Stabilization of the retinal vascular network by reciprocal feedback between blood vessels and astrocytes

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Summary

Development of the retinal vasculature is controlled by a hierarchy of interactions among retinal neurons, astrocytes and blood vessels. Retinal neurons release platelet-derived growth factor (PDGFA) to stimulate proliferation of astrocytes, which in turn stimulate blood vessel growth by secreting vascular endothelial cell growth factor (VEGF). Presumably, there must be counteractive mechanisms for limiting astrocyte proliferation and VEGF production to prevent runaway angiogenesis. Here, we present evidence that the developing vessels provide feedback signals that trigger astrocyte differentiation – marked by cessation of cell division, upregulation of glial fibrillary acidic protein (GFAP) and downregulation of VEGF. We prevented retinal vessel development by raising newborn mice in a high-oxygen atmosphere, which leads, paradoxically, to retinal hypoxia (confirmed by using the oxygen-sensing reagent EF5). The forced absence of vessels caused prolonged astrocyte proliferation and inhibited astrocyte differentiation in vivo. We could reproduce these effects by culturing retinal astrocytes in a low oxygen atmosphere, raising the possibility that blood-borne oxygen itself might induce astrocyte differentiation and indirectly prevent further elaboration of the vascular network.

Key words: Astrocytes, Retina, Blood vessels, PDGF-A, Oxygen, Transgenic mice

Introduction

Retinal astrocytes are believed to be responsible for controlling development of the retinal vasculature in mammals. Astrocytes emerge from the optic nerve head around birth and spread as a proliferating cell population across the inner surface of the retina (Stone and Dreher, 1987; Watanabe and Raff, 1988; Ling et al., 1989; Sandercoe et al., 1999). The resulting network of astrocytes acts as a template for the developing retinal vasculature, which also spreads out from the optic nerve head (Jiang et al., 1995; Zhang and Stone, 1997; Fruttiger, 2002). The precise mechanisms by which retinal astrocytes guide endothelial cells across the retina are not entirely understood but a recent study has shown that the adhesion molecule R-cadherin, expressed by retinal astrocytes, is important for normal retinal vascular development (Dorrell et al., 2002). Retinal astrocytes also express vascular endothelial cell growth factor (VEGF), which promotes endothelial cell proliferation and migration (Alon et al., 1995; Stone et al., 1995; Pierce et al., 1996; Provis et al., 1997). It has been shown that endothelial cells at the leading edge of the vascular plexus possess long filopodia that closely follow the underlying astrocyte scaffold (Dorrell et al., 2002; Gerhardt et al., 2003). The directed extension of these filopodia is mediated via VEGF receptor 2, and is dependent on the correct spatial distribution of VEGF within the retina (Gerhardt et al., 2003). We previously found that disturbances to the astrocyte network strongly affect vascular patterning (Fruttiger et al., 1996). Thus, the final arrangement of retinal blood vessels depends critically on the pre-pattern of astrocytes; factors that influence retinal astrocyte development ultimately also affect retinal vessels.

Retinal astrocytes arise from a population of precursor cells in the optic nerve head that express Pax2 and the platelet-derived growth factor receptor alpha (PDGFRα) (Mudhar et al., 1993; Otteson et al., 1998; Chu et al., 2001; Dakubo et al., 2003). As these astrocyte precursors invade the retina they proliferate rapidly and start to express low levels of GFAP. As they mature, retinal astrocytes become quiescent and display strong GFAP immunoreactivity (Chu et al., 2001; Gariano, 2003). PDGFRα continues to be expressed at all stages of maturation and is important for retinal astrocyte proliferation and migration, being activated mainly by PDGFA homodimers from retinal neurons (Mudhar et al., 1993; Fruttiger et al., 1996). In transgenic mice that overexpress PDGFA under the control of the neuron-specific enolase gene promoter (*NSE-PDGFA* mice), the number of retinal astrocytes is greatly increased, causing a proportional overgrowth of the retinal vasculature (Fruttiger et al., 1996). This indicates that PDGFA is normally a limiting factor for astrocyte proliferation and subsequent angiogenesis. We speculated that if this limitation were overcome by creating an autocrine mitogenic loop in astrocytes, then the astrocytes might proliferate indefinitely and trigger uncontrolled angiogenesis.

We tested this by engineering transgenic mice that express

a *PDGFA* transgene under control of the *GFAP* gene promoter (*GFAP-PDGFA* mice) (Fruttiger et al., 2000), so that astrocytes provide their own PDGFA. Despite dramatic early hyperproliferation of astrocytes and blood vessels, cell proliferation still slowed down and stopped within a week after birth – just as in wild type mice – although final cell numbers were much higher in the transgenics. We found no evidence that expression of the *PDGFA* transgene or its receptor PDGFRα is extinguished in the transgenics, so there must be other factors that limit retinal astrocyte proliferation even when PDGFA is in excess.

In the present study, we tried to identify factors other than PDGF that limit retinal astrocyte proliferation. We noticed that, in neonatal mice, most astrocyte proliferation and VEGF expression occurs in the peripheral retina, ahead of the advancing vasculature. This suggested that blood vessels or their contents might negatively regulate both astrocyte proliferation and VEGF expression. We tested this by raising newborn mice in a high-oxygen atmosphere, which blocks development of retinal blood vessels and consequently leads to tissue hypoxia. This was accompanied by enhanced astrocyte proliferation and VEGF production, suggesting that blood vessels normally limit their own formation through a feedback signal(s) that serves to cut off the VEGF supply. The negative feedback could be mediated by a molecule(s) released from endothelial cells or the blood. We found that astrocyte proliferation and differentiation could be controlled in vitro by manipulating oxygen tension, raising the possibility that oxygen itself might be an active moiety.

