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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

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 activ-

PDGF on a size-exclusion column, competes with PDGF for receptors, and is neutralized by antibodies to PDGF. PDGF dimers can be immunoprecipitated

genesis. We propose that astrocyte-derived PDGF is

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

genitor cells are therefore known as O-2A progenitors (Raff et al., 1983). Apart from the O-2A lineage, the most

cell lineages in the optic nerve, see Raff and Miller, 1984).

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

(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

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

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 differ-

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

ation, it is essential to identify the molecules that mediate

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 fre-

show that pure human and porcine PDGFs are strongly mitogenic for O-2A progenitors in vitro (see also Noble et

conditioned medium is neutralized by anti-PDGF immunoglobulin. In addition, we find that mRNAs encoding the

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

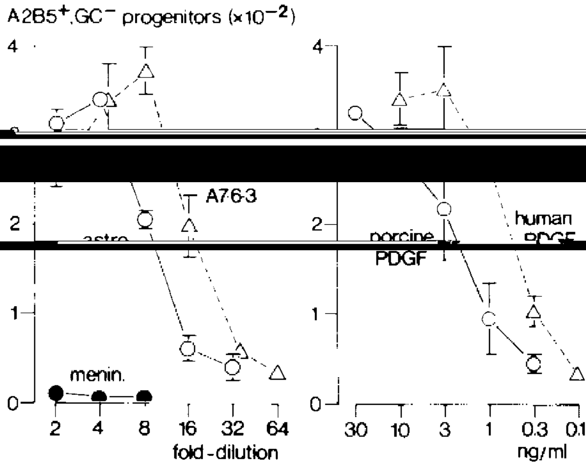


Figure 3. Dose-Response Profiles for Mitogenic Stimulation of O-2A Progenitor Cells by Conditioned Media, and by Purified PDGF

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

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

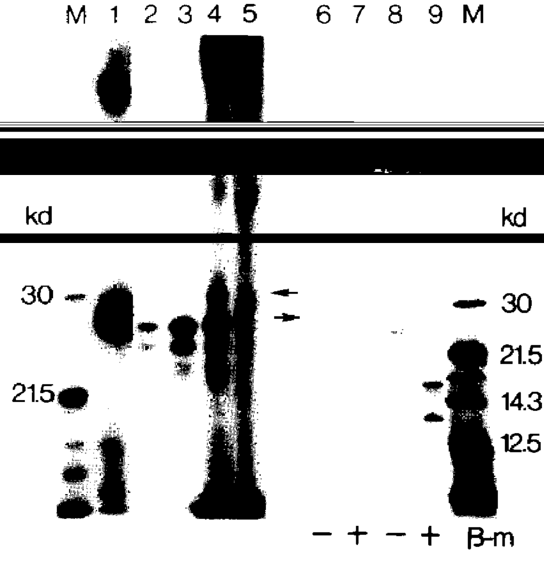
dium tested. The detection limit of this assay is of the order of 1 ng/ml for pure human PDGF, but we do not know how the sensitivity differs between rat and human, or how it

same conditioned media to promote proliferation of O-2A

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.

into the Culture Medium

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



Astrocyte-Conditioned Medium (ACM), with Anti-PDGF Serum

Cultures of primary astrocytes from neonatal rat cerebral cortex were

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

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

PDGF serum, unreduced. Lane 7: same, reduced. Lane 8: human

tated 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 unfractionated (lane 4) astrocyte-conditioned

blet at ∼25 kd (lane 8), depending on the composition of

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%
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 ... the lower of the other two relevant figures (no Ig or control Ig) without correcting for the background in defined medium.

thesize and secrete PDGF dimers into the culture medium.

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 ...

conditioned medium? To answer this question, we attempted ...

lent 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 fac- ...

(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 iso- ... in all of the cell types examined, including type-1 astrocytes and meningeal cells (Figure 5A). The human glioma ...

for other human gliomas by Betsholtz et al. (1986). A7-6-3, ... glioma line (Benda et al., 1968), both contain a single major PDGF A chain transcript of ~1.9 kb, plus other minor ...

sitometry 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 ...

sum of all A chain transcripts in 157 (data not shown). This ...

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.,

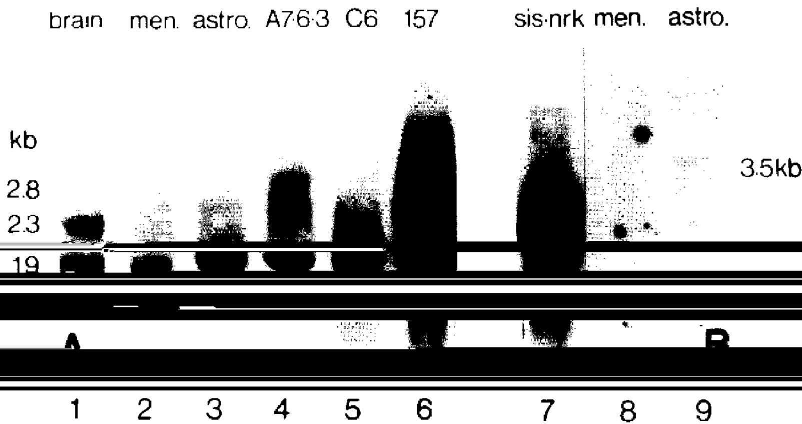


Figure 5. PDGF A and B Chain mRNAs in Various CNS-Derived Cell Types

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.

from neonatal rat. Lane 3, cultures of primary

Lane 4, A7-6-3 rat CNS cells. Lane 5, C6 rat glioma cells. Lane 6, 157 human glioma cells.

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.

have been present in A7-6-3 cells, but nothing could be detected in meningeal cells (Figure 5B, lane 8) or C6 cells

included RNA from the SSV transformed rat cell line, sis-NRK (obtained from P. Stroobant, Ludwig Institute for Can-

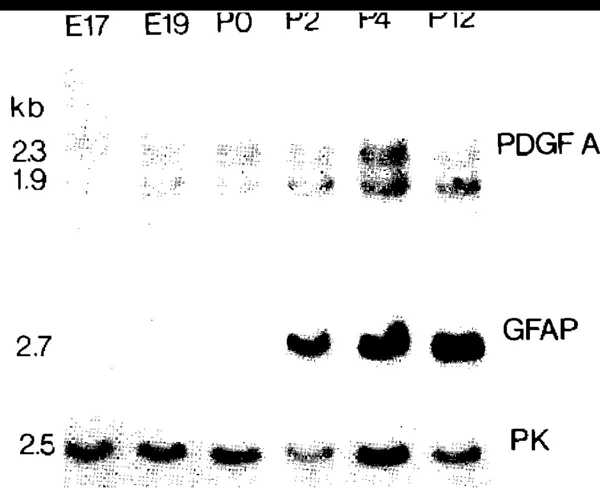


Figure 6. Time Course of Appearance of PDGF A Chain mRNAs in Rat Brain

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

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

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).

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

crease 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

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

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.

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). How-

submitted) provide the first convincing evidence that

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-

the surface of progenitor cells, and it seems reasonable to expect that they also express receptors in vivo. Are

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

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

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

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; alterna-

tive 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 ex-

(AB; Hammacher et al., submitted) and porcine PDGF (BB; Stroobant and Waterfield, 1984) are potent mitogens

field, 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

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

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