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Cell Death and Control of Cell Survival in the Oligodendrocyte Lineage

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Summary

tissues, but the causes of these normal cell deaths are
normal oligodendrocyte death by up to 90% and dou-
results suggest that a requirement for survival signals

Cell death occurs in most animal tissues at some stage of

1980; Ellis et al., 1991), but the mechanisms of death are unknown. Despite the prevalence of normal cell death,

penheim, 1991). In the case of sympathetic and some sensory neurons, it is thought that death occurs because

eral and central nervous systems and is thought to serve at least two functions: to ensure an appropriate numerical

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, 1986), but the type of glial cells that die and the mechanisms responsible for their death are unclear, although some have been identified as

trophic factors, little effort has been made to define factors

the developing rat optic nerve.

teractions that control the proliferation and differentiation

derived growth factor (PDGF) A chain and thus are a likely

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

dendrocyte development in vitro occurs constitutively, type-2 astrocyte development in vitro depends on cell–cell

dendrocytes, while PDGF acts as a survival factor for O-2A

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		
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	70 ± 8	43 ± 13	27 ± 8	9 ± 6
b-FGF (10 ng/ml)	87 ± 10	21 ± 12	6 ± 6	52 ± 10	30 ± 8	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

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

of PDGF and IGFs. To our knowledge, this is the first direct

from other cells to survive in culture and that oligodendro-

O-2A progenitor cells were purified from a suspension of

immunopanning to greater than 99.95% purity, as assessed by immunostaining (see Experimental Procedures). The cells were plated in microwells in

Gate (B–S) medium containing bovine serum albumin (BSA), putrescine, thyroxine, triiodothyronine,

ing cells on the bottom of the well was assessed after about

phase-contrast microscope, it was found that few cells

shown). The addition of serum-free and insulin-free B–S

hese, removed by immunopanning) (optic nerve condi-

surviving cells at each time point (Table 1, O-2A Progenitor

nerve secrete factors in culture that promote the surivival of O-2A progenitor cells.

(O-2A Progenitor Cells) IGF-1, IGF-2, PDGF, or a concentration of insulin (5 μg/ml) high enough to activate IGF-1

at each of the three time points tested. Basic fibroblast

mal growth factor (EGF) had none. Remarkably, nearly

The effects of IGFs, PDGF, and insulin on cell number

cell proliferation. As shown in Table 2, these factors both increased the number of surviving cells and proportionally

statistically different from the numbers in medium alone. Although PDGF did induce bromodeoxyuridine (BrdU) in-

(see below), little proliferation was observed in the micro-

To ensure that our assessment of cell viability and death

was close agreement between the two assays of cell viability for all of the conditions tested and each of the three time

The morphology of the cells that died by 18 hr in the ab-

died by programmed cell death rather than by necrosis (Wyllie et al., 1980). Both by phase-contrast microscopy

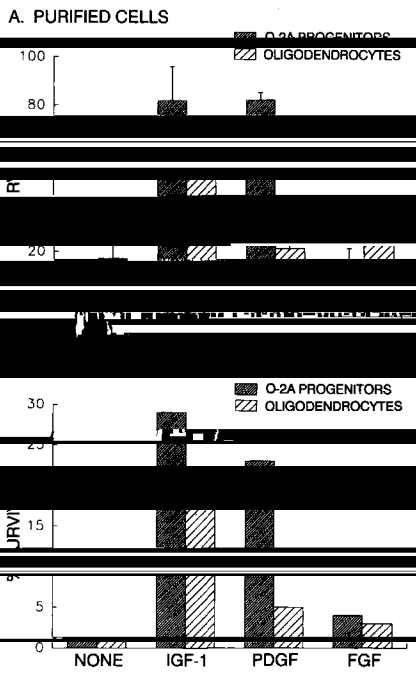

Figure 1. Survival of Purified and Single O-2A Progenitor Cells and Oligodendrocytes In Vitro

Oligodendrocyte 67113 0 Growth

separate experiments, values from four wells were averaged in each experiment.

were cultured in microwells containing 10 μl of insulin-free B–S medium in the presence or absence of growth factors. Survival was as-

progenitor cells or oligodendrocytes by the number of total wells con-

pooled and represent observations on 80 to 200 cells studied in each condition. Very similar results were obtained when single cells were

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 chroma-

When purified progenitor cells cultured in the absence

in the surface activity, the cells rounded up and shrank

died by programmed cell death, cycloheximide increased cell survival in the absence of survival factors, although

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

tected, however, in cultures containing the same number

were deprived of interleukin-2 for 20 hr (data not shown).

