Expression of cell adhesion molecules and vascular endothelial growth factor in experimental choroidal neovascularisation in the rat

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Abstract

Aims—To investigate the longevity and reproducibility of choroidal neovascularisation (CNV) induced by krypton laser photocoagulation in the rat. The presence of cell adhesion molecules (CAMs) and vascular endothelial growth factor (VEGF) during the development of CNV was also studied.

Methods—67 pigmented rats underwent retinal photocoagulation by krypton laser. The eyes were examined by either single or serial fluorescein angiography at 3 days, 1, 2–3, 4–5, 7–8, and 12 weeks post photocoagulation. The expression of CAMs (ICAM-1, E-selectin, and CD44) and VEGF post photocoagulation was studied by immunohistochemistry.

Results—CNV related fluorescein leakage appeared in 46.4% of 766 laser spots delivered to the 58 eyes that were tested at 2–3 weeks post treatment. The ratio of hyper-fluorescent laser sites did not change significantly at 8 weeks post laser. The number of leaky spots was independent of the total number of lesions delivered to each eye (at 2–3 weeks post laser 10–15 spots/eye: 44% and 25–30 spots/eye: 49%; $t=0.7673$; $p=0.3903$). Nine eyes were followed by serial angiography between 2 and 12 weeks. The laser spots with fluorescein leakage at 2 weeks (51.5%) remained leaky at 12 weeks (51.5%). Histopathologically, macrophage accumulation peaked at 5 days and CNV was firstly observed at 1 week post photocoagulation. ICAM-1, E-selectin, CD44, and VEGF were maximally induced at 3–5 days post laser photocoagulation, and were localised to RPE, choroidal vascular endothelial, and inflammatory cells. VEGF was also detected in intravascular leucocytes at the sites of laser lesions.

Conclusions—These studies demonstrated that krypton laser photocoagulation can be successfully used to produce lesions similar to those of human CNV. The response induced remained present for an extended period of time (12 weeks), thus offering a potential model to screen candidate CNV inhibitory agents. In addition, it is proposed that the expression of ICAM-1, E-selectin, CD44, and VEGF before new vessel formation might be linked to the initiation of CNV.

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Choroidal neovascularisation (CNV), a major complication of age related macular degeneration (ARMD), is characterised by the formation of a choroid derived neovascular membrane.¹ The process starts with endothelial cell activation at sites of local degradation of the basement membrane. The endothelial cells then migrate, proliferate, and differentiate to form a capillary sprout followed by the formation of a neovascular membrane.² Histopathology and immunohistochemistry have demonstrated that the cell types present within subretinal neovascular membranes include retinal pigment epithelial (RPE) cells, myofibroblasts, fibroblast-like cells, inflammatory cells, vascular endothelial cells, and pericytes.^{3–5}

Although the primary trigger of the development of CNV is poorly understood, cytokines and growth factors have been proposed to play an important role in the advancement of CNV. In the process of angiogenesis, the activation of endothelium by cytokines is an early pathophysiological response, as it alters the haemostatic functions of the vascular lining cells.⁶ Cell adhesion molecules (CAMs) and integrins are important sites of cell to cell communication, mediating cell adhesion, and signal transduction. During inflammation, they allow leucocytes to interact with endothelial cells and thus regulate the adherence and migration of endothelial cells into the surrounding tissue.⁶ A number of known CAMs, including intercellular adhesion molecule-1 (ICAM-1), E-selectin (CD62E), and CD44, have recently been proposed to be involved in neovascularisation.^{6–8}

The potential role of growth factors in ocular neovascularisation has been recognised for almost half a century,⁹ and several growth factors, such as the fibroblast growth factors, insulin-like growth factor, and vascular endothelial growth factor (VEGF) have been proposed to be involved.^{10–11} However, only VEGF seems to fulfil the criteria of an universal mediating molecule for ocular neovascularisation.¹² VEGF is an ischaemia induced glycoprotein with both angiogenic and vasopermeability activity. It is also secreted by a variety of activated cell types and is a highly specific mitogen for vascular endothelial cells.^{12–13}