Materials and methods

Transgenic mice

The creation of transgenic mice expressing PDGFA under GFAP promoter control has been described previously (Fruttiger et al., 2000). In brief, human PDGFA cDNA, encoding the 'short' alternative splice form (Pollock and Richardson, 1992), was cloned into an expression plasmid containing 2.2 kb of 5′-flanking sequence derived from the human GFAP gene (Brenner et al., 1994) and used to generate transgenic mice.

Retinal whole-mount preparations

Eyes were fixed briefly in 2% (w/v) paraformaldehyde (PFA) in phosphate-buffered salt solution (PBS) and then dissected in 2×PBS. Retinae were flattened after making radial incisions and then stored in methanol at –20°C. After recovery from methanol, retinae were fixed for 5 minutes in 4% PFA in PBS and washed in PBS before further use.

Hypoxia staining with EF5

Mouse pups were injected with 50 μl of EF5 solution (10 mM in PBS) and sacrificed 2 hours later. Retinae were prepared as described above but methanol fixation was replaced with fixation in 4% (w/v) PFA in PBS for 10 minutes at room temperature.

Immunohistochemistry on retinal whole-mount preparations

Retinal wholemounts were incubated in PBS containing 5% fetal bovine serum (FBS) and 0.5% Triton X-100 for one hour at room temperature. Incubations with antibodies (diluted in PBS containing 1% FBS and 0.1% Triton X-100) were carried out overnight at 4°C (primary antibodies) and for 3 hours at room temperature (secondary antibodies). Antibodies used were mouse anti-GFAP (clone G-A-5, Sigma), rabbit anti-GFAP (gift from Martin Raff), rabbit anti-mouse collagen type IV (Biogenesis, Poole, UK), rabbit anti-Pax2 (Covance Research Product, Princeton, USA), Cy3-conjugated ELK3.51 (monoclonal antibody against EF5 adducts, provided by C. J. Koch, University of Pennsylvania, Philadelphia, USA), Alexa Fluor 488 and 594 anti-rabbit IgG, and Alexa Fluor 594 anti-mouse IgG (Molecular Probes, Eugene, USA).

In situ hybridization of retinal wholemounts

Retinal wholemounts were prepared as described above, then digested slightly for 5 minutes in proteinase K (80 μg/ml in PBS containing 1.3% SDS) followed by fixation for 5 minutes in 4% PFA and 0.2% glutaraldehyde in PBS. After a brief wash in PBS, retinae were pre-incubated in hybridization buffer (Jensen and Wallace, 1997) for 10 minutes at 65°C and then incubated with RNA probes diluted in hybridization buffer at 65°C overnight. Probe labeling with digoxigenin-UTP and visualization of RNA hybrids with alkaline phosphatase-conjugated anti-digoxigenin antibodies was carried out according to the manufacturer's instructions using NBT/BCIP as a colour reagent (Roche Diagnostics GmbH, Mannheim, Germany). For combined in situ hybridization and immunohistochemistry, antibody labeling was performed after the in situ hybridization protocol was completed. When double labeling for blood vessels and BrdU incorporation, we first incubated retinae with an anti-collagen type IV antibody, fixed with PFA (4% in PBS for 10 minutes), treated with 6 M HCl containing 1% Triton X-100 for 45 minutes, and then stained with a mouse anti-BrdU antibody (hybridoma supernatant BU209) (Magaud et al., 1989).

In situ hybridization on tissue sections

Whole eyes were fixed in 4% PFA in PBS overnight at 4°C, cryoprotected in 20% (w/v) sucrose in PBS, embedded in OCT compound (Raymond and Lamb, Sussex, UK), frozen and stored at –70°C. Cryosections (15 μm) were collected on Vectabond (Vector Laboratories, Burlingham, USA)-coated slides and air-dried for two hours. Digoxigenin-labeled probes diluted in hybridization buffer were applied directly to sections and hybridized overnight at 65°C. Subsequent visualization of RNA hybrids was carried out as described above.

In situ hybridization on cultured cells

Cultured cells were fixed for 30 minutes in 4% PFA in 5% acetic acid, dehydrated through an ascending series of alcohols and incubated in xylene for 10 minutes. After rehydrating in a descending alcohol series, cells were digested for 10 minutes at 37°C in 0.1 M HCl containing 0.1% pepsin, washed in PBS and post fixed in 1% PFA in PBS for 10 minutes. Cells were dehydrated again in an ascending series of alcohols, air-dried and then processed according to the same protocol described above for tissue sections.

Cell culture

Retinae were dissected in PBS and then incubated for 30 minutes at 37°C in Dulbecco's minimum essential medium (DMEM) containing 1% (w/v) collagenase D (from *Clostridium* histolyticum) and 0.5 mg/ml papain. DNase (1 mg/ml) and 10% FBS were added before cells were dissociated by gentle trituration through a fire-polished Pasteur pipette. Cells were washed and re-dissociated in DMEM containing 10% FBS and then plated on poly D-lysine coated coverslips in 24-well plates at a density of 1.5×10^6 cells/well and incubated at 37°C, 5% CO_2. For hypoxic culture, an oxygen controller (PRO-OX 110; Biospherix, New York, USA) regulated N_2 influx into the tissue culture incubator to achieve 1.5% O_2.