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

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

was similar to that of O-2A progenitor cells under the same conditions, and we could not find evidence for DNA degra-

four experiments (data not shown).

To determine whether newly formed oligodendrocytes which still express PDGF receptors (Hart et al., 1989a

cells were cultured for 24 hr in B–S medium containing

to induce rapid differentiation into oligodendrocytes (Raff et al., 1983b). The cells were then removed from the cul-

ture dish and tested for survival. Less than 1% of these

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.

insulin (5 μg/ml), T3, T4, PDGF (10 ng/ml), CT, CM, and bFGF (10 ng/ml). The cells in which PDGF

(data not shown).

Survival of Single O-2A Lineage Cells in Microculture

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

Factors Added	Number of Cells After 18 hr		Number of Cells After 66 hr	
	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

[redacted]

[redacted]

[redacted]

Raff, 1985). None of the single O-2A progenitor cells or oligodendrocytes survived for 1 day without insulin, and [redacted]

dendrocytes (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 (Wolf et al., 1982b).

[redacted]

a measure of oligodendrocyte differentiation. As shown in Table 2, PDGF, but not IGF-1 or high insulin, induced DNA [redacted]

of PDGF.

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 oligo-

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-

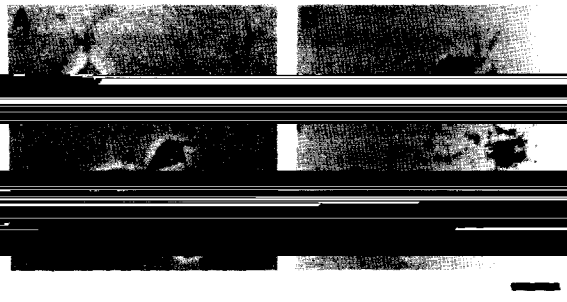

Figure 2. Assays of Survival

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 [redacted] blue reaction product. Bar, 20 μm.

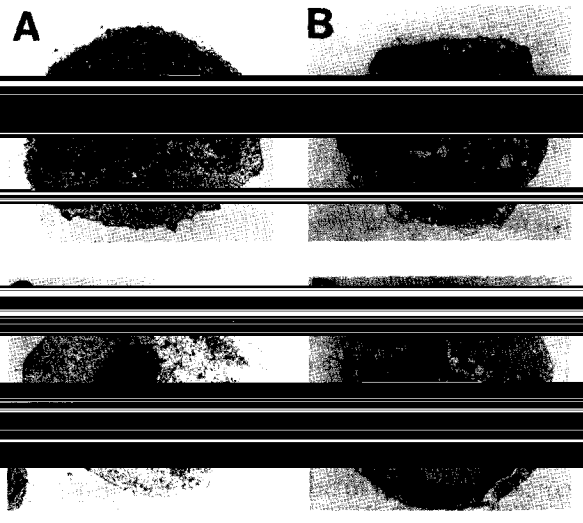

Figure 3. Ultrastructure of Dead Cells

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 pro- [redacted] μm; (D) 2.3 μm.

Table 3. Influence of Insulin and IGF on BrdU Incorporation and Oligodendrocyte Differentiation in Cultures

	Process-Bearing Cells	Flat Cells
None	37 ± 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

Approximately 40,000 purified O-2A progenitor cells were plated in 100 [illegible] 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.