The expression of VEGF has recently been demonstrated in surgically removed neovascular membranes from eyes with neovascular ARMD^{3–14–15} and in experimental CNV induced by krypton laser photocoagulation.^{16–17} Likewise, a correlation between ICAM-1,

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E-selectin, and CD44, and ocular neovascularisation has been shown.^{18–20} In a study of laser induced ocular inflammation Richardson and co-workers suggested the changes in CAM expression regulate the migration and activation of RPE and inflammatory cells.²¹ However, at this stage it remains unknown whether changes in CAMs and, additionally, VEGF expression precedes or possibly regulates the development of CNV. Laser photocoagulation has been previously used to induce both wound repair and CNV responses in a number of animal models.^{16 17 21–25} However, the results vary depending on the laser techniques used, and the optimum CNV model is yet to be established.

This study aimed to investigate the longevity and reproducibility of a krypton laser induced CNV model in the rat. In addition, the location and dynamic expression of ICAM-1, E-selectin, CD44, and VEGF were studied during CNV development.

Materials and methods

MODEL OF CNV

Sixty seven dark Agouti pigmented rats at 8–10 weeks of age were anaesthetised by intramuscular xylazine (6 mg/kg, Bayer AG, Germany)/ketamine (50 mg/kg, Lambert Company, USA) injection. The pupils were dilated with 2.5% phenylephrine (Chauvin Pharmaceuticals Ltd, Romford, Essex) and 1% tropicamide (Alcon, Belgium). Krypton laser irradiation (647.1 nm, Coherent Radiation System, CA, USA) was delivered through a Zeiss slit lamp with a hand held coverslip serving as a contact lens. The laser spots were placed separately using a setting of 100 μ m diameter, 0.1 second duration, and 150 mW intensity. In 58 eyes, 10–15 laser burns were arranged in each eye, surrounding the optic nerve at the posterior pole. In the remaining nine eyes, 15–30 laser spots were delivered onto the retina with the same protocol and the eyes were followed by serial fluorescein angiography. Fluorescein angiography was performed from 3 days to 3 months after laser photocoagulation through intraperitoneal or intravenous injection of 0.1–0.2 ml of 10% sodium fluorescein. A 60° angle of view and high resolution photography of the rat fundus was obtained by a simple modification of a Canon CF-60ZA retinal camera (Kawasaki, Kawagawa, Japan) as previously described.²⁶ All procedures adhered to the guidelines described in the ARVO statement of the use of animals in ophthalmic and vision research.

HISTOPATHOLOGY

A total of 58 animals were sacrificed after single fluorescein angiography at 1, 3, 5 days and 1, 2, 3, 4, 5, 7, and 12 weeks after laser photocoagulation. At least two eyes were taken for each time interval. The enucleated eyes were fixed in 2.5% glutaraldehyde with 0.1 M phosphate buffer saline (PBS) at pH 7.4 for 24 hours. Retinas containing laser lesions were identified and selected under a dissecting microscope. Laser lesions that had been associated with fluorescein leakage were also

identified in some eyes. The selected tissues were post fixed with 1% osmium tetroxide for 1 hour, dehydrated with a series of graded ethanols, then embedded in epoxy resin. Sections of 3 or 4 μ m were cut and stained with 1% toluidine blue.

