PDGF Mediates a Neuron–Astrocyte Interaction in the Developing Retina

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Summary

Astrocytes invade the developing retina from the optic nerve head, over the axons of retinal ganglion cells (RGCs). RGCs express the platelet-derived growth factor A-chain (PDGF-A) and retinal astrocytes the PDGF alpha-receptor (PDGFRα), suggesting that PDGF mediates a paracrine interaction between these cells. To test this, we inhibited PDGF signaling in the eye with a neutralizing anti-PDGFRα antibody or a soluble extracellular fragment of PDGFRα. These treatments inhibited development of the astrocyte network. We also generated transgenic mice that overexpress PDGF-A in RGCs. This resulted in hyperproliferation of astrocytes, which in turn induced excessive vasculogenesis. Thus, PDGF appears to be a link in the chain of cell–cell interactions responsible for matching numbers of neurons, astrocytes, and blood vessels during retinal development.

Introduction

During development of the vertebrate eye, cells from several different sources come together in a coordinated fashion to form the final structure. The cells of the neural retina and pigmented epithelium are derived from the neural tube, whereas the eye lens is formed from the skin of the embryo as a result of inductive interactions between the skin epithelium and the underlying optic stalk. Other components of the eye, for example, the ciliary muscles and vascular system, are of mesenchymal or neural crest origin. For these diverse tissue elements to assemble correctly requires an intricate network of cell–cell communication. This is well illustrated by cell ablation experiments in transgenic mice. For example, if the cells of the eye lens are killed as they develop (by expressing a toxic gene product under the control of a lens-specific gene promoter), many other parts of the eye are secondarily affected and the whole eye is absent or much reduced in size (Kaur et al., 1989; Breitman et al., 1989; Landel et al., 1988). Likewise, ablating the pigmented epithelium has wide-ranging effects on development of the eye as a whole (Raymond and Jackson, 1995).

The retina itself is composed of cells with different developmental origins, whose numbers must presumably be matched to one another by cell–cell interactions. Most cells of the neural retina, such as photoreceptors, neurons, and Müller glia, are generated by multipotent neuroepithelial precursors that reside near the outer surface of the retina (Turner and Cepko, 1987; Wetts and Fraser, 1988; Holt et al., 1988; Turner et al., 1990). In contrast, retinal astrocytes originate from the optic stalk and migrate across the inner surface of the retina, starting from the optic nerve head around the day of birth (Stone and Dreher, 1987; Ling and Stone, 1988; Watanabe and Raff, 1988; Ling et al., 1989). The migrating astrocytes form a glial network that spreads radially in close association with the axons of retinal ganglion cells (RGCs). Patent blood vessels develop in the wake of the migrating astrocytes (Ling and Stone, 1988; Watanabe and Raff, 1988; Ling et al., 1989), presumably as a result of interactions between astrocytes and endothelial cells (Laterra et al., 1990; for a review, see Chang-Ling, 1994). Retinal astrocytes have been shown to make vascular endothelial cell growth factor (VEGF, also known as vascular permeability factor, VPF) (Alon et al., 1995), which is thought to be crucial for vascular development (Leung et al., 1989; Millauer et al., 1993; Peters et al., 1993; Stone et al., 1995; for a review, see Klagsbrun and Soker, 1993). However, the factors that control the astrocyte invasion of the retina and development of the astrocyte network are unknown.

We recently found that platelet-derived growth factor (PDGF) and its receptors are expressed in the developing rodent retina (Mudhar et al., 1993), suggesting that PDGF might be important in retinal development. PDGF is a covalent dimer of A- and B-chains (AA, BB, or AB) and exerts its biological effects through two closely related tyrosine kinase receptors (PDGFRα and PDGFRβ) (for review, see Heldin and Westermark, 1989). The two receptors have different ligand-binding specificities; PDGFRα binds all three dimeric isoforms of PDGF, while PDGFRβ binds PDGF-BB and, to a lesser extent, PDGF-AB, but not PDGF-AA. Cells in the walls of blood vessels in the retina (Mudhar et al., 1993) and elsewhere in the CNS (Smits et al., 1989; Koyama et al., 1994b) express PDGFRβ or PDGF-B (or both), suggesting that PDGF-BB might mediate local interactions among vascular cells. Furthermore, RGCs express PDGF-A and retinal astrocytes express PDGFRα, leading us to suggest that PDGF-AA might mediate a short-range paracrine interaction between RGCs and astrocytes during development (Mudhar et al., 1993).
To test this idea, we experimentally manipulated PDGF-A expression in the developing eye. We inhibited PDGF activity in vivo using a soluble extracellular fragment of PDGFRα that can neutralize the activity of all isoforms of PDGF and also with a neutralizing anti-PDGFRα monoclonal antibody. In both cases, development of the astrocyte network was retarded, strongly suggesting that PDGF mediates a developmental interaction between RGCs and astrocytes. In addition, we overexpressed PDGF-A in retinal neurons in transgenic mice. These transgenic animals displayed a striking hyperplasia of retinal astrocytes with their associated blood vessels, which, in older animals, bore a close resemblance to human proliferative retinopathy. These results emphasize the obligatory relationships among neurons, astrocytes, and vasculature in the retina, and suggest that PDGF is an important component of the signaling network that coordinates growth of these cells during development.