Proliferation assays

Proliferation of cultured cells was assessed by adding BrdU to culture medium (final concentration 10 μM) two hours before cells were fixed with 2% PFA in PBS. Retinal astrocytes were then stained using rabbit

polyclonal antibodies against GFAP or Pax2. Cells were further fixed using 4% PFA in PBS for 10 minutes and then exposed to 6 M HCl containing 1% Triton X-100 for 45 minutes. The pH was neutralized with 0.1 M $Na_2B_4O_7$ (pH 8.5) and cells were incubated with mouse anti-BrdU antibody (Magaud et al., 1989), which was subsequently visualized with Alexa Fluor 488 anti-mouse IgG. In order to measure proliferation in vivo, mouse pups were injected subcutaneously with BrdU (50 μg per gram body weight) two hours before animals were sacrificed. Retinae where dissected, dissociated, cultured overnight, fixed with 2% PFA in PBS and then stained as described above. For each data point at least three coverslips were prepared and counted (~200 astrocytes/coverslip). BrdU-labeling index was calculated as the proportion of GFAP- or Pax2-positive cells that were also BrdU positive.

Hyperoxia exposure in vivo

P0 mice with their mother were exposed to increased oxygen levels in a modified, airtight cage. Oxygen concentrations were measured with a sensor placed inside the cage and regulated by an oxygen controller (PRO-OX 110). A small fan was installed inside the cage to mix inflowing pure oxygen with cage air. Cage air was removed and replaced with normal room air at a constant rate (approximately 6 cage volumes per hour) to prevent build-up of carbon dioxide and humidity. Pups were removed from the hyperoxic chamber for injection with BrdU and then returned for two hours before sacrificing.

Results

Negative control of retinal astrocyte proliferation in vivo

Expressing PDGF-A under *GFAP* promoter control in transgenic mice provides retinal astrocytes with a potentially inexhaustible supply of dimeric PDGFAA. Immunostaining with an antibody against GFAP in whole-mount retinal preparations revealed a dramatic increase of retinal astrocyte numbers in *GFAP-PDGFA* mice compared with in wild-type mice one week after birth (postnatal day 7, P7) (Fig. 1A,B). Instead of a fine network, there was a dense mat of astrocytes. However, despite the autocrine mitogenic loop, the astrocyte population did not grow indefinitely. The size and density of the astrocyte mass did not increase further between one and four weeks after birth (P7-P28; Fig. 1C,D). To assess cell division directly, we measured the bromodeoxyuridine (BrdU)-labeling index of GFAP-positive astrocytes (Fig. 1E). After a two-hour pulse with BrdU in vivo, retinae were dissected, dissociated and cultured overnight. GFAP-positive cells had a high BrdU-labeling index in both wild type and *GFAP-PDGFA* newborn mice, but in both cases this declined to almost zero by P8. At earlier times the labeling index was higher in *GFAP-PDGFA* mice than in wild-type mice, explaining the greater accumulation of retinal astrocytes in the transgenics. The fact that astrocyte proliferation ceased around the same time in both wild-type and *GFAP-PDGFA* mice points to the existence of a negative control mechanism that slows and stops cell division on a predefined schedule, regardless of the rate of production of PDGFA in vivo.

A trivial explanation for this observation might be that PDGFA and/or PDGFRα expression is downregulated in retinal astrocytes during the first postnatal week. However, in situ hybridization showed that the transgene (human *PDGFA*) was still strongly expressed at P10 in the astrocyte layer of *GFAP-PDGFA* retinae (Fig. 1F,G). Likewise, *PDGFRa* mRNA continued to be expressed by retinal astrocytes at P14 (Fig. 1H), and PDGFRα immunoreactivity was still present on the surface of retinal astrocytes at P8 (not shown). As in a previous study (Fruttiger et al., 2000), we were unable to detect PDGFA protein by immunolabeling at any age but, as argued then and in view of further data presented below, this is likely to be a technical problem of detecting inherently low concentrations of secreted PDGFA in the extracellular space.

Retinal astrocyte differentiation correlates with the presence of blood vessels

We assessed BrdU incorporation in retinal whole-mount preparations to gain a better understanding of the spatial distribution of proliferating retinal astrocytes in vivo. Retinal astrocytes were identified by in situ hybridization for *PDGFRa*

