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Oxygen-induced retinopathy: a model for vascular pathology in the retina

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Abstract

Ischaemic vascular disease in the retina may either leave retina permanently ischaemic with slow degradation of vision, or alternatively lead to proliferative vascular disease, which can also destroy vision. To investigate the molecular and cellular mechanisms that contribute to this pathology a mouse model has been studied extensively. The model is based on the exposure of mouse pups to hyperoxia during a phase when their retinal vasculature is still developing. This leads to capillary depletion, and upon return to room air, results in retinal ischaemia and proliferative vascular disease in the retinal vasculature (oxygen-induced retinopathy (OIR)). Numerous studies using this OIR model have revealed that the regulation of angiogenic factors and the influence of inflammatory cells play a pivotal role in the vascular pathogenesis. It has also been demonstrated in the OIR model that proliferative vascular disease is not the only possible outcome of ischaemia-induced angiogenesis in the retina, but that ischaemic areas in the retina can be revascularised with healthy blood vessels. Therefore, understanding the factors that control the balance between pathological and healthy angiogenesis in the OIR model may have important implications for human retinal ischaemic disease.
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Hypoxia in the retina

In the retina, hypoxia may occur as a result of vascular disruption caused by various pathologies, such as hyperglycaemia in diabetes, thrombosis in vein occlusions or developmental delays in retinopathy of prematurity (ROP). Normally, hypoxia is a key driving force inducing a vascular response, whereby insufficiently perfused tissue is revascularised by the sprouting of new capillaries from pre-existing vessels. In theory, hypoxia in the eye should therefore result in compensatory revascularisation to replenish vessel starved areas. However, for reasons not well understood, the revascularisation is sometimes not successful, leading to abnormal vessels—so-called 'neovascularisation'—instead of healthy, new capillaries. This is a major vision-threatening complication in many ischaemic retinopathies because the abnormal vessels leak, cause oedema and exert tractional forces, causing in worst-case scenarios retinal detachment.

The most popular model to study abnormal angiogenesis in the retina is the oxygen-induced retinopathy (OIR) model in mice.[1] One-week-old mouse pups are exposed to hyperoxia, which obliterates capillaries in the retina. Upon return to room air, the retina becomes hypoxic and triggers a vascular repair response, which then results in the formation of neovascular tufts towards the vitreous, a hallmark of ischaemic retinopathies in human pathologies. Although the pathogenesis of vascular damage in different human ischaemic retinopathies varies considerably, the final neovascular stage is very similar in all of these retinopathies. The tuft formation is often referred to as 'pathological angiogenesis' and has made the OIR model a key tool in addressing vascular pathology in ischaemic retinopathies. There are other hyperoxia–hypoxia mouse models (eg, based on cyclic oxygen levels),[2] and other species have also been examined.[3–6] However, the model by Smith *et al*[1] has become the

protocol of choice because it is reproducible and easily quantifiable.[7,8] Furthermore, the study of molecular mechanisms involved is greatly facilitated by the genetic tools available in mice.

Vaso-obliteration and regeneration in OIR

The OIR model consists of two stages. During the first stage, 7-day-old (P7) mouse pups are exposed to 75% oxygen until P12. In mice the retinal vasculature normally starts to develop around birth and is fully mature around 3 weeks after birth.[9] This means at P7 the mouse retinal vasculature is still immature and susceptible to hyperoxia damage. The hyperoxia primarily targets capillaries adjacent to arteries in the centre of the retina (the location where the highest oxygen concentrations are to be expected) leading to a rapid (within 24 h)[10] expansion of the capillary free zones around arteries and leaving behind a functional network in the periphery, supplied by radial arterioles and venules.[11] This obliteration of capillaries may be caused by the direct, toxic effect that high oxygen levels can have on endothelial cells,[12,13] but alternative mechanisms are also possible.

