A Role for TGF-β in Oligodendrocyte Differentiation

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Abstract. Oligodendrocyte-type-2 astrocyte (O-2A) glial progenitor cells undergo a limited number of mitotic divisions in response to PDGF before differentiating into oligodendrocytes, the myelin-forming cell of the CNS. We examined the mechanism limiting O-2A proliferation, and demonstrate that these cells secrete an inhibitor of cell proliferation that can be neutralized with antibodies to TGF-β. O-2A cells also secrete an inhibitory activity that cannot be neutralized with TGF-β antibodies. O-2A progenitor cultures express TGF-β1 isoform and its transcript, while oligodendrocyte cultures express TGF-β1, β-2, and β-3 isoforms. Both recombinant TGF-β1 and O-2A conditioned medium inhibit the proliferation of O-2A pro-

genitor cells cultured in the presence of PDGF, and this inhibition can be partially neutralized with polyclonal TGF-β antibodies. Thus, TGF-β produced by O-2A cells may limit PDGF-driven mitosis and promote oligodendrocyte development. TGF-β is a less potent inhibitor of O-2A proliferation when these cells are cultured in the presence of bFGF, suggesting that bFGF interferes with TGF-β signaling. Thus, the production of TGF-β by cells in the O-2A lineage may account for the distinct effects of PDGF and bFGF on O-2A progenitor cell proliferation. Moreover, our results suggest that TGF-β may be an important mediator of oligodendrocyte differentiation.

DURING vertebrate neural development, factors which control the cell cycle regulate the commitment of precursor cells into the variety of differentiated neuronal and glial cell types. Differentiated cells in the mature brain are postmitotic, and are generated from precursor cells which arise in the germinal subventricular neural epithelium. These precursors retain a remarkable degree of phenotypic plasticity (Gray et al., 1988; Price and Thurlow, 1988; Turner and Cepko, 1987; Turner et al., 1990; Wetts and Fraser, 1988), and commitment to a specific lineage appears to be regulated by signals present in their environment (Le Douarin, 1986; McConnell, 1988; Price, 1989). We have investigated the nature of the signals controlling the proliferation and differentiation of a glial precursor cell that generates oligodendrocytes, the myelin-forming cell in the central nervous system (CNS).[1]

Oligodendrocytes elaborate multiple processes that contact axons and form a compact membranous sheath, the myelin internode, that facilitates saltatory axonal conduction. Oligodendrocytes arise from precursor cells that originate in the subventricular zone of the forebrain and the ventral spinal cord (Levine and Goldman, 1988; Pringle et al., 1992; Reynolds and Wilkin, 1988; Warf et al., 1991). These progenitor cells are multipotential in culture, forming either oligodendrocytes or type-2 astrocytes under different culture conditions, and are termed the oligodendrocyte-type-2 astrocyte (O-2A) progenitor cell (Raff et al., 1983). O-2A progenitors persist in the adult CNS, where they are termed the O-2A^adult progenitor cell (Wren et al., 1992).

In culture, O-2A progenitor cells proliferate in response to several polypeptide growth factors, including basic FGF and PDGF, and differentiate into postmitotic oligodendrocytes upon withdrawal of mitogens (Raff, 1989). FGF is mitogenic for oligodendrocyte precursor cells (Besnard et al., 1989; Bögler et al., 1990; Eccleston and Silberberg, 1985; McKinnon et al., 1990; Noble et al., 1988; Saneto and de Vellis, 1985). FGF also blocks oligodendrocyte differentiation, by maintaining O-2A progenitors in the cell cycle, and sensitizes these cells to PDGF, by upregulating their PDGF receptors (McKinnon et al., 1990). PDGF, in turn, induces a bipolar state associated with cell motility, and is chemotactic for O-2A progenitor cells (Armstrong et al., 1990; Bögler et al., 1990; McKinnon et al., 1993; Noble et al., 1988; Small et al., 1987).

While PDGF is mitogenic for O-2A progenitor cells (No-

1. *Abbreviations used in this paper*: bp, base pairs; CM, conditioned medium; CNS, central nervous system; GC, galactocerebroside; O-2A, oligodendrocyte-type-2 astrocyte; OG, oligodendrocyte; RT, reverse transcriptase.

ble et al., 1988; Richardson et al., 1988), its effects are distinct from bFGF in that PDGF triggers only a limited number of divisions before differentiation (Raff et al., 1988). The normal in vivo timing of oligodendrocyte differentiation can be reconstituted in O-2A cultures in vitro by addition of type-1 astrocytes, type-1 astrocyte-conditioned medium, or PDGF which is produced by astrocytes in vitro (Noble and Murray, 1984; Raff et al., 1985, 1988; Richardson et al., 1988). Thus, PDGF is said to drive a "clock" regulating the timing of oligodendrocyte development in vitro (Raff et al., 1988). PDGF is produced by both astrocytes and by neurons in vivo (Sasahara et al., 1991; Yeh et al., 1991), indicating that interactions between these cell types may regulate the timing of oligodendrocyte development in vivo.

O-2A progenitor cells in culture have been shown to produce molecules which inhibit their own proliferation (Louis et al., 1992b) and promote oligodendrocyte differentiation (Levi et al., 1991), suggesting that their differentiation may be induced by factors acting in either an autocrine or paracrine manner. We have examined whether these signals could be related to the TGF-β family of molecules, which includes five members with a high degree of sequence conservation (TGF-β's 1-5) with significant homology to an extended superfamily of molecules including the activins, inhibins, Müllerian inhibiting substance, decapentaplegic, bone morphogenic factors, and vgl (Massagué, 1990; Miller et al., 1989b). The TGF-β's are produced by glial cells in vitro and in vivo (da Cunha et al., 1993; da Cunha and Vitković, 1992; Flanders et al., 1991), have both inductive and inhibitory activities on cell proliferation, and are important regulators of differentiation in a variety of systems (Massagué, 1990; Roberts and Sporn, 1990; Sporn et al., 1987).

In this study, we demonstrate that cultured O-2A lineage cells express transcripts encoding specific isoforms of the TGF-β's, that these proteins can be detected in cultured cells by immunostaining, and that these cells secrete into their culture medium a growth inhibitory activity that can be neutralized with antibodies to TGF-β. Both recombinant TGF-β1 and O-2A conditioned media can inhibit the proliferation of O-2A progenitor cells in response to PDGF. The ability of TGF-β to inhibit bFGF-driven proliferation of O-2A cells is significantly reduced. Our results may account for the distinct effects of bFGF and PDGF on O-2A progenitor cell proliferation, and suggest that TGF-β could be either an autocrine or paracrine mediator of oligodendrocyte differentiation.

