STUDIES ON THE CONTROL OF OLIGODENDROCYTE DIFFERENTIATION IN VITRO

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ABSTRACT

The question of why many types of cells stop dividing and differentiate in the continuing presence of growth factors is a fundamental one in animal development. In the rat optic nerve, bipotential O-2A progenitor cells give rise to oligodendrocytes and type-2 astrocytes on a precise schedule. Previous studies suggest that platelet-derived growth factor (PDGF) plays an important part in timing oligodendrocyte development and that the timing of oligodendrocyte differentiation is secondary to an intrinsic timing mechanism that controls when O-2A progenitor cells become mitotically unresponsive to PDGF (Raff et al., 1988). This thesis examines the molecular basis of this intrinsic timing mechanism in O-2A progenitor cells in vitro and looks at the relationship between mitosis and oligodendrocyte differentiation in vivo.

O-2A progenitor cells have α PDGF receptors, suggesting that these cells respond directly to PDGF. The receptors are initially retained when progenitor cells stop dividing in vitro and differentiate into oligodendrocytes. This indicates that receptor loss is not the reason that progenitor cells become mitotically unresponsive to PDGF.

Many O-2A progenitor cells, and newly formed oligodendrocytes which are no longer mitotically responsive to PDGF, show an increase in cytosolic Ca^{2+} in response to PDGF. A combination of a Ca^{2+} ionophore plus a phorbol ester mimics the effect of PDGF, both in stimulating O-2A

progenitor cell division and in reconstituting the normal timing of oligodendrocyte differentiation in culture, whereas the same drug combination does not stimulate newly formed oligodendrocytes to proliferate. These findings suggest that at least one reason why newly formed oligodendrocytes do not respond mitotically to PDGF is that there is a block or deficiency downstream from some of the early intracellular events that follow activation of the PDGF receptor.

PDGF increases the expression of Fos and Jun in newly formed oligodendrocytes in vitro, suggesting that at least one intracellular signalling pathway to the nucleus is activated by PDGF in these cells even though PDGF does not stimulate them to synthesize DNA.

O-2A progenitor cells in the developing optic nerve, like those in optic nerve cultures, usually stop synthesizing DNA at least 6-12 hours before they express galactocerebroside, an early surface marker of differentiated oligodendrocytes, suggesting that in vivo differentiation follows loss of mitotic responsiveness, just as it does in vitro.

CONTENTS

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ABBREVIATIONS

4α-PDD	- 4α-phorbol 12,13-didecanoate
ACM	- astrocyte conditioned medium
AP-1	- activator protein 1
A-kinase	- cyclic AMP-dependent protein kinase
BrdU	- bromodeoxyuridine
BSA	- bovine serum albumin
Bt	- biotin
CNS	- central nervous system
C-kinase	- protein kinase C
CSF	- colony stimulating factor
DAG	- diacylglycerol
dbcAMP	- dibutyryl cyclic adenosine monophosphate
DMEM	- Dulbecco's modified Eagle's medium
E	- embryonic
EGF	- epidermal growth factor
FCS	- fetal calf serum
FGF	- fibroblast growth factor
Fl	- fluorescein
GC	- galactocerebroside
GFAP	- glial fibrillary acidic protein
GGF	- glial growth factor
GM cells	- granulocyte-macrophage precursor cells
HLH	- helix-loop-helix
Id	- inhibitor of DNA binding
Ig	- immunoglobulin
IL-3	- interleukin 3

IP_3 - inositol trisphosphate

MBP - myelin basic protein

MEM - minimal Eagle's medium

O-2A - oligodendrocyte-type-2 astrocyte

P - postnatal

PBS - phosphate-buffered saline

PDB - phorbol 12,13 dibutyrate

PDGF - platelet-derived growth factor

PDL - poly-D-lysine

PI kinase - phosphatidyl-inositol kinase

PIP_2 - phosphatidyl-inositol bisphosphate

PMA - phorbol 12-myristate 13-acetate

P^2P - proliferation potential protein

Ran-2 - rat neural antigen-2

Rd - rhodamine

TR - Texas red

CHAPTER 1

GENERAL INTRODUCTION

The study of the biological process of building a vertebrate brain is perhaps the most demanding scientific problem that man has considered. The mature mammalian brain is unique in its complexity: it contains an impressive variety of different neuronal and glial cell types organized into a hierarchical network which, in humans, is comprised of an estimated 10^{12} cells. The task of understanding the formation of the central nervous system (CNS) becomes even more daunting when one considers its development from a relatively modest origin in the embryo.

The neural plate is the first neural structure to arise in the vertebrate. It appears as a monolayer of morphologically uniform, pseudo-stratified neuroepithelial cells in the midline ectoderm of the embryo (Sauer, 1935a,b). Transplant experiments in Xenopus suggest that the neural plate is induced by signals from a specialized region of the underlying mesoderm called the notochord (Spemann, 1938). Signals from the mesoderm are also thought to play an important part in determining the future character of the different regions of the neural tube. If, for example, a section of anterior mesoderm is transplanted to a posterior site in the embryo, the overlying posterior ectoderm develops subsequently in the pattern characteristic of a normal anterior neural plate.

After a restricted period of cell division, the lateral edges of the neural plate arch towards the midline and fuse to form the neural tube (Sauer 1935a; Fujita, 1963; Rakic, 1974). It is this structure that gives rise

eventually to the many hundreds of different classes and subclasses of neurones and the three major types of macroglial cells (oligodendrocytes, astrocytes and ependymal cells) in the adult CNS. Anatomical and autoradiographic studies using tritiated thymidine have defined four distinct zones in the neural tube (His, 1889; Sauer and Walker, 1959; Sidman et al., 1959; Langman et al., 1966). Beginning at the rostral end of the tube, there is a marked proliferation of cells at the inner surface, the ventricular zone. At the time of neural tube closure the first postmitotic primitive neurones (neuroblasts) are generated in the ventricular zone. These neuroblasts then migrate outwards to form the intermediate zone, where some cells stay and begin to extend processes and interconnect, whilst others continue to migrate to the outermost surface of the neural tube which is called the marginal zone. A second germinal region is formed by the migration of cells from the ventricular zone and lies between it and the intermediate zone. This is the subventricular zone and it is thought to give rise to some classes of neurones and macroglial cells.

Studies using glial-cell-specific markers have suggested that the differentiation of macroglial cells begins early in development, in the ventricular and subventricular zones, before neuronogenesis has stopped (Levitt et al., 1981). Also at this early stage, a class of non-proliferative elongated cells, called the radial glial cells, is first recognized. These cells span the neural

tube from the ventricular zone to the marginal zone and they are thought to form a scaffold along which the postmitotic neuroblasts migrate to their final positions in the CNS (Rakic, 1972). After neuronal migration is finished, radial glial cells disappear from most regions of the CNS. It has been suggested that radial glial cells give rise to astrocytes (Schmechel and Rakic, 1979), but this has not been directly shown.

How is the transformation of this relatively simple collection of neuroepithelial cells into the mature CNS controlled? It is helpful to divide the question into two parts: how is the cellular diversity generated, and how do the neurones become organized into complex, interconnected layers and nuclei? This thesis is concerned with the first part of the problem - the cellular and molecular mechanisms underlying the production of cellular diversity. A pre-requisite for the investigation of how an individual cell chooses a particular developmental fate and, equally important, how its differentiation is timed, is the establishment of the plan of a cell's ancestry in relation to other CNS cells by the study of cell lineage. Cell lineage studies identify precursor cells and determine which are pluripotential, i.e. can give rise to more than one cell type, so that the developmental choice points of cells can be recognized. A variety of powerful and complementary techniques have been used to establish cell lineage relationships in the CNS of a range of animals.

The study of neural diversification in invertebrates

is ideally suited to a genetic approach. The nematode Caenorhabditus elegans (C. elegans) is transparent, has a short reproductive cycle, and can reproduce by self-fertilization (reviewed in Kenyon, 1988). It also has a simple and invariant nervous system, consisting of 302 neurones, each identifiable under the light microscope. By a combination of direct observation and the use of cell markers such as horseradish peroxidase and fluorescent dyes, a complete description of the cellular anatomy and lineage at both the light and electron microscopic level has been assembled (Sulston and Horvitz, 1977; Sulston et al., 1983). This has allowed the study of multicellular processes at the level of single cells in living animals by selective cell ablation and mutational and genetic analysis. 800 genes have been identified, including genes that control development, and almost the entire genome has been physically mapped (reviewed in Wood, 1988). More importantly, these approaches have established many general developmental principles which, subsequently, have been shown to be applicable to higher species.

The compound eye of Drosophila has been another productive model for the study of neural development (reviewed in Rubin, 1988; Ready, 1989). The fly eye develops from a simple neuroepithelial monolayer into a stereotyped collection of repetitive units, called ommatidia, consisting of a precise collection of photoreceptors and auxiliary cells. Anatomical studies have again produced a detailed description of cell behaviour

during ommatidial development and this has allowed a genetic and molecular analysis of the processes involved to be carried out.

Until recently, it was difficult to study directly the development of the higher vertebrate CNS, mainly because the genetic approaches that are so useful in invertebrates could not be used to establish the more complex lineage relationships between the more numerous cell types. However, the exploitation of cell culture systems (see below) and the advent of gene transfer by microinjection and retroviral infection have provided important insights into the development of even the most complex areas of the mammalian CNS.

Firstly, both types of gene transfer techniques can be used to generate transgenic animals which promise, in the near future, to allow in situ genetic manipulation of the mammalian CNS on an unprecedented scale (reviewed in Jaenisch, 1988). Several strategies can be applied. By microinjecting foreign DNA into a pronucleus of a fertilized mammalian egg or using retroviruses (see below) to infect embryos, the expression of inserted marker genes has been used to follow the fate of cells in the growing animal. Moreover, these techniques potentially allow the ablation of any cell type for which a specific enhancer or promoter can be used to express a DNA sequence causing cell death, such as the one that encodes the A chain of diphtheria toxin.

Secondly, retroviruses can be used to introduce stable

genetic markers into cells at appropriate developmental stages both *in vivo* and *in vitro*. These viruses have a polymerase enzyme called reverse transcriptase which generates a DNA copy of the viral RNA that inserts into the host cell genome (reviewed in Varmus, 1988). Once a retrovirus has infected a cell, it integrates randomly into the host genome so that the viral genome is inherited without dilution by all the progeny of that cell. This random integration ensures that every integration site is unique and that cells within a given clone can be identified from cells in other clones by restriction enzyme analysis. For lineage studies, retroviral structural and replication genes are replaced by a marker gene, such as the *E. coli lacZ* gene which encodes a β-galactosidase. Thus, the virus is engineered genetically both to produce a marker that is detectable histochemically and to be replication defective so that infected cells cannot obscure clonal analysis by generating new virus to spread horizontally. Since the vertebrate CNS was first studied with retrovirus 4 years ago, new information has been obtained on cell lineage in the retina, cerebral cortex, optic tectum, and peripheral nervous system (reviewed in Sanes, 1989).

If retrovirus is injected into the rat eye during the first postnatal week, for example, and the retina stained for the introduced cell marker 4 weeks later, it is found that the clones that have developed can contain almost any combination of the cell types generated postnatally,

including photoreceptors, neurones and Müller glial cells (Price et al., 1987; Turner and Cepko, 1987). From analysis of hundreds of clones it is suggested that a single type of progenitor cell gives rise to all of the cell types in the adult rat retina, except for astrocytes and microglial cells, and, moreover, that this cell remains pluripotential up to its final mitotic division. Retroviral studies of cell clones in the embryonic cerebral cortex of the rat have also identified a multipotential precursor cell that appears to retain the ability to give rise to the majority of the cortical neurones, and possibly to the glial cells of the white matter, even very late in development (Price and Thurlow, 1988). However, the cerebral cortex differs from the retina in that the cortex also seems to contain a second type of precursor cell that is committed to giving rise to only grey matter astrocytes from a very early stage in development.

From all the above, and other similar studies, it has been established that the timing and direction of development at the cellular level, in species from lowest invertebrates to higher mammals, is the result of an interplay between two basic processes: (1) cell-cell interactions in which the fate of a precursor cell remains undecided until a late stage in development and is controlled by signals from neighbouring cells and (2) cell-intrinsic programmes where precursor cells give rise to pre-ordained progeny through autonomous programmes of division and differentiation. There are convincing examples

of both processes at all stages in CNS development although, in most cases, little is known about the underlying molecular mechanisms.

Intrinsic cellular programmes were first described in the early stages of invertebrate development. Isolated cells or regions from embryos of the ascidian, Halocynthia roretzi, differentiate in vitro as they would had they remained in the intact embryo (reviewed in Slack, 1983). Similarly, in some cases where single cells within a living larval C. elegans are ablated with a laser microbeam only the progeny of the killed cells are absent in the adult and the fate of the neighbouring cells is unaltered (Sulston and Horvitz, 1977; Sulston and White, 1980). However, other studies in C. elegans indicate an important role for cell-cell interactions: for example, the anchor cell in the developing gonad induces three neighbouring cells to form a vulva; if the anchor cell is killed the neighbouring cells develop into hypodermal cells (Sternberg and Horvitz, 1986). This illustrates that development, even in a simple organism, is the result of the cooperation between cell-intrinsic and cell-extrinsic mechanisms.

Morphological and genetic studies using markers to trace cell lineage suggest that the fate of a cell in the fly retina seems to depend mainly on short range cell-cell interactions (reviewed in Ready, 1989). Several eye mutants have been identified which have been useful in the study of the molecular mechanisms that control cell diversification. For example, in the sevenless mutant the seventh member

(R7) of the cluster of eight cells in the mature ommatidium is absent. A cell joins the cluster at the correct time and place to become R7, but becomes a cone cell instead. Mosaic analysis reveals that the cues specifying R7 operate normally, but that mutant cells seem unable to respond to them. The sevenless gene has been cloned. From its sequence and the predicted structure of its protein product, it is speculated that sevenless is a receptor on the surface of a pluripotential precursor cell. This receptor would bind a ligand produced by neighbouring cells and transduce a signal required for the selective gene activation that commits the cell to the R7 pathway.

It can be difficult, however, to disentangle the relative contributions of cell-intrinsic programmes and cell-cell interactions in the living animal, even though the underlying cell lineage is known and mutants deficient in cellular control genes have been characterized. Despite intensive study very few of the molecules involved in cell-cell signalling in the fly eye or in C. elegans have been identified. Another approach is to study the development of CNS cells in culture. While the main disadvantage of this method is the loss of the complex three-dimensional interrelationships of cells in situ, it has the overwhelming advantage of allowing the direct manipulation of the cellular environment. Raff and his colleagues have used this cell biological approach to study the later stages of cell diversification in one of the simplest regions of the rat CNS, the optic nerve (reviewed in Raff,

1989; Miller et al., 1989a). Cell-type-specific markers have been identified, cell lineage defined, and progress has been made in describing some of the cellular and molecular mechanisms underlying cyto-differentiation.

The optic nerve develops from an extension of the optic stalk. It contains mainly myelinated retinal ganglion cell axons, which project to the midbrain without branching, but no neuronal cell bodies. Earlier studies with the electron microscope suggested that the optic nerve neuroepithelium gives rise to two major classes of glial cells in the adult optic nerve, oligodendrocytes and astrocytes (Skoff et al., 1976a; 1976b), which form the axonal support network of the nerve. Oligodendrocytes are the myelinating cells of the CNS and are essential for the rapid and efficient saltatory conduction of action potentials along CNS axons. The function of astrocytes is less certain, although anatomical studies have shown that they extend processes to three sites: to the nerve surface, forming a glial limiting sheath, to blood vessels, forming a perivascular sheath, and to the nodes of Ranvier (ffrench-Constant and Raff, 1986; Miller et al., 1989b). The optic nerve also contains microglial cells, meningeal cells, and vascular endothelial cells, none of which develops from the neuroepithelium.

In vitro, three distinct types of differentiated macroglial cells can be identified in the perinatal optic nerve. Oligodendrocytes appear as multi-processed cells with small cell bodies and are the only cells that are

surface-labelled with antibody against galactocerebroside (GC) (Raff et al., 1978; Ranscht et al., 1982), the major glycolipid in myelin. Antibodies against glial fibrillary acidic protein (GFAP) (Bignami et al., 1972), which is the major subunit of glial filaments that are restricted to astrocytes in the rat CNS (Raff et al., 1979), recognize two classes of astrocytes, named type-1 and type-2 astrocytes. These two classes have been characterized by their different morphology, growth characteristics and antigenic phenotype (Raff et al., 1983a). Most type-1 astrocytes in cultures of optic nerve are flat, non-process bearing cells, are stimulated to proliferate by glial growth factor (GGF) (Lemke and Brockes, 1984) and epidermal growth factor (EGF) (Cohen, 1962), and are surface-labelled by the monoclonal antibody Rat neural antigen-2 (Ran-2; a glycoprotein) (Bartlett et al., 1981) but not by the monoclonal antibody A2B5 or tetanus toxin, both of which bind to specific gangliosides (Eisenbarth et al., 1979). On the other hand, most type-2 astrocytes in culture are multi-processed, are not stimulated to divide by either GGF or EGF (Raff et al., 1983a), and are surface-labelled by A2B5 antibodies and tetanus toxin (Raff et al., 1983a) but not by anti-Ran-2 antibodies in short-term culture (Raff et al., 1984a).

In vivo these three macroglial cells develop on a predictable schedule. Studies using antibodies to label cells in freshly-dissociated suspensions of optic nerve from rats of different ages indicate that type-1 astrocytes

first appear at embryonic day 16 (E16), oligodendrocytes at the time of birth (E21 = P0), and type-2 astrocytes in the second postnatal week (Miller et al., 1985). Similar cells also develop on the same schedule in cultures of embryonic rat brain (Abney et al., 1981; Williams et al., 1985) suggesting that the timing of their differentiation is independent of morphogenesis.

Experiments using bulk cultures of optic nerve cells have provided convincing evidence that oligodendrocytes and type-2 astrocytes develop from a common, bipotential, glial progenitor cell called the oligodendrocyte-type-2 astrocyte (O-2A) progenitor cell with the antigenic phenotype $A2B5^+/GC^-/Ran\text{-}2^-/GFAP^-$, while type-1 astrocytes arise from a separate precursor cell, which are $Ran\text{-}2^+/A2B5^-/GC^-/GFAP^-$ (Raff et al., 1983a; 1984a). With the use of antibodies to eliminate $A2B5^+$ cells by pre-treating dissociated optic nerve cells with A2B5 antibody and rabbit complement before plating, no oligodendrocytes or type-2 astrocytes develop, although normal numbers of type-1 astrocytes are found (Raff et al., 1983b; 1984a). Furthermore, if optic nerve cells are surface-labelled in suspension with either anti-Ran-2 antibody or A2B5 antibody and then allowed to differentiate in culture (Raff et al., 1984a), the great majority of oligodendrocytes and type-2 astrocytes, but very few type-1 astrocytes, are found to develop from $A2B5^+/Ran\text{-}2^-$ cells. Conversely, type-1 astrocytes, but not oligodendrocytes or type-2 astrocytes, develop from $Ran\text{-}2^+$ cells. Thus, these results suggest that there are two

separate glial cell lineages in the optic nerve.

There is indirect evidence that oligodendrocytes or their precursors are able to migrate long distances in the CNS. For example, when pieces of normal mouse brain are transplanted into the brains of mutant shiverer mice that are unable to synthesize myelin basic protein (MBP), MBP^+ myelin is found long distances from the grafts (La Chapelle et al., 1984). Following this, several studies have provided circumstantial evidence that O-2A progenitor cells do not develop in the optic nerve but migrate into it from a germinal zone(s) elsewhere in the brain. Time-lapse microcinematographic studies have shown that O-2A progenitor cells in culture, identified by their characteristic bipolar morphology, are highly motile until they differentiate into oligodendrocytes, at which point locomotion stops (Small et al., 1987). In E16 rats, O-2A progenitor cells are found only at the chiasm-end of the nerve, at birth they are found in much smaller numbers at the eye-end of the nerve compared to the chiasm-end, and it is not until the second postnatal week that progenitors are evenly distributed along the length of the nerve (Small et al., 1987). Type-1 astrocytes, however, first develop at the eye-end of the optic nerve, making it unlikely that the gradient of O-2A progenitor cells in the opposite direction reflects a gradient of differentiation along the nerve (Small et al., 1987). These studies suggest that the neuroepithelial cells of the optic stalk give rise only to type-1 astrocytes.

The identification of a CNS progenitor cell that is bipotential *in vitro* has allowed the investigation of the molecular mechanisms responsible for the differentiation of this cell. The first clues as to how the direction and timing of differentiation are controlled have been provided by comparing the behaviour of O-2A progenitor cells *in vivo* and *in vitro*. Whereas autoradiographic studies have shown that O-2A progenitor cells *in vivo* proliferate extensively before differentiating into postmitotic oligodendrocytes (Skoff *et al.*, 1976a,b; Noble and Murray, 1984) or type-2 astrocytes (Miller *et al.*, 1985), O-2A progenitor cells dissociated from the developing optic nerve and placed in culture stop dividing prematurely and differentiate within 2 days: if cultured in 10% fetal calf serum (FCS) they become type-2 astrocytes; if cultured without FCS they become oligodendrocytes (Raff *et al.*, 1983b, 1984b). These findings indicate that both the timing and direction of O-2A progenitor cell differentiation can be dramatically influenced by environmental signals.

The oligodendrocyte pathway of differentiation seems to be the constitutive pathway, which is triggered automatically when a progenitor cell is deprived of signals from other cells: if a single progenitor cell is cultured alone in a microwell, it stops dividing prematurely and differentiates into an oligodendrocyte (Temple and Raff, 1985). Type-2 astrocyte differentiation *in vitro*, by contrast, requires the presence of inducing factors, some of which are released by type-1 astrocytes and begin to

operate in the second postnatal week (Temple and Raff, 1985; Hughes and Raff, 1987; Hughes et al., 1988; Lillien et al., 1988; Lillien and Raff, 1990). Thus, both cell-cell interactions and an intrinsic cellular programme contribute to the process of cell diversification in the optic nerve.

Several growth factors have been identified that appear to take part in the cell-cell interactions during glial cell development in the rat optic nerve (reviewed in Raff, 1989). Platelet-derived growth factor (PDGF) plays an important role both in promoting O-2A progenitor cell proliferation (Noble et al., 1988; Richardson et al., 1988; Pringle et al., 1989) and in timing oligodendrocyte differentiation in vitro (Raff et al., 1988). The progenitor cells are thought to have a built-in timing mechanism that determines when the cells lose mitotic responsiveness to PDGF, and as a consequence, differentiate into oligodendrocytes (Raff et al., 1985; Temple and Raff, 1986; Raff et al., 1988). The loss of mitotic responsiveness to growth factor is associated with the terminal differentiation of precursor cells in many in vitro systems, although little is known about the molecular mechanisms that underlie this process. In this thesis, I examine these mechanisms in O-2A progenitor cells in vitro and look at the relationship between mitosis and oligodendrocyte differentiation in vivo.

OBJECTIVES

(1) To examine the molecular basis of the intrinsic timing mechanism that causes O-2A progenitor cells to stop dividing and differentiate into oligodendrocytes in the continuing presence of PDGF by:

(a) establishing whether PDGF acts directly on O-2A progenitor cells or indirectly via an intermediate cell.

(b) studying the nature and distribution of PDGF receptors on optic nerve cells.

(c) manipulating intracellular signalling pathways in both progenitor cells and oligodendrocytes.

(d) examining cytoplasmic and nuclear responses to PDGF in both progenitor cells and oligodendrocytes.

(2) To determine whether O-2A progenitor cells <u>in vivo</u> stop dividing before they differentiate into oligodendrocytes, just as they do <u>in vitro</u>, by studying DNA synthesis.

CHAPTER 2

PDGF RECEPTORS ON CELLS OF THE O-2A LINEAGE

Introduction

The normal timing of oligodendrocyte differentiation can be reconstituted *in vitro* by co-culturing embryonic O-2A progenitor cells with purified type-1 astrocytes, or by adding medium conditioned by type-1 astrocytes (ACM) (Noble and Murray, 1984; Raff *et al.*, 1985). Under these conditions the progenitor cells continue to divide, oligodendrocytes first differentiate on the equivalent of the day of birth, and new postmitotic oligodendrocytes continue to develop from dividing progenitor cells for at least two weeks, just as they do *in vivo* (Raff *et al.*, 1985). It is thus clear that type-1 astrocytes play an important role in the development of O-2A lineage cells. These observations give rise to two main questions. How do type-1 astrocytes stimulate O-2A progenitor cells to divide and how do they influence the timing of oligodendrocyte development in culture? Four lines of evidence suggest that these effects of type-1 astrocytes are mediated by platelet-derived growth factor (PDGF). (1) Purified PDGF can replace type-1 astrocytes or ACM, both in stimulating the proliferation of O-2A progenitor cells (Noble *et al.*, 1988; Richardson *et al.*, 1988) and in reconstituting the normal timing of oligodendrocyte development in culture (Raff *et al.*, 1988). (2) Cultures of purified type-1 astrocytes secrete PDGF and contain messenger RNA encoding the A chains of PDGF (Richardson *et al.*, 1988). (3) If ACM is fractionated by gel filtration, the mitogenic activity

for O-2A progenitor cells is found to be in the same fractions as radiolabelled PDGF (Richardson et al., 1988). (4) Anti-PDGF antibodies inhibit the ability of ACM, both to stimulate O-2A progenitor cell proliferation (Noble et al., 1988; Richardson et al., 1988) and to reconstitute the normal timing of oligodendrocyte development in vitro (Raff et al., 1988). Although it remains to be demonstrated that PDGF functions in these ways in vivo, it is apparently present in the developing optic nerve: extracts of developing optic nerve stimulate O-2A progenitor cells to divide in culture and most of this activity is inhibited by anti-PDGF antibodies (Raff et al., 1988).

How does PDGF contribute to the normal timing of oligodendrocyte differentiation? Experiments in vitro suggest that PDGF keeps O-2A progenitor cells dividing until an intrinsic timer in these cells initiates the process leading to oligodendrocyte differentiation (Raff et al., 1985). For example, clonal analyses show that, when stimulated to divide by type-1 astrocytes or PDGF, the descendants of a single progenitor cell usually differentiate more or less synchronously after the same number of divisions (Temple and Raff, 1986; Raff et al., 1988). The nature of the timer is unknown, but it apparently controls when O-2A progenitor cells become unresponsive to PDGF: even if an excess of PDGF is added to cultures of embryonic optic nerve, progenitor cells stop dividing and differentiate into oligodendrocytes on schedule (Raff et al., 1988).

