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PDGF A chain homodimers drive proliferation of bipotential (O-2A) glial progenitor cells in the developing rat optic nerve

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The bipotential glial progenitor cells (O-2A progenitors), which during development of the rat optic nerve give rise to oligodendrocytes and type 2 astrocytes, are stimulated to divide in culture by platelet-derived growth factor (PDGF), and there is evidence that PDGF is important for development of the O-2A cell lineage *in vivo*. We have visualized PDGF mRNA in the rat optic nerve by *in situ* hybridization, and its spatial distribution is compatible with the idea that type 1 astrocytes are the major source of PDGF in the nerve. We can detect mRNA encoding the A chain, but not the B chain of PDGF in the brain and optic nerve, suggesting that the major form of PDGF in the central nervous system is a homodimer of A chains (PDGF-AA). PDGF-AA is a more potent mitogen for O-2A progenitor cells than is PDGF-BB, while the reverse is true for human or rat fibroblasts. Fibroblasts display two types of PDGF receptors, type A receptors which bind to all three dimeric isoforms of PDGF, and type B receptors which bind PDGF-BB and PDGF-AB, but have low affinity for PDGF-AA. Our results suggest that O-2A progenitor cells possess predominantly type A receptors, and proliferate during development in response to PDGF-AA secreted by type 1 astrocytes.

Key words: CNS/glial cells/PDGF/rat development/receptors

Introduction

The optic nerve carries axons from ganglion neurons in the retina to the brain. It contains three types of post-mitotic glial cells—oligodendrocytes and two types of astrocytes—but no neural cell bodies, making the nerve one of the simplest parts of the central nervous system (CNS) (for reviews see Miller *et al.*, 1989a; Raff, 1989).

In the developing rat optic nerve there are bipotential glial progenitor cells (O-2A progenitors), which give rise to both oligodendrocytes, which myelinate CNS axons, and type 2 astrocytes, which contact the axons between adjacent myelinated regions at nodes of Ranvier (ffrench-Constant and Raff, 1986; Miller *et al.*, 1989b). The O-2A progenitors are thought to migrate into the optic nerve from a germinal zone in the brain (Small *et al.*, 1987). Small numbers of O-2A progenitors are found in the nerve as early as embryonic day 15 (E15), and they continue to proliferate for several weeks after this (Skoff *et al.*, 1976a,b), some differentiating into oligodendrocytes starting on the day of birth (Miller *et al.*, 1985), and others into type 2 astroctyes starting in the second postnatal week (P7 – P10) (Miller *et al.*, 1985). Apart from the O-2A cell lineage, the most abundant glial cells in the optic nerve are type 1 astrocytes, which are derived from a separate precursor cell (Raff *et al.*, 1984). Type 1 astrocytes first appear in the optic nerve at E16 (Miller *et al.*, 1985), probably by differentiating from the neuroepithelial cells which comprise the optic stalk (Small *et al.*, 1987).

When dissociated embryonic optic nerve cells are cultured in defined medium, the normal timing of oligodendrocyte development is disrupted; the O-2A progenitor cells stop dividing and differentiate within a day or two into oligodendrocytes, regardless of the age of the animal from which they were taken (Raff *et al.*, 1985). Normal timing can be restored by culturing the optic nerve cells in medium conditioned by type 1 astrocytes: now the O-2A progenitors continue to divide and first differentiate into oligodendrocytes on the *in vitro* equivalent of the day of birth (Raff *et al.*, 1985). Proliferation and differentiation into oligodendrocytes continues for several weeks in culture, just as *in vivo* (Noble and Murray, 1984; Raff *et al.*, 1985; Dubois-Dalcq, 1987). Thus, type 1 astrocytes provide a mitogen(s) which both stimulates O-2A progenitors to divide and prevents their premature differentiation in culture. We recently provided evidence that this mitogen is a form of platelet-derived growth factor (PDGF), and proposed that astrocyte-derived PDGF is crucial for the development of the O-2A cell lineage *in vivo* (Richardson *et al.*, 1988). There is now compelling evidence in favour of this idea (see Discussion).

The majority of PDGF isolated from human platelets consists of heterodimers of A and B chains (Hammacher *et al.*, 1988), but homodimers can also form. For instance, PDGF isolated from porcine platelets resembles a homodimer of B chains (Stroobant and Waterfield, 1984), while some osteosarcoma and glioma cell lines secrete AA homodimers (Heldin *et al.*, 1986; Nistér *et al.*, 1988). Also, human fibroblasts possess two distinct types of PDGF receptors (Claesson-Welsh *et al.*, 1988; Gronwald *et al.*, 1988; Hart *et al.*, 1988; Heldin *et al.*, 1988). The type A receptor binds all three dimeric isoforms of PDGF, while the type B receptor binds mainly PDGF-BB, and PDGF-AB at reduced affinity, but has very low affinity for PDGF-AA (Claesson-Welsh *et al.*, 1988; Escobedo *et al.*, 1988; Gronwald *et al.*, 1988; Hart *et al.*, 1988; Heldin *et al.*, 1988). Competitive [^{125}I]PDGF binding experiments (this paper) indicate that rat fibroblasts also possess analogous type A and type B receptors.

We previously found (Richardson *et al.*, 1988) that although mRNA encoding the PDGF A chain could be readily detected in cultured type 1 astrocytes and in perinatal rat brain, we could not demonstrate convincingly the presence of B chain mRNA. We have now examined PDGF

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mRNA expression in the developing rat optic nerve directly by *in situ* hybridization, and again find evidence for A chain, but not B chain expression. Taken together, these results suggest that PDGF-AA is the predominant PDGF isoform in the optic nerve, and in the CNS in general. PDGF-AA is highly mitogenic for O-2A progenitor cells, as potent as PDGF-AB and 5- to 10-fold more potent than PDGF-BB. In contrast, PDGF-AA is less potent than either PDGF-AB or PDGF-BB in stimulating DNA synthesis in human or rat fibroblasts. Together with [^{125}I]PDGF binding studies (Hart *et al.*, 1989), our data suggest that O-2A progenitor cells, unlike fibroblasts, possess predominantly type A PDGF receptors, and proliferate during development in response to PDGF-AA homodimers secreted by type 1 astrocytes.