Results

Sequestering PDGF in the Developing Eye with the Extracellular Domain of PDGFRα

We wanted to test whether inhibiting PDGF signaling can inhibit normal development of the retinal astrocyte network. We therefore engineered a soluble extracellular fragment of PDGFRα as a neutralizing agent for all dimeric isoforms of PDGF (see Experimental Procedures) and inserted it into a replicating COS cell vector. A Myc epitope tag, recognized by monoclonal antibody 9E10 (Evan et al., 1985), was inserted in-frame with the truncated PDGFRα at its extreme carboxy terminus. The final construct was named pRα17, and the encoded polypeptide Rα17. The construct was electroporated into COS cells, which were fixed 72 hr later and labeled with monoclonal antibody 9E10. A proportion of the cells was labeled, giving intense intracellular labeling of the secretory apparatus (endoplasmic reticulum, Golgi apparatus, and cytoplasmic vesicles) (Pollock and Richardson, 1992; see Figure 2A). COS cells transfected with pRα17 were metabolically labeled with [35S]amino acids and the cell lysate and supernatant were immunoprecipitated with monoclonal antibody 9E10, followed by polyacrylamide gel electrophoresis and autoradiography. The immunoprecipitates from both cell supernatant (Figure 1A, lane 1) and cell lysate (data not shown) contained high molecular weight polypeptides that were absent from control immunoprecipitations (lane 4). To test the PDGF-binding ability of the secreted Rα17 polypeptide, pRα17 and a similar plasmid encoding the short splice variant of human PDGF-A (Pollock and Richardson, 1992) were electroporated separately into cultured COS cells, which were subsequently incubated with [35S]amino acids. The cell culture media were collected and coincubated overnight at 4°C to allow PDGF-AA to bind to the truncated Rα17 receptor. The supernatants were then immunoprecipitated with an antiserum raised against pure human PDGF (R and D Systems). A large proportion of the PDGF-AA and the Rα17 truncated receptor coprecipitated in this experiment (Figure 1A, lane 2), demonstrating that the Rα17 polypeptide is capable of binding to PDGF-AA homodimers in solution, with an affinity high enough to withstand the physiological salt washes performed during the immunoprecipitation protocol.

To assess the ability of the Rα17 truncated receptor to inhibit the mitogenic effect of PDGF, NIH 3T3 cells were cultured in the presence of PDGF-AA, PDGF-BB, PDGF-AB, or basic fibroblast growth factor (bFGF) together with different concentrations of conditioned medium from COS cells expressing Rα17. DNA synthetic activity was measured by 3H-thymidine incorporation. DNA synthesis induced by all three dimeric isoforms of PDGF was inhibited, in a dose-dependent manner, by COS cell medium containing Rα17 but not by control cell medium (Figure 1B). The mitogenic activity of bFGF was unaffected by Rα17. These results are consistent with the known ligand-binding properties of intact PDGFRα, which can bind and be activated by all three dimeric isoforms of PDGF (Heldin et al., 1988; Gronwald et al., 1988; Hart et al., 1988). The inhibitory effect of Rα17 on 3H-thymidine incorporation could in turn be overcome by increasing the concentration of PDGF in the medium (data not shown), demonstrating that the PDGF-neutralizing effect of Rα17 is saturable. Under the conditions of our experiments, Rα17-conditioned medium neutralized between 1 and 3 ng/ml PDGF-AA. A similar truncated PDGFRα was previously shown to inhibit ligand-induced receptor phosphorylation (Yu et al., 1994).

COS cells, transfected with pRα17 and injected unilaterally into the eyes of newborn rats, persisted and continued to express the Myc-tagged Rα17 truncated receptor for several days in vivo (Figures 2B and 2C). Typically, they formed a compact bolus of cells that remained attached to the posterior surface of the lens. The size of the bolus indicated that the COS cells had continued to proliferate in the eye following injection. On postnatal day 5 (P5), injected and contralateral uninjected eyes were dissected and their retinae processed for whole-mount histochemistry with a monoclonal antibody against glial fibrillary acidic protein (GFAP, a specific marker for astrocytes). Eyes that showed signs of having been damaged by the injection procedure (e.g., blood in the vitreous) were rejected, as were eyes that did not appear to have a COS cell tumor and eyes in which the COS cells had invaded the retina (approximately half of the injected eyes were analyzed). There were no significant differences in the radii of retinae from eyes injected with mock-transfected COS cells versus uninjected eyes (data not shown), implying that the COS cells did not inhibit overall growth of the eye. In order to quantify the effect on astrocyte migration, the retinal whole-mounts were divided in six sectors and the distance of the most peripheral astrocyte to the optic nerve head was measured in each sector. The average of these six values was taken as a measure of the overall extent of the astrocyte network. For each experimental situation (pRα17-transfected COS cells, mock-transfected COS cells, contralateral eyes with no COS cell injections), nine
retinae were examined. Careful observation revealed a slight rotational asymmetry in the normal distribution of astrocytes in uninjected eyes at P5. In retinae from eyes that had been injected with mock-transfected COS cells, the GFAP immunoreactivity reflected the normal distribution of astrocytes (Figure 2D). The radius of the astrocyte network (estimated as described above) was only slightly reduced, by 5% ± 3.9% (mean ± SD, n = 9), in mock-transfected COS cell–injected eyes when compared with contralateral uninjected eyes.

In retinae from eyes that had received COS cells expressing R\textsubscript{17}, development of the astrocyte network was clearly perturbed (Figure 2E). The effect was not uniform across the retina, being greater in one half of the retina than the other. In those retinal sectors that were most affected, the morphology of the network was altered (compare Figures 2D and 2E), having a less intricate branching pattern compared with control retinae. In addition, the extent of the retinal astrocyte network was dramatically reduced in the more affected half of the retina. To quantify this effect, we compared the radial distances covered by astrocytes in the two most affected sectors of each retina that was exposed to R\textsubscript{17}-transfected COS cells with the two shortest distances measured in each retina that was exposed to mock-transfected COS cells; we found a reduction in the R\textsubscript{17}-treated retinae of 31% ± 9.6% (n = 9, p < 0.01 Student’s t-test). However, part of these measured distances had already been traveled by the astrocytes before birth, prior to the COS cell injections. When the extent of the astrocyte network in normal newborn rats was subtracted, we found that the distance migrated by astrocytes during the course of the experiment was reduced by 51% ± 18.3% (n = 9) as a result of exposure to R\textsubscript{17}.