These observations raise a number of questions about the response of O-2A progenitor cells to PDGF. Does PDGF act directly on progenitor cells or indirectly via an intermediary cell? What is the nature of the PDGF receptor expressed by the PDGF-responsive cells in the optic nerve? Does the initial loss of mitotic responsiveness to PDGF reflect a loss of PDGF receptors on O-2A progenitor cells, or is the block in the response pathway distal to the receptors? Two types of PDGF receptors have been identified on cells of the human foreskin fibroblast line AG 1523 (Heldin et al., 1988): α PDGF receptors bind both PDGF A- and B-chains with high affinity and, therefore, bind all three forms of dimeric PDGF (AA, BB and AB), while β receptors bind PDGF B- but not A-chains, and therefore, bind PDGF-BB with high affinity, PDGF-AB with lower affinity, and do not bind PDGF-AA (Heldin et al., 1988; Hart et al., 1988; Escobedo et al., 1988a; Gronwald et al., 1988; Claesson-Welsh et al., 1988). Both α- (Matsui et al., 1989) and β- (Yarden et al., 1986; Claesson-Welsh et al., 1988; Gronwald et al., 1988) PDGF receptors have now been cloned. Further work using purified PDGF receptors has suggested that a given PDGF dimer binds to, and cross-links, two receptor molecules (Heldin et al., 1989), and studies looking at the effect of PDGF-BB on actin reorganization in fibroblasts have indicated that PDGF receptors function as dimers (Hammacher et al., 1989). It is proposed that both PDGF-AA and -AB dimers can crosslink α PDGF receptors to other α or β receptors to form $\alpha\alpha$ and

$\alpha\beta$ receptor complexes, and PDGF-BB can crosslink β to other β or α receptors, and α to other α receptors to form $\beta\beta$, $\alpha\beta$, and $\alpha\alpha$ receptor complexes (Seifert et al. 1989).

In this chapter, I have attempted to answer the above questions by analyzing the nature and distribution of PDGF receptors on optic nerve cells in vitro, using ^{125}I-PDGF. I show that O-2A progenitor cells express PDGF receptors (which resemble the α receptors on human fibroblasts in their ability to bind all three forms of PDGF), suggesting that PDGF acts directly on these cells. I also show that many newly formed oligodendrocytes express PDGF receptors although they do not divide in response to PDGF; this makes it unlikely that receptor loss is the reason that progenitor cells initially lose their mitotic responsiveness to PDGF.

Results

^{125}I-PDGF binds mainly to O-2A lineage cells in perinatal optic nerve cultures as determined by autoradiography

Newborn (P0) optic nerve cells were cultured for 1 day, incubated with ^{125}I-human PDGF (hPDGF) (33 ng ml^{-1}), and then labelled with the monoclonal A2B5 antibody to identify the O-2A lineage cells (Raff et al., 1983b; Temple and Raff, 1986), the monoclonal anti-galactocerebroside (GC) antibody to identify the oligodendrocytes (Raff et al., 1978) or with anti-glial fibrillary acidic protein (GFAP) antiserum to identify astrocytes (Bignami et al., 1972). Cells were judged to be radiolabelled if there were more than 10 silver grains over them. 61 ± 2% of O-2A progenitor cells and 50 ± 1% of oligodendrocytes, but no type-1 astrocytes, were radiolabelled (Fig. 2.1). Less than 5% of the non-macroglial cells (mainly meningeal cells, macrophages and endothelial cells) were radiolabelled. Therefore, most of the cells that bound ^{125}I-PDGF belonged to the O-2A cell lineage (Table 2.1). The percentage of radiolabelled O-2A progenitor cells increased with increasing concentrations of ^{125}I-hPDGF, reaching a plateau at around 30 ng ml^{-1} (Fig. 2.2). To demonstrate the specificity of binding, the cells were incubated with 33 ng ml^{-1} of ^{125}I-hPDGF in the presence of a hundred-fold excess of unlabelled hPDGF, basic fibroblast growth factor (bFGF) or epidermal growth factor (EGF). While bFGF and EGF had no detectable effect on the

binding, unlabelled hPDGF greatly reduced the percentage of radiolabelled cells (Table 2.1) as well as the average number of silver grains over each radiolabelled cell: for O-2A progenitors the average number of grains/cell fell from greater than 30 to 17 ± 5 (average of 100 cells). It was not possible to count the number of grains when more than about 30 were present over a cell.

Freshly isolated O-2A progenitor cells also bind radiolabelled PDGF

To determine whether PDGF receptors are present on O-2A progenitor cells *in vivo* as well as *in vitro*, cells were dissociated from P0 optic nerves without trypsin and immediately incubated with ^{125}I-hPDGF (33 ng ml^{-1}), with or without a hundred-fold excess of unlabelled hPDGF. A mean of 66 ± 1% of the A2B5^{+} cells were radiolabelled in the absence of unlabelled PDGF, while 11 ± 2% were radiolabelled and the average number of silver grains overlying the cells was lower in its presence (200 cells were counted on each of 3 coverslips). When P0 optic nerves were dissociated with trypsin in the normal way, and then immediately incubated with ^{125}I-hPDGF, no radiolabelled cells were seen, indicating that the PDGF receptors on optic nerve cells are trypsin-sensitive, as previously shown for other cell types (Bowen-Pope and Ross, 1985).

Scatchard plot analysis of ^{125}I-hPDGF binding to O-2A lineage cells in culture

The interaction between PDGF and its receptors was quantified by Scatchard analysis of binding data obtained on optic nerve cultures by gamma-counting. There are at least two problems with such studies on optic nerve cultures: it is difficult to obtain large numbers of cells as dissociation of one optic nerve yields about 10^4 cells (compared with PDGF binding studies using fibroblasts where 10^6 cells are used for each point in the analysis; Heldin et al., 1982) and the cultures contain a number of different cell types. It has been shown previously that relatively few type-1 astrocytes are released from ≥ postnatal day-7 (P7) optic nerves by the dissociation procedure used (Miller et al., 1985). Therefore, in order to maximize the number of O-2A progenitor cells for analysis, I used postnatal day-7 (P7) optic nerve cells cultured for one day with hPDGF (1 ng ml^{-1}). This low concentration of PDGF would not be expected to significantly downregulate PDGF receptors (Heldin et al., 1982), but is sufficient to keep O-2A progenitor cells dividing and to prevent their premature differentiation into oligodendrocytes (Noble et al., 1988; Richardson et al., 1988). Each of these cultures contained about 4000 O-2A progenitor cells, about 1500 oligodendrocytes, and no detectable type-2 astrocytes. When the cultures were incubated in ^{125}I-hPDGF and studied by autoradiography, 84 ± 3% of the O-2A progenitors and 43 ±

4% of the oligodendrocytes were radiolabelled; non O-2A lineage cells accounted for less than 7% of the radiolabelled cells. Therefore, it is likely that more than 90% of the binding of ^{125}I-hPDGF assessed by gamma-counting represented binding to O-2A progenitor cells and oligodendrocytes.

When cultures of P7 optic nerve cells were incubated with increasing concentrations of ^{125}I-hPDGF and then assayed by gamma-counting, the binding tended to saturate at 25 - 40 x 10^{-10} M and was half-maximal at 3 - 4 x 10^{-10} M (Fig. 2.3A). The Scatchard plot of the binding data (Scatchard, 1949) fitted a single straight line (Fig. 2.3B), suggesting a single class of receptor. The apparent K_d was about 3.45 x 10^{-10} M and the average number of receptors per cell was about 2.2 x 10^5 (Fig. 2.3B). To assess the influence of 10% FCS on the binding of ^{125}I-hPDGF, a 3-point assay was done in the absence of FCS: the binding increased about 1.4-fold in the absence of FCS (Fig. 2.4).

O-2A progenitor cells have α-like PDGF receptors

The results obtained with ^{125}I-PDGF-AA and ^{125}I-PDGF-BB in autoradiography experiments on 1-day-old cultures of P0 optic nerve cells (Table 2.2) were very similar to those obtained with ^{125}I-hPDGF (see Table 2.1): with all three ligands 60 - 65% of the O-2A progenitor cells were radiolabelled and 86 - 90% of the radiolabelling was displaceable by an excess of hPDGF. Moreover, in gamma-

counting experiments on 1-day-old cultures of P0 optic nerve cells, the binding of ^{125}I-PDGF-AA and the binding of ^{125}I-PDGF-BB were similar and both ligands were displaced about equally (range 84 - 92%) by both unlabelled PDGF-AA and unlabelled PDGF-BB (Table 2.3). These results suggest that the PDGF receptors on rat O-2A lineage cells resemble the α PDGF receptors described previously on human fibroblasts; these receptors bind the three PDGF dimers about equally while β receptors on fibroblasts bind PDGF-BB better than PDGF-AB, and do not bind PDGF-AA (Heldin et al., 1988; Hart et al., 1988; Escobedo et al., 1988a; Gronwald et al., 1988; Claesson-Welsh et al., 1988).

To further characterize the binding specificity of the PDGF receptors on O-2A progenitor cells, I carried out receptor downregulation experiments. Binding of a PDGF dimer to its cell-surface receptors leads to the depletion of PDGF binding sites by both receptor occupation and internalization and degradation of the ligand-receptor complex (Heldin et al., 1982). This phenomenon is called downregulation and it has been used previously to identify the type of PDGF receptor present on fibroblasts (Heldin et al., 1988; Hart et al., 1988). Preincubation of P0 optic nerve cultures with either unlabelled PDGF-AA or unlabelled PDGF-BB for 1 hour at 37 °C (followed by acid washing to remove any receptor-bound unlabelled PDGF exposed on the cell surface) reduced the proportion of O-2A progenitor cells that bound ^{125}I-PDGF-AA or ^{125}I-PDGF-BB by 60 - 80%, as assessed by autoradiography (Table 2.4). PDGF-AA was just

as efficient as PDGF-BB in down-regulating the receptors that bound ^{125}I-PDGF-BB, confirming that the PDGF receptors on O-2A progenitor cells resemble the α PDGF receptor on human fibroblasts, at least in terms of their ligand specificity.

Some oligodendrocytes and type-2 astrocytes that develop in culture express PDGF receptors

To determine if the loss of O-2A progenitor cell mitotic responsiveness to PDGF results from a loss of PDGF receptors, P0 optic nerve cells were cultured in a low, but mitogenic, concentration (1 ng ml^{-1}) of unlabelled hPDGF. After 1 day, the binding of ^{125}I-hPDGF to oligodendrocytes was determined by autoradiography. Presumably all of the oligodendrocytes in these cultures will have differentiated on schedule, having lost responsiveness to PDGF (Raff et al., 1985, 1988; and see below). Yet in 3 experiments, 35 out of 73 oligodendrocytes (47 ± 4%) were radiolabelled, suggesting that the loss of PDGF receptors is not a prerequisite for oligodendrocyte differentiation. When P0 optic nerve cells were studied after 3 days in culture without PDGF to maximize the differentiation of oligodendrocytes, only 12 ± 1% of the oligodendrocytes were radiolabelled, suggesting that oligodendrocytes lose PDGF receptors with time.

In order to study PDGF receptor expression on type-2 astrocytes, P7 optic nerve cells were cultured for 3 days

in 10% FCS to induce most of O-2A progenitor cells to differentiate into type-2 astrocytes (Raff et al., 1983b, 1984b). The cells were then incubated with ^{125}I-hPDGF and analyzed by autoradiography. In 3 experiments, 74 ± 6% of the type-2 astrocytes were radiolabelled. Thus like oligodendrocytes, most type-2 astrocytes retain PDGF receptors, at least initially, after they differentiate in culture.

Oligodendrocytes do not divide in response to PDGF

In order to determine whether PDGF can stimulate newly formed oligodendrocytes to synthesize DNA, P0 optic nerve cells were cultured with hPDGF (2 ng ml^{-1}) for 24 hours and for the last 12 hours of the culture period were exposed to the thymidine analogue bromodeoxyuridine (BrdU), which is incorporated into replicating DNA (Gratzner, 1982). The cells were then double-labelled by indirect immunofluorescence with the anti-GC antibody to identify the oligodendrocytes and with monoclonal anti-BrdU antibody to identify cells that had incorporated BrdU into DNA (Magaud et al., 1988). Although more that 15% of the cells in the cultures were $BrdU^+$, all of the oligodendrocytes were $BrdU^-$ (239 oligodendrocytes were examined in 6 cultures). These results suggest that PDGF is not mitogenic for newly formed oligodendrocytes, even though many of them have PDGF receptors on their surface.

When BrdU was added to cultures for 24 hours, 5% of

the oligodendrocytes were $BrdU^+$; these presumably developed from O-2A progenitor cells that had incorporated BrdU before they differentiated into oligodendrocytes.

Figure 2.1

Immunofluorescence autoradiographs of ^{125}I-PDGF binding to cells in 1-day-old cultures of P0 optic nerve. Cells were incubated in ^{125}I-hPDGF, stained with A2B5 antibody followed by G anti-MIg-Rd (A-C) or with anti-GC antibody followed by G anti-MIgG3-Fl (D-F), and then photographed using phase contrast (B,C,E,F), rhodamine (A) or fluorescein (D) optics. Note that three of the four A2B5$^+$ O-2A progenitor cells shown in (A) are seen to be radiolabelled when viewed at different planes of focus in (B) and (C) [arrows]; one A2B5$^-$ cell is also radiolabelled. The GC$^+$ oligodendrocyte shown in (D) is seen to be radiolabelled when viewed at different depths of focus in (E) and (F), while the GC$^-$cells in the field are not radiolabelled. Scale bar, 20 μm.

Figure 2.1

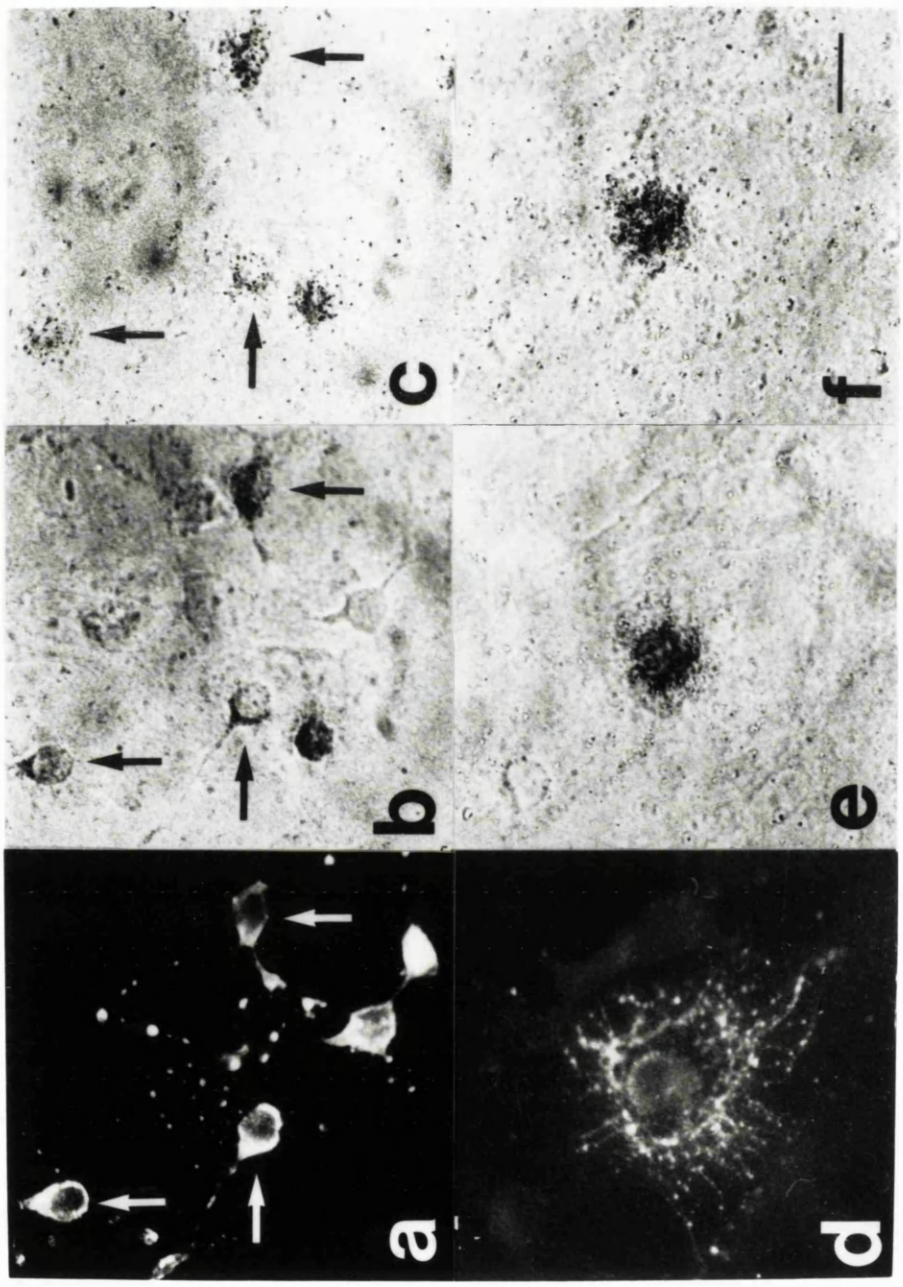


Figure 2.2

Binding of ^{125}I-hPDGF to O-2A progenitor cells in 1-day-old cultures of P0 optic nerve, as determined by autoradiography. The results are expressed as means ± SEM of 3 cultures. At those points where error bars are not shown in this and the following Figures, the SEM was less than the diameter of the symbol.

Fig. 2.2

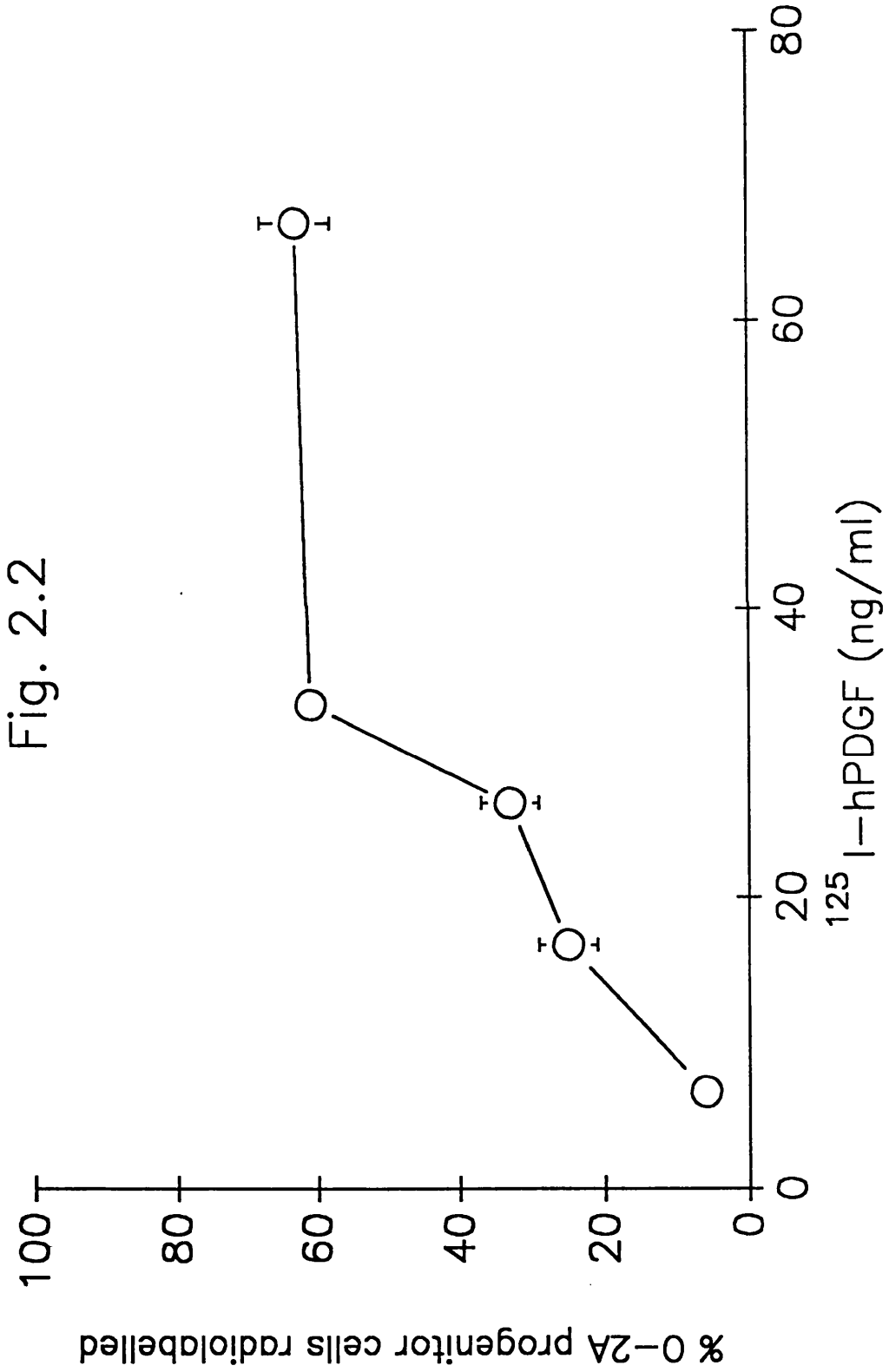

% 0-2A progenitor cells radiolabelled
100
80
60
40
20
0
0
20
40
60
80
^{125}I-hPDGF (ng/ml)

Figure 2.3

Scatchard analysis of the binding of ^{125}I-hPDGF to 1-day-old cultures of P7 optic nerve cells, as determined by gamma-counting. (A) The concentration dependence of ^{125}I-hPDGF binding. Non-specific binding (<7%), as determined by adding a 500-fold molar excess of unlabelled hPDGF, was subtracted from the results, which are expressed as means ± SEM. (B) Scatchard plot of data shown in (A).

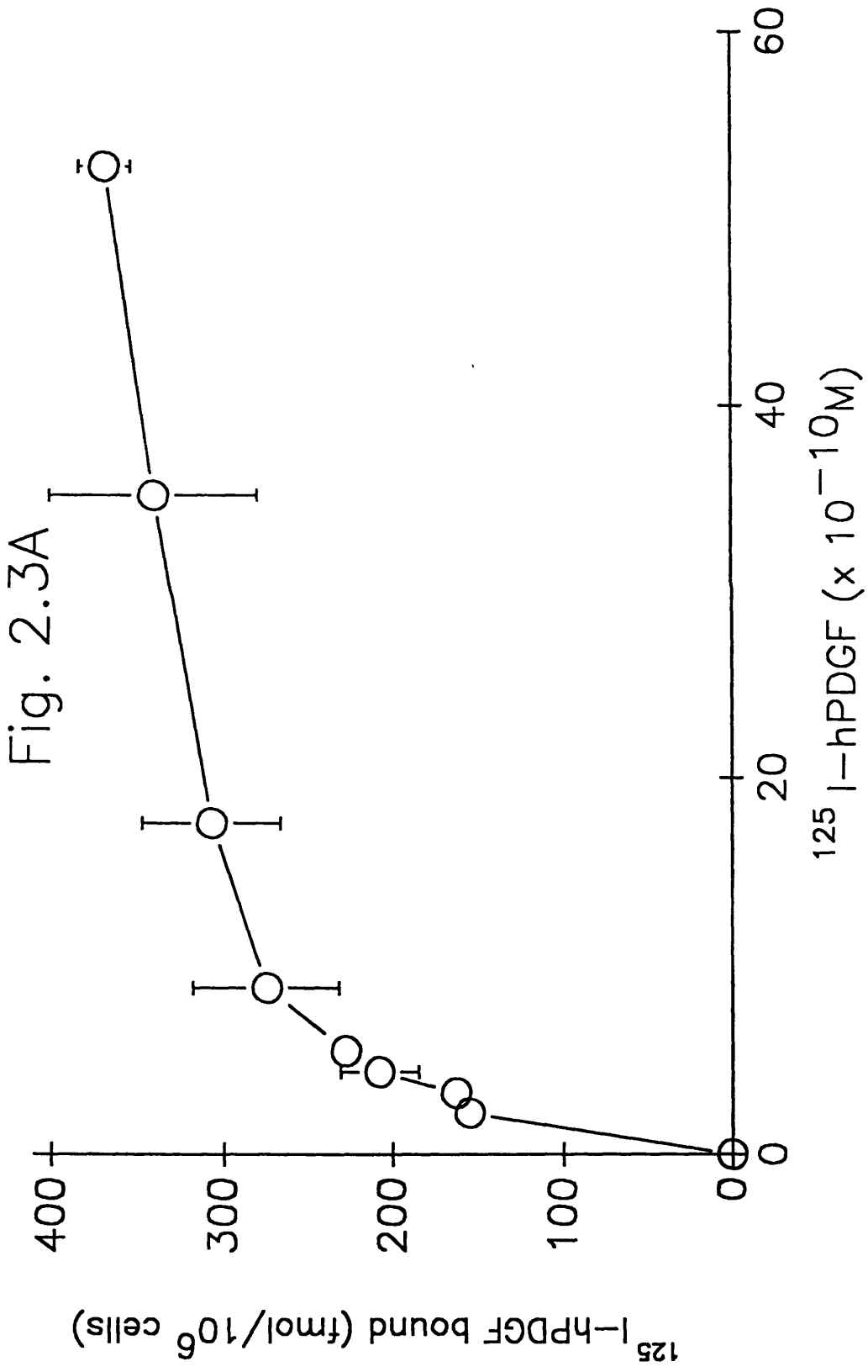

400
300
200
100
0
0
20
40
60
^{125}I–hPDGF bound (fmol/10^6 cells)
^{125}I–hPDGF (x 10^{-10}M)

