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A Role for Platelet-Derived Growth Factor in Normal Gliogenesis in the Central Nervous System

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Summary

The bipotential progenitor cells (O-2A progenitors) that produce oligodendrocytes and type-2 astrocytes in the developing rat optic nerve are induced to proliferate in culture by type-1 astrocytes. Here, we show that the astrocyte-derived mitogen is platelet-derived growth factor (PDGF). PDGF is a potent mitogen for O-2A progenitor cells in vitro. Mitogenic activity in astrocyte-conditioned medium comigrates with PDGF on a size-exclusion column, competes with PDGF for receptors, and is neutralized by antibodies to PDGF. PDGF dimers can be immunoprecipitated from astrocyte-conditioned medium, and mRNA encoding PDGF is present in rat brain throughout gliogenesis. We propose that astrocyte-derived PDGF is crucial for the control of myelination in the developing central nervous sytem.

Introduction

In the neonatal rat optic nerve there are bipotential glial progenitor cells, which during postnatal development give rise either to oligodendrocytes, the myelin-producing cells of the central nervous system (CNS), or type-2 astrocytes, which contact nerve axons at the gaps ("nodes of Ranvier") between adjacent myelinated regions ("internodes") (ffrench-Constant and Raff, 1986a). The bipotential progenitor cells are therefore known as O-2A progenitors (Raff et al., 1983). Apart from the O-2A lineage, the most abundant glial cells in the embryonic and neonatal optic nerve are type-1 astrocytes, which are derived from a different precursor cell (Raff et al., 1984a; for a review of cell lineages in the optic nerve, see Raff and Miller, 1984). There is evidence that type-1 astrocytes are derived from the neuroepithelial cells that form the optic stalk, while the O-2A progenitor cells appear to migrate into the developing optic nerve from elsewhere in the CNS (Small et al., 1987). There are no neural cell bodies in the nerve, only axons from the retinal ganglion neurons projecting to the brain.

Type-1 astrocytes first appear in the rat optic nerve around embryonic day 16 (E16), and oligodendrocytes on the day of birth (E21) (Skoff et al., 1976a, 1976b; Miller et al., 1985). O-2A progenitors continue to divide and differentiate into oligodendrocytes for several weeks after this (Skoff et al., 1976a, 1976b), and some progenitors even persist into adulthood (ffrench-Constant and Raff, 1986b). Starting in the second postnatal week, some O-2A progenitors differentiate into type-2 astrocytes (Miller et al., 1985). This strict developmental sequence is disrupted when dissociated optic nerve cells are cultured in defined medium. Then all the O-2A progenitor cells stop dividing and differentiate within 48 hr into oligodendrocytes, regardless of the age of the animal from which they were derived (Raff et al., 1985). Type-2 astrocytes do not develop in these cultures unless an inducing factor is present (Raff et al., 1983; Hughes and Raff, 1987).

Correct timing of oligodendrocyte development can be restored in culture by growing embryonic optic nerve cells in ≤0.5% FCS on a monolayer of type-1 astrocytes, or in astrocyte-conditioned medium (Raff et al., 1985). Under these conditions O-2A progenitors are stimulated to divide, and first differentiate into oligodendrocytes at the in vitro equivalent of the day of birth. Proliferation and differentiation into oligodendrocytes continue for several weeks in culture (Noble and Murray, 1984; Raff et al., 1985; Dubois-Dalcq, 1987), just as in vivo. Thus, type-1 astrocytes provide a mitogen(s) that can keep O-2A progenitors dividing and prevent their premature differentiation. To understand the mechanisms that control O-2A cell differentiation, it is essential to identify the molecules that mediate this crucial cell–cell interaction.

Several polypeptide growth factors and their receptors are expressed in the CNS (for reviews, see Gospodarowicz, 1984; Westermark et al., 1985; Korsching, 1986; Gammeltoft et al., 1987). One of these, platelet-derived growth factor (PDGF), was of particular interest to us since PDGF receptors seem to be restricted to cells of mesenchymal and glial origin (Heldin et al., 1981c), and PDGF is frequently expressed at high levels in human gliomas (Eva et al., 1982; Betsholtz et al., 1986), suggesting a role for PDGF in the control of normal glial cell growth (for a review of PDGF biology, see Ross et al., 1986). In this article, we show that pure human and porcine PDGFs are strongly mitogenic for O-2A progenitors in vitro (see also Noble et al., submitted), that type-1 astrocytes in culture secrete PDGF dimers, and that mitogenic activity in astrocyte-conditioned medium is neutralized by anti-PDGF immunoglobulin. In addition, we find that mRNAs encoding the PDGF A and B chains are present in primary cultures of type-1 astrocytes, and in neonatal rat brain at the time when O-2A progenitor cells are proliferating in vivo (Skoff et al., 1976a, 1976b). Our findings strongly suggest that PDGF is secreted by type-1 astrocytes and plays a key role

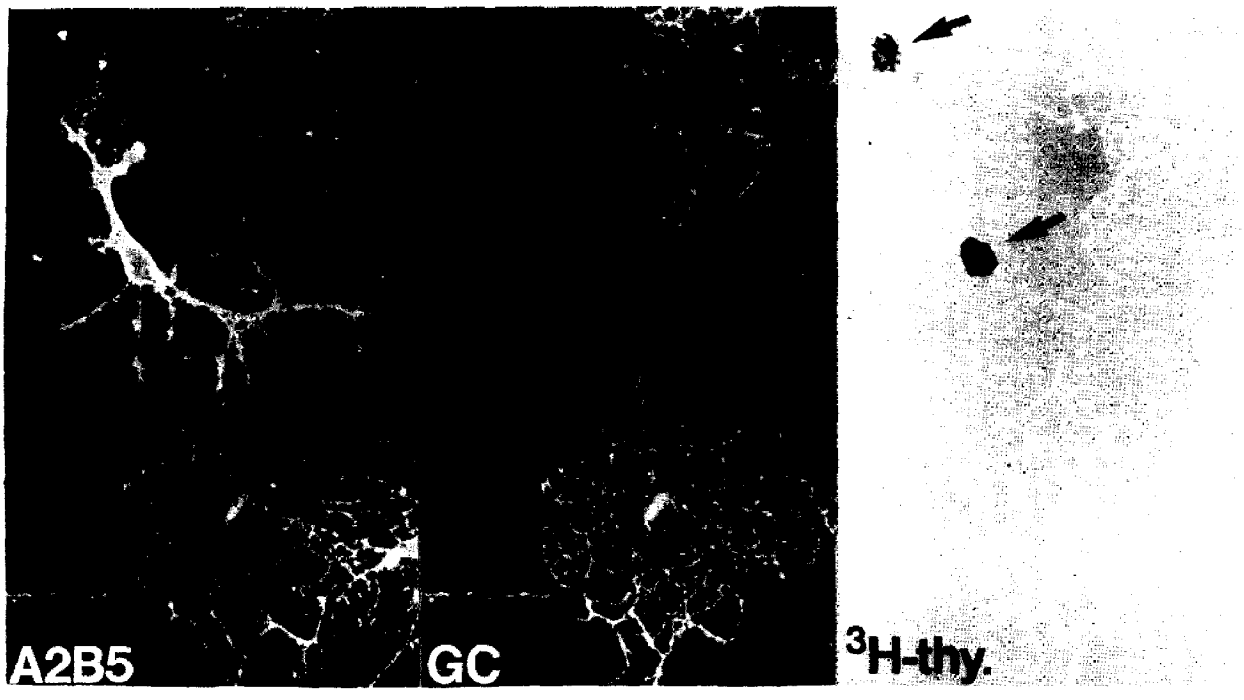

Figure 1. Immunofluorescence Microscopy and [^{3}H]Thymidine Autoradiography of P7 Rat Optic Nerve Cells in Culture