Results

mRNA encoding the PDGF A chain, but not the B chain, is present in the developing rat optic nerve

We previously showed by Northern blot analysis that mRNA encoding the PDGF A chain is present in cultured type-1-like astrocytes purified from neonatal rat cerebral cortex, and in rat brain from E17 to adulthood (Richardson *et al.*, 1988), but there was no direct evidence that PDGF mRNA is present in the perinatal optic nerve where we know that O-2A progenitor cells are proliferating rapidly. We have therefore extended our observations to the developing optic nerve, by *in situ* hybridization.

We first confirmed the specificity of our probes for PDGF A and B chains, by performing *in situ* hybridization on cell lines known to contain PDGF mRNA (Figure 1). Cell line sis-NRK (obtained from P.Stroobant, Ludwig Institute for Cancer Research, London) is a simian sarcoma virus (SSV)-transformed normal rat kidney line which expresses high levels of v-*sis* transcripts (Richardson *et al.*, 1988). v-*sis* is the viral homologue of c-*sis*, the cellular PDGF B chain gene (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983). As expected, we obtained a clear autoradiographic signal following *in situ* hybridization with a ^{35}S-labelled single-stranded RNA probe complementary to the PDGF B chain mRNA ('antisense' probe, Figure 1a), but not with a probe homologous to the mRNA ('sense' probe, Figure 1b). Cell line 157 (obtained from M.Noble, Ludwig Institute for Cancer Research, London) is a human glioma line which expresses high levels of PDGF A chain mRNAs (Richardson *et al.*, 1988). As predicted, we obtained a positive *in situ* hybridization signal using the antisense A chain probe (Figure 1c), but no significant signal over background with the sense A chain probe (Figure 1d).

Cryosections (10 μm nominal thickness) of rat optic nerves were prepared, and subjected to *in situ* hybridization with the PDGF A and B chain probes. Figure 2 shows our results with P8 optic nerves; similar results were obtained with newborn and adult nerves (not shown). Figure 2a shows a longitudinal section through the mid-region of a P8 nerve, stained with toluidine blue and photographed under bright-field illumination. Figure 2b shows an autoradiograph of the same section viewed in dark-field, following *in situ* hybridization with the PDGF A chain antisense probe. Developed silver grains are distributed more or less uniformly over the entire optic nerve, with perhaps a narrow region of slightly higher density just beneath the surface of the nerve. Pretreatment of sections with ribonuclease A, before hybridization with the antisense probe, reduced the signal to near background levels (Figure 2c). Hybridization with the A chain sense probe without prior ribonuclease treatment gave no signal over background (Figure 2d). No

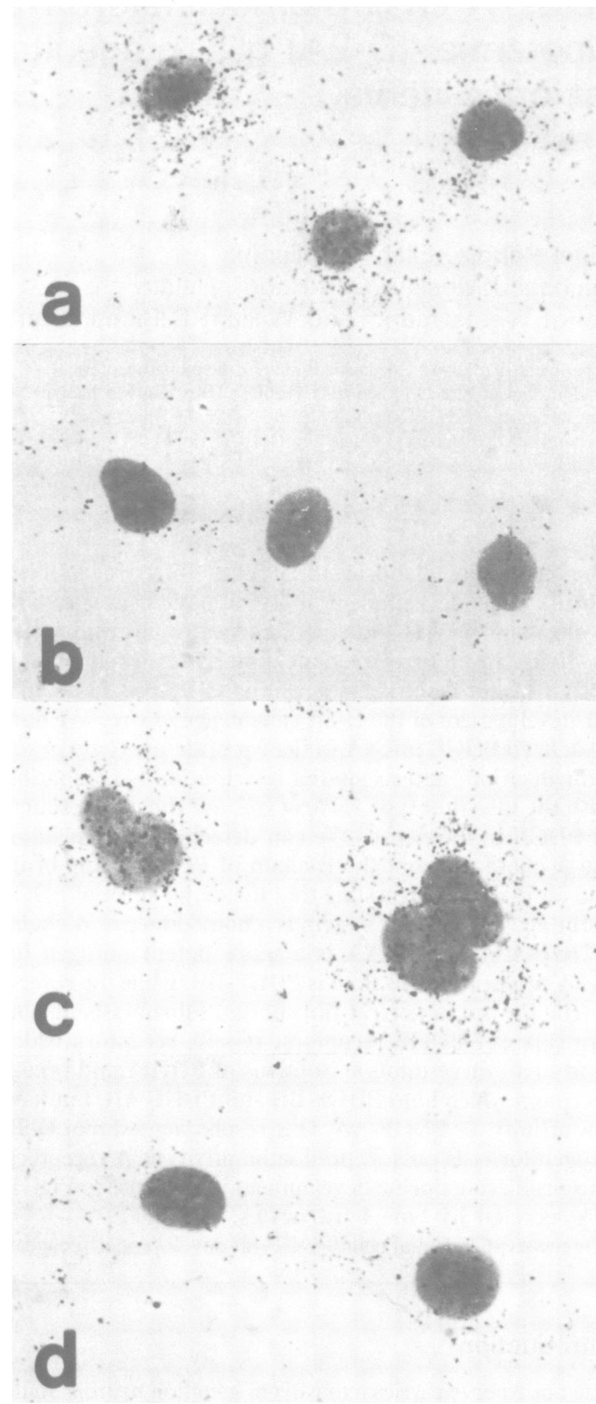

Fig. 1. Testing the specificity of our *in situ* hybridization probes. Cell lines sis-NRK (SSV-transformed rat fibroblasts) and 157 (a human glioma line) were grown on glass coverslips and subjected to *in situ* hybridization and autoradiography, using ^{35}S-labelled single-stranded RNA probes. sis-NRK cells (**a** and **b**) contain high levels of PDGF B chain-related transcripts, while 157 cells (**c** and **d**) contain high levels of PDGF A chain mRNAs. sis-NRK cells give a positive autoradiographic signal with the B chain antisense probe (**a**) but not with the B chain sense probe (**b**). 157 cells give a positive signal with the A chain antisense probe (**c**), but not with the A chain sense probe (**d**). The cells have been stained with toluidine blue to reveal their nuclei, and viewed by bright-field microscopy.