Fig. 2.3A

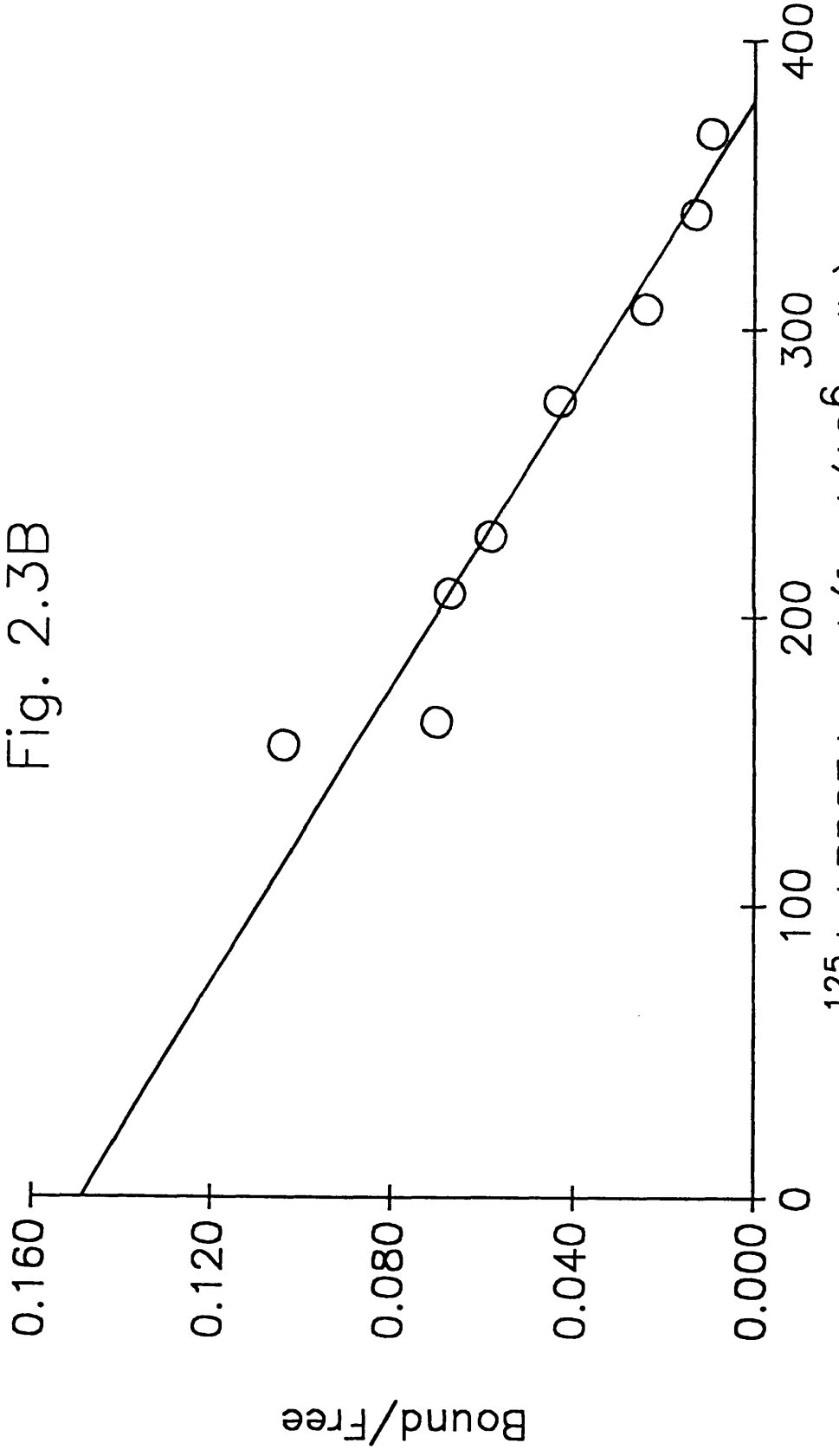

Bound/Free
0.160
0.120
0.080
0.040
0.000
0
100
200
300
400
^{125}I-hPDGF bound (fmol/10^6 cells)

Fig. 2.3B

Figure 2.4

Effect of 10% FCS on binding of ^{125}I-hPDGF to 1-day-old cultures of P7 optic nerve cells, as determined by gamma-counting. (a) Binding in serum-free medium (▲--▲). (b) Binding in medium containing 10% FCS (O--O). Non-specific binding (<5%), as determined by adding a 500-fold molar excess of unlabelled hPDGF, was subtracted from the results, which are expressed as means ± SEM.

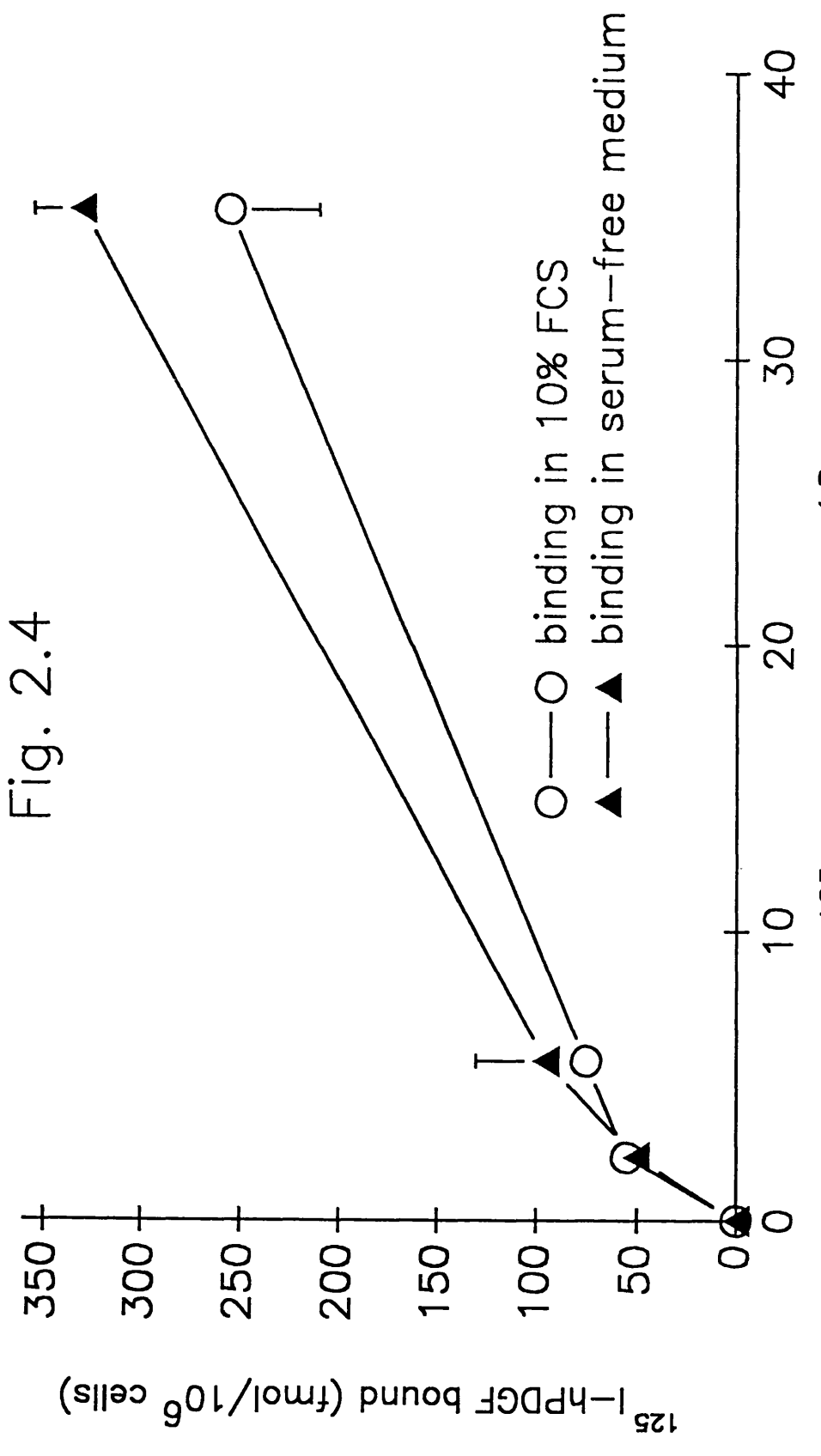


Fig. 2.4
^{125}I–hPDGF bound (fmol/10^6 cells)
350
300
250
200
150
100
50
0
0
10
20
30
40
^{125}I–hPDGF (x 10^{-10}M)
O—O binding in 10% FCS
▲—▲ binding in serum–free medium

Table 2.1 Binding of ^{125}I-hPDGF to cells in 1-day-old cultures of P0 optic nerve, as determined by autoradiography

	Percentage of cells radiolabelled	
Cell type	In absence of unlabelled PDGF	In presence of unlabelled PDGF
Type-1 astrocytes	0	0
O-2A progenitor cells	61 ± 2	7 ± 2
Oligodendrocytes	50 ± 1	8 ± 2
Non-macroglial cells	<5	<1

The cells were incubated in ^{125}I-hPDGF (33 ng ml^{-1}), with or without a 100-fold excess of unlabelled hPDGF, immunolabelled, and processed for autoradiography as described in Materials and methods. In this and the following 3 tables the results are expressed as means ± SEM of at least 3 cultures.

Table 2.2 Binding of ^{125}I-PDGF-AA and ^{125}I-PDGF-BB to O-2A progenitor cells in 1-day-old cultures of P0 optic nerve, as determined by autoradiography

Unlabelled hPDGF added	Percentage of O-2A progenitor cells radiolabelled with	
	^{125}I-PDGF-AA	^{125}I-PDGF-BB
No	65 ± 2	63 ± 2
Yes	9 ± 5	6 ± 3

Cells were incubated in ^{125}I-PDGF (33 ng ml^{-1}), with or without a 100-fold excess of unlabelled hPDGF, immunolabelled, and processed for autoradiography as described in Materials and methods.

Table 2.3 Effects of unlabelled PDGF-AA and PDGF-BB on the binding of ^{125}I-PDGF-AA and ^{125}I-PDGF-BB to 1-day-old cultures of P0 optic nerve, as determined by gamma-counting

Unlabelled PDGF added	Percentage of maximal binding of ^{125}I-PDGF-AA	Percentage of maximal binding of ^{125}I-PDGF-BB
None	100	100
AA	8 ± 4	15 ± 8
BB	13 ± 7	16 ± 3

Cells were incubated in ^{125}I-PDGF (33 ng ml^{-1}), with or without a 100-fold excess of unlabelled PDGF, as described in Materials and methods. The average binding in the absence of unlabelled PDGF was taken as 100% and was 890 cts min^{-1} per culture for ^{125}I-PDGF-AA and 600 cts min^{-1} for ^{125}I-PDGF-BB. When this experiment was performed, the specific activities of ^{125}I-PDGF-AA and ^{125}I-PDGF-BB had fallen to about half their original value and were about 26,000 cts min^{-1} ng^{-1} and about 21,000 cts min^{-1} ng^{-1}, respectively.

Table 2.4 Effects of downregulation of PDGF receptors by unlabelled PDGF-AA and PDGF-BB on binding of ^{125}I-PDGF-AA and ^{125}I-PDGF-BB to O-2A progenitor cells in 1-day-old cultures of P0 optic nerve, as determined by autoradiography

Downregulation with unlabelled PDGF (100ng ml^{-1})	Percentage of O-2A progenitor cells radiolabelled with	
	^{125}I-PDGF-AA	^{125}I-PDGF-BB
None	33 ± 3	38 ± 6
PDGF-AA	7 ± 3	9 ± 2
PDGF-BB	11 ± 4	17 ± 2

The cells were pre-incubated with either unlabelled PDGF-AA or unlabelled PDGF-BB for 1 hour, and then incubated in ^{125}I-PDGF-AA or ^{125}I-PDGF-BB (33 ng ml^{-1}), immunolabelled, and processed for autoradiography as described in Materials and methods.

Table 2.5 Antisera

TYPE	ANTIBODY	ABBREVIATION	DILUTION	REFERENCE/DONOR
Monoclonal	A2B5		Ascites Fluid 1:100 or 1:200	Eisenbarth et al., 1979
	Anti-Bromodeoxyuridine	BrdU	Ascites Fluid 1:2 or 1:5	Magaud et el., 1988
	Anti-Bromodeoxyuridine-Fluorescein	BrdU-Fl	1:2	Beckton Dickinson
	Anti-Galactocerebroside	GC	Ascites Fluid 1:100 or 1:200	Rancht et al., 1982
Polyclonal	Anti-Fos		1:1000	Hunt et al., 1987
	Anti-glial fibrillary acid protein	Anti-GFAP	1:100	Pruss, 1979
	Anti-Jun		1:500	Bos et al., 1988
Conjugates	Goat anti-mouse Ig-rhodamine	G anti-MIg-Rh	1:100	Cappel
	Goat anti-mouse Ig-flourescein	G anti-MIg-Fl	1:100	Cappel
	Goat anti-mouse IgG3-fluorescein	G anti-MIgG3-Fl	1:100	Nordic
	Goat anti-mouse IgG3-rhodamine	G anti-MIgG3-Rh	1:100	Nordic
	Donkey anti-rabbit-Ig-biotin	D anti-RIg-Bt	1:100	Amersham
	Sheep anti-rabbit-Ig-fluorescein	S anti-RIg-Fl	1:100	Wellcome
	Sheep anti-mouse-Ig-biotin	S anti-MIg-Bt	1:100	Amersham

Discussion

PDGF probably acts directly on O-2A progenitor cells

I have demonstrated that most O-2A progenitor cells in the developing rat optic nerve have PDGF receptors on their surface. Moreover, most of the cells in cultures of newborn rat optic nerve that have detectable PDGF receptors are O-2A lineage cells: type-1 astrocytes and the great majority of non-macroglial cells in these cultures did not bind detectable amounts of ^{125}I-PDGF. These findings suggest that PDGF acts directly on O-2A progenitor cells to stimulate their proliferation and prevent their premature differentiation into oligodendrocytes (Richardson et al., 1988; Noble et al., 1988; Raff et al., 1988).

Scatchard analysis of PDGF binding to mainly O-2A lineage cells in P7 optic nerve cultures suggests that these cells have a single class of high affinity receptors with a K_d of at most 3.5×10^{-10} M. While this value falls within the reported range of K_ds (10^{-9} - 10^{-11} M) for PDGF receptors on other cell types (Bowen-Pope and Ross, 1985), it is about 20-fold greater than the concentration of PDGF required for half-maximal stimulation of O-2A progenitor cell proliferation in culture (about 0.2×10^{-11} M) (Richardson et al., 1988). While at least some of this discrepancy is probably caused by technical problems in the binding assays, such as the presence of 10% FCS and perhaps a loss of labelled ligand during washing, it raises the

possibility that O-2A progenitor cells, like human fibroblasts (Heldin et al., 1981), have spare PDGF receptors, so that only a small fraction of the receptors on a cell need to be occupied to produce a maximal proliferative response.

Dissociation of bound ^{125}I-PDGF during the multiple washes required for immunostaining may account, at least in part, for the finding that only 60-65% of the O-2A progenitor cells in the newborn optic nerve were labelled by the radioactive PDGF in autoradiographs; about 80% of progenitor cells in the newborn optic nerve can be stimulated by PDGF in culture to synthesize DNA (M.C. Raff, personal communication). Scatchard analysis of the binding data generated by gamma-counting without immunolabelling indicated that each O-2A lineage cell, on average, has about 2.2×10^5 receptors (which is similar to the estimate of 3×10^5 PDGF receptors on human foreskin fibroblasts in culture, Heldin et al., 1981), while with immunolabelling the maximum number of silver grains detected by autoradiography over a radiolabelled cell was only about 100. Thus, the combination of immunostaining and autoradiography was relatively inefficient at detecting PDGF receptors and the proportions of radiolabelled cells determined in these studies should be taken as minimal estimates.

O-2A progenitor cells express α-like PDGF receptors

Two types of PDGF polypeptides, A-chains and B-chains, have been described (Johnsson et al., 1982), each encoded by a separate gene (Dalla Favera et al., 1982; Betsholtz et al., 1986). At least three forms of PDGF occur naturally, each consisting of two polypeptide chains bound together by disulphide bonds (Johnsson et al., 1982): human platelets, for example, contain mainly PDGF-AB (Hammacher et al., 1988b), some human osteosarcoma (Heldin et al., 1986), melanoma (Westermark et al., 1986) and glioma (Hammacher et al., 1988a) cell lines make PDGF-AA, while PDGF isolated from porcine platelets resembles PDGF-BB (Stroobant and Waterfield, 1984). In addition, there are two types of PDGF receptors that were first distinguished on human fibroblasts by their size, ligand specificity, and by reactivity with monoclonal antibodies (Heldin et al., 1988; Hart et al., 1988). The α PDGF receptor binds all three PDGF dimers, whereas the β receptor binds PDGF-BB with high affinity, PDGF-AB with lower affinity, and does not bind PDGF-AA (Heldin et al., 1988; Hart et al., 1988; Escobedo et al., 1988a; Claesson-Welsh et al., 1988). DNA sequencing studies indicate that there are two distinct PDGF receptor proteins: an α type (Matsui et al., 1989) and a β type (Yarden et al., 1986; Claesson-Welsh et al., 1988; Gronwald et al., 1988) that are both single-pass, transmembrane tyrosine kinases, like several other growth factor receptor proteins (Yarden and Ullrich, 1988).

Two findings in the present study indicate that, in terms of ligand specificity, the PDGF receptors on O-2A progenitor cells in the newborn rat optic nerve resemble fibroblast α PDGF receptors. First, iodinated PDGF-AA, PDGF-BB and hPDGF all bound about equally to O-2A progenitor cells, and the binding could be displaced about equally by an excess of unlabelled PDGF-AA, PDGF-BB or hPDGF. Second, preincubation with either unlabelled PDGF-AA or PDGF-BB were equally effective at downregulating most of the PDGF receptors on O-2A progenitor cells, as assessed by the binding of either ^{125}I-PDGF-AA or ^{125}I-PDGF-BB in autoradiographs.

Separate functional studies indicate that α PDGF receptors are mainly, and perhaps exclusively, responsible for mediating the proliferative response of O-2A progenitor cells to PDGF: PDGF-AA is at least as potent as PDGF-AB in stimulating progenitor cell proliferation in cultures of P7 optic nerve, whereas PDGF-BB is 5- to 10-fold less potent (Pringle et al., 1989). Type-1 astrocytes in culture (Richardson et al., 1988) and probably in vivo (Pringle et al., 1989) make PDGF A-chain mRNA but little, if any, B-chain mRNA. Also newborn rat brain O-2A progenitor cells express α PDGF receptor protein, but not β receptor protein, on Western blot analysis (McKinnon et al., 1990). All of these findings, taken together with the present binding studies, make a persuasive case for a crucial role for PDGF-AA acting via α PDGF receptors, in the stimulation of O-2A progenitor cell proliferation in the developing rat

optic nerve. The rat CNS O-2A progenitor cell is the first known cell to express only the α PDGF receptor. By contrast, only the β receptors seem to mediate the proliferative response of human fibroblasts (Heldin et al., 1988; Hart et al., 1988).

Receptor loss is not the reason O-2A progenitor cells become unresponsive to PDGF

Although O-2A progenitor cells are stimulated to proliferate by PDGF in cultures of perinatal optic nerve, they do not proliferate indefinitely. Even in the presence of an excess of PDGF, the progenitor cells eventually stop dividing and (if the concentration of FCS is less than 1%) differentiate into oligodendrocytes that are resistant to the mitogenic effects of PDGF (Raff et al., 1988). The present results suggest that the molecular mechanism responsible for this loss of mitogenic responsiveness is not the loss of PDGF receptors: at least half of the oligodendrocytes that developed in cultures of newborn optic nerve, either in the presence or absence of PDGF, had readily detectable PDGF receptors, even though these cells no longer divided in response to PDGF. It is thus likely that the block in mitotic responsiveness to PDGF lies downstream from the receptors. On the other hand, the proportion of oligodendrocytes that had PDGF receptors decreased about four-fold over three days in culture, suggesting that these cells eventually lose their PDGF

receptors. When O-2A progenitor cells were induced by 10% FCS to differentiate into type-2 astrocytes (Raff et al., 1983b), most of these newly formed astrocytes also retained their PDGF receptors.

In summary, I have provided evidence that O-2A progenitor cells respond directly to PDGF by means of α-like PDGF receptors and that receptor loss is not the reason that these cells eventually lose mitogenic responsiveness to PDGF.

Materials and methods

Cell culture

Optic nerves were dissected from decapitated P0 or P7 Sprague-Dawley rats and dissociated into single cells using a method modified from Miller *et al.* (1985). The nerves were cut into small pieces and incubated in air for 30 min at 37 °C in 1 ml of minimal Eagle's medium (MEM) with 0.02 M Hepes buffer (MEM-Hepes) and an equal volume of trypsin (type III, Sigma; 0.025% w/v) and collagenase (type 1A, Sigma; 0.04% w/v) in Ca^{2+}- and Mg^{2+}-free Dulbecco's modified Eagle's medium (DMEM) containing bovine serum albumin (fatty acid-free, Sigma; 0.25% w/v) (CMF-DMEM-BSA). After a second incubation for 15 min at 37 °C in 1 ml of the trypsin and collagenase solution and 1 ml EDTA (0.02% w/v), the nerves were dissociated in 1 ml DNAase I (Sigma; 0.04% w/v) and soyabean trypsin inhibitor (Sigma; 0.05% w/v) in CMF-DMEM-BSA by pipetting 10 times through a Pasteur pipette, followed by drawing gently 3 times through a 21-gauge and then a 23-gauge needle on a 2 ml syringe. The resulting cell suspension was washed in DMEM containing 10% FCS and centrifuged at 2500 rpm for 5 min. The supernatant was removed and the cells were then re-suspended in 1 ml DMEM containing D-glucose (5.6 mg ml^{-1}), bovine insulin (5 μg ml^{-1}), human transferrin (100 μg ml^{-1}), BSA (100 μg ml^{-1}), progesterone (60 ng ml^{-1}), putrescine (16 μg ml^{-1}), sodium selenite (40 ng ml^{-1}), L-thyroxine (40 ng ml^{-1}), and 3,3',5,tri-iodo-L-thyronine (30 ng ml^{-1}) (supplemented

DMEM), as modified from Bottenstein and Sato (1979) and described previously (Lillien et al., 1988).

Approximately 20,000 - 40,000 cells were plated in 20 µl drops on poly-D-lysine-coated (PDL) glass coverslips, which were placed in 24-well tissue culture plates (Falcon). The cells were allowed to adhere to the coverslips for 30 min at 37 °C in 5% CO_2 and supplemented DMEM with 0.5% FCS was added to a final volume of 0.5 ml. In some cases the cultures were treated with hPDGF (1 ng ml^{-1}). To study fresh suspensions of newborn optic nerve cells, the nerves were dissociated with EDTA and collagenase, in the absence of trypsin.

Growth factors

The major component of hPDGF is a heterodimer of one A and one B chain with variable proportions of the homodimers (Hammacher et al., 1988b). The hPDGF, PDGF-AA and PDGF-BB used in my binding studies (both labelled and unlabelled) were gifts from Carl Heldin. hPDGF was purified from human platelets and contained about 70% PDGF-AB and about 30% PDGF-BB. Recombinant PDGF-AA and PDGF-BB were purified from supernatants of yeast cells transfected with recombinant DNA vectors containing the coding sequence for the human PDGF A chain or B chain, as described previously (Östman et al., 1989). The hPDGF used to treat cultures was of unknown dimeric composition and was obtained from R and D Systems. bFGF and EGF were obtained from Collaborative Research. hPDGF and PDGF-BB were labelled with ^{125}I by the Bolton-

Hunter method (Bolton and Hunter, 1973) to a specific activity of about 37,000 cts min^{-1} ng^{-1} and about 57,000 cts min^{-1} ng^{-1}, respectively. PDGF-AA was labelled with ^{125}I by the chloramine-T method (Hunter and Greenwood, 1962) to a specific activity of about 64,000 cts min^{-1} ng^{-1}. Some experiments were carried out using human ^{125}I-PDGF of unknown dimeric composition (specific activity about 40,000 cts min^{-1} ng^{-1}) obtained from PDGF Inc.

Autoradiography

Cultures of optic nerve cells were washed once in DMEM containing 10% FCS, 0.1% BSA, and made up to pH 7.4 (binding buffer). The cells were then incubated for 1 hour at room temperature in 30 μl binding buffer containing ^{125}I-PDGF at a concentration of 33 ng ml^{-1}, unless otherwise stated. This concentration of ^{125}I-PDGF was at least 20-fold higher than the concentration of PDGF in 10% FCS. FCS was used in the binding buffer because it helped the cells retain their original morphology during the radiolabelling procedure; in this way morphology as well as antigenic phenotype could be used to distinguish the various cell types in the autoradiographs. In competition studies, 100-fold excess of unlabelled hPDGF, bFGF or EGF were added with the ^{125}I-PDGF.

For downregulation experiments, the cells were preincubated for 1 hour at 37 °C with 100 ng ml^{-1} of unlabelled PDGF-AA, PDGF-BB, or with the vehicle used to reconstitute the lyophilized PDGF (10 mM-acetic acid with

1 mg ml^{-1} BSA). Before being exposed to ^{125}I-PDGF as above, the cells were washed once in binding buffer containing 20 mM-acetic acid, pH 3.75 (in order to dissociate receptor-bound unlabelled PDGF at the cell surface) and then in binding buffer.

After radiolabelling, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4), which was added during the last 4 minutes of incubation with ^{125}I-PDGF. The cells were then washed three times in minimal Eagle's medium buffered with 0.02 M-Hepes (MEM-Hepes) and containing 10% FCS and 0.1% sodium azide, and once in MEM-Hepes. Washing before fixation gave much poorer cell preservation and did not significantly reduce non-specific binding. The cells were then immunolabelled, as described below, and the coverslips were mounted (with the cells uppermost) on glass slides using Fluorospar (BDH Ltd.). One day later, the slides were dipped in K2 emulsion (Ilford, diluted 1:2 in distilled water containing 1% glycerol heated to 43 °C), fan-dried at room temperature, and exposed for 6 days at 4 °C before they were developed in Contrast-FF (Ilford, diluted 1:4 in distilled water containing 1% glacial acetic acid) for 4 minutes at 16 °C and fixed in Hypam (Ilford, diluted 1:4 in distilled water) for 5.5 minutes at 16 °C. A second coverslip was mounted in Citifluor (City University, London) on top of the cells and was sealed with clear nail varnish. The cells were examined on a Zeiss Universal fluorescence microscope using a x63 oil-immersion objective and photographed using Kodak Tri-X

film (ASA 400). The macroglial cell types were identified by their characteristic morphologies and antigenic phenotypes: astrocytes were labelled by antibodies against GFAP (Bignami et al., 1972), oligodendrocytes by anti-GC antibody (Raff et al., 1987) and O-2A progenitor cells by the A2B5 monoclonal antibody (Raff et al., 1983b). Unless otherwise stated, each experiment was carried out in three separate cultures and at least 100 cells were counted on 3 coverslips in each experiment.