Optic nerve cells were grown in defined medium supplemented with 0.5% FCS and Superose 12 fractionated astrocyte-conditioned medium (see Figure 2). [^{3}H]thymidine (2 μCi/ml final concentration) was added to the cultures from 18 to 32 hr after plating, and the cells were fixed and stained with monoclonal antibodies A2B5 and GC (see text), followed by appropriate fluorescent second antibodies. The stained cells were processed for autoradiography (see Experimental Procedures) and developed after 3 days. The figure shows cells cultured with the most active Superose fraction (number 31, see Figure 2). An (A2B5$^+$, GC$^-$) O-2A progenitor cell lies between two GC$^+$ oligodendrocytes. Of these, only the progenitor cell has incorporated [^{3}H]thymidine (lower arrow). An unstained flat cell, possibly a type-1 astrocyte, has also incorporated radiolabel (upper arrow). Approximately 45% of O-2A progenitor cells incorporated [^{3}H]thymidine when cultured with Superose fraction 31 or PDGF (Table 1). Mature GC$^+$ oligodendrocytes never incorporated [^{3}H]thymidine in our experiments.

in controlling the proliferation and differentiation of O-2A progenitors in the developing rat optic nerve.

Results

The Astrocyte-Derived Mitogen Comigrates with PDGF on a Size-Exclusion Column

We fractionated astrocyte-conditioned medium on a Superose 12 FPLC size-exclusion column (Pharmacia), and tested individual fractions for their ability to promote proliferation of O-2A progenitor cells in cultures of neonatal rat optic nerve cells. Cells plated on glass coverslips were cultured in defined medium containing transferrin and insulin (see Experimental Procedures), supplemented with 0.5% fetal calf serum (FCS) and a portion of each column fraction. In some experiments, the cells were fixed after 3 days in culture and stained with monoclonal antibodies A2B5 (Eisenbarth et al., 1979) and anti-galactocerebroside (GC) (Raff et al., 1978), followed by appropriate fluorescent second antibodies, to allow O-2A progenitors (A2B5$^+$,GC$^-$) and oligodendrocytes (GC$^+$) to be identified in a fluorescence microscope (Figure 1). In defined medium containing ≤0.5% FCS, most O-2A progenitors in optic nerve cultures stop dividing and differentiate within 1 or 2 days into oligodendrocytes (Raff et al., 1985); however in the presence of mitogens derived from type-1 astrocytes some of the O-2A progenitors continue to divide a number of times before differentiating (Noble and Murray, 1984; Raff et al., 1985; Temple and Raff, 1986). Therefore, the number of O-2A progenitors remaining after 3 days in culture provides an estimate of the amount of mitogen in the medium. According to this progenitor cell counting assay, the mitogenic activity in astrocyte-conditioned medium migrated on Superose 12 as a single trailing peak with an apparent molecular mass of ~18 kd (Figure 2, upper panel).

In other experiments, DNA synthesis in O-2A progenitors was measured by ^{3}H-thymidine autoradiography (Figure 1). Table 1 shows the proportions of O-2A progenitors that incorporated ^{3}H-thymidine when grown in the presence of a portion of the most active Superose 12 column fraction (number 31, see Figure 2), compared to an inactive fraction (number 28) or 10 ng/ml of pure human PDGF. A significant proportion (about 45%) of O-2A progenitors growing in the presence of fraction 31 or pure PDGF incorporated ^{3}H-thymidine (Table 1), indicating that the increased numbers of O-2A progenitors depicted in Figure 2 (upper panel) are produced by cell divisions, rather than by an inhibitory effect on growth and differentiation, for example. Therefore, only the simpler progenitor cell counting assay was used in subsequent experiments.

For comparison, ^{125}I-labeled human PDGF was applied to the Superose 12 column under the same conditions. The elution profile, shown in Figure 2 (lower panel),

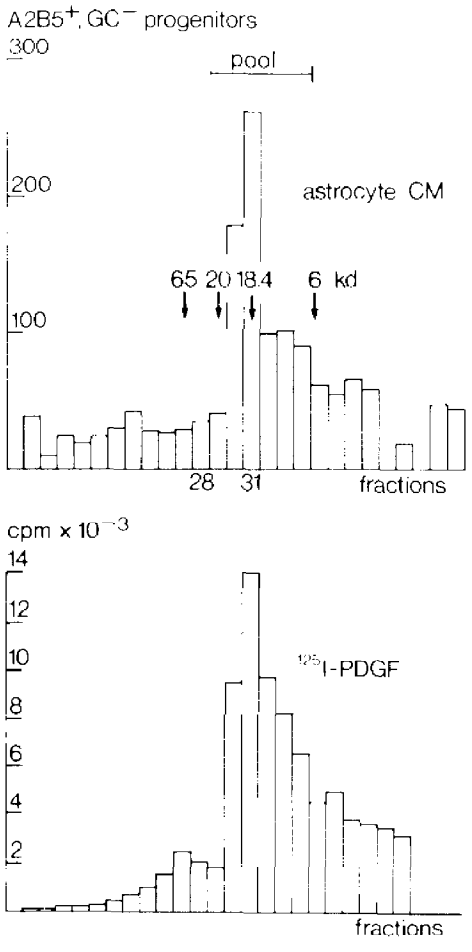

Figure 2. Superose 12 Size-Exclusion Chromatography of Astrocyte-Conditioned Medium

We concentrated astrocyte-conditioned medium by ammonium sulfate precipitation (see Experimental Procedures) and separated it on a Superose 12 (Pharmacia) column equilibrated with 0.2 M ammonium acetate (pH 7.0). A portion of each column fraction was tested for its ability to stimulate proliferation of O-2A progenitor cells in cultures of P7 rat optic nerve, by counting progenitor cell numbers after 3 days in culture (see Results). Mitogenic activity migrated as a single trailing peak with an apparent molecular weight of ~18 kd (upper panel). On the same column under identical conditions, ^{125}I-labeled dimeric human PDGF had the same mobility (lower panel).

was very similar to that of the astrocyte-derived mitogen, with the peaks falling in precisely the same fraction. Human PDGF has a molecular mass of ~30 kd (Heldin et al., 1981a; also see Figure 4), and is a heterodimer of A and B chains, of ~17 and ~14 kd, respectively (Hammacher et al., submitted). It is probable that the anomalous apparent molecular mass on Superose 12 is a consequence of interaction between the hydrophobic PDGF molecule and the column matrix.