Hyperoxia does not affect the larger, more mature veins and arteries projecting from the centre to the periphery. Similarly, capillaries in older mice (ie, P21)—where the retinal vasculature is fully developed—are also resistant to the effects of hyperoxia.[14] It is therefore very likely that hyperoxia interferes with developmental processes. For example, hyperoxia drives down expression of vascular endothelial growth factor (VEGF),[11,15] which can lead to reduced endothelial cell survival in immature vessels.[16] In contrast, in the fully matured retinal vasculature endothelial cell survival has become independent of VEGF.[1,17]

It is important to note that the retina does not become hypoxic when the mice are exposed to hyperoxia, despite the large capillary obliteration in the retinal centre. This can be shown with a stain that visualises hypoxia in tissue.[18] The system is based on the *in vivo* administration of the nitroimidazole EF5, which is reduced in hypoxic tissue and then binds covalently to the proteins. These protein adducts can be detected in fixed tissue with fluorescently labelled antibody. Hyperoxia-treated mice that have been injected with EF5 while still in hyperoxia show no signs of hypoxia despite extensive capillary depletion in the centre of the retina (Figure 1a).

The second stage of the OIR model is initiated when the mouse pups are removed from the hyperoxia and placed in normoxia. The retina becomes immediately hypoxic (Figure 1b) because in the capillary-depleted centre of the retina there is only sufficient oxygen in the immediate vicinity of the remaining radial vessels, creating dark shadows in the intense hypoxia stain (Figure 1b). The retinal vasculature responds to the pronounced hypoxia in several ways (Figure 1c). Vascular sprouting is initiated from the remaining capillaries in the periphery and from the veins. In contrast, arteries do not develop angiogenic sprouts. Instead they become tortuous, a phenomenon also observed in ROP (plus disease). Some of the newly forming vascular sprouts fail to regenerate the capillary network and form neovascular tufts towards the vitreous (arrowheads in Figure 1c). Understanding why these tufts occur and why physiological revascularisation fails in the OIR model may have direct translational implications, because it could answer a key ophthalmic question as to why in some human pathologies ischaemic zones fail to be revascularised.

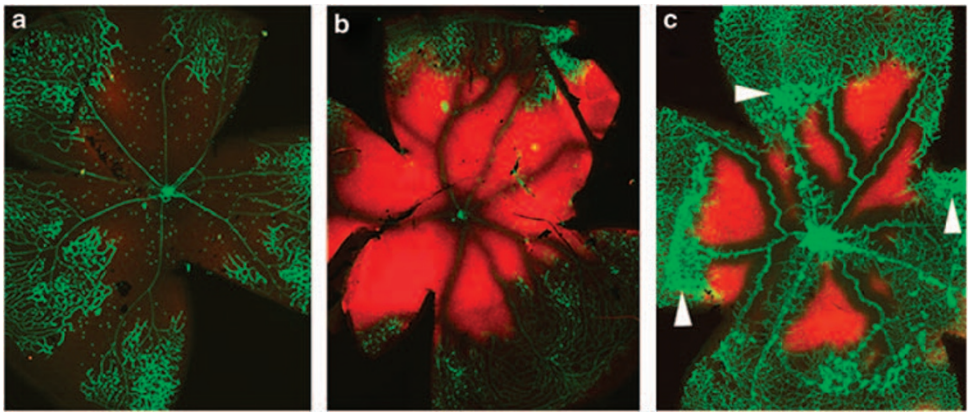

Figure 1 Retinas from C57BL/6 mice during OIR stained with a hypoxia (red) and a vascular stain (green, lectin). Little hypoxia is visible at P12 when the animals are still exposed to hyperoxia despite extensive capillary obliteration (a). Two hours after return to room air the avascular areas become strongly hypoxic (b). After 5 days (P17) the retina is still hypoxic (c) and neovascular tufts have formed (arrowheads).