Systemically Delivered Anti-PDGFR\alpha Immunoglobulin Perturbs Development of the Retinal Astrocyte Network

As another means of inhibiting PDGF signaling, we used a rat monoclonal anti-mouse PDGFR\alpha (antibody APA5) that competes with PDGF-AA and PDGF-BB for binding to PDGFR\alpha on Balb/c-3T3 cells (Takakura et al., 1996) and inhibits PDGF-AA-induced responses in Balb/c-3T3 cells and cultured fetal liver cells (N. T., unpublished data). Newborn mice were injected subcutaneously with 50 \( \mu \)g of column-purified APA5 Ig once each day for 3 days, then they were processed for whole-mount immunohistochemistry with anti-GFAP and anti-collagen IV. Littermates that had been injected with 50 \( \mu \)g of monoclonal rat anti-mouse c-Kit Ig (antibody ACK2) or with vehicle alone served as controls. To test whether the subcutaneously injected antibodies reached the eye and bound to retinal astrocytes, we performed whole-mount immunohistochemistry with FITC-conjugated goat-anti-rat IgG (Figures 3A–3C). In mice injected with APA5, the
Figure 2. Neutralizing PDGF with an Extracellular Fragment of the PDGFRα Inhibits Formation of the Retinal Astrocyte Network during Normal Development

(A) Immunofluorescence micrograph of a COS cell transfected with pRα17 (see Experimental Procedures) and labeled in vitro with monoclonal 9E10 and fluorescent secondary antibodies. Isolated cells or clusters of cells within the cell mass label strongly for Rα17 truncated receptor.

(B) Micrograph of a cryosection through an eye containing an implant of COS cells that were transfected with pRα17. The cells were injected at P0 and the eye processed for microscopy on P5. The arrow points to the bolus of COS cells adhering to the back of the lens (L). The dark granules within the COS cell mass are Rα17-expressing cells visualized by 9E10 immunolabeling followed by an immunoperoxidase detection system.

(C) High magnification micrograph of a COS cell mass like that in (B), labeled with antibody 9E10 followed by fluorescent secondary antibodies. Isolated cells or clusters of cells within the cell mass label strongly for Rα17 truncated receptor.

(D) and (E) Composite immunofluorescence micrographs of whole-mount retinae, labeled with anti-GFAP to visualize the retinal astrocyte network.

(D) Part of a normal P5 retina.

(E) Part of a retina from an eye injected at P0 with COS cells expressing Rα17 and processed for microscopy at P5. The extent of the astrocyte network is much reduced and the fine structure of the network is coarser. Scale bars, 500 μm.

anti-rat IgG-FITC conjugate clearly outlined the astrocyte network (Figure 3A), whereas in mice treated with ACK2 or PBS, no staining above background was observed (Figures 3B and 3C). Mice treated with ACK2 showed reduced skin pigmentation as described previously (Nishikawa et al., 1991), indicating that the control antibody was active. We were able to localize rat IgG immunoreactivity in ACK2-injected mice on an unidentified subset of retinal cells located near the optic nerve head at P5 (data not shown), demonstrating that ACK2 had reached the retina.

The retinal astrocyte network (revealed by GFAP immunolabeling) in P3 mice injected with APA5 was strikingly perturbed in morphology and reduced in radial...
than normal (Figure 3). As in the Rx17 experiment described above, the effect was more pronounced in one half of the retina than in the other. It is likely that inherent asymmetrical properties of the developing retina are the root cause of the uneven effect of systemic APA5 injection (and, by extrapolation, Rx17 treatment too). In addition to the disturbed morphology of the astrocyte network found in APA5-injected mice, there was also a reduction in the extent of astrocyte migration. Combining data from three independent experiments, the average radial distance migrated over all six sectors of the APA5-treated retinae ($n = 13$) was reduced by $20\% \pm 1.2\%$ compared with ACK2-treated retinae ($n = 10$).

There is believed to be a close link between the development of retinal astrocytes and blood vessels (see below), so we also examined the retinal vasculature in APA5-injected eyes. There was possibly a small inhibitory effect on the vasculature, but this was much less pronounced than the effect on astrocytes and we did not attempt quantitation. Vascular cells do not express PDGFRα so a direct effect of the antibody on these cells is not expected.

Transgenic Mice That Overexpress PDGF-A in Neurons

We generated transgenic mice that express PDGF-A in subsets of central and peripheral neurons under transcriptional control of the rat neuron-specific enolase (NSE) gene promoter (Forss-Petter et al., 1990). The transgene contained a human PDGF-A cDNA engineered to encode the “short” alternative-splice isoform of PDGF-A with a Myc epitope tag appended to the carboxy terminus (hPDGF-A; Figure 4A) (Pollock and Richardson, 1992). The “short” PDGF-A isoform is freely diffusible in the extracellular fluid, since it lacks the extracellular matrix-binding motif present at the carboxy terminus of the “long” PDGF-A isoform (LaRochelle et al., 1991; Östman et al., 1991; Khachigian et al., 1992; Raines and Ross, 1992). In addition to the NSE-PDGF-A mice, we also generated transgenic mice expressing a closely related transgene encoding an endoplasmic reticulum (ER)-gated retained form of hPDGF-A (PDGF-AKDEL; Figure 4A). Seven NSE-PDGF-A founders and four NSE-PDGF-AKDEL founders were obtained. Four of the NSE-PDGF-A founders (A5-64, -72, -75, -82) and all of the NSE-PDGF-AKDEL founders (A10-3, -21, -23, -26) transmitted the transgene to their first offspring and transgenic lines were established. The transgene copy numbers of the lines were all in the range 1–10; the line used for all the experiments described in this paper (A5-75) had about five transgene copies per genome. We used a reverse transcriptase (RT)–PCR approach to examine expression of transgene-derived hPDGF-A mRNA in the retinae of each line (e.g., Figure 4B). The PCR primers were chosen to amplify PDGF-A transcripts across the Myc epitope sequences near the 3’ end of the transgene. Thus, transgene-derived human PDGF-A transcripts could be distinguished unambiguously from endogenous mouse transcripts by the size of the PCR product after Southern blotting and probing with a PDGF-A-specific oligonucleotide. Repробing the Southern blot with
The transgene consists of human PDGF-A coding sequences (1.0 kb) with a Myc epitope tag (44 bp) at its carboxy terminus, under the control of the rat NSE gene promoter (1.8 kb) and SV40 polyadenylation site. A second closely related transgene also had an oligonucleotide encoding an endoplasmic reticulum (ER) retention signal followed by a stop codon (KDEL) inserted immediately downstream of the Myc tag. See Experimental Procedures for construction details.

