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Cell Death and Control of Cell Survival in the Oligodendrocyte Lineage

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Summary

Dead cells are observed in many developing animal tissues, but the causes of these normal cell deaths are mostly unknown. We show that about 50% of oligodendrocytes normally die in the developing rat optic nerve, apparently as a result of a competition for limiting amounts of survival signals. Both platelet-derived growth factor and insulin-like growth factors are survival factors for newly formed oligodendrocytes and their precursors in culture. Increasing platelet-derived growth factor in the developing optic nerve decreases normal oligodendrocyte death by up to 90% and doubles the number of oligodendrocytes in 4 days. These results suggest that a requirement for survival signals is more general than previously thought and that some normal cell deaths in nonneural tissues may also reflect competition for survival factors.

Introduction

Cell death occurs in most animal tissues at some stage of their development (Glucksman, 1951). These normal or programmed cell deaths are thought to involve the activation of a suicide program in the cells that die (Wyllie et al., 1980; Ellis et al., 1991), but the mechanisms of death are unknown. Despite the prevalence of normal cell death, there has been remarkably little work on the control of cell survival, especially when compared with the enormous effort that has been devoted to studying the control of cell proliferation. Neurons are an exception. It has long been recognized that many neurons in the developing vertebrate nervous system die soon after they are formed (Hamburger and Levi-Montalcini, 1949; Cowan et al., 1984; Oppenheim, 1991). In the case of sympathetic and some sensory neurons, it is thought that death occurs because the developing cells compete for limiting amounts of nerve growth factor (NGF) released by their target cells, so that only a proportion of the neurons receive enough NGF to survive (Levi-Montalcini, 1987; Purves, 1988; Barde, 1989). Competition for target cell–derived survival (neurotrophic) factors is believed to occur widely in the developing peripheral and central nervous systems and is thought to serve at least two functions: to ensure an appropriate numerical match between synaptically connected cells, and to eliminate inappropriate neuronal projections (Cowan et al., 1984).

Some glial cells also die during the development of the vertebrate central nervous system (CNS) (Smart and Leblond, 1961; Pannese and Ferrannini, 1967; Hildebrand, 1971; Sturrock, 1979; Korr, 1980; Knapp et al, 1986; Jackson and Duncan, 1988), but the type of glial cells that die and the mechanisms responsible for their death are unclear, although some have been identified as oligodendrocytes (Mori and Leblond, 1970; Hildebrand, 1971). Compared with the intensive search for neurotrophic factors, little effort has been made to define factors that might be required for glial cell survival. A standard approach to define novel neurotrophic factors has been to study the influence of tissue extracts, cell culture supernatants, and known signaling molecules on the survival of purified populations of neurons in culture (Barde et al., 1983). Here we have adopted a similar strategy to look for survival factors for oligodendrocytes and their precursors, using cultures of either single or purified cells isolated from the developing rat optic nerve.

We and others have previously studied the cell–cell interactions that control the proliferation and differentiation of glial precursors called O-2A progenitor cells, which, depending on the culture conditions, can develop in vitro into either oligodendrocytes or type-2 astrocytes (Raff, 1989; Richardson et al., 1990; Noble et al., 1991). Type 1 astrocytes, the first glial cells to differentiate in the developing rat optic nerve (Miller et al., 1985), produce platelet-derived growth factor (PDGF) A chain and thus are a likely source of the AA homodimer form of PDGF (Richardson et al., 1988; Pringle et al., 1989). PDGF–AA binds to PDGF α receptors on O-2A progenitor cells (Hart et al., 1989b; Pringle et al., 1992) and thereby stimulates these cells to proliferate (Noble et al., 1988; Richardson et al., 1988). O-2A progenitor cells, however, cannot divide indefinitely in response to PDGF; they have an intrinsic mechanism that causes them to stop dividing and differentiate into oligodendrocytes after a maximum of about eight cell divisions in vitro (Raff et al., 1985, 1988; Temple and Raff, 1986). Once formed, oligodendrocytes start to lose their PDGF receptors (McKinnon et al., 1990). Whereas oligodendrocyte development in vitro occurs constitutively, type-2 astrocyte development in vitro depends on cell–cell interactions (Lillien and Raff, 1990).

We now show that the survival of O-2A progenitor cells and oligodendrocytes in vitro also depends on signals from other cells. We show that insulin-like growth factors (IGFs) act as survival factors for O-2A progenitor cells and oligodendrocytes, while PDGF acts as a survival factor for O-2A progenitor cells and those newly formed oligodendrocytes that have just begun to differentiate and still express PDGF α receptors. The growth factors appear to promote survival by suppressing an active death program in the glial cells.

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Table 1. Survival of Purified O-2A Lineage Cells in Culture

	O-2A Progenitor Cells			Oligodendrocytes		
	Percentage of Cells Surviving After			Percentage of Cells Surviving After		
Factors Added	18 hr	42 hr	66 hr	18 hr	42 hr	66 hr
None	31 ± 11	5 ± 4	2 ± 2	34 ± 4	18 ± 7	4 ± 4
ONCM (1:1)	85 ± 12	70 ± 8	55 ± 5	90 ± 12	74 ± 9	55 ± 6
Insulin (5 μg/ml)	100	78 ± 8	65 ± 13	100	79 ± 12	55 ± 17
IGF-1 (100 ng/ml)	101 ± 8	81 ± 10	70 ± 12	98 ± 4	79 ± 7	58 ± 9
IGF-2 (100 ng/ml)	92 ± 5	70 ± 11	56 ± 12			
PDGF (10 ng/ml)	95 ± 13	82 ± 12	79 ± 8	43 ± 13	27 ± 8	9 ± 6
bFGF (10 ng/ml)	54 ± 19	21 ± 12	9 ± 5	52 ± 10	36 ± 9	17 ± 2
EGF (10 ng/ml)	33 ± 9	10 ± 3	4 ± 2	32 ± 8	16 ± 3	2 ± 2
Cycloheximide (0.1 μg/ml)	77 ± 14	5 ± 1	1 ± 1	67 ± 13	14 ± 5	3 ± 1

Approximately 1000 purified cells were plated in 10 μml of B–S medium without insulin in Terasaki microplates; about 200 cells adhered to the bottom of the well. After 30 min, 1 μl of DMEM, or DMEM containing the appropriate factor, was added. The number of live cells on the bottom of the microwell was counted in an inverted phase-contrast microscope at the times indicated. The means of quadruplicate wells in each experiment were calculated and expressed as a percent of the mean value of the number of cells surviving in 5 μg/ml insulin at 18 hr. The results shown are means ± S. D. of at least three separate experiments.

Lastly, we show that about 50% of newly formed oligodendrocytes in the developing rat optic nerve normally die, apparently as a result of a competition for limiting amounts of PDGF and IGFs. To our knowledge, this is the first direct demonstration that developing glial cells require signals from other cells to survive in culture and that oligodendrocytes die in large numbers during normal development.

Results

Survival of Purified O-2A Progenitor Cells in Culture

O-2A progenitor cells were purified from a suspension of postnatal day 7 (P7) rat optic nerve cells by sequential immunopanning to greater than 99.95% purity, as assessed by immunostaining (see Experimental Procedures). The cells were cultured in Terasaki microwells in serum-free and insulin-free, modified Bottenstein and Sato (B–S) medium containing bovine serum albumin (BSA), selenium, putrescine, thyroxine, tri-iodothyronine, transferrin, and progesterone. When the number of surviving cells on the bottom of the well was assessed after about 18, 42, and 66 hr by counting the live cells in an inverted phase-contrast microscope, it was found that few cells survived in this medium (Table 1, O-2A Progenitor Cells). Even when the cells were cultured at five times higher density than usual, the majority died within 18 hr (data not shown). The addition of serum-free and insulin-free B–S medium that had been conditioned for 2 days by a culture of P7 optic nerve cells (from which O-2A lineage cells had been removed by immunopanning) (optic nerve–conditioned medium [ONCM]) greatly increased the number of surviving cells at each time point (Table 1, O-2A Progenitor Cells), suggesting that the non-O-2A lineage cells in optic nerve secrete factors in culture that promote the surivival of O-2A progenitor cells.

To determine which signaling molecules could act as survival factors for O-2A progenitor cells, we tested a number of purified growth factors. As can be seen in Table 1 (O-2A Progenitor Cells) IGF-1, IGF-2, PDGF, or a concentration of insulin (5 μg/ml) high enough to activate IGF-1 receptors greatly increased the number of surviving cells at each of the three time points tested. Basic fibroblast growth factor (bFGF) had only a small effect, and epidermal growth factor (EGF) had none. Remarkably, nearly identical results were obtained when the various factors were tested in Dulbecco's modified Eagle's medium (DMEM) without the B–S additives, so that most purified O-2A progenitor cells survived for 3 days in cultures that contained IGF-1 or PDGF as the only added protein (Figure 1A).

The effects of IGFs, PDGF, and insulin on cell number were mainly due to effects on cell survival, rather than on cell proliferation. As shown in Table 2, these factors both increased the number of surviving cells and proportionally decreased the number of dead cells, so that the total numbers of cells in the factor-containing microwells were not statistically different from the numbers in medium alone. Although PDGF did induce bromodeoxyuridine (BrdU) incorporation in purified O-2A progenitor cells at high density (see below), little proliferation was observed in the microwells, perhaps because of the low cell density.

To ensure that our assessment of cell viability and death by phase-contrast microscopy was accurate, we also assessed cell survival with the MTT assay (Mosmann, 1983), which measures mitochondrial function (Figure 2). There was close agreement between the two assays of cell viability for all of the conditions tested and each of the three time points; an example is shown in Table 2.