Immunofluorescence staining

All antibodies used in this thesis are listed in Table 2.5.

After fixation with 4% paraformaldehyde in PBS, but before dipping in emulsion, cells were surface-stained, either with A2B5 monoclonal antibody (Eisenbarth et al., 1979; ascites fluid diluted 1:100) followed by rhodamine-coupled goat anti-mouse Ig (G anti-MIg-Rd, Cappel, diluted 1:100) or with monoclonal anti-GC antibody (Ranscht et al., 1982) followed by fluorescein-coupled, class-specific, goat anti-mouse IgG3 (G anti-MIgG3-Fl, Nordic, diluted 1:100). The cells were then postfixed in methanol at -20 °C for 2 minutes. In some experiments, cells were stained with anti-GFAP antiserum (Pruss, 1979). In these cases the cells were not fixed with paraformaldehyde: after washing, they were fixed in methanol at -20 °C for 2 minutes, and then stained with rabbit anti-GFAP serum (diluted 1:1000) followed by fluorescein-coupled sheep anti-rabbit Ig (S anti-RIg-Fl, Wellcome, diluted 1:100).

Cross-competition studies and Scatchard analysis

For cross-competition experiments, 1-day-old cultures of P0 optic nerve cells were incubated with ^{125}I-PDGF-AA or ^{125}I-PDGF-BB, with or without a 100-fold excess of unlabelled PDGF-AA or PDGF-BB, in 30 μl PBS containing 1 mg ml^{-1} BSA, 0.01 mg ml^{-1} $CaCl_2.2H_2O$ and 0.01 mg ml^{-1} $MgSO_4.7H_2O$, pH 7.4 (PBS-BSA). Binding was terminated by washing three times in PBS-BSA at 4 °C. The cells were then lysed with 30 μl of an aqueous solution of 20 mM-Hepes, pH 7.4, containing 1% Triton X-100, 10% (vol/vol) glycerol, and BSA (0.1 mg ml^{-1}) for 30 minutes at room temperature. The radioactivity of the lysate was then counted in a gamma-counter (Nuclear Enterprises).

For Scatchard analysis, 1-day-old cultures of P7 optic nerve cells were incubated with ^{125}I-hPDGF, as for autoradiography, but the cells were not fixed: they were washed three times in MEM-Hepes with 10% FCS and 0.1% sodium azide, once in MEM-Hepes, and then lysed as above. Residual bound ^{125}I-PDGF was solubilized from the coverslips with 0.3 M-sodium hydroxide and the lysates were counted in a gamma-counter.

Bromodeoxyuridine incorporation

P0 optic nerve cells were cultured for 1 day with hPDGF (2 ng ml^{-1}) and BrdU (Boehringer) was added to a final concentration of 5 x 10^{-5}M for the entire period, or for the last 6-12 hours. After fixation with 4% paraformaldehyde in PBS for 2 minutes, the cells were then stained with anti-

GC antibody followed by G anti-MIgG3-Fl, and then postfixed in acid-alcohol (5% glacial acetic acid: 95% ethanol) at -20 °C for 10 minutes. They were then treated with 2 N-HCl to denature the nuclear DNA (Yong and Kim, 1987), followed by 0.1 M-$Na_2B_4O_7$, pH 8.5, both for 10 minutes at room temperature, and then labelled with monoclonal anti-BrdU antibody (Magaud et al., 1988) (culture supernatant diluted 1:5 in PBS containing 1% Triton X-100 and 2% FCS) followed by G anti-MIg-Rd (diluted 1:100 in the same solution). The cells were mounted and examined as described above.

CHAPTER 3

PDGF AND INTRACELLULAR SIGNALLING IN THE TIMING OF OLIGODENDROCYTE DIFFERENTIATION

Introduction

What is the nature of the timer that causes O-2A progenitor cells to stop dividing in the continuing presence of PDGF? The most obvious possibility is that a molecule required for the proliferative response to PDGF either decreases with time, or becomes diluted with each successive cell division, until it falls below a threshold level (Raff et al., 1985; Temple and Raff, 1986). In view of the evidence presented in Chapter 2 that many newly formed oligodendrocytes still express large numbers of PDGF receptors (Hart et al., 1989a) it is unlikely that receptor loss is responsible for the loss of mitotic responsiveness to PDGF. A second possibility is that the loss of responsiveness to PDGF results from uncoupling of the PDGF receptor from an intracellular signalling pathway.

In 3T3 cells, PDGF activates multiple intracellular signalling pathways (Figure 3.1)(Rozengurt, 1986; Williams, 1989), including the inositol phospholipid pathway (Habenicht et al., 1981; Matuoka et al., 1988; Meisenhelder et al., 1989) that leads to protein kinase C (C-kinase) activation (Rozengurt et al., 1983a) and an elevation of cytosolic Ca^{2+} (Ives and Daniel, 1987), and the cAMP pathway (Rozengurt et al., 1983b) that leads to the activation of cAMP-dependent protein kinase (A-kinase). In addition, PDGF receptors are tyrosine kinases (Yarden et al., 1986; Claesson-Welsh et al., 1988; Gronwald et al., 1988; Matsui et al., 1989), which phosphorylate both themselves and

other target proteins in the cell (Escobedo et al., 1988b; Williams, 1989), including the tyrosine kinase c-src (Ralston and Bishop, 1985), a phosphatidylinositol kinase (Coughlin et al., 1989), and the serine/threonine kinase Raf-1 (Morrison et al., 1988). It is not known how these various intracellular signalling pathways lead to cell division.

In the present chapter I have examined the possibility that O-2A progenitor cells become mitotically unresponsive to PDGF because their PDGF receptors become uncoupled from the inositol phospholipid or cyclic AMP signalling pathways. Because the other signalling pathways activated by PDGF are poorly understood, they were not specifically investigated. I show that, although newly formed oligodendrocytes do not respond mitotically to PDGF, PDGF can stimulate a rapid increase in cytosolic Ca^{2+} in these cells, as well as in progenitor cells. I also show that O-2A progenitor cells can be stimulated to divide and differentiate into oligodendrocytes on schedule when their cytosolic Ca^{2+} levels are raised with a Ca^{2+} ionophore and, at the same time, their C-kinases are activated by a phorbol ester; the same combination of drugs, however, does not stimulate newly formed oligodendrocytes to synthesize DNA, even in the presence of dibutyryl cyclic AMP (dbcAMP) or PDGF. Finally, I demonstrate that PDGF, but not the combination of Ca^{2+} ionophore and phorbol ester, is still mitogenic for O-2A progenitor cells in which C-kinase has been downregulated. Taken together, these findings suggest

that the reason O-2A progenitor cells become mitotically unresponsive to PDGF cannot be solely that their PDGF receptors are functionally uncoupled from early intracellular signalling events; there must be a block in the intracellular mitotic response pathways downstream from these events.

Results

PDGF causes a rapid increase in cytosolic Ca^{2+} in newly formed oligodendrocytes

Many newly formed oligodendrocytes have large numbers of PDGF receptors even though they are mitotically unresponsive to PDGF (see Chapter 2 and Hart et al., 1989a). To determine if the PDGF receptors on these cells are functional, I studied the effect of PDGF on cytosolic Ca^{2+} levels in P2 optic nerve cells that were cultured for 1 day and then loaded with fura-2AM. The cytosolic free Ca^{2+} concentration was determined in individual cells before and after PDGF was added to the cultures. The oligodendrocytes were initially identified by their characteristic morphology, and the assignments were later checked by labelling with anti-GC antibodies (Raff et al., 1978). In 12 experiments, 12/22 GC^+ oligodendrocytes showed a rise in cytosolic free Ca^{2+} when PDGF (2 ng ml^{-1}) was added. In some of these cases the increase was small (< 100 nM), occurred slowly (reaching a peak in > 200 seconds), or quantitative data were incomplete. Of the 17 cells where quantitative data were available, 5 showed an increase in cytosolic free Ca^{2+} (from 82 ± 19 nM to 277 ± 15 nM) within 21-200 seconds after addition of PDGF. An example is shown in Figure 3.2 (the cytosolic Ca^{2+} concentration fell to resting level [47 nM] 584 seconds after the addition of PDGF). Although there was a mixture of strongly and weakly

GC^+ oligodendrocytes in these cultures, all of the oligodendrocytes that showed a Ca^{2+} response were weakly GC^+, suggesting that they had only recently differentiated. This is consistent with the findings in the previous chapter that oligodendrocytes tend to lose PDGF receptors as they mature (Hart et al., 1989a). In control experiments, no change in cytosolic free Ca^{2+} levels was observed in 20/20 oligodendrocytes after addition of the vehicle used to dissolve the hPDGF. Thus PDGF receptors on at least some newly formed oligodendrocytes could be activated by PDGF to cause an increase in cytosolic Ca^{2+}.

As expected, some of the O-2A progenitor cells in these cultures (identified by immunolabelling with A2B5 antibody; Raff et al., 1983b; Temple and Raff, 1986) also showed a Ca^{2+} response to PDGF, although fewer cells responded than anticipated. In 12 experiments, 13/52 progenitor cells showed a response, and in the 48 cells in which quantitative data were complete, 8 showed a rise in cytosolic free Ca^{2+} (from 64 ± 8 nM to 152 ± 33 nM) within 28-266 seconds after addition of PDGF. In control experiments, 25/25 progenitor cells showed no response when vehicle was added to the cultures without PDGF. The low proportion of progenitor cells that responded to PDGF (25%), compared with the much higher proportion (about 70-80%) that would be expected to have PDGF receptors (see Chapter 2 and Hart et al., 1989a), was probably due to the sensitivity of these cells to the damaging effects of loading with fura-2AM (see Materials and methods). O-2A

progenitor cells have been found to be unusually sensitive to sub-optimal culture conditions and to various physical and chemical manipulations (Martin Raff, personal communication).

64/64 type-1 astrocytes showed no detectable change in cytosolic free Ca^{2+} levels after addition of PDGF (from an average resting level of 87 ± 6 nM). These results are consistent with the finding in Chapter 2 that type-1 astrocytes do not have PDGF receptors (Hart et al., 1989a).

A combination of PDB and A23187 stimulates O-2A progenitor cells to synthesize DNA

To study the effects of manipulating intracellular signalling pathways on DNA synthesis in O-2A progenitor cells, I cultured optic nerve cells from newborn (P0) rats in serum-free medium for 2 days in the presence of various agents. Bromodeoxyuridine (BrdU) was added for the last 24 hours of the culture period and the cells were then double-labelled by indirect immunofluorescence with the A2B5 monoclonal antibody to identify the progenitor cells and with monoclonal anti-BrdU antibody to identify cells that had incorporated BrdU into DNA (Magaud et al., 1988) (Figure 3.3).

When P0 optic nerve cells were cultured in the absence of drugs, less than 5% of the O-2A progenitor cells incorporated BrdU. When added individually, none of the following agents had a detectable effect on BrdU

incorporation in progenitor cells (data not shown): arachidonic acid (10^{-7} - 10^{-4} M), dibutyryl-cyclic AMP (dbcAMP, 10^{-6} - 10^{-3}M), forskolin (an activator of adenylate cyclase, 10^{-8} - 10^{-3} M), phorbol 12,13 dibutyrate (PDB, a phorbol ester that activates C-kinase, 10^{-12} - 10^{-6}M), or A23187 (a Ca^{2+} ionophore, 10^{-12} - 10^{-6}M). A combination of PDB and A23187, however, did stimulate progenitor cells to synthesize DNA, with a maximal effect (50 ± 6%) at concentrations of 10^{-8} M and 10^{-9}M, respectively (Figure 3.4). This stimulation almost equalled the effect of 2 ng ml^{-1} PDGF (62 ± 4%), which has been shown previously to be a potent mitogen for O-2A progenitor cells in culture (Noble et al., 1988; Richardson et al., 1988; Pringle et al., 1989). When 4α-phorbol 12,13-didecanoate (4α-PDD, 10^{-12} - 10^{-6}M), an inactive isomer of PDB, was used in place of PDB, no stimulation was seen in the presence of A23187 (10^{-9}M) (not shown), suggesting that the observed effect of PDB (when used with A23187) depended on the specific activation of C-kinase. Another phorbol ester, phorbol 12-myristate 13-acetate (PMA, 10^{-9}M), when used together with A23187 (10^{-9}M), also stimulated 51 ± 2% of O-2A progenitor cells to synthesize DNA, but only if 0.5% fetal calf serum (FCS) was added to the medium. I have no explanation for this difference between PDB and PMA.

A23187 (10^{-9}M) or PDB (10^{-8}M) were without effect when they were combined with forskolin (10^{-8} - 10^{-3}M) or dbcAMP (10^{-6} - 10^{-3}M) (not shown), suggesting that the activation of A-kinase, in combination with either an increase in

cytosolic Ca^{2+} or the activation of C-kinase, does not stimulate DNA synthesis in these cells.

PDB and A23187 act directly on O-2A progenitor cells to stimulate DNA synthesis

It was important to determine whether PDB and A23187 were acting directly on O-2A progenitor cells to stimulate DNA synthesis, or whether they were acting indirectly via another cell type. It was possible, for example, that the drugs were stimulating type-1 astrocytes to secrete PDGF, which in turn stimulated the progenitor cells to synthesize DNA. Three kinds of experiments were carried out to address this problem.

First the responses of progenitor cells to a combination of PDB and A23187 were studied in bulk cultures of optic nerve cells containing few, if any, type-1 astrocytes. This was achieved by using low density cultures (< 5000 cells per coverslip) of optic nerve cells from postnatal day 15 (P15) rats; it has been shown previously that relatively few type-1 astrocytes are released from ≥ P7 optic nerves by the dissociation procedure used (Miller et al., 1985). In 3 experiments on P15 cells, 34 ± 5% of the O-2A progenitor cells were stimulated to incorporate BrdU by the combination of PDB and A23187, compared to less than 1% in control cultures; in sister cultures, PDGF stimulated 23 ± 6% of the progenitor cells to incorporate BrdU. There were no type-1 astrocytes (as determined by

anti-GFAP staining and morphology; Raff et al., 1983a) in any of these cultures, although GFAP$^-$ fibroblast-like cells accounted for about 5% of the population.

In a second set of experiments the possibility of an indirect effect mediated by released PDGF was studied by the use of anti-PDGF antibodies. In 3 experiments, the addition of goat anti-PDGF antibodies (90 μg ml^{-1} of IgG) to 2-day-old bulk cultures of P7 optic nerve cells did not inhibit the ability of PDB plus A23187 to stimulate O-2A progenitor cells to synthesize DNA, even though the same concentration of anti-PDGF antibodies almost completely neutralized the effect of 4 ng ml^{-1} PDGF (Table 3.1).

To further assess whether PDB and A23187 acted directly on O-2A progenitor cells to stimulate them to synthesize DNA, single-cell cultures were prepared in microwells from P7 optic nerve by limiting dilution, as previously described (Hughes and Raff, 1987). In microwells that contained only a single O-2A progenitor cell, as assessed by morphology and immunofluorescence staining with A2B5, PDB plus A23187 stimulated BrdU incorporation almost to the same extent as PDGF (30% and 38% respectively: Table 3.2), suggesting that all of these agents act directly on the progenitor cells to induce DNA synthesis.

A combination of PDB and A23187 reconstitutes normal timing of oligodendrocyte differentiation in culture

To determine whether PDB plus A23187 could also

stimulate progenitor cell proliferation and reconstitute the normal *in vivo* timing of oligodendrocyte differentiation *in vitro*, I cultured E18 optic nerve cells for 1, 2 and 3 days with PDB and A23187. A small amount of FCS (0.5%) was added to the medium since it improved cell survival. As described previously (Raff *et al.*, 1985, 1988), in cultures maintained without added mitogens, many O-2A progenitor cells prematurely stopped dividing and differentiated into oligodendrocytes (identified by staining with anti-GC antibody), while in cultures maintained in PDGF, O-2A progenitor cells continued to divide and the first oligodendrocytes appeared after 3 days *in vitro*, which is the equivalent to the day of birth, when the first oligodendrocytes develop *in vivo* (Miller *et al.*, 1985). As shown in Table 3.3, cultures maintained in PDB plus A23187 behaved similarly to those maintained in PDGF; progenitor cells continued to proliferate and the first oligodendrocytes were seen after 3 days *in vitro*.

A combination of PDGF, PDB and A23187 does not stimulate newly formed oligodendrocytes to synthesize DNA

To determine if newly formed oligodendrocytes could be stimulated to synthesize DNA in response to a combination of PDB and A23187 (with or without PDGF), cultures of P0 optic nerve cells were maintained in these reagents and double-labelled with anti-GC and anti-BrdU antibodies after 2 days *in vitro*, having been exposed to BrdU for the last

24 hours of the culture period. In 3 out of 3 experiments, no oligodendrocytes were induced to incorporate BrdU. Adding dbcAMP (10^{-6} - 10^{-3} M) together with these agents did not effect this result. Thus newly formed oligodendrocytes, which have been shown in Chapter 2 not to synthesize DNA in response to PDGF (Hart et al., 1989a) also do not respond to a combination of PDB and A23187, regardless of whether PDGF and dbcAMP are present.

Downregulation of C-kinase does not inhibit the ability of PDGF to stimulate O-2A progenitor cells to synthesize DNA

To determine whether the mitotic effect of PDGF on O-2A progenitor cells requires the activation of C-kinase, I cultured P0 optic nerve cells for 12 hours at 37°C in a high concentration of PDB (10^{-6} M) to downregulate C-kinase (Collins and Rozengurt, 1984). The cultures were then washed and maintained for an additional 36 hours with either PDGF (2 ng ml^{-1}) or PDB (10^{-8} M) plus A23187 (10^{-9} M). BrdU was added for the last 24 hours of the culture period. Whereas downregulation of C-kinase decreased the DNA synthesis response of O-2A progenitor cells to PDB plus A23187 from 46 ± 3% to 3 ± 1%, it did not affect their response to PDGF (62 ± 6% compared with 58 ± 5% in control cultures) (Table 3.4). Thus, the ability of PDGF to stimulate O-2A progenitor cells to synthesize DNA does not appear to depend on the activation of C-kinase.

Figure 3.1

Activation of intracellular signalling pathways in 3T3 cells in response to PDGF.

Figure 3.1

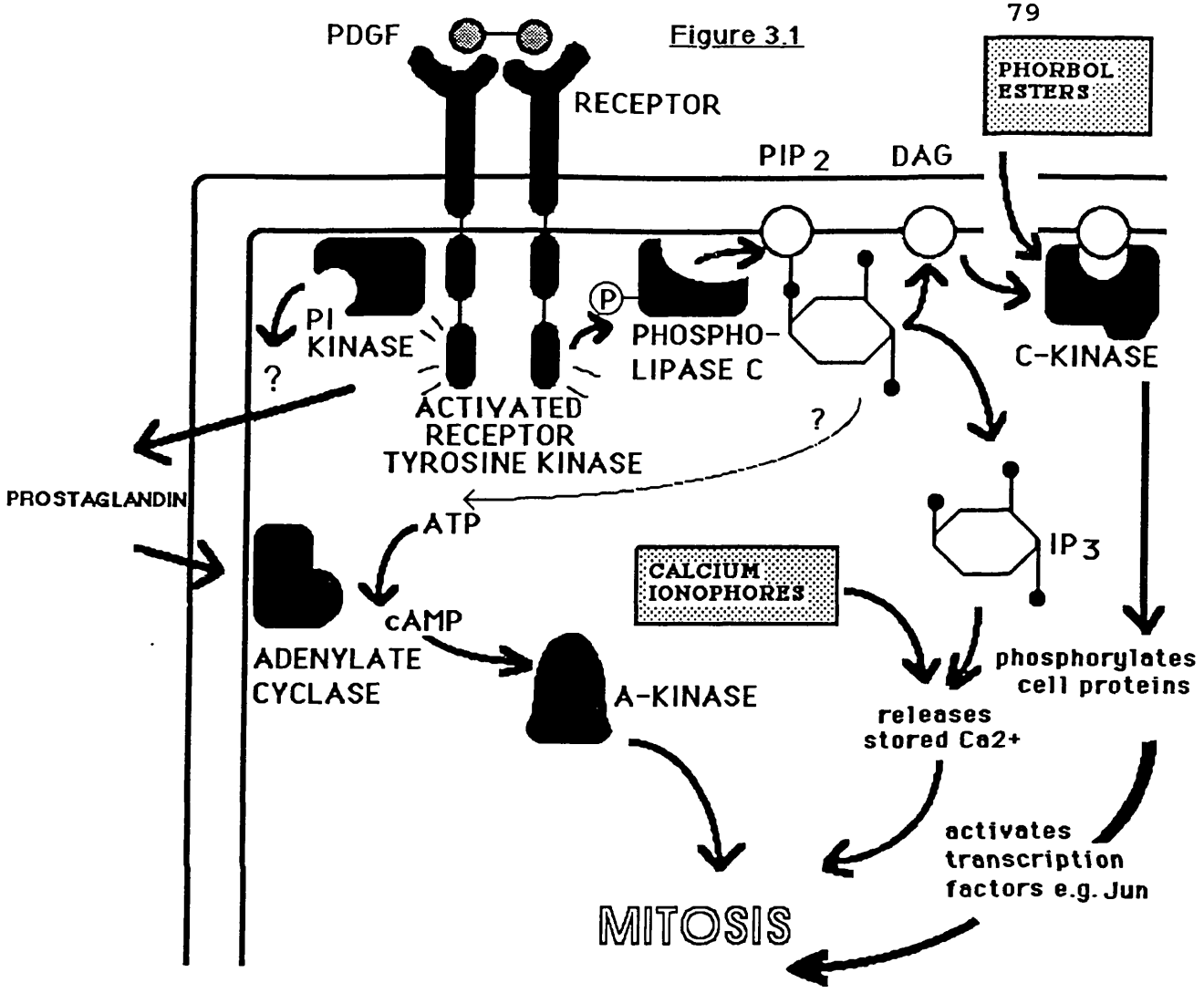

PDGF
RECEPTOR
PHORBOL ESTERS
PIP 2
DAG
PI KINASE
?
P
PHOSPHO-LIPASE C
C-KINASE
ACTIVATED RECEPTOR TYROSINE KINASE
?
PROSTAGLANDIN
ATP
IP 3
CALCIUM IONOPHORES
cAMP
ADENYLATE CYCLASE
A-KINASE
phosphorylates cell proteins
releases stored Ca2+
activates transcription factors e.g. Jun
MITOSIS

Figure 3.2

Effect of PDGF on cytosolic Ca^{2+} in a newly formed oligodendrocyte in culture. P2 optic nerve cells were cultured in serum-free medium for 1 day and then loaded with fura-2AM. The cytosolic free Ca^{2+} concentration was determined in this cell before and after PDGF (2 ng ml^{-1}) was added to the culture. The oligodendrocyte was initially identified by morphology and was found to be weakly stained after the culture was labelled with anti-GC antibody.

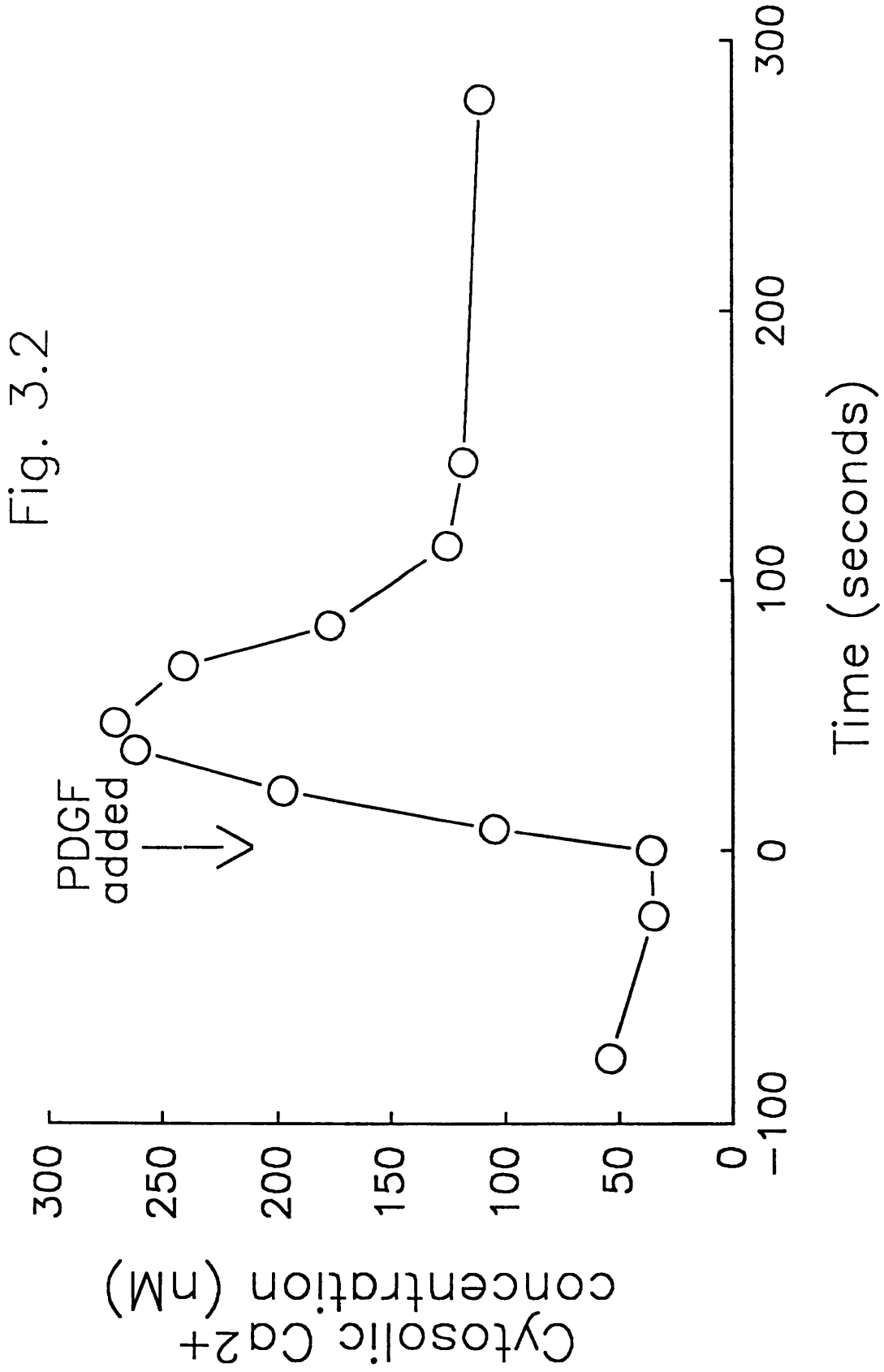

Cytosolic Ca2+
concentration (nM)
300
250
200
150
100
50
0
−100
0
100
200
300
Time (seconds)
PDGF
added

Fig. 3.2

Figure 3.3

Immunofluorescence micrographs of O-2A progenitor cells stimulated to incorporate BrdU in response to A23187 plus PDB. P7 optic nerve cells were cultured in serum-free medium for 2 days with A23187 (10^{-9} M) plus PDB (10^{-8} M). BrdU was added for the last 24 hours of the culture period and the cells were double-labelled with A2B5 antibody followed by S anti-MIg-biotin and then streptavidin-Fl, and then with anti-BrdU antibody followed by G anti-MIg-Rd. The cells were photographed using phase contrast (a), fluoroscein (b), or rhodamine (c) optics. One of the two $A2B5^+$, process-bearing progenitor cells shown in (a) and (b) is seen to be $BrdU^+$ in (c). Scale bar, 20 μm.