Astrocyte-Conditioned Medium Competes with PDGF for Receptors on Human Foreskin Fibroblasts

Serum-free conditioned media were collected from cultures of primary rat cortical astrocytes, primary rat meningeal cells, and some rat and human CNS cell lines. The conditioned media were tested for PDGF-like molecules by their ability to compete with ^{125}I-labeled human PDGF for receptors on the surface of human foreskin fibroblasts (Nistér et al., 1984). The human glioma cell line 157 (provided by M. Noble, Ludwig Institute for Cancer Research, London) secreted the highest amounts of PDGF receptor competing activity (Table 2). A7-6-3, a rat CNS cell transformed by a retrovirus carrying the SV40 large T gene (H. Geller and M. Dubois-Dalcq, unpublished data), also secreted substantial receptor competing activity. The amount of receptor competing activity in astrocyte-conditioned medium was variable, but we readily detected activity in two out of the three batches that we tested (Table 2). On the other hand, there was no activity in any of the three batches of primary meningeal cell-conditioned me-

Table 1. [^{3}H]Thymidine Incorporation in O-2A Progenitors

Addition to Culture Medium[a]	^{3}H-Labeled O-2A Progenitor Cells
astrocyte CM (1:5)	28% (22/78)
astrocyte CM (1:10)	34% (29/86)
Superose fraction 28 (1:20)[b]	5% (2/42)
Superose fraction 31 (1:20)[b]	47% (68/144)
human PDGF (5 ng/ml)	45% (77/170)
no addition	0% (0/16)

[a] Modified medium of Bottenstein and Sato (1979). See Experimental Procedures.

[b] See Figure 2.

^{3}H-thymidine autoradiography was performed (see Experimental Procedures) on P7 optic nerve cultures grown in defined medium supplemented as shown in the table. Before processing for autoradiography, the cultures were doubly surface-stained with monoclonal antibodies A2B5 and GC, and, after exposure, the numbers of (A2B5$^+$, GC$^-$) O-2A progenitor cells, with and without silver grains, were counted under a fluorescence microscope (see Figure 1). Shown are the percentages of progenitor cells that incorporated [^{3}H]thymidine, with the actual numbers observed (means of duplicate coverslips) in parentheses.

Table 2. Mitogenic Effect, and PDGF Receptor Competing Abilities of Conditioned Media

Source of Medium	Effect on ^{125}I-PDGF Binding (% Displacement)	Fold-Dilution at Half Maximal Mitogenic Activity
157 human glioma cells	73 (82,75,61)	128
A7-6-3 rat CNS cells	34 (45,21,35)	16
primary astrocytes	20 (27,0,32)	8
primary meninges	−5 (−1,0,−13)	no activity
DMEM	0	no activity
human PDGF (10 ng/ml)	57 (48,79,43)	20 (0.5 ng/ml)

Conditioned media were collected as described in Experimental Procedures. Three independent batches of media were tested, undiluted, for their ability to compete with human ^{125}I-PDGF for receptors on human foreskin fibroblasts (Nistér et al., 1984). The individual results (parentheses) and the mean are listed in the center column. The third batch of each conditioned medium was tested for mitogenic effect on O-2A progenitors in cultures of P7 rat optic nerve. The fold-dilution required for half-maximal response, shown in the right-hand column, was determined in each case from a dose-response profile like those in Figure 3. There is good correspondence between the PDGF receptor competing ability and the mitogenic activity in each sample. The activity in astrocyte-conditioned medium is equivalent to ~4 ng/ml human PDGF.

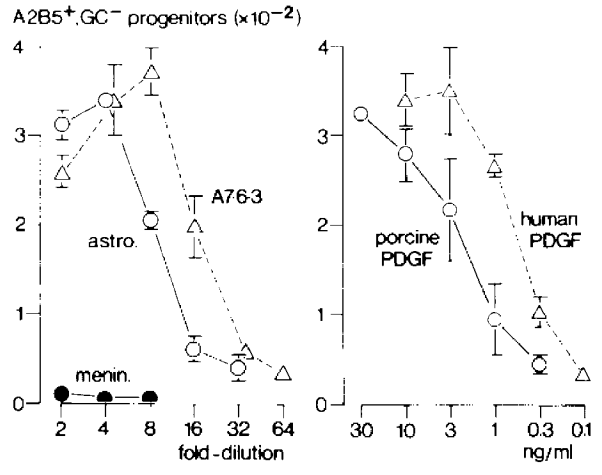

Figure 3. Dose-Response Profiles for Mitogenic Stimulation of O-2A Progenitor Cells by Conditioned Media, and by Purified PDGF

Media conditioned for 48 hr by primary cultures of astrocytes from neonatal rat cerebral cortex, by primary cultures of rat meningeal cells, or by A7-6-3 rat CNS cells were tested at various dilutions for mitogenic effect on O-2A progenitors in P7 rat optic nerve cultures, by counting progenitor cell numbers after 3 days in culture (see Results). Plotted in the figure are the means of duplicate experiments; vertical bars represent the difference between individual cell counts. The mitogenic response is dose-dependent and decreases from near-maximal to near-background over a 4- to 8-fold concentration range. Pure human or porcine PDGF elicited a similar mitogenic response, but activity diluted out over an ~30-fold concentration range. The concentration of human PDGF at half-maximal activity is ~0.5 ng/ml (17 pM).

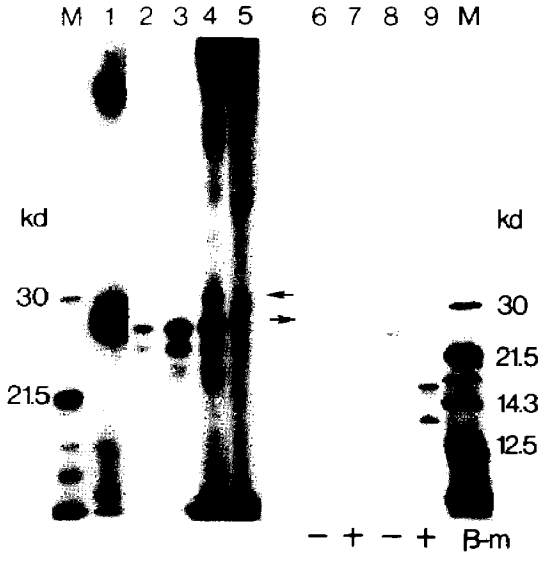

Figure 4. Immunoprecipitation of ^{35}S-Cysteine-Labeled Proteins from Astrocyte-Conditioned Medium (ACM), with Anti-PDGF Serum

Cultures of primary astrocytes from neonatal rat cerebral cortex were incubated overnight in serum-free medium containing ^{35}S-cysteine (see Experimental Procedures). The medium was collected and half of it applied to a Superose 12 FPLC column. The fractions around the peak of mitogenic activity were combined ("pool" in Figure 2), and immunoprecipitated with anti-PDGF serum or control serum, followed by Staphylococcus A. The remaining unfractionated ACM was treated in the same way. The precipitates were run on a 13% polycrylamide-SDS gel (left panel) or a 17% gel (right panel), either with or without prior reduction with β-mercaptoethanol (see below). ^{125}I-labeled human PDGF was also precipitated for comparison. Left panel: no β-mercaptoethanol. Lane 1: human PDGF, anti-PDGF serum. Lane 2: Superose-fractionated ACM, anti-PDGF serum. Lane 3: Superose-fractionated ACM, control serum. Lane 4: unfractionated ACM, anti-PDGF serum. Lane 5: unfractionated ACM, control serum. A protein band at ~30 kd (arrowhead) is precipitated by anti-PDGF serum, but not control serum. Right panel: samples unreduced or reduced by β-mercaptoethanol as indicated. Lane 6: Superose-fractionated ACM, anti-PDGF serum, unreduced. Lane 7: same, reduced. Lane 8: human PDGF, unreduced. Lane 9: same, reduced. The ~30 kd protein precipitated from ACM (lane 6, arrowhead) yields two bands at ~14 kd and ~17 kd when reduced by β-mercaptoethanol. Lanes m: protein molecular weight markers.

dium tested. The detection limit of this assay is of the order of 1 ng/ml for pure human PDGF, but we do not know how the sensitivity differs between rat and human, or how it differs for different molecular forms of PDGF.