Mechanism of O-2A Progenitor Cell Death

The morphology of the cells that died by 18 hr in the absence of survival factors was consistent with their having died by programmed cell death rather than by necrosis (Wyllie et al., 1980). Both by phase-contrast microscopy and by fluorescence microscopy after propidium iodide staining, the nucleus was usually seen to be shrunken, highly condensed, and sometimes fragmented. By elec-

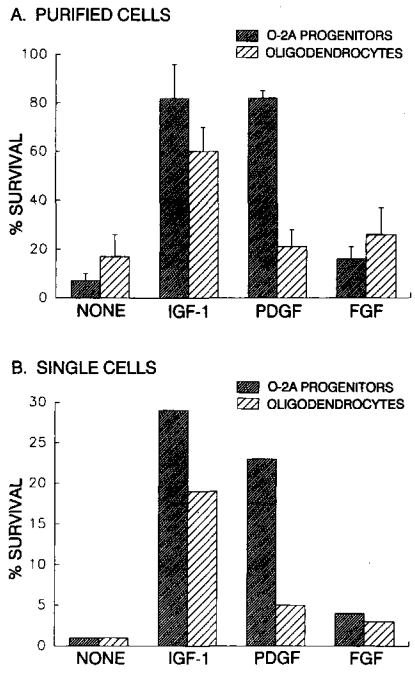

Figure 1. Survival of Purified and Single O-2A Progenitor Cells and Oligodendrocytes In Vitro

(A) Cells were purified from P7 optic nerves, and approximately 1000 cells were plated per microwell in 10 μl of DMEM without insulin. Growth factors were added 30 min later, and the number of live cells per well was assessed both by phase microscopic appearance or by the MTT assay after 18 hr. The results are expressed as a percent of the survival in high insulin at 18 hr, and are the mean ± S. D. of three separate experiments; values from four wells were averaged in each experiment.

(B) Single cells from unfractionated cell suspensions of P7 optic nerve were cultured in microwells containing 10 μl of insulin-free B–S medium in the presence or absence of growth factors. Survival was assessed after 1 day in vitro by dividing the number of surviving O-2A progenitor cells or oligodendrocytes by the number of total wells containing a single live cell at 1 hr minus the number of wells containing a live non-O-2A lineage cell. Since this denominator included all dead cells, some of which were presumably non-O-2A lineage cells, these results underestimate survival. The values of all experiments were pooled and represent observations on 80 to 200 cells studied in each condition. Very similar results were obtained when single cells were cultured in DMEM rather than B–S medium; for example, at 1 day in IGF-1 in DMEM, the survival was 35%.

tron microscopy, the chromatin was usually seen to be compacted and segregrated into sharply defined masses (Figures 3B, 3C, and 3D), although margination of chromatin was observed in only about 10% of the cells (Figure 3D); the cytoplasm frequently contained multiple vacuoles (Figures 3B and 3D).

When purified progenitor cells cultured in the absence of survival factors were observed by phase-contrast microscopy using time-lapse video recording, individual dying cells were seen to undergo morphological changes consistent with programmed cell death; after a period of intense surface activity, the cells rounded up and shrank.

Consistent with the possibility that factor-deprived cells died by programmed cell death, cycloheximide increased cell survival in the absence of survival factors, although only for 1 day (Table 1, O-2A Progenitor Cells). On the other hand, in three separate experiments, DNA extracted from purified O-2A progenitor cells that were cultured in the absence of survival factors for 13–38 hr showed no evidence of DNA degradation into oligonucleosome-size fragments, which is often observed in programmed cell death (Wyllie, 1980). DNA fragmentation was easily detected, however, in cultures containing the same number of interleukin-2-dependent cytotoxic T lymphocytes that were deprived of interleukin-2 for 20 hr (data not shown).

Survival of Purified Oligodendrocytes in Culture

The survival of purified galactocerebroside (GC)-positive oligodendrocytes from P7 optic nerve was studied in the same way as described above for O-2A progenitor cells. As was the case for O-2A progenitor cells, most oligodendrocytes died within 18 hr when cultured in insulin-free B–S medium or DMEM, even when cultured at five times the usual cell density. ONCM, IGF-1, high concentrations of insulin, and cycloheximide greatly enhanced cell survival, whereas bFGF had only a weak effect, and EGF had little effect (Table 1, Oligodendrocytes; see Figure 1A). In contrast with O-2A progenitor cells, however, PDGF had relatively little effect on oligodendrocyte survival (Table 1, Oligodendrocytes; see Figure 1A). The results were similar whether or not recently formed A2B5$^+$ oligodendrocytes were eliminated from the starting population by an additional immunopanning step, and whether cell survival was assessed by phase-contrast microscopy or by the MTT assay (data not shown). The morphology of the dead oligodendrocytes after 18 hr in the absence of survival factors was similar to that of O-2A progenitor cells under the same conditions, and we could not find evidence for DNA degradation into oligonucleosome-size fragments in four out of four experiments (data not shown).

To determine whether newly formed oligodendrocytes, which still express PDGF receptors (Hart et al., 1989a, 1989b), could be saved by PDGF, purified O-2A progenitor cells were cultured for 24 hr in B–S medium containing high insulin, conditions that have previously been shown to induce rapid differentiation into oligodendrocytes (Raff et al., 1983b). The cells were then removed from the culture dish and tested for survival. Less than 1% of these cells incorporated BrdU in response to PDGF, suggesting that they had become oligodendrocytes and were no longer progenitor cells. As was the case for O-2A progenitors, PDGF had a significant survival effect on these cells, even though it no longer induced them to synthesize DNA. At 42 hr after replating into microwells, the results of two separate experiments, expressed as the percent of survival in high insulin, were the following: no insulin, 68, 68; insulin (5 μg/ml), 100, 100; PDGF (10 ng/ml), 85, 86; and bFGF (10 ng/ml), 57, 67. The period during which PDGF could save newly formed oligodendrocytes was brief; when purified O-2A progenitor cells were cultured for 48 hr instead of 24 hr, they could no longer be saved by PDGF (data not shown).

Survival of Single O-2A Lineage Cells in Microculture

The most convincing way to demonstrate that a survival

Table 2. Various Measures of Cell Survival in Cultures of Purified O-2A Progenitor Cells in B–S Medium

	Number of Cells After 18 hr		Number of Cells After 66 hr	
Factors Added	Live Cells by Phase Contrast	Dead Cells by Phase Contrast	Live Cells by Phase Contrast	Live Cells by MTT Assay
None	90 ± 18	145 ± 25	6 ± 2	6 ± 3
Insulin (5 μg/ml)	199 ± 40	44 ± 7	133 ± 4	137 ± 7
IGF-1 (100 ng/ml)	207 ± 38	40 ± 5	135 ± 16	138 ± 20
PDGF (10 ng/ml)	201 ± 29	50 ± 8	164 ± 21	170 ± 22

Purified O-2A progenitor cells were cultured as described in Table 1. After 18 hr, the total numbers of live and dead cells were counted by phase-contrast microscopy. At 66 hr, the numbers of live cells were recounted by phase contrast, and then 1 μl of MTT (5 mg/ml) was added, and the numbers of cells whose mitochondria were able to reduce the MTT to a dark blue reaction product were assessed by bright-field microscopy. The results shown are means ± S. D. of quadruplicate cultures.

factor acts directly is to study its effects on single cells in isolation. Single cells were micromanipulated from unfractionated cell suspensions prepared by trypsin dissociation of P7 optic nerves, as previously described (Temple and Raff, 1985). None of the single O-2A progenitor cells or oligodendrocytes survived for 1 day without insulin, and few survived in bFGF, whereas there was substantial survival in IGF-1 (see Figure 1B). PDGF promoted survival of single O-2A progenitor cells, but had little effect on oligodendrocytes (see Figure 1B).

In single-cell experiments, only 7%–25% of the surviving O-2A progenitors had divided once by 1 day (as expected, since 20% of the O-2A progenitor cells were in S phase at the time of isolation); no cells divided more than once in any of the conditions tested, even after 4 days of culture (data not shown). This result further illustrates that O-2A progenitor cells at low density do not divide much in the presence of PDGF, although they do incorporate BrdU (Hart et al., 1989b).

Influence of IGF-1 on Purified O-2A Progenitor Cell Proliferation and Differentiation into Oligodendrocytes

To study the influence of IGF-1 (or high insulin) on O-2A progenitor cell proliferation and differentiation into oligodendrocytes, we cultured purified progenitor cells in B–S medium on glass coverslips in the presence or absence of various combinations of PDGF, insulin, and IGF-1. After 2 days, we measured the uptake of BrdU into DNA as a measure of DNA synthesis and the expression of GC as a measure of oligodendrocyte differentiation. As shown in Table 3, PDGF, but not IGF-1 or high insulin, induced DNA synthesis in O-2A progenitor cells, and the presence of IGF-1 or high insulin had little influence on the proportion of cells that took up BrdU or expressed GC in the presence of PDGF.

Cell Death in the Developing Optic Nerve

To determine whether O-2A lineage cells normally die during development, we analyzed optic nerves that had been perfusion fixed, frozen, cut longitudinally into 8 μm sections, and labeled with propidium iodide to stain nuclear DNA. Dead (pyknotic) cells were identified with phase-contrast optics by their shrunken, phase-dark appearance (Figure 4A) and with fluorescence optics by their con-

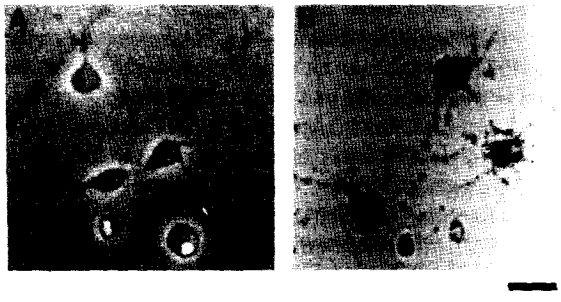

Figure 2. Assays of Survival

Survival of the purified O-2A progenitor cells cultured in microwells was assessed either by phase-contrast microscopy (A) or by the MTT assay (B). In each figure, two typical dead cells and three live cells are shown. In (B), viable cells metabolized the MTT into an insoluble dark blue reaction product. Bar, 20 μm.