Materials and Methods

Primary Glial Cell Culture

Primary cortical glial cultures were established from 2-d old Sprague-Dawley rat pups (Taconic Farms Inc., Germantown, NY) as previously described (Behar et al., 1988), and were maintained in DMEM (4.5 g/L D-glucose) supplemented with 25 μg/ml gentamycin and 10% FBS (Hyclone Labs., Logan, UT). Optimal growth was obtained by refeeding every 3 d and by using sera which was not heat inactivated. O-2A progenitor cells were isolated from monolayer cultures after 8-10 d by shaking on a rotary platform (180 rpm) at 37°C overnight (McCarthy and de Vellis, 1980), and enriched to >95% purity of O-2A progenitor cells by reverse panning of cells coated with monoclonal antibody A2B5 ascites fluid (Behar et al., 1988). Cells were plated at 140,000 cells per 12-mm poly-L-lysine–coated coverslip (Bellco Glass, Inc., Vineland, NJ), or 2 × 10⁶ cells per 100-mm Falcon dish, in DMEM with 10% FBS. After 18 h the cultures were refed

with control medium (DMEM supplemented with 50 μg/ml transferrin, 30 nM sodium selenite, 30 nM triiodothyronine, 50 ng/ml bovine insulin, and 0.5% FBS) (Eccleston and Silberberg, 1984), plus 5 ng/ml bFGF or 5 ng/ml PDGF-AA (Upstate Biotechnology, Inc., Lake Placid, NY). Growth factors were replenished every 36-48 h.

Immunocytochemistry

Cells growing on glass coverslips were fixed in 2% paraformaldehyde, and then processed for immunocytochemistry as described (Behar et al., 1988). Antibodies for glial lineage analysis included monoclonals A2B5 (Eisenbarth et al., 1979), O1 and O4 (Sommer and Schachner, 1981), and RmAb (Ranscht et al., 1982). A2B5 and O4 recognize surface antigens expressed during progenitor cell maturation (Bansal et al., 1989; Dubois-Dalcq, 1987; Gard and Pfeiffer, 1990). The O1 antibody is specific for the oligodendrocyte differentiation marker galactocerebroside (GC) (Raff et al., 1978), while RmAb recognizes GC, sulfatide, and related glycolipids (Bansal et al., 1989).

For TGF-β immunoreactivity, cells were fixed in Bouin's fixative for 20 min, and then with methanol (−20°C, 10 min), and then blocked in 10% normal goat sera for 30 min. TGF-β antibody staining was done using 10 μg/ml rabbit anti-TGF-β antibody (R&D Sys., Inc., Minneapolis, MN), 20 μg/ml turkey anti-TGF-β1 antisera (Genzyme Corp., Cambridge, MA), or 2 μg/ml affinity purified rabbit anti-TGF-β2 and -β3 antipeptide antisera (Flanders et al., 1991) provided by Kathy Flanders and Anita Roberts, National Cancer Institute. Cells on glass coverslips were incubated with primary antibodies at room temperature for 60 min, and TGF-β immunoreactivity was detected by incubating the coverslips for 60 min with 15 μg/ml of appropriate secondary antibody, either donkey anti-rabbit Ig (Amersham Corp., Arlington Heights, IL) or rabbit anti-turkey IgG (Zymed Labs, Inc., S. San Francisco, CA) antibodies conjugated to biotin, followed by incubation for 30 min with 1:1,000 dilution of peroxidase conjugated streptavidin (Zymed Labs., Inc.). Peroxidase activity was detected using an aminoethyl carbazole substrate kit (Zymed Labs., Inc.) as described by the manufacturer. No immunoreactivity was detectable when cells under any of the culture conditions were incubated in absence of primary antibodies. Coverslips were mounted on glass slides using Fluromount-G (Southern Biotech, Inc., Birmingham, AL) and photographed with Kodak 400 ASA Tri-X film under phase-contrast and bright-field optics.

RT-PCR Analysis

RNA was extracted from cultured cells in the presence of guanidinium isothiocyanate, and then isolated by centrifugation through CsCl (Chirgwin et al., 1979). Reverse transcriptase (RT) reactions were performed using 10 μg total cell RNA and 20 pmol d(T)₁₅ as described (McKinnon et al., 1993). RNA plus primers were heated at 95°C for 3 min, annealed at 37°C for 5 min, and then at room temperature for 5 min, and then incubated at 37°C for 60 min in 20 μl of 10 mM Tris hydrochloride (pH 8.3), 50 mM KCl-1.5 mM MgCl₂, 1 mM deoxynucleoside triphosphates and 1.5 U/μl of Moloney MuLV-RT (Stratagene Inc., San Diego, CA). Reactions were terminated by heating at 95°C for 10 min. PCR contained 150 pmol of primer pairs and 2 μl (∼1 μg) of cDNA in 50 μl of 10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.4 mM dNTPs, and .05 U/μl of Taq DNA polymerase (Perkin-Elmer, Norwalk, CT), and subjected to 30 cycles at 94°C for 1.5 min, 55°C for 1.5 min, 72°C for 3 min using a DNA thermocycler (Perkin Elmer). DNA fragments were resolved by electrophoresis on 4-20% acrylamide gradient gels (Novex, San Diego, CA).

Oligonucleotide primers were synthesized on a 380A DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). Primers were based on published nucleotide sequences for rat TGF-β1 (Qian et al., 1990), mouse TGF-β2 (Miller et al., 1989a), human TGF-β2 (de Martin et al., 1987), mouse TGF-β3 (Denhez et al., 1990), human TGF-β3 (ten Dijke et al., 1988), and rat cyclophilin clone 1B15 (Danielson et al., 1988). TGF-β primers were selected using the PCR primer selection program (Lowe et al., 1990). To optimize their utility, primers were selected from regions with maximal conservation between the published sequences for rodent and human TGF-β isoforms using the GAP alignment program of the Sequence Analysis Software Package, Version 5 (University of Wisconsin Genetics Computer Group, Madison, Wisconsin).

DNA Sequence Analysis

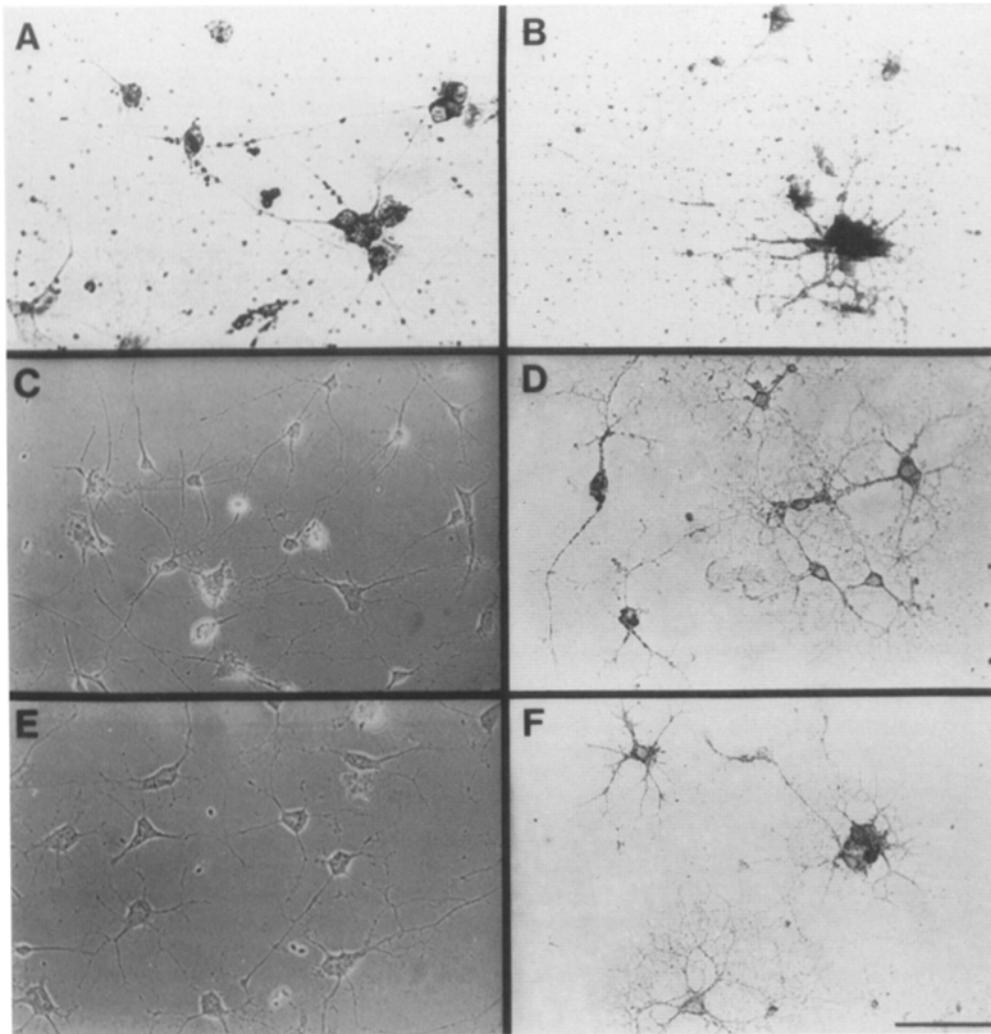The identity of the PCR products amplified by TGF-β2 and TGF-β3 primer