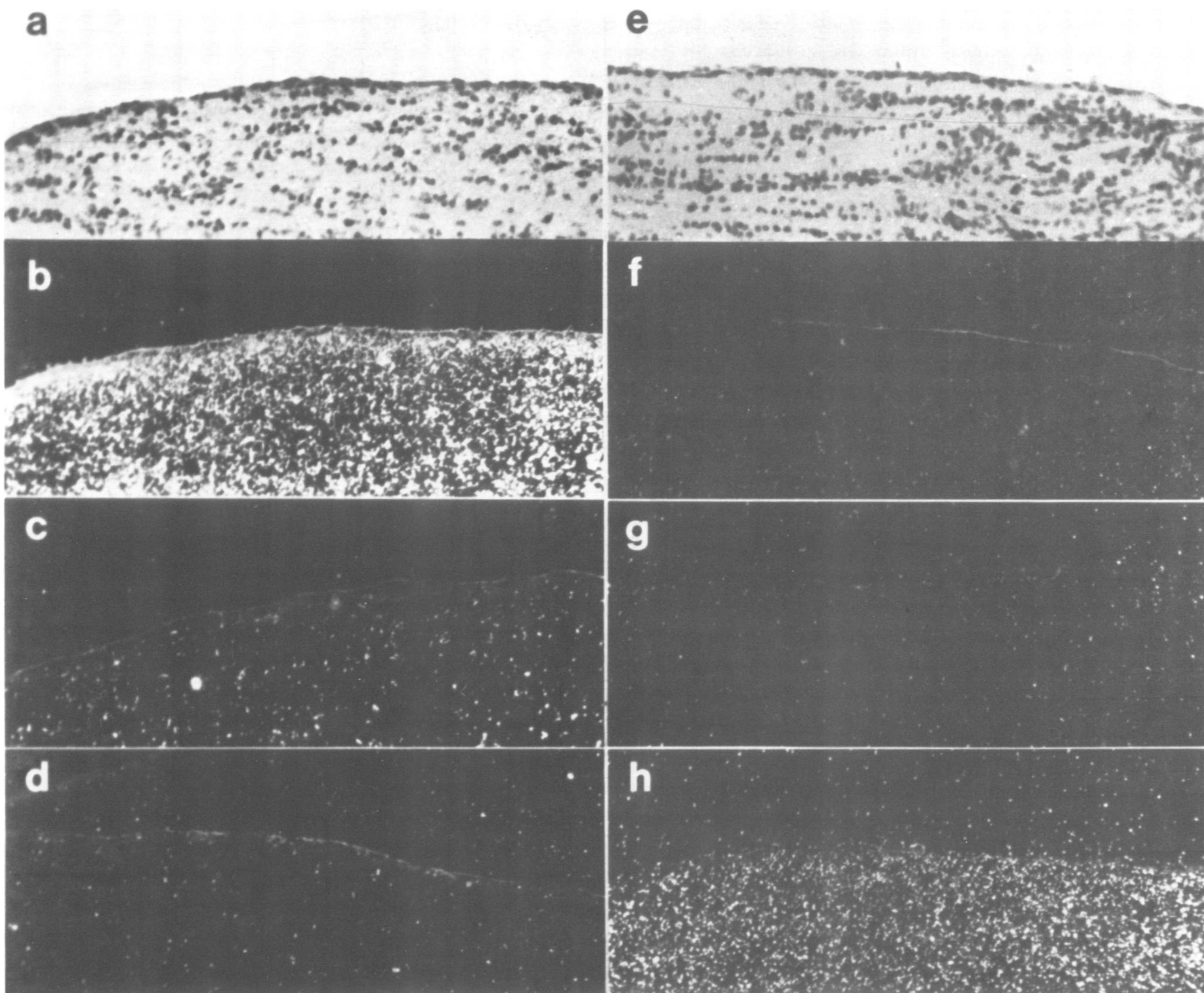

Fig. 2. Expression of PDGF mRNAS in P8 rat optic nerves. Cryosections (10 μm thick) were subjected to *in situ* hybridization and autoradiography, using ^{35}S-labelled single-stranded RNA probes, then counterstained with toluidine blue and viewed by bright-field (**a** and **e**) or dark-field light microscopy. (**a**) Bright-field micrograph of a longitudinal section through the mid-region of a P8 nerve, hybridized with the PDGF A chain antisense probe. (**b**) Same section viewed in dark-field to reveal autoradiographic signal. (**c**) Similar section, pretreated with RNase A before hybridization with the PDGF A chain antisense probe. (**d**) Section hybridized with the PDGF A chain sense probe. (**e**) Section hybridized with the PDGF B chain antisense probe, viewed in bright-field. (**f**) Same section, viewed in dark-field. (**g**) Section hybridized with the PDGF B chain sense probe. (**h**) Section hybridized with an antisense probe for GFAP mRNA. Positive signals are obtained only with the PDGF A chain and GFAP antisense probes.

signal was obtained with either antisense or sense probes for the PDGF B chain (Figure 2e–g).

All the sections illustrated in Figure 2 were prepared and processed together, under as similar conditions as possible, so the results convincingly demonstrate that PDGF A chain mRNA is present in the developing optic nerve, and also strongly suggest that PDGF B chain mRNA is absent, or present at very low levels in comparison with the A chain, as we reported before for the brain as a whole (Richardson *et al.*, 1988). Since PDGF-like mitogenic activity can be found in extracts of rat optic nerves (Raff *et al.*, 1988), and PDGF dimers are secreted by type 1 astrocytes in culture (Richardson *et al.*, 1988), we conclude that the major form of PDGF in the nerve is probably a homodimer of A chains (PDGF-AA).

To compare the distribution of PDGF A chain mRNA with the distribution of type 1 astrocytes in the P8 optic nerve (type 2 astrocytes have not yet started to develop at this age), we performed *in situ* hybridizations with a probe for mRNA encoding the astrocyte-specific intermediate filament protein, glial fibrillary acidic protein (GFAP). The exposed silver grains were distributed uniformly over the entire nerve (Figure 2h). Prior treatment with ribonucleases abolished the signal, and the sense probe gave no signal (not shown). Thus the distributions of PDGF A chain mRNA and GFAP mRNA are similar, consistent with the idea that type 1 astrocytes may be the major source of PDGF in the optic nerve.

