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 system.

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 Hatt, 1987).

Correct timing of oligodendrocyte development can be restored in culture by growing embryonic optic nerve cells in a 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 (Iedlin et al., 1981c), and PDGF is frequently expressed at high levels in human gliomas (Eva et al., 1982; Betcholtz 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 [3H]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). [3H]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 [3H]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 [3H]thymidine when cultured with Superose fraction 31 or PDGF (Table 1). Mature GC+ oligodendrocytes never incorporated [3H]thymidine in our experiments.

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 [3H]thymidine autoradiography (Figure 1). Table 1 shows the proportions of O-2A progenitors that incorporated [3H]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 [3H]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, [3H]labeled human PDGF was applied to the Superose 12 column under the same conditions. The elution profile, shown in Figure 2 (lower panel),

A2B5
GC
3H-thy
PDGF, in the CNS

A2BS gc progenitors

PDGF, in the CNS

A2BS gc progenitors

Figure 7 Superose 17 S17e-Exclusion Chromatography of Astmryte-

We concentrated astrocyte-conditioned medium by ammonium sulfate

preparation (see Experimental Procedures) and separated it on a Su-

perase 12 (Pharmacia) column equilibrated with 0.2 M ammonium acc-

tate pH 7.0). A portion of each column fraction was tested for its abil-

ity 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 we 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) 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 Superase 12 is a consequence of interac-

tion between the hydrophobic PDGF molecule and the column matrix.

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

<table>
<thead>
<tr>
<th>Addition to Culture Medium</th>
<th>[3H]-Labeled O-2A Progenitor Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>astrocyte CM (1:5)</td>
<td>28% (22/78)</td>
</tr>
<tr>
<td>astrocyte CM (1:10)</td>
<td>54% (25/46)</td>
</tr>
<tr>
<td>Superose fraction 6 (1:20)</td>
<td>54% (25/46)</td>
</tr>
<tr>
<td>Superose fraction 31 (1:20)</td>
<td>47% (66/144)</td>
</tr>
<tr>
<td>human PDGF (5 ng/ml)</td>
<td>45% (77/170)</td>
</tr>
<tr>
<td>no addition</td>
<td>0% (0/16)</td>
</tr>
</tbody>
</table>

1 Modified medium of Bottenstein and Sato (1979). See Experimental

Procedures

2 See Figure 2

[3H]Thymidine autoradiography was performed (see Experimental

Procedures) on P7 optic nerve cultures grown in defined medium sup-
mplemented as shown in the table. Before processing for autoradiogra-
phy, the cultures were doubly surface-stained with monoclonal antibo-
dies A2BS and GC, and, after exposure, the numbers of A2BS . GC . O-2A progenitor cells, with and without silver grains, were count-
ed under a fluorescein microscope (see Figure 1). Shown are the percentages of progenitor cells that incorporated [3H]thymidine, with the actual numbers observed (means of duplicate coverslips) in parentheses.

Table 2. Mitogenic Effect, and PDGF Receptor Competing

Abilities of Conditioned Media

<table>
<thead>
<tr>
<th>Source of Medium</th>
<th>Effect on [3H]PDGF Binding (Displacement)</th>
<th>Fold-Dilution at Half Maximal Mitogenic Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>157 human glioma cells</td>
<td>73 (52.79.51)</td>
<td>128</td>
</tr>
<tr>
<td>A7-6-3 rat CNS cells</td>
<td>34 (45.21.35)</td>
<td>16</td>
</tr>
<tr>
<td>primary astrocytes</td>
<td>20 (27.0.32)</td>
<td>8</td>
</tr>
<tr>
<td>primary meninges</td>
<td>5 (1.0.13)</td>
<td>no activity</td>
</tr>
<tr>
<td>DMEM</td>
<td>0</td>
<td>no activity</td>
</tr>
<tr>
<td>human PDGF (10 ng/ml)</td>
<td>57 (48.79.43)</td>
<td>29 (0.5 ng/ml)</td>
</tr>
</tbody>
</table>