[illegible] probably an underestimate, since cells in the early stages [illegible] copy. The proportion of dead cells at each age was calculated by dividing the number of dead cells by the total [illegible]

cells was highest in developing optic nerves (Figure 5B); it peaked between P4 and P10 at about 0.25% and de[illegible]

Intraperitoneal injections with the protein synthesis inhibitor cycloheximide, at concentrations previously demonstrated to decrease brain protein synthesis [illegible] 1972), diminished the number of dead cells per section by 91%: control, 13.4 ± 0.65; test, 1.27 ± 0.29 (mean [illegible]

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 [illegible] between P4 and P12, 26/167 pyknotic cells were positive), suggesting that some of the dead cells were oligodendro[illegible]

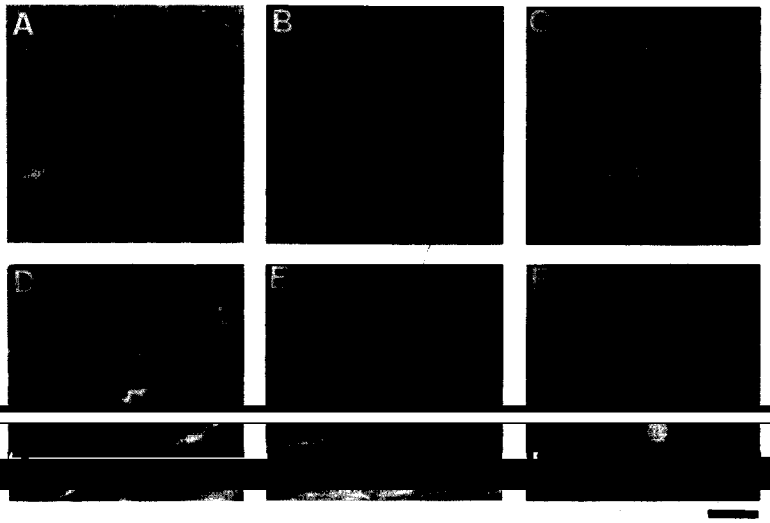

Figure 4. Cell Death in the Developing Rat Optic Nerve

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 oligodendrocyte 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 [illegible] dendrocytes are present just above the dead oligodendrocyte: the one on the right appears [illegible] morphology, however, were not included in the dead cell counts).

[illegible] 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.

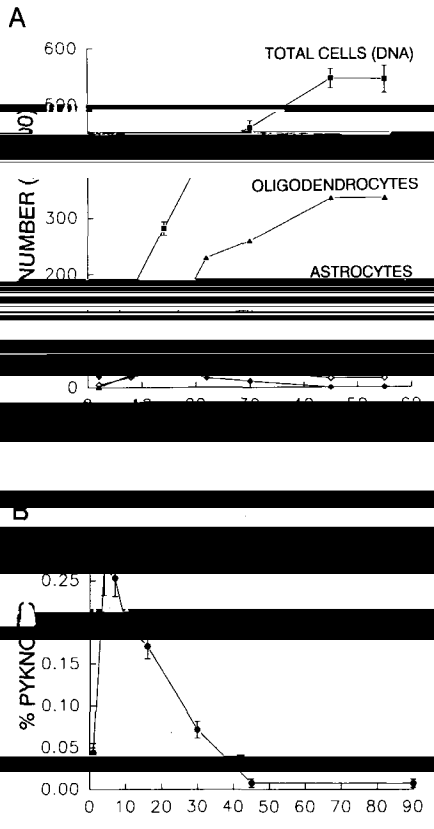

Figure 5. Quantitation of Cell Production and Death in the Developing Rat Optic Nerve

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.

longitudinal section of the nerve (excluding the optic chiasm) by the average number of sections per nerve at a given age; this value was

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 oligo-

(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

the course of programmed cell death (Wyllie et al., 1980).

cell type–specific surface antigens in the optic nerve

which specifically recognize type-1 astrocytes and their

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 spe-

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

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

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

increase cell death, the numbers of dead cells per optic

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

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

mainly newly generated cells died (Figure 5). The interval between S phase of the cell cycle and cell death was deter-