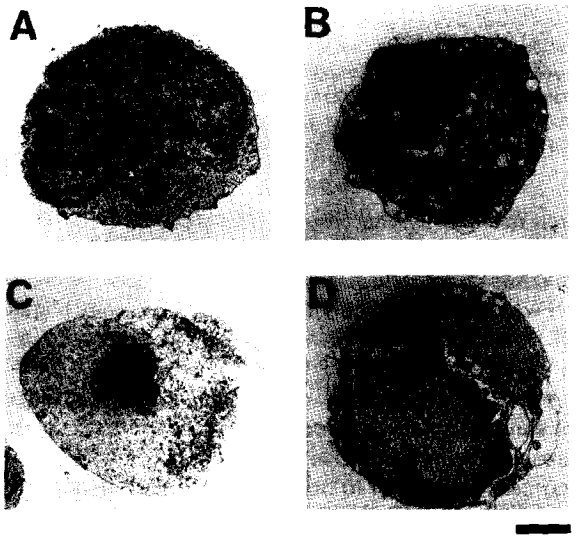

Figure 3. Ultrastructure of Dead Cells

Purified O-2A progenitor cells were cultured for 15 hr in B–S medium with (A) or without (B, C, and D) insulin (5 μg/ml) and were then processed for electron microscopy. Bar, (A) 2.8 μm; (B) 2.6 μm; (C) 4.3 μm; (D) 2.3 μm.

Table 3. Influence of Insulin and IGF on BrdU Incorporation and Oligodendrocyte Differentiation in Cultures of Purified O-2A Progenitor Cells

Factor Added	Percent GC^+ Oligodendrocytes	Percent $BrdU^+$ Cells
None	91 ± 1	0
PDGF (10 ng/ml)	16 ± 2	52 ± 6
Insulin (5 μg/ml)	96 ± 1	0
IGF-1 (100 ng/ml)	97 ± 1	0
PDGF + insulin (5 μg/ml)	16 ± 1	53 ± 1
PDGF + IGF-1	16 ± 1	52 ± 4

Approximately 40,000 purified O-2A progenitor cells were plated in 100 μl of insulin-free B–S medium containing the appropriate factor onto PDL-coated 6 mm glass coverslips. After 24 hr, BrdU (10 μM) was added, and 24 hr later, the cells were fixed and labeled with anti-BrdU and anti-GC antibodies. The results shown are means ± S. E. of three coverslips.

densed and often fragmented nucleus, which stained intensely with propidium iodide (Figure 4B). Such cells were observed in optic nerves at all ages examined.

The number of dead cells per nerve at the time of fixation (estimated by multiplying the number of dead cells per section times the average number of sections per nerve) was about 300–500, depending on the age. This value is probably an underestimate, since cells in the early stages of degeneration are unlikely to be detected by light microscopy. The proportion of dead cells at each age was calculated by dividing the number of dead cells by the total number of cells per nerve, which was calculated from measurements of the total amount of DNA in the nerve (Figure 5A; see Experimental Procedures). The proportion of dead cells was highest in developing optic nerves (Figure 5B); it peaked between P4 and P10 at about 0.25%, and decreased progressively to less than 0.01% in adult animals. Dead cells became rare (<0.01%) from P45 onward, which corresponded closely with the termination of net cell generation in the nerve (Figures 5A and 5B).

Intraperitoneal injections with the protein synthesis inhibitor cycloheximide, at concentrations previously demonstrated to decrease brain protein synthesis by about 90% (Bannon and Goedert, 1984; Deguchi and Axelrod, 1972), diminished the number of dead cells per section by 91%; control, 13.4 ± 0.65; test, 1.27 ± 0.29 (mean ± S. E., n = 3). This concentration of cycloheximide also eliminated more than 90% of the mitotic figures (data not shown).

Identity of Dead Cells in the Optic Nerve

The optic nerve contains mainly oligodendrocytes and their precursors, type-1 astrocytes and their precursors, endothelial cells, and microglia. To determine the identity of the dead cells, optic nerve sections were double labeled with propidium iodide to detect the dead cells and with cell type–specific antibodies to detect the two major cell types, astrocytes and oligodendrocytes. Antibodies to glial fibrillary acidic protein (GFAP), a marker of astrocytes, did not label any of the dead cells. The RIP monoclonal antibody, which specifically recognizes oligodendrocytes (Friedman et al., 1989), labeled about 15% of the dead cells (at ages between P4 and P12, 26/167 pyknotic cells were positive), suggesting that some of the dead cells were oligodendrocytes (see Figure 4C).

The timing of the appearance of the pyknotic cells and their spatial distribution corresponded closely with the on-

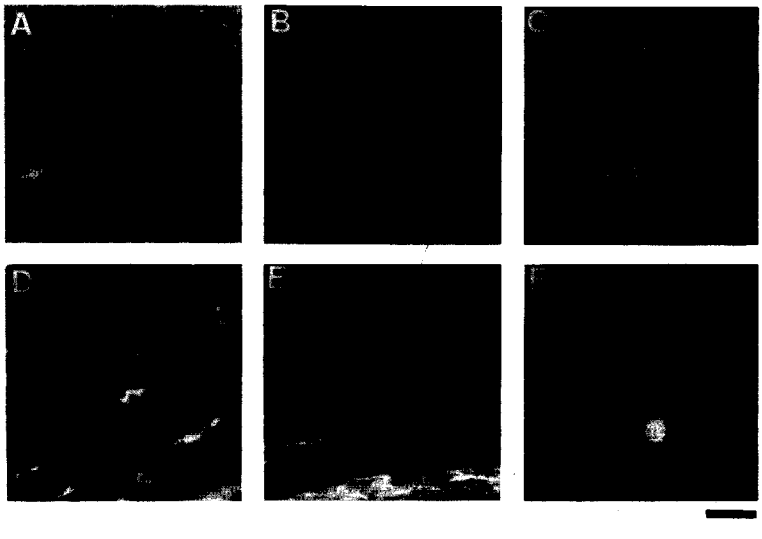

Figure 4. Cell Death in the Developing Rat Optic Nerve

Perfusion-fixed and frozen postnatal optic nerves were sectioned longitudinally and labeled with propidium iodide to stain the nuclei of normal and dead cells. In some cases, the sections were also labeled with antibodies to identify the type of cells that had died.

(A) Typical appearance of a dead cell (arrow) in a P9 optic nerve, visualized with phase-contrast microscopy.

(B) Dead cell (arrow) in P9 optic nerve section stained with propidium iodide.

(C and F) Dead oliogdendrocyte in an optic nerve section from a P5 rat that had received hybridoma cells secreting anti-GC antibodies. The section was stained with FITC-conjugated anti-mouse Ig antibodies to detect the anti-GC antibody (C) and propidium iodide (F). The dead cell (arrow) is GC^+. Two other GC^+ oligodendrocytes are present just above the dead oligodendrocyte: the one on the right appears normal, whereas the one on the left appears to have margination of its chromatin and was probably at an early stage of death (cells with this nuclear morphology, however, were not included in the dead cell counts).

(D) Section from a P7 animal that had received hybridoma cells secreting the A2B5 antibody. The hybridoma cells were injected into the subarachnoid space of P2 rats. FITC-conjugated anti-mouse Ig was used to detect the primary antibody; only the O-2A progenitor cells in the optic nerve are apparently labeled.

(E) Section from a P14 animal that had received hybridoma cells secreting the anti-Thy1-1 antibody on P7; only axons of retinal ganglion cells are specifically labeled. Bar, (A and B) 12 μm; (D and E) 17 μm, (C and F) 6 μm.

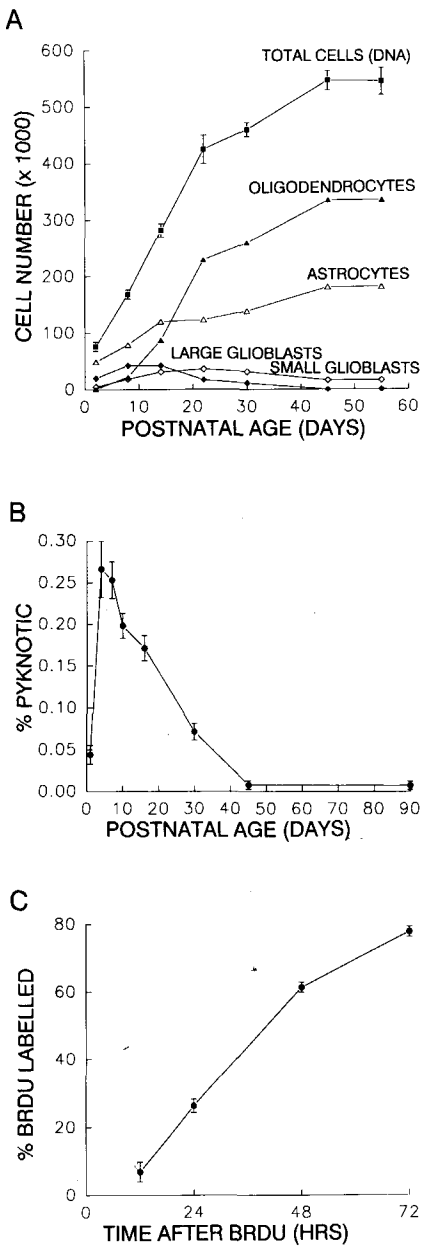

Figure 5. Quantitation of Cell Production and Death in the Developing Rat Optic Nerve

(A) The increase in total cells in the optic nerve with development, calculated from the total amount of DNA in the nerve. Each value represents the mean ± S. E. of four animals. The other four curves represent the estimated number of glial cells and precursors per nerve and were determined by multiplying the total number of cells in the nerve by the proportion of each cell type as determined in electron microscopic sections of rat optic nerves by Vaughn (1969, Figure 18). Small glioblasts are now thought to be O-2A progenitor cells (see Fulton et al., 1992), while large glioblasts are probably type-1 astrocyte precursors.

(B) The proportion of pyknotic nuclei in the optic nerve at different ages. The average instantaneous number of dying cells per nerve was determined by multiplying the average number of pyknotic nuclei in a longitudinal section of the nerve (excluding the optic chiasm) by the average number of sections per nerve at a given age; this value was converted to a percent of total cells by dividing by the number of total cells per nerve.

