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A developmental analysis of oligodendroglial integrins in primary cells: changes in αv-associated β subunits during differentiation

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SUMMARY

We have examined the expression of integrins on primary oligodendroglial cells during the differentiation of the proliferative oligodendrocyte precursor (O-2A progenitor) cell to the postmitotic oligodendrocyte. Cells of the oligodendrocyte lineage expressed a limited repertoire of integrins: α6β1 and αv integrins including αvβ1, αvβ3 and αvβ5, as well as a potentially novel integrin αvβ80kDa. Integrin expression was developmentally regulated; during differentiation αvβ1 was reduced and αvβ5 upregulated. These results suggest that laminin and vitronectin are important extracellular matrix ligands for oligodendrocytes, and provide a rational explanation for previous observations that RGD peptides inhibit the expression of myelin-specific genes. They also suggest a simple model by which switching of integrin β subunits might regulate differentiation. As chimeric β1 integrins with a β5 cytoplasmic domain support proliferation less well than normal β1 integrins (Pasqualini and Hemler (1994), *J. Cell Biol.* 125, 447-460) the switch from αvβ1 to αvβ5 might play a key instructive role in the cessation of proliferation and subsequent differentiation.

Key words: oligodendrocyte, oligodendrocyte precursor, O-2A progenitor cell, integrin, proliferation, differentiation, α6β1, αvβ1, αvβ5

INTRODUCTION

A prominent feature of development in the vertebrate central nervous system (CNS) is myelination, a process that permits the rapid and efficient transmission of electrical impulses along the axon (Huxley and Stampfli, 1949). Myelination is carried out by oligodendrocytes, which are dispersed widely throughout the adult CNS. In contrast, oligodendrocyte precursors (0-2A progenitor cells) arise from restricted areas within the CNS (Warf et al., 1991; Pringle and Richardson, 1993) and, just as in other developmental systems such as the neural crest (Le Douarin, 1982; Marusich and Weston, 1991), it is the extensive proliferation and migration of cells during development that results in the widespread distribution of the differentiated cell type. A number of studies have examined the role of growth factors in the control of oligodendrocyte precursor cell proliferation, migration, survival and differentiation (for reviews see Kiernan and ffrench-Constant, 1993; Barres and Raff, 1994). An important question so far not addressed in such detail, however, is the role of environmental cues from the surrounding extracellular matrix (ECM), which have been shown to control cell behaviour in a wide range of other developmental systems (Adams and Watt, 1993).

One important family of molecules responsible for transducing signals from the ECM to the inside of the cell are the integrins (Hynes and Lander, 1992; Hemler, 1990; Ruoslahti, 1991; Hynes, 1992; Diamond and Springer, 1994). Integrins have been shown to play a role in the regulation of neural cell behaviour during CNS development. Transfection of neuroblasts with a cDNA construct encoding an antisense RNA complementary to the β1 subunit mRNA inhibits the migration of neural cells during development of the tectum in chick (Galileo et al., 1992). Later in development, the loss in response to laminin shown by retinal ganglion cell axons as they reach their tectal targets (Cohen et al., 1986) reflects, at least in part, downregulation of both the level and activity of the laminin integrin receptor α6β1 (de Curtis et al., 1991; Neugebauer and Reichardt, 1991). Integrins have also been implicated specifically in oligodendrocyte differentiation. The ability of RGD peptides to inhibit adhesion and differentiation of oligodendrocytes on astrocyte-derived ECM (Cardwell and Rome, 1988a,b; Malek-Hedayat and Rome, 1994) suggests that integrins play a central role in controlling oligodendroglial behaviour, as many integrins recognize this peptide sequence in their ligands (Pytela et al., 1985a,b; Plow et al., 1985; Cheresh et al., 1989; Vogel et al., 1990; Busk et al., 1992; Koivunen et al., 1993). The mechanisms by which integrins exert these effects are, however, unknown.

