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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

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 promoter), many other

Most cells of the neural retina, such as photoreceptors, neurons, and Müller glia, are generated by multipotential 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, b) (for review, see Heldin and Westermark, 1989). The two receptors have different ligand-binding specificities; PDGFRa binds all three dimeric isoforms of PDGF, while PDGFRb 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 PDGFRb 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 PDGFRa, leading us to suggest that PDGF-AA might mediate a short-range paracrine interaction between RGCs and astrocytes during development (Mudhar et al., 1993).

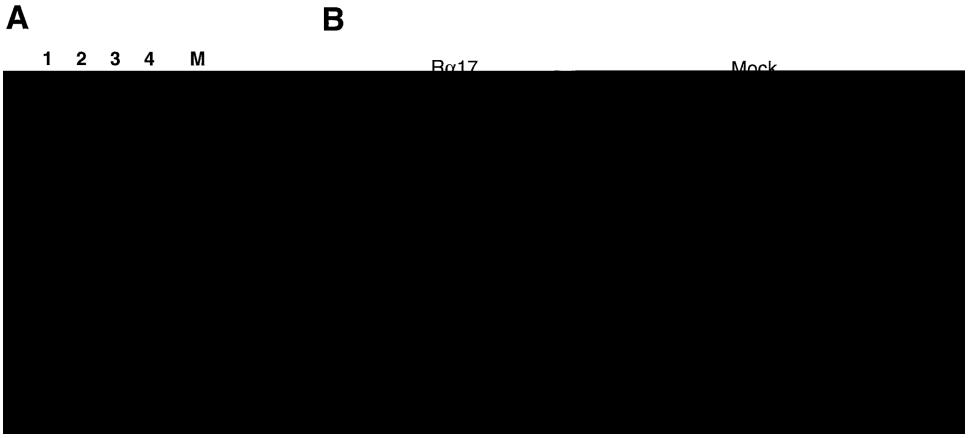

Figure 1. In Vitro Activity of Ra17 Truncated Receptor

(A) Immunoprecipitation of Ra17 truncated receptor bound to PDGF-AA. COS cells were electroporated with pRa17 or an analogous plasmid encoding PDGF-A and metabolically labeled with a ^{35}S-methionine/cysteine mixture. Cell culture supernatants were collected and immunoprecipitated, either with or without being previously coincubated overnight at 4°C with anti–PDGF-AA or 9E10 (anti-c-Myc) antibodies. Precipitates were run on a polyacrylamide gel and visualized by fluorography. Lane 1: Ra17-conditioned medium (CM) precipitated with anti-c-Myc. Lane 2: Ra17-CM, coincubated with PDGF-A-CM, precipitated with anti-PDGF-AA. Lane 3: Control (mock-transfected)-CM, coincubated with PDGF-A-CM, precipitated with anti-PDGF-AA. Lane 4: Ra17-CM precipitated with anti-PDGF-AA. Lane M: molecular weight markers. Upper arrow: position of Ra17 polypeptide. Lower arrow: position of plasmid-encoded PDGF-AA. A proportion of Ra17 and PDGF-AA coprecipitate with anti-PDGF-AA (lane 2).

(B) Neutralization of PDGF isoforms with Ra17 truncated receptor. Subconfluent cultures of NIH 3T3 cells were growth-arrested by serum deprivation. Purified growth factors were added to a fixed concentration, sufficient to stimulate half-maximal mitogenic response, together with different dilutions of conditioned medium from COS cells that had been transfected with plasmid pRa17 (left panel, "Ra17") or with the vector backbone alone (right panel, "mock"). After overnight incubation at 37°C, ^{3}H-thymidine was added to the cultures for 4 hr before solubilizing the cells and determining the amount of TCA-precipitable radioactivity by scintillation counting. Assays were performed in triplicate. The results are expressed as a percentage of the incorporation obtained in response to growth factor alone. Conditioned medium containing Ra17 truncated receptor was able to neutralize all three dimeric isoforms of PDGF, but not bFGF, in a dose-dependent fashion. For further details, see Experimental Procedures.

retinae were examined. Careful observation revealed a

before birth, prior to the COS cell injections. When the extentwas eeisimennseThe

than normal (Figure 3). As in the Ra17 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, Ra17 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 5 13) was reduced by 20% 6 1.2% compared with ACK2-treated retinae (n 5 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 astrocytesonPoretinaosed(hibi-)]TJ0

Figure 4. Structure and Expression of the Human PDGF-A Transgene

(A) 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.