In parallel experiments, we compared the abilities of the same conditioned media to promote proliferation of O-2A progenitor cells by the progenitor cell counting assay used to generate Figure 2 (see previous section). Dose-response curves for conditioned media of astrocytes, A7-6-3 cells, and meningeal cells are shown in Figure 3, with dose-response profiles for human and porcine PDGFs for comparison. The amount of mitogenic activity in each conditioned medium, expressed as the dilution at which activity is half-maximal, is listed in Table 2. For the cell types examined, the mitogenic effect on O-2A progenitor cells correlates well with the level of PDGF-like molecules in the medium, estimated by receptor competing ability. This is consistent with the notion that the predominant active molecule may be a form of PDGF.

Astrocytes Secrete PDGF Dimers into the Culture Medium

We labeled primary astrocytes for 24 hr with ^{35}S-cysteine, and collected the culture medium. Some of the medium was fractionated on Superose 12 as above, and fractions encompassing the peak of activity ("pool" in Figure 2, upper panel) were combined. This pool of fractions, and the remainder of the unfractionated medium, were incubated separately with anti-PDGF serum (Heldin et al., 1981b) or control serum, followed by formalin-fixed Staphylococcus A, and the precipitates subjected to SDS-polyacrylamide gel electrophoresis either with or without prior reduction with β-mercaptoethanol (Figure 4). An unreduced protein of 30 kd (arrows) was precipitated from both fractionated (lane 2) and unfractionated (lane 4) astrocyte-conditioned medium by anti-PDGF serum, but not by control serum (lanes 3 and 5). Pure ^{125}I-labeled human PDGF migrated as a broad band from 26 kd to 30 kd (lane 1), or as a doublet at ~25 kd (lane 8), depending on the composition of the gel matrix. Upon reduction, the 29 kd astrocyte protein was eliminated, and instead two bands at ~14 kd and ~17 kd appeared (compare lanes 6 and 7), which migrated close to the reduced A and B chains of human PDGF (lane 9). We do not know whether the ~14 kd astrocyte polypeptide represents the B chain of PDGF or a partial proteolytic degradation product of the ~17 kd A chain. Definitive identification will require the use of A and B chain–specific an-

Table 3. The Mitogenic Activity in Astrocyte-Conditioned Medium Is Neutralized by Anti-PDGF Immunoglobulin

Addition to Culture Medium[a]	Number of Progenitors Ig: None	Control	αPDGF	Neutralization
(Experiment 1)				
astrocyte CM (1:5)	147	ND	25	83%
astrocyte CM (1:10)	55	85	15	73%
human PDGF (5 ng/ml)[b]	41	36	3	92%
no addition	6	ND	ND	–
(Experiment 2)				
astrocyte CM (1:2)	592	372	38	90%
no addition	19	ND	ND	–
(Experiment 3)				
astrocyte CM (1:5)	72	ND	3	96%
no addition	1.5	ND	ND	–
(Experiment 4) Superose 12 fractions[c]				
fraction 28 (1:20)	1	ND	13	–
fractions 31 + 32 (1:20)	59	ND	13	78%
no addition	9	ND	ND	–

[a] Modified medium of Bottenstein and Sato (1979). See Experimental Procedures.
[b] Nominal concentration.
[c] See Figure 2.
Astrocyte-conditioned media (CM), or column fractions from Superose 12 fractionated astrocyte CM (see Figure 2), were tested for their ability to stimulate proliferation of O-2A progenitor cells in P7 rat optic nerve cultures in the presence of 25 μg/ml rabbit anti-human PDGF Ig, control Ig, or no Ig. Three different batches of astrocyte-conditioned medium were tested (Experiments 1–3), using two independent preparations of anti-PDGF Ig (Experiments 1–2, and Experiments 3–4). Quoted progenitor cell numbers are averages of triplicate (Experiment 1) or duplicate coverslips. The proportion of mitogenic activity which was neutralized by anti-PDGF Ig is listed in the right-hand column. These values are minimum estimates, because they were calculated from the progenitor cell number in the presence of anti-PDGF Ig (α-PDGF) and the lower of the other two relevant figures (no Ig or control Ig) without correcting for the background in defined medium.

tibodies. It appears, however, that primary astrocytes synthesize and secrete PDGF dimers into the culture medium.

The Majority of Mitogenic Activity in Astrocyte-Conditioned Medium Is Neutralized by Anti-PDGF Immunoglobulins

The experiments described above demonstrate that astrocytes secrete PDGF dimers, which are mitogenic for O-2A progenitor cells in cultures of rat optic nerve. Is this the major, or only, mitogen for O-2A progenitors in astrocyte-conditioned medium? To answer this question, we attempted to neutralize the mitogenic activity with antibodies directed against PDGF (Heldin et al., 1981b). An ammonium sulfate Ig fraction of rabbit anti-human PDGF, used at a protein concentration of 25 μg/ml in the culture medium, neutralized over 90% of the mitogenic activity of 5 ng/ml pure human PDGF (Table 3, Experiment 1). In contrast, an equivalent amount of control Ig had no effect. Between 73% and 96% of the mitogenic activity of unfractionated astrocyte-conditioned medium was also neutralized by anti-PDGF, but not control Ig (Table 3, Experiments 1–3). These effects were elicited by Ig prepared from three independent anti-PDGF rabbit sera (see Experimental Procedures). One of these sera (used in Experiments 1 and 2, Table 3) has been characterized by Heldin et al. (1981b), and shown to specifically neutralize PDGF-related mitogens while not affecting epidermal growth factor (EGF) or fibroblast growth factors (FGF). As an additional test of the specificity of our anti-PDGF Ig preparations, we tried to determine whether they would inhibit type-2 astrocyte inducing factor, an activity found in extracts of rat optic nerves (Hughes and Raff, 1987) and also in astrocyte-conditioned medium (Lillien and Raff, personal communication). This activity, which is mediated by an as yet uncharacterized ~25 kd protein (Hughes and Raff, 1987), induces expression of the astrocyte marker, glial fibrillary acid protein (GFAP), in O-2A progenitors in optic nerve cultures. Our anti-PDGF Ig preparations had no inhibitory effect on this activity in optic nerve extracts (data not shown).

At least 70% of the mitogenic activity in the active peak of Superose 12–fractionated astrocyte-conditioned medium was also neutralized by anti-PDGF Ig (Table 3, Experiment 4). These results suggest that a molecule antigenically related to PDGF is essential for the mitogenic effect of type-1 astrocytes on O-2A progenitor cells.

