MODULATORS AND MILIEU IN PRERETINAL NEOVASCULARISATION

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It has long been established that preretinal neovascularisation follows retinal capillary non-perfusion and inner retinal ischaemia,[1,2] but the precise mechanism whereby ischaemia stimulates new vessel growth is unclear. Some consider that hypoxia is the sole aetiological factor in preretinal neovascularisation (independent of growth modulators).[3,4] Others advocate that vasoproliferation is mediated by the release of diffusible biochemical factors from the ischaemic area which induce new vessels to grow from adjacent capillaries and venules.[1,5] The four major events of preretinal neovascularisation (i.e. basement membrane breakdown, endothelial cell migration, endothelial cell proliferation and vessel remodelling) can be modulated by a variety of such soluble factors.[6,7] These modulators, usually peptides, are growth-regulating molecules which have a wide spectrum of biological activities and which exert their actions by binding with specific receptor sites localised in the plasma membrane of target cells.[8,9] Although the majority of these modulators are designated 'growth factors' this is probably a misleading term since they not only modulate cell proliferation but also regulate cell migration, extracellular matrix turnover, the production of adhesion molecules and a variety of other intrinsic biological activities.

The aim of this article is to consider the evidence supporting a crucial role for oxygen in retinal new vessel formation. From an *in vivo* perspective we will consider oxygen profiles in the normal retina and how they change under pathological conditions. From an *in vitro* perspective we will discuss why we believe routine cell culture studies are normally carried out under extremely hyperoxic, non-physiological conditions and how the intrinsic biological activity of retinal microvascular cells changes dramatically when cells are cultured within a more pertinent oxygen environment. Finally, we will consider the origin of ocular growth factors and attempt to reconcile otherwise conflicting hypotheses regarding the pathophysiology of preretinal neovascularisation.

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OXYGEN LEVELS IN THE NORMAL RETINA

The retinal oxygen supply is derived from two sources: the choriocapillaris and the inner retinal capillary net.[10] The choroidal circulation supplies oxygen to the avascular outer retina located between the choriocapillaris and the deep retinal capillaries (primarily the retinal pigment epithelium (RPE), photoreceptors and some neurons in deeper retinal layers). It is estimated that the choriocapillaris normally supplies 60% of the tissue in this avascular area while the other 40% is supplied by the inner retinal capillaries.[10,11]

Standard textbooks of physiology quote the arterial PO_2 as 100 mmHg, venous PO_2 as 40 mmHg and the PO_2 in tissues generally as 35 mmHg. In the retina, the oxygen environment has been determined using micro-electrodes, and studies in animals have demonstrated a non-uniform distribution of oxygen supply and consumption in the various retinal layers. Oxygen tensions are highest in the region of the RPE/photoreceptor complex (up to 70–90 mmHg) while PO_2 values can decrease to as low as 17 mmHg in mid-retina.[12–15] Values at the retina–vitreous interface appear to be intermediate between the two. There is also some topographic variation within the retina and immediately preretinally, PO_2 values being slightly higher adjacent to arterioles than in intervascular areas. Since the values for transretinal oxygen tensions are similar in rabbits, cats, pigs and monkeys it is reasonable to assume that like PO_2 levels are present in the human eye. Intravitreal oxygen tensions in man and rabbit are similar, PO_2 values ranging from 14 to 20 mmHg.[15]

OXYGEN LEVELS IN THE PATHOLOGICAL RETINA

Little is known about changes in retinal oxygen tension

before and during preretinal neovascularisation, i.e. in the context of persisting retinal capillary non-perfusion. However, Maeda *et al.*[16] measured oxygen tensions during diabetic vitrectomy and recorded values of 12.1 mmHg in the mid-vitreous cavity, 31.4 mmHg above neovascular membranes, 14.8 mmHg above the macula and 24.0 mmHg in the subretinal space. The high value above the neovascular tissue probably reflects oxygen delivery without associated consumption. Stefansson *et al.*[17] reported preretinal oxygen tensions of 110 (± 42) mmHg in patients undergoing vitrectomy for proliferative diabetic retinopathy (PDR). The difference in PO_2 levels between the two studies may reflect differences in the oxygen breathing mixtures used; the patients of Maeda *et al.* were breathing normal air (21% oxygen) while those in Stefansson's study were breathing about 30% oxygen.[17]