Figure 3.3

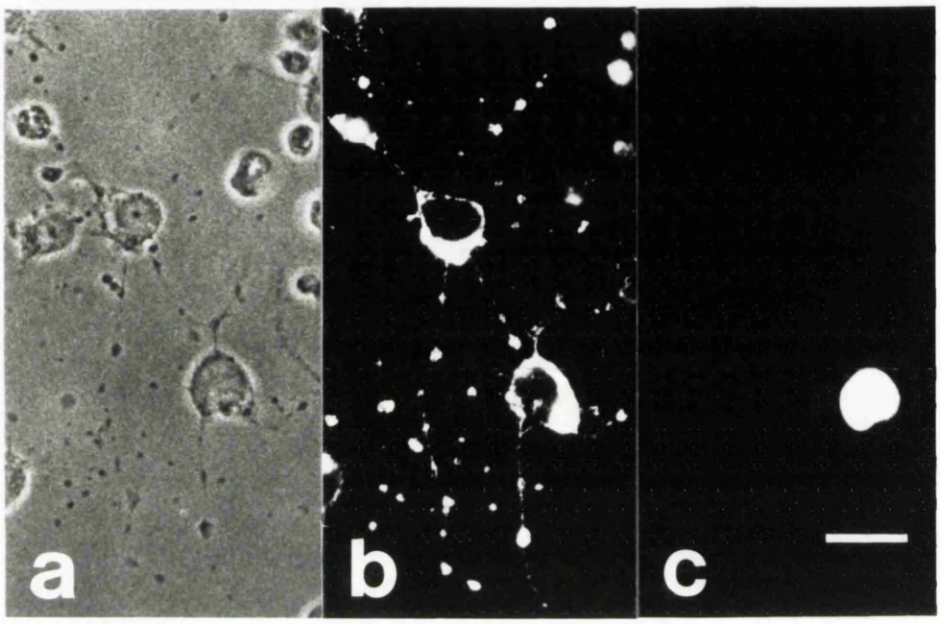


Figure 3.4

Effect of A23187 plus PDB on BrdU incorporation in O-2A progenitor cells in culture. P0 optic nerve cells were cultured in serum-free medium for 2 days without additives or with either A23187 (10^{-9} M) plus varying concentrations of PDB or PDGF (2 ng ml^{-1}). BrdU was added for the last 24 hours of the culture period and the cells were double-labelled with A2B5 and anti-BrdU antibodies. The O-2A progenitor cells were identified as $A2B5^{+}$, process-bearing cells (see Figure 3.3). The results in this Figure and in Tables 3.1 and 3.4 are expressed as mean ± S.E. of at least 3 experiments.

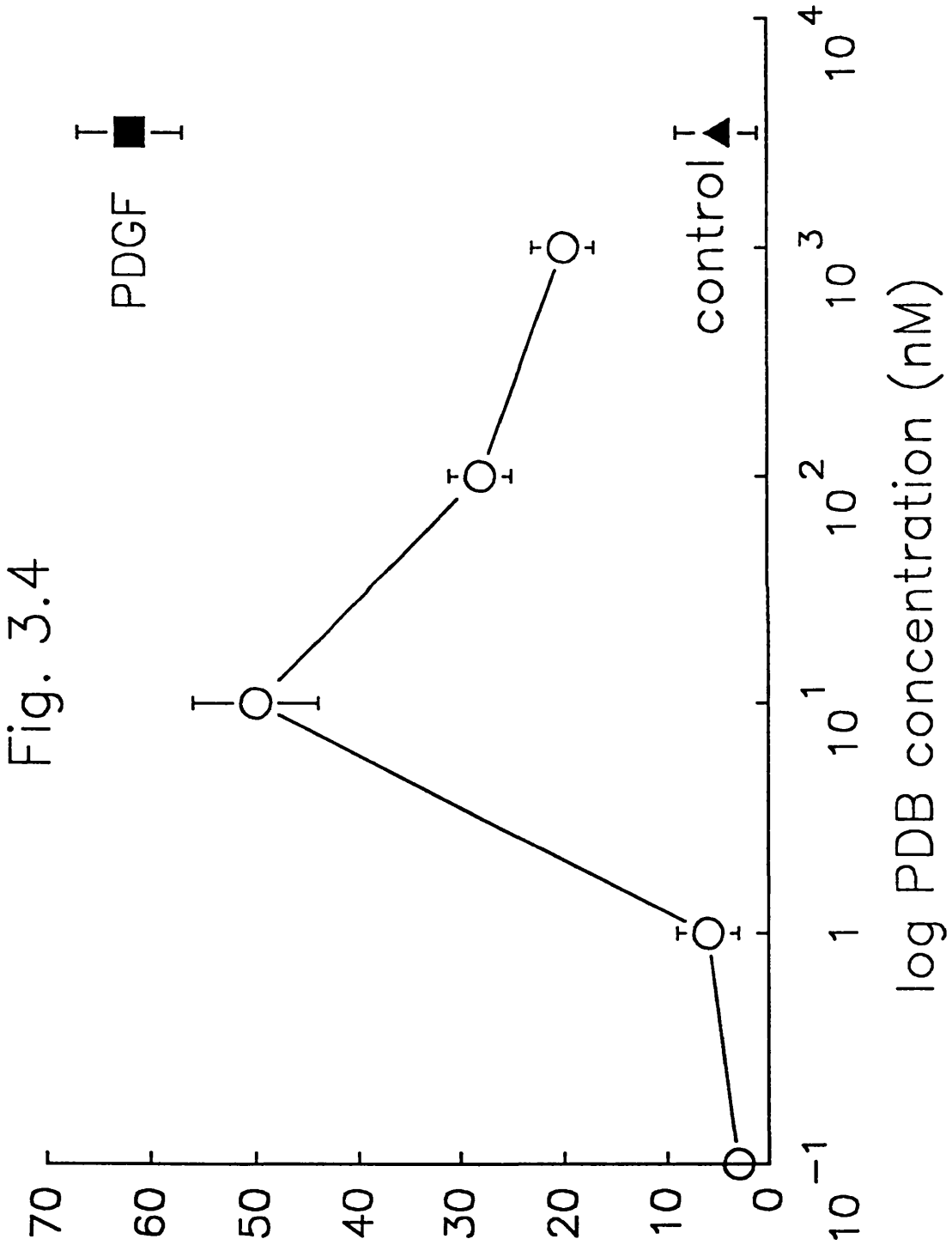

% O-2A progenitor cells incorporating BrdU
70
60
50
40
30
20
10
0
PDGF
control
10^{-1}
1
10^{1}
10^{2}
10^{3}
10^{4}
log PDB concentration (nM)

Fig. 3.4

Table 3.1 Effect of anti-PDGF antibodies on the ability of PDB plus A23187 or of PDGF to stimulate O-2A progenitor cells to incorporate BrdU

Agent added	Anti-PDGF added	% O-2A progenitor cells incorporating BrdU
none	-	6 ± 2
none	+	3 ± 1
PDGF	-	37 ± 2
PDGF	+	9 ± 2
PDB + A23187	-	23 ± 3
PDB + A23187	+	21 ± 1

P7 optic nerve cells were cultured for 2 days with either PDB (10^{-8} M) plus A23187 (10^{-9} M) or hPDGF (4 ng ml^{-1}), in the presence or absence of goat anti-PDGF antibodies (90 μg ml^{-1}). BrdU incorporation was assayed as described in Figure 3.3.

Table 3.2 Effect of PDB plus A23187 on BrdU incorporation by O-2A progenitor cells in single-cell microwell cultures

Agent added	% O-2A progenitor cells incorporating BrdU ($BrdU^+$ progenitor cells/total progenitor cells assessed)
Control	6 (8/127)
PDGF	38 (96/256)
PDB + A23187	30 (54/188)

P7 optic nerve cells were plated in microwells (≤5 cells per well) and cultured for 2 days without additives or with either PDB (10^{-8}M) plus A23187 (10^{-9} M) or hPDGF (2 ng ml^{-1}). BrdU was added for the last 24 hours of the culture period and the cells were quadruple-labelled with A2B5,anti-GC, anti-GFAP and anti-BrdU antibodies. Only microwells that contained a single O-2A progenitor cell ($A2B5^+$, GC^-, $GFAP^-$, process-bearing), and no other cells, were included in the results shown.

Table 3.3 Effect of PDB plus A23187 on O-2A progenitor cell proliferation and the timing of oligodendrocyte development in cultures of E18 optic nerve cells

Days in culture	Cell type	Total number of cells in cultures containing: No additives	PDGF ($2ng\ ml^{-1}$)	PDB($10^{-8}M$)+ A23187($10^{-9}M$)
1	O-2A progenitors	133	173	148
	oligodendrocytes	0	0	0
2	O-2A progenitors	184	299	233
	oligodendrocytes	14	0	0
3	O-2A progenitors	168	522	378
	oligodendrocytes	58	15	18

The results represent the total number of progenitor cells and oligodendrocytes seen in 3 experiments. The cells were double-labelled with A2B5 and anti-GC antibodies. Oligodendrocytes were identified as GC^+ cells, whereas O-2A progenitors were identified as $A2B5^+$, GC^-, process-bearing cells.

Table 3.4 Effect of C-kinase downregulation on the ability of PDGF to stimulate O-2A progenitor cells to incorporate BrdU

Agent added (dose)	Pretreatment with PDB(10^{-6}M)	% O-2A progenitor cells incorporating BrdU
none	-	1 ± 1
none	+	1 ± 1
PDGF (2ng ml^{-1})	-	58 ± 1
PDGF (2ng ml^{-1})	+	62 ± 6
PDB (10^{-8}M) + A23187 (10^{-9}M)	-	46 ± 3
PDB (10^{-8}M) + A23187 (10^{-9}M)	+	3 ± 1

P0 optic nerve cells were cultured for 2 days and BrdU incorporation was assayed as described in Figure 3.3. As indicated, in some cases the cultures were pretreated for 12 hours at 37 °C with 10^{-6} M PDB to downregulate C-kinase.

Discussion

In the present chapter I have continued my effort to understand the molecular nature of the timing mechanism that controls the onset of oligodendrocyte differentiation. Previous studies have suggested that the mechanism depends on an intrinsic timer in each O-2A progenitor cell that operates by limiting the time, or the number of times, that the cell can divide in response to PDGF (Raff et al., 1985, 1988; Temple and Raff, 1986). The question of why many types of cells stop dividing and differentiate in the continuing presence of growth factors is a fundamental one in vertebrate development.

Two types of PDGF receptor proteins have been defined (Claesson-Welsh et al., 1988; Gronwald et al., 1988; Hart et al., 1988; Heldin et al., 1988) - α and β - both of which are single-pass transmembrane tyrosine kinases (Yarden et al., 1986; Claesson-Welsh et al., 1988; Gronwald et al., 1988; Matsui et al., 1989). In Chapter 2, I have provided evidence that cells of the O-2A lineage have α receptors (Hart et al., 1989a; Pringle et al., 1989). Although much more is known about the functional properties of β receptors (Williams, 1989), given that both types of receptor have very similar structures (Claesson-Welsh et al., 1989), it seems likely that they use similar signal-transduction mechanisms. In experiments reported here, I have examined the possibility that O-2A progenitor cells eventually become mitotically unresponsive to PDGF because

their PDGF receptors become functionally uncoupled from the inositol phospholipid or cyclic AMP signalling pathways. The present results suggest that this is not the case.

Using fura-2AM to measure cytosolic free Ca^{2+}, it was demonstrated that PDGF stimulates a rapid increase in cytosolic Ca^{2+} in a proportion of both O-2A progenitor cells, which are mitotically responsive to PDGF, and newly formed oligodendrocytes, which are not. This indicates that, when they are activated, the PDGF receptors on at least some newly formed oligodendrocytes are capable of increasing cytosolic Ca^{2+}. If the increase in Ca^{2+} is mediated by inositol trisphosphate (Berridge, 1988), then this finding would indicate that the PDGF receptors on these newly formed oligodendrocytes are able to activate the inositol phospholipid signalling pathway. Even if the increase in cytosolic Ca^{2+} is mediated by some other signalling pathway (Nånberg and Rozengurt, 1988), the present finding raises the possibility that the block to mitotic responsiveness in these cells lies downstream from the PDGF receptor and some of the early intracellular signalling events that follow its activation.

A second line of experiments strongly supports this possibility. I found that the effects of PDGF on O-2A progenitor cells in culture could be mimicked by both increasing cytosolic Ca^{2+} with A23187 and activating C-kinase with PDB. Single cell experiments showed that both PDGF and the combination of these two drugs act directly on progenitor cells to stimulate DNA synthesis. Not only did

the drugs mimic PDGF in stimulating progenitor cells to synthesize DNA and to proliferate, they also mimicked PDGF in reconstituting the normal *in vivo* timing of oligodendrocyte differentiation in cultures of embryonic optic nerve cells. Thus, the timing mechanism that controls when progenitor cells stop dividing and differentiate into oligodendrocytes apparently operates normally when the cells are stimulated to divide by drugs that bypass both the PDGF receptors and the earliest signalling events that they activate. This suggests that these receptors and events might not be involved in the timing mechanism. Consistent with this view was the finding that the same combination of drugs did not stimulate newly formed oligodendrocytes to synthesize DNA, even in the presence of PDGF; this indicates that these cells are blocked in their mitotic response somewhere distal to the earliest intracellular signalling events in the mitotic pathway.

All of the results discussed thus far are consistent with the possibility that O-2A progenitor cells eventually become unresponsive to PDGF and to the combination of A23187 plus PDB because C-kinase decreases in concentration or is inactivated. To test this possibility indirectly, I treated optic nerve cultures with a high concentration of PDB for 12 hours to downregulate C-kinase in O-2A progenitor cells. Whereas this treatment abolished the ability of the progenitor cells to synthesize DNA in response to A23187 plus PDB, it had no effect on the response of these cells to PDGF. This result suggests that

in O-2A progenitor cells, as in other cells, there are multiple, redundant, intracellular signalling pathways from the plasma membrane to the nucleus that lead to cell division (Rozengurt, 1986). It also suggests that the ability of PDGF to stimulate progenitor cells to synthesize DNA does not depend on C-kinase activation, which makes it unlikely that the failure of PDGF to stimulate newly formed oligodendrocytes to synthesize DNA is due solely to a decrease in or inactivation of C-kinase.

I was not able to stimulate O-2A progenitor cells to synthesize DNA by increasing the intracellular concentration of cyclic AMP with dbcAMP or forskolin, either alone or in combination with A23187 or PDB. Nor was I able to stimulate newly formed oligodendrocytes to synthesize DNA by these treatments, even when they were combined with PDGF, making it unlikely that the mitotic unresponsiveness of these cells is due to a block in generating cyclic AMP.

Taken together, the present results suggest that O-2A progenitor cells normally become mitotically unresponsive to PDGF because they develop a block downstream from the PDGF receptor and some of the early events that follow its activation, including the increase in cytosolic Ca^{2+} and the activation of C-kinase. Although I cannot exclude the possibility that some early events that normally follow PDGF receptor activation (that I did not examine) fail to occur, this could not be the sole cause of the mitotic unresponsiveness.

Materials and methods

Cell culture

Optic nerves were dissected from embryonic day 18 (E18), newborn (P0), postnatal day 2 (P2), P7, or P15 Sprague-Dawley rats and dissociated into single cells using trypsin, EDTA, and collagenase, as previously described (Miller et al., 1985). About 5000 to 20000 cells were cultured at 37 °C in 5% CO_2 on a PDL-coated glass coverslip in 0.5 ml serum-free DMEM containing bovine insulin (5 μg ml^{-1}), human transferrin (50 μg ml^{-1}), sodium selenite (30 nM), and biotin (10 ng ml^{-1})(supplemented DMEM), described previously by Bottenstein (1986).

Cells for culture in microwells were diluted in L-15 medium (Gibco) containing 10% FCS. About 5 cells in 10 μl medium were transferred by pipette to single wells of a Terasaki microculture plate (Falcon), which had previously been coated with PDL and then incubated with supplemented DMEM for 2 hours at 37 °C. The cells were incubated at 37 °C in air for 30 minutes to allow them to adhere, the medium was then changed to astrocyte conditioned medium (ACM: prepared as below) diluted 1:20 with supplemented DMEM, and the plates were then incubated at 37 °C in 5% CO_2.

Preparation of ACM

Purified type-1 astrocyte cultures were prepared by a method modified from Noble and Murray (1984) and Lillien et al. (1988). P0 rat cerebral hemispheres (after removal of

the meninges) were dissociated into single cells as described above for optic nerve cell cultures. The cells were cultured in 4 ml DMEM containing 10% FCS at 37 °C in 5% CO_2 in PDL-coated tissue culture flasks (Falcon) and grown to confluency. After the cells were shaken overnight on a cell rotator to remove the top layer of cells, cytosine arabinoside (10^{-5} M) was added for 24 hours to kill any cells that were dividing rapidly. After washing, the cells were cultured in serum-free supplemented DMEM for at least 1 week, and the medium was collected as ACM; it was used to increase the survival of isolated cells, but had no mitogenic effect at 1:20 dilution.

Downregulation of C-kinase

For C-kinase downregulation experiments (Collins and Rozengurt, 1984), P0 optic nerve cells were cultured on coverslips in supplemented DMEM with a high concentration of phorbol 12,13 dibutyrate (PDB, 10^{-6} M) for 12 hours. The cells were then transferred to supplemented DMEM containing 0.1% BSA for 30 minutes to remove the PDB, then rinsed, transferred to supplemented DMEM, and cultured for an additional 36 hours with either PDB (10^{-8} M) plus the Ca^{2+} ionophore A23187 (10^{-9} M) or human PDGF (2 ng ml^{-1}).

Bromodeoxyuridine incorporation and immunofluorescence staining

Bromodeoxyuridine (BrdU, Boehringer), which is incorporated into replicating DNA (Gratzner, 1982), was added to

cultures of optic nerve cells to a final concentration of 5×10^{-5} M for the last 24 hours of the culture period. After fixation with 4% paraformaldehyde for 2 minutes at room temperature, cells were surface-stained either with monoclonal anti-galactocerebroside (anti-GC) antibody (Ranscht et al., 1982; ascites fluid diluted 1:100) followed by fluorescein-coupled, class specific, goat anti-mouse IgG3 (G anti-MIgG3-Fl; Nordic; diluted 1:100) or with the A2B5 monoclonal antibody (Eisenbarth et al., 1979; ascites fluid diluted 1:100) followed either by fluorescein-coupled goat anti-mouse Ig (G anti-MIg-Fl; Cappel; diluted 1:100) or by biotin-coupled sheep anti-mouse Ig (S anti-MIg-biotin; Amersham; diluted 1:50) followed by fluorescein-coupled streptavidin (streptavidin-Fl; Amersham; diluted 1:50). The antibodies were diluted in Eagle's minimum essential medium buffered with 0.02-M Hepes and containing 10% FCS and 0.2% (w/v) sodium azide. Cells were postfixed in acid-alcohol (5% glacial acetic acid : 95% ethanol) at -20 °C for 10 minutes. They were then treated with 2 N-HCl to denature the nuclear DNA (Yong and Kim, 1987), followed by 0.1 M-sodium borosilicate (pH 8.5), each for 10 minutes at room temperature. The cells were then labelled with monoclonal anti-BrdU antibody (Magaud et al. 1988; culture supernatant diluted 1:2 in phosphate-buffered saline (PBS) containing 1% Triton-X 100 and 2% FCS) followed by G anti-MIg-Rd (diluted 1:100 in the same solution). In some experiments, cells were stained with a rabbit antiserum against glial fibrillary acidic protein

(GFAP, Pruss, 1979; diluted 1:1000). In these cases, the cells were prefixed with 70% ethanol for 30 minutes at -20 °C, the bound anti-GFAP antibodies were detected with fluorescein-coupled sheep anti-rabbit Ig (S anti-RIg-Fl; Wellcome, diluted 1:100), and the cells were then prepared for BrdU labelling as before.

Cells on coverslips were mounted in Citifluor (City University, London) on glass slides and sealed with nail varnish, while cells in microwells were viewed after the addition of PBS containing 0.1% p-phenylenedramine to prevent fading of fluorescence. The cells were then examined on a Zeiss Universal fluorescence microscope using a x40 or x63 oil-immersion objective and photographed using Kodak Tri-X film (ASA 400).

Glial cell types were identified by their characteristic morphologies and antigenic phenotypes: astrocytes were labelled by anti-GFAP antiserum (Bignami et al., 1972), O-2A progenitor cells by A2B5 monoclonal antibody (Raff et al., 1983b), and oligodendrocytes by anti-GC antibody (Raff et al., 1978). Unless otherwise stated, each experiment was carried out on three separate cultures and at least 100 cells were counted on each coverslip.

Measurement of cytosolic Ca^{2+}

Intracellular Ca^{2+} measurements were carried out in collaboration with Stephen Bolsover (Department of Physiology, University College London). P2 optic nerve

cells were cultured for 1 day in supplemented DMEM (without PDGF) on PDL-coated glass coverslips on which a grid had been drawn in indelible ink. The cells were incubated with the tetra-acetoxymethyl ester of the fluorescent Ca^{2+} indicator fura-2 (fura-2AM; 4 μM) in supplemented DMEM for 30 minutes at room temperature. The fura-2AM was aspirated and the cells incubated in L-15 medium for a further 90 minutes at 37 °C in air, before they were examined on a Zeiss fluorescence microscope, adapted to alternate rapidly between the excitation wavelengths of 350 nm and 380 nm. A field of cells was visualized via a television camera connected to an image processor, and the fluorescence was recorded for at least 10 minutes before and 10 minutes after the addition of human PDGF (2 ng ml^{-1}). Free Ca^{2+} levels were calculated for individual cells within the field by computer, as described previously (Silver et al., 1989). In control experiments, the vehicle used to suspend the PDGF (10 mM-acetic acid containing 2 mg ml^{-1} BSA) was added to the cultures. Results are expressed as mean ± S.E. Photographs of the cells were taken on Polaroid Instamatic film, and the cells were then immunolabelled as described above to aid identification; the field of interest was relocated using the photographs and the ink grid on the coverslip.

Although 4 μM was the lowest concentration of fura-2AM that gave adequate loading of O-2A lineage cells, these cells did not tolerate the procedure well: some showed a spontaneous gradual rise in cytosolic free Ca^{2+}

concentration and, in some cases, degenerated before the experiment ended. Such cells were not included in the analysis.

Materials

Human PDGF was obtained from R and D Systems and goat anti-human PDGF antibodies (an IgG fraction prepared by ion-exchange chromatography) were purchased from Collaborative Research. Forskolin, PDB, PMA, 4α-PDD, dbcAMP, A23187, and arachidonic acid were obtained from Sigma, and fura-2AM from Molecular Probes.

CHAPTER 4

PDGF INCREASES THE EXPRESSION OF FOS AND JUN IN NEWLY FORMED OLIGODENDROCYTES THAT HAVE BECOME RESISTANT TO THE MITOGENIC EFFECTS OF PDGF

Introduction

The findings in Chapters 2 and 3 suggest that newly formed oligodendrocytes do not respond mitotically to PDGF because there are blocks or deficiencies downstream to some of the early intracellular signalling events that follow activation of the PDGF receptor.

The study of those parts of signalling pathways that lie closer to the nucleus, or within the nucleus itself, has been helped by the discovery of the transforming oncogenes (v-*oncs*) of the oncogenic retroviruses (reviewed in Hunter, 1985). V-*oncs* cause growth deregulation by producing protein products that mimic normal cellular processes. Almost all v-*oncs* have been found to be altered versions of normal cellular genes which are now collectively called proto-oncogenes because of their potential, when mutated, to induce tumours. Many proto-oncogenes have been characterized using DNA transfection techniques and their protein products have been shown to play important roles at many stages of the normal signalling pathways that control neural cell growth during development (reviewed in Walicke, 1989). For example: growth factors - c-*sis* encodes the PDGF B chain (Devare *et al.*, 1983; Waterfield *et al.*, 1983); growth factor receptors - c-*erb*-B encodes the EGF receptor (Downward *et al.*, 1984); cell membrane-bound enzymes - c-*src* encodes a tyrosine kinase (Takeya and Hanafusa, 1983); membrane/cytoplasmic enzymes - *Raf-1* encodes a serine-

threonine kinase (Bonner *et al.*, 1985) and c-H-*ras*-1 encodes a GTPase (Hurley *et al.*, 1984); c-*fos* and c-*jun* encode gene regulatory proteins.