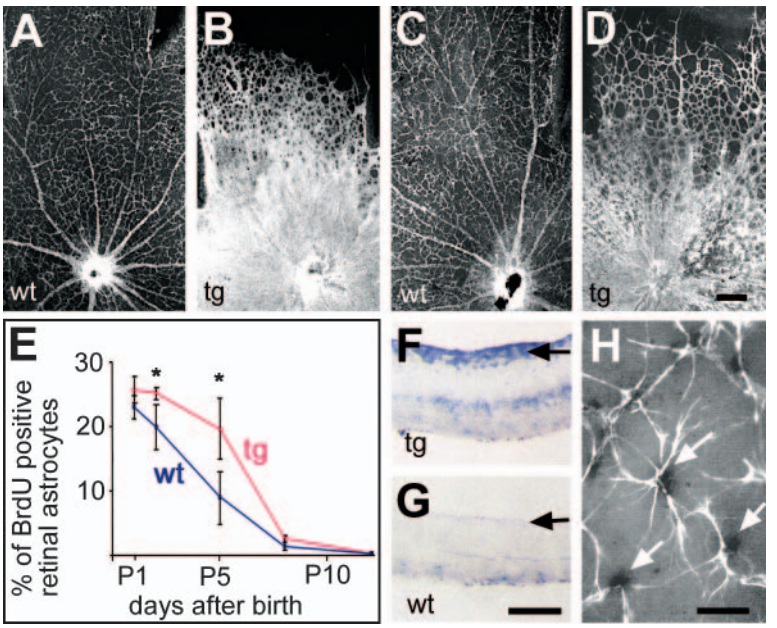

Fig. 1. Retinal astrocytes do not proliferate indefinitely in *GFAP-PDGFA* mice. (A-D) Immunohistochemistry on retinal wholemounts from P7 (A,B) and P28 (C,D) animals with an anti-GFAP antibody show increased retinal astrocyte numbers in *GFAP-PDGFA* transgenic mice (B,D) compared with wild-type mice (A,C). However, retinal astrocytes do not proliferate out of control in transgenic mice (D). (E) Retinal astrocytes in dissociated and overnight-cultured retinae were stained with an anti-GFAP antibody and assessed for BrdU incorporation. Retinal astrocyte proliferation ceases in both wild-type and transgenic mice at about P8. Each data point represents the mean±s.d. from triplicate cultures from four animals (data points labeled with a star are significantly different at a 95% confidence level). (F,G) In situ hybridization of retinal sections from 10-day-old mice reveals that transgene mRNA (human *PDGFA*) can be readily detected in the astrocyte layer (black arrows) in transgenic animals (F) but not in wild-type animals (G). (H) Combined immunohistochemistry and in situ hybridization on retinal wholemounts shows that retinal astrocytes, identified with an anti-GFAP antibody (white staining), express *PDGFRa* mRNA (black staining, white arrows) in 14-day-old wild type animals. Scale bars: 200 μm in A-D,F,G; 20 μm in H.

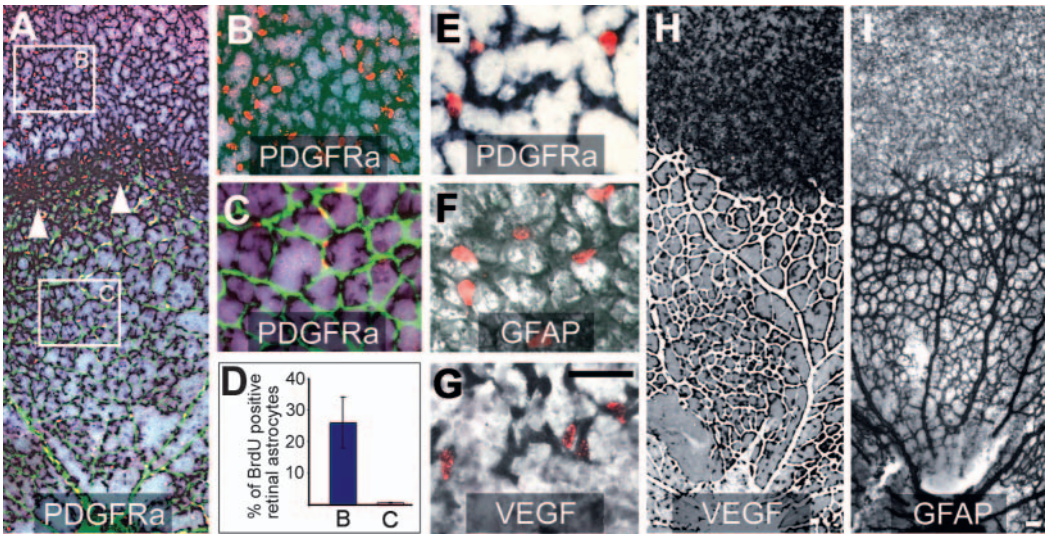

Fig. 2. Retinal vascularization correlates with differentiation of retinal astrocytes. Retinal wholemounts from P5 mice were analyzed for proliferation (A-G) and stained for *PDGFRa* (A-C,E), *VEGF* (G,H) or *GFAP* (F,I) mRNA. Retinal astrocytes were visualized by *PDGFRa* in situ hybridization (dark signal in A-C,E) and cell proliferation was assessed by BrdU incorporation (red nuclei in A-C,E-G). Blood vessels were stained with anti-collagen type IV antibody (green in A,C, white in H). Proliferation of retinal astrocytes is high in peripheral, avascular areas (B) but low in central, vascularized areas (C). This was quantified by counting cells in the avascular (B in panel D) or vascularized (C in panel D) retina in whole-mount preparations double labeled for *PDGFRa* mRNA and BrdU incorporation (data points represent the mean±s.d. from five different animals). High magnification reveals that BrdU incorporation in the peripheral retina is limited to retinal astrocytes identified by *PDGFRa* mRNA (E), *GFAP* mRNA (F) and *VEGF* mRNA (G) expression. *VEGF* mRNA (dark signal in H) is strongly expressed in avascular areas, whereas *GFAP* mRNA (black in I) is expressed strongly in the presence of blood vessels. Upper box in A refers to B, lower box to C. Scale bars: 50 μm.

and blood vessels by immunolabeling for collagen type IV. In P5 wild-type mice, retinal astrocytes in outer regions of the retina, not yet occupied by the expanding vascular network, incorporated BrdU readily (Fig. 2A,B), whereas more central astrocytes, associated with blood vessels, did not incorporate BrdU (Fig. 2A,C). High magnification micrographs of the peripheral avascular area clearly reveal that BrdU labeling is limited to retinal astrocytes, identified by *PDGFRa* (Fig. 2E), *GFAP* (Fig. 2F) and *VEGF* (Fig. 2G) transcripts. We counted BrdU-positive cells in selected areas of five different retinae and found that astrocytes in avascular regions undergo proliferation, whereas they are virtually quiescent in vascularized regions (Fig. 2D). Near the leading edge of the vessel network (arrowheads in Fig. 2A), cell proliferation was pronounced but we were unable to distinguish between retinal astrocytes and vascular cells due to the high cell density in this area. From the distribution of *PDGFRa*-positive cells it appears that the retinal astrocyte network is reorganized by the spreading vasculature as it advances (Fig. 2A). A further effect on retinal astrocytes, apparently caused by the presence of blood vessels, was a marked downregulation of *VEGF* mRNA (Fig. 2H). This is most likely due to differences in tissue oxygenation in vascular and avascular areas of the retina, as it is well known that *VEGF* transcripts are specifically induced by hypoxia and suppressed by normoxia/hyperoxia. Expression levels of *PDGFRa* in astrocytes are unaffected by the presence or absence of blood vessels (Fig. 2A). However, we found an uneven distribution of *GFAP* mRNA (Fig. 2I), which is low in the avascular region but sharply increases in areas covered by vessels. GFAP is upregulated during astrocyte differentiation in the retina (Chu et al., 2001), and it therefore appears that the proximity of blood vessels causes retinal astrocytes to stop dividing and differentiate. This could be mediated by local interactions between astrocytes and vascular cells, or by a diffusible agent carried in the blood.