Figure 1. Immunodetection of TGF-β in primary O-2A cell cultures. (*Left panels*) O-2A progenitor cells cultured in the presence of bFGF and PDGF. (*Right panels*) oligodendrocyte cultures. Cells were immunostained with (*A* and *B*) turkey anti-TGF-β1; (*C* and *D*) rabbit anti-TGF-β2; and (*E* and *F*) rabbit anti-TGF-β3 antisera. *C* and *E* are phase-contrast photomicrographs in order to show the presence of unstained cells; all other panels are bright-field photographs. Bar, 20 μM.

pairs were confirmed by direct DNA sequencing analysis. PCR products were purified using Gene Clean (BIO 101, San Diego, CA) and used as templates for asymmetric PCR amplification (McCabe, 1990). The asymmetric products were dialyzed (Centricon filters; W. R. Grace & Co.-Conn., Beverley, MA), ethanol precipitated, and then sequenced using the Sequinase II protocol (Un. States Biochem. Corp., Cleveland, OH). Sequencing products were resolved by electrophoresis on 6% polyacrylamide-urea gels which were fixed in 10% acid/methanol before drying, and then exposed to Kodak XAR-5 film. The DNA sequences shown represent partial coding regions of TGF-β2 and TGF-β3, and were done twice on PCR fragments generated in separate reactions from cDNA of adult rat brain.

Detection of TGF-β Activity from Conditioned Culture Medium

Growth inhibitory activity in conditioned media (CM) were determined using CCL-64 mink lung cells (Danielpour et al., 1989) or primary O-2A progenitor cells. CCL-64 proliferation assays were carried out in 24 well plates (Costar Corp., Cambridge, MA) containing 10^5 cells/well, in medium containing 0.5% FBS. Cells were incubated either in the presence of TGF-β1 or CM for 26 h, with 0.5 μCi [^{3}H]thymidine (Amersham, specific activity 48 Ci/mmol) present for the final 4 h, and then incorporated radioactivity was determined by liquid scintillation counting as described (Danielpour et al., 1989). TGF-β standard curves were performed in the same medium using recombinant human TGF-β1 and TGF-β2 (R&D Sys., Inc.). All points were done in triplicate, and results are presented as mean ± SEM. 100% incorporation represents thymidine incorporation in the absence of inhibitors. O-2A proliferation assays were carried out in either 24 well (10^5 cells/well) or 96 well (10^4 cells/well) plates. Cells were incubated

in control culture medium (described above) plus indicated growth factors for 24 h, with [^{3}H]thymidine present for the final 7 h. 100% incorporation represents the thymidine incorporation in growth factor-treated cultures in absence of inhibitors.

CM were collected from primary O-2A cells plated in 100-mm dishes as described above. After 18 h in DMEM plus 10% FBS, the cells were refed with control culture medium in absence of growth factors, and CM were collected every 24 h. The first 24-h CM represented O-2A progenitor cell CM (O2A-CM), after which the cells acquired the complex morphology of differentiated oligodendrocytes (OG-CM). The CM were filtered (.45 μ), BSA (1 μg/ml) and protease inhibitors (1 μg/ml each of leupeptin, pepstatin, and aprotinin) were added (Sigma Chemical Co., St. Louis, MO) and the media were stored at -20°C. To activate latent forms of TGF-β, the CM were transiently acidified (10 min at pH 2.0), and then neutralized immediately before use as described (Danielpour et al., 1989). Transient acidification of control medium had no effect on assays with recombinant TGF-β1 standards.

Antibody Neutralization

Four anti-TGF-β neutralizing antibodies have been used in this study, including a rabbit polyclonal anti-TGF-β(1,2,3) neutralizing antibody (R&D Sys., Inc.), a mouse monoclonal anti-TGF-β(1,2,3) antibody (Genzyme, Inc.), and turkey antisera (Danielpour et al., 1989) raised against and specific for TGF-β1 and TGF-β2 (generously provided by Dr. Anita Roberts, National Cancer Institute). Neutralizations were carried out by preincubation of either recombinant TGF-β or CM with antibodies for 30 min at room temperature.
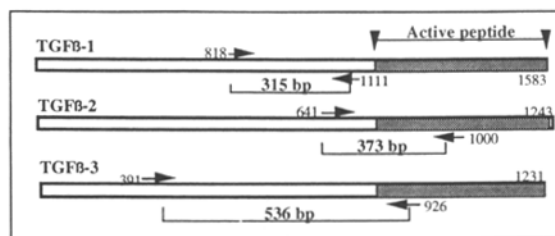
Results

Expression of TGF-β Isoforms during Oligodendrocyte Differentiation

The TGF-βs have potent inhibitory effects on the proliferation of a variety of cell types (Massagué, 1990; Sporn et al., 1987; Roberts and Sporn, 1990). Three isoforms of TGF-β have been characterized in mammalian cells, TGF-β1, TGF-β2, and TGF-β3. We used isoform-specific TGF-β antisera (Flanders et al., 1991) to determine whether the inhibitory activities produced by O-2A lineage cells (Levi et al., 1991; Louis et al., 1992b) could be related to one of these forms of TGF-β. As shown in Fig. 1, both O-2A progenitor cells and oligodendrocytes had detectable immunoreactivity with anti-TGF-β1 antisera (Fig. 1, A and B). Similar results were obtained with rabbit antisera recognizing all three isoforms of TGF-β (data not shown). The immunostaining was primarily cytoplasmic and perinuclear (Flanders et al., 1989). O-2A progenitor cells cultured in the presence of bFGF plus PDGF had no detectable immunoreactivity with TGF-β2 or TGF-β3 antisera (Fig. 1, C and E). Similar results were obtained with freshly isolated O-2A progenitor cells which had not been exposed to these growth factors (data not shown). In contrast, cultured oligodendrocytes were immunopositive with both TGF-β2 (Fig. 1 D) and TGF-β3 (Fig. 1 F) antisera.