The goal of this study was to characterize the different αβ integrin heterodimers present on the cell surface during oligodendroglial differentiation in order to examine of the role of integrin-ECM interactions in this lineage. In order to do this, we have adopted a biochemical approach. Such an approach allows the association of any particular integrin subunit to be determined directly by immunoprecipitation of complexes under non-denaturing conditions that do not break up the αβ

heterodimers. It avoids the disadvantages of immunocytochemical studies or those analysing integrin mRNA expression, neither of which can examine subunit association satisfactorily. It does, however, require large numbers of purified primary cells, which are impossible to obtain in most developmental systems. We have taken advantage of the fact that oligodendrocyte precursors can be purified and grown in sufficiently large numbers in cell culture. In the presence of appropriate mitogens, these cells will migrate (Small et al., 1987; Noble et al., 1988; Armstrong et al., 1990) and proliferate (reviewed in Barres and Raff, 1994) and then differentiate into postmitotic, non-migratory oligodendrocytes, with the timing of this differentiation reflecting that seen in vivo (Raff et al., 1985; Dubois-Dalcq et al., 1986).

We find oligodendroglia express a limited repertoire of integrins of the α6 and αv families. While α6β1 expression is maintained throughout development, we observe changes in the β subunits associated with αv such that αvβ1 is downregulated and αvβ5 upregulated during differentiation. As well as providing evidence of a role for β subunit switching during development, these results suggest a simple model by which integrin expression might play a role in the regulation of proliferation and differentiation in oligodendroglial cells.

MATERIALS AND METHODS

Cell culture

Purified oligodendrocyte precursors were obtained by the method of McCarthy and de Vellis (1980) with minor modifications. Briefly, cerebral cortices from postnatal day 0-2 Sprague-Dawley rats were dissected free of meninges, minced with fine scissors and incubated for 1 hour at 37°C in an enzymatic solution containing 30 U/ml papain (Worthington), 0.24 mg/ml L-cysteine (Sigma) and 40 μg/ml DNAase I type IV (Sigma) in 1 ml MEM-Hepes (as described in Barres et al., 1992). After incubation the supernatant was removed, 1 ml ovomucoid trypsin inhibitor solution added (containing 1 mg/ml trypsin inhibitor (Boehringer Mannheim), 50 μg/ml BSA (Sigma) and 40 μg/ml DNAase I type IV in L-15 media) for 3 minutes, supernatant removed again and a fresh 1 ml ovomucoid solution added. The tissue was then triturated seven times with a Pasteur pipette followed by four times with a 21-gauge needle before centrifugation in 10 ml DMEM supplemented with 10% FCS. Cells were cultured at a density of two brains per flask in DMEM supplemented with 10% FCS (GlobePharm) 4 mM L-glutamine (Sigma) and penicillin/streptomycin (Sigma) at 37°C and 7.5% CO_2. The flasks (Falcon) were precoated with 5 μg/ml poly-D-lysine (Sigma). Media was changed every 3 days. Mouse cells were grown under identical conditions except for the replacement of 10% horse serum in place of 10% FCS. After a period of 10 days the cells separate into two layers: a basal monolayer consisting primarily of astrocytes and some fibroblasts, and a top layer of oligodendrocyte precursors and microglia. The loosely attached microglia were removed during a preliminary 2 hour shake on an orbital shaker (New Brunswick, G24) at 250 revs/minute, after which the supernatant and loose cells were removed and a fresh 10 ml of media added. The flasks were then shaken for 20-24 hours at 250 revs/minute. During this period, many 'top cells' consisting of oligodendrocyte precursors and remaining microglia are shaken free of the monolayer. Microglial contaminants were then removed by plating the supernatant cell mixture onto non-tissue culture Petri dishes (Media) for 20 minutes, during which time the adhesive microglia stick down while the oligodendrocyte precursors remain unattached. The remaining cell suspension was then centrifuged and resuspended in Sato media (DMEM supplemented with bovine insulin (Sigma, 5 μg/ml), human transferrin (Sigma, 50 μg/ml), BSA V (Sigma, 100 μg/ml), progesterone (Sigma, 6.2 ng/ml), putrescine (Sigma, 16 μg/ml), sodium selenite (Sigma, 5 ng/ml), T3 (Sigma, 400 ng/ml), T4 (Sigma, 400 ng/ml), L-glutamine (Sigma, 4 mM), penicillin and streptomycin (Sigma) and 0.5% FCS) and plated onto polyornithine-coated six-well plates (Nunc) or 100 mm Petri dishes (Nunc). The purity of the resulting cell suspension was assessed by morphology as greater than 95% oligodendroglial cells (oligodendrocyes and precursors) at day 1. In this analysis, process-bearing oligodendroglia could be distinguished from fibroblast-like astrocytes and microglia. In secondary culture, precursor cells constitutively differentiate into oligodendrocytes. We confirmed this by immunostaining with a monoclonal antibody (Ranscht et al., 1982) against the oligodendrocyte-specific marker galactocerebroside (GalC) (Raff et al., 1978). We found that that the cells in these cultures were less than 10% GalC+ after 4 hours (i.e. more than 90% precursor cells) but greater than 80% GalC+ at day 7 and greater than 95% GalC+ by day 10. In our experiments to examine oligodendrocyte integrins, secondary cultures were maintained for 7 days before analysis, except in the case of the developmental time course experiments in which some cultures were grown for up to 26 days.