PDGF-AA is a more potent mitogen than PDGF-BB for O-2A progenitor cells

PDGF is a 30 kd disulphide-linked dimer, with the structure AB, AA or BB depending on its source (Hammacher *et al.*, 1988; Nistér *et al.*, 1988). PDGF from human platelets

(hPDGF) is a mixture of dimeric forms, PDGF-AB being the major species (Hammacher *et al.*, 1988). The A and B chains share ~60% amino acid similarity, but they are the products of unlinked genes whose expression is often independently regulated (Betsholtz *et al.*, 1986). When tested in human foreskin fibroblasts, PDGF-AA has a low mitogenic activity compared to either PDGF-AB or PDGF-BB (Heldin *et al.*, 1988; Kazlauskas *et al.*, 1988; Nistér *et al.*, 1988; this paper). PDGF-AA is not inherently defective, however, because PDGF-AA is reported to be a potent mitogen for Swiss mouse 3T3 cells (Kazlauskas *et al.*, 1988). Our conclusion that PDGF-AA is the predominant PDGF isoform in the CNS predicts that PDGF-AA should also be mitogenic for O-2A progenitor cells, so we tested the response of O-2A progenitors to the different dimeric forms of PDGF.

Dissociated cells from P7 rat optic nerves were plated on glass coverslips and cultured in defined medium containing transferrin and insulin, plus 0.5% fetal calf serum (FCS) and various concentrations of PDGF. After 3 days in culture, the cells were fixed and stained with monoclonal antibodies A2B5 (Eisenbarth *et al.*, 1979) and anti-galactocerebroside (GC, Raff *et al.*, 1978; Ranscht *et al.*, 1982), followed by appropriate fluorescent anti-immunoglobulin antibodies, and examined by fluorescence microscopy. When O-2A progenitor cells ($A2B5^+GC^-$) stop dividing in low-serum culture, they differentiate within 1–2 days into oligodendrocytes (GC^+). Thus, the number of O-2A progenitors present after 3 days is a measure of the mitogenic activity in the culture medium. By this criterion, pure PDGF-AB from human platelets is strongly mitogenic for O-2A progenitor cells in P7 optic nerve cultures, the concentration required for half-maximal effect being ~2 ng/ml (Figure 3, upper panel). Recombinant PDGF-AA, purified from yeast cells containing a plasmid encoding the human A chain (A.Östman *et al.*, submitted), was also strongly mitogenic for O-2A progenitors, the half-maximal effect occurring at 2–3 ng/ml. Recombinant PDGF-BB expressed in yeast was also mitogenic for O-2A progenitor cells, but was less active, exerting its half-maximal effect at ~10 ng/ml.

We also examined the abilities of the different PDGF isoforms to stimulate DNA synthesis in O-2A progenitors. Dissociated P7 optic nerve cells were grown for 24 h in defined medium, plus 0.5% FCS and various concentrations of PDGF, then 5-bromodeoxyuridine (BrdU, 10 μM) was added for a further 18 h. The cells were then stained with antibody A2B5 as described above, then with monoclonal antibody against BrdU (Magaud *et al.*, 1988), followed by fluorescent anti-immunoglobulin antibodies. O-2A progenitor cells could be unambiguously identified by their bipolar morphology and $A2B5^+$ phenotype (Figure 4). Cells which had incorporated BrdU were revealed by additional strong nuclear staining (Figure 4). A large proportion (75–90%) of O-2A progenitor cells were stimulated to synthesize DNA by all three PDGF isoforms (Figure 3, lower panel), but PDGF-AB and PDGF-AA exerted their half-maximal effects at 2–3 ng/ml, while PDGF-BB was ~10-fold less active (Figure 3, lower panel).

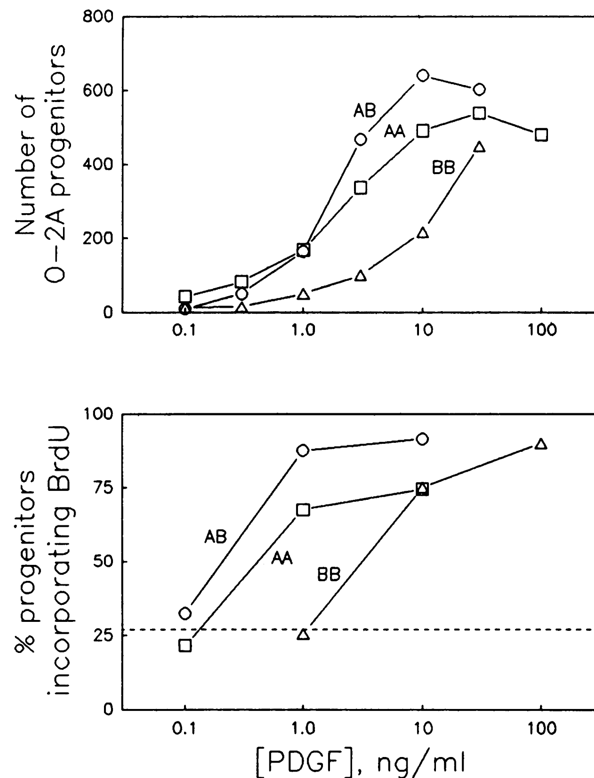

Fig. 3. Mitogenic responses of O-2A progenitor cells to the three dimeric isoforms of PDGF. Dissociated optic nerve cells from P7 rats were grown in wells of a 24-well plate in defined medium plus 0.5% FCS and various concentrations of PDGF-AB, PDGF-AA or PDGF-BB. Two different mitogen assays were used. The upper panel shows mitogenic activities estimated from the total number of O-2A progenitor cells present after 3 days in culture. The lower panel shows the proportion of O-2A progenitor cells which synthesized DNA during the second day of culture, estimated by BrdU incorporation (see Figure 4). Each point represents the average of duplicate experiments; individual measurements differed from the mean by <10%. The dotted line shows BrdU incorporation in the absence of PDGF. Both the progenitor cell counting assay and the BrdU incorporation assay show that for O-2A progenitor cells PDGF-AA is more mitogenic than PDGF-BB (cf. Figure 5).