Cultured Type-1 Astrocytes Contain mRNA Encoding PDGF

We prepared poly(A)-containing RNA from cultured rat astrocytes, and subjected it to Northern blot analysis using ^{32}P-labeled DNA probes specific for PDGF A or B chains. On the same blot, for comparison, we included equivalent quantities of poly(A) RNA from a variety of other rat and human cell types. The A chain probe, a human cDNA isolated by Betsholtz et al. (1986), hybridized with transcripts in all of the cell types examined, including type-1 astrocytes and meningeal cells (Figure 5A). The human glioma line 157 (lane 6) contains high levels of A chain mRNAs at approximately 1.9 kb, 2.3 kb, and 2.9 kb, as described for other human gliomas by Betsholtz et al. (1986). A7-6-3, the transformed rat CNS-derived cell line, and C6, a rat glioma line (Benda et al., 1968), both contain a single major PDGF A chain transcript of ~1.9 kb, plus other minor species (lane 4 and 5). Primary rat astrocytes (lane 3) and meningeal cells (lane 2) contain a similar ~1.9 kb transcript, present at levels comparable to that in A7-6-3. Densitometry of the autoradiographs showed that the level of the 1.9 kb transcript in primary astrocytes or meningeal cells was about 7-fold less than the equivalent transcript in the glioma line 157, and about 20-fold lower than the sum of all A chain transcripts in 157 (data not shown). This result was independent of the stringency of the washing regimen (see Experimental Procedures) and is presumably a reliable estimate of the relative abundance of transcripts in the human and rat cells. mRNA from 3 day old rat brain (lane 1) also contains the 1.9 kb transcript, and additional transcripts similar in size to the 2.3 kb and 2.9 kb human transcripts.

Both astrocytes (Figure 5B) and brain (data not shown) contained very small amounts of a ~3.5 kb transcript that hybridized with the B chain probe, a fragment of the human B chain (c-*sis*) coding sequence (Josephs et al.,

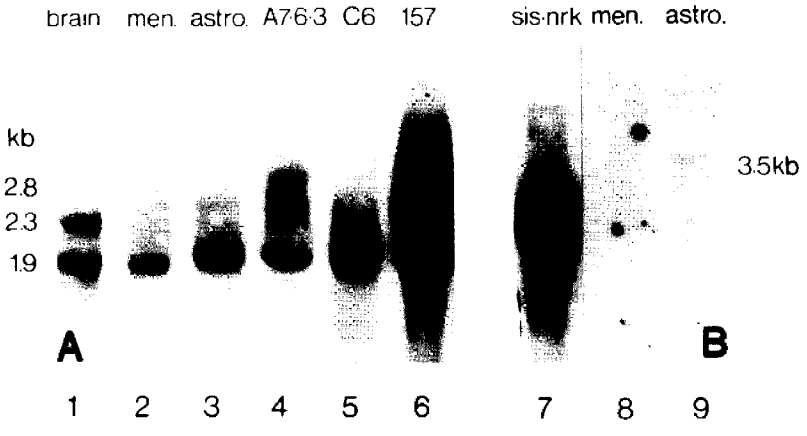

Figure 5. PDGF A and B Chain mRNAs in Various CNS-Derived Cell Types

Poly(A)-containing RNA (15 μg/lane) was electrophoresed on an 1% agarose gel containing formaldehyde, transferred to nylon membrane, hybridized with ^{32}P-labeled DNA probes specific for PDGF A or B chain, and autoradiographed. Left panel: A chain probe. RNA from the following sources: Lane 1, P3 rat brain. Lane 2, cultures of primary meningeal cells from neonatal rat. Lane 3, cultures of primary astrocytes from neonatal rat cerebral cortex. Lane 4, A7-6-3 rat CNS cells. Lane 5, C6 rat glioma cells. Lane 6, 157 human glioma cells. Right panel: PDGF B chain probe. Lane 7, SSV-transformed normal rat kidney cells. Lane 8, primary cultures of rat meningeal cells. Lane 9, primary cultures of rat astrocytes. The exposure time of lane 7 was approximately one-fifth that of lanes 8 and 9.

1984). Trace amounts of an RNA of the same size may also have been present in A7-6-3 cells, but nothing could be detected in meningeal cells (Figure 5B, lane 8) or C6 cells (data not shown). The 157 glioma cells contained small amounts of a ~4 kb B chain–specific transcript (data not shown). As a positive hybridization control for B chain, we included RNA from the SSV-transformed rat cell line, sis-NRK (obtained from P. Stroobant, Ludwig Institute for Cancer Research, London). This cell line contains two very abundant transcripts at about 2 kb and 3 kb, and other less abundant transcripts (Figure 5B, lane 7).

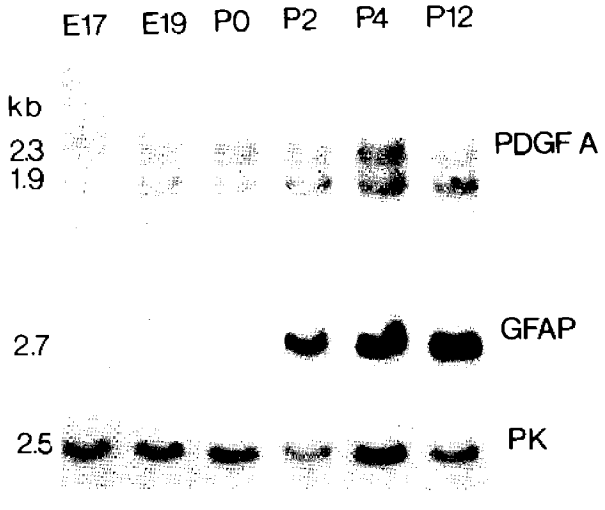

Figure 6. Time Course of Appearance of PDGF A Chain mRNAs in Rat Brain

Poly(A)-containing RNA (10 μg/lane) from the brains of rats of various ages was electrophoresed on an agarose-formaldehyde gel, transferred to nylon membrane, and hybridized to a ^{32}P-labeled DNA probe specific for the PDGF A chain mRNAs. After autoradiographic exposure, the probe was removed by boiling, and the blot rehybridized with a probe specific for glial fibrillary acidic protein (GFAP) mRNA, and then again with a probe for pyruvate kinase (PK) mRNA. PDGF A chain mRNAs and GFAP mRNA both increase several-fold between E17 and E19; PDGF A chain mRNAs then remain at a fairly constant level to P12, whereas GFAP mRNA increases further between P0 and P12, probably reflecting the growth of astrocytic processes. PK mRNA remains at roughly constant levels over the time period examined, and acts as a control for lane loadings. PDGF A chain mRNA levels remain constant up to 2 years of age (data not shown). PDGF B chain mRNAs (3.5 kb and 2.1 kb) were expressed at low, constant levels between E15 and 2 years (data not shown).