Astrocytes were obtained by removing all 'top cells' from a flask and using the basal layer of astrocytes left behind. Fibroblasts were obtained by performing a papain dissociation of meninges (as above) and then culturing the cells in DMEM supplemented with 10% FCS and 4 mM L-glutamine and penicillin and streptomycin. The STO mouse embryonic fibroblast cell line was maintained in the same media.

Antibodies

In immunoprecipitations and western blots, the antibodies were generous gifts from the following: (1) monoclonals; GoH3 (rat IgG1, anti-α6) and 1A10 (mouse IgG1, anti-α6A) from Dr Arnoud Sonnenberg, Amsterdam, Holland (Sonnerberg et al., 1987, Hogervorst et al., 1993); F11 (mouse IgG1, anti-β3) from Dr Michael Horton, ICRF St. Bartholomews, London (Helfrich et al., 1992); 346-11A (rat IgG1, anti-β4) from Dr S. Kennel, Oak Ridge, Tennessee (Kennel et al., 1989); P3G2 (mouse IgG1, anti-αvβ5) from Dr David Cheresh, Scripps Research Institute, San Diego (Wayner et al., 1991) and (2) polyclonals; anti-α1, anti-α2, anti-α3, anti-α4, anti-α5 and anti-αv from Dr Guido Tarone, Milan, Italy, anti-α6A (6845), anti-α6B (382) and anti-β6 (6830 and 6840) from Dr Vito Quaranta, Scripps Research Institute, San Diego (Cooper et al., 1991, Tamura et al., 1991, Sheppard et al., 1990), anti-α8 from Drs Robert Pytela and Lynn Schnapp, University of California, San Francisco; anti-β1 from Dr Richard Hynes, MIT, Boston (Marcantonio and Hynes, 1988); anti-β3 from Dr Kristine Venstrom, University of California, San Francisco. All polyclonal antibodies were raised against peptides derived from the cytoplasmic domains of the individual integrin subunits.

Cell surface labelling and immunoprecipitation

Cell surface molecules were labelled with biotin by removing growth media, washing the cell layer twice with PBS and then labelling with 0.1 mg/ml NHS-LC-Biotin (Pierce) in PBS at 37°C in 7.5% CO_2 for 1 hour. Cell monolayers were then washed three times with cell wash buffer (50 mM Tris-HCL pH 7.5, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) and harvested with a rubber policeman before being washed twice more in suspension. Cells were then lysed in 1% NP40 extraction buffer (cell wash buffer plus 300 μg/ml PMSF, 1 μg/ml pepstatin A, 2 μg/ml aprotinin and 4 μg/ml leupeptin) for 30 minutes on ice, followed by trituration and centrifugation at 14,000 revs/minute at 4°C to remove the insoluble fraction. The supernatants were then precleared by two sequential 2 hour incubations with 30 μl protein A-sepharose (Pharmacia) and 4 μl non-immune rabbit serum/ml cell lysate. Immunoprecipitations were carried out overnight at 4°C on a rotating platform using 1 μl rabbit antisera/250 μl cell lysate. Where