Conditioned media were collected as described in Experimental

Procedures. Three independent batches of media were tested, undiluted, for their ability to compete with human [3H]PDGF for receptors on human

foreskin fibroblasts (Nister 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 re-

quired 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 ac-

tivity in astrocyte-conditioned medium is equivalent to 14 ng/ml human

PDGF.

for receptors on the surface of human foreskin fibroblasts

(Nister 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-

Astrocyte-Conditioned Medium Competes with PDGF

for Receptors on Human Foreskin Fibroblasts

Serum-free conditioned media were collected from cul-

tures of primary rat cortical astrocytes, primary rat menin-

geal cells, and some rat and human CNS cell lines. The conditioned media were tested for PDGF-like molecules by their ability to compete with [3H]labeled dimeric human PDGF.
We labeled primary astrocytes for 24 hr with [35S]-cysteine. The detection limit of this assay is of the order of ~65 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 meningial 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 [35S]-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 unfractonated medium, were incubated separately with anti-PDGF serum (Heldin et al., 1981b) or control serum, followed by formalin-fixed Staphylococcus A. The remaining unfractonated ACM was treated in the same way. The precipitates were run on a 13% polyacrylamide-SDS gel (left panel) or a 17% gel (right panel), either with or without prior reduction with β-mercaptoethanol (see below). [35S]-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: unfractonated ACM, anti-PDGF serum. Lane 5: unfractonated ACM, control serum. A protein band at ~25 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.

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 unfractonated (lane 4) astrocyte-conditioned medium by anti-PDGF serum, but not by control serum (lanes 3 and 5). Pure [35S]-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-
The Majority of Mitogenic Activity in Astrocyte-Conditioned Medium Is Neutralized by Anti-PDGF Immunoglobulins

| Addition to Culture Medium
d | Number of Progenitors | Neutr/ization |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(Experiment 1) astrocyte CM (1:5)</td>
<td>147</td>
<td>ND</td>
</tr>
<tr>
<td>no addition</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>(Experiment 2) astrocyte CM (1:2)</td>
<td>592</td>
<td>372</td>
</tr>
<tr>
<td>no addition</td>
<td>19</td>
<td>ND</td>
</tr>
<tr>
<td>(Experiment 3) astrocyte CM (1:5)</td>
<td>72</td>
<td>ND</td>
</tr>
<tr>
<td>no addition</td>
<td>1.5</td>
<td>ND</td>
</tr>
<tr>
<td>(Experiment 4) Superose 12 fractions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fraction 28 (1:20)</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>fractions 31 + 32 (1:20)</td>
<td>50</td>
<td>ND</td>
</tr>
<tr>
<td>no addition</td>
<td>6</td>
<td>ND</td>
</tr>
</tbody>
</table>


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 (Experiments 1–4) wells. The ratio of progenitor cell numbers was calculated from the number of progenitor cells in the presence of anti-PDGF Ig (or control Ig) and the lower of the two relevant figures (no Ig or control Ig) without correcting for the background in defined medium.

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

Developmental Regulation of PDGF A Chain mRNA in Brain Is Consistent with Its Synthesis by Type-I-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 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 in amount 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-I-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, ~21 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 cells 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., 1083; Doolittle et al., 1985), 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 renaturation into monomers of ~17 kD and ~11 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 CI 2-6; Nister 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 olodendrocytes or their precursors in vitro, including insulin and IGF I (McMorris et al., 1986; Dubois-Dalcq, 1987). Par黔-TGF (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 a 100-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-II-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 enzymically 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 (maternal from three brains into 175 cm² 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 cytochrome c and ampicillin (araC, 10⁻⁶ 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 HNA 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 (maternal from three brains into 175 cm² 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, 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., 1986). 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 (6 ml/75 cm² 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 Nistler et al. (1981). 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 (cultured 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 CaCl₂, 0.001 M MgSO₄, and 0.001 mg/ml MgSO₄·7H₂O. Cells were washed 4°C with binding buffer containing 1% newborn calf serum instead of albumin, then incubated with 50,000 cpm/well [³H]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 100°C in 0.5 ml of 1% Triton X-100, 20 mM HEPES (pH 7.4), 10% w/v glycerol, 0.1 mg/ml human serum albumin, and counting in a gamma counter.