In animals, experimental occlusion of the retinal blood vessels leads to a reduction in intraretinal oxygen tension and Wolbarsht *et al.*[18] reported a drop in preretinal oxygen tension from 20 mmHg to zero after occlusion of the retinal circulation at the optic disc of the cat. Occlusion of retinal blood vessels in the pig leads to the development of local anoxia following arteriolar occlusion[19,20] or local hypoxia following the occlusion of a venule.[21] Stefansson *et al.*[4] observed that temporary occlusion of the retinal circulation in albino rats resulted in a proliferative response with mitotic figures in both the retinal capillary endothelial cells and the pericytes, thus intimating a relationship between retinal oxygenation and cell proliferation *in vivo.*

SCATTER PHOTOCOAGULATION

Scatter photocoagulation is a proven treatment for preretinal neovascularisation.[22] The mechanism by which retinal photocoagulation results in new vessel regression is unclear but a number of theories have been put forward: (1) destruction of the ischaemic retina curtails the production of angiogenic factors,[23] (2) destruction of retinal tissue makes more oxygen available for the hypoxic inner retina[24] and (3) cellular changes after photocoagulation stimulate production of an inhibitory modulator which induces regression of new vessels.[25,26] Several experimental studies have shown that scatter photocoagulation increases retinal oxygen tension and may thus counteract retinal hypoxia.[27–31] Furthermore, Stefansson and colleagues[17] have recently shown that in patients undergoing vitreous surgery for PDR, the oxygen tension was significantly higher over areas of retina that had been treated with laser (140 ± 59 mmHg) than it was over untreated areas (110 ± 42 mmHg).

CONSTRAINTS IN EXPERIMENTAL STUDIES

In the absence of a practical animal model with which to study preretinal neovascularisation, research into the cellular mechanisms of new vessel formation has relied on either *in vivo* new vessel formation in non-retinal vascular beds or the use of *in vitro* culture techniques.[6] Although valuable information on the identity and actions of growth stimulators and inhibitors has been obtained, these studies have largely ignored the role of oxygen in modulating their production and their interactions at the cellular level. The best tool for such studies is cell culture, which allows the effect of oxygen levels on retinal microvascular cell behaviour to be investigated.

OXYGEN LEVELS IN CELL CULTURE MEDIA

Cultured cells are routinely grown in an environment of 95% air/5% CO_2.[32] This air/CO_2 mixture is misleadingly termed 'normoxia' while lower atmospheric oxygen concentrations are regarded as 'hypoxia'. However, measurements of oxygen tensions in growth medium exposed to 'normoxia' (i.e. 95% air/5% CO_2) show a media PO_2 of between 133 and 152 mmHg,[33–37] which is far higher than the highest oxygen tensions recorded in the normal adult retina (70–90 mmHg).[12–15] Thus existing cell culture studies of the physiology of retinal microvascular cells have failed both to mimic the *in vivo* situation and to address the role of hypoxia in cellular function. A further problem is that of the few publications that are available, most have failed to measure the PO_2 levels in the media and claims of achieving hypoxia *in vitro* may well be unfounded.