rat or mouse monoclonal antibodies were used, rabbit anti-rat or rabbit anti-mouse antisera was also added to the tube. This linking antisera was used at the same concentation as the primary antisera (1 in 250), while the monoclonal antibodies were used at the following concentrations: GoH3 (supernatant), 4 in 250; 346-11A (supernatant), 4 in 250; F11 (ascites) 1 in 250 and P3G2 (ascites) 1 in 250. The immune complexes were collected by incubation with 30 μl protein A-sepharose beads for 2 hours after which time the beads were extensively washed five times in immunoprecipitation wash buffer (identical to the cell wash buffer except for 0.5 M NaCl and the addition of 0.1% Tween-20). The immunodepletion experiment was performed by four sequential rounds of incubation with either GoH3 and rabbit anti-rat antisera or non-immune rabbit antisera and removal of complexes with protein A-sepharose beads. After the last round of immunodepletion, the remaining lysate was immunoprecipitated with antisera against the β1 subunit as described above. Integrins were separated from the beads by boiling in non-reducing SDS sample buffer for 5 minutes before being analysed by SDS-PAGE on a 7.5% resolving gel and 4% stacking gel under non-reducing conditions. Proteins were then electroblotted for 3 hours onto nitrocellulose (Hybond-C, Amersham), blocked overnight with 3% B.S.A. in TBS (10 mM Tris-HCL, 0.15 NaCl, pH 8.0) containing 0.1% Tween-20 and detected with streptavidin-HRP (ECL detection system, Amersham) for 1 hour according to manufacturers instructions. In those experiments where we obtained estimates of the expression levels of the β1 and β5 subunits, band intensity was measured by scanning the film with a Microtek scanner linked to a Macintosh computer running Image (NIH) and expressing the result as a ratio of αv intensity within each immunoprecipitate.

For the α6 western blotting experiment, cells were harvested and boiled immediately in SDS sample buffer for 5 minutes. Proteins were then separated on SDS-PAGE under non-reducing conditions, electroblotted onto nitrocellulose as above, and the blots were blocked overnight with 3% BSA in TBS plus 0.1% Tween-20. The α6A subunit was detected using antisera against the a6A subunit (6845) diluted 1:500 in TBS containing 5% normal goat serum followed by incubation with anti-rabbit-HRP (Amersham), diluted 1:1000 in TBS containing 5% normal goat serum.

RESULTS

Analysis of β1 integrins using biotin labelling

In order to analyse integrin expression of cells grown in culture, cell surface molecules were labelled with biotin, membranes lysed with 1% NP40 and immunoprecipitations carried out using a panel of antibodies directed against individual α or β subunits. As a positive control, initial experiments were carried out on astrocytes and fibroblasts, cells known to express a range of β1 integrins (Tawil et al., 1993). Using a panel of anti-α antisera, we found that astrocytes express predominantly α3β1 and α6Aβ1 and minor levels of α1β1 and α5β1, while fibroblasts express predominantly α5β1 with low levels of α1β1 and α3β1 (Fig. 1). In order to demonstrate that the α2 and α4 subunits could be detected using biotin labelling, immunoprecipitations were also performed on JM, a human 'T' cell line obtained from the American Tissue Culture Collection (ATCC). Both these antisera immunoprecipitated bands of the predicted molecular weights for the α2, α4 and β1 subunits (data not shown).

Having established that our biotin-labelling protocol could identify the different integrin subunits, initial experiments were aimed at characterizing the integrin expression profile of differentiated oligodendrocytes. Rat oligodendrocyte precursors were obtained by mechanical shaking of mixed glial cultures followed by microglial subtraction. In secondary culture these cells constitutively differentiate into oligodendrocytes. Using galactocerebroside (GalC) as a marker of differentiation into oligodendrocytes, our secondary cultures were <10% GalC at day 1 postshake, increasing to >80% GalC at day 7 and >95% at day 10. Over the same time period, the cultured oligodendroglial cells changed their morphology from a simple bipolar or tripolar phenotype to a complex branching pattern typical of oligodendrocytes in culture (Raff et al. 1978).

Just like the astrocytes and fibroblasts, we found that oligodendrocytes expressed β1 integrins. Anti-β1 antiserum immunoprecipitated three bands which run at 140, 130 and 110×10^3 M_r under non-reduced conditions on SDS-PAGE (Fig. 2A) The molecular weight of the lowest band and the observation that it shifts up to 130×10^3 M_r upon reduction strongly suggest this is the β1 subunit. In agreement with previous observations (Malek-Hedayat and Rome, 1994), we found that the oligodendrocyte β1 band ran at a lower molecular weight than the β1 subunit expressed by either astrocytes or fibroblasts.

