

Extracellular Matrix-associated Molecules Collaborate with Ciliary Neurotrophic Factor to Induce Type-2 Astrocyte Development

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Abstract. O-2A progenitor cells give rise to both oligodendrocytes and type-2 astrocytes in vitro. Whereas oligodendrocyte differentiation occurs constitutively, type-2 astrocyte differentiation requires extracellular signals, one of which is thought to be ciliary neurotrophic factor (CNTF). CNTF, however, is insufficient by itself to induce the development of stable type-2 astrocytes. In this report we show the following: (a) that molecules associated with the extracellular matrix (ECM) cooperate with CNTF to induce stable type-2 astrocyte differentiation in serum-free cultures. The combination of CNTF and the ECM-associated molecules thus mimics the effect of FCS, which has been shown previously to induce stable type-2 astrocyte differentiation in vitro. (b) Both the ECM-associated molecules and CNTF act directly

on O-2A progenitor cells and can induce them to differentiate prematurely into type-2 astrocytes. (c) ECM-associated molecules also inhibit oligodendrocyte differentiation, even in the absence of CNTF, but this inhibition is not sufficient on its own to induce type-2 astrocyte differentiation. (d) Whereas the effect of ECM on oligodendrocyte differentiation is mimicked by basic fibroblast growth factor (bFGF), the effect of ECM on type-2 astrocyte differentiation is not. (e) The ECM-associated molecules that are responsible for inhibiting oligodendrocyte differentiation and for cooperating with CNTF to induce type-2 astrocyte differentiation are made by non-glial cells in vitro. (f) Molecules that have these activities and bind to ECM are present in the optic nerve at the time type-2 astrocytes are thought to be developing.

THE vertebrate central nervous system (CNS)¹ develops from the neuroepithelial cells that form the neural tube. These cells give rise to a spectacular diversity of nerve cells, as well as to various kinds of supporting (glial) cells. The generation of diverse cell types at specific times and locations is thought to depend on interactions between cells, although little is known about the nature of the signals that presumably mediate these interactions.

To begin to analyze these signals, we have studied the development of a bipotential glial progenitor cell found in the rat CNS. It is called the O-2A progenitor cell because it can differentiate in vitro into either an oligodendrocyte, which makes myelin in the CNS, or a type-2 astrocyte, whose functions are uncertain (reviewed in Raff, 1989). Studies in which antibodies were used to distinguish glial cell types in cell suspensions prepared from rat brain and optic nerve at different ages suggest that O-2A progenitor cells differentiate in vivo on a characteristic schedule, giving rise to oligoden-

drocytes beginning at the time of birth and to type-2 astrocytes beginning in the second postnatal week (Abney et al., 1981; Miller et al., 1985; Williams et al., 1985). This developmental sequence can be reproduced in cultures of perinatal rat brain (Williams et al., 1985; Lillien et al., 1988) or optic nerve cells (Lillien and Raff, 1990). These cultures, therefore, provide experimentally accessible systems to analyze the environmental signals that control the differentiation of O-2A progenitor cells. Cultures of optic nerve cells are especially advantageous because they do not contain neurons; in addition to O-2A lineage cells (oligodendrocytes, type-2 astrocytes, and O-2A progenitor cells), they contain several types of non-O-2A lineage cells, mainly type-1 astrocytes, meningeal cells, macrophages, and endothelial cells.

The differentiation of O-2A progenitor cells into oligodendrocytes is thought to be the default pathway of development, which is triggered automatically when the progenitor cells stop dividing (reviewed in Raff, 1989). O-2A progenitor cells are stimulated to proliferate in vitro by PDGF (Noble et al., 1988), which is secreted by type-1 astrocytes (Richardson et al., 1988), the first glial cells to develop in the optic nerve (Miller et al., 1985). In the absence of PDGF and other environmental signals, progenitor cells stop dividing

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1. **Abbreviations used in this paper:** CNTF, ciliary neurotrophic factor; ECM, extracellular matrix; GC, galactocerebroside; GFAP, glial fibrillary acidic protein; SF-DME, serum-free Dulbecco's modified Eagle's medium.

prematurely and differentiate exclusively into oligodendrocytes (Temple and Raff, 1985; Raff et al., 1988). Even in the presence of PDGF, however, O-2A progenitor cells divide only a limited number of times before an intrinsic timing mechanism in the cells causes them to stop dividing and differentiate into oligodendrocytes (Raff et al., 1988).

In contrast to oligodendrocyte development, the differentiation of O-2A progenitor cells into type-2 astrocytes *in vitro* requires signals made by other types of cells. If an enriched population of O-2A progenitor cells is cultured in PDGF in the absence of serum, for example, oligodendrocytes develop, but type-2 astrocytes do not unless non-O-2A lineage cells are added (Lillien and Raff, 1990); the non-O-2A lineage cells presumably provide the required type-2 astrocyte-inducing signals. There is evidence that one of these inducing signals is CNTF, a 23-kD protein (Manthorpe et al., 1986), which is made *in vitro* by type-1 astrocytes (Hughes et al., 1988; Lillien et al., 1988). The onset of type-2 astrocyte development seems to be regulated, at least in part, by the timed production and release of ciliary neurotrophic factor (CNTF), rather than by the onset of progenitor cell responsiveness to it (Hughes et al., 1988; Lillien et al., 1988). CNTF is not sufficient on its own, however, to induce the development of stable type-2 astrocytes: when O-2A progenitor cells are exposed *in vitro* to CNTF (Hughes et al., 1988), or to extracts of brain cultures (Lillien et al., 1988) or optic nerves (Hughes and Raff, 1987) that contain CNTF (or a CNTF-like protein), they are induced to express the astrocyte-specific molecule glial fibrillary acidic protein (GFAP; Bignami et al., 1972), but only transiently. This observation suggests that additional signals, perhaps nondiffusible, are required for stable type-2 astrocyte development.

We recently reported that stable type-2 astrocytes develop in serum-free cultures of newborn optic nerve cells, beginning ~ 1 wk after the first oligodendrocytes develop, provided that PDGF is added to the cultures (Lillien and Raff, 1990). Thus all of the environmental signals required for stable type-2 astrocyte development must be present in these cultures. In the present study we have used these cultures to characterize the signals that, in addition to CNTF, are necessary for type-2 astrocyte development.

Materials and Methods

Cell Cultures Used for Preparing ECM

Newborn optic nerve cells were dissociated from Sprague-Dawley rats and cultured on 13-mm poly-D-lysine (PDL)-coated glass coverslips for 3–15 d in serum-free, hormone-supplemented Dulbecco's modified Eagle's medium (SF-DME) as previously described (Lillien and Raff, 1990). In some cases the cells were grown in human PDGF (2 ng/ml; R & D Systems, Inc., Minneapolis, MN), which was added every 2–3 d. The culture medium was changed 1 d after plating, and half the medium was replaced every 3–4 d.