PDGF-AA is less mitogenic than PDGF-BB for rat fibroblasts

The striking difference in the responses of rat O-2A progenitor cells and human fibroblasts to PDGF-AA could have been a trivial consequence of our using human forms of PDGF on rat cells. We therefore tested the abilities of the different PDGF isoforms to stimulate [^{3}H]thymidine incorporation in the normal rat kidney fibroblast cell line, NRK (clone 49F) and, for comparison, the human foreskin fibroblast line AG 1523. In confirmation of previous reports (Heldin *et al.*, 1988; Nistér *et al.*, 1988), we found that PDGF-AA has a low mitogenic effect on AG 1523 cells: over the range of PDGF concentrations examined, PDGF-AA stimulated [^{3}H]thymidine incorporation only 4-fold, compared to 15-fold for PDGF-BB (Figure 5, upper panel). A similar trend was observed with NRK cells, only PDGF-AA was relatively more mitogenic for NRK cells than for AG 1523 cells (Figure 5, lower panel). All the dose–response curves of Figures 4 and 5 were generated using the same batches of PDGF-AA and PDGF-BB, so our data demonstrate that in rats, cells of different tissue origin can respond preferentially to either one or the other of the PDGF homodimers.

Rat fibroblasts possess both type A and type B PDGF receptors

A possible explanation for the contrasting response of O-2A progenitor cells and NRK cells to PDGF-AA and PDGF-BB

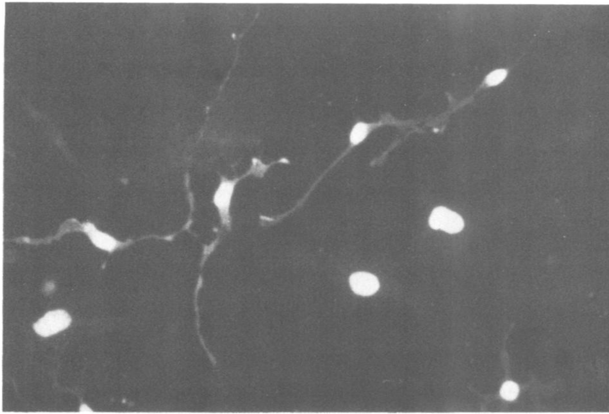

Fig. 4. O-2A progenitor cells synthesize DNA in response to PDGF-AA. Dissociated optic nerve cells from P7 rats were cultured in defined medium, plus 0.5% FCS and 2 ng/ml recombinant human PDGF-AA produced in yeast. BrdU was added to the culture from 24 to 42 h after plating, then the cells were fixed and stained with antibody A2B5 and a monoclonal antibody against BrdU, followed by fluorescent anti-immunoglobulin antibodies, and examined by fluorescence microscopy. In this micrograph, four O-2A progenitor cells can be clearly identified by their bipolar morphology and A2B5-positive processes. All four of these have incorporated BrdU, judging by their brightly fluorescent nuclei. Also in the field are four A2B5-negative cells which have incorporated BrdU.

may be that these cell types possess different populations of PDGF receptors. Competitive receptor binding experiments with O-2A progenitor cells (Hart *et al.*, 1989) indicate that these cells display a single class of PDGF receptors, which bind all three dimeric forms of PDGF and thus resemble the type A receptors on human fibroblasts (Hart *et al.*, 1988; Heldin *et al.*, 1988). We did not know whether NRK cells, like human fibroblasts, possess two types of PDGF receptors with different ligand specificities, so we performed [^{125}I]PDGF binding experiments on NRK cells (Table I). NRK cells were incubated with [^{125}I]PDGF-AA or [^{125}I]PDGF-BB, in the presence or absence of a 100-fold excess of unlabelled PDGF-AA or PDGF-BB, and the amount of bound [^{125}I]PDGF was estimated by gamma counting. Excess unlabelled PDGF-BB competed effectively with both [^{125}I]PDGF-AA and [^{125}I]PDGF-BB for binding to the surface of NRK cells, whereas excess unlabelled PDGF-AA competed with [^{125}I]PDGF-AA but was ineffective against [^{125}I]PDGF-BB. This is the behaviour to be expected if NRK cells, like human fibroblasts, possess both type A receptors (which bind PDGF-AA and PDGF-BB) and type B receptors (which bind PDGF-BB but not PDGF-AA).

Discussion

There is now compelling evidence that PDGF plays an essential role in controlling the proliferation and differentiation of O-2A progenitor cells in the developing optic nerve. First, pure preparations of PDGF, including recombinant PDGF produced in yeast, are strongly mitogenic for O-2A progenitor cells *in vitro* (Noble *et al.*, 1988; Richardson *et al.*, 1988; this paper), and PDGF restores the normal timing of oligodendrocyte development in cultures of embryonic optic nerve cells (Raff *et al.*, 1988). Second, mitogenic activity for O-2A progenitor cells is found in supernatants of optic nerve cultures, and in protein extracts of postnatal optic nerves, and the majority of this activity can be