Expression of transgene-derived mRNA was detected by RT-PCR. Left, diagram showing the predicted structures of the transgene (hPDGF-A) and endogenous (mPDGF-A) mRNAs, and the relative positions of oligonucleotide PCR primers (arrows) and hybridization probes (P1, P2) used for detection. The position of exon 6 (69 bp), which encodes an extracellular matrix binding motif that can be inserted by alternative splicing, is indicated. Right, agarose gel electrophoresis of RT-PCR products generated from line A5-75 transgenic (tg) or wild-type (wt) P3 retinae and a control reaction (−RT) in which reverse transcriptase was omitted from the PCR reaction, Southern blotted, and probed with 32P-labeled probes P1 (detects all PDGF-A mRNA species) or P2 (detects only transgene-derived mRNA). The predicted sizes of the PCR products are 211 bp (“short” mPDGF-A mRNA lacking exon 6), 280 bp (“long” mPDGF-A mRNA including exon 6), or 318 bp (transgenic hPDGF-A mRNA). A series of control experiments established that our RT-PCR reaction conditions were such that the band intensities after blotting were proportional to the amount of mRNA added; band intensities could therefore be compared on a semiquantitative basis. Densitometry of gel lanes indicated that there were about five times as many transgene-derived PDGF-A transcripts as endogenous transcripts in the neural retina of line A5-75, and also that no “long” form mPDGF-A mRNA can be detected in wild-type or transgenic retinae.

Immunofluorescence localization of transgene-derived hPDGF-A in the retina of a P14 transgenic mouse expressing the ER-retained form of hPDGF-A (see [A]). Monoclonal 9E10 (anti-c-Myc) was the primary antibody; FITC-conjugated rabbit-anti-mouse IgG was the secondary antibody. The transgenic retina is on the right, a wild-type littermate on the left. The strongest signal is detected in the cell bodies of retinal ganglion cells (RGC); a weaker signal is detected in the cell bodies of photoreceptor cells in the outer nuclear layer (ONL, arrowheads), and in the inner nuclear layer (INL, arrows). The strong signal in the pigment epithelium (PE) is background autofluorescence. Retinal astrocytes migrate into the retina along the plane of the nerve fibre layer (NFL), which contains the projection axons of RGCs. IPL, inner plexiform layer; OPL, outer plexiform layer; IS, OS inner and outer segments of the photoreceptor cells. Scale bar, 50 μm.

We were unable to detect any immunoreactivity over background in retinae from any of the NSE-PDGF-A mice using antibody 9E10, which recognizes the Myc epitope present in the transgene-encoded hPDGF-A. However, in both of the NSE-PDGF-AKDEL lines, we could easily visualize the encoded polypeptide in the cell bodies of RGCs and, at a lower level, in neurons in the inner and outer nuclear layers (Figure 4C). The protein was abundant in all RGCs from before the day of birth (data not shown) to at least the end of the second postnatal week (Figure 4C) and was still expressed, at a lower level, in the adult (data not shown). The spatial expression pattern, in particular expression in RGCs, was similar to that previously described for an NSE-lacZ transgene.
(Forss-Petter et al., 1990; Seiler and Aramant, 1995) and an NSE-BCL2 transgene (Martinou et al., 1994), demonstrating that the activity of the NSE promoter cassette is not markedly affected in cis by flanking chromosomal sequences at the site of integration. Thus, it seems very likely that the expression pattern of the PDGF-A<sub>ACE</sub> transgene is a faithful representation of the expression pattern of the secreted PDGF-A transgene. We conclude, therefore, that our NSE-PDGF-A transgenic lines had any retinal phenotype between transgene expression (at the mRNA level) and retinal development. This might be a perturbed in the NSE-PDGF-A mice. Immuno-}

Hyperplasia of the Retinal Astrocyte Network in NSE-PDGF-A Mice During normal development, retinal astrocytes migrate radially across the inner surface (i.e., the nerve fiber layer) of the retina from the optic nerve head, starting around the day of birth and reaching the periphery of the retina around postnatal day 5 (P5) in the rat and the mouse (Watanabe and Raff, 1988; Mudhar et al., 1993; M. F., unpublished data). Since retinal astrocytes express PDGFRA<sub>α</sub> (Mudhar et al., 1993), which can be activated by all three dimeric isoforms of PDGF (AA, AB, BB), we expected that development of retinal astrocytes might be perturbed in the NSE-PDGF-A mice. Immuno-}

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Figure 5. Hyperplasia, but Decreased Migration, of Retinal Astrocytes and Blood Vessels in NSE-PDGF-A Transgenic Mice

Retinal astrocytes (A–C) and inner vasculature (D–F) in P4 wild-type (A and D), hemizygous transgenic (B and E), and homozygous transgenic (C and F) littermates were visualized by anti-GFAP labeling (A–C) and anticollagen IV labeling (D–F) of retinal whole-mounts. Only part of each preparation is shown, including the optic nerve head (bottom) and one lobe of the whole-mount. With increasing hPDGF-A transgene dose, the astrocyte network becomes more dense, and advances less far from the optic nerve head. The vasculature in the wild-type retina (D) forms an even network that does not yet extend all the way to the retinal periphery, even though astrocytes have already reached the periphery (compare with [A]). In the hemizygous transgenic animal (E), the retinal vessels form a denser network; there appear to be more and thicker vessels compared with wild-type. Note that the transgenic vascular net extends approximately to the leading edge of the astrocyte net, but does not overtake it (compare with [B]). In the homozygous transgenic retina (F), the vasculature forms a dense mat and extends a relatively short distance from the optic nerve head, closely corresponding to the distribution of astrocytes (compare with [C], which shows the same retina double-labeled for GFAP). Thus, the distribution of blood vessels in the transgenic retinae appears to be determined by the distribution of astrocytes. In (F), note vessels growing away from the retina into the vitreous (arrows). Scale bar, 200 μm.