Developmental Regulation of PDGF A Chain mRNA in Brain Is Consistent with Its Synthesis by Type-1-like Astrocytes In Vivo

We prepared poly(A)-containing RNA from the brains of rats of various ages, from embryonic day 17 (E17) to 2 years. (Conception marks the start of E1, and birth is on E21.) After separation on formaldehyde-agarose gels, we blotted the mRNAs onto nylon membrane and probed for transcripts encoding PDGF A chain (Figure 6) and B chain (data not shown). We also reprobed the same blot for pyruvate kinase mRNA to control for sample loadings, and for GFAP mRNA, an astrocyte-specific marker (Figure 6). PDGF A chain transcripts were present but barely detectable at E15 (data not shown) and E17 (Figure 6), but increased several-fold in amount between E17 and E19 (Figure 6), and thereafter remained at a fairly constant level up to postnatal day 12 (P12; Figure 6) and even up to 2 years of age (data not shown). A single pyruvate kinase transcript of ~2.5 kb was present on the same blot at a roughly constant level at all ages, showing that similar amounts of RNA were loaded in each gel lane. The single ~2.7 kb GFAP transcript was first detected at E17 (at a longer exposure than is shown in Figure 6), but increased several-fold between E17 and E19, coinciding with the increase in PDGF A chain mRNA, and then increased again several-fold after birth. These observations are consistent with the idea that type-1-like astrocytes are a source of PDGF A chain mRNA in brain (see Discussion), although other cell types may also contribute. In addition, we have found that PDGF A chain mRNA is present in calf optic nerves at similar levels to whole brain (data not shown), strongly suggesting that PDGF is also produced by glial cells in the optic nerve.

In contrast to the A chain mRNAs, very low, roughly constant levels of PDGF B chain transcripts at ~3.5 kb,~2.1 kb, and below were present in rat brain from E15 to 2 years of age (data not shown).

Discussion

A Role for PDGF in CNS Development

The aim of the experiments reported in this paper was to identify the growth factor(s) secreted by type-1 astrocytes that induces O-2A progenitor cells from developing rat optic nerve to proliferate in culture. In the absence of any mitogen, the O-2A progenitors promptly stop dividing in culture and differentiate into oligodendrocytes or type-2 astrocytes. Hence, the mitogen seems to be important not only for expanding the pool of progenitor cells, but also for controlling the time and rate of production of differentiated progeny. The in vitro behavior of O-2A progenitor cells isolated from rat brain closely resembles that of their optic nerve counterparts (Behar et al., unpublished data), so it is likely that our conclusions from studies on optic nerve also apply to other myelinated tracts in the CNS.

Our data shows that cultured type-1 astrocytes make and secrete dimeric PDGF, which is essential for their mitogenic effect on O-2A progenitor cells in vitro. Several previous findings have suggested that PDGF is a growth factor for glial cells: it is mitogenic for cell lines of presumed glial origin (Heldin et al., 1981c), some human gliomas secrete PDGF-like molecules and synthesize PDGF mRNAs (Eva et al., 1982; Betsholtz et al., 1986), and intracranial injection of simian sarcoma virus, which encodes an altered form of the PDGF B-chain gene (Waterfield et al., 1983; Doolittle et al., 1983), causes a high frequency of glioblastomas (Deinhardt, 1980). However, the results presented here (see also Noble et al., submitted) provide the first convincing evidence that PDGF plays an important role in normal gliogenesis, and may help explain the involvement of PDGF in glial tumor growth.

The evidence that PDGF plays an active role in development of the O-2A cell lineage in vivo is indirect, but persuasive. First, PDGF is a potent mitogen for O-2A progenitors in vitro (Figure 3 and Table 2; Noble et al., submitted). Most batches of human PDGF that we tested had a half-maximal effect in our assays at ~0.5 ng/ml, presumably reflecting the presence of high affinity PDGF receptors on the surface of progenitor cells, and it seems reasonable to expect that they also express receptors in vivo. Are O-2A progenitors exposed to PDGF in the developing optic nerve? We have shown that type-1-like astrocytes from neonatal rat cerebral cortex secrete PDGF in vitro. Apart from the O-2A lineage, type-1 astrocytes form the majority of cells in the optic nerve during the first two postnatal weeks, and would be expected to have a major influence on the local environment throughout this period, when O-2A progenitor cells are dividing rapidly (Skoff et al., 1976a, 1976b; Miller et al., 1985). Although we cannot be certain that type-1 astrocytes secrete PDGF in vivo, secretion of PDGF does not appear to be a general consequence of placing cells in primary culture, since meningeal cells secrete no detectable PDGF (Table 2) or mitogenic activity for O-2A progenitors (Figure 3 and Table 2).

The time course of appearance of PDGF mRNA in the brain (Figure 6) is also consistent with the notion that type-1 astrocytes are a source of PDGF A chain mRNA in the CNS. The A chain mRNAs are barely detectable at E17, just after the time that small numbers of type-1 astrocytes first appear in the brain (Abney et al., 1981) and optic nerve (Skoff et al., 1976a; 1976b; Miller et al., 1985), and increase several-fold between E17 and E19 when GFAP mRNA first becomes obvious. Thereafter, the A chain mRNAs remain at relatively constant levels into adulthood. The dramatic rise in GFAP mRNA after birth probably reflects the combined effects of astrocyte proliferation and elaboration of astrocytic processes. PDGF B chain mRNAs, in contrast to the A chain mRNAs, are present at very low, constant levels at all ages from E15 to adulthood (data not shown), suggesting that astrocytes may not be the major source of B chain mRNA in brain.

Taken together, our observations argue strongly that PDGF is secreted by type-1 astrocytes in vivo, and is responsible for the proliferation of O-2A progenitor cells in the developing optic nerve. Formal proof of this would require the localization of PDGF mRNA or protein in the nerve in situ and, ultimately, a means of specifically eliminating secretion of PDGF from type-1 astrocytes in a living embryo.

What Are the Contributions of the A and B Chains?

The ~30 kd PDGF dimers immunoprecipitated from astrocyte-conditioned medium (Figure 4) dissociate on reduction into monomers of ~17 kd and ~14 kd. These could represent the A and B chains, respectively; alternatively, the smaller polypeptide could be a partial degradation product of the ~17 kd presumptive A chain. In other experiments (data not shown), we have found that the relative amount of the ~14 kd component is reduced: this could mean either that the relative proportions of A and B chains are not fixed, possibly because astrocyte PDGF is a mixture of dimeric forms, or it could reflect a variable degree of proteolysis during isolation. This latter interpretation is perhaps more consistent with the very low B chain mRNA levels in astrocytes (Figure 5B). Further experiments using antibodies specific for the A or B chains should resolve this uncertainty. Both pure human PDGF (AB; Hammacher et al., submitted) and porcine PDGF (BB; Stroobant and Waterfield, 1984) are potent mitogens for O-2A progenitor cells (Figure 3 and Noble et al., submitted), as they are for fibroblasts (Stroobant and Waterfield, 1984). It has recently been discovered that AA dimers secreted by a human clonal glioma line (U-343 MGa CL2:6; Nistér et al., 1988) have little mitogenic effect on human foreskin fibroblasts; most of the mitogenic activity is carried by a small and previously undetected component of AB and BB dimers secreted by the same cells (Hammacher et al., submitted). It is not yet known whether O-2A progenitor cells are also unresponsive to AA dimers. We need to answer this question, and establish the structure of the PDGF dimers from astrocytes, in

order to evaluate the relative importance of the A and B chains for O-2A progenitor proliferation.