Integrin α subunit expression on oligodendrocytes

In order to characterize the α subunits associated with oligo-

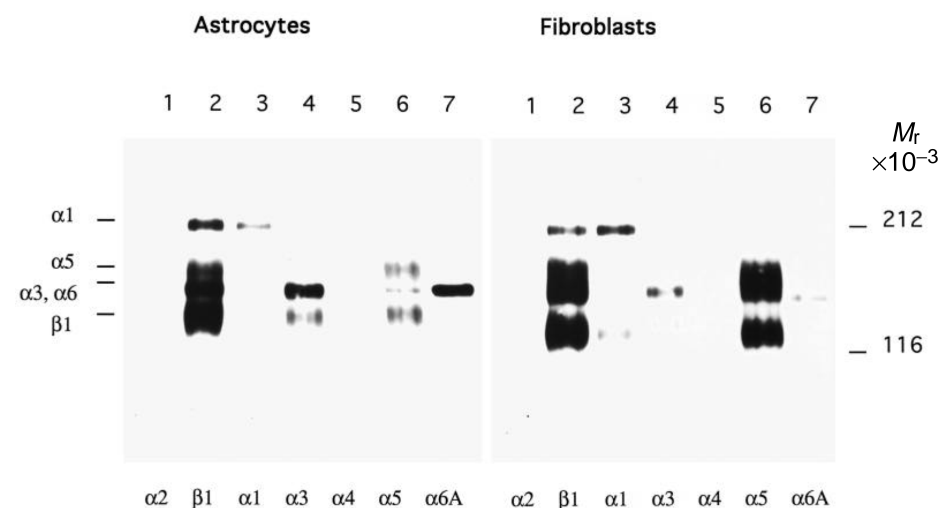

Fig. 1. Analysis of β1 integrins on astrocytes and fibroblasts. Immunoprecipitations of biotin-labelled cell surface proteins were performed as described in Materials and methods, after which the proteins were separated on non-reducing gels. The left-hand panel shows astrocytes immunoprecipitated with the antisera shown below the figure, while the right-hand panel shows the same analysis performed on fibroblasts. Note that astrocytes express predominately α3β1 and α6Aβ1, with low levels of α5β1 and α1β1. Fibroblasts express predominately α5β1 and low levels of α1β1 and α3β1. M_r markers are shown on the right side of both gels ($\times10^{-3}$).