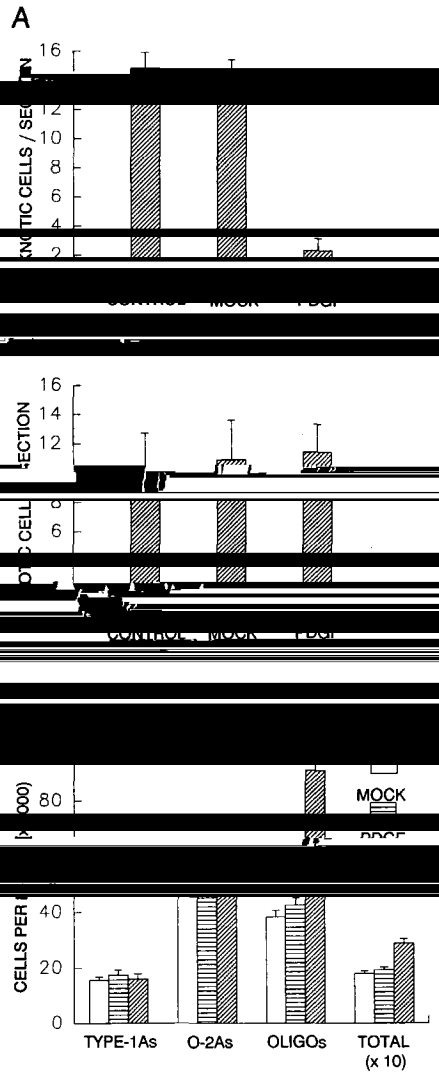

Figure 6. The Effect of Increasing the Concentration of PDGF in the Developing Optic Nerve

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

(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 chemically the percent of astrocytes, O-2A progenitor cells, and oligo-

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

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

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

plantation 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

differentiation within the

tions of COS cells expressing PDGF, but was unaffected

(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

number of GFAP$^+$ type-1 astrocytes was not changed by PDGF delivery, the number of A2B5$^+$ O-2A progenitor cells

which have PDGF α receptors (Pringle et al., 1992).

O-2A Lineage Cells Require Signals from

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

alter their response to mitogens (Fraser and Katz, 1975). Although a variety of approaches have been used to purify

O-2A progenitor cells (Raber et al., 1989; Aloisi et al., 1990; Bögler 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 se-

we have obtained O-2A lineage cells of greater than

By using both single cell cultures and cultures of pure

to survive on their own. Conditioned medium from cultures of non-O-2A lineage cells from optic nerve (containing

cytes, endothelial cells) allows the O-2A progenitor cells

mating that non-O-2A lineage cells in the optic nerve se-

vitro.

cytes, either IGF-1 or PDGF is sufficient for short-term

proteins; for more mature oligodendrocytes, IGF-1, but not

change as O-2A progenitor cells differentiate into oligo-

been shown to be present on cultured O-2A progenitor cells and oligodendrocytes (McMorris et al., 1986; Baron

for both oligodendrocytes and progenitor cells in the presence of high insulin (Eccleston and Silberberg, 1985; Bo-

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 devel-

shown to make PDGF in culture (Richardson et al., 1988)

the developing optic nerve, IGF-1 and IGF-2 proteins and

(Bondy et al., 1990; Hoffman et al., 1990; Drago et al., 1991). Anti-IGF-1 antibodies stain glial cells in the developing rat optic nerve and ganglion cells in the developing

only between P1 and P45, which corresponds to the period

CNS is made primarily by the choroid plexus and lepto-

meninges 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 oligoden-

shown to stimulate some cells to make IGF-1 (Clemmons

PDGF promotes the survival of O-2A progenitor cells by

IGFs Are Not Mitogens for O-2A Progenitor Cells

portant roles in regulating mammalian growth (Mathews

still unclear whether they do so by primarily promoting cell

IGF-1 or high insulin is a required component of defined media for the growth of most cell types in culture (Barnes

normal cells, mixed cell cultures were used, so that it is

Studies of the effects of IGF-1 on O-2A progenitor and

McMorris and his colleagues were the first to show that

genitor cells growing in the presence of inhibited astrocytes led them to conclude that IGF-1 acts in two