We also examined the expression of TGF-β transcripts in O-2A cultures, using isoform-specific oligodeoxynucleotide primers (Fig. 2 a) to detect TGF-β transcripts by PCR amplification. RNA was isolated from cells growing under different culture conditions, reverse transcribed into cDNA using oligo(dT) as a primer, and then amplified by PCR using specific TGF-β primer pairs. The predicted PCR products of 315, 373, and 536 bp for TGF-β1, TGF-β2, and TGF-β3, respectively, were detected after PCR amplification of rat brain cDNA (Fig. 3, lane 1). Digestion of the rat TGF-β1 PCR product with PstI endonuclease generated restriction fragments of 250 and 65 bp (data not shown), as predicted from the DNA sequence (Qian et al., 1990). The identity of the rat TGF-β2 and TGF-β3 PCR products, generated using primers corresponding to conserved regions of mouse and human TGF-β sequences, were confirmed by direct DNA sequence analysis (Fig. 2 b). For TGF-β2, the sequenced region was 93 and 91% homologous to mouse and human TGF-β2, respectively. For TGF-β3, the rat sequence was 95 and 88% homologous to mouse and human TGF-β3, respectively. For TGF-β2, there were five amino acid substitutions and for TGF-β3 there was one change relative to the human sequence (Fig. 2 b).

These TGF-β isoform-specific primer pairs were used to examine the expression of TGF-β transcripts in cultured O-2A lineage cells, as shown in Fig. 3. O-2A progenitor cells maintained in the presence of bFGF expressed only TGF-β1 transcripts (Fig. 3, lane 3), while oligodendrocyte cultures expressed transcripts encoding all three TGF-β isoforms (Fig. 3, lane 4), in agreement with the immunohistochemical analysis shown in Fig. 1. The additional PCR products seen with the TGF-β2 primer pairs in oligodendrocyte cultures, which were not seen in rat brain cDNA using the same primer pairs, have not been characterized. For comparison, we also examined the expression of TGF-β transcripts in cultured type-1 astrocytes, a macroglial cell type that is distinct from the O-2A lineage. As predicted by immunohistochemi-

cal analysis (Saad et al., 1991), these cells expressed transcripts encoding TGF-β2 (Fig. 3, lane 2). We did not detect TGF-β1 transcripts in type-1 astrocytes (da Cunha and Vitković, 1992) cultured under these conditions.

O-2A Lineage Cells Release TGF-β into Their Culture Medium

We used the sensitive CCL-64 proliferation inhibition assay (Danielpour et al., 1989) to determine whether the TGF-βs made by O-2A lineage cells were secreted into their culture medium. As shown in Fig. 4, conditioned medium from both primary O2A progenitor cells and from oligodendrocytes
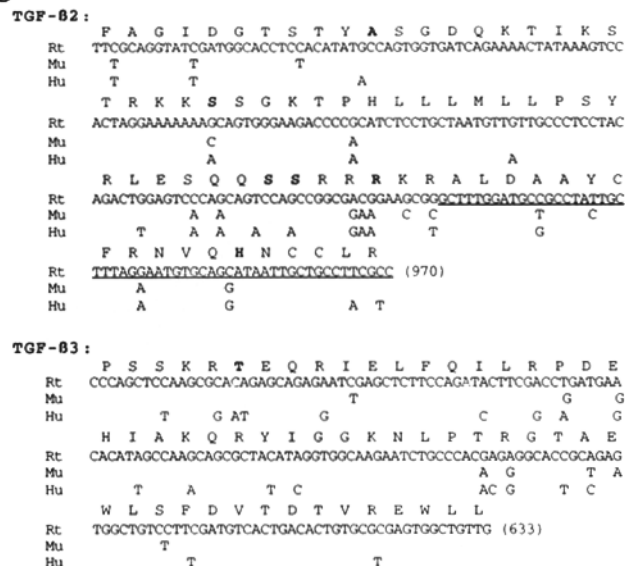

Figure 2. (a) Isoform-specific primer pairs for reverse transcriptase-PCR (RT-PCR) analysis of TGF-β transcripts in O-2A lineage cells. Numbers refer to the 5' nucleotide positions for rat TGF-β1 and mouse TGF-β2 and TGF-β3 primers, and the predicted size of each PCR product in bp. Arrows indicate the position of each primer relative to the coding regions for precursor (*boxed*) and active peptide (*shaded box*) TGF-β isoforms. (b) Sequence of rat TGF-β2 and -β3 PCR products generated from rat brain by RT-PCR. Numbers in parentheses refer to corresponding positions of the published sequence of mouse TGF-β2 and TGF-β3, and the start of the mature TGF-β2 is underlined. The translated amino acid sequence is shown above, with positions differing from human shown in bold. The nucleotide sequence of murine and human TGF-β2 and β3 (variant positions only) are shown below the rat sequence. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers X71904 (rat TGF-β2) and X71903 (rat TGF-β3).
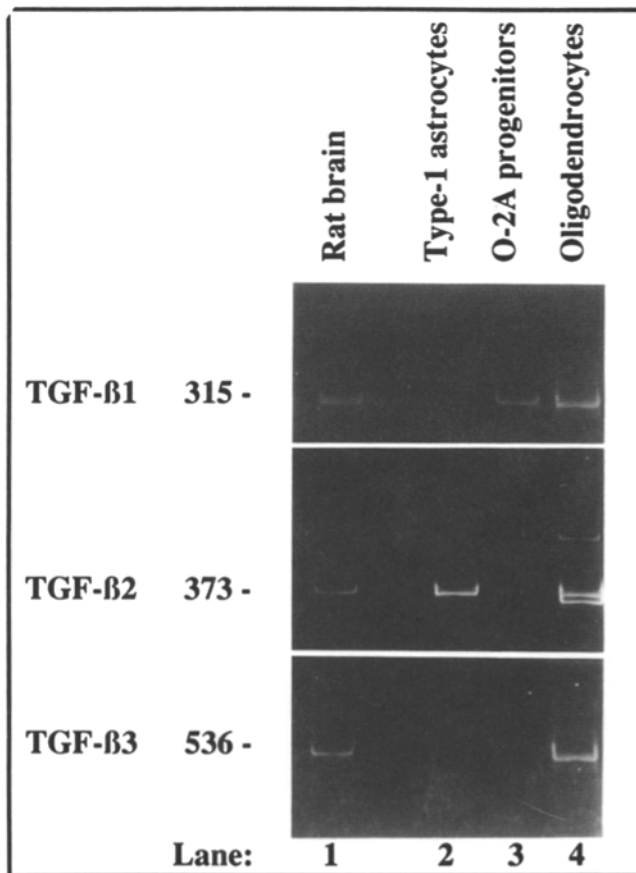
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Figure 3. Expression of TGF-β transcripts in O-2A lineage cells. RT-PCR analysis of RNA (1 μg each) from adult rat brain (lane 1), cultured type-1 astrocytes (lane 2), O-2A progenitor cells (lane 3), and oligodendrocytes (lane 4). RNA was reverse transcribed into cDNA, amplified using TGF-β isoform-specific primer pairs, and then the DNA products were resolved by electrophoresis on 4-20% acrylamide gels and visualized after ethidium bromide staining. Shown are the regions of the gels with detectable PCR products. DNA sizes are in bp.

contained an activity which produced a dose-dependent inhibition of CCL-64 mink lung epithelial cell proliferation. The inhibitory activity of 50%-CM was equivalent to that of 400 pg/ml recombinant TGF-β1. The majority of the inhibitory activity present in both O2A-CM and OG-CM was neutralized by preincubation with rabbit anti-TGFβ serum (Table I). This serum, raised against recombinant TGF-β and capable of neutralizing all three isoforms of TGF-β, neutralized 90% of the activity of 500 pg/ml recombinant human TGF-β1 when used at a concentration of 20 μg/ml (Table I). With four independent preparations of OG-CM, this serum neutralized from 51 to 85% of the CCL-64 inhibitory activity present. Comparable results were obtained with a monoclonal anti-TGF-β(1,2,3) neutralizing antibody (data not shown). Thus, the major CCL-64 inhibitory activity present in these glial CM represent a form of TGF-β recognized by these specific antibodies.