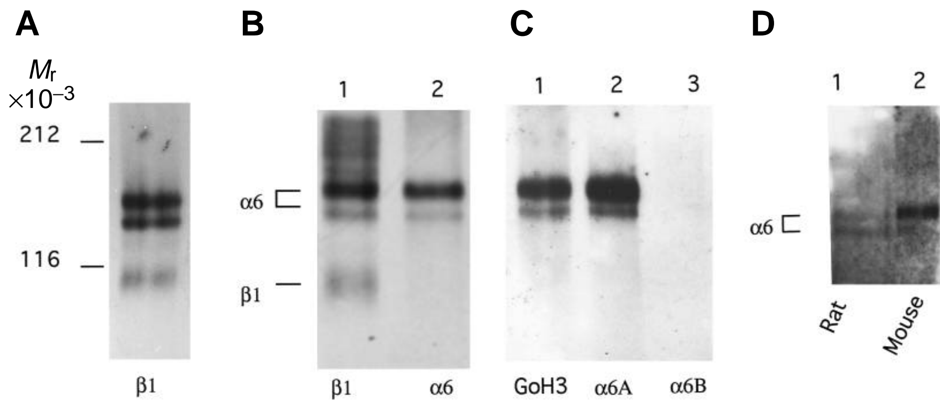

Fig. 2. (A) β1 integrin expression on rat oligodendrocytes. Immunoprecipitations of biotin-labelled cell surface proteins were performed as described in Materials and methods, after which the proteins were separated on non-reducing gels. Note that 3 bands are pulled down; the lowest band corresponds to β1 as discussed in the text. (B) β1 integrin expression on mouse oligodendrocytes. Immunoprecipitations were performed with antiserum against β1 (lane 1) and the GoH3 monoclonal antibody against α6 (lane 2) and run under non-reducng conditions. Note that both the α subunits associated with β1 also immunoprecipitate with the anti-α6 antibody. (C) Expression of the alternatively spliced forms of α6 in mouse oligodendrocytes. Immunoprecipitations were performed with the GoH3 monoclonal antibody (lane 1) and with antisera against α6A (lane 2) and α6B (lane 3), and then run under non-reducing conditions. GoH3 would be expected to immunoprecipitate both forms of α6; however, note that the splice-specific antisera show that α6A but not α6B is expressed by the oligodendrocytes. (D) Western blot analysis of α6 expression on rat and mouse oligodendrocytes. Oligodendrocytes were boiled in SDS-sample buffer immediately after harvesting, separated by SDS-PAGE and then electroblotted onto nitrocellulose prior to detection with the α6A antiserum. Note that both rat and mouse cells express two forms of α6A.

dendrocyte β1 integrin, we performed immunoprecipitation analysis using a panel of antisera raised against the cytoplasmic domains of α1, α2, α3, α4 and α5. All of these well-characterized α subunits associate with β1 (Hynes, 1987). In contrast to the results with astrocytes and fibroblasts, immunoprecipitation of oligodendrocytes with these antibodies failed to produce any bands on the gel (data not shown). As all of these antisera do pull down bands in other cell types (Fig. 1 and data not shown), we conclude that oligodendrocytes do not express significant levels of α1, α2, α3, α4 and α5 on their cell surface.

Five other α subunits have been described as associating with β1 integrin; α6, α7, α8, α9 and αv (Hemler et al., 1988; Song et al., 1992; Bossy et al., 1991; Palmer et al., 1993; Bodary and McLean, 1990; Vogel et al., 1990). We were able to examine α6, α8 and αv directly in this study. In order to examine α6 expression, we used a monoclonal antibody (GoH3) which recognizes mouse α6. Just like the rat cells, mouse oligodendrocytes immunoprecipitated with the anti-β1 antiserum gave a pattern of three bands at 140, 130 and 110×10^3 M_r under non-reducing conditions. Fig. 2B shows that the anti-α6 antibody gave an identical pattern of bands to that seen with the anti-β1 antiserum. These results show that oligodendrocytes express α6β1 integrins.

Previous work has shown that there are two forms of the α6 subunit that arise from alternative splicing of the cytoplasmic domain (Hogervorst et al., 1991; Cooper et al., 1991). Using antisera specific for the two forms, we found that oligodendrocytes express α6A but not α6B (Fig. 2C). We were surprised to find that the two α subunits running 10×10^3 M_r apart were both α6A. One possibility is that the lower band represented a degradation product. In order to address this question, oligodendrocytes were rapidly harvested within 2 minutes and immediately boiled in SDS-sample buffer to inactivate any protease activity. Samples were then separated on SDS-PAGE and western blotted using antibodies specific for the α6A subunit. We found that both rat and mouse oligodendrocytes gave an identical pattern of α6 bands as the immunoprecipitation experiments (Fig. 2D), suggesting that the α6 heterogeneity is not a result of degradation but represents a real heterogeneity of α6 at the oligodendrocyte cell surface.

Because the α6 integrin subunit is known to associate with both β1 and β4 subunits (Hemler et al., 1989; Kajiji et al., 1989), we investigated the possibility that oligodendrocytes might express α6β4 in addition to α6β1. However, immunoprecipitations performed with an anti-β4 monoclonal antibody failed to show any bands, even when the oligodendroglia were left for up to 2 weeks in secondary culture. Therefore, we conclude that the oligodendrocytes in this system express α6 only in association with β1.