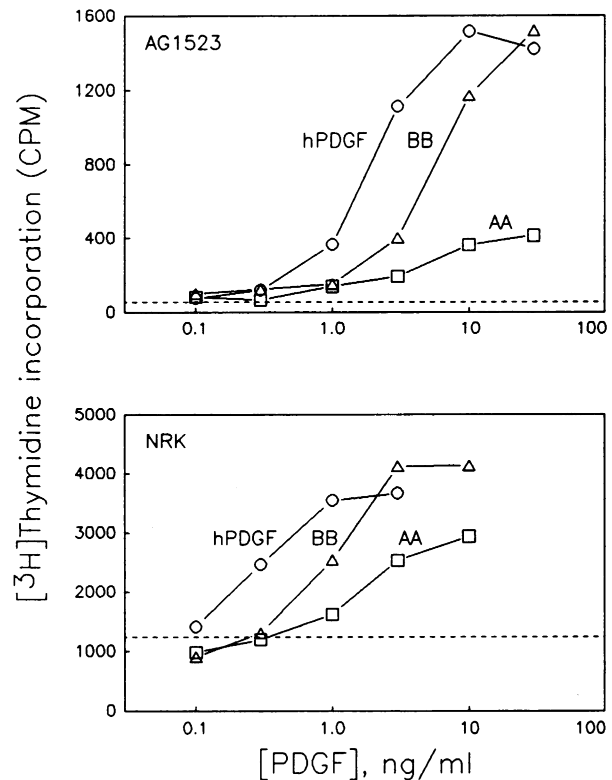

Fig. 5. Mitogenic responses of human and rat fibroblasts to the three dimeric isoforms of PDGF. Cultures of human foreskin fibroblasts (AG 1523) and normal rat kidney fibroblasts (NRK clone 49F) were incubated under serum-free conditions for 3 days, then various concentrations of PDGF were added and incubation continued for 24 h. At the end of this period, [^{3}H]thymidine was added, and 4 h later the amount of incorporated (TCA-precipitable) radiolabel was estimated by scintillation counting. Each point is the average of duplicate experiments; individual measurements differed from the mean by $<15\%$. Dotted lines indicate the incorporation in the absence of PDGF. For both human and rat fibroblasts, PDGF-AA is less mitogenic than hPDGF or PDGF-BB (cf. Figure 3). hPDGF in this experiment consisted of 70% PDGF-AB, 30% PDGF-BB. Pure PDGF-AB gave a very similar result (not shown).

Table I. Binding of [^{125}I]PDGF to rat fibroblasts

Unlabelled competitor	[^{125}I]PDGF bound, % of maximum	
	[^{125}I]PDGF-AA	[^{125}I]PDGF-BB
None	100 (539)	100 (1696)
PDGF-AA	40	105
PDGF-BB	36	31

^{125}I-labelled PDGF-AA or PDGF-BB was allowed to bind to the surface of NRK cells at 4°C, in the presence or absence of a 100-fold excess of unlabelled PDGF-AA or PDGF-BB. Bound [^{125}I]PDGF was determined by gamma counting. Binding is expressed as a percentage of the binding in the absence of unlabelled competitor. The means of two independent experiments are tabulated. Numbers in parentheses are the ^{125}I c.p.m. bound (mean of both experiments).

neutralized by antibodies against human PDGF (Raff *et al.*, 1988). Furthermore, we show in this paper that mRNA encoding the PDGF A chain is present in the developing optic nerve (Figure 2). Finally, [^{125}I]PDGF binding studies (Hart *et al.*, 1989) demonstrate that O-2A progenitor cells from newborn rat optic nerves possess specific, high-affinity receptors for PDGF.

What is the cellular source of PDGF in the optic nerve?

Several lines of evidence point to type 1 astrocytes, which are the major cell type in the perinatal rat optic nerve (Miller *et al.*, 1985). Cultured astrocytes from newborn rat cerebral cortex, which closely resemble type 1 astrocytes in optic nerve cultures, synthesize and secrete PDGF dimers into the culture medium (Richardson *et al.*, 1988). Astrocyte-conditioned medium is mitogenic for O-2A progenitor cells (Noble and Murray, 1984), and prevents premature differentiation of embryonic O-2A progenitors *in vitro* (Raff *et al.*, 1985), and both these activities are abolished by antibodies to PDGF (Raff *et al.*, 1988; Richardson *et al.*, 1988). The experiments reported here further strengthen the notion that type 1 astrocytes produce PDGF in the developing optic nerve. By *in situ* hybridization, we have demonstrated the presence of PDGF A chain mRNA in optic nerves of P8 rats (Figure 2), and also in neonatal and adult nerves (not shown). The spatial distribution of PDGF mRNA (nearly uniform throughout the nerve), while it is not distinctive, is similar to that of mRNA encoding GFAP, an astrocyte-specific intermediate filament protein. With the PDGF A chain probe, there was usually a narrow layer of slightly higher grain density just beneath the surface of the nerve, which may reflect the frequent siting of astrocyte cell bodies in this region (Miller *et al.*, 1985). This feature is barely discernible in Figure 2 but in other sections was more obvious. We did not notice a similar dense layer with the GFAP probe, but since GFAP is an intracellular protein, while PDGF is secreted, we would not necessarily expect their mRNAs to reside in the same regions of the cell; PDGF may be translated preferentially in the cell body near the Golgi, while GFAP may be translated throughout the cell, including the cell processes. Subcellular localization of specific mRNAs has been described in other cell types (Trapp *et al.*, 1987; Fontaine *et al.*, 1988).

We have been able to detect readily PDGF A chain mRNA by Northern blot analysis of RNA from cultured cortical astrocytes and whole rat brain (Richardson *et al.*, 1988), and by *in situ* hybridization in perinatal rat optic nerves (this paper), but we have never been able to demonstrate convincingly mRNA encoding the B chain. Since it is thought that PDGF is mitogenic only as a dimer, we conclude that the predominant isoform of PDGF in the CNS is PDGF-AA. Two alternatively spliced human PDGF A chain mRNAs have been identified from cDNA clones. The longer form contains an extra 69 bp at the 3′ end of the coding sequence (Betsholtz *et al.*, 1986; Collins *et al.*, 1987; Tong *et al.*, 1987), which results in a protein with a highly basic 15 amino acid extension at its carboxyl terminus. We do not yet know the detailed structure of the PDGF A chain mRNA or protein in the rat CNS.

PDGF-AA has a low mitogenic activity when assayed on human fibroblasts (Heldin *et al.*, 1988; Nistér *et al.*, 1988; this paper), but is a potent mitogen for Swiss mouse 3T3 cells (Kazlauskas *et al.*, 1988). We have shown here that PDGF-AA is also strongly mitogenic for rat O-2A progenitor cells, about as potent as PDGF-AB and 5- to 10-fold more potent than PDGF-BB (Figure 3). These differences in the mitogenic response of cells to PDGF-AA are not solely a consequence of using human PDGF isoforms on rodent cells, because although we found that rat NRK fibroblasts also responded to PDGF-AA, for these cells PDGF-AA was a less potent mitogen than either PDGF-AB or PDGF-BB (Figure 5). These data demonstrate that different cell types of a single animal species can display distinct preferences for one or other of the PDGF homodimers.