Cultures of non-O-2A lineage cells were prepared by treating fresh suspensions of newborn optic nerve cells with mAb A2B5 (Eisenbarth et al., 1979) and anti-galactocerebroside (GC) antibodies in the presence of rabbit complement for 45 min at 37°C, as previously described (Lillien and Raff, 1990). After washing, $\sim 5,000$ cells were cultured in SF-DME for 10 d. No O-2A lineage cells (oligodendrocytes or O-2A progenitor cells) were found when such cultures were stained for GC and A2B5 as described below.

Cultures enriched for O-2A lineage cells were prepared by dissociating P8 optic nerve cells and culturing $\sim 5,000$ cells in SF-DME for 10 d. PDGF was added to obtain cultures enriched for both O-2A progenitor cells and oligodendrocytes, but was omitted to obtain cultures enriched mainly for oligodendrocytes.

Cultures enriched for type-1 astrocytes or meningeal cells were prepared from the cerebral hemispheres of newborn rats as previously described (Lillien et al., 1988). The cells were removed from flasks with trypsin (0.1%) and EDTA (0.02%), and, after washing, $\sim 10,000$ cells were plated on PDL-coated coverslips in DME containing 10% FCS. Cells were either maintained in 10% FCS for 10 d or switched to SF-DME 1 d after plating; no significant difference was observed in the ECMs prepared from cultures grown in the two conditions.

Cultures of macrophages were prepared from meningeal cell cultures in the following way. Flasks of meningeal cells were not fed for 1–2 wk, after which time large numbers of macrophages (identified by the presence of cell-surface Fc receptors, Raff et al., 1979) appeared on top of the meningeal cell monolayer. The macrophages were removed by shaking the flask, and 10,000 of them were cultured on PDL-coated coverslips in SF-DME containing 0.5% FCS.

Cultures of bovine aortic endothelial cells (kindly provided by M. Noble, Ludwig Institute, London, who obtained the cells from J. Folkman, Harvard Medical School; Vlodavsky et al., 1987) were cultured (after multiple passages) on PDL-coated coverslips in DME containing 10% calf serum. Half of the medium was replaced with SF-DMEM 1 d after plating and every 3–4 d thereafter.

Matrigel was obtained from Collaborative Research, Inc. (Waltham, MA) and was prepared as a substratum as described by Stemple et al. (1988).

Preparation of ECM

After the culture medium was aspirated, the cells were removed from the coverslips by treatment with 20 mM ammonia containing 0.5% Triton X-100 for 2–3 min at room temperature, and the coverslips were washed in the same solution for another 2–3 min. The ECM was washed twice with either 150 mM or 2 M NaCl (in 2 mM phosphate buffer, pH 7.5) for 30 min and then rinsed repeatedly in DME. The ECM was kept at 37°C and used within 3–4 h. Similar results were obtained when the cells were removed with 20 mM ammonia alone.

Cells Used for Assaying the Effects of CNTF and ECM

Because FCS induces type-2 astrocyte differentiation *in vitro* (Raff et al., 1983), we performed all of these experiments in serum-free medium. Newborn and embryonic day 18 (E18) optic nerve cells were dissociated as previously described (Raff et al., 1988; Lillien and Raff, 1990), and 3,000–5,000 cells were cultured in PDGF and/or CNTF (2 ng/ml) in SF-DME, either on PDL-coated coverslips or on ECM-coated coverslips. The CNTF was purified from rat sciatic nerve as previously described (Stockli et al., 1989). The culture medium was replaced 1 d after plating and fresh PDGF and/or CNTF was added. The medium change at 1 d was important because it removed the endogenous CNTF-like activity that is released by the cultured cells during the first day *in vitro*, presumably in response to the dissociation procedure (Lillien et al., 1988).

Cultures enriched for O-2A progenitor cells were prepared from P7–9 optic nerves after partial removal of the meninges. Approximately 20,000–40,000 cells were cultured on PDL-coated 35-mm plastic tissue culture dishes (Falcon Labware, Oxnard, CA) in SF-DME containing PDGF and bovine brain FGF (10 ng/ml; R & D). M. Noble and his colleagues have observed that this combination of growth factors prevents the differentiation of O-2A progenitor cells into oligodendrocytes and keeps them dividing (Bögler et al., 1990). Culture medium and growth factors were replaced 1 d after plating, and fresh PDGF and bFGF were added every other day. Cells were used within 1 wk of plating. They were removed from the culture dish with trypsin and EDTA and then washed; 500–1,000 cells were used in each assay culture. At the time the cells were used, $\sim 85\%$ were O-2A lineage cells, and, of these, 5–20% were GC⁺ oligodendrocytes.

Immunofluorescence

Cultures were fixed in 4% paraformaldehyde for 5 min at room temperature and then double- or triple-stained by indirect immunofluorescence as previously described (Lillien et al., 1988). Briefly, for triple labeling, the cells were first labeled with anti-GC mAb (ascites fluid, 1:100; Ranscht et al., 1982) followed by class-specific goat anti-mouse IgG3 coupled to fluorescein (G anti-MIgG3-FI; Nordic Immunology, Tilburg, The Netherlands; 1:100), and then with mAb A2B5 (ascites fluid, 1:100; Eisenbarth et al., 1979) followed by G₂ anti-mouse immunoglobulin coupled to rhodamine (G anti-MIg-Rd, Cappel Laboratories, Malvern, PA; 1:100); after fixation with

acid/alcohol at -20°C for 10 min, the cells were labeled with rabbit anti-glial fibrillary acidic protein (GFAP) antiserum (1:1,000; Pruss, 1979) followed by sheep anti-rabbit Ig-FI (Sh anti-RIG-FI; Burroughs Wellcome Co., Research Triangle Park, NC; 1:100). For double labeling, the cells were labeled with either A2B5 or anti-GC antibody followed by G anti-MIg-Rd and then processed for GFAP staining as above. Labeled cultures were examined with a Zeiss Universal fluorescence microscope using a $40\times$ objective and photographed using Tri-X film (ASA 400).

Microcultures

60-well Terasaki plates (Nunc, Roskilde, Denmark) were seeded with either newborn optic nerve cells in SF-DME or type-1 astrocytes in DME containing 10% FCS; 1,000 cells were seeded in $10\ \mu\text{l}$. ECM was prepared after 10–14 d as described above. Approximately five enriched O-2A progenitor cells were then added to each microwell in $10\ \mu\text{l}$ of SF-DME, containing 2 ng/ml CNTF where appropriate. Most microwells contained one to two cells. The cells were double-labeled with A2B5 and anti-GFAP antibodies and viewed as previously described (Temple and Raff, 1985).