EFFECT OF OXYGEN ON *IN VITRO* CELL PROLIFERATION

Rosen *et al.*[36] demonstrated that the proliferation of retinal microvascular cells *in vitro* is dependent on oxygen concentration. The proliferation of retinal microvascular endothelial cells, retinal pericytes and Tenon's fibroblasts was greatest at a media PO_2 of 82 mmHg and decreased as the media PO_2 increased. The decrease in the proliferative ability of cells exposed to higher oxygen concentrations could be reversed if the cells were returned to lower oxygen environments. In addition, it was observed that endothelial cells were more sensitive to changes in the oxygen environment than were either pericytes or fibroblasts. An extension of this study has examined cell proliferation of these cell types at a media PO_2 of less than 82 mmHg (Boulton *et al.* unpublished). Cells exposed to a PO_2 of 30–50 mmHg exhibited similar growth characteristics to those exposed to a PO_2 of 82 mmHg while 14–17 mmHg resulted in a proliferative capacity lower than at either 82 or 133 mmHg; no cell cytotoxicity was observed. A typical example is shown for Tenon's fibroblasts in Fig. 1a. The response of these cells to low oxygen appears to be a general phenomenon since a similar result has been reported for a variety of non-ocular cell types.[33–35,38] Whether oxygen acts directly or indirectly to modulate cell behaviour is unclear. However, the studies described below support an indirect mechanism through which oxygen can modulate cells via the production of, and response to, growth factors.