Two types of PDGF receptors are present on human fibroblasts (Claesson-Welsh *et al.*, 1988; Gronwald *et al.*, 1988; Hart *et al.*, 1988; Heldin *et al.*, 1988). The type A receptor binds to all three dimeric forms of PDGF, while the type B receptor binds mainly PDGF-BB, and PDGF-AB at lower affinity, but has very low affinity for PDGF-AA. The [^{125}I]PDGF binding data in Table I indicate that rat fibroblasts possess analogous type A and type B receptors. DNA sequence analysis of the human and mouse type B receptor genes predicts a typical transmembrane tyrosine kinase receptor, with a cytoplasmic 'split' tyrosine kinase domain (Yarden *et al.*, 1986; Claesson-Welsh *et al.*, 1988; Gronwald *et al.*, 1988). As yet, little is known about the structure or function of the type A PDGF receptor.

Recent competitive binding and receptor down-regulation studies (Hart *et al.*, 1989) indicate that rat O-2A progenitor cells probably possess only type A PDGF receptors. It seems likely, therefore, that the preference of O-2A progenitor cells for PDGF-AA, and fibroblasts for PDGF-BB, is determined by the different compositions of their receptor populations. The degree to which cells respond to PDGF-AA may in general depend on the number of type A receptors on their surface (Kazlauskas *et al.*, 1988). However, one observation that remains to be explained (Hart *et al.*, 1989) is that when O-2A progenitor cells differentiate into oligodendrocytes *in vitro*, they retain their PDGF receptors for some time, but lose the ability to divide in response to PDGF.

Materials and methods

In situ hybridization

Our *in situ* hybridization procedure was essentially that described by Lawrence and Singer (1985), with minor modifications as described below. Optic nerves dissected from newborn, P8 or adult rats were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), for 2–3 h at 20°C. The nerves were immersed for 2–3 h in 0.5 M sucrose in PBS, and frozen in a drop of OCT embedding compound (BDH), in an aluminium foil boat floating on liquid N_2. Frozen sections (10 μm nominal thickness) were cut, and collected on freshly prepared poly-L-lysine-coated glass microscope slides. Sections were dried for 2 h at 20°C, fixed with 4% paraformaldehyde in PBS for 15 min, extracted with 0.2 M HCl for 20 min (to remove basic proteins), and submerged in 2 × SSC for 30 min at 70°C (1 × SSC is 150 mM NaCl, 15 mM Na citrate, pH 7). The sections were then incubated in a 0.125 mg/ml solution of predigested pronase (Sigma, type XIV), for 20 min at 20°C, and the digestion was arrested by rinsing for 30 s in PBS containing 0.2% (w/v) glycine, followed by several washes in PBS. At this point control sections were treated with 100 μg/ml RNase A in 0.5 M NaCl, 10 mM Tris–HCl, pH 7.6, 1 mM EDTA for 1 h at 37°C. All sections were postfixed in 4% paraformaldehyde in PBS for 15 min at 20°C, then acetylated for 10 min at 20°C in a freshly prepared 25 mM solution of acetic anhydride in 0.1 M triethanolamine, pH 8.0, washed briefly in PBS and dehydrated in a series of ascending concentrations of ethanol (1 min each in 30, 60, 80, 95, 100% v/v ethanol/water). Slides were allowed to dry before prehybridizing with non-radioactive α-thio UTP (500 nM) (Bandtlow *et al.*, 1987) in hybridization solution [0.3 M NaCl, 10 mM Tris–HCl, 10 mM $NaPO_4$, pH 6.8, 5 mM EDTA, 0.02% (w/v) Ficoll 400, 0.02% (w/v) polyvinyl pyrolidone (PVP), 0.02% (w/v) BSA (Sigma fraction V), 10% (w/v) dextran sulphate, 0.1 mg/ml yeast tRNA, 10 mM dithiothreitol (DTT) and 50% (v/v) deionized formamide]. Incubation was from 3 to 4 h at 50°C. The sections were washed for 30 min at 50°C in wash solution (hybridization solution minus dextran sulphate and yeast tRNA), dehydrated through ascending concentrations of ethanol and air dried.

^{35}S-labelled RNA probes (see below) were heated for 5 min at 80°C in hybridization buffer, chilled on ice, and 10–25 μl of this solution was applied to each slide under a siliconized glass coverslip. The slides were incubated in a humid chamber for 18–24 h at 50°C. Coverslips were removed by submerging in wash solution for 30 min at 50°C; sections were washed in the same buffer at 50°C for a further hour and then at 65°C

for 30 min. This was followed by digestion with RNase A (20 μg/ml) in 0.5 M Nacl, 10 mM Tris−HCl, pH 7.6, 1 mM EDTA for 30 min at 37°C. Finally the slides were washed for 30 min at 65°C in wash solution, then 30 min at 45°C in 2 × SSC, then 30 min at 45°C in 0.1 × SSC. The sections were dehydrated through ascending concentrations of ethanol in 0.25 M ammonium acetate, and air dried. For autoradiography, the slides were coated with Ilford K5 nuclear emulsion, and exposed for between 1 and 3 weeks in the dark at 4°C. After developing in Kodak D-19, and fixing, the sections were counterstained in 0.02% (w/v) toluidine blue, dehydrated in ethanol, cleared with xylene and mounted for examination by bright-field and dark-field microscopy.

In situ hybridization of cultured cell lines was performed essentially as described above, but using shorter incubation times: 10 min fixation steps in 4% paraformaldehyde, 5 min extraction in 0.2 M HCl followed by 10 min in 2 × SSC at 70°C, 5 min in pronase (40 μg/ml). Hybridization and initial washes (i.e. prior to RNase treatment) were at 37°C instead of 50°C and 65°C.