Preparation of Optic Nerve Extract in 2 M NaCl

Extracts of optic nerves from 3–5-wk-old rats were prepared as previously described (Hughes and Raff, 1987) except that 2 M NaCl was used. The extracts were dialyzed extensively against 150 mM NaCl, centrifuged at 80,000 g for 30 min to remove material that had precipitated, and then stored at -70°C . We refer to this extract as 2M-ONE.

Adsorption of 2M-ONE, CNTF, and bFGF to 2 M NaCl-treated ECM

ECM that had been treated with 2 M NaCl was incubated in DME alone or in DME containing 2M-ONE (150–250 $\mu\text{g}/\text{ml}$ total protein), CNTF (10 ng/ml), or bFGF (100 ng/ml) for 3 h at 37°C . The ECM was rinsed in DME before enriched O-2A progenitor cells were added.

Results

CNTF-treated O-2A Progenitor Cells Express GFAP Transiently and Then Become Oligodendrocytes

As reported previously (Hughes et al., 1988), when cells from newborn optic nerve were exposed to CNTF, 20–30% of the O-2A progenitor cells (identified by their characteristic morphology and staining with the A2B5 mAb; Temple and Raff, 1986) began to express GFAP after 1 d in vitro, but, by 3 d in vitro, O-2A lineage cells expressing GFAP were no longer seen (Table I). After 2 d in vitro, >95% of the GFAP⁺ O-2A lineage cells in such cultures were found to express a mixed oligodendrocyte/type-2 astrocyte phenotype, being GC⁺ and GFAP⁺ (Table I). This finding suggests that the cells that are induced by CNTF to express GFAP transiently do not die or dedifferentiate into O-2A progenitor cells but instead go on to become oligodendrocytes.

PDGF Antagonizes the Transient Effect of CNTF

We showed previously that stable type-2 astrocytes develop in cultures of newborn optic nerve cells grown in serum-free medium, but only if PDGF is added to the medium (Lillien and Raff, 1990). The requirement for PDGF raised the possibility that stable type-2 astrocyte differentiation in culture might be induced by a combination of CNTF and PDGF. When tested directly, however, the combination of CNTF and PDGF did not induce O-2A progenitor cells to differentiate into stable type-2 astrocytes; instead, PDGF antagonized the ability of CNTF to induce progenitor cells to express GFAP transiently (Table I). This suggests that PDGF

Table I. Signals Required for Stable vs. Transient Type-2 Astrocyte Differentiation

Culture treatment	O-2A lineage cells expressing a type-2 astrocyte phenotype (A2B5 ⁺ , GFAP ⁺) after			GFAP ⁺ O-2A lineage cells expressing a mixed oligodendrocyte/type-2 astrocyte phenotype (GC) after		
	1 d	2 d	3 d	1 d	2 d	3 d
	%			%		
CNTF	22 ± 2	14 ± 3	0	5 ± 5	96 ± 4	ND
CNTF + PDGF	6 ± 2	ND	2 ± 1	ND	ND	ND
CNTF + ECM	38 ± 5	ND	79 ± 5	ND	ND	0

Newborn optic nerve cells were cultured for 1–3 d and then double-labeled with either A2B5 and anti-GFAP or anti-GC and anti-GFAP antibodies. The ECM was prepared from 9–12-d-old cultures of newborn optic nerve cells. O-2A lineage cells were recognized by their antigenic phenotype (A2B5⁺) and/or their characteristic process-bearing morphologies. In the absence of CNTF and ECM, <1% of the O-2A lineage cells expressed GFAP after 1, 2, and 3 d in vitro. In this and in Tables II, III, and V the results are expressed as means ± SE of at least three experiments.

is required for type-2 astrocyte development in serum-free cultures of newborn optic nerve cells only because it keeps O-2A progenitor cells proliferating (and thereby prevents their premature differentiation into oligodendrocytes) until endogenous type-2 astrocyte-inducing signals are produced by the cultured cells.

A Combination of CNTF and ECM-associated Molecules Induces Stable Type-2 Astrocyte Differentiation In Vitro

Although stable type-2 astrocytes develop after 8–10 d in serum-free cultures of newborn optic nerve cells in the presence of PDGF (Lillien and Raff, 1990), supernatants prepared from these cultures at this time only induce O-2A progenitor cells in vitro to express GFAP transiently rather than to develop into stable type-2 astrocytes (unpublished observations). The same is true for supernatants prepared from embryonic brain cell cultures (Lillien and Raff, 1988) and for saline extracts prepared from such cultures (Lillien et al., 1988) and from optic nerve (Hughes and Raff, 1987), all at the time type-2 astrocytes are developing. These findings suggest that diffusible signals are not sufficient to induce stable type-2 astrocyte differentiation. We therefore looked for

Table II. O-2A Progenitor Cells Respond Prematurely to Type-2 Astrocyte-inducing Signals

Age of rats	Culture treatment	O-2A lineage cells expressing a type-2 astrocyte phenotype after 3–4 d in vitro
		%
E18	ECM + CNTF	100
E18	ECM + CNTF + PDGF	100
Newborn	ECM + CNTF	79 ± 5
Newborn	ECM + CNTF + PDGF	61 ± 3

Optic nerve cells from newborn or embryonic day 18 (E18) rats were cultured on ECM prepared from 10-d-old cultures of newborn optic nerve cells. The cultures were triple-labeled with anti-GC, A2B5, and anti-GFAP antibodies. A type-2 astrocyte phenotype was defined as a process-bearing cell that was A2B5⁺ and GFAP⁺.