numbers of astrocytes rather than, for example, more extensive GFAP+ processes on the same number of cells, we enzymatically dissociated individual retinae from P3 and P6 wild-type and transgenic (A5-75) retinae and labeled them in suspension with anti-GFAP antibodies followed by FITC-conjugated second antibodies. The cells were also incubated with propidium iodide (PI) to label nuclear DNA and analyzed in a fluorescence-activated cell scanner (FACS). Representative primary FACS data for one wild-type and one hemizygous transgenic mouse are shown in Figures 6A and 6B, and combined data from several animals are plotted in Figures 6D and 6E (details in the figure legend). A control in which the primary antibody (anti-GFAP) was omitted is shown in Figure 6C. Cells with GFAP labeling greater than background (boxed regions in the FACS plots of Figures 6A–6C) were counted as a proportion of the total retinal cell population. There were more than twice the normal number of GFAP+ astrocytes in the transgenic retinae at P3, and about four times the normal number at P6 (Figure 6D). In the transgenic retinae, there was also a higher proportion of astrocytes in the S, G2, and M phases of the cell cycle (15.6% ± 2.4% compared with 10.4% ± 1.0% in wild type, means ± SDs). This suggests that proliferation of astrocytes is enhanced in the transgenic retinae. We cannot yet say whether this effect accounts completely for the increased number of astrocytes in the transgenic retinae. For example, it is possible that increased survival of astrocytes also contributes to the phenotype.

Adult NSE-PDGF-A Mice Display a Phenotype Resembling Human Proliferative Retinopathy

In normal adult retinae, astrocytes are only found on the inner surface and never deep within the retina (Figure 7A). In adult transgenics, the astrocytes formed an abnormally tight mesh on the inner surface of the retina (data not shown) and, in addition, penetrated into the neural retina to form abnormal extensions of the net throughout the retina and even as deep as the retinal pigment epithelium (Figure 7B).

In adult wild-type mice, blood vessels are limited to well defined planes at the inner surface of the retina and in the plexiform layers (arrows in Figure 7C), where they border the inner nuclear layer (Engerman and Meyer, 1965; Connolly, 1991). The outer vascular network develops during the second postnatal week by budding from the inner vasculature, and is independent of astrocytes (Engerman and Meyer, 1965; Stone et al., 1995). In adult hemizygous transgenics, blood vessels penetrated into all layers of the retina right out to the photoreceptor layer (arrows in Figure 7D). Confocal microscopy revealed that they were colocalized with GFAP+ glial cells, most likely astrocytes (data not shown). The lamellar
suggests that PDGF-A might mediate a short-range paracrine interaction between these two cell types during normal development (Mudhar et al., 1993). The experiments reported here were designed to test whether such an interaction does occur and, if so, what the nature of the interaction might be. We attempted to inhibit PDGF signaling through PDGFRα on astrocytes, both with a blocking anti-PDGFRα antibody and also with a soluble extracellular fragment of PDGFRα. Both treatments resulted in significant but incomplete inhibition of astrocyte migration and a reduced branching pattern of the astrocyte network. Because we cannot tell to what extent PDGF signaling was inhibited in our experiments, we cannot deduce whether PDGF is wholly or only partly responsible for astrocyte proliferation/migration during development. It is possible that development of the astrocyte network depends solely on PDGF and that our inhibitory regimes were inadequate to reveal this. It is perhaps more likely that PDGF normally acts in concert with other factors whose individual contributions to astrocyte development are relatively small but whose combined effects are cumulative. If so, eliminating any one of them would not necessarily have a catastrophic effect.

To gain further information about the activity of PDGF-A in the retina, we generated transgenic mice that express PDGF-A under control of the NSE promoter. Although we were unable to visualize the transgene-derived secreted PDGF-A directly in situ, we showed, in two independent lines of transgenic mice, that an ER-retained form of PDGF-A under NSE control is expressed strongly in RGCs and less strongly in other retinal neurons. This is in accord with experience of other NSE-driven transgenes (Forss-Petter et al., 1990; Seiler and Aramant, 1995; Martinou et al., 1994), demonstrating that the activity of the NSE promoter cassette in the retina is robust, reproducible, and refractory to cis-effects of the transgene or neighboring chromosomal sequences. The ER-retained PDGF-A transgene did not elicit any retinal phenotype, unlike the secreted PDGF-A transgene, providing strong genetic evidence that the phenotype of our NSE-PDGF-A mice results from synthesis and secretion of the encoded PDGF-A polypeptide. Therefore, our transgenic mice demonstrate that increasing the supply of PDGF-A, most likely from RGCs (which also express endogenous PDGF-A), dramatically enhances accumulation of neighboring retinal astrocytes and their associated blood vessels. FACS analysis showed that the proportion of astrocytes containing more than a diploid complement of DNA is also increased. One possible interpretation of this observation is that the time the cells spend in G1 is reduced in proportion to the time spent in S/G2 and M, that is, the cell cycle is accelerated. Alternatively, the proportion of astrocytes that is actively engaged in the cell cycle might be increased in the transgenic mice. At present, we cannot distinguish between these possibilities. Nevertheless, we can conclude from the present data that PDGF-A overproduction in the transgenic mice has a positive effect on retinal astrocyte proliferation. It is possible that there is also an effect on astrocyte survival.

The fact that increased levels of PDGF-A stimulate
astrocyte proliferation demonstrates that neither PDGF-A nor any other mitogenic factor is available in the normal developing mouse retina at a concentration that is saturating for astrocyte proliferation. It follows that the number of astrocytes that develop in the retina could potentially be determined by the rate of supply of PDGF-A, which in turn depends on the number of RGCs. It is known that the final number of RGCs, in common with many other neuronal populations, depends on survival signals from their target cells. It is also strongly suspected that the number of vascular cells that develop in the retina depends on astrocytes (see below), and the data presented here support this idea. Therefore, there appears to be a hierarchy of sequential cell–cell interactions among vascular cells, astrocytes, RGCs and their target cells, the purpose of which is to ensure that each cell population develops in proportion to the others. We suggest that PDGF-AA is an important link in this chain of number-matching interactions, acting as an RGC-derived mitogen for astrocytes and thereby controlling the proportion of astrocytes to RGCs.