In various cell types, PDGF induces the rapid expression of several nuclear proto-oncogenes, including the immediate-early genes c-*fos* (Cochran *et al.*, 1984; Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Müller *et al.*, 1984; Almendral *et al.*, 1988) and c-*jun* (Ryder and Nathans, 1988), which encode the proteins Fos and Jun, respectively. It has been shown that Fos (or one of its relatives) binds to Jun (or one of its relatives) to form a heterodimeric transcription factor called activator protein 1 (AP-1; Rauscher *et al.*, 1988). By binding to specific regulatory elements in various genes, AP-1 is thought to play an important part in the control of cell proliferation and differentiation (Curran and Franza, 1988; Bartel *et al.*, 1989), although the genes and mechanisms involved are not known.

In the present chapter, I have used rabbit antisera and indirect immunofluorescence to detect Fos and Jun in both O-2A progenitor cells and newly formed oligodendrocytes in culture. I show that PDGF increases the expression of Fos and Jun in both cell types, even though PDGF does not stimulate newly formed oligodendrocytes to synthesize DNA. I also show that O-2A progenitor cells *in vivo* usually withdraw from the cell cycle at least 6-12 hours before they express galactocerebroside (GC), an early marker of oligodendrocyte differentiation (Mirsky *et al.*,

1980; Dubois-Dalcq et al., 1986).

Results

PDGF induces the expression of Fos and Jun in newly formed oligodendrocytes

To study the expression of Fos and Jun in newly formed oligodendrocytes, I cultured P1 optic nerve cells in serum-free medium for 1 day and then added PDGF (2 ng ml^{-1}), or the vehicle used to dissolve PDGF, for 1 (for Fos) or 2 hours (for Jun). Fos and Jun were found to be expressed maximally at these respective times (not shown) which are consistent with the times reported for maximal expression of Fos (1 hour; Müller et al., 1984) and Jun (2 hours; Bos et al., 1988) in 3T3 cells in response to mitogens, as determined by immunoprecipitation. The cells were then fixed and double-labelled for immunofluorescence with anti-GC antibody to identify the oligodendrocytes (Raff et al., 1978) and with either anti-Fos or anti-Jun antibodies. The labelled cells were counted under blind conditions. The proportions of oligodendrocytes that showed nuclear staining with anti-Fos and anti-Jun antibodies (Figure 4.1) were consistently greater in PDGF-treated cultures than in control cultures, although the control levels in the absence of PDGF were high, especially in the case of Jun (Figure 4.2a). Lower control levels of Jun in oligodendrocytes were seen in cultures of P7 optic nerve cells treated in the same way: in 6 experiments, 42 ± 6% of oligodendrocytes showed nuclear staining with anti-Jun

antibodies in response to PDGF compared to 23 ± 3% in control cultures.

To determine if any newly formed oligodendrocytes were stimulated to synthesize DNA in response to PDGF in these conditions, I exposed cultures of P1 optic nerve cells to BrdU and analyzed its incorporation into DNA with a monoclonal anti-BrdU antibody (Gratzner, 1982; Magaud et al., 1988); the cultures were also labelled with anti-GC antibody to identify the oligodendrocytes. In 9 experiments, no BrdU incorporation was seen in over 3000 oligodendrocytes that were treated with both PDGF and BrdU for the last 24 hours of the 2 day culture period, after being deprived of exogenous PDGF for the first 24 hours. Thus, PDGF stimulated newly formed oligodendrocytes to increase their expression of Fos and Jun, but did not induce these cells to synthesize DNA.

When cultures of P1 optic nerve cells were studied after 3 days in vitro in the absence of PDGF, the proportion of oligodendrocytes that showed nuclear staining with anti-Fos antibodies in response to a 1 hour exposure to PDGF, or with anti-Jun antibodies in response to a 2 hour exposure to PDGF was smaller than that seen after 1 day in vitro (Figure 4.2b). This result is consistent with the finding in Chapter 2 that oligodendrocytes tend to lose PDGF receptors with time in culture (Hart et al., 1989a).

PDGF induces the expression of Fos and Jun in O-2A progenitor cells

To study the expression of Fos and Jun in O-2A progenitor cells, I cultured newborn (postnatal day 0 = P0) optic nerve cells in serum-free medium for 1 day and then studied their response to PDGF as described above. O-2A progenitor cells were identified as GC^- cells with a characteristic bipolar or multipolar morphology (Raff et al., 1983b; Temple and Raff, 1986). As shown in Figure 4.2c, the proportions of O-2A progenitor cells that showed nuclear staining with either antiserum were always greater in cultures treated with PDGF than in control cultures that were treated with the vehicle used to dissolve the PDGF. To determine whether O-2A progenitor cells were induced to synthesize DNA in response to PDGF in these circumstances, cultures of P0 optic nerve cells were treated with both PDGF and BrdU for the last 24 hours of culture, after being deprived of exogenous PDGF for the first 24 hours. The cells were then double-labelled with A2B5 antibody (Eisenbarth et al., 1979), to stain the O-2A lineage cells (Raff et al., 1983b), and monoclonal anti-BrdU antibody. In 6 experiments, 15 ± 6% of the O-2A lineage cells ($A2B5^+$ process-bearing cells) in the PDGF-treated cultures incorporated BrdU, whereas only 1 ± 1% did so in control cultures that were not exposed to PDGF. As PDGF does not stimulate oligodendrocytes to synthesize DNA in vitro (Noble et al., 1988; Hart et al., 1989a, and see above) and

type-2 astrocytes do not develop in these serum-free short-term cultures (Raff et al., 1988; Lillien and Raff, 1990), the A2B5$^+$, BrdU$^+$ process-bearing cells in the PDGF-treated cultures must have been O-2A progenitor cells.

GC is first expressed 6-12 hours after O-2A progenitor cells in vivo stop synthesizing DNA

It has been shown previously that O-2A progenitor cells in cultures of perinatal optic nerve cells stop synthesizing DNA 12-24 hours before they express GC (Noble and Murray, 1984; Hart et al., 1989b). To determine whether this is also the case in vivo, I injected BrdU (1 mg g^{-1}) into P2 rats and sacrificed them 1, 6, 12, and 24 hours later. I prepared optic nerve cells and labelled them in suspension with anti-GC antibody to identify oligodendrocytes, or A2B5 antibody to identify O-2A lineage cells. I allowed the cells to settle on a PDL-coated glass coverslip and then fixed and labelled them with anti-BrdU antibody. As none of the A2B5$^+$ cells in these suspensions are GFAP$^+$ and less than 10% are GC$^+$ (Miller et al., 1985), the great majority of the A2B5$^+$ cells were probably O-2A progenitors. Although many A2B5$^+$/BrdU$^+$ cells were seen as early as 1 hour after an injection of BrdU, GC$^+$/BrdU$^+$ cells were not seen until 12 or 24 hours after such an injection; no GC$^+$/BrdU$^+$ cells were found after 1 or 6 hours, even though at least 3000 GC$^+$ cells were scanned at each time point (Figure 4.3).

I also injected BrdU into P2, P7 and P10 rats,

sacrificed them after 1 hour and cultured cells from the optic nerve on coverslips for 3 hours before double-labelling them for GC and BrdU. Combining the results of at least 3 experiments at each age, 0/4000 GC^+ cells incorporated BrdU in P2 rats, 3/16000 GC^+ cells did so in P7 rats and 3/12000 GC^+ cells did so in P10 rats. Although the significance of these small numbers of double-labelled cells is unclear, my findings indicate that, in the developing rat optic nerve, most GC^+ oligodendrocytes do not divide and, in the great majority of cases, GC is first expressed at least 6-12 hours after O-2A progenitor cells stop synthesizing DNA.

Figure 4.1

Immunofluorescence micrographs of PDGF-stimulated oligodendrocytes expressing Jun or Fos protein. P1 optic nerve cells were cultured for 1 day. PDGF was added for either the last 1 hour (for Fos) or for the last 2 hours (for Jun) of the culture period and the cells were double-labelled with anti-GC antibody followed by G anti-MIgG3-Fl (a - d), and then with anti-Jun (a, b) or anti-Fos antibodies (c, d), followed by D anti-RIg-Bt and streptavidin-TR (a - d). The cells were photographed using fluoroscein (a, c) or Texas red (b, d) optics. The GC^+ oligodendrocyte shown in (a) is seen to be Jun^+ in (b) and the 3 GC^+ oligodendrocytes in (c) are seen to be Fos^+ in (d). Scale bar, 20 μm.

Figure 4.1

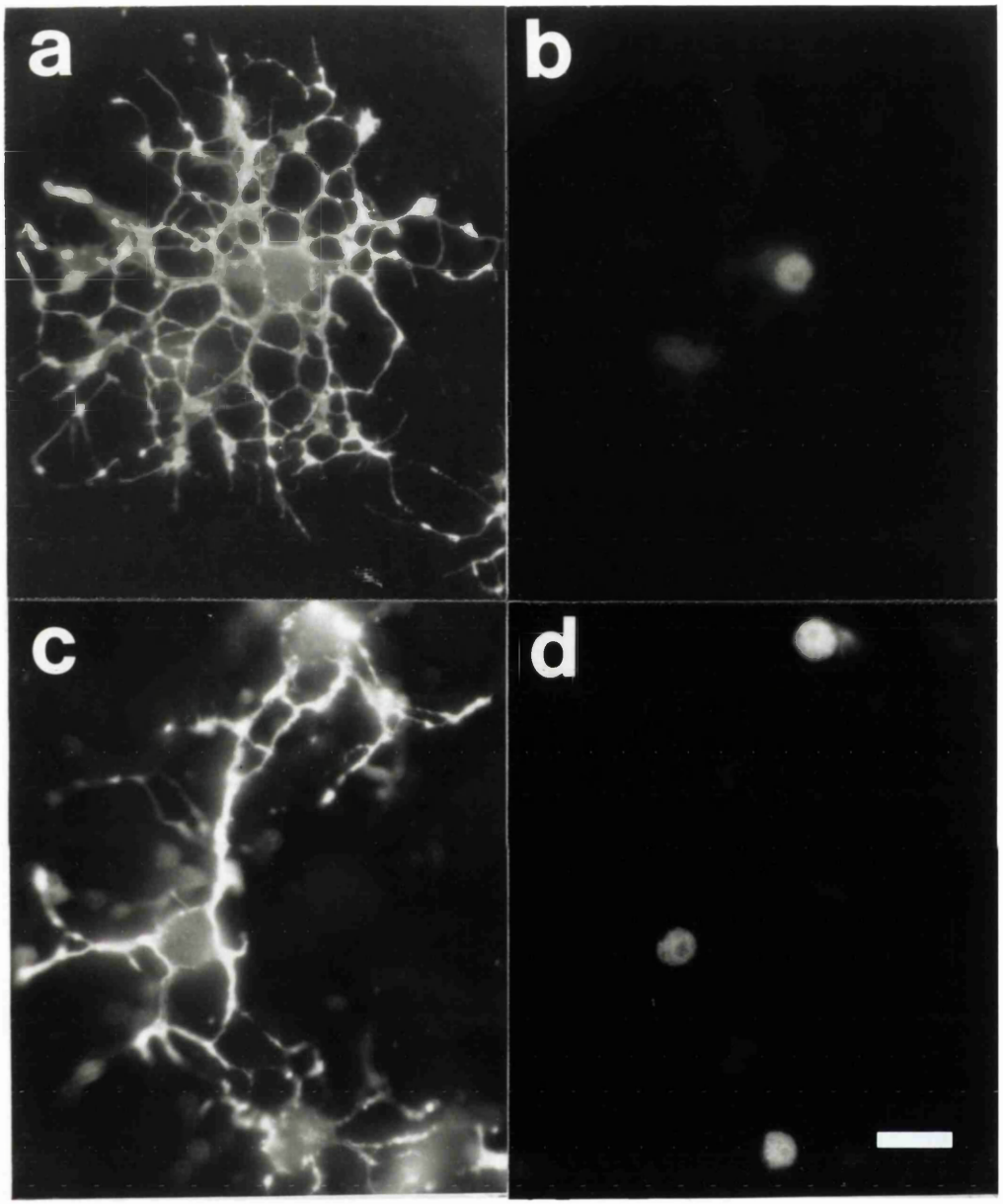


Figure 4.2

Effects of PDGF on the expression of Fos and Jun proteins in O-2A lineage cells in 1-day-old (a and c) or 3-day-old (b) cultures of P1 (a and b) or P0 (c) optic nerve cells. PDGF was added for either the last 1 hour (for Fos) or for the last 2 hours (for Jun) of the culture period and the cells were labelled with anti-GC (to identify oligodendrocytes), and then anti-Jun or anti-Fos antibodies. O-2A progenitor cells in (c) were identified by their characteristic morphology and GC^- phenotype. The results are expressed as means ± SEM of 9 cultures.

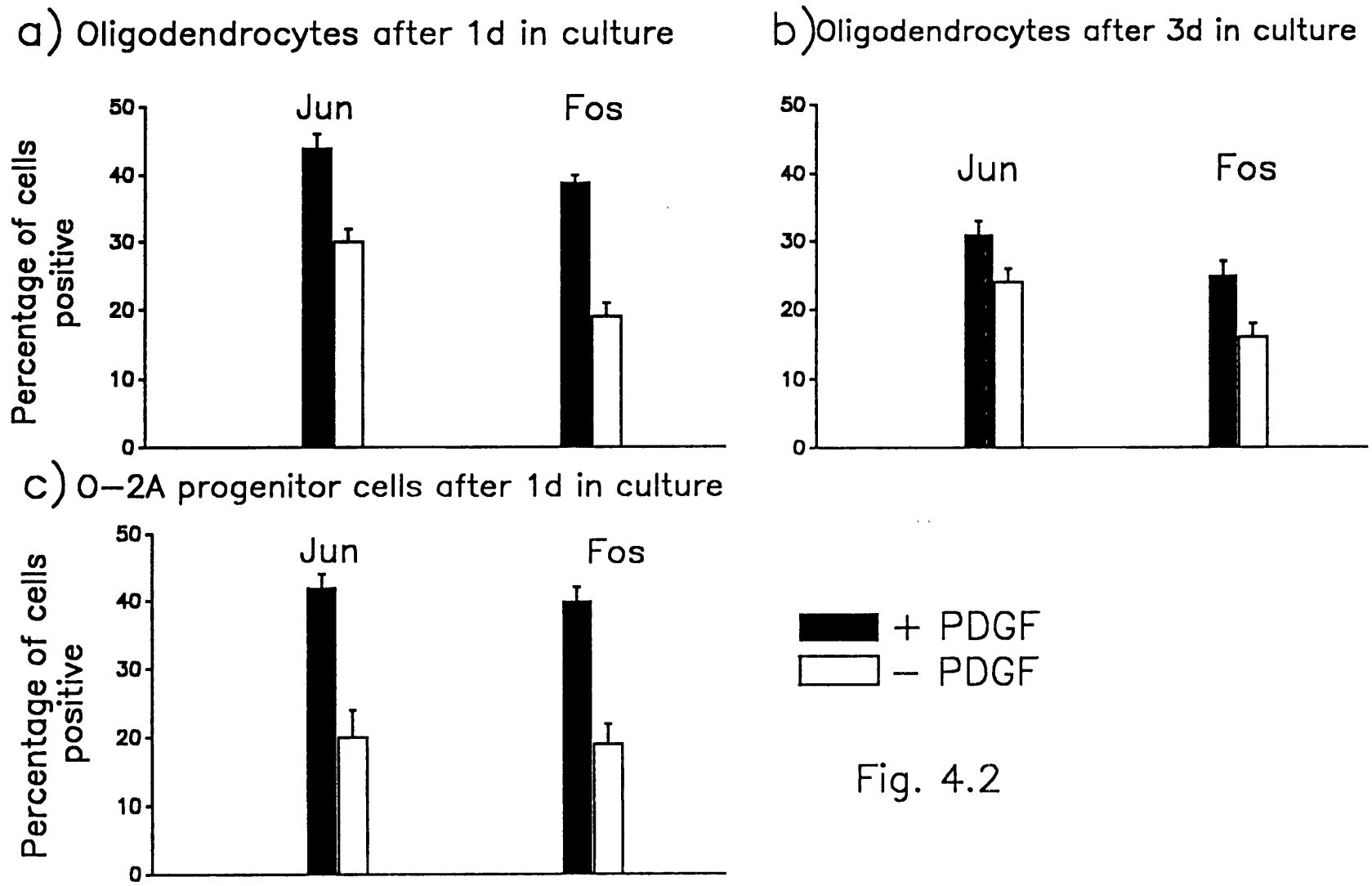
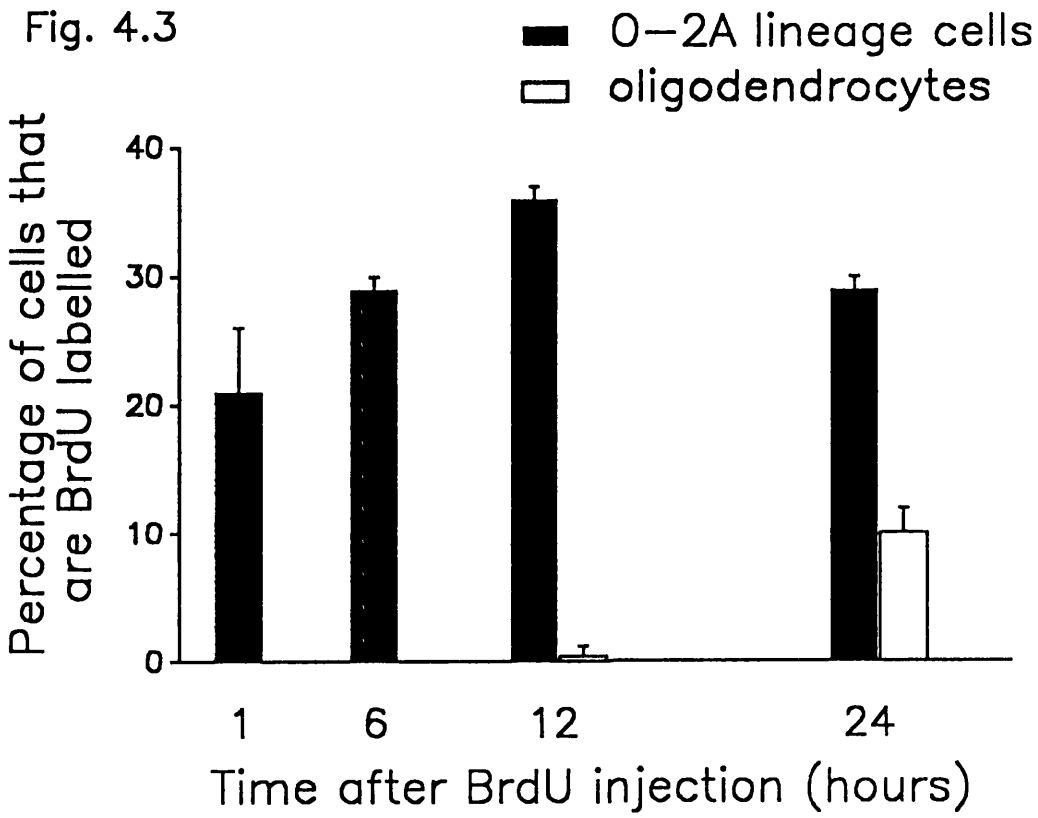

a) Oligodendrocytes after 1d in culture
b) Oligodendrocytes after 3d in culture
c) O-2A progenitor cells after 1d in culture
Percentage of cells positive
Jun
Fos
0
10
20
30
40
50
+ PDGF
– PDGF

Fig. 4.2

Figure 4.3

BrdU incorporation in O-2A lineage cells *in vivo*. BrdU was injected into P2 rats and animals were sacrificed after 1, 6, 12, or 24 hours. After dissociation, optic nerve cells were immunolabelled in suspension for either A2B5 (to identify O-2A lineage cells) or GC (to identify oligodendrocytes), plated on coverslips for 30 min, and then immunolabelled for BrdU. The results are expressed as means ± SEM of 6 cultures and 2 experiments.

Fig. 4.3


O—2A lineage cells
oligodendrocytes
Percentage of cells that are BrdU labelled
40
30
20
10
0
1
6
12
24
Time after BrdU injection (hours)

Discussion

The basis for this thesis (Hart et al., 1989a, 1989b) is the hypothesis that the timing of oligodendrocyte differentiation depends on an intrinsic mechanism in the O-2A progenitor cell that controls when the cell becomes mitotically unresponsive to PDGF (Raff et al., 1988). The hypothesis assumes that progenitor cells stop dividing before they differentiate into oligodendrocytes, and previous studies have indicated that this is the case in vitro: O-2A progenitor cells stop dividing in optic nerve cultures 12-24 hours before they express GC (Noble and Murray, 1984; Hart et al., 1989a), an early marker of oligodendrocyte differentiation (Mirsky et al., 1980; Dubois-Dalcq et al., 1986).

Does the same sequence of events also happen in vivo? Using electron microscopic autoradiography, Skoff et al., (1976a), showed that oligodendrocytes in the rat optic nerve do not become labelled after a short pulse of ^{3}H-thymidine. In these studies, however, oligodendrocytes could only be detected (by ultra-structural criteria) after the 5th postnatal day, whereas it was subsequently shown that GC^{+} oligodendrocytes first appear in small numbers in the rat optic nerve at birth (Miller et al., 1985), suggesting that electron microscopy might not be a reliable way of detecting the earliest-formed oligodendrocytes. By injecting P2 rats with BrdU, I have demonstrated in the present study that, at this age at least, few, if any, GC^{+}

oligodendrocytes in the optic nerve divide, and that O-2A progenitor cells stop synthesizing DNA at least 6-12 hours before they become GC^+ oligodendrocytes. Thus, in trying to understand the molecular basis for why O-2A progenitor cells become mitotically unresponsive to PDGF, it seems reasonable to use newly formed GC^+ oligodendrocytes in the neonatal optic nerve as surrogates for postmitotic O-2A progenitor cells: whereas the former cells are relatively easy to identify, the latter cells are not.

In P7 and P10 rats, I found that a small fraction (<1%) of GC^+ oligodendrocytes in the optic nerve was labelled by a 1 hour pulse of BrdU. Skoff et al., (1989) have reported that small numbers of GC^+ oligodendrocytes become labelled in the postnatal rat CNS after a 1 hour pulse of ^{3}H-thymidine, and Sturrock and his colleagues (Sturrock and McRae, 1980; Sturrock, 1981) have reported that occasional oligodendrocytes with apparent connections to myelin sheaths can divide during development. The significance of these small numbers of dividing oligodendrocytes seen during development is unclear. Fibroblast growth factor (FGF) has been reported to stimulate some GC^+ oligodendrocytes to synthesize DNA in vitro (Saneto and de Vellis, 1985; Eccleston and Silberberg, 1985; Wren et al., 1990). It is possible, therefore, that FGF stimulates the occasional GC^+ oligodendrocyte to proliferate in vivo. Whatever the significance and mechanism(s) of this oligodendrocyte proliferation turn out to be, there can be little doubt

that the great majority of GC^+ oligodendrocytes in the developing rat optic nerve do not divide and are produced instead from rapidly dividing GC^- progenitor cells.

The question of why many types of cells stop dividing and differentiate in the continuing presence of growth factors is a fundamental one in animal development. In the present study, I have explored the molecular basis of the intrinsic timing mechanism in O-2A progenitor cells that is thought to control the onset of their mitotic unresponsiveness to PDGF (Raff et al., 1988). Studies on newly formed oligodendrocytes (in Chapters 2 and 3), which do not synthesize DNA in response to PDGF (Hart et al., 1989a), have suggested that this unresponsiveness is the result of blocks or deficiencies in the intracellular mitotic signalling pathways, and that these blocks or deficiencies, at least in part, lie downstream of the PDGF receptor and some of the early steps that follow its activation, such as the increase in cytosolic Ca^{2+} and the activation of C-kinase (Hart et al., 1989a and b).

Using immunohistochemical techniques, I have now shown that PDGF increases nuclear Fos and Jun, both in O-2A progenitor cells and newly formed oligodendrocytes. It is not clear why only 15-20% of these cells showed an increase. One reason might be that the control levels of expression were high; another might be that the immunofluorescence assay was non-quantitative and probably insensitive, especially as all cells showed some background nuclear staining with the rabbit antisera. Despite these

limitations, the PDGF-induced increases were significant and observed consistently.

The observation that PDGF increases nuclear Fos and Jun in at least some newly formed oligodendrocytes suggests that PDGF must activate at least one intracellular signalling pathway in these cells that couples PDGF receptor activation to an elevation of Fos and Jun levels in the nucleus, even though these cells are resistant to the mitogenic effects of PDGF. With respect to PDGF receptor number (Hart et al., 1989a), PDGF-induced increase in cytosolic Ca^{2+} (Hart et al., 1989b), or PDGF-induced increase in Fos and Jun, I have not been able to detect significant differences between O-2A progenitor cells, which proliferate in response to PDGF, and newly formed oligodendrocytes, which do not.

It is not clear what roles Fos and Jun play in the mitotic response of O-2A progenitor cells to PDGF. In 3T3 cells, PDGF stimulates high levels of c-fos expression within minutes (Greenberg and Ziff, 1984), although other mitogens can apparently stimulate these cells to proliferate without inducing a detectable increase in c-fos expression (Rozengurt and Sinnett-Smith, 1987). In some cases, c-fos induction seems to play an important part in the mitogenic effects of growth stimulators (Cochran et al., 1984; Kruijer et al., 1984; Müller et al., 1984; Holt et al., 1986). Using c-fos antisense-RNA-expressing plasmids, for example, it has been shown that c-fos expression is necessary for quiescent fibroblasts to pass

from G_0 to G_1 in response to serum stimulation (Nishikura and Murray, 1987; Riabowol *et al.*, 1988). In addition, increasing c-*fos* expression by transfection with plasmids encoding the wild-type protein has been shown to cause increased proliferation of the transfected cells (Miller *et al.*, 1984). The role of c-*jun* in the regulation of cell proliferation has been less well characterised. In most cell types studied, however, c-*jun* expression is rapidly induced by mitogens (Lamph *et al.*, 1988; Quantin and Breathnach, 1988; Ryder and Nathans, 1988; Ryseck *et al.*, 1988) and mitogen treatment also rapidly co-induces both c-*fos* and c-*jun* (Rauscher *et al.*, 1988) leading to an increase in AP-1 complexes (Curran and Franza, 1988; Rauscher *et al.*, 1988). It has been suggested that the components of the AP-1 complexes change with time in response to an extracellular stimulus and that different complexes have different gene regulatory functions (Curran and Franza, 1988).