THE EFFECT OF OXYGEN ON THE PRODUCTION OF GROWTH MODULATORS

Despite the hypothesis by Michaelson[5] that retinal hypo-

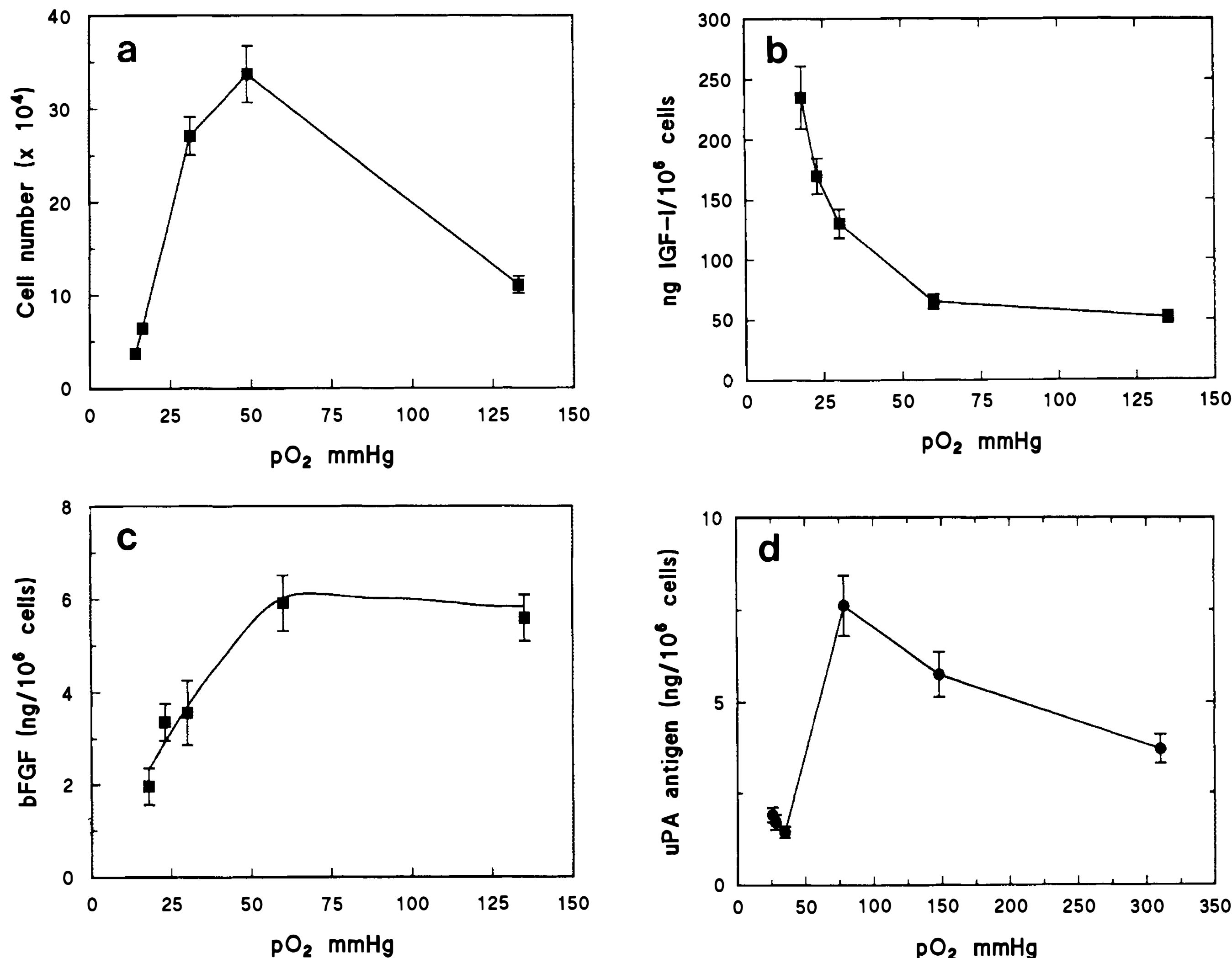

Fig. 1. *Graphs showing the effect of oxygen on* in vitro *cell behaviour. (a) Comparison of the proliferative capacity of Tenon's capsule fibroblasts exposed to varying oxygen concentrations. Cell counts were performed 3 days post plating. (b) Levels of IGF-I in serum-free conditioned media from confluent cultures of pericytes exposed to varying oxygen concentrations for 48 hours. IGF-I levels were determined by radioimmunoassay. (c) Intracellular bFGF levels in confluent cultures of pericytes exposed to varying oxygen concentrations for 48 hours. Cell lysates were prepared by exposing monolayers to Triton X-100/NH_4OH and bFGF levels were determined by radioimmunoassay. (d) Levels of uPA in plasma-containing conditioned media (Dulbecco's modified Eagle's medium + 7.5% human platelet-poor plasma) from confluent cultures of RPE cells exposed to varying oxygen concentrations for 48 hours. uPA levels were determined by an ELISA kit (Biopool, Sweden). Vertical bars indicate SEM* (n>6).

xia results in the production of diffusible biochemical factors which mediate new vessel growth, and the subsequent identification and characterisation of a plethora of such factors,[6-9] the effect of hypoxia *per se* on the production of such growth modulators has received little attention.

By way of background, in non-ocular tissues the levels of platelet-derived growth factor (PDGF) in the culture media overlying pulmonary and aortic endothelial cells have been shown to increase under hypoxia as compared with normoxia.[39] A similar response was seen in the production of cytokine neutrophil attractant activity by pulmonary endothelial cells.[40] Conversely exposure of aortic and microvascular endothelial cells to hypoxia (14 mmHg) resulted in decreased levels of endogenous basic fibroblast growth factor (bFGF) when compared with cells grown in 'normoxia' (145 mmHg).[35] The production of tissue plasminogen activator (tPA) and its inhibitor PAI-1 (factors which modulate extracellular matrix turnover) were also influenced by the oxygen tension in the media.[41,42]

Recent studies in this laboratory have begun to elucidate the effect of oxygen on the production of growth modulators by retinal cells. We have shown that cultured retinal microvascular endothelial cells, pericytes and RPE cells release insulin-like growth factor-I (IGF-I), which is a potent mitogenic and metabolic modulator of many cell types,[43] and that production increases towards hypoxia (Fig. 1b). Furthermore, the response to IGF-I in many tissues is modulated by high-affinity IGF binding proteins (IGFBPs) of which several forms exist. A whole host of cell types have been shown to produce IGFBPs, and these binding proteins can either inhibit or enhance local IGF-I action.[43] Pilot studies using western ligand blot analysis have demonstrated cell-specific differences in the types of

IGFBP synthesised by retinal microvascular endothelial cells, retinal pericytes, RPE and Tenon's fibroblasts cells. With the exception of endothelial cells production of IGFBPs is sensitive to oxygen concentration. The production of some IGFBPs is 5- to 8-fold greater at a PO_2 of 18 mmHg than at 150 mmHg and the reverse profile is evident for other IGFBP isoforms.