An unexpected finding was that, despite the considerable increase in astrocyte numbers in the transgenics, migration away from the optic nerve head was retarded. Superficially, it might appear that stimulating and inhibiting PDGF signaling have similar effects on the astrocyte network but this is not so; inhibiting PDGF signaling with the receptor 17 truncated receptor or APA5 anti-PDGFRα Ig resulted in a less extensive and more sparse astrocyte network, whereas increasing PDGF supply in the transgenics resulted in a less extensive but much denser network. PDGF-AA has been reported to either stimulate or inhibit cell migration, depending on its concentration and the cell type under investigation (Ferns et al., 1990; Siegbahn et al., 1990; Uren et al., 1994; Koyama et al., 1994a; Iguchi et al., 1995). For example, migration of Swiss 3T3 fibroblasts in vitro is maximal at relatively low PDGF-AA concentrations (3 ng/ml) and completely abolished at higher concentrations (12 ng/ml), whereas the proliferative response to PDGF-AA is monophasic and reaches a plateau at comparatively high concentrations (25 ng/ml) (Abedi et al., 1995). PDGF-AA has also been reported to antagonize PDGF-BB-stimulated cell migration (Siegbahn et al., 1990; Koyama et al., 1992). It seems likely that the signal transduction pathways that stimulate cell proliferation and migration interact but, in general, the relationship between proliferation and migration is poorly understood. An alternative explanation for the altered distribution of astrocytes in the transgenic retinas might be that this reflects an altered expression pattern of PDGF-A. We think that this is less likely, because the postnatal expression pattern of our ER-retained form of PDGF-A and other NSE-driven transgenes (Forss-Petter et al., 1990; Martinou et al., 1994; Seiler and Aramant, 1995) is rather similar to the expression pattern of endogenous PDGF-A (Mudhar et al., 1993). Both transgene-derived and endogenous PDGF-A are expressed in the great majority of RGCs all the way from optic nerve head to the retinal periphery, and in other retinal neurons in the inner and outer nuclear layers.

A striking aspect of the transgenic phenotype was the close correspondence between the distribution patterns of retinal astrocytes and blood vessels. In wild-type mice, the astrocytes migrate from the optic nerve head slightly ahead of the developing vascular network. In transgenic retinas, where astrocyte migration was retarded, the leading edge of the blood vessels caught up with but did not overtake the astrocyte front. This was true even in homozygous transgenic retinas, where astrocyte migration was severely inhibited. This is consistent with the view that physical proximity of astrocytes is required for retinal vasculogenesis. Retinal astrocytes express PDGFRα and probably respond directly to the transgene-encoded hPDGF-A. However, vascular cells express PDGFRβ (Mudhar et al., 1993)
and would not be expected to respond directly to PDGF-AA (Heldin et al., 1988; Coats et al., 1994). In keeping with this expectation, neither pericytes nor endothelial cells respond to PDGF-AA in vitro (D’Amore and Smith, 1993). Therefore, vascular cells in the transgenic retinae are probably stimulated by secondary signals from the PDGF-AA-responsive astrocytes. A strong candidate for an astrocyte-derived vasculogenic factor is VEGF/VPF (Barinaga, 1995). This factor is synthesized by retinal astrocytes in vivo (Stone et al., 1995), and is regulated by oxygen tension both in vivo and in vitro. Thus, experimentally induced hypoxia results in up-regulation of VEGF mRNA and stimulates vasculogenesis (Shweiki et al., 1992; Alon et al., 1995; Pierce et al., 1995; Stavri et al., 1995; Stone et al., 1995), whereas hyperoxia has the reverse effect (Alon et al., 1995). We did not investigate VEGF expression in our transgenic mice, but the fact that the overextended retinal vasculature was leaky and released blood into the retina is consistent with the known activities of VEGF/VPF (Conolly, 1991).

In adult NSE-PDGF-A transgenic retinae, astrocytes were distributed throughout the depth of the retina, including the inner and outer plexiform layers and as far out as the retinal pigment epithelium. This is in striking contrast to wild-type retinae, in which astrocytes are found only at the inner retinal surface (Stone et al., 1995). It seems likely that this is a consequence of overexpression of the NSE-PDGF-A transgene in the retina (Fors-Rasmussen et al., 1991). The outer retinal layers thus seem thinner, in contrast to what one would expect to see if there had been normal development and subsequent degeneration of the NSE-PDGF-A transgene in the retina (De Juan, 1990; Hosada et al., 1993; Alon et al., 1995; PDGFRα, 1990). Therefore, our transgenic mice might model a naturally occurring human disease state and could prove useful for testing potential treatments for some aspects of the proliferative retinopathies.

**Experimental Procedures**

**Construction of pRc17 Plasmid Expression Vector**

A full-length cDNA encoding truncated PDGRF-α (Lee et al., 1990) was obtained from R. Reed (John Hopkins, Baltimore). A 1.7 kb fragment of this cDNA was obtained following digestion with EcoRI and NotI, and inserted into a COS cell expression vector based on pHyK (Pollock and Richardson, 1992), which contains the adenovirus major late promoter, polyadenylation site from the herpesvirus type-1 thymidine kinase gene, and simian virus 40 origin of replication. This vector can replicate autonomously in COS cells under the influence of the endogenous SV40 large-T antigen. The pRc17 insert includes a small amount of 5′ noncoding sequence, followed by the initiation codon and coding sequence up to, but not including, the transmembrane domain. The mode of construction also placed a c-Myc epitope tag in-frame at the carboxy terminus of the encoded receptor fragment, so that it could be recognized by monoclonal antibody 9E10 (Evan et al., 1985). The final construct was named pRc17, and the encoded polypeptide Rc17.