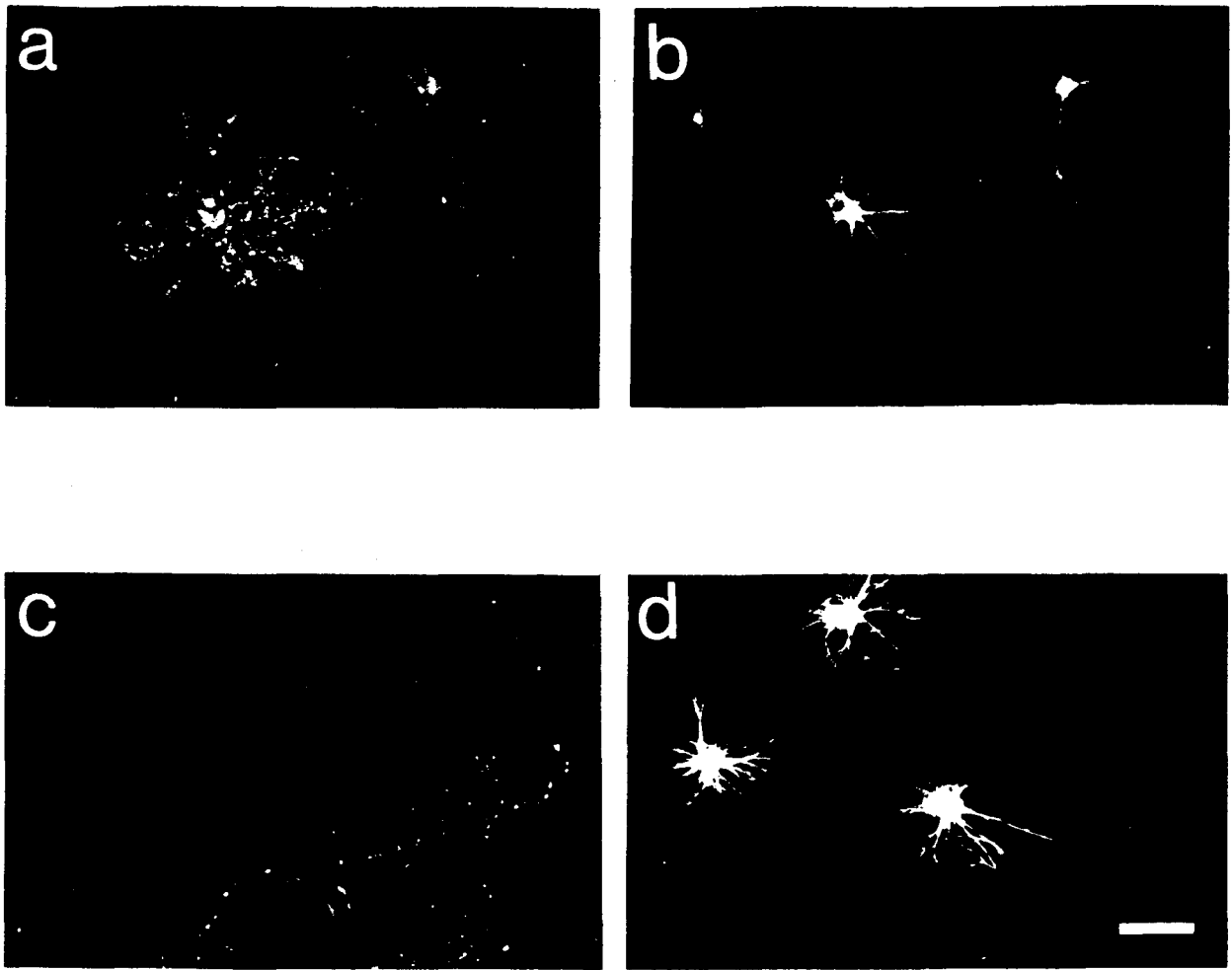


Figure 1. Immunofluorescence micrographs of type-2 astrocytes induced by CNTF and ECM. Enriched O-2A progenitor cells were cultured in CNTF for 6 d on ECM prepared from 14-d-old cultures of newborn optic nerve cells. Cells in two cultures are shown: in one the cells were labeled for A2B5 (*a*) and GFAP (*b*); in the other the cells were labeled for GC (*c*) and GFAP (*d*). Note that the type-2 astrocytes are A2B5⁺, GFAP⁺, and GC⁻, whereas the GC⁺ oligodendrocyte shown in *c* is GFAP⁻ (*d*). Bar, 25 μ m.

a nondiffusible inducing signal in cultures of newborn optic nerve cells grown *in vitro* for 9–15 d, a time when stable type-2 astrocytes were found to develop in these cultures (Lillien and Raff, 1990). We began by testing the ECM produced by these cultures; although we shall follow the conventional practice of using the term ECM to refer to the material left behind on the culture dish after the cells were removed with ammonia and Triton X-100, we cannot exclude the possibility that the relevant signalling molecules were associated with residual cell fragments.

When cells from newborn optic nerve were cultured in the presence of CNTF on ECM prepared from cultures of newborn optic nerve cells, the majority of O-2A progenitor cells differentiated into stable, GC⁻ type-2 astrocytes by 3 d *in vitro* (Table I). If, instead of CNTF, a saline extract of 3–4-wk-old optic nerve (Hughes and Raff, 1987) was used in combination with ECM, similar results were observed (not shown). Even after 1 d *in vitro*, more O-2A lineage cells expressed GFAP when they were exposed to both ECM and CNTF than when they were exposed to CNTF alone (Table I). When cells from embryonic day 18 (E18) optic nerves were cultured on ECM in the presence of CNTF, all of the

O-2A progenitors differentiated into type-2 astrocytes by 3 d *in vitro* (Table II), indicating that O-2A progenitor cells are responsive to both CNTF and ECM long before they normally differentiate into type-2 astrocytes. Whereas PDGF strongly antagonized the transient effects of CNTF alone (see Table I), it antagonized the effects of the combination of CNTF and ECM only weakly or not at all (Table II).

CNTF and ECM Act Directly on O-2A Progenitor Cells to Induce Type-2 Astrocyte Development

To study further the signal associated with the ECM made by optic nerve cultures and to identify its cellular source, we used an enriched population of O-2A progenitor cells as test cells. These cells were prepared from optic nerve cultures that had been maintained in the continuous presence of PDGF and bFGF. For the most part, these enriched O-2A progenitor cells responded to CNTF and ECM in the same way as O-2A progenitor cells in cultures of perinatal optic nerve cells: when cultured on ECM in the presence of CNTF for 3 or 6 d, the majority differentiated into GC⁻, A2B5⁺, GFAP⁺ type-2 astrocytes (Fig. 1 and Table III), whereas when cultured on ECM without CNTF, or in CNTF without

Table III. Responses of Enriched O-2A Progenitor Cells to CNTF and ECM

Substratum	CNTF	O-2A lineage cells expressing a type-2 astrocyte phenotype after		
		1 d	3 d	6 d
			%	
PDL	-	0	0	ND
PDL	+	4 ± 1	0	ND
ECM	-	<1	<1	ND
ECM	+	42 ± 9	63 ± 6	91 ± 1

Enriched O-2A progenitor cells were cultured on PDL or on ECM that was prepared from 9-12-d-old cultures of newborn optic nerve cells. After 1-6 d, the cultures were triple-labeled as in Table II, or double-labeled with anti-GC and anti-GFAP antibodies, and type-2 astrocytes were identified either as A2B5⁺, GFAP⁺ process-bearing cells, or as GC⁻, GFAP⁺ process-bearing cells, respectively. The results were not significantly different with the two staining procedures.