Parallel studies on the effect of oxygen on bFGF (a potent endothelial cell mitogen[8]) have shown that endogenous levels of this growth factor decrease as the PO_2 levels are reduced from 75 to 18 mmHg (Fig. 1c). These results are similar to those reported by Shreeniwas *et al.*[35] Initial studies indicate a similar profile for transforming growth factor-beta (TGF-β), with secreted levels decreasing from normoxia to hypoxia.

Finally, studies on RPE cells suggest that oxygen may modulate the production of plasminogen activators and their inhibitors by microvascular cells.[44] Production of urokinase plasminogen activator (uPA) by RPE cells was greatest at a PO_2 level of approximately 75 mmHg but uPA secretion decreased if the PO_2 levels were increased or reduced (Fig. 1d). In contrast PAI-1 production was lowest at a PO_2 of 35 mmHg and increased at lower or higher oxygen tensions.

EFFECT OF OXYGEN ON *IN VITRO* RESPONSES TO GROWTH MODULATORS

In the only study to date on the effect of oxygen on the response of endothelial cells to growth modulators, Shreeniwas *et al.*[35] observed that exposure of cultured aortic or adrenal cortex capillary endothelial cells immediately after subculturing led to a dose-dependent decrease in cell growth once PO_2 values below 47 mmHg were reached. However, when exogenous bFGF was added to the hypoxic cultures (PO_2 = 14 mmHg), growth was stimulated in a dose-dependent manner with a maximal 12-fold increase above growth in unstimulated hypoxic controls. A similar response was observed with aortic endothelial cells but the level of stimulation was not as great. Furthermore, receptor binding studies demonstrated a 300% increase in high-affinity bFGF receptors in hypoxic cultures as compared with those exposed to 147 mmHg PO_2. Thus PO_2 can affect the response to growth modulators (probably via receptor up-regulation) as well as growth modulator production.

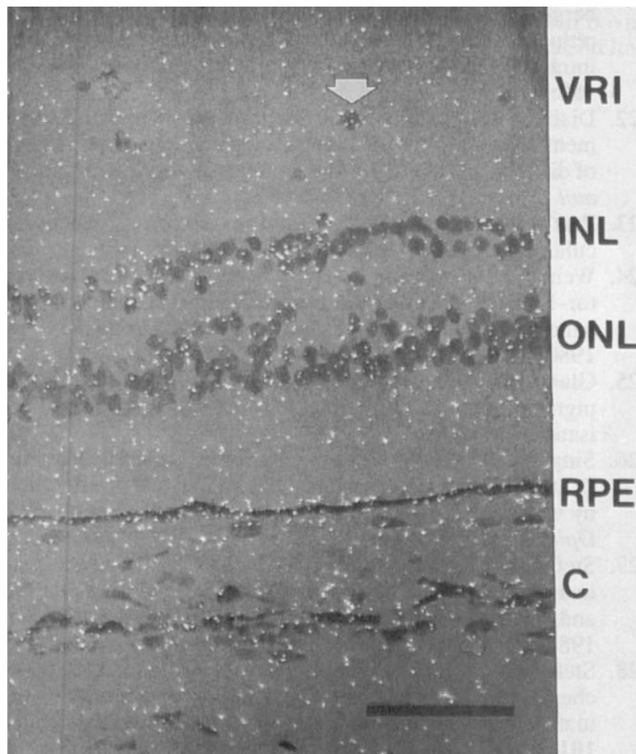

Fig. 2. *Expression of IGF-I mRNA in a section of normal human retina viewed by epi-polarisation microscopy. Low-level labelling can be seen in the inner and outer nuclear layers (INL and ONL respectively), retinal pigment epithelium (RPE), choroid (C) and occasional ganglion cells (arrow). VRI denotes the vitreous–retina interface. Scale bar represents 50 μm.*