It has been reported that the PDGF A chain itself is heterogeneous, some of the A chain in glioma cells having a carboxy-terminal extension not found on A chain from normal endothelial cells (Tong et al., 1987; Collins et al., 1987), but it is not known if the longer form is specific for glial cells, or related to tumor growth. It will therefore be interesting to determine the detailed structure of the A chain mRNA and its encoded protein from primary astrocytes.

Are Other Growth Factors Required for O-2A Proliferation?

It is well known that growth factors can act together to exert synergistic effects on cells (Rozengurt, 1986). Several growth factors other than PDGF are reported to stimulate proliferation and differentiation of oligodendrocytes or their precursors in vitro, including insulin and IGF I (McMorris et al., 1986; Dubois-Dalcq, 1987), FGF (Eccleston and Silberberg, 1985; Saneto and de Vellis, 1985), and IL-2 (Saneto et al., 1986; Benveniste and Merrill, 1986; Saneto et al., 1987). We have shown (Ballotti et al., 1987) that cultured rat astrocytes synthesize IGF I mRNA, but there is as yet no evidence for its secretion. We might not have detected an effect of IGF I in the experiments reported here, since the culture medium (see Experimental Procedures) contains insulin (50 ng/ml), which will interact with IGF receptors (Gammeltoft et al., 1987). It is not yet known if astrocytes secrete FGF or IL-2, or other growth factors that may act on O-2A progenitors.

Is there sufficient PDGF in astrocyte-conditioned medium to account for all of its mitogenic activity? From the information in Figure 3 and Table 2, we estimate that the mitogenic activity in astrocyte-conditioned medium is equivalent to approximately 4 ng/ml of pure human PDGF. This is within the limits suggested by receptor competition assay (Table 2), so it seems possible that most of the mitogenic activity can be accounted for by PDGF alone. On the other hand, it is curious that the slopes of the dose-response curves for astrocyte-conditioned medium and pure PDGF are different: the activity of pure PDGF rises from near-background to near-maximal levels over an ~30-fold range of concentrations, while the equivalent range for astrocyte-conditioned medium is 4- to 8-fold (Figure 3). This may indicate that PDGF from rat astrocytes is subtly different from pure human or porcine PDGFs, or that other factors secreted from astrocytes modify its activity.

Apart from its mitogenic properties, PDGF is known to be a chemoattractant for several cell types, including glial cells (Bressler et al., 1985; Harvey et al., 1987). Since O-2A progenitor cells are highly motile when cultured in the presence of astrocyte-conditioned medium (Small et al., 1987) or PDGF (Noble et al., submitted), and are thought to migrate into the developing optic nerve from elsewhere in the CNS (Small et al., 1987), it is possible that PDGF plays a dual role by stimulating proliferation *and* migration of O-2A progenitors in the developing nerve. This could have implications not only for normal development, but also for the repair of demyelinating damage in the CNS.

Experimental Procedures

Primary Astrocyte and Meningeal Cell Cultures

Cultures of type-1-like astrocytes from neonatal rat cerebral cortex were established by a modification of the method of McCarthy and de Vellis (1980). Cerebral cortices from 2 day old rats, with the meningeal membranes removed, were coarsely minced, digested with trypsin (0.025% w/v) for 30 min at 37°C, dissociated by trituration through a Pasteur pipet, and cultured in DMEM plus 10% FCS and antibiotics (material from three brains to one 75 cm^2 flask), until the cells were confluent (about 1 week). The cultures were then vigorously agitated overnight at 37°C (180 revolutions/minute on a horizontal rotating platform, radius of rotation 2 cm) to shake off cells growing on top of the monolayer of flat cells. After shaking, the remaining cells were grown for 24 hr at 37°C and then treated for 24 hr with cytosine arabinoside (araC, 10^{-5} M) to preferentially kill rapidly dividing cells such as fibroblasts. The cultures were passaged once (1 to 4) and grown to near confluence (about 1 week). At this stage the cultures consisted of at least 98% GFAP-positive, fibronectin-negative flat cells with the morphology of type-1 astrocytes, and were used for collection of conditioned medium or for RNA extraction (see below).

Meningeal membranes, removed in the course of preparing astrocyte cultures, were trypsinized (see above) and put into culture in DMEM plus 10% FCS and antibiotics (meninges from three brains into one 75 cm^2 flask). The cultures were confluent within a week, when they were passaged 1 to 4. After a further week, the cells were again confluent and were used for collecting conditioned medium, or for RNA extraction. These meningeal cell cultures contained less than 1% contaminating GFAP-positive cells.

Cell Lines and Conditioned Media

The 157 human glioma cell line was isolated by Dr. M. Noble from autopsy material. The A7-6-3 rat cell line was isolated by infecting optic nerve cells, growing on a monolayer of type-1 astrocytes, with a retrovirus carrying the large T gene of simian virus 40. From the resultant mixture of transformed cells, a series of clonal lines was derived, including A7-6-3 (H. Geller and M. Dubois-Dalcq, unpublished data). Both 157 and A7-6-3 are GFAP-negative. Cell line C6 was derived from a chemically induced rat brain tumor (Benda et al., 1968). About 5% of cells in C6 cultures are GFAP-positive. All cell lines were maintained in DMEM plus 5% FCS and antibiotics. For collection of conditioned medium, semi-confluent monolayers of cell lines or primary cell cultures were washed three times with serum-free DMEM, and incubated at 37°C for 48 hr in serum-free DMEM (8 ml/75 cm^2 flask). Sometimes two batches of conditioned medium were collected from primary cells, by returning the cells to 10% FCS-containing medium for 2 days between successive collections.

PDGF Receptor Competition Assays

PDGF receptor competition assays were performed essentially as described previously by Nistér et al. (1984). Human foreskin fibroblasts (AG 1523, obtained from the Human Mutant Cell Repository, Institute for Medical Research, Camden, NJ), grown in 12-well Linbro plates, were incubated with 0.5 ml conditioned medium (buffered with 20 mM HEPES [pH 7.4]) or pure human PDGF in binding buffer, for 1 hr at 4°C. Binding buffer is PBS containing 1 mg/ml human serum albumin, 0.01 mg/ml $CaCl_2.2H_2O$, and 0.01 mg/ml $MgSO_4.7H_2O$. Cells were washed at 4°C with binding buffer containing 1% newborn calf serum instead of albumin, then incubated with 50,000 cpm/well ^{125}I-labeled human PDGF (specific activity 70,000 cpm/ng, labeled by Bolton-Hunter procedure) for 2 hr at 4°C. Cells were then washed five times in binding buffer, and cell-associated radioactivity determined by extracting the cells for 20 min at 20°C in 0.5 ml of 1% Triton X-100, 20 mM HEPES (pH 7.4), 10% v/v glycerol, 0.1 mg/ml human serum albumin, and counting in a gamma counter.

Superose 12 Chromatography

For mitogenic assay, 50 ml of astrocyte-conditioned medium (see above) was concentrated 100-fold by precipitation with 80% w/v ammonium sulfate, and dialyzed against 0.2 M ammonium acetate (pH 7.0).