Blocking retinal vascularization delays retinal astrocyte differentiation

To test the effects of blood vessels on retinal astrocytes, we prevented retinal vascularization in newborn mice during the first postnatal week by keeping them in a high oxygen (80% O_2) atmosphere. In a previous study we showed that this causes vaso-obliteration and VEGF downregulation in a small central area surrounding the optic nerve head (Claxton and Fruttiger, 2003) (arrow in Fig. 3G). In newborn mice, where retinal vessels are just emerging from the optic nerve head, the conditions in this small central area seem to prevent vessels from entering the rest of the retina. Hence, continued hyperoxia exposure prevents retinal vascularization up to at least P8 (Fig. 3A,B). This leads to upregulation of *VEGF* in the majority of retinal astrocytes (Fig. 3G), despite the high levels of oxygen in inhaled air. On the face of it, this might appear paradoxical; however, oxygen delivery in tissues depends critically on the presence of blood vessels, so it is not unexpected that avascular retinae should become hypoxic (and upregulate *VEGF*) despite the higher than normal atmospheric oxygen. We confirmed the hypoxic state of the retina in avascular retinae by using the EF5 oxygen sensing system (see the following section).

We found that in the avascular, hypoxic retinae, BrdU incorporation by astrocytes is much higher than in age-matched controls (Fig. 3A-D), suggesting that the absence of vessels keeps retinal astrocytes in a proliferating state. *GFAP* mRNA levels remain low in the majority of retinal astrocytes (Fig. 3H), consistent with the notion that the arrival of blood vessels normally induces cell cycle exit and differentiation.

An interesting exception to this rule is the small area in the immediate vicinity of the optic nerve head. In this localized region astrocytes downregulated *VEGF*, upregulated *GFAP* (arrows in Fig. 3G,H) and did not incorporate BrdU (Fig. 3F), in stark contrast to astrocytes in the remainder of the retina. Thus, astrocytes in this region behave as if they are

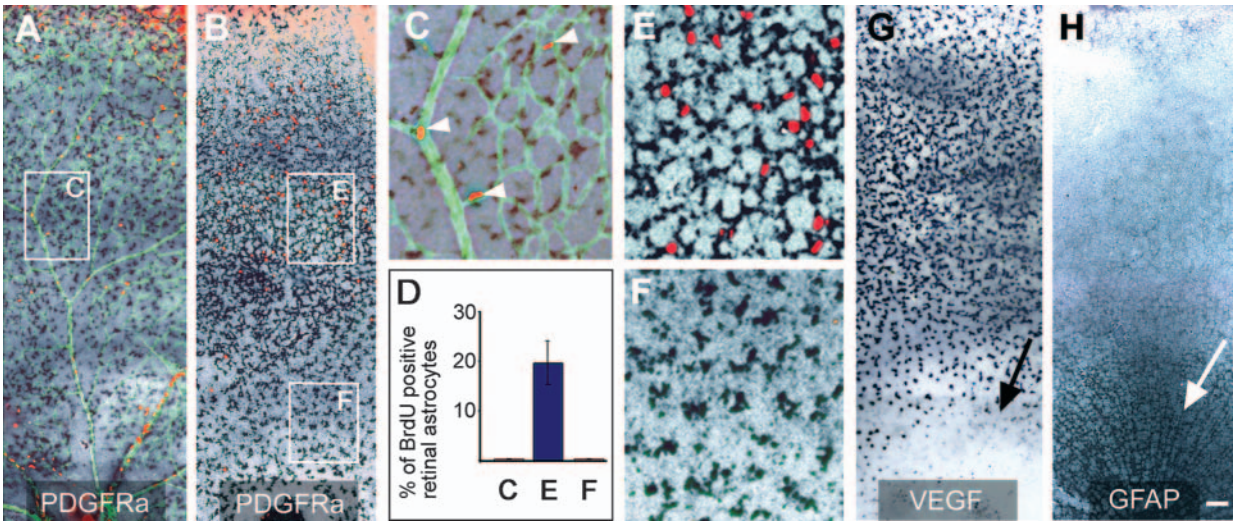

Fig. 3. Inhibition of vascular growth in vivo prevents astrocyte differentiation. In situ hybridization for *PDGFRa* (A-C,E,F), *VEGF* (G) or *GFAP* (H) was combined with immunostaining for anti-collagen type IV (green in A-C,E,F) and anti-BrdU (red in A-C,E,F) on retinal wholemounts. Exposure of mouse pups to 80% O_2 from P0-P8 prevents retinal vascularization (B,E-H) that normally occurs in control animals (A,C). Proliferating cells in control animals are limited to vessels (arrowheads, C), whereas many retinal astrocytes are proliferating in animals lacking retinal blood vessels (E). However, retinal astrocytes are quiescent in the very center of the retina (F). Proliferation of retinal astrocytes was quantified by counting cells in whole-mount retinae (D). Data points represent the mean±s.d. (from four different animals) of cells counted in normoxic animals (C in panel D), and in hyperoxic animals in the periphery (E in panel D) or the center (F in panel D) of the retina. In the central area, *VEGF* mRNA expression is low (arrow, G) and *GFAP* mRNA expression is high (arrow, H). Box in A refers to C; boxes in B refer to E and F. Scale bar: 100 μm in A,B,G,H.

experiencing normoxia, despite the absence of retinal vessels. A likely explanation is that the optic nerve head is oxygenated directly by the major hyaloid artery that passes along the optic nerve, through the central retina to the lens (Claxton and Fruttiger, 2003). Note that this hyaloid artery is not visible in our whole-mount preparations.