We also used isotype-specific neutralizing antisera (Danielpour et al., 1989) to examine whether different isoforms of TGF-β were secreted into the O-2A culture medium. The specificity of these antisera was determined using recom-
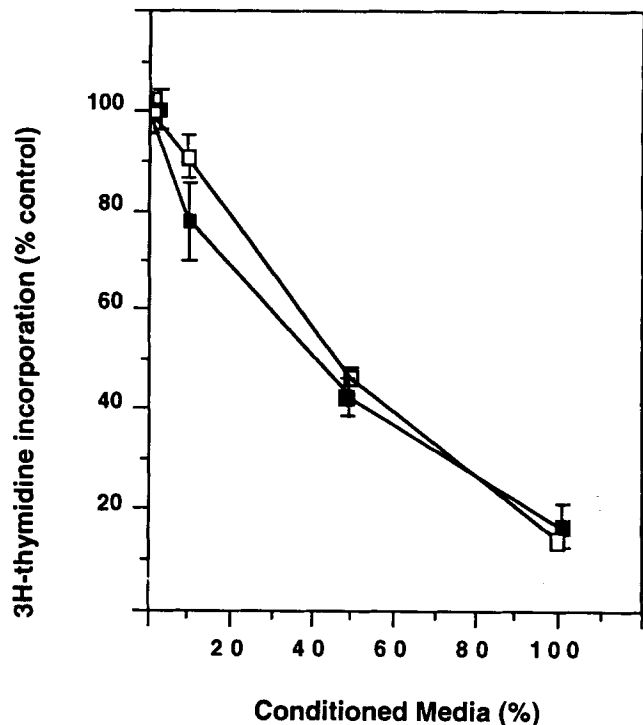

Figure 4. Inhibition of CCL64 proliferation with glial cell conditioned medium. Incorporation of [³H]thymidine into CCL64 mink lung epithelial cells, cultured for 24 h in the presence of conditioned medium collected from cultures of O-2A progenitor cells (O2A-CM, open symbols) and oligodendrocytes (OG-CM, closed symbols). The CM was transiently acidified in order to activate any latent TGF-β. Values represent the mean ± SEM of triplicate samples, and are representative of four independent assays. 100% incorporation (DMEM+0.5% FBS, in absence of CM) represents 62,328 ± 2,883 cpm.

binant TGF-β1 and TGF-β2. Polyclonal turkey anti-TGF-β1 antisera neutralized recombinant TGF-β1 with no cross-reactivity with TGF-β2, anti-TGF-β2 antisera had the opposite reactivity, and monoclonal anti-TGF-β(1,2,3) antibody neutralized all isoforms of TGF-β (Fig. 5). Using these reagents, CM from O-2A progenitor cell cultures were partially neutralized with both anti-TGF-β1 and with anti-TGF-β2 sera (Fig. 5). The activity present in oligodendrocyte CM was partially neutralized with anti-TGF-β2 but not anti-TGF-β1 antisera (Fig. 5). Anti-TGF-β(1,2,3) antibody gave the best neutralization of OG-CM (Fig. 5), suggesting that both TGF-β2 and TGF-β3 isoforms are released into oligodendrocyte-conditioned medium.

O-2A Lineage Cells also Secrete an Inhibitory Activity That Is Not Neutralized by TGF-β Antibodies

The TGF-β's are secreted from cells in a latent form, requiring proteolytic processing in order to be biologically active (Roberts and Sporn, 1990; Massagué, 1990). To determine if inhibitory activities other than latent forms of TGF-β were produced by O-2A lineage cells, we examined CM both with and without prior transient acidification (Fig. 6). O-2A progenitor cells were cultured without mitogens, allowing the cells to differentiate into oligodendrocytes, and conditioned medium was collected every 24 h for 6 d. Acidified CM collected from day 1 cultures (O2A-CM) gave 86% inhibition

Table I. Neutralization of CCL-64 Growth Inhibitory Activity with TGF-β Antibody

	Thymidine incorporation, cpm (% control)	
	−Antibody	+Antibody
TGF-β1 pg/ml		
0	22,533 ± 8,877 (100%)	−
100	11,822 ± 2,212 (52%)	22,993 ± 2,584 (102%)
250	6,445 ± 29 (28.6%)	24,067 ± 2,180 (107%)
500	1,666 ± 671 (7.4%)	9,068 ± 185 (87%)
Conditioned medium		
Expt. 1 O2A-CM	6,015 ± 314 (27%)	16,811 ± 1,977 (75%)
OG-CM	5,279 ± 226 (23%)	16,589 ± 1,211 (74%)
Expt. 2 O2A-CM	4,219 ± 442 (23%)	8,099 ± 789 (44%)
OG-CM	718 ± 29 (4%)	11,199 ± 132 (61%)
Expt. 3 OG-CM	645 ± 123 (6.7%)	8,452 ± 417 (88%)
Expt. 4 OG-CM	3,097 ± 1,309 (10%)	22,477 ± 1,548 (73%)

Inhibition of CCL-64 proliferation by recombinant TGF-β1 and by conditioned medium from O-2A progenitor cells and oligodendrocytes. The CM (100% vol) was transiently acidified to activate latent TGF-β activity, and then preincubated in the presence or absence of 20 μg/ml rabbit anti-TGF-β(1,2,3) neutralizing antisera. Results are expressed as the mean ± SD of [³H]thymidine incorporation (cpm) in triplicate samples, and values in parentheses represent percent control thymidine incorporation in CCL-64 cells in the absence of CM.

of CCL-64 cell proliferation, equivalent of 1,000 pg/ml TGF-β activity. There was a relatively constant level of inhibitory activity in acidified CM from day 2 through 6 (OG-CM) in these cultures (Fig. 6, *closed symbols*). In day 1 cultures, ~50% of this activity was detectable without prior acidification of the CM (Fig. 6, *open symbols*). The levels of inhibitory activity in nonacidified CM decreased after day 1 (Fig. 6, *open symbols*), coincident with oligodendrocyte differentiation.