IMMUNOHISTOCHEMISTRY

For immunohistochemistry, four to five eyes were taken at each interval as mentioned above. The enucleated eyes were fixed in 4% paraformaldehyde in 0.1 M PBS for 4–6 hours. After fixation, the eyes were washed in PBS, then PBS with increasing concentrations of sucrose as previously described.²⁷ The following day eyes were bisected posterior to the limbus and their anterior segments and lenses removed. Some eye cups were embedded in Tissue Tek OCT and snap frozen using liquid nitrogen. The others were placed in 50% ethanol for 2 hours, followed by 70% ethanol at 4°C overnight, then embedded in paraffin. Cryostat sections of 10–12 μ m thickness were cut at –20°C, air dried and stored at –70°C until use. Paraffin sections of 6 μ m thickness were cut and stored at 4°C. All sections were mounted on 2% 3-aminopropyl-triethoxysilane (Sigma, St Louis, MO, USA, Code A-3648) coated slides. Before immunohistochemistry, some paraffin sections were deparaffinised, stained with haematoxylin and eosin, and observed using a light microscope. The monoclonal antibodies of mouse anti-rat CD68 (1:500), ICAM-1 (1:500), CD44 (1:5), and mouse against human E-selectin (1:100) were obtained from Serotec Ltd, Kidlington, Oxford. The antibodies were diluted in 0.1 M PBS pH 7.4 containing 1% bovine serum albumin (BSA). Paraffin sections were deparaffinised in xylene and brought to PBS through graded ethanol baths. As a result of the dense pigmentation, it is difficult to detect immunopositive stained cells in the choroid. We employed a bleaching procedure²⁸ to detect signals within the choroid and RPE layer. Briefly, the sections were bleached by a 30–45 minute incubation in 0.25% KMnO₄ solution followed by 0.1% oxalic acid for 5 minutes. The possible effect of bleaching was evaluated for each antibody by comparing the immunostaining before and after bleaching. All incubations were carried out in a moist chamber at room temperature unless otherwise specified. Sections were first preincubated with 10% swine serum in PBS for 20 minutes, given 3 $\times$ 5 minute washes in PBS, then incubated with primary antibody at 4°C overnight. The sections were then washed and incubated with Universal Dako Lasb 2 (Dako, CA, USA, Code K0609) biotinylated secondary antibody for 30 minutes. After 3 $\times$ 5 minutes washes in PBS, sections were treated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase, then incubated with streptavidin peroxidase for 30 minutes. Peroxidase was developed with 3-amino-9-ethylcarbazol (AEC, Sigma, St Louis, MO, USA, Code A-6926) for 10 minutes to yield a red reaction product. Most sections were

Table 1 Development of choroidal neovascularisation (CNV) evidenced by single fluorescein angiography (FA) post krypton laser photocoagulation

Time post laser	Eyes	Laser spots	CNV evidenced by FA	Rate of leaky spots
3 Days	10	113	5	4.4%
2-3 Weeks	31	431	200	46.4%
4-5 Weeks	7	77	46	59.7%
8 Weeks	10	145	65	44.8%

lightly counterstained with Mayer's haematoxylin before mounting.

For VEGF immunostaining, paraffin sections were treated and bleached as described above. After blocking with 10% normal goat serum and incubated with rabbit anti-human VEGF IgG (1:100, Santa Cruz, CA, USA) at 4°C overnight. This antibody was raised against amino terminal epitope of human VEGF mapping at residues 1-20 and recognises the 121, 165, and 189 amino acid splice variants of VEGF. The sections were washed in TRIS buffered solution (TBS) and incubated with alkaline phosphatase conjugated goat anti-rabbit IgG (1:200, Vector Labs, Burlingame, CA, USA) for 1 hour. Colour detection was carried out by fast red TR/Naphthol AS-MX (Sigma, St Louis, MO, USA) for 20 minutes. Sections were counterstained and mounted as described above. In negative controls, the primary antibodies were replaced by normal rabbit or mouse IgG (1 µg/µl, dilution 1:100, Sigma, St Louis, MO, USA) or omission of the primary antibody.

The immunostaining for CD68, ICAM-1, E-selectin, CD44, and VEGF was performed in three or four sections from three or four different animals at each time interval, and repeated twice for each procedure. In each laser spot the percentage of positively stained cells was assessed under a microscope (magnification ×400). The immunostaining was graded as ± (very weak signal, 1-3 cells/view), + (weak signal, 4-6 cells/view), ++ (medium signal, 7-10 cells/view), and +++ (strong signal, >10 cells/view) by two masked observers.