ECM, the cells that differentiated developed into oligodendrocytes and not type-2 astrocytes (Table III; see Fig. 2). The enriched O-2A progenitor cells differed from progenitor cells in cultures of perinatal optic nerve, however, in that few of them responded even transiently to CNTF in the absence of ECM, although many responded within 24 h to CNTF in the presence of ECM (Table III).

To determine whether CNTF and the ECM-associated

Table IV. Effect of CNTF and ECM on Single O-2A Progenitor Cells

ECM prepared from cultures of	CNTF	Fraction of O-2A lineage cells expressing a type-2 astrocyte phenotype after 3 d
Optic nerve cells	-	0/42
Optic nerve cells	+	64/117
Type-1 astrocytes	+	0/97

Individual O-2A progenitor cells were added (by limiting dilution) to micro-wells containing ECM produced by either newborn optic nerve cells or type-1 astrocytes. The cells were double-labeled with A2B5 and anti-GFAP antibodies. On average, there were 1.5 cells/well; 32 of the wells with optic nerve cell ECM contained a single O-2A lineage cell as the only visible cell in the well, and 17 of these were GFAP⁺.

molecules act directly on O-2A progenitor cells, rather than indirectly via other cell types, we cultured individual progenitor cells in microwells for 3 d. As shown in Table IV, isolated O-2A progenitor cells cultured on ECM prepared from newborn optic nerve cell cultures developed into type-2 astrocytes in the presence, but not in the absence, of CNTF. Thus both CNTF and the ECM-associated molecules act directly on O-2A progenitor cells to induce type-2 astrocyte differentiation.

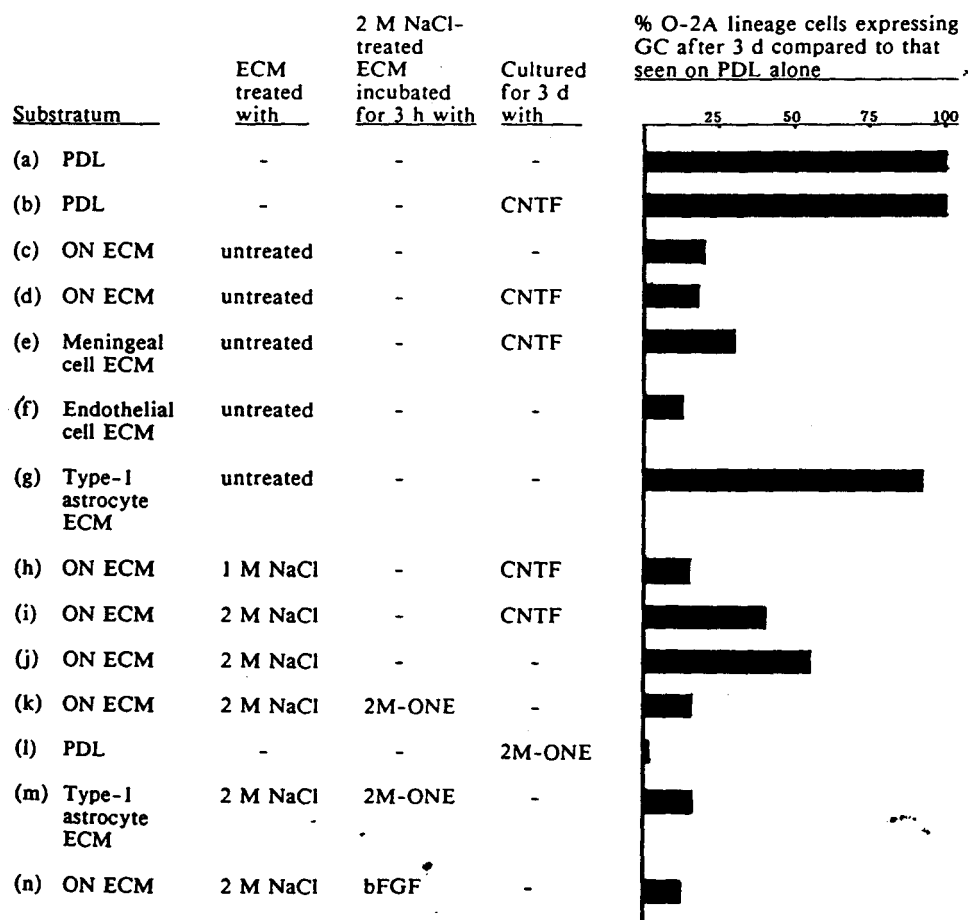


Figure 2. ECM-associated signals that inhibit oligodendrocyte differentiation. Enriched O-2A progenitor cells were cultured on PDL or ECM for 3 d and then triple-labeled for GC, A2B5, and GFAP. The proportion of O-2A lineage cells that differentiated into GC⁺ oligodendrocytes was determined and compared to that seen when the cells were cultured on PDL alone. More than 95% of the O-2A lineage cells were GC⁺ when cultured on PDL alone. In this and the following figure, ECM was prepared from 9-12-d-old cultures, and the results are expressed as means ± SE of at least three experiments. ON, optic nerve.

Table V. Cellular Sources of the Nondiffusible Type-2 Astrocyte-inducing Signal

Substratum	O-2A lineage cells expressing a type-2 astrocyte phenotype after 3 d	%	O-2A lineage cells expressing a type-2 astrocyte phenotype after 3 d compared with that seen with 9-12 d ON ECM
ECM from newborn ON cells + 9-12 d in vitro	63 ± 6		100
ECM from newborn ON cells + 4 d in vitro	4 ± 1		7
Newborn ON cells + 12 d in vitro	89 ± 1		140
ECM from non-O-2A lineage cells of newborn ON	53 ± 3		110
ECM from meningeal cells	42 ± 4		95
ECM from type-1 astrocytes	0		0
ECM from meningeal macrophages	0		0
ECM from oligodendrocytes	0		0
ECM from oligodendrocytes plus O-2A progenitor cells	0		0
Matrigel	0		0
ECM from bovine aortic endothelial cells	72 ± 10		104

Enriched O-2A progenitor cells were grown in the presence of CNTF on PDL, ECM prepared from various types of cells, or on unextracted cultures of newborn optic nerve cells. Except where indicated, ECM was prepared from 9-12-d-old cultures. After 3 d, the cells were triple-labeled for GC, A2B5, and GFAP and the proportion of O-2A lineage cells that differentiated into A2B5⁺, GFAP⁺ type-2 astrocytes was determined and compared with that seen with cells cultured in CNTF on ECM prepared from 9-12-d-old cultures of newborn optic nerve cells, which varied between 35 and 93% in different experiments. ON, optic nerve.