To determine if the activity present in nonacidified conditioned medium from O-2A progenitor cell (day 1) cultures was a form of TGF-β, we attempted to neutralize this activity using anti-recombinant TGF-β antibodies. As shown in Table II, the activity present in nonacidified CM was not affected by preincubation with TGF-β antibodies. Incubation of acid-

treated CM with these antibodies reduced the level of total inhibitory activity to that of nonacidified CM (Table II). Thus, there are two inhibitory components present in O2A-CM. One (latent TGF-β) is seen upon acidification of the CM and is neutralized by anti-TGF-β antibodies. A second component active in nonacidified CM cannot be neutralized with these antibodies. This second activity could represent a modified form of TGF-β, distinct from the recombinant
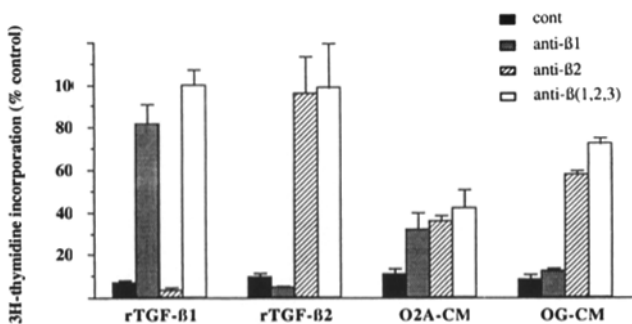

Figure 5. Neutralization of CCL-64 growth-inhibitory activities in conditioned medium from O-2A progenitor cells (*O2A-CM*) and oligodendrocytes (*OG-CM*). The CM (100% vol) was transiently acidified to activate latent TGF-β activity, and then preincubated in the presence or absence of turkey anti-TGF-β1 (*stippled bars*), turkey anti-TGF-β2 (*striped bars*), or mouse monoclonal anti-TGF-β(1,2,3) neutralizing antibodies (*open bars*). (*rTGF-β1*, *rTGF-β2*) recombinant TGF-β1 and -β2. Results are the average (± SEM) from two independent experiments in triplicate, and are expressed as percent of control [³H]thymidine incorporation in CCL-64 cells in absence of CM. 100% incorporation represents 46,029 ± 270 cpm.
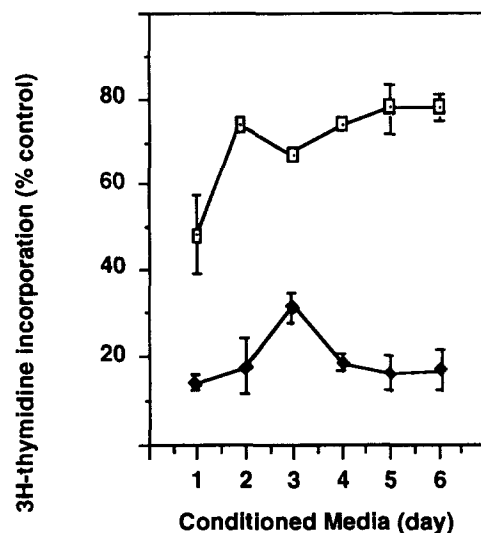

Figure 6. Levels of total and latent TGF-β inhibitory activity in O-2A culture medium. Inhibition of proliferation of CCL64 cells with conditioned medium, collected every 24 h over a 6-d period, from O-2A cultures. Conditioned medium (100% vol) was assayed both with (*closed symbols*) and without (*open symbols*) acidification in order to detect total inhibitory activity present and inhibitory activities other than latent TGF-β, respectively. Results represent the mean ± SEM of triplicate samples from one experiment, and were repeated in two separate assays. O-2A progenitor cells were undifferentiated at day 1, and began to acquire the phenotype of differentiated oligodendrocytes by day 2–3 in these cultures. 100% incorporation represents 62,328 ± 2,883 cpm.

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Table II. A Component of O-2A Conditioned Medium Cannot be Neutralized by TGF-β Antibody

	Thymidine incorporation (% control)	
	− Antibody	+ Antibody
Untreated CM	74.4 ± 4.9	72.9 ± .52
Acid-treated CM	30.4 ± 5.0	68.1 ± 4.4

Inhibition of CCL-64 proliferation by O2A-CM (100% vol), preincubated in the presence and absence of monoclonal anti-TGF-β(1,2,3) neutralizing antibody. Acid-treated CM: CM was transiently acidified to activate latent TGF-β activity. Results are expressed as the mean ± SEM of [³H]thymidine incorporation in triplicate samples, expressed as percent of control incorporation (in DMEM+0.5% FBS) in absence of CM (52,404 ± 789 cpm).

TGF-βs used to raise these neutralizing antibodies, or could represent an inhibitory molecule that is distinct from TGF-β.

TGF-β1 Inhibits O-2A Progenitor Cell Proliferation and Promotes Oligodendrocyte Differentiation

O-2A progenitor cells have a limited proliferative capacity when cultured in the presence of PDGF (Raff et al., 1988). We examined whether the production and accumulation of TGF-β in their culture media could account for this limited proliferation. O-2A progenitor cells were cultured with increasing concentrations of either recombinant TGF-β1 (Fig. 7 a) or oligodendrocyte-conditioned medium (Fig. 7 b), and the incorporation of thymidine was determined after 24 h. The level of DNA synthesis in O-2A progenitor cells cultured in the presence of PDGF was inhibited in a dose-dependent manner by addition of increasing concentrations of recombinant TGF-β1, with half-maximal inhibition observed upon addition of 1 ng/ml (Fig. 7 a). DNA synthesis was also inhibited by addition of both nonacidified and acidified OG-CM (Fig. 7 b). The level of inhibition observed with acidified CM (2−, Fig. 7 b) was approximately twice that seen with nonacidified CM (1−, Fig. 7 b). The inhibitory activity of nonacidified CM was not affected by preincubation with TGF-β antibodies (2+, Fig. 7 b), while the inhibitory activity of acidified CM was decreased to a level equivalent to nonacidified CM by preincubation with these antibodies (2+, Fig. 7 b).

TGF-β also affected the rate at which O-2A progenitor cells differentiated into oligodendrocytes in the presence of PDGF, as measured by emergence of the oligodendrocyte-specific differentiation marker GC. After 48 h in the presence of PDGF, 38% of O-2A cells which were labeled with the O4 antibody had the characteristic branched morphology of differentiated oligodendrocytes and were labeled with the monoclonal antibody RmAb (Fig. 8, A and B). After 48 h in PDGF plus TGF-β1, in contrast, 62% of O4⁺ cells were also RmAb⁺ (Fig. 8, C and D). Similar results were obtained using the anti-GC antibody O1 (data not shown). In the presence of PDGF, O-2A progenitor cells (O4⁺GC⁻ cells) had a predominantly bipolar morphology with long processes (Fig. 8 A, arrows), characteristic of PDGF-induced migratory cells (McKinnon et al., 1993). In the presence of PDGF and TGF-β1, in contrast, the O4⁺GC⁻ cells had a multibranched appearance of nonmigratory cells (Fig. 8 C, arrows), suggesting that TGF-β also prevented the PDGF-induced motility of these cells.