Preparation of ^{35}S-labelled RNA probes

Single-stranded RNA probes were generated by *in vitro* transcription as described by Cox *et al.* (1986). A 681 bp *Sac*I−*Hin*dIII fragment encompassing most of the coding region of a human PDGF A chain cDNA (Betsholtz *et al.*, 1986) was cloned into plasmid pGEM3 (Promega Biotec). A 839 bp *Pst*I−*Avr*II coding region fragment of a human PDGF B chain cDNA (Josephs *et al.*, 1984) was subcloned into pGEM1 (Promega). A 1200 bp *Hin*dIII−*Kpn*I coding fragment of a mouse GFAP cDNA (Lewis *et al.*, 1984) was subcloned into pGEM3. Using bacteriophage T7 or SP6 RNA polymerases (both obtained from Promega), [α-^{35}S]UTP (Amersham), and linear DNA templates, ^{35}S-labelled 'sense' or 'antisense' RNA run-off transcripts (2×10^8 c.p.m./μg) were generated *in vitro*. Reaction conditions were as recommended by Promega. The full length transcripts were reduced in size by limited alkaline hydrolysis to an average length of 150 bp, estimated by comparison with DNA size markers on a formaldehyde-containing agarose gel (Maniatis *et al.*, 1982). This radiolabelled probe was hybridized to cryosections (see above), using ~5 ng RNA in 25 μl hybridization buffer per slide.

PDGF

hPDGF was isolated from human platelets, as described by Heldin *et al.* (1987). The particular batch used in the experiments of Figure 5 contained 70% PDGF-AB and 30% PDGF-BB. Pure PDGF-AB was further purified from hPDGF by metal ion chromatography as described by Hammacher *et al.* (1988); PDGF-AA and PDGF-BB were purified to apparent homogeneity from supernatants of yeast cells containing plasmids encoding the human A or B chains respectively (A.Östman *et al.*, submitted). The A chain corresponded to the 'short' form (Betsholtz *et al.*, 1986; Collins *et al.*, 1987; Tong *et al.*, 1987). The concentrations of all PDGF preparations was determined by amino acid composition analysis.

PDGF-AA was ^{125}I-labelled by the chloramine T method (Hunter and Greenwood, 1982) to a sp. act. of 35 000 c.p.m./ng. PDGF-BB was labelled by the method of Bolton and Hunter (1973), to a sp. act. of 25 000 c.p.m./ng.

Optic nerve cultures

Cultures of optic nerve cells from P7 rats were prepared as described previously (Miller *et al.*, 1985). Briefly, optic nerves were dissociated with trypsin, collagenase and EDTA, and plated on poly-D-lysine coated glass coverslips (5000 cells/coverslip) in the defined medium of Bottenstein and Sato (1980), modified as described by Richardson *et al.* (1988). The medium was supplemented with 0.5% FCS and PDGF where appropriate. Cells were usually cultured for 3 days without replenishing the growth medium.

Immunofluorescence and BrdU incorporation

For counting O-2A progenitors, optic nerve cultures were pre-fixed with 2% paraformaldehyde in Hepes-buffered DMEM for 10 min at room temperature. The cells were stained with a mixture of monoclonal antibodies A2B5 (IgM: Eisenbarth *et al.*, 1980) and anti-GC (IgG3: Raff *et al.*, 1978), followed by a mixture of rhodamine-labelled goat anti-mouse IgM and fluorescein-labelled goat anti-mouse IgG3 class specific antibodies (Nordic). The cells were post-fixed in 4% paraformaldehyde in PBS and mounted for fluorescence microscopy.

For DNA synthesis assays, optic nerve cells were cultured for 24 h in the presence or absence of PDGF. Then 5-bromodeoxyuridine (Boehringer) was added (10 μM final concentration), and the cells cultured for a further 24 h. The cells were pre-fixed as above and stained with A2B5, followed by fluorescein-labelled goat anti-mouse Ig. After fixing in methanol at −20°C, the cells were incubated with 2 M HCl for 10 min at room temperature and then with 0.1 M sodium borate (pH 8.5) for a further 10 min. The cultures were then stained with a monoclonal antibody against BrdU (BU20a; Magaud *et al.*, 1988) followed by rhodamine-labelled goat anti-mouse Ig. After fixing with 4% paraformaldehyde in PBS, the coverslips were mounted for fluorescence microscopy.

[^{3}H]Thymidine incorporation assays

Our mitogen assay is similar to that described by Assoian *et al.* (1983). Normal rat kidney fibroblasts (cell line NRK, clone 49F, obtained from P.Stroobant) (De Larco and Todaro, 1978), and human foreskin fibroblasts (cell line AG 1523, purchased from the Human Mutant Cell Repository, Institute for Medical Research, Camden, NJ, USA) were plated in 15 μl droplets, containing 2000 cells, in the centre of the wells of a 24-well culture plate. The cell number was kept low to ensure an excess of PDGF molecules to receptors. After the cells had attached, the wells were flooded with DMEM plus 10% FCS and incubated overnight. The next day, the cells were washed three times with 1 ml of serum-free medium. The culture medium was replaced with 0.5 ml DMEM plus 0.5% bovine plasma (Gibco: heat inactivated and dialysed against DMEM), and the cells were incubated for 3 days, after which time an island of confluent cells had formed in the centre of each well. PDGF was added, and the cells were cultured for a further 18 h. [^{3}H]thymidine (Amersham, 2 Ci/mmol; 4 μCi/ml final concentration) was added and the cells incubated for another 4 h. The medium was removed, and the cells fixed for 10 min in 0.5 ml of ice-cold 5% trichloroacetic acid (TCA). The fixed cells were washed three times with 1 ml cold 5% TCA and air dried. The precipitates were solubilized with 0.2 ml 0.5 M NaOH for 30 min at 37°C, and counted in a scintillation counter.

[^{125}I]PDGF binding

NRK cells were plated in 24-well dishes in DMEM plus 10% FCS and grown to confluence. The cells were washed twice with Hepes-buffered DMEM supplemented with 0.25% (w/v) BSA (binding buffer), then 0.5 ml binding buffer was added to each well. [^{125}I]PDGF-AA or [^{125}I]PDGF-BB (0.5 ng/ml) was added, with or without a 100-fold excess of unlabelled PDGF-AA or PDGF-BB. The cultures were incubated at 4°C for 6 h, washed three times with cold binding buffer, solubilized in 1% (v/v) Triton X-100 with 0.1% (w/v) BSA, and counted in a gamma counter.

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