Results

FUNDUS PHOTOGRAPHY AND FLUORESCIN ANGIOGRAM

Krypton laser photocoagulation at the chosen settings produced laser burns of moderate whiteness, that were usually accompanied by central bubble formation, with or without localised intraretinal or choroidal haemorrhage (Fig 1A). It was found that intraperitoneal injection of fluorescein was superior to tail vein injection

as it consistently provided an image of good contrast with wide field and high resolution. The presence of CNV related fluorescein leakage was recognised as a focal hyperfluorescent spot²³ with progressive leakage during the angiogram. Minor fluorescein leakage was detected in 4.4% of laser spots at 3 days post laser photocoagulation. This early fluorescein leakage was probably due to the presence of persisting unsealed laser injuries (Table 1). Except for these occasional leaky sites no fluorescein leakage was present in the majority of eyes (Fig 1B). CNV related fluorescein leakage appeared at 2-3 weeks post laser treatment in 200 of 431 (46.4%) of the originally delivered laser spots. The ratio of hyperfluorescent laser sites did not change significantly during the 8 week follow up period (Table 1). The number of leaky spots at 2-3 weeks post laser was independent of the total number of lesions delivered (10-15 spots/eye (average 11 spots/eye, n=17): 83/189 (44%); 25-30 spots/eye (average 22 spots/eye, n=6): 67/136 (49%); $r=0.7673$; $p=0.3903$). To monitor the development of leakage in individual laser spots nine eyes were followed in a time course. Those laser spots which developed hyperfluorescent leakage by 2-3 weeks post laser treatment (Fig 1C) maintained fluorescein leakage for the rest of the 3 months follow up period (Fig 1D and Table 2).

HISTOPATHOLOGY

The development of CNV started with the disruption of the Bruch's membrane and the RPE layer (Fig 2A). At 1-3 days after laser photocoagulation, RPE cells showed migration, proliferation, and multilayering (Fig 2B, open arrow). The accumulation of leucocytes was evident immediately around the region damaged by laser photocoagulation. Based on their morphological appearance²⁹ the infiltrating cells were identified as neutrophils (Fig 2B, small arrow) and monocyte macrophages (Fig 2B, arrowhead). By 5 days post laser treatment, the inflammatory cells predominantly consisted of pigment laden macrophages (data not shown). CNV was first observed at 1 week post photocoagulation. Pigment laden macrophages were commonly observed around and within the region of neovascularisation and occasionally they were located at the advancing front of the choroidal neovascular network (Fig 2C, star). The identification of the infiltrating cells was determined using a specific marker (CD68) for macrophages. There was no CD68 related signal present in the normal retina (data not shown). The number of CD68 positive cells

Table 2 Time course of the development of choroidal neovascularisation (CNV) as followed by fluorescein angiography (FA)

Eye	Laser spots	CNV evidenced by FA (weeks)					
		1	2-3	4	5	7	12
1	24	6	9	9	9	9	9
2	26	8	18	18	18	18	18
3	15	6	7	7	7	7	7
4	17	6	11	12	12	12	12
5	18	7	10	10	10	10	10
6	12	8	10	10	10	10	10
7	21	3	11	12	11	11	11
9	30	6	8	8	7	7	7
Total 9	163	50 (30.6%)	84 (51.5%)	86 (52.7%)	84 (51.5%)	84 (51.5%)	84 (51.5%)

Figure 1 Choroidal neovascularisation induced by krypton laser photocoagulation in sample 3 of Table 2. (A) Fundus photograph demonstrating the 15 laser spots delivered. Laser spots were photographed at 3 days post photocoagulation and were consecutively numbered. (B–D) Fluorescein angiograms of the same animal taken at different time points after photocoagulation. (B) At 3 days post photocoagulation no fluorescein leakage was seen; however, some non-staining window defects were noted (arrows). (C) At 2 weeks post photocoagulation fluorescein leakage was detected in seven of the 15 laser spots. (D) At 3 months post photocoagulation the same laser spots maintained fluorescein leakage.

constantly increased and peaked at 1 week post photocoagulation. The presence of CD68 positive cells persisted for the whole observation period of 8 weeks with a widespread distribution in the choroid, subretinal space, and inner retina at the lesion sites (Fig 2D). Histological examination of 10 laser spots which demonstrated fluorescein leakage during angiography confirmed the presence of CNV in all of these spots. The majority of neovascular membranes were situated in the subretinal space without intrusion into the neural retina (Fig 2E) and they included many new vessels (Fig 2F).