SOURCE OF GROWTH MODULATORS IN THE EYE

Although there is considerable evidence implicating a role for growth modulators in normal retinal homeostasis and in proliferative disorders of the vitreous, there is little evidence to indicate whether these molecules are transcribed locally or are of systemic origin. *In situ* hybridisation studies in this laboratory have confirmed the transcription of one such modulator, IGF-I, in the normal human retina. Low levels of expression were observed in the inner and outer nuclear layers, the RPE and choroid (Fig. 2). In addition, expression could also be observed in some ganglion cells and cone photoreceptor cells. The autocrine/paracrine role of this factor in retinal homeostasis is at present unclear.

With respect to growth modulator production in proliferative disorders of the vitreous, mRNA expression of IGF-I, IGF-II, TGF-β and epithelial growth factor (EGF) can all be observed in epiretinal membranes excised during vitrectomy. The message can either be generalised throughout the membrane or specifically located in certain

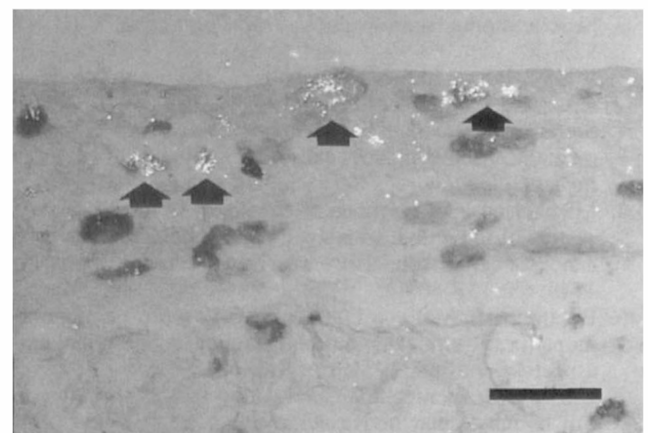

Fig. 3. *Expression of IGF-I mRNA in a section of a late-stage PDR epiretinal membrane viewed by epi-polarisation microscopy. Heavy labelling can be seen associated with some cells towards the edge of the membrane (arrows). Scale bar represents 30 μm.*

cells within the tissue (Fig. 3). In all cases mRNA expression in the membranes was higher than that seen in the normal retina. Although the full implications of these results have yet to be appreciated they do suggest that autocrine/paracrine mechanisms may be a significant component in proliferative disorders in the vitreous.

CONCLUSIONS

It is becoming increasingly evident that retinal and retinal vascular homeostasis are dependent on a fine balance between growth stimulators and inhibitors. Immunohistochemistry and/or *in situ* hybridisation studies clearly demonstrate that aFGF, bFGF, IGF-I, TGF-β, tPA and uPA are all expressed in the normal retina[45–48] and that the levels of these modulators are often dramatically increased during retinal pathologies.[49–52] *In vitro* studies from this and other laboratories suggest that the production of, and response to, such growth modulators is influenced by the local oxygen environment. The variability of this responsiveness (i.e. some increase towards hypoxia, some decrease towards hypoxia, while others exhibit an intermediate profile) suggests that several growth modulators may have an individual role in the ultimate neovascular response. Furthermore, the identification of mRNA expression for these growth factors in the normal retina and in epiretinal membranes suggests an autocrine/paracrine action. Further investigations are underway in an attempt to elucidate the interplay between growth modulators and oxygen, and its importance in the cascade of events leading to preretinal neovascularisation.

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