As most of the steps in the intracellular mitotic signalling pathways from the PDGF receptor to the nucleus, as well as the subsequent nuclear events, are unknown, it is difficult to speculate on why newly formed oligodendrocytes fail to synthesize DNA in response to PDGF even though at least some of them show an increase in nuclear Fos and Jun in response to the growth factor. One possibility is that all of the signalling pathways from the PDGF receptors to the nucleus operate normally in these cells, but there is a block or deficiency distal to the

immediate-early genes such as c-fos and c-jun. Another possibility is that the immediate-early gene response is incomplete in these cells, either because of a change in the nucleus or because some of the signalling pathways to the nucleus are defective. As more is learned more about the cytosolic signalling pathways and the nuclear responses, it should be possible to determine why newly formed oligodendrocytes do not proliferate in response to PDGF, which should in turn provide clues to the molecular nature of the intrinsic timing mechanism that initiates oligodendrocyte differentiation.

Materials and methods

Materials

Human PDGF was obtained from R and D Systems. Rabbit anti-Fos antiserum was a gift from Gerard Evan and rabbit anti-Jun antiserum was a gift from Tim Bos. The specificities of these antisera have been described previously (Hunt et al., 1987; Bos et al., 1988).

Cell culture

Optic nerves were removed from newborn (P0) or P1 Sprague-Dawley rats and dissociated into single cells as described previously (Miller et al., 1985, and see Chapter 2). About 20,000 cells were cultured at 37 °C in 5% CO_2 on PDL-coated glass coverslips in 0.5 ml serum-free DMEM containing various additives (glucose, insulin, transferrin, BSA, progesterone, putrescine, sodium selenite, thyroxine and tri-iodothyronine), as modified from Bottenstein and Sato (1979) and described previously (Lillien et al., 1988, and see Chapter 2).

Fos and Jun expression

Optic nerve cells were cultured in supplemented DMEM for 1 day, or in supplemented DMEM with 0.5% FCS for 3 days (to improve cell survival), and PDGF (2 ng ml^{-1}) was added for the last 1 hour (for Fos expression) or 2 hours (for Jun expression) of the culture period. In control cultures, the vehicle used to suspend the PDGF (10 mM-acetic acid

containing 2 mg ml^{-1} BSA) was added instead of PDGF. Monoclonal anti-GC antibody (Ranscht et al., 1982; ascites fluid at a final dilution of 1:200) was added for the last 30 minutes of the culture period. The cells were then fixed with 4% paraformaldehyde in PBS for 15 minutes at 4 °C, and then simultaneously labelled for 2 hours at room temperature with either rabbit anti-Fos antibody (diluted 1:1000), or rabbit anti-Jun antibody (diluted 1:500), and with fluorescein-coupled, class specific, goat anti-mouse IgG3 (G anti-MIgG3-Fl; Nordic, diluted 1:100) to detect the anti-GC antibody. The cells were then treated with biotin-coupled donkey anti-rabbit Ig (D anti-RIg-Bt; Amersham, diluted 1:100) followed by streptavidin coupled to Texas red (streptavidin-TR; Amersham, diluted 1:100). Fos and Jun antibodies, and the conjugates used to visualize their binding, were diluted in PBS containing 0.5% Triton-X 100, 1% BSA (w/v), and 0.02% sodium azide (w/v).

After staining, the cells on coverslips were mounted in Citifluor (City University, London) on glass slides and sealed with nail varnish. The cells were examined in a Zeiss Universal fluorescence microscope using a x40 or x63 objective and photographed using Kodak Tri-X film (ASA 400).

Glial cell types were identified by their characteristic morphologies and antigenic phenotypes (Raff et al., 1978; Raff et al., 1983b).

Bromodeoxyuridine incorporation

BrdU (Boehringer) was added to cultures of optic nerve cells to a final concentration of 5 x 10^{-5} M for the last 24 hours of the culture period. After fixation with 4% paraformaldehyde for 2 minutes at room temperature, cells were surface-stained with either anti-GC antibody followed by G anti-MIgG3-Fl or A2B5 antibody (Eisenbarth et al., 1979; ascites fluid at a final dilution of 1:200) followed by fluoroscein-coupled goat anti-mouse Ig (G anti-MIg-Fl, Cappel; diluted 1:100). The antibodies were diluted in Eagle's minimum essential medium buffered with 0.02 M-Hepes (MEM-Hepes) and containing 10% FCS and 0.2% sodium azide (MEM-Hepes-FCS). After surface-staining, the cells were postfixed in acid-alcohol (5% glacial acetic acid : 95% ethanol) at -20 °C for 10 minutes. They were then treated with 2 N-HCl to denature the nuclear DNA (Yong and Kim, 1987), followed by 0.1 M-sodium borosilicate (pH 8.5), both for 10 minutes at room temperature. The cells were then labelled with the Bu 20a monoclonal anti-BrdU antibody (Magaud et al., 1988; culture supernatant diluted 1:2 in PBS containing 1% Triton-X 100, 2% FCS, and 0.02% sodium azide), followed by rhodamine-coupled G anti-MIg (G anti-MIg-Rd, Cappel; diluted 1:100 in the same solution).

For studies of BrdU incorporation in vivo, BrdU (1 mg 10 g^{-1} diluted in MEM-Hepes) was injected intraperitoneally into P2, P7 or P10 rats. After various times, optic nerve cells were prepared as described above and either labelled in suspension or after 3 hours in

culture. For suspension labelling, cells were incubated in anti-GC or A2B5 antibody (diluted 1:100 in MEM-Hepes containing 10% FCS, 20% normal goat serum, and 0.02% sodium azide [w/v]) followed by either G anti-MIgG3-Fl or G anti-MIg-Fl (both diluted 1:100 in the same solution), respectively. The cells were then washed once in MEM-Hepes-FCS, plated on PDL-coated coverslips and allowed to adhere for 30 minutes. They were then stained for BrdU as above. For staining after 3 hours in culture, cells on coverslips were fixed and surfaced-stained with anti-GC antibody followed by rhodamine-coupled G anti-MIgG3 (Nordic, diluted 1:100) as described above for optic nerve cell cultures. The cells were prepared for BrdU staining as above and labelled with fluorescein-coupled monoclonal anti-BrdU antibody (Becton Dickinson; diluted 1:10 in PBS containing 0.5% Triton-X 100, 1% BSA, and 0.02% sodium azide).

GENERAL DISCUSSION

The aim of this work was to elucidate the molecular basis of the intrinsic timing mechanism in O-2A progenitor cells that controls both the loss of mitotic responsiveness to PDGF and, as a consequence of this, the timing of oligodendrocyte differentiation. In this chapter, I discuss the implications and possible uses of my findings and I review what is known about the mechanisms underlying terminal differentiation associated with loss of proliferative capacity in some other cell systems.

The nature and distribution of PDGF receptors on optic nerve cells

The binding data presented in Chapter 1, taken together with other studies (Richardson et al., 1988; Pringle et al., 1989; McKinnon et al., 1990), provides evidence that PDGF-AA acts via α PDGF receptors to stimulate O-2A progenitor cell proliferation in the developing rat CNS.

The O-2A progenitor is the first cell known to express α without β PDGF receptors. Schwann cells express β but not α PDGF receptor mRNA and specific antibodies reveal the expression of the β receptor protein (Weinmaster and Lemke, 1990) suggesting that these cells express only the β receptor. 3T3 cells and human and rat fibroblasts, by contrast, express both α and β PDGF receptors, as assessed by binding studies (Heldin et al., 1988; Hart et al., 1988; Kazlauskas et al., 1988; Pringle et al., 1989). Furthermore, binding data also suggest that the mitotic

sensitivity of a cell to the three isoforms of PDGF is determined exclusively by the relative numbers of the two types of PDGF receptor expressed by the cell (Kazlauskas et al., 1988; Seifert et al., 1989). It seems likely, therefore, that the preference of O-2A progenitor cells for PDGF-AA is explained by their α only receptor phenotype. These cells would be ideal for studying the signal transduction pathways activated by the α receptor; so far, all studies of PDGF-activated signalling pathways have involved cells with β receptors.

The combination of autoradiography and immunofluorescence suggested that O-2A progenitors and their newly differentiated progeny are the only glial cells in vitro in the developing optic nerve to express α PDGF receptors; the receptors seem to be eventually lost by differentiated oligodendrocytes (Chapter 1). Progress in the study of O-2A progenitors in vivo has been limited by the lack of specific markers to distinguish these cells. Therefore, it would be worth investigating whether antibodies against the α PDGF receptors can be used to mark O-2A progenitor cells in vivo, in which case they could be used to study the distribution and origins of these cells in situ. There is indirect evidence that O-2A progenitors migrate into the optic nerve from germinal centres in the brain (Small et al., 1987), but direct evidence is needed.

The molecular basis of the intrinsic timing mechanism in O-2A progenitor cells

Manipulation of the inositol phospholipid and cAMP signalling pathways by drugs, in both O-2A progenitor cells and oligodendrocytes, suggested that O-2A progenitor cells lose their mitotic responsiveness to PDGF, at least in part, because of changes in the intracellular mitotic response pathways that develop downstream from the early intracellular signalling events following activation of the PDGF receptor. Despite being unable to divide or synthesize DNA in response to PDGF, newly formed oligodendrocytes seem to possess PDGF receptors which are indistinguishable from those on proliferating O-2A progenitor cells by all the criteria I have investigated; the receptors are present at similar levels on both types of cell, and can stimulate a similar increase in cytosolic Ca^{2+} as well as the expression of Fos and Jun proteins following PDGF binding. Therefore, at least one signalling pathway from the PDGF receptor to the nucleus remains open in newly formed oligodendrocytes. While my findings cannot exclude the possibility that either some signalling pathways to the nucleus are defective in these cells or the immediate-early gene response is incomplete, they raise the possibility that the block or deficiency in PDGF-stimulated mitosis might lie distal to the points at which Fos and Jun act in the nucleus. I will now consider approaches that might help to distinguish between these different mechanisms.

It is now possible to obtain purified cultures of O-

2A progenitor cells or oligodendrocytes (Wren et al., 1990). Therefore, the full range of molecular and biochemical techniques, which have been so useful in the study of the cascade of early signalling events elicited by PDGF in 3T3 cells (reviewed in Rozengurt, 1986; Williams, 1989), can now be applied quantitatively to O-2A lineage cells. This approach should make it possible to determine whether changes that develop in the cytosolic signalling pathways contribute to the failure of newly formed oligodendrocytes to divide in response to PDGF.

It also needs to be established whether Fos and Jun are essential for the PDGF-induced proliferation of O-2A progenitor cells. The only convincing way to do this is to inhibit the expression of these genes (alone and in combination) in O-2A progenitor cells in the presence of PDGF and to look for any reduction in progenitor cell proliferation. Oligonucleotides that are complimentary to the leader sequence of the mRNAs encoding c-fos and c-jun can be synthesized and used to try to inhibit specifically the expression of these genes in O-2A progenitor cells in the presence of PDGF. This technique has been used to inhibit the expression of a number of genes, including c-myc (Heikkila et al., 1987), and it has the great advantage that oligonucleotides can penetrate living cells and achieve high intracellular levels; anti-sense RNA constructs, by contrast, which can also be used to inhibit endogenous gene expression, have to be transfected into cells using viral vectors (Nishikura and Murray, 1987).

Alternatively, as oligonucleotides often inhibit gene expression non-specifically, affinity-purified anti-Fos and anti-Jun antibodies can be injected into single O-2A progenitor cells to inhibit the effects of these proteins (Riabowol et al., 1988).

The idea that the changes that underlie the loss of proliferative response to mitogens that accompanies terminal differentiation lie distal to the early events in the intracellular signalling pathways has also been proposed in a number of other in vitro cell systems, for example, the terminal differentiation of haemopoietic precursor cell lines in response to colony stimulating factors (CSFs), myoblast cell lines in response to FGF, the development of senescence in human fibroblasts, and terminal differentiation in murine mesenchymal stem cells and human keratinocytes.

Three distinct CSFs that stimulate bipotential granulocyte-macrophage (GM) precursor cells to divide and form colonies of mature neutrophils and macrophages in culture have been defined (reviewed in Ihle, 1990). One of these factors, granulocyte CSF (G-CSF), stimulates GM cells to divide and differentiate into neutrophils. However, neutrophils retain specific cell surface receptors for G-CSF, suggesting that receptor loss is not the reason that these cells become mitotically unresponsive to G-CSF. Interestingly, a number of interleukin 3 (IL-3) dependent cell lines have been developed and one of these, 32DCL, differentiates into mature granulocytes in response to G-

CSF (Dexter et al., 1980). If a combination of IL-3 and G-CSF is added to the culture medium, these cells continue to divide and do not differentiate. On the other hand, if these cells are transformed with the oncogene v-abl, the cells proliferate and do not differentiate despite the presence of G-CSF. These findings suggest that continued proliferation in this system inhibits the ability of G-CSF to induce differentiation.

Recent studies have uncovered a similar phenomenon in O-2A progenitor cells. The combination of PDGF and bFGF causes most, but not all, progenitor cells in vitro to proliferate continuously and prevents their differentiation into oligodendrocytes (Wren et al., 1990). Thus, it seems that the combination of PDGF and bFGF can over-ride the timing mechanism that normally limits the proliferation of these cells to PDGF alone, and that ensures that oligodendrocyte differentiation begins on schedule.

The mouse myoblast cell line MM14 is stimulated to divide indefinitely by FGF. If FGF is removed from the culture the cells stop dividing, express a variety of muscle-specific genes, including MyoD, and fuse to form multinucleate myotubes (Clegg et al., 1987). These effects are not reversed by re-exposure to FGF. At least in its response to FGF, this cell line mimics the behaviour of primary mouse myoblasts in culture (Linkhart et al., 1980). Studies using ^{125}I-FGF show that MM14 cells express FGF receptors and that these receptors are retained for 24 hours after the cells have stopped dividing and begun to

differentiate in response to the removal of FGF from the culture medium (Olwin and Hauschka, 1988); thus, the loss of receptors seems not to be responsible for the development of mitogenic unresponsiveness to FGF. The differentiated MM14 cells eventually lose both FGF (Olwin and Hauschka, 1988) and EGF (Lim and Hauschka, 1984) receptors. As quiescent MM14 cells, differentiation-defective MM14 cells, and differentiated BC3H1 muscle cells (a line that fails to become post-mitotic after differentiation) all retain their FGF receptors, it has been suggested that the loss of growth factor receptors is a control process for maintaining terminally differentiated skeletal muscle cells in a postmitotic state (Olwin and Hauschka, 1988).

An alternative approach to the study of the control of differentiation in MM14 cells has been the construction of heterokaryons between postmitotic MM14 cells and other cells by polyethylene glycol-mediated cell fusion (Clegg and Hauschka, 1987). If postmitotic MM14 cells are fused with various quiescent nonmyogenic cells and the fused cells are treated with mitogen-rich medium within 6 hours, the MM14 nuclei synthesize DNA. However, if postmitotic MM14 cells are fused to proliferating MM14 myoblasts, even heterokaryons containing a nuclear ratio of three myoblast nuclei to one postmitotic MM14 nucleus do not synthesize DNA in response to FGF. These results suggest that mitosis is not irreversibly blocked in skeletal muscle cells but, instead, the postmitotic state of these cells is mediated

by cytoplasmic inhibitors. As it is now possible to obtain purified populations of O-2A progenitor cells or oligodendrocytes, it would be interesting to do similar fusion experiments between O-2A progenitor cells and oligodendrocytes to determine if the postmitotic state of the oligodendrocyte or the proliferative state of the progenitor cell dominates in such hybrids. My results, for example, do not exclude the possibility that a cytoplasmic inhibitor is present in oligodendrocytes.

Recently, the cDNA encoding a novel inhibitor of DNA binding proteins (Id) has been cloned (Benezra et al., 1990). This protein can specifically associate with three members of a family of proteins involved in transcriptional regulation (MyoD, E12 and E47) which share a highly conserved sequence motif called helix-loop-helix (HLH) and an adjacent basic (B) region. The Id protein also has an HLH domain but lacks an adjacent B region. HLH proteins combine to form homo- and heterodimeric transcription factors that bind to regulatory elements in various genes and are thought to play an important part in cell differentiation. Id RNA levels decrease in several cell lines at the time of induction of differentiation. Moreover, transfection experiments indicate that over-expression of Id inhibits MyoD-dependent activation of a muscle-specific enzyme. It is thought that in the muscle differentiation pathway Id acts as a negative regulator by competing with MyoD for binding to E12 and E47; by forming non-functional heterodimers with these proteins, Id

prevents MyoD and other HLH proteins from binding to E12 and E47 and therefore prevents differentiation until an appropriate stimulus is provided. Fos and Jun also form a heterodimeric transcription factor, called AP-1 (Curran and Franza, 1988), and the dimerization and subsequent DNA binding specificity appear to be mediated by a "leucine zipper" motif and an adjacent basic region, in a manner that is analogous to the HLH family. It is possible, therefore, that there might be leucine zipper proteins that lack an adjacent basic domain and, therefore, can negatively regulate this class of transcriptional activators.

Senescent human fibroblasts in culture cannot be stimulated to synthesize DNA by any combination of growth factors or mitogens, although they retain functional receptors for some of these growth factors (reviewed in Phillips et al., 1987). In addition, these cells have been shown to display a variety of intracellular responses to growth factors, including an increase in expression of the c-myc and c-H-ras proto-oncogenes (Rittling et al., 1986), even though the cells do not proliferate. Recently, such cells have been shown to have a markedly impaired c-fos response to serum, phorbol esters, cAMP, and EGF, suggesting that the loss of mitotic responsiveness, unlike that in O-2A progenitor cells, at least in part, might be due to selective repression of c-fos (Seshardi and Campisi, 1990).

A subset of highly conserved basic ribonuclear

proteins, called proliferation potential proteins (P^2Ps), have been identified in 3T3 T cells and human keratinocytes by Western blotting with monoclonal antibodies against heterogenous ribonuclear core proteins (Minoo et al., 1989). When these cells undergo terminal differentiation, they show a marked reduction in the expression of P^2Ps, suggesting that these proteins may mediate post-transcriptional control of the processing of specific RNAs required for cell proliferation.

The molecular mechanisms responsible for either cell proliferation or terminal differentiation are not known. As indicated above, it seems likely that a large number of genes might be regulated at both the transcriptional and post-transcriptional levels and that proteins encoded by some of these genes themselves act to regulate the expression of other genes. It would be surprising if the regulation of proliferation and differentiation of O-2A progenitor cells were less complex.

In conclusion, the present work has failed to define the molecular mechanisms that control when O-2A progenitor cells become mitotically unresponsiveness to PDGF, although it has excluded some mechanisms. Unlike most of the other systems I have discussed above, O-2A progenitor cells are not cell lines and they can behave in vitro much like they do in vivo, which makes them especially suitable for the study of normal differentiation. The ability to produce large numbers of O-2A progenitor cells and oligodendrocytes should now make it possible to extend these studies in ways

that were not previously feasible. As more is learned about the cytosolic signalling pathways and the nuclear events involved in the control of cell proliferation and differentiation in general, it should be possible to arrive at a detailed molecular explanation for why O-2A progenitor cells become mitotically unresponsive to PDGF.

REFERENCES

Abney, E., Bartlett, P. F. and Raff, M. C. (1981). Astrocytes, ependymal cells and oligodendrocytes develop on schedule in dissociated cell cultures of embryonic brain. Dev. Biol., **83**, 301-310.

Almendral, J. M., Sommer, D., MacDonald-Bravo, H., Burckhardt, J., Perera, J. and Bravo, R. (1988). Complexity of the early genetic response to growth factors in mouse fibroblasts. Mol. Cell Biol., **8**, 2140-2148.

Bartel, D. P., Sheng, M., Lau, L. F. and Greenberg, M. E. (1989). Growth factors and membrane depolarization activate distinct programs of early response gene expression: dissociation of fos and jun induction. Genes Dev., **3**, 304-313.

Bartlett, P. F., Noble, M., Pruss, R. M., Raff, M. C. and Williams, C. A. (1981). Rat neural antigen-2 (Ran-2): A cell surface antigen on astrocytes, ependymal cells, Müller cells and leptomeninges defined by a monoclonal antibody. Brain Res., **204**, 339-351.

Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L. and Weintraub, H. (1990). The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell, **61**, 49-59.

Berridge, M. J. (1988). Inositol lipids and calcium signalling. Proc. R. Soc. Lond. B. Biol. Sci., **234**, 359-378.

Betsholtz, C., Johnsson, A., Heldin, C.-H., Westermark, B., Lind, P., Urdea, M. S., Eddy, R., Shows, T. B., Philpott, K., Mellor, A. L., Knott, T. J. and Scott, J. (1986). cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumour cell lines. Nature, **320**, 695-699.

Bignami, A., Eng, L., Dahl, D. and Uyeda, C.T. (1972). Localisation of the glial fibrillary acidic protein in astrocytes by immunofluorescence. Brain Res., **43**, 429-435.

Bolton, A. E. and Hunter, W. M. (1973). The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Biochem. J., **133**, 529-539.

Bonner, T. I., Kerby, S. B., Sutrave, P., Gunnell, M. A., Mark, G. and Rapp, U. R. (1985). Structure and biological activity of human homologs of the raf/mil oncogene. Mol. Cell Biol., **5**, 1400-1407.

Bos, T. J., Bohmann, D., Tsuchie, H., Tjian, R. and Vogt, P. K. (1988). v-jun encodes a nuclear protein with enhancer binding properties of AP-1. Cell, **52**, 705-712.

Bottenstein, J. E. (1986). Growth requirements in vitro of oligodendrocyte cell lines and neonatal rat brain oligodendrocytes. Proc. Natl. Acad. Sci. USA., **83**, 1955-1959.

Bottenstein, J. E. and Sato, G. H. (1979). Growth of a rat neuroblastoma cell line in serum free supplemented medium. Proc. Natl. Acad. Sci. USA., **76**, 514-517.

Bowen-Pope, D. F. and Ross, R. (1985). Methods for studying the platelet-derived growth factor receptor. Methods in Enzym., **109**, 69-100.

Claesson-Welsh, L., Eriksson, A., Morén, A., Severinsson, L., Ek, B., Östman, A., Betsholtz, C. and C.-H. Heldin. (1988). cDNA cloning and expression of a human platelet-derived growth factor (PDGF) receptor specific for B-chain-containing PDGF molecules. Mol. Cell Biol., **8**, 3476-3486.

Claesson-Welsh, L., Hammacher, A., Westermark, B., Heldin, C.-H. and Nistér, M. (1989). Identification and structural analysis of the A-type receptor for platelet-derived growth factor. J. Biol. Chem., **264**, 1742-1747.

Clegg, C. H. and Hauschka, S. D. (1987). Heterokaryon analysis of muscle differentiation: regulation of the postmitotic state. J. Cell Biol., **105**, 937-947

Clegg, C. H., Linkhart, T. A., Olwin, B. B. and Hauschka, S. D. (1987). Growth factor control of skeletal muscle differentiation: commitment to terminal differentiation occurs in G_1 phase and is repressed by fibroblast growth factor. J. Cell Biol., **105**, 949-956.

Cochran, B. H., Zullo, J., Verma, I. M. and Stiles, C. D. (1984). Expression of the c-fos gene and of a fos-related gene is stimulated by platelet-derived growth factor. Science, **226**, 1080-1082.

Cohen, S. (1962). Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the newborn animal. J. Biol. Chem., **237**, 1555-1562.

Collins, M. K. L. and Rozengurt, E. (1984). Homologous and heterologous mitogenic desensitization of Swiss 3T3 cells to phorbol esters and vasopressin: role of receptor and postreceptor steps. J. Cell. Physiol., **118**, 133-142.

Coughlin, S. R., Escobedo, J. A. and Williams, L. T. (1989). Role of phosphatidylinositol kinase in PDGF receptor signal transduction. Science, **243**, 1191-1194.

Curran, T. and Franza Jr, B. R. (1988). Fos and Jun: The AP-1 connection. Cell, **55**, 395-397.

Dalla Favera, R., Gallo, R. C., Giallongo, A. and Croce, C. M. (1982). Chromosomal localisation of the human homologue (c-sis) of the simian sarcoma virus oncogene. Science, **218**, 686-688.

Devare, S. G., Reddy, E. P., Law, J. D., Robbins, K. and Aaronson, S. (1983). Nucleotide sequence of the simian sarcoma virus genome: demonstration that its acquired cellular sequences encode the transforming gene product $p28^{sis}$. Proc. Natl. Acad. Sci. USA., **80**, 731-735.

Dexter, T. M., Garland, J., Scott, D., Scolnick, E. and Metcalf, D. (1980). Growth of factor-dependent hemopoietic precursor cell lines. J. Exp. Med., **152**, 1036-1047.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M. D. (1984). Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature, **307**, 521-527.

Dubois-Dalcq, M., Behar, T., Hudson, L. and Lazzarini, R. A. (1986). Emergence of three myelin proteins in oligodendrocytes cultured without neurons. J. Cell Biol., **102**, 384-392.

Eccleston, P. A. and Silberberg, D. H. (1985). Fibroblast growth factor is a mitogen for oligodendrocytes in vitro. Dev. Brain Res., **21**, 315-318.

Eisenbarth, G. S., Walsh, F. S. and Nirenburg, M. (1979). Monoclonal antibody to a plasma membrane antigen of neurons. Proc. Natl. Acad. Sci. USA., **76**, 4913-4917.

Escobedo, J. A., Barr, P. J. and Williams, L. T. (1988b). Role of tyrosine kinase and membrane-spanning domains in signal transduction by the platelet-derived growth factor receptor. Mol. Cell Biol., **8**, 5126-5131.

Escobedo, J. A., Navankasatussas, S., Cousens, L. S., Coughlin, S. R., Bell, G. T. and Williams, L. T. (1988a). A common PDGF receptor is activated by homodimeric A and B forms of PDGF. Science, **318**, 474-475.

ffrench-Constant, C. and Raff, M. C. (1986). The oligodendrocyte-type-2 astrocyte cell lineage is specialized for myelination. Nature, **323**, 335-338.

Fujita, S. (1963). The matrix cell and cytogenesis in the developing central nervous system. J. Comp. Neurol., **120**, 37-42.

Gratzner, H. G. (1982). Monoclonal antibody to 5-bromo and 5-iododeoxyuridine: A new reagent for detection of DNA replication. Science, **318**, 474-475.