IMMUNOSTAINING OF ICAM-1, E-SELECTIN, CD44, AND VEGF

While the generation of laser burns required the use of pigmented animals, the presence of

melanin in the RPE layer and the choroid caused difficulties in the interpretation of the histochemical data. To overcome these obstacles, bleaching of melanin was implemented and the intensity of the relevant signals compared in bleached and unbleached samples. It was found that bleaching did not significantly effect ICAM-1, E-selectin, CD44, and VEGF generated signal intensity (data not shown).

The results of the immunohistochemical analysis are summarised in Table 3. In the normal rat retina, there was no ICAM-1 related signal present in the choroid, RPE, or the retina other than a weak signal in the external limiting membrane (ELM), internal limiting membrane (ILM), and the ganglion cell layer (data not shown). ICAM-1 expression was

Figure 2 Histopathological studies after laser photocoagulation. (A) One week after photocoagulation, new vessels (small arrows) occurred with the disruption of Bruch's membrane (large arrows) ($\times 400$). (B) Three days post photocoagulation, RPE cells (open arrows) showed migration, proliferation, and multilayering. The infiltrating cells predominantly consisted of neutrophils (arrows) and small number of monocyte macrophages (m, arrowhead) ($\times 1000$). (C) Three months post photocoagulation, the neovascular network (arrows) disrupted the sealed RPE layer and invaded into the neural retina. Pigment laden macrophages (m) were observed around and within the region of neovascularisation and one macrophage (star) was located at the advancing front of the neovascular network ($\times 1000$). (D) Six weeks post photocoagulation, numerous CD68 positive macrophages (arrows) were observed in the laser photocoagulated area ($\times 400$). (E) Three months after photocoagulation, a section from the eye of Figure 1D demonstrates a fully developed neovascular membrane (star) ($\times 100$). (F) Higher magnification of (E) demonstrated numerous new vessels (arrows) within the neovascular membrane ($\times 400$). (A) and (C) Resin embedded, stained with toluidine blue. (B), (E), and (F) Paraffin sections stained with haematoxylin and eosin. (D) Cryostat section counterstained with haematoxylin.

Figure 3 Immunohistochemical studies after laser photocoagulation. (A) Immunostaining for ICAM-1 at 3 days after photocoagulation ($\times 400$). (B) Immunostaining for E-selectin, 5 days after photocoagulation ($\times 400$). (C) Immunostaining for CD44, 5 days after photocoagulation ($\times 400$). In A–C, positive staining was localised to choroidal vascular endothelial cells (arrows), infiltrating cells (arrowheads), and RPE cells (open arrows). The star in (A) indicates the photocoagulated site. (D) The normal rat retina demonstrated very weak immunoreactivity to VEGF in RPE (arrow) and choroidal layer ($\times 200$). (E–H) Immunostaining for VEGF at 5 days after photocoagulation ($\times 400$). Following photocoagulation, positive staining was localised to the choroidal vascular endothelial cells (E, arrows), accumulating infiltrating cells (F, arrowheads), and the proliferating RPE cells (G, open arrows). No VEGF immunostaining was detected in the foci of lesion where neovascularisation has been enveloped by the proliferating cells (H). Arrow is pointing to a new vessel in the middle of a neovascular membrane.