Mechanisms of neovascular tuft formation

Exposing mouse pups to continuous hyperoxia from P7 to P27 (ie, without a return to room air at P12) paradoxically leads to normal vascular regeneration after the initial vessel depletion. Interestingly, this occurs in the complete absence of pathological neovascularisation, suggesting vascular pathology in OIR might be a consequence of the sudden onset of hypoxia and an overexpression of hypoxia-inducible growth factors when the mice are returned to room air.[14] VEGF, in particular, has been intensively studied in this context. It is strongly upregulated upon return to room air and, because VEGF inhibition with antibodies or soluble VEGF receptor fragments can block neovascularisation, VEGF seems to be a major contributor towards neovascular tuft formation.[19–21] It is plausible that the intense hypoxia causes unphysiologically high levels of VEGF which is known to perturb vascular development.[22] But there are also other factors that have been shown to have a role in neovascular pathologies in the retina. For example, neovascular tuft formation is reduced in mice that lack insulin-like growth factor 1, its receptor or insulin-like growth factor-binding protein-3.[23–25] Similarly, erythropoietin and angiopoietin-2 have been implicated in OIR,[26,27] and there is a rapidly expanding list of other genes that are involved. However, how these genes precisely affect cellular behaviour and how the different cell types (neurons, glia, inflammatory and vascular cells) in the retina interact with each other in OIR is still only partially understood.

Retinal astrocytes critically support the development of the retinal vasculature and are therefore likely to modulate angiogenesis during OIR. They are a potent source of VEGF and provide an important template for growing retinal vessels during development.[9] Retinal astrocytes can be lost during hypoxia and it has been shown that rescue of these cells (eg, via intraocular injection of various factors) normalises retinal revascularisation.[28] A previous study has also shown that treatment with an angiotensin II type-1 receptor antagonist (Valsartan), can improve glia survival and reduce vitreal neovascularisation.[29]

Inflammatory cells, such as lymphocytes, macrophages, and microglia have also been shown to crucially contribute to neovascular pathogenesis. Pioneering work, aimed at blocking specifically the VEGF isoform 164 ($VEGF_{164}$), showed that VEGF not only acts on endothelial cells but can also regulate lymphocyte behaviour in OIR.[30] Moreover, the severity of neovascularisation is reduced by anti-inflammatory reagents (eg, dexamethasone, ibuprofen) or genetic defects targeting the inflammatory system (eg, tumour necrosis factor alpha receptor, monocyte chemotactic protein-1, and macrophage inflammatory protein-1 alpha knockout mice).[31–34] Furthermore, there are other factors that influence the vascular response in OIR, such as nitric oxide, oxidative stress, and dietary omega-3-poly-unsaturated fatty acids, all of which have been linked to inflammatory signalling pathways.[35–37]

However, the precise role of immune cells in OIR is not clear yet. On the one hand depletion or inactivation of resident microglia can reduce pathological neovascularisation.[38,39] On the other hand intravitreal injection of bone marrow-derived cells can suppress neovascularisation and promote revascularisation.[40] It may not be the presence or absence of microglia cells, but rather their activation status, that ultimately affects the outcome of vascular regeneration in the retina. Using interleukin-10 (Il10) deficient mice a recent study has shown that Il10 does not influence macrophage influx but rather regulates their angiogenic function by increasing their VEGF and nitric oxide production.[41]
A further differentiation of the inflammatory response may also depend on whether it is dominated by resident, retinal microglia or invading, bone marrow-derived cells.[42] The later population may contribute to angiogenesis via the secretion of factors. Alternatively, the bone marrow may also contribute to so-called endothelial progenitor cells (EPCs), which might be directly incorporated into growing vessels.[43] EPCs are still a controversial cell population,[44] but they have recently been implicated in ischaemic retinal vascular disease,[45,46] and potentially hold exciting therapeutic promise.[47]

A number of gene expression analyses carried out on OIR tissue have further expanded the list of potential players.[48–50] Apart from classical inflammatory and vascular genes, these studies have also revealed other responding signalling pathways (such as non-classic regulators of angiogenesis[51]) and provide a valuable resource to further elucidate the workings of OIR.