Superose 12 Chromatography

For mitogen 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 3H-PDGF and 3H-insulin showed that <30% of these polypeptides were recovered in the precipitate. The concentrate was loaded on a Superose 12 size-exclusion FPLC column (Phar- macia) 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).

32P-Labeling and Immunoprecipitation of Proteins

For 32P-labeling of secreted proteins, confluent primary astrocyte monolayers were incubated for 24 hr in serum-free, cysteine-free DMEM containing 50 μM 32P-cysteine (Amersham, 1000 Ci/mmol). Medium from one 75 cm² flask (∼2 × 10⁶ 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 samples were incubated overnight with normal rabbit serum followed by formalin-fixed Staphylococcus A (Cowan I strain) Bethesda Research Laboratories, then incubated with polyclonal rabbit antihuman-PDGF serum (Heldin et al., 1986b) and Staphylococcus A. An equivalent amount of untracinated 32P-labeled astrocyte supernatant was immunoprecipitated in parallel. One-quarter of each immunoprecipitated medium, precipitated by a lane of a 13% (bisacrylamide, 3:1 w/w) polyacrylamide-SDS gel, either with or without reduction by boiling for 3 min in 0.4 M dithiothreitol, was processed for fluorography. The gels were processed for fluorography by immersion in Enhance (New England Nuclear), then incubated with polyclonal rabbit anti-mouse IgG class-specific antibodies (Nordic) diluted with DNA probes labeled with 32P to a specific activity of ∼2 × 10⁸ cpm/μg by random priming (Feinberg and Vogelstein, 1983). Blots were washed under conditions of high stringency (0.1 x SSC at 53°C) according to the manufacturer’s instructions. The PDGF A chain probe was an ∼600 bp HpaI fragment containing the coding sequence of the human cDNA isolated by Belshoitz et al. (1986). The PDGF B chain probe was an ∼840 bp PstI-Avrl fragment of plasmid pSM7 (Josephs et al., 1984), containing the human c-sis coding sequence. The GFAP probe was an ∼500 bp SacI coding sequence fragment of plasmid pG1 (Lewis et al., 1984), which contains a mouse GFAP cDNA. The pyruvate kinase (PK) probe was an ∼1600 bp PvuII-Apal fragment of plasmid pk5000 (Lonberg and Gilbert, 1983), which encodes chicken muscle PK.

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References


RNA Extraction and Analysis

Total cell RNA was prepared from cultured cells by the inurn 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 Genescreen Plus nylon membrane (New England Nuclear), the RNA was hybridized with DNA probes labeled with 32P to a specific activity of ∼2 × 10⁸ cpm/μg by random priming (Feinberg and Vogelstein, 1983). Blots were washed under conditions of high stringency (0.1 x SSC at 53°C) according to the manufacturer’s instructions. The PDGF A chain probe was an ∼600 bp HpaI fragment containing the coding sequence of the human cDNA isolated by Belshoitz et al. (1986). The PDGF B chain probe was an ∼840 bp PstI-Avrl fragment of plasmid pSM7 (Josephs et al., 1984), containing the human c-sis coding sequence. The GFAP probe was an ∼500 bp SacI coding sequence fragment of plasmid pG1 (Lewis et al., 1984), which contains a mouse GFAP cDNA. The pyruvate kinase (PK) probe was an ∼1600 bp PvuII-Apal fragment of plasmid pk5000 (Lonberg and Gilbert, 1983), which encodes chicken muscle PK.


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