(C) The interval between S phase and cell death was determined by injecting developing animals with BrdU and following the appearance of the BrdU-labeled pyknotic nuclei.

set and localization of oligodendrocyte differentiation. At P4, the distribution of RIP$^+$ oligodendrocytes is graded along the length of the optic nerve, with most of the oligodendrocytes located in the chiasm-third of the nerve (B. Friedman, unpublished data), whereas the distributions of type-1 astrocytes and O-2A progenitor cells are not graded (Small et al., 1987). At this age, we observed a gradient of pyknotic cells that colocalized with the distribution of RIP$^+$ cells: about 80% of the pyknotic cells were found in the chiasm-third of the nerve, and only rare pyknotic cells were found in the eye-third. By P7, the gradients of RIP$^+$ cells and of pyknotic cells were no longer apparent.

GFAP and the RIP antigen are intracellular proteins and therefore might be digested during the death process; the plasma membrane, by contrast, is preserved until late in the course of programmed cell death (Wyllie et al., 1980). There are several monoclonal antibodies directed against cell type–specific surface antigens in the optic nerve. These include anti-RAN-2, A2B5, and anti-GC antibodies, which specifically recognize type-1 astrocytes and their precursors, O-2A progenitor cells, and oligodendrocytes, respectively (Bartlett et al., 1981; Raff et al., 1978, 1983a, 1983b; Ranscht et al., 1982). Hitherto, these antibodies have not been generally useful for labeling tissue sections; RAN-2 is destroyed by fixation, A2B5 antibody is only specific when applied to cell surfaces, and GC is destroyed by drying. To avoid these problems, hybridoma cells secreting these antibodies were injected into the subarachnoid space of P2-P8 rats (Schnell and Schwab, 1990). The cells seeded the meningeal surfaces and secreted antibodies into the cerebrospinal fluid, from where the antibodies were distributed to cell surfaces in the optic nerve; specific labeling of type-1 astrocytes, O-2A progenitor cells, oligodendrocytes, and axons was achieved within 2–3 days with anti-RAN-2, A2B5, anti-GC, and anti-Thy-1 antibodies, respectively (see Figures 4D and 4E). These antibodies were also distributed to cell surfaces throughout much of the brain (data not shown).

Using this procedure, we found that at P5, 91% (202/221) of the dead cells were GC$^+$ oligodendrocytes (see Figures 4C and 4F) and 17% (9/54) were A2B5$^+$ cells—either O-2A progenitor cells or just formed oligodendrocytes. Dead cells were not labeled with anti-RAN-2, anti-Thy-1-1, or anti-Thy-1-2 antibodies, or with fluorescein-conjugated anti-mouse Ig antibodies alone, suggesting that they were not type-1 astrocytes or their precursors, endothelial cells, or microglia, and that the anti-GC labeling was specific. To be certain that the antibodies did not increase cell death, the numbers of dead cells per optic nerve section were also counted: although the numbers were not increased by any of the antibodies, there was a 75% decrease with the anti-GC antibody, possibly because antibody-coated dead cells were phagocytosed more rapidly.

Determination of the Interval between S Phase and Cell Death in the Optic Nerve

The sharp decrease in cell death at P45, when net cell generation ceased in the optic nerve, suggested that mainly newly generated cells died (Figure 5). The interval between S phase of the cell cycle and cell death was deter-

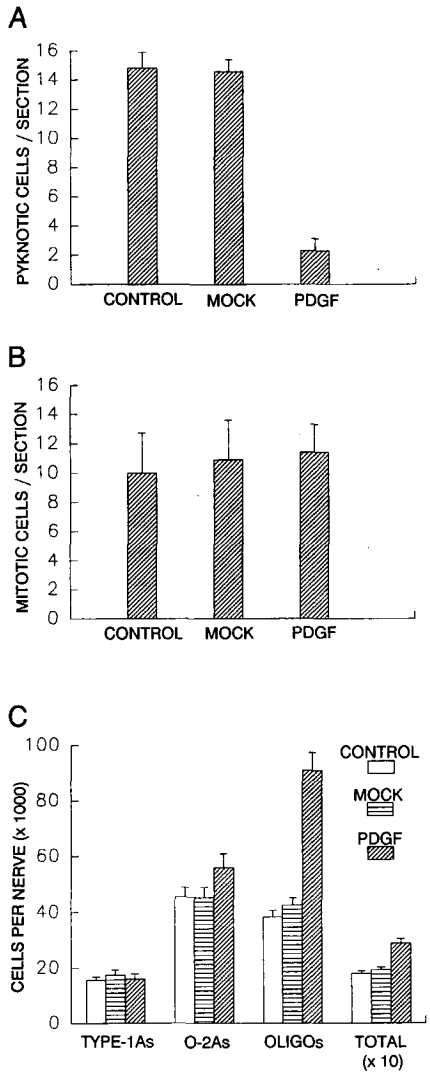

Figure 6. The Effect of Increasing the Concentration of PDGF in the Developing Optic Nerve

COS cells, which had been transiently transfected with a plasmid expression vector encoding the human PDGF-A chain, were injected into the subarachnoid space of a postnatal animal at P8, and the optic nerves were examined at P12. Values represent means ± S. E., n = 4 animals.

(A) The number of dead cells per propidium iodide–stained longitudinal section of optic nerve was determined in control animals that received no cell injection, in animals that received mock-transfected COS cells, and in animals that received PDGF-expressing COS cells.

(B) The number of mitotic figures per longitudinal section was determined, as described in (A).

(C) The total number of cells and the number of cells of each type per optic nerve was determined in control and test animals by measuring the DNA and by dissociating the cells and determining immunohistochemically the percent of astrocytes, O-2A progenitor cells, and oligodendrocytes. These immunohistochemical values were multiplied by the total number of cells per nerve (determined by DNA measurement) to give the number of each cell type per nerve.

mined by following the appearance of labeled dead cells in P15 optic nerves after three intraperitoneal injections of BrdU were given over 16 hr (which labeled 75% of O-2A progenitor cells). Although only small numbers of pyknotic nuclei were BrdU labeled immediately after the third injection, by 48 hr, most of the pyknotic nuclei were BrdU labeled (Figure 5C). The proportion reached a plateau value of about 75%, which corresponded closely with the proportion of O-2A progenitor cells that was labeled by the pulse, suggesting that virtually all of the dead oligodendrocytes were born no longer than 2–3 days prior to their death.

Effect of Increased PDGF on Cell Death in the Optic Nerve

To test the possibility that oligodendrocyte death in vivo reflects a competition for limiting amounts of survival factors such as PDGF, we delivered extra PDGF into the developing optic nerve. This was achieved by the transplantation of COS cells, which were transiently transfected with a plasmid vector designed to express PDGF-A chain, into the subarachnoid space of P8 rats. The expressed PDGF also contained a Myc epitope tag so that it could be detected using an anti-Myc monoclonal antibody (Evan et al., 1985). Four days after the COS cell injection, the optic nerves were examined. When sections of the nerves were stained with the anti-Myc antibody, large numbers of brightly labeled COS cells were seen overlying the optic chiasm; there was also diffuse staining within the nerve, suggesting the presence of high amounts of PDGF in the extracellular fluid of the nerve.

The average number of dead cells per section was decreased by about 85% in animals that had received injections of COS cells expressing PDGF, but was unaffected in the control animals that received mock-transfected cells (Figure 6A). The average number of mitotic figures per section, however, was similar in control and test animals (Figure 6B), as was the proportion of O-2A progenitor cells that incorporated BrdU 90 min after a single intraperitoneal injection (about 20%, data not shown).

The total number of cells per nerve, determined by measuring the amount of DNA, was increased by 50% in the PDGF-treated animals compared with normal or control animals (Figure 6C). To determine which cell types contributed to this increase, optic nerves were dissociated and stained with various cell type–specific antibodies; the number of $GFAP^+$ type-1 astrocytes was not changed by PDGF delivery, the number of $A2B5^+$ O-2A progenitor cells was increased by about 20%, and the number of GC^+ oligodendrocytes was increased more than 2-fold (Figure 6C). There was also an increase in the number of cells that were unlabeled by any of the antibodies in the PDGF-treated animals; these may have been meningeal cells, which have PDGF α receptors (Pringle et al., 1992).

Discussion

O-2A Lineage Cells Require Signals from Other Cells to Survive in Culture

To determine whether a cell requires signals from other cells to survive, it is important to study single or highly purified cells. Even small numbers of contaminating cells can dramatically alter the behavior of purified cells; the presence of one contaminating antigen-presenting cell per 10,000 purified T lymphocytes, for example, can greatly alter their response to mitogens (Habu and Raff, 1977). Although a variety of approaches have been used to purify

O-2A progenitor cells (Behar et al., 1988; Aloisi et al., 1990; Bogler et al., 1990), including immunopanning (Stallcup and Beasley, 1987; Gard and Pfeiffer, 1989; Dutly and Schwab, 1991), in no case has the purity achieved been better than 90%. By combining positive and negative selection steps in a sequential immunopanning procedure, we have obtained O-2A lineage cells of greater than 99.95% purity with a yield of greater than 95%.

By using both single-cell cultures and cultures of pure O-2A progenitor cells or oligodendrocytes, we show that these cells require signals from other cells to survive in vitro, even when cultured at high density, they are unable to survive on their own. Conditioned medium from cultures of non-O-2A lineage cells from optic nerve (containing mainly type-1 astrocytes, meningeal cells, and to a lesser extent endothelial cells) allow the O-2A progenitor cells and oligodendrocytes to survive for at least 3 days, suggesting that non-O-2A lineage cells in the optic nerve secrete survival factors for O-2A lineage cells, at least in vitro.

IGFs and PDGF Act As Survival Factors for O-2A Lineage Cells in Culture

For O-2A progenitor cells and newly formed oligodendrocytes, either IGF-1 or PDGF is sufficient for short-term survival in the absence of other signaling molecules or proteins; for more mature oligodendrocytes, IGF-1, but not PDGF, is sufficient. Thus, the requirements for survival change as O-2A progenitor cells differentiate into oligodendrocytes. IGF-2 or a high concentration of insulin mimics the effect of IGF-1, presumably because they both activate IGF-1 receptors (Sara and Hall, 1990), which have been shown to be present on cultured O-2A progenitor cells and oligodendrocytes (McMorris et al., 1986; Baron-Van Evercooren et al., 1991). Although bFGF is mitogenic for both oligodendrocytes and progenitor cells in the presence of high insulin (Eccleston and Silberberg, 1985; Bogler et al., 1990; McKinnon et al., 1990), it is not mitogenic in the absence of insulin (unpublished data) and has only a weak survival-promoting effect on its own. EGF has little survival-promoting effect on either cell type.