Neovascularisation *vs* revascularisation

So far much research has been focused on the mechanisms that drive pathological neovascularisation in the retina whereas healthy revascularisation has received less attention. It is intriguing that the two processes are usually inversely correlated; when healthy vascular regeneration is increased, neovascular tufts are reduced. However, whether these two vascular phenotypes are two independent processes or whether they are alternative outcomes of the same process is not entirely clear. Early OIR experiments were quantified by counting in transverse sections the number of nuclei that have breached the inner limiting membrane. Crucially

this does not measure the extent of healthy revascularisation efficiently. Only in recent years retinal whole mount analysis has become the norm for the OIR model,[7,8] allowing for the quantification of the area with neovascular tufts, as well as the size of the vessel depleted area, which gives an indication of revascularisation.

Efficient revascularisation reduces hypoxia in the retina and it is plausible that consequently pathological neovascular tuft formation is reduced. In fact, it could be argued that most 'treatments' that have been shown to inhibit vascular pathology without blocking healthy revascularisation in the OIR model, do not directly block angiogenesis but rather promote healthy vessels growth and that the reduction of neovascular pathology is a consequence of reduced hypoxia. For example, it is possible that standard VEGF inhibition (eg, with antibodies) does not completely abolish, but simply reduces VEGF concentrations to physiological levels that are conducive with normal vascular outgrowth, thereby reducing hypoxia.

Interestingly, this balance between physiological revascularisation and vascular pathology in the OIR model is also strongly strain dependant. In C57BL/6J mice neovascular growth is profuse and regeneration minimal (at P17) whereas in BALB/cByJ mice the retinal vasculature efficiently regenerates in the complete absence of neovascular tufts.[40] A similar finding was made in Brown–Norway and Sprague–Dawley rats.[52] Backcross analysis between two other rat strains (Albino Fischer 344 and Dark Agouti) revealed an association between ocular pigmentation and neovascular growth in the retina, whereby more pigmented animals displayed more severe pathology.[53] An association between pigmentation and ROP severity has also been established in humans, but intriguingly in this case stronger pigmentation seems to be protective, leading to less severe disease phenotypes.[54–56]

Relevance for human disease

The better we understand the factors and cellular behaviour that lead to neovascular pathology in OIR, the more potential options we will have for therapeutic interference in human ischaemic retinopathy. However, it will be important to establish whether the same mechanisms are at work in human disease as in the OIR model. In humans the retinal vasculature develops before birth. Therefore, hyperoxia only affects the retinal vasculature of premature babies. Thus, there are obvious similarities between human ROP and mouse OIR. In both systems the retinal vasculature is immature and developing, and in both cases VEGF and IGF1 have a role. Early data suggests that anti-VEGF therapy may hold some promise in the treatment of ROP (although randomised trial data is still outstanding).[57] It is also clear that blocking the action of VEGF can reduce vascular pathology in the OIR model, but the differences between OIR and ROP should also be noted. In the mouse OIR model hyperoxia is the key pathological insult, whereas in ROP oxygen is only one of several factors that have a role in disease pathology. Newborn, hyperoxia exposed mice are in principle healthy organisms with only transient retinal vasculature pathology,[1,14] whereas the most severe cases of ROP tend to also have complications in other organ systems.[54]

By the same logic, caution has to be applied when extrapolating findings from mouse OIR experiments to human proliferative diabetic retinopathy. The vascular pathology (vitreal neovascularisation) seen in the human condition strikingly resembles the tufts that form in mouse OIR, and at the moment the OIR model remains the only mouse model that allows us to investigate proliferative vascular disease in the retina. Nevertheless, it is important to keep in mind that one occurs in healthy, newborn mice whereas the other plays out in diabetic adult individuals with underlying general microvascular disease. Thus, to address the question, why diabetic ischaemia is not resolved by regenerative vascular growth but leads to neovascular pathology, it would be useful to have a model of retinal ischaemia that also includes systemic aspects of diabetes such as reduced function of EPCs and microvascular dysfunction.

Why normal revascularisation does not occur in OIR is an important question because, unlike in cancer biology, in ophthalmology we are not trying to prevent all vascular growth. In fact, to alleviate ischaemia in the retina the opposite is required. Thus, the success of future treatments of retinal neovascular disease in humans will not depend on simply blocking angiogenesis *per se*, but rather on our ability to shift pathological neovascularisation towards healthy revascularisation.

Conflict of interest

The authors declare no conflict of interest.

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