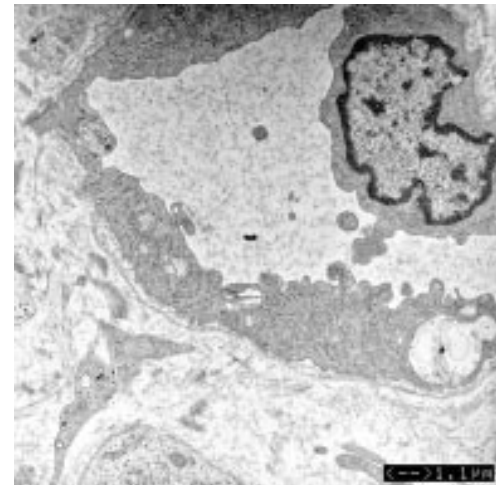


Figure 12 Immature capillary of a CNV after PDT. The endothelium contains abundant rough endoplasmic reticulum, some intact mitochondria, and a thin basal lamina. Collagen fibrils and parts of fibroblasts surrounding the vessel.

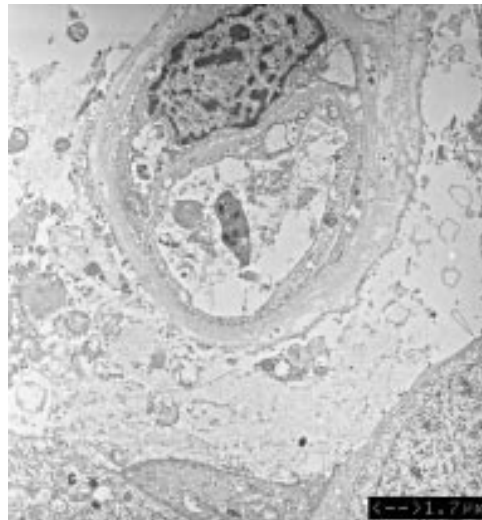


Figure 11 Thrombotic capillary with structurally damaged endothelium and pericyte of a CNV after PDT. Note the fibrin and debris in the lumen and the split basal lamina. Cellular debris is also in the fibrillar matrix around the vessel.

(Fig 11). The neovascular endothelium showed ultrastructural damage such as vacuolisation and fragmentation of neovascular endothelium. Most nuclei of the endothelial cells were pyknotic. The mitochondria were destroyed, myelin figures were frequent, and the endothelial basal lamina was split (Fig 12). Some vessels with immature endothelial cells contained intact red blood cells. The retinal pigment epithelium cells showed damaged cytoplasm with vacuoles in some parts.

Small collagen fibrils, scattered fibroblastic cells, and cellular debris were present as described above.

### Discussion

Recently, randomised clinical trials<sup>9 10</sup> have demonstrated the beneficial effect of photodynamic therapy in patients with predominantly classic subfoveal CNV secondary to AMD. In that study most of the patients had evidence of fluorescein leakage from the CNV following PDT after repeated treatment with PDT.<sup>10</sup> The

explanation for fluorescein leakage from the CNV includes recanalisation of vessels or new vessel formation.<sup>14</sup> Since PDT may have no influence on the underlying mechanism that originally caused the CNV, the new vessel formation may be the result of the original pathology. Other explanations include a local inflammatory reaction with release of cytokines and angiogenic factors or impairment of choriocapillary perfusion resulting in a hypoxic stimulus for new vessel formation.<sup>15</sup>

In two patients with initially predominant classic CNV secondary to AMD we removed the neovascular complex surgically within 4 months after a single photodynamic therapy with verteporfin. Clinically, both patients showed reperfusion and enlargement of the neovascular complex 3 months after initial treatment. Patient 1 had experienced a severe visual loss within 3 months after initial PDT treatment. Since only 5% of the treated cases undergoing photodynamic therapy as reported by the TAP study group<sup>10</sup> experience a severe visual loss, the CNV in this patient may be more aggressive than in most patients with exudative AMD.

We used light microscopy for the analysis of cellular components (RPE cells, vascular endothelium, fibrocytes, macrophages, and photoreceptors) as well as extracellular components (collagen, fibrin). Additionally, immunohistochemical staining was applied for characterisation of the collagen type.<sup>13</sup>

Both specimens contained a fibrovascular membrane located mainly between the RPE and the neurosensory retina. Additionally, a minor fibrovascular component was found under the RPE. These features are consistent with type 2 CNV with retinal pigment epithelium on the outer side and photoreceptor outer segments on the inner side.<sup>16</sup> However, the exact histopathological interpretation of the surgically excised specimen is often difficult. Distortion of the specimens can only be overcome by complete serial sectioning. Additionally, incomplete removal of the membrane

cannot be excluded. The type 2 characteristics of the excised membranes challenge the concept that CNV in AMD grows primarily under the RPE (type 1).<sup>2 16-19</sup> However, some authors pointed out that CNV in AMD are similar to those seen in myopia, presumed ocular histoplasmosis and multifocal choroiditis except for the presence of laminar deposits.<sup>20</sup> Recent studies<sup>20-23</sup> have shown that subretinal neovascularisation is a more common component of CNV in AMD than previously thought. Especially, classic CNV in AMD may have a predominant subretinal fibrovascular component and represent in the majority of cases type 2 neovascular membranes.<sup>22</sup>

The cellular components in these two specimens included retinal pigment epithelium, inflammatory cells, vascular endothelium, and extravasated red blood cells. The membranes contained occluded and perfused vessels. Occluded vessels were characterised by thrombotic material within the lumen (Fig 6), whereas perfused vessels contained intact red blood cells. This is consistent with the clinical appearance of perfusion of the CNV and significant leakage in fluorescein angiography.

With electron microscopy we noted ultrastructural damage of the neovascular endothelium in occluded vessels. In these vessels the endothelial basal lamina was partly split, several endothelial cells contained pyknotic nuclei, and the majority of mitochondria were more or less destroyed. The extracellular matrix was rich in collagen fibrils of small and medium calibre. Occasionally we observed growing capillaries characterised by immature endothelium, rich in organelles. The retinal pigment epithelium showed significant degeneration. These included cytoplasm with vacuoles and damaged mitochondria.

Our findings are consistent with previous reports on surgically excised choroidal neovascular membranes. Previous electron microscopic studies<sup>20 23-26</sup> have shown that many of these cells contain microfilaments, leading to the conclusion that these are myofibroblasts or fibroblasts. Therefore, our findings may be non-specific and cannot definitely be attributed to PDT. The observed damage of the endothelial cells has been described in animal models of photodynamic therapy of choroidal neovascularisation.<sup>8 27</sup>

In this paper we report light and electron microscopic findings of choroidal neovascular membranes after PDT treatment. The findings suggest that the evidence of fluorescein leakage from the CNV and enlargement of the neovascular complex following PDT could be related to new vessel growth and rescanalisation of occluded vessels. Additionally, we observed RPE disturbances in our specimens. This finding may be related to the original pathology or could indicate that PDT treatment may result in RPE atrophy.

Proprietary interest: None.

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*Br J Ophthalmol* 2001 85: 1086-1091  
doi: 10.1136/bjo.85.9.1086

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