Not All Cell Types Make the Relevant ECM-associated Molecules

In the experiments described so far, ECM was prepared from newborn optic nerve cell cultures that were maintained, with or without PDGF, in the absence of serum for 9-15 d (the presence or absence of PDGF did not affect the activity of the ECM [not shown]). ECM prepared from similar cultures but after only 3-4 d in vitro was much less effective in inducing stable type-2 astrocyte differentiation (Table V). Intact cells from 12 d-old cultures of newborn optic nerve cells (which were grown without PDGF and, therefore, did not contain type-2 astrocytes) were at least as effective as the ECM prepared from such cultures (Table V); in these cultures, many of the type-2 astrocytes that developed after the addition of enriched O-2A progenitor cells did not appear to be in contact with other cells, suggesting that the nondiffusible signalling molecules are associated with the ECM and not just with cell surfaces. This finding also suggests that the active components in the ECM are normally extracellular and not merely released from intracellular sites as a result of the cell removal procedure used to prepare the ECM.

To begin to identify the cellular source(s) of the ECM-associated molecules, we first eliminated the O-2A lineage population from newborn optic nerve cells by treatment with A2B5 and anti-GC antibodies in the presence of rabbit complement. ECM prepared from such cultures of non-O-2A lineage cells was as active as that prepared from cultures of unfractionated newborn optic nerve cells (Table V). This non-O-2A lineage population contains mainly type-1 astrocytes and meningeal cells, but also some endothelial cells and macrophages. When cultures enriched for specific types of non-O-2A lineage cells were tested, ECM from meningeal cells was found to support stable type-2 astrocyte differentia-

tion in the presence of CNTF, whereas ECM from cultures of cortical type-1 astrocytes (Tables IV and V) or of meningeal macrophages (Table V) was not effective. ECM prepared from cultures enriched for O-2A lineage cells (either oligodendrocytes or oligodendrocytes plus O-2A progenitor cells) were also ineffective, as was Matrigel, an ECM preparation from mouse sarcoma cells (Table V). ECM prepared from cultures of multiply passaged bovine aortic endothelial cells was as effective as ECM from meningeal cultures and newborn optic nerve cells (Table V). These results suggest that the ECM-associated molecules that collaborate with CNTF to induce type-2 astrocyte development are made by nonglial cells.

ECM-associated Molecules Inhibit Oligodendrocyte Differentiation Even in the Absence of CNTF

In all of the cases we tested, ECM did not induce type-2 astrocyte differentiation unless CNTF was added to the cultures (see Table III, for example). All of the ECMs that collaborated with CNTF to induce type-2 astrocyte differentiation, however, inhibited the differentiation of O-2A progenitor cells into oligodendrocytes even without the addition of CNTF. Whereas in the absence of ECM >95% of O-2A progenitor cells differentiated into GC⁺ oligodendrocytes within 3 d in vitro (Fig. 2 a), in the presence of ECM prepared from optic nerve cultures (and in the absence of CNTF), <30% of O-2A progenitor cells differentiated into oligodendrocytes within 3 d (Fig. 2 c). ECM made by meningeal cells and endothelial cells also inhibited oligodendrocyte differentiation (Fig. 2, e and f), whereas ECM prepared from type-1 astrocyte cultures did not (Fig. 2, g). CNTF, in the absence of ECM, did not inhibit oligodendrocyte differentiation (Fig. 2 b).

Substratum	ECM treated with	2 M NaCl-treated ECM incubated for 3 h with	Cultured for 3 d with
(a) ON ECM	untreated	-	CNTF
(b) ON ECM	0.5 M NaCl	-	CNTF
(c) ON ECM	1 M NaCl	-	CNTF
(d) ON ECM	2 M NaCl	-	CNTF
(e) ON ECM	2 M NaCl	2M-ONE	CNTF
(f) PDL	-	-	2M-ONE + CNTF
(g) ON ECM	2 M NaCl	-	2M-ONE + CNTF
(h) Matrigel	-	-	2M-ONE + CNTF
(i) Type-1 astrocyte ECM	2 M NaCl	-	2M-ONE + CNTF
(j) ON ECM	2 M NaCl	CNTF	-
(k) ON ECM	2 M NaCl	-	bFGF + CNTF

ECM Activity Is Removed by Treatment with 2 M NaCl

To begin to characterize the signaling molecules associated with the ECM made by optic nerve cell cultures, we tried to remove the molecules by treating the ECM with increasing concentrations of NaCl. Whereas treatment with 0.5 M NaCl had little if any effect (Fig. 3 *b*), treatment with 1 M NaCl removed approximately half of the type-2 astrocyte-inducing activity (Fig. 3 *c*), but had no effect on the ability of the ECM to inhibit oligodendrocyte differentiation (Fig. 2 *h*). Treatment with 2 M NaCl removed 60–80% of both activities (Figs. 2 *j* and 3 *d*), as did 3 M NaCl (not shown). Both the type-2 astrocyte-inducing (Fig. 3 *e*) and oligodendrocyte-inhibiting activities (Fig. 2 *k*) were restored if the salt-treated ECM was incubated with an extract of optic nerves from 3–4-wk-old rats made in 2 M NaCl. This optic nerve extract (2M-ONE) did not induce stable type-2 astrocyte differentiation when applied in the absence of the 2 M NaCl-treated ECM, even in the presence of extra CNTF (Fig. 3 *f*), although it did inhibit oligodendrocyte differentiation in the absence of ECM (Fig. 2 *l*). Thus type-2 astrocyte differentiation requires the combination of CNTF and 2 M NaCl-extractable molecules in ECM, which must either be bound to the ECM to act or be presented in combination with an additional ECM-associated signal that is not extracted by 2 M NaCl. Surprisingly, 2M-ONE was not able to activate either Matrigel or ECM prepared from cultures of type-1 astrocytes to collaborate with CNTF to induce type-2 astrocyte differentiation (Fig. 3, *h* and *i*). 2 M NaCl-treated type-1 astrocyte ECM, however, could be activated by 2M-ONE to inhibit oligodendrocyte differentiation (Fig. 2 *m*). CNTF itself did not bind in an active form to ECM prepared from optic nerve cultures, whether or not the ECM was first treated with 2 M NaCl (Fig. 3 *j*), excluding the possibility that the ECM acts simply to concentrate or present CNTF to O-2A progenitor cells.