It seems likely that neighboring cell types supply survival factors to O-2A lineage cells in the optic nerve, as they do in vitro. PDGF activity (Raff et al., 1988) and PDGF mRNA (Pringle et al., 1989) have been demonstrated in the developing rat optic nerve, and type-1-like astrocytes have been shown to make PDGF in culture (Richardson et al., 1988), suggesting that astrocytes are a major source of PDGF in the developing optic nerve. IGF-1 and IGF-2 proteins and mRNAs have also been demonstrated in the developing and adult rat CNS (Rotwein et al., 1988; Lievre et al., 1991), and IGF-1 is made by both neurons and glial cells in culture (Ballotti et al., 1987; Rotwein et al., 1988; Drago et al., 1991). Anti-IGF-1 antibodies stain glial cells in the developing rat optic nerve and ganglion cells in the developing rat retina (Hannson et al., 1989); interestingly, the IGF-1 immunoreactivity was detected in optic nerve glial cells only between P1 and P45, which corresponds to the period of oligodendrocyte generation (see Figure 5). IGF-2 in the CNS is made primarily by the choroid plexus and leptomeninges and is present in the cerebrospinal fluid in much higher concentrations than IGF-1 (Sara and Carlsson-Skwirut, 1990).

It seems likely, then, that PDGF, IGF-1, and IGF-2 all promote the survival of O-2A progenitor cells and oligodendrocytes in the developing optic nerve. As PDGF has been shown to stimulate some cells to make IGF-1 (Clemmons, 1985; Clemmons and Shaw, 1986), it is possible that PDGF promotes the survival of O-2A progenitor cells by stimulating them to make IGF-1 or IGF-2 for themselves. If so, PDGF must be able to induce a single cell to produce enough IGF to save itself in microculture.

IGFs Are Not Mitogens for O-2A Progenitor Cells

Although it is clear that both IGF-1 and IGF-2 play important roles in regulating mammalian growth (Mathews et al., 1988; Sara and Hall, 1990), for the most part, it is still unclear whether they do so by primarily promoting cell survival or cell proliferation. While it has been found that IGF-1 or high insulin is a required component of defined media for the growth of most cell types in culture (Barnes and Sato, 1980), most studies of IGF effects on growth in vivo and in vitro have not specifically examined the influence of IGFs on cell survival. Moreover, in most studies of normal cells, mixed cell cultures were used, so that it is uncertain whether IGF-1 was acting directly or indirectly. Studies of the effects of IGF-1 on O-2A progenitor and oligodendrocyte development illustrate these difficulties.

McMorris and his colleagues were the first to show that IGF-1 increases the numbers of oligodendrocytes that develop in culture (McMorris et al., 1986). Their subsequent studies on microcultures of rat brain–derived O-2A progenitor cells growing in the presence of irradiated cortical astrocytes led them to conclude that IGF-1 acts in two ways to promote oligodendrocyte development: it promotes proliferation of O-2A progenitor cells, and it induces these cells to become committed to develop into oligodendrocytes (McMorris and Dubois-Dalcq, 1988; McMorris et al., 1990). In contrast, in the present study, we find that IGF-1 (or high insulin) is not mitogenic for optic nerve O-2A progenitor cells, nor does it significantly enhance PDGF-induced DNA synthesis or promote oligodendrocyte differentiation in the presence or absence of PDGF. Its main effect is to promote the survival of both O-2A progenitor cells and oligodendrocytes. Our differing conclusions might reflect differences in the way the experiments were performed: McMorris and his colleagues studied the effects of IGF-1 (or high insulin) on O-2A progenitor cells in the presence of other cell types, so that the effects of IGF-1 on progenitor cell proliferation and differentiation might have been indirect; for example, IGF-1 might have increased the generation of O-2A progenitors from contaminating pre-O-2A cells. More importantly, McMorris and Dubois-Dalcq (1988) did not examine the effects of IGF-1 on the survival of O-2A progenitor cells or oligodendrocytes; by increasing the survival of these cells, IGF-1 or high insulin would be expected to increase the number of oligodendrocytes that develop in culture. As in the case of oligodendrocytes and their precursors, IGFs promote survival and not proliferation of vertebrate CNS neurons

(Bottenstein et al., 1980; Aizenman and deVellis, 1987; Svzric and Schubert, 1990) and neuroepithelial cells (Drago et al., 1991).

Approximately 50% of Oligodendrocytes Die during Normal Optic Nerve Development

Although degenerating glial cells have long been observed in the developing CNS, the identity of these cells has been uncertain. Hildebrand (1971) provided evidence, on the basis of ultrastructural studies of developing cat white matter, that some degenerating cells were oligodendrocytes. In the present study, we show that most of the dying cells in the developing optic nerve are newly formed oligodendrocytes. First, the RIP monoclonal antibody, which specifically recognizes oligodendrocytes, labels 15% of the pyknotic cells, while an anti-GC monoclonal antibody labels more than 90% of the pyknotic cells; none of the pyknotic cells is labeled by anti-GFAP or anti-RAN-2 antibodies, which recognize astrocytes. The low proportion of dead cells that are labeled by the RIP antibody compared with that labeled by anti-GC antibody might reflect either a relatively later appearance of the RIP antigen during oligodendrocyte differentiation or the destruction of the antigen in dying cells. Second, at P4, a gradient of pyknotic cells is observed that colocalizes with a gradient of newly formed oligodendrocytes, with most of these cells in the chiasm-third of the nerve. Third, when normal cell death in the optic nerve is decreased by experimentally increasing PDGF levels, it is mainly the number of oligodendrocytes that correspondingly increases (see below). Fourth, BrdU pulse-chase experiments show that the majority of the pyknotic cells go through S phase 12–60 hr prior to their death, indicating that most of the oligodendrocytes (which are postmitotic cells) that die have recently been born. Cell death in the optic nerve is observed throughout the time-course of oligodendrocyte generation from P1 to P45, indicating that oligodendrocyte death is not simply a secondary response to retinal ganglion cell death, which is completed by P4 in albino rats (Potts et al., 1982).

It is possible to estimate the number of newly formed oligodendrocytes that normally die, from the increase in oligodendrocytes that occurs when PDGF is increased in the optic nerve by the transplantation of transfected COS cells. Because the increase in PDGF does not increase cell proliferation, and only slightly increases the number of O-2A progenitor cells, the bulk of the increase in oligodendrocyte number can be accounted for by the decrease in their death. During the 4 days of PDGF treatment, there is an increase of 40,000 oligodendrocytes, suggesting that at least 10,000 oligodendrocytes normally die daily between P8 and P12.

To determine the proportion of newly formed oligodendrocytes that die, it is necessary to know how many new oligodendrocytes are generated daily. To estimate this, we first determined the total number of cells produced daily by measuring the amount of DNA in the optic nerves at different ages (see Figure 5A). Vaughn (1969) has determined the proportions of the different types of glial cells in the rat optic nerve at various ages using electron microscopy. By multiplying these percentages (Vaughn, 1969, Figure 18) by the total number of cells in the nerve determined by DNA analysis, we can estimate the number of each cell type and their rate of generation throughout development (see Figure 5A). Between P8 and P12, about 10,000 (±2,000) surviving oligodendrocytes are produced daily, which is about the same as the number that die daily during this period, so that approximately 50% (±10%) of newly formed oligodendrocytes must die in the optic nerve during this time.

We can estimate the proportion of oligodendrocytes that die at other times in the following way. Although about 10,000 cells die per day in the optic nerve between P8 and P12, only about 400 pyknotic cells are present in the optic nerve at any moment. This suggests a clearance time of about 1 hr, once a pyknotic cell is seen. If the clearance time remains constant after P12, one can estimate the rate of cell death in older nerves from the numbers of pyknotic cells. About 480 dead cells per optic nerve are present at P16 and about 320 at P30, which represents about 11,500 and 7,700 dead cells per optic nerve per day, respectively. Surviving oligodendrocytes are generated at about 15,000 and 4,600 cells per day at P16 and P30, respectively (see Figure 5A), which corresponds to a death rate of about 43% and 64%. Thus, the proportion of newly generated oligodendrocytes that die seems to average about 50% throughout optic nerve development. This is similar to the amount of death in many vertebrate neuronal populations during development (Cowan et al., 1984; Oppenheim, 1991), although oligodendrocyte death (like oligodendrocyte generation) is spread over a much longer period.

Oligodendrocyte Death Seems to Reflect a Competition for Limiting Amounts of Survival Factors In Vivo

Our findings suggest that O-2A lineage cells compete for limiting amounts of survival factors produced by their neighbors in the developing CNS, just as developing neurons are thought to compete for limiting amounts of neurotrophic factors produced by their target cells. Experimentally increasing the amounts of neurotrophic factor during development rescues neurons that would normally die by naturally occurring cell death; NGF rescues chick sensory and sympathetic neurons (Hamburger et al., 1981), and BDNF rescues quail nodose ganglion neurons (Hofer and Barde, 1988). Thus far, it has not been possible to deliver trophic factors to the normal developing CNS because of the early formation of the blood–brain barrier. To overcome this problem we implanted PDGF-secreting cells into the CNS, using a modification of a method described by Schnell and Schwab (1990) for implanting antibody-secreting cells.

Using this method, we found that increasing the amount of PDGF available decreases the death of O-2A lineage cells in the developing optic nerve by about 85% and correspondingly increases the number of surviving oligodendrocytes. This finding suggests that limiting amounts of PDGF may normally regulate the survival and death of newly formed oligodendrocytes in the developing optic nerve. As neurotrophic factors are generally defined as substances that participate directly in the regulation of

naturally occurring neuronal death and are present in vivo in limiting amounts (Barde, 1988, 1989), it seems reasonable to conclude that PDGF is a "gliatrophic" factor for newly formed oligodendrocytes. Although PDGF is largely thought of as a mitogen for many types of nonneural cells, our findings suggest that it may also act as a survival factor for some of these cells.