motes 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

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

performed: McMorris and his colleagues studied the ef-

the presence of other cell types, so that the effects of IGF-1

rating pro-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 oligodendro-

oligodendrocytes that develop in culture. As in the case

survival and not proliferation of vertebrate CNS neurons

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

Approximately 50% of Oligodendrocytes Die

Although degenerating glial cells have long been observed

uncertain. Hildebrand (1971) provided evidence, on the

in the developing optic nerve are newly formed oligoden

pyknotic cells, while an anti-GC monoclonal antibody la-

a relatively later appearance of the HNK antigen during oligodendrocyte differentiation or the destruction of the

cells is observed that colocalizes with a gradient of newly

PDGF levels, it is mainly the number of oligodendrocytes

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

any response to retinal ganglion cell death, which is

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

cell proliferation and only slightly increases the number

dendrocyte 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

tween P8 and P12.

To determine the proportion of newly formed oligoden-

drocytes that die, it is necessary to know how many new

oligodendrocytes are generated daily. To estimate this, we

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

copy by multiplying these percentages (Vaughn, 1969;

Figure 1B) 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 pro-

die daily during this period, so that approximately 50%

optic nerve during this time.

time remains constant after P12, one can estimate the rate

cells. About 490 dead cells per optic nerve are present at

and 4,000 cells per day at P16 and P30, respectively (see Figure 5A), which corresponds to a death rate of about

oligodendrocytes that die seems to average about 50%

1991), although oligodendrocyte death (like oligodendro-

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

rons are thought to compete for limiting amounts of neuro-

tally 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

Barde, 1988). Thus far, it has not been possible to deliver

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

secreting cells.

Using this method, we found that increasing the amount

cells in the developing optic nerve by about 85% and cor-

drocytes. This finding suggests that limiting amounts of PDGF may normally regulate the survival and death of newly formed oligodendrocytes in the developing optic

substances that participate directly in the regulation of

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 oligoden-

size, brain DNA, and the amount of brain myelin are all

McMorris et al., 1990; Mozell and McMorris, 1991). The

Why is it that PDGF levels in the developing optic nerve

cytes but are much less so for the survival and proliferation

al., 1989b; McKinnon et al., 1990; Pringle et al., 1992),

just the number of oligodendrocytes to the number of

Purves, 1988; Oppenhein, 1991) and in evolution (Purves,

that oligodendrocytes are evenly spaced along the length

number of O-2A lineage cells in the cut nerve was decreased 8-fold compared with the uncut nerve.

Precursors Die by Programmed Cell Death

It is increasingly believed that most normal cell deaths in invertebrate and vertebrate development depend on the

ing 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

gesting that NGF normally promotes survival by inhibiting

tic, but not invariable, features: the dying cells usually

fragments (Wyllie, 1980; Wyllie et al., 1980; Clarke, 1990;

Our findings suggest that developing O-2A progenitor

and in vitro, the morphology of the dead cells is character-

How General Is the Requirement and Competition for Survival Factors?

our studies and those of others (Perry et al., 1983; Cowan et al., 1984; Oppenheim, 1991) indicate, the dead cells are

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 death, like the death of many developing neurons, seems

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

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

(Rall, 1982).

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

experiments) or were generously provided by Mats Lake of Kabigen (used for purified cell experiments). EGF and insulin were purchased

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

The purification procedure was based on previously described immu-

wysocki and Sato, 1978; Barres et al., 1988). All important aspects of

Preparation of Panning Plates

Secondary antibodies were affinity-purified goat anti-mouse IgM (μ

(H+L chain–specific, Accurate). Primary monoclonal antibodies were

(IgG, Bartlett et al., 1981), and anti-GC antibody (IgG, Hansen 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

supernatant 1:4 (two IgG dishes), or anti-GC supernatant 1:4 (one IgG

containing BSA (1 mg/ml; Sigma A4161), which blocked the nonspe-

Optic nerves (from postnatal rats (ages P6 to P10) were dissected from

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

50 rats, was resuspended in 7 ml of L15 Air Medium (GIBCO) con-

μm pore size; Teflo). In order to deplete type-1 astrocytes and menin-

geal cells (as well as microglia and macrophages which stick via their

step was first placed on the BAN-2 plate for 40 min at room tempera-

face area to all cells). The nonadherent cells were transferred to the second BAN-2 plate for 20 min, after which the nonadherent cells were