Control experiments with ^{125}I-PDGF and ^{125}I-insulin showed that ~30% of these polypeptides were recovered in the precipitate. The concentrate was loaded on a Superose 12 size-exclusion FPLC column (Pharmacia) equilibrated with 0.2 M ammonium acetate, and run at 0.5 ml/min. Fractions (0.5 ml) were collected, and twice evaporated to dryness in a rotary drier. The pellets were dissolved in DMEM containing 1 mg/ml bovine serum albumin (BSA), and each fraction was tested for mitogenic activity on O-2A progenitor cells in cultures of P7 rat optic nerve (see below).

^{35}S-Labeling and Immunoprecipitation of Proteins

For ^{35}S-labeling of secreted proteins, confluent primary astrocyte monolayers were incubated for 24 hr in serum-free, cysteine-free DMEM containing 50 μCi/ml ^{35}S-cyteine (Amersham, 1000 Ci/mmol). Medium from one 75 cm^2 flask (~2 × 10^6 cells) was concentrated by precipitation with 80% ammonium sulfate, and subjected to Superose 12 chromatography as described above. Fractions 29–34 (see Figure 2) were combined, concentrated in a rotary evaporator, and redissolved in 10 mM acetic acid, 1 mg/ml BSA. After diluting in PBS (pH 7), the sample was preincubated overnight with normal rabbit serum followed by formalin-fixed Staphylococcus A (Cowan I strain) Bethesda Research Laboratories), then incubated with polyclonal rabbit anti-human-PDGF serum (Heldin et al., 1981b) and Staphylococcus A. An equivalent amount of unfractionated ^{35}S-labeled astrocyte supernatant was immunoprecipitated in parallel. One-quarter of each immunoprecipitate was electrophoresed in a lane of a 13% (bisacrylamide: acrylamide, 1:30 w/w) or a 17% (bisacrylamide:acrylamide, 1:300) polyacrylamide-SDS gel, either with or without reduction by boiling for 3 min in 0.45 M β-mercaptoethanol. The gels were processed for fluorography (Bonner and Laskey, 1974), dried and exposed to presensitized Kodak XAR5 film at −70°C for 2 weeks.

Optic Nerve Cultures

Cultures of optic nerve cells were prepared essentially as described previously (Miller et al., 1985). All culture reagents were bought from Sigma. Optic nerves were dissected from Sprague-Dawley rat pups and treated with trypsin (type III, 0.05% w/v) and collagenase (type IA, 0.04% w/v) in the presence of BSA (fatty acid-free, 0.25% w/v) for 1 hr at 37°C. EDTA was added to a final concentration of 0.01% w/v and incubation continued for a further 30 min. The nerves were washed in DMEM plus 10% FCS, suspended in DMEM containing soybean trypsin inhibitor (0.05% w/v), DNAase I (0.04 mg/ml) and 5% FCS, and dissociated by gently drawing through a 23-gauge needle (five times up-and-down). Cells were plated in 20 μl droplets on poly(D) lysine coated glass coverslips (~5000 cells/coverslip) in a 24-well microtiter plate, allowed to attach for 30 min at 37°C, and then flooded with 400 μl of a modified Bottenstein and Sato (1980) medium, with or without conditioned medium, PDGF, or Superose column fractions. Our modified Bottenstein and Sato medium consists of DMEM supplemented as follows: D-glucose (5.7 mg/ml), bovine insulin (50 ng/ml), BSA (0.1 mg/ml), human transferrin (0.1 mg/ml), progesterone (62 ng/ml), putrescine chloride (1.6 μg/ml), sodium selenite (40 ng/ml), L-thyroxine (40 ng/ml), 3,3',5,triiodo-L-thyronine (30 ng/ml). Penicillin (100 U/ml) and streptomycin (100 μg/ml) were also added. Purified PDGF from human or porcine platelets was obtained from R and D Systems Inc., (Minneapolis, MN), and from R. Ross (University of Washington, Seattle, WA). Cells were routinely cultured for 3 days without replenishing the medium.

Antibody Neutralization Assays

Anti-human-PDGF immunoglobulins, prepared by differential ammonium sulfate precipitation of polyclonal rabbit sera (Heldin et al., 1981a), were obtained from R and D Systems Inc. (Minneapolis, MN), from Collaborative Research (Bedford, MA), and from C.-H. Heldin (Uppsala, Sweden). To neutralize the mitogenic activity of PDGF, these antibodies were added to optic nerve cultures at a final concentration of 25 or 40 μg/ml, at the time of addition of PDGF or conditioned media. Neither PDGF nor anti-PDGF Ig were replenished during the 3 day culture period.

Immunofluorescence and Autoradiography

For counting O-2A progenitor cell numbers, optic nerve cultures were prefixed in 2% w/v paraformaldehyde in HEPES-buffered DMEM for 20 min at room temperature. The cells were then incubated in a mixture of mouse monoclonal antibodies A2B5 (IgM; Eisenbarth et al., 1979) and anti-galactocerebroside (IgG3; Raff et al., 1978), followed by a mixture of rhodamine-labeled rabbit anti-mouse-IgM and fluorescein-labeled rabbit anti-mouse-IgG3 class-specific antibodies (Nordic). The cells were post-fixed in 4% w/v paraformaldehyde in PBS, and mounted in 90% glycerol, 10% PBS containing 2.5% w/v DABCO antifade reagent (1,4-Diazabicyclo[2,2,2]octane; BDH) for fluorescence microscopy.

For autoradiography, optic nerve cultures on coverslips were incubated for 20 hr after plating, then ^{3}H-thymidine (Amersham, 2 Ci/mmol) was added to the medium at a final concentration of 2 μCi/ml. After a further 20 hr incubation, the cells were fixed and stained as above, and the coverslips permanently mounted cell-side-up on glass microscope slides. The slides were dipped with photographic emulsion (Ilford K5 nuclear emulsion, 50% w/v in water), dried, and exposed for 3 days. The autoradiographs were developed in an Ilford Contrast FF developer, mounted as for fluorescence microscopy, and examined by two-channel fluorescence and bright field microcopy (Figure 1).

RNA Extraction and Analysis

Total cellular RNA was prepared from cultured cells by the lithium chloride-urea method of Auffray and Rougeon (1980), and enriched for poly(A)-containing RNA by oligo(dT)-cellulose chromatography. Poly(A)-RNA (10 or 15 μg per lane, estimated by absorbance at 260 nm) was denatured and electrophoresed on a 1% agarose gel containing formaldehyde (Maniatis et al., 1982). After transfer to Gene Screen Plus nylon membrane (New England Nuclear), the RNA was hybridized with DNA probes labeled with ^{32}P to a specific activity of ~2 × 10^8 cpm/μg by random priming (Feinberg and Vogelstein, 1984). Blots were washed under conditions of high stringency (0.1× SSC at 53°C) according to the manufacturer's instructions. The PDGF A chain probe was an ~800 bp RsaI fragment containing the coding sequence of the human cDNA isolated by Betsholtz et al. (1986). The PDGF B chain probe was an ~840 bp PstI–AvrII fragment of plasmid pSM1 (Josephs et al., 1984), containing the human c-*sis* coding sequence. The GFAP probe was an ~500 bp SacI coding sequence fragment of plasmid G1 (Lewis et al., 1984), which contains a mouse GFAP cDNA. The pyruvate kinase (PK) probe was an ~1600 bp PvuII–ApaI fragment of plasmid pPK300 (Lonberg and Gilbert, 1983), which encodes chicken muscle PK.

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