Our observations suggest that IGF-1 and IGF-2 are also present in the optic nerve in limiting amounts; either alone is sufficient to prevent oligodendrocytes and their precursors from dying in vitro, yet many newly formed oligodendrocytes die in vivo. A more direct demonstration that IGFs are normally limiting in the CNS is the finding that brain size, brain DNA, and the amount of brain myelin are all increased in mice that carry a human IGF-1 transgene and, as a result, express increased levels of IGF-1 mRNA and protein in the brain (Mathews et al., 1988; Carson, 1990; McMorris et al., 1990; Mozell and McMorris, 1991). The increase in the numbers of various nonneural cell types in these transgenic mice indicates that IGFs are also present in limiting amounts outside the nervous system (Mathews et al., 1988).

Why is it that PDGF levels in the developing optic nerve are limiting for the survival of newly formed oligodendrocytes but are much less so for the survival and proliferation of O-2A progenitor cells? It seems likely that this vulnerability of newly formed oligodendrocytes reflects the progressive loss of PDGF receptors from these cells (Hart et al., 1989b; McKinnon et al., 1990; Pringle et al., 1992), which makes them less able to compete for PDGF. But why should oligodendrocytes that survive for 2–3 days no longer be at risk? Our data do not distinguish among three possibilities: more mature oligodendrocytes might become better at competing for IGFs; they might become responsive to a new survival factor; or they might entirely lose dependence on survival factors.

Why are twice as many oligodendrocytes generated than are needed? One possibility is that cell death resulting from a competition for survival factors helps to adjust the number of oligodendrocytes to the number of axons requiring myelination, just as competition for neurotrophic factors is thought to adjust the number of presynaptic cells to the number of postsynaptic cells requiring innervation—both in development (Cowan et al., 1984; Purves, 1988; Oppenhein, 1991) and in evolution (Purves, 1988). The same mechanism could also help to ensure that oligodendrocytes are evenly spaced along the length of the axon, with death occurring mainly in regions of oligodendrocyte crowding. In both cases, axons would somehow regulate either the availability of survival factors or the sensitivity of O-2A lineage cells to the factors. Consistent with such axonal regulation, David et al. (1984) found that 1 week after neonatal optic nerve transection, the number of O-2A lineage cells in the cut nerve was decreased 8-fold compared with the uncut nerve.

Newly Formed Oligodendrocytes and Their Precursors Die by Programmed Cell Death

It is increasingly believed that most normal cell deaths in invertebrate and vertebrate development depend on the activation of a suicide program in the cells that die (Wyllie et al., 1980; Oppenheim et al., 1990; Ellis et al., 1991). The most direct evidence comes from genetic studies in the nematode Caenorhabditis elegans, where loss of function mutations in either of two genes (*ced*-3 or *ced*-4) prevent almost all the somatic cell deaths that normally occur during development (Ellis et al., 1991). Indirect evidence comes from experiments in which inhibitors of RNA or protein synthesis prevent or delay cell death; when embryonic rat sympathetic neurons are cultured in the absence of NGF, for example, they die within 24–48 hr; the cells survive for days without NGF, however, if RNA or protein synthesis inhibitors are added to the culture medium, suggesting that NGF normally promotes survival by inhibiting an active suicide program in the cells (Martin et al., 1988). In addition to sensitivity to RNA and protein synthesis inhibitors, programmed cell death has two other characteristic, but not invariable, features: the dying cells usually undergo a characteristic sequence of morphological changes (known as apoptosis) that include cytoplasmic and nuclear condensation; and the chromatin is often cleaved by endonucleases into oligonucleosome-sized fragments (Wyllie, 1980; Wyllie et al., 1980; Clarke, 1990; Kerr and Harmon, 1991).

Our findings suggest that developing O-2A progenitor cells and oligodendrocytes undergo active programmed cell death when they die, either naturally in vivo, or when they are deprived of survival factors in vitro. Both in vivo and in vitro, the morphology of the dead cells is characteristic of apoptosis, and cycloheximide inhibits the cell death (although the in vivo cycloheximide experiments are difficult to interpret). Moreover, when followed by time-lapse video recording, dying factor-deprived progenitor cells exhibit morphological changes characteristic of programmed cell death. Thus IGF-1 and PDGF might promote the survival of O-2A lineage cells by suppressing a suicide program in these cells. We could not, however, detect evidence of DNA fragmentation in dying O-2A progenitors or oligodendrocytes, suggesting either that DNA fragmentation is not an important part of the death mechanism in these cells or that our methods were insufficiently sensitive to detect it. DNA fragmentation, however, seems not to be an invariabe feature of programmed cell death (Schwartz et al., 1991; Lockshin and Zakeri, 1991).

How General Is the Requirement and Competition for Survival Factors?

Normal cell death, comparable with the amount we have observed in sections of developing optic nerve, has been described in most tissues at some stage in their development (Glucksman, 1951). Because the number of dead cells seen at any one time is usually small, it has generally been assumed that only small numbers of cells die. But as our studies and those of others (Perry et al., 1983; Cowan et al., 1984; Oppenheim, 1991) indicate, the dead cells are cleared so rapidly by phagocytosis that even when there is a large amount of cell death, the number of dead cells seen in a section will be small. Thus, it is possible that as many as 50% or more of the cells generated in various nonneural tissues die during normal development, just as

is the case for neurons and oligodendrocytes in the nervous system.

The mechanisms responsible for most normal cell deaths are unknown. Our evidence that oligodendrocyte death, like the death of many developing neurons, seems to result from a competition for limiting amounts of survival factors raises the possibility that some normal cell deaths in nonneural tissues may also reflect such a competition. Like neurons and oligodendrocytes, hematopoietic cells undergo programmed cell death if deprived of signaling molecules in culture (Duke and Cohen, 1986; Koury and Bondurant, 1990; Williams et al., 1990; Cohen, 1991). Moreover, some endocrine-dependent cells in adult animals undergo programmed cell death if deprived of the hormone they require (Wyllie et al., 1984; Krypaniou and Isaacs, 1988). Thus, it might be a general rule that vertebrate cells need signals from other cells to avoid killing themselves, just as they need signals from other cells to proliferate; this arrangement might be exploited in tissues throughout the body to control cell number and to eliminate misplaced cells, just as in the developing nervous system (Raff, 1992).

Experimental Procedures

Animals and Materials

Sprague–Dawley rats were obtained from the breeding colony of the Imperial Research Cancer Fund. Recombinant human PDGF–AA and bFGF were purchased from Peprotech. Recombinant human IGF-1 and IGF-2 were purchased from Boehringer Mannheim (for single cell experiments) or were generously provided by Mats Lake of Kabigen (used for purified cell experiments). EGF and insulin were purchased from Sigma.

Purification of O-2A Progenitor Cells and Oligodendrocytes by Sequential Immunopanning

The purification procedure was based on previously described immunopanning purification protocols for other cell types (Mage et al., 1977; Wysocki and Sato, 1978; Barres et al., 1988). All important aspects of the panning purification procedures are summarized below; a more detailed protocol is available on request.

Preparation of Panning Plates

Secondary antibodies were affinity-purified goat anti-mouse IgM (μ chain–specific, Accurate) and affinity-purified goat anti-mouse IgG (H+L chain–specific, Accurate). Primary monoclonal antibodies were the A2B5 antibody (IgM, Eisenbarth et al., 1979), anti-RAN-2 antibody (IgG, Bartlett et al., 1981), and anti-GC antibody (IgG, Ranscht et al., 1982). Petri dishes (10 cm; Falcon) were incubated with 10 ml of Tris buffer solution (50 mM, [pH 9.5]) with 50 μg of secondary antibody, either anti-IgM or anti-IgG, for 12 hr at 4°C. Each dish was then washed three times with 8 ml of phosphate-buffered saline (PBS) and incubated with 5 ml of either A2B5 ascites at 1:2000 (one IgM dish), anti-RAN-2 supernatant 1:4 (two IgG dishes), or anti-GC supernatant 1:4 (one IgG dish) for at least 1 hr at room temperature. The antibodies were diluted in HEPES-buffered minimal Eagle's medium (MEM/HEPES, GIBCO) containing BSA (1 mg/ml; Sigma A4161), which blocked the nonspecific adherence of cells to the panning plates. After the antibody solution was removed, the plates were washed three times with PBS, and PBS was left on the plates until use.

Preparation of Cell Suspensions

Optic nerves from postnatal rats (ages P6 to P10) were dissected from just posterior to the optic foramen to the optic chiasm, and a single cell suspension was prepared, essentially as described by Huettner and Baughman (1986). Briefly, the tissue was minced and incubated at 37°C for 75 min in a papain solution (30 U/ml; Worthington) in MEM/HEPES containing L-cysteine as described. The tissue was then triturated sequentially with 21 and 23 gauge needles in a solution containing ovomucoid (2 mg/ml; Boehringer–Manheim) and BSA (1 mg/ml) to yield a suspension of single cells.

Immunopanning Procedure

The optic nerve cell suspension, prepared from optic nerves from 20–50 rats, was resuspended in 7 ml of L15 Air Medium (GIBCO) containing insulin (5 μg/ml; Sigma) and filtered through Nitex mesh (15 μm pore size, Tetko). In order to deplete type-1 astrocytes and meningeal cells (as well as microglia and macrophages, which stick via their Fc receptors to the first Ig-coated panning dish used), the cell suspension was first placed on the RAN-2 plate for 30 min at room temperature, with brief and gentle agitation after 15 min (each panning step included agitation every 15 min to ensure access of the panning surface area to all cells). The nonadherent cells were transferred to the second RAN-2 plate for 30 min, after which the nonadherent cells were transferred to the GC plate for 45 min to deplete the oligodendrocytes. The nonadherent cells were transferred to the A2B5 dish to deplete the O-2A progenitor cells. The plates containing the adherent GC^+ oligodendrocytes and $A2B5^+$ O-2A progenitors were washed 8 times with 6 ml of PBS or MEM/HEPES, with moderately vigorous agitation, to remove all antigen-negative nonadherent cells. The progress of nonadherent cell removal was monitored under an inverted phase-contrast microscope, and washing was terminated when only adherent cells remained.