If 2 M NaCl-treated ECM prepared from optic nerve cul-

% O-2A lineage cells expressing a type-2 astrocyte phenotype after 3 d compared to that seen with ON ECM plus CNTF

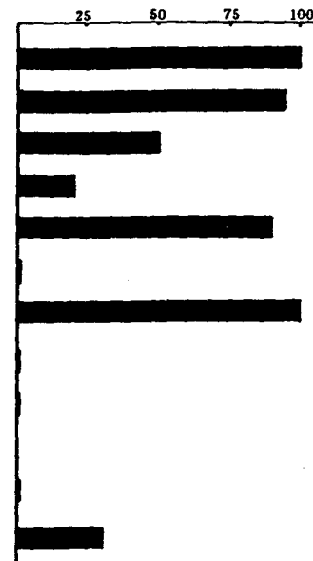


Figure 3. Removing and reconstituting ECM-associated signals that induce type-2 astrocyte differentiation in the presence of CNTF. Enriched O-2A progenitor cells were cultured for 3 d and then either double-labeled for GC and GFAP or triple-labeled for GC, A2B5, and GFAP. The proportion of O-2A lineage cells that differentiated into cells with a type-2 astrocyte phenotype (process-bearing cells that were either A2B5⁺, GFAP⁺, or GC⁻, GFAP⁺) was determined and compared with that seen with cells cultured on ON ECM (prepared from 9–12-d-old cultures of newborn optic nerve cells) in the presence of CNTF, which varied between 35 and 93% in different experiments.

tures was incubated in 100 ng/ml of bFGF, its ability to inhibit oligodendrocyte differentiation was completely restored (Fig. 2 *n*). This concentration of bFGF, however, did not restore the ability of salt-washed ECM to collaborate with CNTF in inducing type-2 astrocyte differentiation, even when the bFGF was continuously present in the culture (Fig. 3 *k*).

Discussion

Previous studies suggested that CNTF is only one part of a system of extracellular inducing signals required for the development of type-2 astrocytes (Hughes et al., 1988; Lillien et al., 1988). We have now shown that O-2A progenitor cells can be induced to differentiate *in vitro* into stable type-2 astrocytes by a combination of CNTF and a nondiffusible signal that is associated with the extracellular matrix produced by cultures of optic nerve cells. Single-cell experiments indicate that both signals act directly on O-2A progenitor cells. The ECM-associated signal has two effects on O-2A progenitor cells: it promotes type-2 astrocyte differentiation, and it inhibits oligodendrocyte differentiation. Whereas the first effect requires CNTF, the second does not.

Timing Mechanism of Type-2 Astrocyte Development

The development of O-2A progenitor cells into oligodendrocytes appears not to require environmental inducing signals (Temple and Raff, 1985). Both the choice and timing of oligodendrocyte development seem to depend instead on an intrinsic mechanism that limits progenitor cell proliferation in response to PDGF; oligodendrocyte differentiation is thought to follow automatically as a consequence of the progenitor cell's withdrawal from the cell cycle (Raff et al., 1988; Hart et al., 1989).

Type-2 astrocytes, like oligodendrocytes, begin to differentiate on a predictable schedule both *in vivo* (Miller et al.,

1985; Williams et al., 1985) and in vitro (Williams et al., 1985; Lillien et al., 1988; Lillien and Raff, 1990). In principle, the timing of type-2 astrocyte development could reflect the timed production of inducing signals (environmental control), the timed responsiveness to these signals (intrinsic control), or both. Previous work demonstrated that O-2A progenitor cells can respond prematurely, although transiently, to CNTF (Hughes et al., 1988). In this study we have shown that perinatal O-2A progenitor cells can respond to the combination of CNTF and ECM by differentiating into stable type-2 astrocytes at least a week before they normally would do so in vivo. This combination of type-2 astrocyte-inducing signals is not inhibited by PDGF, although, in the absence of exogenous ECM, PDGF antagonizes the transient response to CNTF. These and previous observations (Hughes and Raff, 1987; Hughes et al., 1988; Lillien et al., 1988; Lillien and Raff, 1990) suggest that O-2A progenitor cells are responsive to type-2 astrocyte-inducing signals before the cells reach an intrinsic proliferation limit and that the timing of type-2 astrocyte development depends exclusively on when the inducing signals appear. In previous studies it was shown that the production of CNTF in the developing optic nerve (Hughes et al., 1988) and in cultures of brain cells (Lillien et al., 1988) coincides with the development of type-2 astrocytes. In this study we found that the signal associated with the ECM also increases with time in cultures of newborn optic nerve cells. It is not clear whether this increase results from the timed production of the signal or from the steady accumulation of the signal with time in vitro; in either case, it probably contributes to the timing of type-2 astrocyte development in optic nerve cultures. We have not yet determined when the ECM-associated signal first appears in the developing optic nerve.

Cellular Source of the ECM-associated Signal

The differentiation of O-2A progenitor cells into type-2 astrocytes in vitro was previously shown to require non-O-2A lineage cells (Lillien and Raff, 1990), which, in optic nerve cultures, include type-1 astrocytes, meningeal cells, macrophages, and endothelial cells. This observation is now explained as non-O-2A lineage cells appear to be the source of both CNTF and the ECM-associated signal, at least in vitro. Type-1 astrocytes were previously shown to be a source of CNTF (Lillien et al., 1988), but this study suggests that they do not make the ECM-associated inducing signal: ECM produced by cultures of cortical type-1 astrocytes did not induce type-2 astrocyte differentiation in the presence of CNTF. Whereas ECM produced by cultures of macrophages or O-2A lineage cells was also ineffective at inducing type-2 astrocyte development in the presence of CNTF, ECM produced by cultures of cerebral meningeal cells or bovine aortic endothelial cells was as effective as ECM produced by cultures of mixed optic nerve cells. The meningeal cultures were morphologically heterogeneous and might well have contained endothelial cells, as the meninges used to prepare the cultures were rich in blood vessels. It remains to be determined whether CNS endothelial cells also produce the ECM-associated type-2 astrocyte-inducing signal, and whether these cells are the source of the signal in vivo. As meningeal cultures were found previously not to produce CNTF-like activity (Lillien et al., 1988), it seems that at least two types

of non-O-2A lineage cells must collaborate to induce type-2 astrocyte development in vitro.