**Electroporation of COS Cells**

DNA-encoding truncated rat PDGRF-α (Rc17) or a similar plasmid encoding the short splice-variant of human PDGF-A (Pollock and Richardson, 1992) was introduced into cultured COS cells by electroporation. The cells were grown to about 70% confluence in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS), trypsinized, and washed twice in HBS (20 mM Hepes [pH 7.05], 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 6 mM D-glucose), and resuspended in HBS at a concentration of 8 × 106 cells/ml. We mixed 2 × 105 cells with 15 μg of DNA and electroporated with a BioRad apparatus at 320 V and 125 mF, giving a time constant of 4.9 μs. After electroporation, the cells were grown in DMEM containing 10% FCS for 48 hr on plastic tissue culture dishes prior to intravitreal injection (see below) or on glass coverslips for immunocytochemistry with anti-c-Myc monoclonal antibody 9E10.

**Metabolic Radiolabeling of COS Cells**

About 5 × 106 electroporated COS cells were plated into 25 cm2 tissue culture dishes and grown for 48 hr before washing once with phosphate-buffered saline (PBS). The culture medium was replaced with cysteine- and methionine-free DMEM containing 10% dialysed fetal calf serum (FCS) and 60 μCi/ml 35S-cysteine/methionine mixture (Translabel, Amersham). After 18 hr incubation, the cell supernatant was centrifuged at 25,000 × g for 20 min and stored at 4°C before use.

**Immunoprecipitation and Gel Electrophoresis**

35S-labeled supernatants from COS cells expressing truncated rat PDGRF-α, or human PDGF-A, were preincubated with 10-3 volume of normal mouse serum for 1 hr at 4°C, then with protein A-Sepharose for 1 hr, followed by centrifugation at 15,000 × g for 10 min. The Rc17 supernatant was then incubated with an equal volume of either DMEM or PDGF-A supernatant for 16 hr at 4°C. The supernatants were immunoprecipitated with either mouse monoclonal antibody 9E10 or rabbit antiserum raised against PDGF from human platelets (R and D Systems, Minneapolis). After precipitation of immune complexes with protein A-Sepharose, they were electrophoresed on a 10% nonreducing polyacrylamide gel containing SDS and visualized by fluorography (Boner and Laskey, 1974).

**3H-Thymidine Incorporation Assays**

Approximately 2 × 105 NIH 3T3 cells were plated in each well of a 24-well tissue culture plate and cultured overnight in DMEM containing 10% FCS. After two washes with DMEM, the cells were growth-arrested by incubation in DMEM containing 0.25% FCS for 30 hr. DMEM-containing recombinant PDGF homodimers or bFGF...
against PDGFR clonal antibody 9E10 (Evan et al., 1985) were used for immunohistochemistry on cryosections. The monoclonal antibody (IgG 2a) against mouse c-Kit (clone A2K2; Nishikawa et al., 1991) was used as a negative control for the effects of APA5. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibodies, FITC-conjugated anti-rat antibodies, FITC-conjugated anti-rabbit antibodies, and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated anti-rabbit antibodies (Sigma Immuno Chemicals) were used as secondary antibodies.

Production of Transgenic Mice
Mice that overexpress PDGF-A in neurons under control of the NSE gene promoter were generated at the UMDS Transgenic Unit, The Rayne Institute, St. Thomas's Hospital, London SE1 7EH. The transgene consisted of a partial human PDGF-A CDNA (Betsholtz et al., 1986) engineered to encode the “short” alternative splice PDGF-A isoform (Tong et al., 1987; Bonthron et al., 1988; Rorsman et al., 1988) with a Myc epitope tag (Evan et al., 1985) at its carboxy terminus, fused to the rat NSE promoter (Forss-Petter et al., 1990) and the SV40 early polyadenylation site (Figure 4A). In brief, the SacII site upstream of the initiation codon in plasmid “PDGF-A+ TAG” (Pollock and Richardson, 1992) was converted to a HindIII site by T4 DNA ligase. The HindIII–SspI fragment from pdGF-A cDNA and the SV40 early polyadenylation site were ligated into the HindIII site of the NSE lacZ fusion gene in plasmid “NSElacZ” (Forss-Petter et al., 1990), leaving the SV40 poly(A)-addition site excised and used to replace the lac-Z fusion gene in plasmid “NSElacZ” (Forss-Petter et al., 1990), leaving the SV40 poly(A)-addition site intact. The intact human PDGF-A under the control of 1.8 kb of rat NSE promoter sequences and the SV40 early poladenylation site. The entire NSE-PDGF-SV40 cassette was purified as a linear EcoRI fragment for injection into mouse oocytes (C57Bl/6 × CBA 11 hybrids).

Identification and Genotyping of Transgenic Animals
Tail clipplings were digested overnight at 55°C in a buffer containing 50 mM Tris (pH 8), 10 mM EDTA, 100 mM sodium chloride, 1% (w/v) SDS, and 50 mg/ml proteinase K (Sigma). Following RNAase A treatment (100 μg/ml, 1 hr at 37°C), ammonium acetate was added to a final concentration of 2 M, chilled on ice, and centrifuged to precipitate proteins. DNA in the supernatant was precipitated with 0.6 vol of cold isopropanol and washed with 70% ethanol. The DNA pellet was dissolved in water overnight at 4°C. Yields were estimated from absorbance at 260 nm, and DNA aliquots (10 μg) were digested with restriction enzymes and subjected to Southern blot analysis.

The blots were hybridized with a PDGF-A chain cDNA probe radiolabeled by random priming (Feinberg and Vogelstein, 1984).