Removing the Purified Adherent Cells from the Plates

A trypsin solution (0.125%, Sigma) was prepared from a 20× stock stored at −70°C in Ca^{2+}- and Mg^{2+}-free DMEM containing sodium bicarbonate (25 mM). Cells on each panning dish were incubated in 4 ml of this solution for 10 min at 37°C in a 5% CO_2 incubator. The cells were then dislodged by gentle pipetting, monitoring progress under the microscope. The trypsin solution containing the cells was combined with 8 ml of DMEM containing 20% heat-inactivated fetal calf serum (FCS) and spun at 800 × g for 10 min. To wash away remaining traces of FCS, the pellet was resuspended in 6 ml of MEM/HEPES containing BSA (0.5%, Sigma crystalline grade, A4161); it was important to use crystalline BSA in this step, as crude BSA contains IGF-1 (Svrzic and Schubert, 1990) and promoted O-2A progenitor cell survival (data not shown).

In some experiments, the purified GC^+ oligodendrocytes were further treated by an addtional panning step to eliminate $A2B5^+$ newly formed oligodendrocytes; some O-2A progenitor cells that expressed small amounts of GC were probably also removed in this step. This was done by transferring the GC^+ cell fraction to an A2B5 panning dish (A2B5 at 1:500) for 45 min to deplete the $A2B5^+$ cells and retaining the nonadherent cells. The results for oligodendrocytes were similar whether or not the $A2B5^+$ cells were removed.

To prepare pure populations of newly formed oligodendrocytes, the O-2A progenitor cells were cultured in B–S medium (modified from Bottenstein and Sato, 1979, as previously described; Lillien and Raff, 1990) containing insulin (5 μg/ml) for 24 or 48 hr in a 35 mm tissue culture dish (Falcon). The cells were then trypsinized off the dish, as above.

Purity and Yield of the Purified Oligodendrocytes and O-2A Progenitor Cells

To assess the purity of the panned cells, 15,000 cells were plated onto 6 mm poly-D-lysine (PDL) (10 μg/ml, 130K; Sigma)-coated glass coverslips in 96-well tissue culture plates (Falcon) in 100 μl of serum-free B–S medium containing insulin (5 μg/ml). The coverslips were stained with A2B5, anti-GC, anti-RAN-2, or anti-Thy-1-1 (MRC-OX-7) monoclonal antibodies (all as supernatant diluted 1:1) and with rabbit anti-macrophage (Axell), anti-GFAP (Pruss, 1979), and anti-vimentin antisera (Hynes and Destree, 1978) (all diluted 1:100) to assess their purity. The cells were greater than 99.9% pure; fewer than one in 2000 cells were $vimentin^+$ fibroblast-like cells. The yield of purified progenitors was about 17,000 cells per P7 animal, which was about 40% of the total O-2A progenitor cells present in the optic nerves at this age and greater than 95% of the O-2A progenitors isolated by the papain dissociation procedure. The yield of purified oligodendrocytes was about 7,000 cells per P7 animal, which was about 40% of the total oligodendrocyte cells present in the optic nerves at this age and greater than 95% of oligodendrocytes isolated by the papain dissociation procedure.

Cell Survival Assays

Phase Contrast

Approximately 1000 purified O-2A progenitor cells or oligodendrocytes were cultured in 10 μl of B–S medium or in DMEM in Terasaki mi-

croplates (Falcon). About 200 cells adhered to the bottom of the well, and 98% of the O-2A progenitor cells and 80% of the oligodendrocytes were viable when tested at 1 hr by the MTT assay (see below). Thirty minutes after plating, 1 μl of DMEM, or DMEM containing a specific growth factor, was added to each well. An optimal concentration of each factor was initially determined in titration experiments. The survival effects of these growth factors were similar when used at 10-fold higher concentrations than used in the experiments shown in Table 1 and Figure 1A (data not shown). In experiments where conditioned medium was added to the microwells, 5 μl of medium was removed after 30 min, and 5 μl of conditioned medium was immediately added to each well. The B–S medium was prepared with a highly purified, crystalline grade of BSA (Sigma, A4161) in order to avoid contaminating survival factors (see above). The number of surviving cells on the bottom of the well was assessed after about 18, 42, and 66 hr by counting the live cells in an inverted, phase-contrast microscope. In the case of O-2A progenitor cells, at 18 hr, in all of the conditions studied, most of the surviving cells still had the characteristic morphology of progenitor cells, whereas by 42 hr, in most conditions studied, the majority of cells had acquired the morphology of oligodendrocytes, except in PDGF where most still had the morphology of O-2A progenitor cells.

MTT Survival Assay

The MTT survival assay was performed as described by Mosmann (1983). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) was dissolved in PBS at 5 mg/ml and sterilzed by passage through a Millipore filter (0.22 μm). This stock solution was added, 1 μl to 10 μl of medium, to all wells of the microculture plate, and the plates were incubated at 37°C for 2 hr. Viable cells with active mitochondria cleave the tetrazolium ring into a visible dark blue formazan reaction product. The viable cells in each well were counted by bright-field microscopy.

Preparation of Conditioned Medium from Panned Non-O-2A Lineage Cells from Optic Nerve (ONCM)

Optic nerve suspensions prepared from P6 to P10 rats were depleted of O-2A progenitor cells, oligodendrocytes, microglia, and macrophages by panning sequentially on A2B5 and anti-GC panning dishes (see above), and finally, on an anti-RAN-2 dish, to which meningeal cells and type-1 astrocytes selectively adhered. Five milliliters of B–S medium (without insulin) was added and conditioned for 4 days; the ONCM was frozen in 0.2 ml aliquots until use.

BrdU Incorporation and Oligodendrocyte Differentiation In Vitro

Approximately 40,000 purified O-2A progenitor cells were plated onto 6 mm, PDL-coated glass coverslips in 100 μl of insulin-free B–S medium containing no added factors, low insulin (20 ng/ml), high insulin (5 μg/ml), or truncated IGF-1 (100 ng/ml), or PDGF (10 ng/ml) in the combinations listed in Table 3. After 24 hr, BrdU (10 μM) was added, and 24 hr later, the cells were fixed and labeled with anti-BrdU and anti-GC antibodies (see below). In control experiments, BrdU did not influence the percentage of cells that differentiated into GC^+ oligodendrocytes (data not shown).

Micromanipulation and Culture of Single Optic Nerve Cells

Microculture

For reasons that are unclear, O-2A progenitor cells that were dissociated in papain and purified by sequential immunopanning did not extend processes in single-cell culture, and, therefore, their viability was difficult to assess. Instead, optic nerve cell suspensions were prepared from P7 rats using trypsin, EDTA, and collagenase, as previously described (Miller et al., 1985), and single cells were micromanipulated as described by Temple and Raff (1985). In brief, the cells were resuspended in 1 ml of L15 Air medium containing 0.1% BSA, allowed to settle for 2 min, and transferred to a small bacteriological Petri dish. The cells were viewed with a Leitz Diavert inverted microscope with phase-contrast optics. Single cells were picked from the suspension using a hand-pulled 10 μl micropipet and mouth suction and transferred in a small volume (<0.5 μl) into Terasaki wells (Falcon) coated with salt-extracted extracellular matrix (ECM, see below) and containing 10 μl of B–S medium and the growth factors to be tested. Cells were fed by replacing half of the culture medium every 2 days. The ECM was used routinely because it enhanced the adherence of the cells to the bottom of the wells, but similar results were obtained when ECM was omitted.

Preparation of ECM

Purified, cortical, type-1-like astrocyte cultures were prepared by a method modified from Noble and Murray (1984) and Lillien et al. (1988). Newborn rat cerebral hemispheres were dissociated into single cells, which were cultured in DMEM containing 10% FCS until they were confluent. Cells were shaken overnight on a cell rotator to remove the top layer of cells, and cytosine arabinoside (10 μM) was added for 24 hr to kill any rapidly dividing cells. To prepare ECM, the cells were removed from the flasks with trypsin (0.1%) and EDTA (0.02%), and 1000 cells were plated into PDL-coated wells of Terasaki plates. The cells were grown in DMEM containing 10% FCS until confluent and were then removed from the wells by treating with 20 mM ammonia containing 0.5% Triton X-100 for 2 min at room temperature several times. To strip the matrix of growth factors, the plates were flooded four times with 5 ml of 2 M NaCl in 2 mM phosphate buffer (pH 7.5). The resulting ECM was washed with DMEM (10 μl per well) for 3 hr at 37°C in a 5% CO_2 incubator, and the medium was replaced every 30 min.

Cell Counting and Immunofluorescence

The survival of single cells in microculture was assessed daily by phase-contrast microscopy as described above. Only wells that contained single cells 1 hr after plating were studied. O-2A progenitor cells and oligodendrocytes were identified by their characteristic morphologies (Raff et al., 1978, 1983a, 1983b; Temple and Raff, 1986), and, in some cases, cell assignments were confirmed by immunolabeling with the anti-GC, A2B5, and anti-GFAP antibodies, as previously described (Temple and Raff, 1986).

Time-Lapse Video Recording

Approximately 100,000 cells were plated onto the center of a PDL-coated 35 mm tissue culture dish in 1 ml of insulin-free B–S medium. The dish was placed on the stage of an inverted Zeiss microscope and maintained at 37°C in a humid 95% air, 5% CO_2 environment; the cells were viewed using phase-contrast microscopy. Time-lapse recordings were made using a CCD video camera coupled to a time-lapse video tape recorder (Panasonic Model AG6720A), which acquired images at a rate of one image every 6 s. At the same time, images were also captured once every 4 min by a computer-controlled image processor (Matrox MVP-AT); these images were signal averaged and enhanced and then later transferred to videotape to make a high speed summary of the recording period. After 24 hr of recording, the process of death was observed from the videotapes by following the fates of individual cells.