Retinal oxygen tension measured with the hypoxia marker EF5

In order to visualize oxygenation in whole-mount retinae we used the EF5 hypoxia marker system (Koch, 2002). Mouse pups were injected with the EF5 reagent, which is reduced and covalently bound to cellular macromolecules under hypoxic conditions. The drug-macromolecule adducts are then detected with specific antibodies. In P6 mice the peripheral, not yet vascularized retina shows a clear increase in EF5 staining when compared with the central, vascularized retina (Fig. 4A,B), indicating a low oxygen concentration in the avascular retina. In mice raised for 6 days in 80% oxygen, the retina remains avascular and displays strong EF5 staining throughout (Fig. 4C,D) with the exception of a small circular area in the center (arrow in Fig. 4D). Thus EF5 staining confirms the existence of retinal hypoxia in a pattern that correlates closely with *VEGF* mRNA distribution (compare Fig. 3H).

Oxygen tension affects proliferation and differentiation of cultured retinal astrocytes

In order to test the effects of oxygen directly, neonatal mouse retinae were dissociated and cultured in different oxygen atmospheres (1.5% and 20% O_2). After four days in vitro, we performed in situ hybridization and detected a strong *GFAP* signal in normoxic cells but only a very weak signal in hypoxic cells (Fig. 5A,B). Conversely – and as expected – *VEGF* mRNA levels were low in normoxic cells but high in hypoxic cells (Fig. 5C,D). Thus, retinal astrocytes seem to respond to oxygen in vitro in the same way as they respond to the proximity of blood vessels in vivo.

We tested whether oxygen levels can also influence the proliferation rates of cultured retinal astrocytes. We plated dissociated isolated retinae on coverslips and used anti-GFAP antibodies to identify retinal astrocytes in the mixed cultures. Double labeling with antibodies against Pax2 (Chu et al., 2001) confirmed that the GFAP-positive cells were retinal astrocytes (Fig. 5E,F). First, we cultured newborn (P1) and P7 retinal cells in an ambient (20% O_2) atmosphere and pulsed with BrdU for two hours before fixing and double immunolabeling the cells for BrdU and GFAP (inset in Fig. 5G). After 24 hours in vitro, P1 astrocytes had a high BrdU-labeling index, whereas P7 astrocytes were virtually quiescent (Fig. 5G), mimicking the proliferation state of most astrocytes in vivo at this age. The BrdU-labeling index of P1 astrocytes dropped with time in culture, so that after four days in vitro proliferation had almost completely stopped (Fig. 5G). Similar results were obtained when we used antibodies against Pax2 instead of GFAP to identify retinal astrocytes (Fig. 5H). We were unable to prevent the decline in proliferation rate by adding additional PDGFAA or a variety of other purified polypeptide growth factors to the medium. Nor did we prevent the decline when we eliminated vascular cells from the P1 cultures by removing the central, vessel-containing region around the optic nerve head before dissociating and culturing the cells (not shown). However, when we cultured P1 retinal cells in a hypoxic atmosphere (1.5% O_2), we found that astrocyte proliferation did not fall as rapidly as it did in

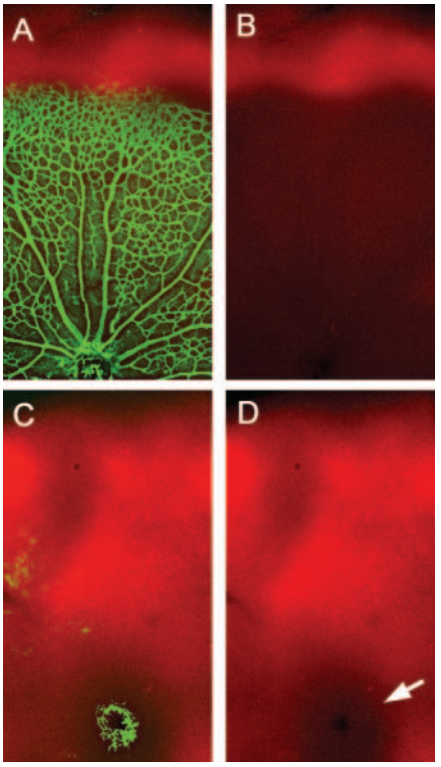

Fig. 4. Avascular areas of the retina are hypoxic. EF5 staining (red) visualizes hypoxia in retinal wholemounts from P6 mice raised in normal atmosphere (A,B) and 80% oxygen (C,D). Blood vessels were stained with anti-collagen type IV antibody (green staining in A,C). Panels B and D are identical to A and C but with the green channel omitted. The arrow in D indicates a small, circular normoxic area in the centre of the retina.