RT–PCR
Retinae were dissected from wild-type and transgenic littermates and homogenized in guanidinium isothiocyanate, and total cellular RNA was prepared as described previously (Chomczynski and Sacchi, 1987). This was treated with 400 units/ml RNase-free DNase 1 (Pharmacia) for 15 min at 37°C, then phenol/chloroform-extracted and precipitated with ethanol. Total RNA (2 μg) was reverse-transcribed into cDNA using the Superscript premplification system (Gibco-BRL). PDGF-A sequences were then specifically amplified by PCR (25 cycles of the following): 94°C, 30 s; 55°C, 2 min; 72°C, 2 min; terminating with 72°C, 5 min). The PCR primers were as follows: 5′-GGCTGCAGTGAGGTAGAAAGAG3′ (upstream) – 5′-TCA CGGAAGGAGAACAGAC-3′ (downstream). These primers hybridized to both endogenous (mouse) and transgenic (human) PDGF-A sequences without mismatch. RNA titration experiments showed that the RT–PCR reaction was semi quantitative under these conditions (i.e., operating in the linear range). PCR products were separated on a 2% (w/v) agarose gel, which was Southern blotted onto nylon membrane (Zetaprobe, BioRad) and probed with 32P-labeled (using polynucleotide kinase) oligonucleotide probes against either PDGF-A or the Myc epitope tag (see Figure 4). The oligonucleotide probe against PDGF-A was an equimolar mixture of two 39-mers, 5′-AAGCACCATACTAGATCTGTTACGGAAAATGCTCA CAGCGCA3′ (mouse) and 5′-AGGACCGCTACATAGATGGTCCAGG

**Antibodies**
For whole-mount preparations, a mouse monoclonal antibody raised against pig glial fibrillary acidic protein (GFAP) (Sigma Immuno Chemicals) and a polyclonal rabbit antibody raised against murine collagen IV (Biogenesis, England) were used. A polyclonal rabbit anti-GFAP antibody (Pruss, 1979) was used in flow cytometry. The lacto anti-GFAP antibody and monoclonal antibody 9E10 (Evans et al., 1985) were used for immunohistochemistry on cryosections. The monoclonal antibody (IgG 2a) against mouse PDGFRα (clone APA5) was raised against recombinant mouse PDGFRα in rat and competes with PDGF for binding to PDGFRα in vitro (Takakura et al., 1996). Hybridoma culture supernatants were precipitated with saturated ammonium sulfate at 50% (v/v) concentration. The precipitated antibody was further purified by anion-exchange chromatography (Cleardin et al., 1985). A monoclonal rat antibody (IgG 2a) against mouse c-Kit (clone A2K2; Nishikawa et al., 1991) was used as a negative control for the effects of APA5. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibodies, FITC-conjugated anti-rat antibodies, FITC-conjugated anti-rabbit antibodies, and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated anti-rabbit antibodies (Sigma Immuno Chemicals) were used as secondary antibodies.

Histology and Immunocytochemistry
For whole-mount preparations, eyes were removed and given a brief fixation in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS). The retinae were subsequently dissected and fixed in ice-cold methanol. After incubation in PBS containing 50% fetal calf serum (FCS) and 1% (w/v) Triton X-100 for 30 min at room temperature, the retinae were incubated overnight at room temperature in primary antibody, washed for 1 hr in PBS, incubated for 3 hr at room temperature in fluorescent secondary antibody, washed for 1 hr, and mounted in Citifluor anti-fade reagent (City University, London).

All antibodies were diluted in PBS containing 10% FCS. For cryosections, eyes were removed and fixed in 4% (w/v) paraformaldehyde in PBS for 1 hr and cryoprotected in 30% (w/v) solution of sucrose in cold methanol. After incubating in PBS containing 50% fetal calf serum (FCS) and 1% (w/v) Triton X-100 for 30 min at room temperature, the retinae were incubated overnight at room temperature in primary antibody, washed for 1 hr in PBS, incubated for 3 hr at room temperature in fluorescent secondary antibody, washed for 1 hr, and mounted in Citifluor anti-fade reagent (City University, London). The incorporated radioactivity was estimated by scintillation counting in Aquasol (Dupont).
AATGTAACACCGCA-3′ (human). The probe for Myc sequences was a 44-mer oligonucleotide, 5′-CTCAAGAAAGCATCTTTGTTAGAGA-3′. The blots were exposed and scanned using a BioRad GS-250 Molecular Imager with a BI screen to quantitate band intensities.

Flow Cytometry
Retinae were dissected and collected in Mg2+/Ca2+-free EarI’s basal salt solution (EBSS; Gibco) and incubated for 1 hr at 37°C in 0.125% (w/v) trypsin (Sigma) in EBSS followed by 30 min at 37°C in 0.125% (w/v) type I collagenase (Sigma) in DMEM. Cells were mechanically dissociated by trituration through a yellow Eppendorf tip in PBS containing 20% FCS, 6 mM MgCl2, and 50 mg/ml DNase I (Sigma). The cells were fixed for 30 min in 70% ethanol on ice and labeled with rabbit anti-GFAP and FITC-conjugated anti-rabbit antibodies in PBS containing 10% FCS. To analyze DNA content, 1 mg/ml RNase A was present during the first antibody incubation (anti-GFAP) and 25 μg/ml propidium iodide (PI; Sigma) during the second (anti-rabbit). Cells were analyzed by flow cytometry in a fluorescence-activated cell scanner (FACS) (Becton-Dickinson) to quantitate GFAP-immunoreactivity and cellular DNA content.

Acknowledgments
We would like to thank the other members of our respective laboratories for ideas, advice, and practical help. We would particularly like to thank Derek Davis for help with FACS, Frank Grosveld for help and advice on transgenics, Frank Walsh and Alex Harper (UMDS Transgenic Unit, St. Thomas’s Hospital, London SEI 7EH) for transgenic mouse production. This research was supported by the UK Medical Research Council, the Multiple Sclerosis Society for ideas, advice, and practical help. We would particularly like to thank Derek Davis for help with FACS, Frank Grosveld for help and advice on transgenics, Frank Walsh and Alex Harper (UMDS Transgenic Unit, St. Thomas’s Hospital, London SEI 7EH) for transgenic mouse production. This research was supported by the UK Medical Research Council, the Multiple Sclerosis Society for ideas, advice, and practical help. We would particularly like to thank Derek Davis for help with FACS, Frank Grosveld for help and advice on transgenics, Frank Walsh and Alex Harper (UMDS Transgenic Unit, St. Thomas’s Hospital, London SEI 7EH) for transgenic mouse production. This research was supported by the UK Medical Research Council, the Multiple Sclerosis Society for ideas, advice, and practical help.

Received July 22, 1996; revised October 16, 1996.

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