(B) Expression of transgene-derived mRNA was detected by RT–PCR. Left, diagram showing the predicted structures of the transgenic (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 (2 RT) in which reverse transcriptase was omitted from the PCR reaction, Southern blotted, and probed with ^{32}P-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 h468(6),)0462(e71(bpbe8(60)-1.2T0 -1.2fction)-1.58(dSE)-29
PCR p(P1,ion)-260()-2]TJs oncodwe31(21ckisu21(r3on))-86h5PCRp4P1,in

(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_{KDEL} transgene is a faithful representation of the expression pattern of the secreted PDGF-A transgene. We conclude, therefore, that our NSE-PDGF-A transgenic mice synthesize the encoded PDGF polypeptide in RGCs and other retinal neurons but that this does not accumulate to a detectable degree either inside cells or in the extracellular space following secretion. This conclusion is strongly supported by phenotypic analysis of the NSE-PDGF-A mice (see below). Note that the expression pattern of transgene-derived PDGF-A is not dissimilar to the endogenous pattern of PDGF-A expression (Mudhar et al., 1993). Both are expressed in the great majority littermates of one of the lines, called A5-75. At P4, the homozygous animals displayed a retinal astrocyte phenotype that was clearly more severe than that of the hemizygotes (Figures 5B and 5C). The homozygotes (which have a double complement of transgenes and, presumably, correspondingly higher PDGF-A expression) had a much denser mat of GFAP^{1} astrocyte processes than either the hemizygotes or wild-types (Figures 5A–5C). The radial spread of astrocytes from the optic nerve head was less in the homozygotes than in the hemizygotes at P4, and less in the hemizygotes than in wild-type mice (Figures 5A–5C). At P4, for example, the average radius of the astrocyte net was reduced by 41% 6 10.6% (n 5 10) in heterozygotes and by 59% 6 1.3% (n 5 5) in homozygotes compared with wild type. Possible reasons for this effect are discussed later. However, despite the fact that astrocyte migration was retarded in A5-75 mice during early postnatal develop-

suggests that PDGF-A might mediate a short-range par-

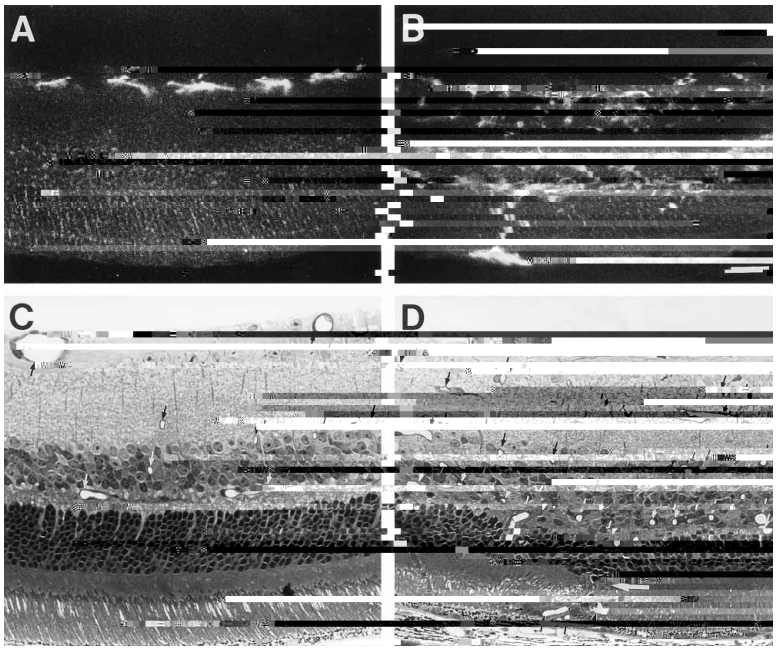

Figure 7. Retinae of Adult NSE-PDGF-A Transgenic Mice Have Similarities to Human Proliferative Retinopathies

Sections through adult wild-type (A and C) and hemizygous transgenic (B and D) retinae were immunolabeled with anti-GFAP to reveal the distribution of astrocytes (A and B) or stained with methylene blue and photographed in bright-field to show retinal morphology (C and D). Whereas astrocytes in the wild-type retina (A) are restricted to the inner surface (nerve fiber layer), astrocytes are distributed through all retinal layers right out to the pigment epithelium in the transgenic retina (B). This aberrant distribution of astrocytes is reflected by the vasculature in the transgenics. Whereas blood vessels in the wild-type retina are restricted to the nerve fiber layer and to the inner and outer faces of the inner nuclear layer (small arrows in [C]), they are more abundant and are distributed throughout the depth of the transgenic retina (small arrows in [D]). Presumably because of this vascular invasion of the retina, the normal lamellar organization of the retina is disrupted in places (e.g., large arrow in [D]). Scale bars, 50 μm.

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

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 retinae 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 endogeneous PDGF-A (Mudhar

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, 1991). 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.

(all human sequence, from Peprotech, New York), or PDGF-AB purified by metal ion chromatography from human platelets (Hammacher et al., 1988; a generous gift from Carl-Henrik Heldin), with or without medium conditioned by COS cells expressing Ra17, was added to the cells, which were cultured for a further 16 hr at 37°C before the addition of 1–3 μCi of ^{3}H-thymidine. The concentrations PDGFRa in vitro (Takakura et al., 1996). Hybridoma culture-supernatants were precipitated with saturated amonium sulfate at 50% (v/v) concentration. The precipitate was further purified by anion-exchange chromatography (Clezardin et al., 1985). A monoclonal rat antibody (IgG 2a) against mouse c-Kit (clone ACK2; Nishikawa et al., 1991) was used as a negative control for the effects of APA5.

AATGT**A**ACACGCCA-3

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