Nature of the ECM-associated Signal

There are numerous reports of the influence of ECM components on the growth and differentiation of a variety of cell types; some of these effects are mediated by the structural components of the ECM itself (for example, Panayotou et al., 1989; Adams and Watt, 1989), whereas others are mediated by signalling molecules bound to the ECM (for example, Vlodavsky et al., 1987; Baird and Ling, 1987; Gordon et al., 1987; Rogelj et al., 1989). The functional significance of ECM-bound signaling molecules is not clear. In the case of bFGF, which is active in a soluble state, association with the ECM has been reported to stabilize the protein (Gospodarowicz and Cheng, 1986; Saksela et al., 1988), and it has been suggested that ECM might serve as a storage site for FGF (Baird and Ling, 1987; Vlodavsky et al., 1987). Treatment of ECM with high concentrations of NaCl causes bound factors such as bFGF to dissociate (Baird and Ling, 1987; Vlodavsky et al., 1987), and it might also release some intrinsic matrix components as well (for example, Lindahl and Höök, 1978; Timpl and Rohde, 1979). Although we refer to the nondiffusible signal that influences O-2A progenitor cell development as ECM associated, many ECM-associated molecules are also bound to cell surfaces, and we cannot exclude the possibility that the nondiffusible signal we have studied is also cell-surface associated. We found that when O-2A progenitor cells were grown (in the presence of CNTF) on 12-d-old cultures of newborn optic nerve cells, rather than on ECM prepared from such cultures, many of the type-2 astrocytes that developed were located entirely on the substratum between the optic nerve cells. This finding suggests that the nondiffusible signal that collaborates with CNTF to induce type-2 astrocyte differentiation is not exclusively associated with cell surfaces. By contrast, studies of a nondiffusible signal made by 3T3 cells that stimulates the proliferation and development of hemopoietic stem cells have shown that the stem cells respond only when in contact with the 3T3 cells and not when located on the ECM produced by these cells (Roberts et al., 1987).

To begin to characterize the ECM-associated molecules that inhibit oligodendrocyte differentiation and act in conjunction with CNTF to induce type-2 astrocyte differentiation, we treated ECM made by optic nerve cultures with 2 M NaCl. This treatment largely removed both the type-2 astrocyte-inducing activity and the oligodendrocyte differentiation inhibiting activity. Both activities were restored by incubation of the salt-treated ECM with an extract of optic nerve made in 2 M NaCl. This finding suggests that the nondiffusible signal, like CNTF (Hughes et al., 1988), is present in the optic nerve at the time type-2 astrocytes are developing.

Does the same ECM-associated molecule both induce type-2 astrocyte differentiation and inhibit oligodendrocyte differentiation? Four findings suggest that the two activities are mediated by different molecules. (a) When ECM produced by cultures of newborn optic nerve cells was treated with 1 M NaCl, much more type-2 astrocyte-inducing activity was removed than oligodendrocyte-inhibiting activity. (b) The active component(s) in 2 M NaCl extracts of optic nerve appears to have to be associated with ECM to induce type-2

astrocyte differentiation but not to inhibit oligodendrocyte differentiation. (c) The addition of bFGF to 2 M NaCl-washed ECM reconstituted the ability of the ECM to inhibit oligodendrocyte differentiation, but it did not reconstitute the ability of the ECM to collaborate with CNTF to induce type-2 astrocyte differentiation. (d) Whereas ECM made by type-1 astrocyte cultures expressed neither activity, treatment of this ECM with 2 M NaCl extracts of optic nerve conferred oligodendrocyte inhibiting activity but not type-2 astrocyte-inducing activity. A parsimonious interpretation of these findings is that bFGF, or a related protein, is responsible for the oligodendrocyte inhibitory effect of the ECM while a different molecule is responsible for the type-2 astrocyte-inducing effect. Preliminary observations indicate that the ECM-associated type-2 astrocyte-inducing activity is sensitive to trypsin but not to collagenase, suggesting that the relevant signaling molecule is itself a noncollagenous protein, or is bound to such a protein.

Mechanism of Action of the ECM-associated Molecules

The presence of ECM appears to change the response of perinatal O-2A progenitor cells to CNTF from a transient to a stable one. Several observations, however, suggest that even the transient response to CNTF requires some ECM. When enriched O-2A progenitor cells were exposed to CNTF for 24 h, few responded in the absence of ECM, although many responded in the presence of ECM. By contrast, 20–30% of the O-2A progenitor cells in cultures of newborn optic nerve responded transiently to CNTF in the absence of exogenous ECM, although many more responded in the presence of ECM. As newborn optic nerve cultures contain a high proportion of non-O-2A lineage cells (Lillien and Raff, 1990), which are the source of the ECM-associated signals, it is possible that these cells produce enough ECM to support a transient response to CNTF but not enough to support a stable response. A correlation between the proportion of non-O-2A lineage cells present and the degree of transient response to CNTF was observed previously (although its significance was not recognized): the transient response to CNTF was found to be lower in cultures of P7 optic nerve cells, which contain relatively few non-O-2A lineage cells, than in cultures of perinatal optic nerve cells, which contain many such cells (Hughes and Raff, 1987).

There are other instances where the combination of diffusible and non-diffusible extracellular signals are required for a cellular response. For example, the response of blood neutrophils to tumor necrosis factor requires an integrin-mediated interaction with ECM proteins (Nathan et al., 1989), and the survival of embryonic sensory neurons in vivo is promoted by the combination of brain-derived neurotrophic factor and laminin, but not by brain-derived neurotrophic factor alone (Kalcheim et al., 1987). In neither of these cases is the mechanism of the collaboration understood. Similarly, it is not clear how CNTF and the ECM-associated molecules collaborate to induce type-2 astrocyte development, although it is clear that the ECM does not simply bind CNTF and present it to O-2A progenitor cells, as we could not detect CNTF activity in ECM even after the ECM was treated with exogenous CNTF. Our finding that ECM inhibits oligodendrocyte differentiation but does not

induce type-2 astrocyte development in the absence of CNTF demonstrates that the inhibition of the default pathway of O-2A progenitor cell development is not sufficient on its own to induce type-2 astrocyte differentiation; positive inducing signals are still required. It is possible, however, that the inhibition of oligodendrocyte differentiation is an essential part of the induction process.

The most important conclusion of this study is that both CNTF and ECM-associated molecules are required to induce O-2A progenitor cells to develop into type-2 astrocytes in serum-free cultures. It is not known whether, in inducing stable type-2 astrocyte development in vitro, FCS mimics the effects of these diffusible and nondiffusible signals, or whether it induces their production or release; previous single-cell experiments suggest that FCS can act both directly and indirectly on progenitor cells to induce them to differentiate into type-2 astrocytes (Temple and Raff, 1985). It is also uncertain how our findings in vitro relate to glial cell differentiation in vivo; it is clear, however, that both signals required for type-2 astrocyte development in vitro are present in the optic nerve at the time type-2 astrocytes are thought to be developing.

We thank M. Noble and J. Folkman for the bovine aortic endothelial cells and I. Mason, A. Mudge, and J. Voyvodic for helpful comments on the manuscript. L. E. Lillien was supported by the American Multiple Sclerosis Society and by the Wellcome Trust.

Received for publication 14 March 1990 and in revised form 1 May 1990.

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