We examined whether the TGF-βs produced by O-2A cells inhibited the PDGF-driven proliferation of these cells. O-2A progenitor cells were cultured in media containing PDGF, in either the presence or absence of TGF-β neutralizing antibodies, and thymidine incorporation was measured after 72 h (Table III). In the absence of TGF-β antibodies, thymidine incorporation in PDGF-containing cultures was reduced relative to bFGF-treated cultures, indicating that the cells were exiting the cell cycle and beginning to differentiate (Table III). Addition of TGF-β antibodies to PDGF-treated cultures increased the level of DNA synthesis to levels seen with bFGF (Table III). These antibodies also increased the level of DNA synthesis in O-2A cultures in the absence of mitogens, but had no significant effect on cultures incubated in the presence of bFGF (Table III). These results suggest that the latent forms of TGF-β secreted by O-2A cells (Figs. 6 and 7 b) are biologically activated in these cultures, as determined in a 72 h differentiation assay (Table III). The mechanism by which these molecules are activated in this
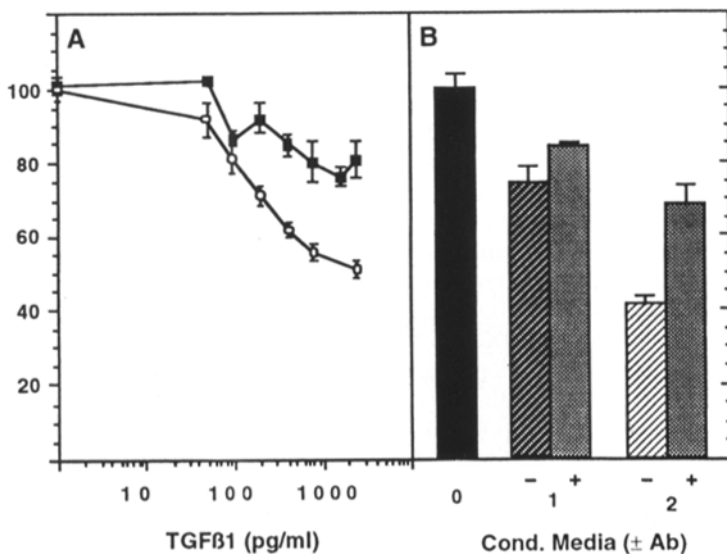

Figure 7. Inhibition of PDGF-driven proliferation of primary rat O-2A glial progenitor cells by recombinant TGF-β (A) and by oligodendrocyte-conditioned medium (B). (A) Incorporation of [³H]thymidine into O-2A progenitor cells cultured for 24 h in the presence of 5 ng/ml PDGF-AA (*open symbols*) or 5 ng/ml bFGF (*closed symbols*), with increasing concentrations of recombinant TGF-β1. Values represent the mean ± SEM of triplicate samples from three independent assays. 100% incorporation for PDGF represents 11,632 ± 571 cpm, and for bFGF represents 12,100 ± 247 cpm. (B) Thymidine incorporation in O-2A cells cultured in the presence of nonacidified (1) or acidified (2) oligodendrocyte-conditioned medium (75% vol), with (+) or without (−) preincubation with rabbit anti-TGF-β neutralizing antisera. Values expressed as % control represent the mean ± SEM of triplicate samples, representative of two assays, and 100% incorporation in control cultures (0) represents 3,903 ± 244 cpm.
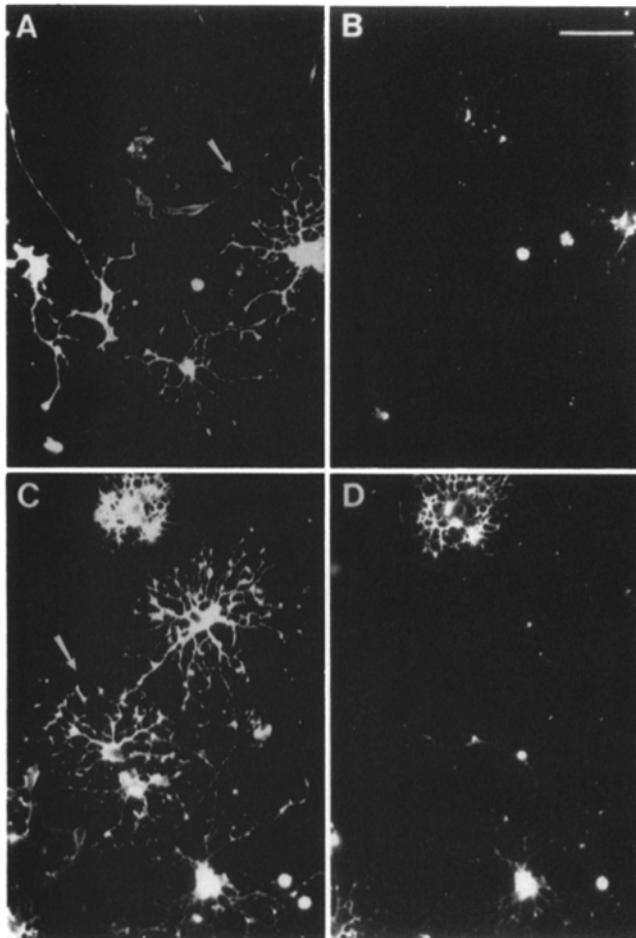
Figure 8. Immunocytochemical analysis of O-2A cells under different culture conditions. Cultures were stained with monoclonal antibodies recognizing antigens present on O-2A progenitor cells (O4 antibody, *left panels*) and differentiated oligodendrocytes (RmAb, *right panels*). Cells were cultured for 48 h in control medium containing 5 ng/ml PDGF-AA (*A* and *B*) or PDGF plus 2 ng/ml TGF-β1 (*C* and *D*). In the presence of PDGF, O-2A progenitor (RmAb⁻) cells had a predominantly bipolar morphology (*A, arrow*), while in the presence of TGF-β1 plus PDGF, these cells had a highly branched morphology (*C, arrow*). Bar, 20 μM.

system remains to be elucidated. Thus, TGF-β produced by O-2A lineage cells may limit their proliferative response to PDGF.

Our results also suggest that bFGF may counteract the inhibitory effects of TGF-β on cell proliferation. Recombinant TGF-β1 was less potent in inhibiting the proliferation of O-2A progenitor cells cultured in the presence of bFGF (Fig. 7 *a*). At 2 ng/ml TGF-β1, bFGF-driven mitosis was inhibited by 20% relative to control values, while PDGF-driven mitosis was inhibited by 55% (Fig. 7 *a*). bFGF also decreased the growth inhibitory effect of TGF-β1 on CCL-64 cells; in the presence of PDGF-AA (or PDGF-BB), TGF-β1 gave 50% inhibition of proliferation at 250 pg/ml, while in the presence of bFGF comparable inhibition required a fourfold higher concentration (1 ng/ml) of TGF-β1. Thus, bFGF attenuated the growth inhibitory effects of TGF-β1, both in CCL-64 epithelial cells and in primary O-2A glial progenitor cells.

Table III. Proliferation of O-2A Cells in the Presence of TGF-β Neutralizing Antibodies

	Thymidine incorporation (% control)	
	− Antibody	+ Antibody
No mitogen	50.7 ± 16.0	90.0 ± 3.6
bFGF	100.0 ± 13.0	113.4 ± 14.6
PDGF-AA	78.1 ± 11.3	107.1 ± 10.4
bFGF+PDGF	111.7 ± 12.9	106.8 ± 15.4

Proliferation of O-2A progenitor cells in the presence and absence of TGF-β neutralizing antisera. Results are expressed as the mean ± SEM of [³H]thymidine incorporation in triplicate samples, representative of three independent assays, and are expressed as percent of control incorporation (in media containing bFGF) in absence of CM (4,640 ± 604 cpm).