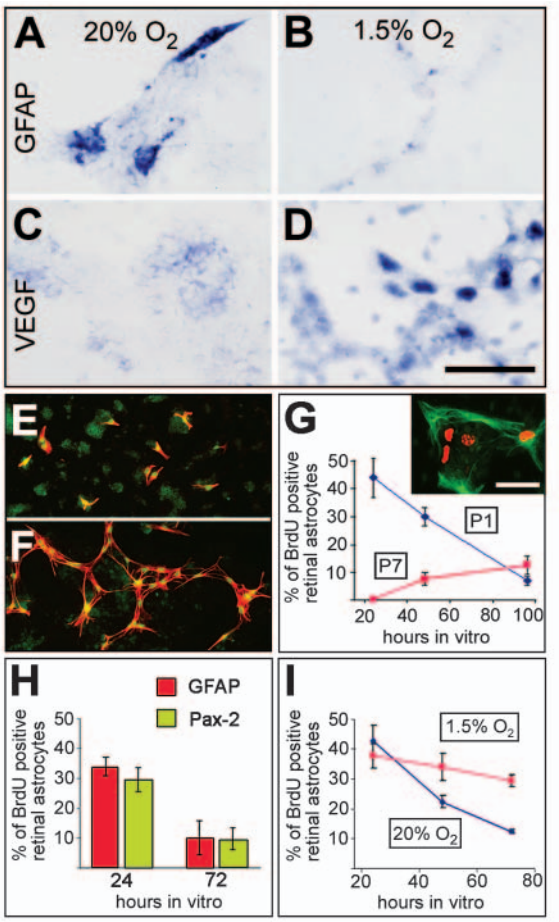

Fig. 5. Normoxia induces differentiation of cultured retinal astrocytes. Cultures of dissociated retinal cells from P1 animals were maintained for 4 days in vitro at 20% oxygen and 1.5% oxygen. In situ hybridization revealed high *GFAP* (A) and low *VEGF* mRNA levels in normoxic culture conditions (C), and, conversely, low *GFAP* and high *VEGF* mRNA levels in hypoxic conditions (B,D). Double immunolabeling (E,F) shows that the GFAP-positive (red) cell population is identical to the Pax2-positive (green) cell population after 24 (E) and 72 (F) hours in culture. (G) Assessment of BrdU incorporation (red, inset) in GFAP-positive cells (green, inset) reveals high proliferation in retinal astrocytes from P1 but a reduction in proliferation over time in culture, whereas retinal astrocytes from P7 animals were quiescent throughout the culture period. (H) The proliferation decrease in P1 retinal astrocytes in culture is independent of the staining method (anti-GFAP or -Pax2 labeling) used to identify retinal astrocytes. (I) However, culturing P1 retinal cells in 1.5% O_2 largely prevents the decline in astrocyte proliferation seen under normoxic conditions. Scale bars: 50 μm.

normoxic cultures, but remained elevated throughout the culture period (Fig. 5I).

Discussion

A lot of research has focused on local factors that control blood vessel growth. However, little is known about the reverse: how blood vessels affect development of surrounding tissue. In this study, we provide evidence that blood vessels exert a negative growth control over retinal astrocytes and induce astrocyte differentiation. This creates a negative-feedback loop that limits the number of retinal astrocytes and, in turn, the density of blood vessels (as astrocytes induce vessel formation) (Fig. 6). This reciprocal feedback makes for an inherently stable and reproducible pattern of vascular development.

In a previous study we overexpressed *PDGFA* in retinal neurons in transgenic mice. This led to a large increase in the number of retinal astrocytes and a corresponding overgrowth of the retinal vasculature (Fruttiger et al., 1996). Nevertheless, a stable steady state ensued and overall retinal architecture was preserved. This suggested to us that astrocytes might proliferate until such a time as the astrocyte population as a whole consume PDGFA (by receptor binding and internalization) as fast as it is supplied by neighbouring neurons. The extracellular concentration of PDGF would then inevitably fall and astrocyte proliferation would cease. We have presented evidence elsewhere that such a mechanism might apply to the control of oligodendrocyte progenitor cell number in the developing spinal cord (van Heyningen et al., 2001). One prediction of this 'supply and demand' model is that, by creating an autocrine mitogenic loop, one might prevent demand from outstripping supply and allow cells to proliferate indefinitely. However, we found that this did not happen when we engineered an autocrine PDGFA mitogenic loop in retinal astrocytes (*GFAP-PDGFA* mice; this paper). This led us to identify the proximity of blood vessels as an important

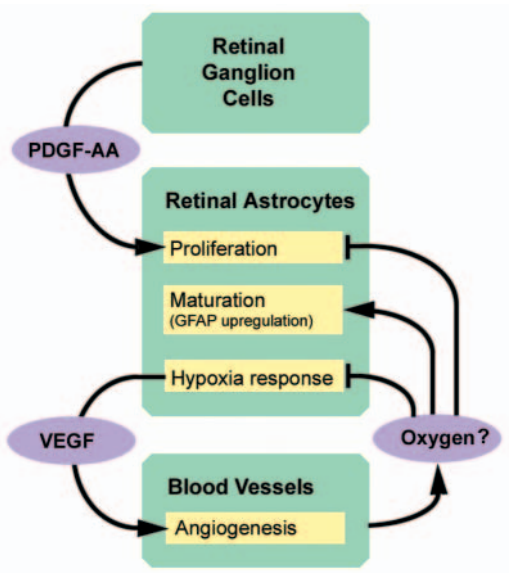

Fig. 6. Diagram showing interactions between different retinal cell types during retinal vasculature development. Hypoxic retinal astrocytes secrete VEGF and induce blood vessel growth. This leads to increased oxygen tension, which acts as a negative feedback on the hypoxia response in retinal astrocytes. Blood vessels also inhibit proliferation and stimulate maturation of retinal astrocytes. It is possible that these latter interactions are also mediated by oxygen.

overriding factor for inhibiting retinal astrocyte proliferation. Our findings raise the question of whether retinal astrocytes will proliferate unchecked in *GFAP-PDGFA* mice if we can simultaneously prevent development of the retinal vasculature. To test this, we tried to block vessel growth in neonatal *GFAP-PDGFA* mice by raising them in an 80% O_2 atmosphere – but without success, perhaps because the greatly increased number of retinal astrocytes in the transgenics provided too strong an angiogenic stimulus to be overcome by hyperoxia.