Greenberg, M. E. and Ziff, E. B. (1984). Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature, **311**, 433-438.

Gronwald, R. G. K., Grant, F. J., Haldeman, B. A., Hart, C. E., O'Hara, P. J., Hagen, F. S., Ross, R., Bowen-Pope, D. F. and Murray, M. J. (1988). Cloning and expression of a cDNA coding for the human platelet-derived growth factor receptor; evidence for more than one receptor class. Proc. Natl. Acad. Sci. USA., **85**, 3435-3439.

Habenicht, A. J. R., Glomset, J. A., King, W. C., Nist, C., Mitchell, C. D. and Ross, R. (1981). Early changes in phosphatidylinositol and arachidonic acid metabolism in quiescent Swiss 3T3 cells stimulated to divide by platelet-derived growth factor. J. Biol. Chem., **256**, 12329-12335.

Hammacher, A., Hellman, U., Johnson, A., Östman, A., Gunnarsson, K., Westermark, B., Wasteson, A. and Heldin, C.-H. (1988b). A major part of platelet-derived growth factor purified from human platelets is a heterodimer of one A and one B chain. J. Biol. Chem., **263**, 16493-16498.

Hammacher, A., Mellström, K., Heldin, C.-H. and Westermark, B. (1989). Isoform-specific induction of actin reorganization by platelet-derived growth factor suggests that the functionally active receptor is a dimer. EMBO J., **8**, 2489-2495.

Hammacher, A., Nistér, M., Westermark, B. and Heldin, C.-H. (1988a). A human glioma cell line secretes three structurally and functionally different PDGF dimers. Eur. J. Biochem., **176**, 179-186.

Hart, C. E., Forstrom, J. W., Kelly, J. D., Seifert, R. A., Smith, R. A., Ross, R., Murray, M. J. and Bowen-Pope, D. F. (1988). Two classes of PDGF receptor recognise different isoforms of PDGF. Science, **240**, 1529-1531.

Hart, I. K., Richardson, W. D., Heldin, C.-H., Westermark, B. and Raff, M. C. (1989a). PDGF receptors on cells of the oligodendrocyte-type-2 astrocyte (O-2A) cell lineage. Development, **105**, 595-603.

Hart, I. K., Richardson, W. D., Bolsover, S. R. and Raff, M. C. (1989b). PDGF and intracellular signalling in the timing of oligodendrocyte differentiation. J. Cell Biol., **109**, 3411-3417.

Heikkila,R., Schwab, G., Wickstrom, E., Loke, S. L., Pluznik, D. H., Watt, R. and Neckers, L. M. (1987). A c-myc antisense oligodeoxynucleotide inhibits entry into S phase but not progress from G_0 to G_1. Nature, **328**, 445-449.

Heldin, C.-H., Bäckström, G., Östman, A., Hammacher, A., Rönnstrand, L., Rubin, K., Nistér, M. and Westermark, B. (1988). Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. EMBO J., **7**, 1387-1393.

Heldin, C.-H., Ernlund, A., Rorsman, C. and Rönnstrand, L. (1989). Dimerization of B-type platelet-derived growth factor receptors occurs after ligand binding and is closely associated with receptor kinase activation. J. Biol. Chem., **264**, 8905-8912.

Heldin, C.-H., Johnsson, A., Wennergren, S., Wernstedt, C., Betsholtz, C. and Westermark, B. (1986). A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A chains. Nature, **319**, 511-514.

Heldin, C.-H., Wasteson, A. and Westermark, B. (1982). Interaction of platelet-derived growth factor with its fibroblast receptor. J. Biol. Chem., **275**, 4216-4211.

Heldin, C.-H., Westermark, B. and Wasteson, A. (1981). Specific receptors for platelet-derived growth factor on cells derived from connective tissue and glia. Proc. Natl. Acad. Sci. USA., **78**, 3664-3668.

His, W. (1889). Die neuroblasten und deren entstehung im embryonalen mark. Abhandl. Kgl. Sachs. Ges. Wiss. Math. Phys. Kl., **15**, 313-372.

Holt, J. T., Gopal, T. V., Moulton, A. D. and Nienhuis, A. W. (1986). Inducible production of c-fos antisense RNA inhibits 3T3 cell proliferation. Proc. Natl. Acad. Sci. USA., **83**, 4794-4798.

Hughes, S. M., Lillien, L. E., Raff, M. C., Rohrer, H. and Sendtner, M. (1988). Ciliary neurotrophic factor induces type-2 astrocyte differentiation in culture. Nature, **335**, 70-73.

Hughes, S. M. and Raff, M. C. (1987). An inducer protein may control the timing of fate switching in a bipotential glial progenitor cell in the rat optic nerve. Development, **101**, 157-167.

Hunt, S. P., Pini, A. and Evan, G. (1987). Induction of c-fos-like protein in spinal cord neurones following sensory stimulation. Nature, **328**, 632-634.

Hunter, T. (1985). Oncogenes and growth control. TIBS., **7**, 275-280.

Hunter, W. M. and Greenwood, F. C. (1962). Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature, **194**, 495-496.

Hurley, J. B., Simon, M. I., Teplow, D. B., Robishaw, J. D. and Gilman, A.G. (1984). Homologies between signal transducing G proteins and ras gene products. Science, **226**, 860-867.

Ihle, J. N. (1990). Lymphokine regulation of hematopoietic cell development. In Immunophysiology (Oppenheim, J.J. and Shevach, E. M., eds.). New York; Oxford University Press, pp. 190-229

Ives, H. E. and Daniel, T. O. (1987). Interrelationship between growth factor-induced pH changes and intracellular Ca^{2+}. Proc. Natl. Acad. Sci. USA., **84**, 1950-1954.

Jaenisch, R. (1988). Transgenic animals. Science, **240**, 1468-1474.

Johnsson, A., Heldin, C.-H., Westermark, B. and Wasteson, A. (1982). Platelet-derived growth factor: Identification of constituent polypeptide chains. Biochem. Biophys. Res. Commun., **104**, 66-74.

Kazlauskas, A., Bowen-Pope, D., Seifert, R., Hart, C. E. and Cooper, J. A. (1988). Different effects of homo- and heterodimers of platelet-derived growth factor A and B chains on human and mouse fibroblasts. EMBO J., 7, 3727-3735.

Kenyon, C. (1988). The nematode Caenorhabditis elegans. Science, **240**, 1448-1452.

Kruijer, W., Cooper, J. A., Hunter, T. and Verma, I. M. (1984). Platelet-derived growth factor induces rapid but transient expression of the c-fos gene and protein. Nature, **312**, 711-716.

La Chapelle, F., Gumple, M., Baluac, M., Jacque, C., Due, P. and Baumann, N. (1984). Transplantation of CNS fragments into the brain of shiverer mutant mice: Extensive myelination by implanted oligodendrocytes. 1. Immunological studies. Dev. Neurosci., **6**, 325-334.

Langman, J., Guerrant, R. and Freeman, B. (1966). Behaviour of neuroepithelial cells. J. Comp. Neurol., **127**, 399-412.

Lamph, W. W., Wamsley, P., Sassone-Corsi, P. and Verma, I. M. (1988). Induction of proto-oncogene JUN/AP-1 by serum and TPA. Nature, **334**, 629-631.

Lemke, G. E. and Brockes, J. P. (1984). Identification and purification of glial growth factor. J. Neurosci., **4**, 75-83.

Levitt, P,. Cooper, M. L. and Rakic, P. (1981). Co-existence of neuronal and glial precursor cells in the cerebral ventricular zone of the fetal monkey: an ultrastructural immunoperoxidase analysis. J. Neurosci., **1**, 27-39.

Lillien, L. E. and Raff, M. C. (1990). Analysis of the cell-cell interactions that control type-2 astrocyte development in vitro. Neuron, 4, 525-534.

Lillien, L. E., Sendtner, M., Rohrer, H., Hughes, S. M. and Raff, M.C. (1988). Type-2 astrocyte development in rat brain cultures is initiated by a CNTF-like protein produced by type-1 astrocytes. Neuron, 1, 485-494.

Lim, R. W. and Hauschka, S. D. (1984). A rapid decrease in epidermal growth factor-binding capacity accompanies the terminal differentiation of mouse myoblasts in vitro. J. Cell Biol., 98, 739-747.

Linkhart, T. A., Clegg, C. H. and Hauschka, S. D. (1980). Control of mouse myoblast commitment to terminal differentiation by mitogens. J. Supramol. Struct., 14, 483-498.

Magaud, J. P., Sargent, I. and Mason, D.Y. (1988). Detection of human white cell proliferative responses by immunoenzymatic measurement of bromodeoxyuridine uptake. J. Immunol. Methods, 106, 95-100.

Matsui, T., Heidaran, M., Miki, T., Popescu, N., LaRochelle, W,. Kraus, M., Pierce, J. and Aaronson, S. (1989). Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. Science, **243**, 800-804.

Matuoka, K., Fukami, K., Nakanishi, O., Kawai, S. and Takenawa, T. (1988). Mitogenesis in response to PDGF and bombesin abolished by microinjection of antibody to PIP_2. Science, **239**, 640-643.

McKinnon, R. D., Matsui, T., Dubois-Dalcq, M. and Aaronson, S. (1990). FGF modulates the PDGF-driven pathway of oligodendrocyte development. Neuron, in press.

Meisenhelder, J., Gill, P.-G., Rhee, G. G. and Hunter, A. (1989). Phospholipase C-τ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. Cell, **57**, 1109-1122.

Miller, A. D., Curran, T. and Verma, I. M. (1984). c-fos protein can induce cellular transformation: a novel mechanism of activation of a cellular oncogene. Cell, **36**, 51-60.

Miller, R. H., David, S., Patel, R., Abney, E. R. and Raff, M. C. (1985). A quantitative immunohistochemical study of macroglial cell development in the rat optic nerve: *in vivo* evidence for two distinct astrocyte lineages. *Dev. Biol.*, **111**, 35-41.

Miller, R. H., ffrench-Constant, C. and Raff, M. C. (1989a). The macroglial cells of the rat optic nerve. *Ann. Rev. Neurosci.*, **12**, 517-534.

Miller, R. H., Fulton, B. P. and Raff, M. C. (1989b). A novel type of glial cell associated with nodes of Ranvier in rat optic nerve. *Eur. J. Neurosci.*, **1**, 172-180.

Minoo, P. Sullivan, W., Soloman, L.R. Martin, T. E., Toft, D. O. and Scott, R.E. (1989). Loss of proliferative potential during terminal differentiation coincides with the decreased abundance of a subset of heterogenous ribonuclear proteins. *J. Cell Biol.*, **109**, 1937-1946.

Mirsky, R., Winter, J., Abney, E. R., Pruss, R. M., Gavrilovic, J. and Raff, M. C. (1980). Myelin-specific proteins and glycolipids in rat Schwann cells and oligodendrocytes in culture. *J. Cell Biol.*, **84**, 483-494.

Morrison, D.K., Kaplan, D. R., Rapp, U. and Roberts, T. M. (1988). Signal transduction from membrane to cytoplasm: growth factors and membrane-bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity. Proc. Natl. Acad. Sci. USA., 85, 8855-8859.

Müller, R., Bravo, R., Burckhardt, J. and Curran, T. (1984). Induction of c-fos gene and protein by growth factors precedes activation of c-myc. Nature, 312, 716-720.

Nånberg, E. and Rozengurt, E. (1988). Temporal relationship between inositol polyphosphate formation and increases in cytosolic Ca^{2+} in quiescent 3T3 cells stimulated by platelet-derived growth factor, bombesin and vasopressin. EMBO J., 7, 2741-2747.

Nishikura, K. and Murray, J. M. (1987). Antisense RNA of proto-oncogene c-fos blocks renewed growth of quiescent 3T3 cells. Mol. Cell Biol., 7, 639-649.

Noble, M. and Murray, K. (1984). Purified astrocytes promote the in vitro divisions of a bipotential glial progenitor cell. EMBO J., 3, 2243-2247.

Noble, M., Murray, K., Stroobant, P., Waterfield, M. D. and Riddle, P. (1988). Platelet-derived growth factor promotes division and motility and inhibits premature differentiation of the oligodendrocyte-type-2 astrocyte progenitor cell. Nature, **333**, 562-565.

Olwin, B. B. and Hauschka, S. D. (1988). Cell surface fibroblast growth factor and epidermal growth factor receptors are permanently lost during skeletal muscle terminal differentiation in culture. J. Cell Biol., **107**, 761-769.

Östmann, A., Backström, G., Fong, M., Betsholtz, G., Wernstedt, C., Hellman, U., Westermark, B., Valenzuela, P. and Heldin, C.-H. (1989). Expression of 3 recombinant homodimeric isoforms of PDGF in Saccharomyces cerevisiae: Evidence for differences in receptor binding and functional activities. Growth Factors, **1**, 271-281.

Phillips, P. D., Pignolo, R. J. and Cristofalo, V. J. (1987). Insulin-like growth factor-1: specific binding to high and low affinity sites and mitogenic action throughout the lifespan of the WI-38 cell. J. Cell. Physiol., **133**, 135-143.

Price, J. and Thurlow, L. (1988). Cell lineage in the rat cerebral cortex: a study using retroviral-mediated gene transfer. Development, **104**, 473-482.

Price, J., Turner, D. and Cepko, C. (1987). Lineage analysis in the vertebrate nervous system by retrovirus -mediated gene transfer. Proc. Natl. Acad. Sci. USA., **84**, 156-160.

Pringle, N., Collarini, E. J., Mosley, M. J., Heldin, C.-H., Westermark, B. and Richardson, W.D. (1989). PDGF A chain homodimers drive proliferation of bipotential (O-2A) glial progenitor cells in the developing rat optic nerve. EMBO J., **8**, 1049-1056.

Pruss, R. (1979). Thy-1 antigen on astrocytes in long term cultures of rat central nervous system. Nature, **280**, 688-690.

Quantin, B. and Breathnach, R. (1988). Epidermal growth factor stimulates transcription of the c-jun proto-oncogene in rat fibroblasts. Nature, **334**, 538-539.

Raff, M. C. (1989). Glial cell diversification in the rat optic nerve. Science, **243**, 1450-1455.

Raff, M. C., Abney, E. R., Cohen, J., Lindsay, R. and Noble, M. (1983a). Two types of astrocytes in cultures of developing rat white matter: differences in morphology, surface gangliosides, and growth characteristics. J. Neurosci., **6**, 1289-1300.

Raff, M. C., Abney, E. R. and Fok-Seang, J. (1985). Reconstruction of a developmental clock *in vitro*: a critical role for astrocytes in the timing of oligodendrocyte differentiation. *Cell*, **42**, 61-69.

Raff, M. C., Abney, E. R. and Miller, R. H. (1984a). Two glial cell lineages diverge prenatally in rat optic nerve. *Dev. Biol.*, **106**, 53-60.

Raff, M. C., Fields, K. l., Hakomori, S.-I., Mirsky, R., Pruss, R. M. and Winter, J. (1979). Cell type-specific markers for distinguishing and studying neurones and the major classes of glial cells in culture. *Brain Res.*, **174**, 283-308.

Raff, M. C., Lillien, L. E., Richardson, W. D., Burne, J. F. and Noble, M. (1988). Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. *Nature*, **333**, 562-565.

Raff, M. C., Miller, R. H. and Noble, M. (1983b). A glial progenitor cell that develops *in vitro* into an astrocyte or an oligodendrocyte depending on culture medium. *Nature*, **303**, 390-396.

Raff, M. C., Mirsky, R., Fields, K. L., Lisak, R. P., Dorfman, S. H., Silberberg, D. H., Gregson, N. A., Leibowitz, S. and Kennedy, M. (1978). Galactocerebroside: a specific cell surface antigenic marker for oligodendrocytes in culture. Nature, **274**, 813-816.

Raff, M. C., Williams, B. P. and Miller, R. H. (1984b). The in vitro differentiation of a bipotential glial progenitor cell. EMBO J., **3**, 1857-1864.

Rakic, P. (1972). Mode of cell migration to the superficial layers of the fetal monkey neocortex. J. Comp. Neurol., **145**, 61-84.

Rakic, P. (1974). Neurons in the rhesus monkey visual cortex: Systematic relation between time of origin and eventual disposition. Science, **183**, 425-427.

Ralston, R. and Bishop, J. M. (1985). The product of the protooncogene c-src is modified during the cellular response to platelet-derived growth factor. Proc. Natl. Acad. Sci. USA., **82**, 7845-7849.

Ranscht, B., Clapshaw, P. A., Price, J., Noble, M. and Seifert, W. (1982). Development of oligodendrocytes and Schwann cells studied with a monoclonal antibody against galactocerebroside. Proc. Natl. Acad. Sci. USA., **79**, 2709-2713.

Rauscher, F. J., Cohen, D. R., Curran, T., Bos, T. J., Vogt, P. K., Bohmann, D., Tjian, R. and Franza Jr, B. R. (1988). Fos-associated protein p39 is the product of the jun proto-oncogene. Science, **240**, 1010-1016.

Ready, D. F. (1989). A multifaceted approach to neural development. TINS, **12**, 102-110.

Riabowol, K. T., Vosatka, R. J., Ziff, E. B., Lamb, N. J. and Feramisco, J. R. (1988). Microinjection of fos-specific antibodies blocks DNA synthesis in fibroblast cells. Mol. Cell Biol., **8**, 1670-1676.

Richardson, W. D., Pringle, N., Mosley, M. J., Westermark, B.and Dubois-Dalcq, M. (1988). A role for platelet-derived growth factor in normal gliogenesis in the central nervous system. Cell, **53**, 309-319.

Rittling, S. R., Brooks, K. M., Cristofalo, V.J., and Baserga, R. (1986). Expression of cell-cycle dependent genes in young and senescent WI-38 fibroblasts. Proc. Natl. Acad. Sci. USA., **83**, 3316-3320.

Rozengurt, E. (1986). Early signals in the mitogenic response. Science, **243**, 161-166.

Rozengurt, E., Rodriguez-Pena, M. and Smith, K. A. (1983a). Phorbol esters, phospholipase C, and growth factors rapidly stimulate the phosphorylation of a M_r 80,000 protein in intact quiescent 3T3 cells. Proc Natl. Acad. Sci. USA., **80**, 7244-7248.

Rozengurt, E. and Sinnett-Smith, J. W. (1987). Bombesin induction of c-fos and c-myc proto-oncogene in Swiss 3T3 cells: significance for the mitogenic response. J. Cell. Physiol., **131**, 218-225.

Rozengurt, E., Stroobant, P., Waterfield, M. D., Druel, T. F. and Keehan, M. (1983b). Platelet-derived growth factor elicits cyclic AMP accumulation in Swiss 3T3 cells: role of prostaglandin production. Cell, **34**, 265-272.

Rubin, G. M. (1988). Drosophila melanogaster as an experimental organism. Science, **240**, 1453-1459.

Ryder, K. and Nathans, D. (1988). Induction of protooncogene c-jun by serum growth factors. Proc. Natl. Acad. Sci. USA., **85**, 8464-8467.

Ryseck, R.-P., Hirai, S. I., Yaniv, M. and Bravo, R. (1988). Transcriptional activation of c-jun during the G_0/G_1 transition in mouse fibroblasts. Nature, **334**, 535-537.

Sanes, J. R. (1989). Analyzing cell lineage with a recombinant retrovirus. TINS, **12**, 21-28.

Saneto, R. P. and de Vellis, J. (1985). Characterization of cultured rat oligodendrocytes proliferating in serum-free, chemically defined medium. Proc. Natl. Acad. Sci. USA., **82**, 3509-3513.

Sauer, F. C. (1935a). Mitosis in the neural tube. J. Comp. Neurol., **62**, 337-405.

Sauer, F. C. (1935b). The cellular structure of the neural tube. J. Comp. Neurol., **63**, 13-23.

Sauer, M. E. and Walker, B. E. (1959). Radiographic study of interkinetic nuclear migration in the neural tube. Proc. Soc. Exp. Biol. Med., **101**, 557-560.

Scatchard, G. (1949). The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. Sci., **51**, 660-672.

Schmechel, D. E. and Rakic, P. (1979). A Golgi study of radial glial cells in the developing monkey telencephalon: morphogenesis and transformation into astrocytes. Anat. Embryol., **156**, 115-152.

Seifert, R. A., Hart, C. E., Phillips, P. E., Forstrom, J. W., Ross, R., Murray, M. J. and Bowen-Pope, D. F. (1989). Two different subunits associate to create isoform-specific platelet-derived growth factor receptors. J. Biol. Chem., **264**, 8771-8778.

Seshardi, T. and Campesi, J. (1990). Repression of c-fos transcription and an altered genetic program in senescent human fibroblasts. Science, **247**, 205-208.

Sidman, R. L., Miale, I. L. and Feder, N. (1959). Cell proliferation and migration in the primitive ependymal zone: an autoradiographic study of histogenesis in the nervous system. Exp. Neurol., **1**, 322-333.

Silver, R. A., Lamb, A. G. and Bolsover, S. R. (1989). Elevated cytosolic calcium in the growth cone inhibits neurite elongation in neuroblastoma cells: correlation of behaviourial states with cytosolic calcium concentration. J. Neurosci., **9**, 4007-4020.

Skoff, R. P., Knapp, P. E. and Ghandour, M. S. (1989). Neuroglial cell lineage: In vivo and in vitro differences. Soc. Neurosci. Abstr., **15**, 14 (Neuroglia, section 10.6).

Skoff, R., Price, D. and Stocks, A. (1976a). Electron microscopic autoradiographic studies of gliogenesis in rat optic nerve. I. Cell proliferation. J. Comp. Neurol., **169**, 291-312.

Skoff, R., Price, D. and Stocks, A. (1976b). Electron microscopic autoradiographic studies of gliogenesis in rat optic nerve. II. Time of origin. J. Comp. Neurol., **169**, 313-333.

Slack, J. M. W. (1983). From egg to embryo: determinative events in early development. Cambridge; Cambridge University Press.

Small, R. K., Riddle, P. and Noble, M. (1987). Evidence for migration of oligodendrocyte-type-2 astrocyte progenitor cells into the developing rat optic nerve. Nature, **328**, 155-157.

Spemann, H. (1938). Embryonic development and induction. New Haven; Yale University Press. (Reprinted, New York; Garland, 1988).

Sternberg, P. W. and Horvitz, H. R. (1986). Pattern formation during vulval development in C. elegans. Cell, **44**, 761-772.

Stroobant, P. and Waterfield, M. D. (1984). Purification and properties of porcine platelet-derived growth factor. EMBO J., **2**, 2963-2967.

Sturrock, R. R. and McRae, D. A. (1980). Mitotic division of oligodendrocytes which have begun myelination. J. Anat., **131**, 577-582.

Sturrock,R. R. (1981). Electron microscopic evidence for mitotic division of oligodendrocytes. J. Anat., **132**, 429-432.

Sulston, J. E. and Horvitz, H. R. (1977). Postembryonic cell lineages of the nematode Caenorhabditis elegans. Dev. Biol., **56**, 110-156.

Sulston, J. E., Schierenberg, E., White, J. G. and Thompson, J. N. (1983). The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev. Biol., **100**, 64-119.

Sulston, J. E. and White, J. G. (1980). Regulation and cell autonomy during development of Caenorhabditis elegans. Dev. Biol., **78**, 577-597.

Takeya, T. and Hanafusa, H. (1983). Structure and sequence of the cellular gene homologous to the RSV src gene and the mechanism for generating the transforming virus. Cell, **32**, 881-890.

Temple, S. and Raff, M. C. (1985). Differentiation of a bipotential glial progenitor cell in single cell microculture. Nature, **313**, 223-225.

Temple, S., and Raff, M. C. (1986). Clonal analysis of oligodendrocyte development in culture: evidence for a developmental clock that counts cell divisions. Cell, **44**, 773-779.

Turner, D. and Cepko, C. (1987). Cell lineage in the rat retina: a common progenitor for neurons and glia persists late in development. Nature, **328**, 131-136.

Varmus, H. (1988). Retroviruses. Science, **240**, 1427-1435.

Walicke, P. A. (1989). Novel neurotrophic factors, receptors, and oncogenes. Ann. Rev. Neurosci., **12**, 103-126.

Waterfield, M. D., Scrace, G. T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C.-H., Huang, J. S. and Druel, T. F. (1983). Platelet -derived growth factor is structurally related to the putative transforming protein p28^{sis} of simian sarcoma virus. Nature, **304**, 35-39.

Weinmaster, G. and Lemke, G. (1990). Cell-specific cyclic AMP-mediated induction of the PDGF receptor. EMBO J., **9**, 915-920.

Westermark, B., Johnsson, A., Paulsson, Y., Betsholtz, C., Heldin, C.-H., Herlyn, M., Rodeck, U. and Koprowski, H. (1986). Human melanoma cell lines of primary and metastatic origin express the genes encoding the chains of platelet-derived growth factor (PDGF) and produce a PDGF-like growth factor. Proc. Natl. Acad. Sci. USA., **83**, 7197-7200.

Williams, B. P., Abney, E. R. and Raff, M. C. (1985). Macroglial cell development in embryonic rat brain: studies using monoclonal antibodies, fluorescence activated cell sorting and cell culture. Dev. Biol., **112**, 126-134.

Williams, L. T. (1989). Signal transduction by the platelet-derived growth factor receptor. Science, **243**, 564-1570.

Wood, W. B. ed. (1988). The nematode Caenorhabditis elegans. Cold Spring Harbor; Cold Spring Harbor Laboratory.

Wren, D., Bogler, O., Barnett, S. C. and Noble, M. (1990). Co-application of PDGF plus FGF promotes continuous proliferation, and inhibits differentiation, of oligodendrocyte-type-2 astrocyte progenitor cells. Proc. Natl. Acad. Sci. USA., in press.

Yarden, Y., Escobedo, J. A., Kuang, W. J., Yang-Feng, T. L., Daniel, T. O., Tremble, P. M., Chen, E. Y., Ando, M. E., Harkins, R. N., Franke, U., Fried, V. A., Ullrich, A. and Williams, L. T. (1986). Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. Nature, 323, 226-232.

Yarden, Y. and Ullrich, A. (1988). Growth factor receptor tyrosine kinases. Ann. Rev. Biochem., 57, 443-478.

Yong, V. W. and Kim, S. U. (1987). A new double-labelling immunofluorescence technique for the determination of proliferation of human astrocytes in culture. J. Neurosci. Methods, 21, 9-16.