Table 3 Immunohistochemical detection of cell adhesion molecules (ICAM-1, E-selectin and CD44) and vascular endothelial growth factor (VEGF) in laser treated rats

Tissue	Time post laser	ICAM-1	E-selectin	CD44	VEGF
Retinal layer	Untreated	ELM, ILM, GL	–	ELM, ILM, GL	–
	3–5 days	ELM, ILM, GL	–	ELM, ILM, GL	–
	2 weeks	ELM, ILM, GL	–	ELM, ILM, GL	–
RPE cells	Untreated	–	–	–	±
	3–5 days	++	+	++	+++
	2 weeks	+	–	–	+
Choroidal layer	Untreated	–	–	–	±
	3–5 days	+++ VEC, IF,	+ VEC, IF	+++ VEC, IF	+++ VEC, IVL, IF
	2 weeks	–	–	±	+

ELM = external limiting membrane; ILM = internal limiting membrane; GL = ganglion cell layer; VEC = vascular endothelial cells; IF = infiltrating cells; IVL = intravascular leucocytes. – no signal; ± very weak signal, 1–3 cells/view; + weak signal, 4–6 cells/view; ++ medium signal, 7–10 cells/view; +++ strong signal, >10 cells/view.

induced in RPE cells and the choroidal layer at the site of laser injury around 3–5 days post photocoagulation (Fig 3A). There was a strong immunostaining within the choroid. The signal appeared to be localised to the choroidal vascular endothelial cells (Fig 3A, arrows) and to infiltrating cells (Fig 3A, arrowhead). In addition, an increased staining was observed in the RPE cells (Fig 3A, open arrows). Two weeks after photocoagulation, the RPE cells remained immunoreactive but all signal disappeared from the choroid (data not shown). The presence of E-selectin could not be demonstrated in the normal retina (data not shown). However, it was detected in the choroidal layer and in the RPE layer at 3–5 days after photocoagulation. The immunostaining localised to choroidal vascular endothelial cells (Fig 3B, arrow), infiltrating cells (Fig 3B, arrowhead) and to the RPE layer (Fig 3B, open arrow). There was no E-selectin related signal detected at 2 weeks after photocoagulation in any cells. The presence of CD44 was demonstrated in the ELM and ILM layers, and in the ganglion cells of the normal retina, but was undetectable in the RPE or choroid before laser photocoagulation. CD44 related signals were detected in vascular endothelial (Fig 3C, arrows) and infiltrating cells (Fig 3C, arrowheads) at 3 and 5 days after photocoagulation. CD44 was also present in a few RPE cells (Fig 3C, open arrows) and in the choroid, but this signal was only transient, and had disappeared by about 2 weeks after laser treatment (data not shown). The normal rat retina demonstrated very weak immunoreactivity to VEGF in RPE and the choroidal layer (Fig 3D). Photocoagulation induced a significant increase in VEGF immunoreactivity, with maximum immunostaining visible at 3 and 5 days after treatment. The positive staining was observed in choroidal vascular endothelial cells (Fig 3E, arrow), intravascular leucocytes (data not shown), areas of infiltrating cell accumulation (Fig 3F,) and proliferating RPE cells (Fig 3G). The VEGF immunostaining was significantly weaker at 2 weeks after laser photocoagulation in all cell types. No VEGF immunostaining was detected in the foci of laser lesions in which the neovascularisation (Fig 3H, arrow) was enveloped by proliferating cells. All immu-

nostaining experiments were accompanied by appropriate controls, and none of the negative controls generated detectable immunoreactivity (data not shown). In all cases (Table 3), the increased immunoreactivity of CAMs and VEGF preceded the development of fluorescein leakage that was seen in the laser lesions (Tables 1 and 2).

Discussion

These studies demonstrated that krypton laser photocoagulation can be successfully used to produce lesions similar to those of human CNV. The fluorescein leakage at individual spots remained present for 3 months and was independent of the total number of laser lesions. In our hands, the number of lesions developing CNV was higher than has been previously reported,²³ possibly due to improvement of the fundus photography technique allowing high resolution and a wider field.²⁶ The experimentally induced CNV was observed actively growing through a disrupted Bruch's membrane and was accompanied by RPE proliferation and inflammatory cell infiltration. The pigment laden macrophages, which appeared by 3–5 days post photocoagulation, seemed to precede the growth and invasion of the neovascular network. Previous investigations, both clinical and experimental, have suggested that activated macrophages which are known to release proteases, growth factors, and monokines³⁰ take part in the pathological process of CNV.^{15 23 25 29 31 32}