DNA Fragmentation Assay

Purified O-2A progenitor cells and oligodendrocytes were cultured on the final panning dish for 13–38 hr in B–S medium without insulin. The dish was rinsed with 5 ml of TNE buffer (10 mM Tris–HCl [pH 8.0], 150 mM sodium chloride, 10 mM EDTA), and the cells were then harvested, using a cell scraper, into a final volume of 70–100 μl of TNE. Three volumes of lysis buffer containing TNE, SDS (0.2%), proteinase K (100 μg/ml; Sigma), and RNAase A (50 μg/ml; Sigma) were added, and the lysate incubated at 55°C for 2.5 hr. The DNA was extracted with TNE-saturated phenol, followed by phenol:chloroform:isoamylalcohol (25:24:1), and finally by chloroform:isoamylalcohol (24:1). The DNA, with 1 μl of carrier tRNA (1 mg/ml), was precipiated in 0.25 M sodium acetate and 2 vol of 95% ethanol at −20°C overnight. The DNA was pelleted at 12,000 × g at 4°C for 20 min, washed twice with 70% ethanol, once with 95% ethanol, air dryed and resuspended in TE (10 mM Tris, 1 mM EDTA [pH 7.4]). Electrophoresis of the DNA was performed in a 1.5% agarose gel for 2.5 hr at 80 V in Tris–borate buffer (pH 7.5). The DNA was visualized by staining with ethidium bromide.

Electron Microscopy

Purified O-2A progenitor cells were cultured on PDL-coated glass coverslips in B–S medium with or without insulin (5 μg/ml). After 15 hr in culture, the cells were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at 37°C for 60 min. After rinsing with 0.1 M PB three times over 30 min, the cells were postfixed with 1% osmisum tetroxide in veronal acetate buffer (VAB), rinsed three times over 30 min in VAB,

and stained with 0.5% uranyl acetate in VAB, all at 4°C in the dark. The cells were washed three times over 30 min in distilled water, dehydrated in a graded series of alcohols, and embedded in Epon. Thin sections were cut on an LKB ultratome, counterstained with lead citrate, and examined using a JEOL 100-CX II electron microscope at 80 kV.

Quantitation of DNA in Optic Nerves

Optic nerves were cut immediately anterior to the chiasm. The optic nerve arachnoid was removed by pulling the nerve out of the optic canal by the eyeball, and the nerve was cut behind the eyeball. In this way, the optic nerve was isolated with only a thin layer of tightly adherent pial cells. Two optic nerves were put into an Eppendorf tube containing 400 to 800 μl of tissue digestion buffer containing TES (10 mM Tris–HCl, 50 mM EDTA, 0.1% SDS) and proteinase K (200 μg/ml). The nerves were minced in this buffer into small pieces with a pair of small dissecting scissors and incubated at 55°C for 36 hr, with vortexing every 12 hr. The Eppendorf tubes were spun for 30 s at high speed in an Eppendorf centrifuge, and the final volumes measured. The amounts of DNA in the optic nerve digests were measured using the fluorimetric method of LaBarca and Paigen (1980; also see Brunk et al., 1979), which is based on the enhancement of fluorescence seen when the dye bisbenzimidazole (Hoechst 33258) binds to DNA. The DNA values were converted to cell number by dividing by 6.6 pg of DNA per cell (Wilson et al., 1987). The values at P2, P8, and P14 were corrected by 30%, 20%, and 10% to account for cells in S phase that have double the diploid amount of DNA. Treatment of the optic nerve digests with DNAase removed all of the fluorescence enhancement. DNA standard curves were prepared using the same percent of SDS present in the DNA sample added.

Propidium Iodide Labeling and Preparation of Cryostat Sections of Optic Nerve

Postnatal rats were anesthetized with ether and perfused with 4% paraformaldehyde. The optic nerves were incubated in 4% paraformaldehyde at 4°C overnight and transferred to 30% sucrose in PBS until equilibrated. The nerves were frozen in O. C. T. compound (Miles) and cut into 8 μm longitudinal sections with a Bright cryostat. The sections were collected onto gelatinized glass microscope slides, air dried, post-fixed in 70% ethanol for 10 min at −20°C, and stained with propidium iodide (4 μg/ml) solution in MEM/HEPES containing DNAase-free RNAase A (100μg/ml) for 30 min at 37°C (Rodriguez-Tarduchy et al., 1990). The slides were washed three times in PBS and mounted in Citifluor (City University, London, England).

The number of pyknotic nuclei per section was determined by averaging the number of pyknotic cells counted in five optic nerve sections per animal. Only clearly condensed or fragmented nuclei were counted as pyknotic. Most pyknotic nuclei ranged from about 2–4 μm in diameter, which was several times smaller than the thickness of the section (8 μm).

BrdU Incorporation and Immunofluorescence Staining

To label cells in S phase in vivo, postnatal rats were injected intraperitoneally with BrdU (0.1 mg/g body weight; Boehringer Mannheim), which is incorporated into replicating DNA (Gratzner, 1982). The animals were sacrificed 90 min later, and optic nerve cell suspensions were prepared and cultured for several hours as above. After fixation with 4% paraformaldehyde for 90 s at room temperature, and a 15 min incubation in 50% goat serum containing 1% BSA and 100 mM L-lysine to block nonspecific binding, cells were surface stained either with monoclonal anti-GC antibody (supernatant used at 1:1) followed by fluorescein-coupled goat anti-mouse IgG3 (Nordic; 1:100) or with A2B5 antibody (supernatant diluted 1:1) followed by fluorescein-coupled goat anti-mouse IgM (μ chain–specific, Accurate). Cells were post-fixed in 70% ethanol at −20°C for 10 min, incubated in 2 M HCl for 10 min to denature the nuclear DNA, followed by 0.1 M sodium borate (pH 8.5) for 10 min. The cells were then incubated in 50% goat serum containing 0.4% Triton X-100 for 30 min and labeled with monoclonal anti-BrdU antibody (ascites, 1:100; Magaud et al., 1988), followed by rhodamine-coupled goat anti-mouse IgG1 (Nordic, 1:100). In some experiments, cells were stained with rabbit anti-GFAP antiserum (diluted 1:100); in this case, the cells were fixed with acid–alcohol for 10 min at −20°C and blocked with goat serum as above. The anti-GFAP antibodies were detected with fluorescein-coupled goat anti-rabbit IgG (H+L chain–specific, Accurate). The coverslips were mounted in Citifluor on glass slides and sealed with nail varnish, examined in a Zeiss Universal fluoresence microscope, and photographed using Kodak Tri-X film (ASA 400). The sections shown in Figures 4C and 4F were examined with an MRC-600 laser-scanning confocal imaging system in conjunction with a Nikon Optophot microscope.

Glial cell types were identified by their characteristic morphologies and antigenic phenotypes; astrocytes were labeled by anti-GFAP antiserum, O-2A progenitor cells by A2B5 antibody (Raff et al., 1983b), and oligodendrocytes by anti-GC antibody (Raff et al., 1978).

BrdU Pulse-Chase Experiments

Twelve P15 rats were treated with three intraperitoneal injections of BrdU (0.1 mg/g) given every 8 hr. At 12, 24, 48, and 72 hr after the first injection, three animals were perfused with 70% ethanol. The optic nerves were removed and incubated in 70% ethanol for 4 hr and then in 30% sucrose, both at 4°C. Cryostat sections were prepared and labeled with propidium iodide as described above. At each time point, the average number of dead cells per section was determined; these were not increased over those of control animals that had not recieved BrdU, indicating that the dose of BrdU used was not toxic.

Cycloheximide Treatment

P22 rats were treated with cycloheximide in doses that have previously been shown to result in nearly complete inhibition of brain protein synthesis (Bannon and Goedert, 1984; Deguchi and Axelrod, 1972). The cycloheximide was dissolved in water, and 1.5 mg/kg was administered intraperitoneally every 12 hr for 48 hr (also see Oppenheim et al., 1990).

Transfection of COS Cells with a PDGF Plasmid Expression Vector

Plasmid PHYK, which encodes chicken lysozyme under the control of the adenovirus-2 major late promoter was obtained from H. Pelham (Munro and Pelham, 1987). The lysozyme coding sequences were replaced by a cDNA encoding the human PDGF–A chain (Betsholtz et al., 1986). Subsequently, a double-stranded oligonucleotide coding for 10 aa corresponding to the c-Myc epitope recognized by the 9E10 monoclonal antibody (Evan et al., 1985), followed by a termination codon, was inserted at the StuI site close to the C-terminus of the PDGF–A chain coding region to give plasmid PHYKA5.

COS-7 cells, grown to 70% confluence in 75 cm^2 flasks were washed three times with DMEM. A 5 ml solution containing 7 μg of the plasmid PHTKA5 in 3.75 ml of DMEM and 1.25 ml of DEAE Dextran (1 mg/ml) was added to the flask for 60 min. The solution was aspirated, and the cells incubated in 10 ml of DMEM containing chloroquine (10 mg/ml) for 3 hr at 37°C. This solution was aspirated, and 10 ml of DMEM containing 10% FCS was added for 18 hr at 37°C. Mock-transfected cells were prepared in the same way, except no plasmid was added.

To determine whether the COS cells secreted factors that stimulated the survival or proliferation of O-2A progenitor cells, purified progenitors were cultured in insulin-free B–S medium conditioned by either the transfected or mock-transfected COS cells. Conditioned medium from mock-transfected COS cells had no detectable survival or mitogenic activity for the O-2A progenitor cells, whereas conditioned medium from the transfected COS cells induced both survival and mitogenesis comparable with medium containing 10 ng/ml PDGF (data not shown).

Transplantation of Hybridoma and COS Cells

Hybridoma cells secreting antibodies or COS cells secreting PDGF–AA were transplanted into the brain according to the method of Schnell and Schwab (1990). The cells were trypsinized from a 75 cm^2 flask and washed as described above. They were resuspended in 1 ml of MEM/HEPES and spun for 10 s at high speed in an Eppendorf microcentrifuge to form a compact pellet. The pellet was resuspended in MEM/HEPES to a concentration of 1 to 2 million cells per 3 μl. Under ether anesthesia, 3 μl of the cell suspension was slowly injected through the right frontal skull into the subarachnoid space (rather than directly into the brain as described by Schnell and Schwab, 1990) of P2 to P8 rats, using a 10 μl Hamilton syringe. The rats were sacrificed after 4

days, at which time the success rate of the transplants was 100%. The transplanted cells were normally rejected after about 10 days.

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