In the present study we also present evidence suggesting that tissue oxygenation might be the critical factor that drives differentiation of retinal astrocytes. In vitro, low oxygen kept retinal astrocytes in a proliferating, immature state, whereas increased oxygen inhibited their proliferation and induced a more mature, GFAP-positive phenotype. From our experiments it is not possible to determine whether oxygen levels affect astrocytes directly or indirectly; for example, by stimulating the release of differentiation factors from other retinal cells. It has been shown that leukemia inhibitory factor (LIF) secreted from endothelial cells can induce cultured, GFAP-negative astrocyte precursors from optic nerve to differentiate into GFAP-positive astrocytes (Mi et al., 2001). An analogous paracrine interaction between endothelial cells and astrocytes in the retina is possible but, at least in our in vitro experiment, seems unlikely because our retinal cultures contained very few, if any, endothelial cells (as assessed by in situ hybridization with probes against VEGFR1/2; data not shown). Moreover, we have failed to see any effects of anti-LIF antibodies on retinal astrocytes in our culture system (data not shown). However, we cannot exclude the possibility that other secreted factors might mediate the effects of oxygen in our retinal cultures.

Manipulation of tissue oxygen levels in living mice is not as straightforward as in cell culture. It is well established that exposing newborn mouse pups to an 80% oxygen-containing atmosphere prevents retinal vessel development. The conventional explanation is that high atmospheric oxygen leads to high tissue oxygen levels, resulting in the downregulation of VEGF and consequent angiogenesis failure. This is a tenable explanation in situations where blood vessels have already become established; in mouse retinae one week or more after birth, for example. In such cases, the high atmospheric oxygen can be directly transported into the retina via the blood. However, the situation described in the present paper is very different; in newborn mice retinal vessels have not yet developed so the majority of the retina is never exposed to oxygenated blood and consequently remains hypoxic, despite higher than normal atmospheric oxygen.

This explains why VEGF is not downregulated across the retinae of newborn mice exposed to hyperoxia, but presents an apparent paradox as to why the high VEGF levels do not trigger angiogenesis as normal. The answer probably lies at the very centre of the retina around the hyaloid artery. Exposure to hyperoxia does cause local downregulation of *VEGF* mRNA around the hyaloid artery, because this central region becomes normoxic or hyperoxic, whereas the remainder of the (as yet avascular) retina remains hypoxic, as we confirmed by EF5 labeling. This presumably forms a hyperoxic barrier around the hyaloid vessel that prevents radial sprouting (angiogenesis).

It could be argued that the changes in astrocyte proliferation and GFAP expression that we observe in normoxic and hyperoxic retinae result from tissue oxygenation per se, rather than from cell-cell interactions between vascular cells and astrocytes (Fig. 6). A link between oxygen and astrocyte differentiation was also suggested by a previous study (Zhang et al., 1999), but those authors proposed that oxygen was acting indirectly via other retinal cells. As discussed above, our results do not exclude such indirect mechanisms. It is also possible that multiple factors, including oxygen, might mediate the differentiating effects of retinal blood vessels.

Oxygen levels in most mammalian tissues range between 1% and 5% (8-38 mm of Hg partial pressure) (Silver and Erecinska, 1998). Therefore, the 20% O_2 that cells experience under standard cell culture conditions is highly non-physiological. In general, culturing primary cells under hypoxic conditions might more closely mimic their natural environment and allow them to behave more nearly as they do in vivo. Under conditions of 'physiological hypoxia' a multitude of genes are differentially regulated (Maltepe and Simon, 1998), so it is quite plausible that oxygen levels might affect more integrative aspects of cell behaviour, such as proliferation and differentiation. There are some concrete examples of this. Cultured CNS precursor cells and T lymphocytes both display enhanced proliferation and survival under hypoxia (Studer et al., 2000; Hale et al., 2002), and isolated neural crest stem cells show a wider differentiation potential (Morrison et al., 2000). Cultured human cytotrophoblasts (specialized placental cells) proliferate in low oxygen but differentiate in high oxygen, mimicking their response as they migrate towards blood vessels in vivo (Genbacev et al., 1997; Adelman et al., 2000). Early embryonic development is dominated by physiological hypoxia, whereas late embryonic development is characterized by a mosaic of normoxic and hypoxic regions giving rise to oxygen gradients

(Maltepe and Simon, 1998). Therefore, many cell types develop and differentiate in an environment of changing oxygen concentrations and it seems likely that more examples will be found of cells maturing in response to rising oxygen levels.

How do our studies of retinal astrocytes apply to other regions of the CNS? Astrocytes in the gray matter of the brain or spinal cord are usually GFAP-negative, apart from those that specifically contact blood vessels, which express GFAP strongly (Bignami and Dahl, 1974). It is possible that astrocytes in contact with blood vessels are induced to express GFAP by differentiation factors secreted by vascular cells or, alternatively, because they experience higher oxygen concentrations than astrocytes further away from vessels. Sequence analysis of the *GFAP* promoter region reveals several hypoxia response elements (not shown), but to date it has not been established how or whether these affect *GFAP* transcription in vivo.

In future it will be interesting to explore the developmental relationships between astrocytes, blood vessels and oxygen in the brain and spinal cord. It is also an intriguing possibility that reciprocal feedback between vessel-inducing cells and their associated vessels might govern vascular development in other parts of the embryo outside the CNS.

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