The nonadherent cells were transferred to the A2B5 dish to deplete

oligodendrocytes and A2B5$^-$ O-2A progenitors were washed 6 times

aberrant cell removal was monitored under an inverted phase

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

were then dislodged by gentle pipetting, monitoring progress under the microscope. The trypsin solution containing the cells was combined with 9 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

portant to use crystalline BSA in this step, as crude BSA contains

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

To prepare pure populations of newly formed oligodendrocytes, the

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

above.

Purity and Yield of the Purified GC$^+$ Oligodendrocytes

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

monoclonal antibodies (all as supernatant diluted 1:1) and with rabbit anti-macrophage (Axell), anti-GFAP (Bruce, 1979), and anti-vimentin

purity. The cells were greater than 99.9% pure; fewer than one in

papain dissociation procedure. The viability of purified oligodendrocytes

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 90% 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 higher concentrations than used in the experiments shown in Table 1 and Figure 1A (data not shown). In experiments where conditioned after 30 min, and 5 μl of conditioned medium was immediately added ing survival factors (see above). The number of surviving cells on the counting live cells in an inverted phase-contrast microscope. In tor cells.

MTT Survival Assay

The MTT survival assay was performed as described by Mosmann sade through a Millipore filter (0.22 μm). This stock solution was added, mitochondria cleave the tetrazolium ring into a visible dark blue

phages 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

BrdU Incorporation and Oligodendrocyte Differentiation

Approximately 40,000 purified O-2A progenitor cells were plated onto 6 μg/ml), or truncated IGF-1 (100 ng/ml), or PDGF (10 ng/ml) in the 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

tend processes in single-cell culture, and therefore, their viability was 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 which were cultured in DMEM containing 10% FCS until they were hr to kill any rapidly dividing cells. To prepare ECM, the cells were cells were grown in DMEM containing 10% FCS until confluent and containing 0.5% Triton X-100 for 2 min at room temperature several

Cell Counting and Immunofluorescence

The survival of single cells in microculture was assessed daily by phase-contrast microscopy as described above. Only wells that con- gies (Raff et al., 1978, 1983a, 1983b; Temple and Raff, 1986), and, in (Temple and Raff, 1986).

Approximately 100,000 cells were plated onto the center of a PDL- 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 and then later transferred to videotape to make a high-speed summary of the recording period. After 24 hr of recording, the presence of death cells.

Purified O-2A progenitor cells and oligodendrocytes were cultured on 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 acetate and 2 vol of 95% ethanol at −20°C overnight. The DNA was

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% osmium tetroxide in veronal-acetate buffer (VAB), rinsed three times over 30 min in VAB,

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

al., 1979), which is based on the enhancement of fluorescence seen

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

present in the DNA sample added.

Propidium Iodide Labeling and Preparation

paraformaldehyde. The optic nerves were incubated in 4% paraformal-

cut into 8 μm longitudinal sections with a Bright cryostat. The sections

ium iodide (4 μg/ml) solution in MEM/HEPES containing DNAase-free RNAase A (100 μg/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

nearly with BrdU (0.1 mg/g body weight; Boehringer Mannheim), which

prepared and cultured for several hours as above. After fixation with 4% paraformaldehyde for 90 s at room temperature, and a 15 min

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

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 IgG (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

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

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

terse intraperitoneally every 12 hr for 48 hr (also see Oppenheim et

Transfection of COS Cells with a PDGF Plasmid

replaced by a cDNA encoding the human PDGF–A chain (Betsholtz et al., 1986). Subsequently, a double-stranded oligonucleotide coding for

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 cm² 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

for 3 hr at 37°C. This solution was aspirated, and 10 ml of DMEM

To determine whether the COS cells secreted factors that stimulated

genic activity for the O-2A progenitor cells, whereas conditioned medium from the transfected COS cells induced both survival and mito-

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 cm² flask and washed as described above. They were resuspended in 1 ml of MEM/

fuge 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

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

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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