The current study suggests an overexpression of ICAM-1, E-selectin, CD44, and the resulting infiltration after laser photocoagulation may be of significance in the pathogenesis of CNV. ICAM-1 is involved in leucocyte endothelial adhesion and leucocyte migration through its receptor lymphocyte function associated antigen-1 (LFA-1).^{33 34} ICAM-1 is known to be expressed by proliferating endothelial cells in the epiretinal membranes of eyes with proliferative diabetic retinopathy,^{18 19} and has been found in retinal and choroidal vascular endothelial cells, and RPE cells stimulated by inflammation.^{35 36} Likewise, it has been shown that soluble E-selectin induces chemotaxis of vascular endothelial cells through a sialyl Lewis-X dependent mechanism.⁷ In addition CD44 regulates endothelial cell migration, proliferation, and closely correlates with corneal neovascularisation.^{8 20} The mechanism of these factors could therefore be either via products released by the migrating inflammatory cells, or the direct angiogenic function of E-selectin and CD44. It has been hypothesised that CAMs on the endothelial cells are shed when leucocytes bind to endothelial cells. The shed molecules then bind to adjacent endothelial cells, and possibly lead to direct angiogenic effects as well as mediating the inflammatory response.⁷

Our experiments showed that the accumulating inflammatory cells and proliferating RPE cells express VEGF at the site of the laser lesions for a limited period of time. Besides being produced by macrophages,^{15–17 30} lymphocytes, and peripheral mononuclear cells,³⁷

VEGF was also expressed in retinal intravascular leucocytes.³⁸ VEGF expressed by intravascular leucocytes may directly induce endothelial cell activation, leucocyte endothelial adhesion, and enhance vascular permeability. As hypoxia has been identified as the main VEGF stimulating factor,³⁹ the expression of VEGF in this CNV model could be due to a reduction in the local choroidal vascular blood supply after laser photocoagulation.¹⁷ VEGF stimulates macrophage migration, increases the levels of ICAM-1 and E-selectin, and facilitates lymphocyte recognition of new vessels.⁴⁰⁻⁴¹ Based on these facts, VEGF may induce macrophages and lymphocytes to migrate into the laser lesions in both a paracrine and autocrine fashion. Overall, the upregulation of VEGF production by a variety of cells in the laser site might induce endothelial cell proliferation which can result in new vessel formation. However, it is important to note that the expression of VEGF was temporary. By the time the neovascular network was enveloped by proliferating RPE cells VEGF was not present in the foci of photocoagulated lesions. The absence of VEGF expression is in agreement with previous reports where the envelopment of choroidal new vessels by RPE cells prevents the continuation of neovascularisation.⁴² RPE cells have been proposed to have a bidirectional effect on the proliferation of vascular endothelial cells.^{3-5, 22-29, 42-43} This bidirectional effect is well demonstrated by the results of numerous photocoagulation studies.^{17, 22, 42-44} The delivery of limited number of laser spots has been showed to induce active neovascularisation^{16, 17, 23, 24} even without breaking the Bruch's membrane.²⁵ In contrast with these findings, it is well documented that pan-retinal photocoagulation reverses active neovascularisation in clinical diabetic retinopathy.⁴⁵ The inhibition of new vessel growth by panretinal photocoagulation is not well understood. A recent study demonstrated that scatter photocoagulation induces the expression of CAMs²¹ which have been shown to be linked to inflammation^{33, 34} and angiogenesis.^{6, 7} Further studies to identify factors responsible for the bidirectional effect of RPE cells are essential as they might offer clues to the pathogenesis of CNV.

In summary, krypton laser photocoagulation have been used successfully to produce lesions similar to those of human CNV. The lesions demonstrated fluorescein leakage which remained present for 3 months. In addition, our study demonstrated that in case of the delivery of limited number of laser spots (<30) which break the Bruch's membrane, the upregulation of CAMs arises together with VEGF production and precedes the development of new vessels. Therefore, we propose that transient over-expression of CAMs and VEGF correlates with the vascular endothelial cell activation and, in turn, might be linked to the initiation of CNV-like lesions in this model.

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