Discussion

We have demonstrated that O-2A lineage cells express transcripts encoding specific isoforms of TGF-β, are immunoreactive with anti-TGF-β antibodies, and secrete into their culture medium a growth inhibitory activity which is neutralizable with anti-TGF-β antisera. O-2A progenitor cells produce TGF-β1, and as these cells differentiate into oligodendrocytes, we observed a relative increase in latent forms of TGF-β, the emergence of TGF-β2 and TGF-β3 transcripts and immunoreactive protein, and a decrease in the levels of a second uncharacterized inhibitory activity. Further, both recombinant TGF-β and TGF-β produced by O-2A cultures inhibits the proliferation of O-2A progenitor cells and promotes oligodendrocyte differentiation. This inhibition of proliferation is 2.5-fold greater when O-2A progenitors are cultured in the presence of PDGF than in the presence of bFGF. Our results suggest that TGF-β produced by O-2A lineage cells could have autocrine (self) or paracrine (neighboring cells) inhibitory effects to limit the number of mitosis occurring in O-2A progenitor cells cultured in the presence of PDGF.

Our data indicate that the majority of the growth-inhibitory activity in O-2A progenitor cell conditioned medium represents a latent form of TGF-β. The lowest level of neutralization we obtained with anti-TGF-β antisera eliminated ∼50% of the CCL-64 inhibitory activity in O2A-CM (Table I). The remaining activity could represent a form of TGF-β not recognized by antibodies raised against recombinant TGF-β, or a distinct (non-TGF-β) inhibitory molecule. This second inhibitory component did not require acidification in order to be active in the CCL-64 growth inhibition assay, and its levels were substantial in O-2A progenitor cell conditioned medium and were reduced as these cells differentiated into oligodendrocytes (Fig. 6). This second activity has not been further characterized.

Oligodendrocyte cultures secreted predominantly latent forms of TGF-β, which required transient acidification in order to be detected in the CCL-64 assay. Since the TGF-βs produced during oligodendrocyte differentiation could be neutralized with TGF-β antibodies (Table III), O-2A lineage cells may be capable of activating the latent forms of TGF-β produced in this culture system. The process whereby latent TGF-β is activated is not well understood, although there is evidence that plasminogen activator may be involved (Flaumenhaft et al., 1992). The role of other neuronal and glial

cell types in the activation of latent TGF-β produced by O-2A cells remains to be determined.

Levi et al. (1991) have reported that O-2A conditioned medium contains a high molecular mass (>30 kDa) factor capable of inducing oligodendrocyte differentiation. Louis et al. (1992b) have also described a growth inhibitory activity produced by CG-4 cells, a rat CNS cell line with the phenotypic properties of O-2A progenitor cells (Louis et al., 1992a). Similarly, the myelin-forming Schwann cells of the peripheral nervous system secrete a growth-inhibitory activity which may be a proteolytic fragment of fibronectin (Muir et al., 1990; Muir and Manthorpe, 1992). Thus, TGF-β may be only one of several molecules acting in an autocrine manner to regulate the growth of myelin-forming cells.

While both bFGF and PDGF promote O-2A proliferation, only bFGF can sustain this proliferation and thus block differentiation (McKinnon et al., 1990). In the presence of PDGF, these cells undergo only a limited number of mitosis before differentiation, referred to as a PDGF-driven molecular "clock" (Raff et al., 1988). It has been proposed that this represents the dilution of some critical intracellular molecule in the PDGF signal transduction pathway (Temple and Raff, 1986; Raff et al., 1988; Raff, 1989). The ability of TGF-β1 to inhibit the PDGF-driven mitosis of O-2A progenitor cells (Fig. 7) suggests that cell-extrinsic factors may also limit the proliferative response of these cells to PDGF. TGF-β also appeared to inhibit the effects of PDGF on cell motility, as determined by the absence of bipolar motile cells in the presence of both PDGF and TGF-β1 (Fig. 8 c). TGF-β can negatively regulate the expression of PDGF alpha-receptors in other cell types (Gronwald et al., 1989; Battegay et al., 1990). Thus, O-2A progenitor cells may also downregulate PDGF alpha-receptor expression in response to TGF-β.

If the limited proliferation of O-2A progenitor cells in the presence of PDGF is due to either autocrine or paracrine effects of TGF-β, then their extended proliferation in the presence of bFGF may interfere with either the production of TGF-β or the ability of TGF-β to inhibit their proliferation. Our results suggest that bFGF did not affect the expression or release of TGF-β1 by O-2A progenitor cells. However, the inhibitory effects of TGF-β were attenuated in the presence of bFGF, for both O-2A progenitor cells (Fig. 7 a) and for CCL-64 mink lung epithelial cells. Thus, one mechanism by which bFGF blocks O-2A progenitor cell differentiation (McKinnon et al., 1990) may be to interfere with a TGF-β–mediated pathway of oligodendrocyte differentiation.

Antagonistic effects for bFGF and TGF-β have been described in several systems, and may be a general feature of vertebrate development. For example, TGF-β inhibits the bFGF-induced commitment of neural-crest cells to melanogenesis (Stocker et al., 1991). TGF-β also inhibits bFGF-induced endothelial cell proliferation, migration, and production of plasminogen activator (Baird and Durkin, 1986; Fräter-Schröder et al., 1986; Heimark et al., 1986; Müller et al., 1987; Saksela et al., 1987) through a complex loop in which plasminogen activator leads to the activation of latent TGF-β, which in turn inhibits plasminogen activator (Flaumenhaft et al., 1992). FGF and TGF-β may also have synergistic effects in some developmental systems, such as in mesoderm induction (Kimelman and Kirschner, 1987).

Another mechanism by which bFGF and TGF-β have an-

tagonistic effects could be through receptor modulation. Both ligands bind with high affinity to specific transmembrane receptors with intrinsic tyrosine (FGF) or serine-threonine (TGF-β) kinase activity (Aaronson, 1991; Lin et al., 1992; Ullrich and Schlessinger, 1990), as well as to lower affinity receptors (Massagué, 1992; Yayon et al., 1991). The type III TGF-β receptor betaglycan (López-Casillas et al., 1991; Massagué, 1992; Wang et al., 1991; Yayon et al., 1991) can also bind bFGF (López-Casillas et al., 1991), and bFGF downregulates one TGF-β receptor subtype on endothelial cells (Fafeur et al., 1990). Thus, the antagonistic effects of bFGF and TGF-β on O-2A progenitor cells could also result from receptor downregulation.

Our in vitro studies suggest a role for TGF-β in the development of the myelin-forming cells in the CNS. The finding that TGF-β2 and TGF-β3 are present in the developing CNS (Flanders et al., 1991; Unsicker et al., 1991) provides support for this postulate. Whether any of these factors influence oligodendrocyte development in vivo remains to be elucidated. If TGF-β is produced by oligodendrocytes and/or their precursors in vivo, this would support the concept of an internally timed cell autonomous regulatory mechanism that limits the proliferation and controls the differentiation of these cells in vivo. However, the observations that TGF-β is present in neurons (Flanders et al., 1991) would suggest that the interaction of oligodendrocyte precursor cells with their axonal targets could also be a key regulatory step in the cascade of events initiating oligodendrocyte differentiation.

Our studies further indicate that a hierarchy of polypeptide growth factors influence the proliferation and rate of differentiation of O-2A glial progenitor cells in culture. TGF-β suppresses the mitogenic stimulus of PDGF, limiting the extent of PDGF-driven proliferation and allowing differentiation. In contrast, bFGF limits the inhibitory effects of TGF-β and allows sustained proliferation, preventing the cells from differentiating. Inherent in these observations is the principle that the context of growth factor presentation (Nathan and Sporn, 1991; Roberts and Sporn, 1990) greatly influences their biological effects on the cells under study.

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