Immunoprecipitation with antiserum raised against the cytoplasmic domain of αv showed that oligodendrocytes also expressed αv integrins. Three bands were observed: 140×10^3 M_r, the expected size for the αv subunit and two other bands at 80 and 90×10^3 M_r (Fig. 3). Of these two bands, the 80×10^3 M_r band was dominant. As αv has been described as associating with a range of β subunits (β1, β3, β5, β6 and β8), it seemed likely that these lower bands were β subunits. Further experiments supported this interpretation. First, immunopre-

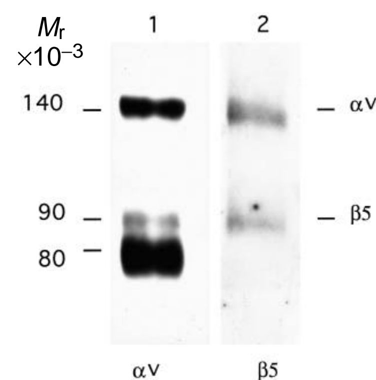

Fig. 3. The αv expression profile of rat oligodendrocytes. Immunoprecipitations of biotin-labelled cell surface proteins were performed as described in Materials and methods, after which the proteins were separated on non-reducing gels. αv integrins were immunoprecipitated with anti-αv antiserum (lane 1) and the P3G2 antibody against αvβ5 (lane 2). Note that the anti-αv antiserum pulls down 3 bands, with αv running at 140×10^3 M_r and the two associated β subunits at 90 and 80×10^3 M_r. The αvβ5 immunopreciptation identifies the 90×10^3 M_r band as β5.

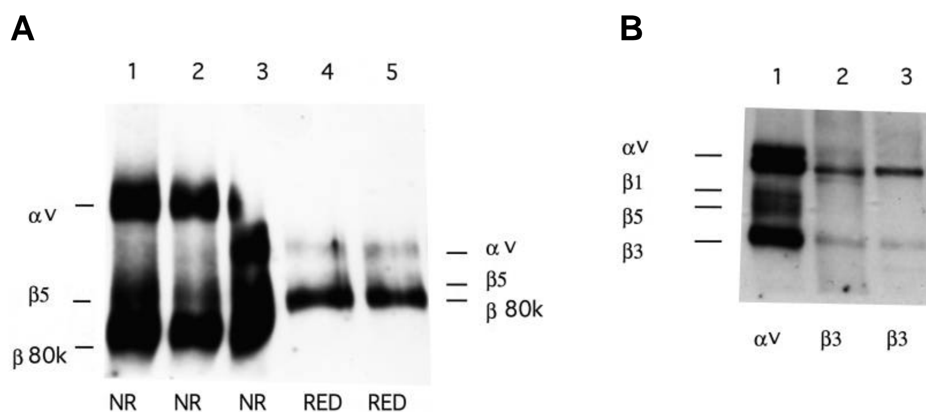

Fig. 4. Characterization of the 80×10^3 M_r subunit associated with αv on oligodendrocytes. (A) Identical αv immunoprecipitates from biotin-labelled oligodendrocytes (as shown in Fig. 3, lane 1) run under non-reducing (lanes 1-3) and reducing (lanes 4-5) conditions. These were run side by side so as to demonstrate the shift in mobility. Note that upon reduction the 80×10^3 M_r β-subunit shifts in mobility by at least 10×10^3 M_r, as it now runs just above non-reduced β5 (90×10^3 M_r). (B) The immunoprecipitations with αv (lane 1) run alongside immunoprecipitations with an anti-β3 antiserum (lane 2) and F11, a monoclonal antibody against β3 (lane 3) under non-reducing conditions. Note that oligodendrocytes express αvβ3 and that β3 runs within the dominant 80×10^3 M_r band in the αv immunoprecipitates.

cipitation using a monocolonal anti-αvβ5 (P3G2) brought down the 90×10^3 M_r band and the αv subunit (Fig. 3), confirming that oligodendrocytes express αvβ5 and that the 90×10^3 M_r band was β5. Second, upon reduction, the 80×10^3 M_r band follows the expected behaviour of an integrin β subunit by shifting up ($10\text{-}15\times10^3$ M_r) to a higher apparent molecular weight. This is shown in Fig. 4A, in which the same αv immunoprecipitation has been run in either non-reduced or reducing conditions. Based on molecular weight, the possible candidates for this putative 80×10^3 M_r β subunit are β3, β6, β8 or a novel β subunit. Immunoprecipitations with anti-β6 antiserum were negative. The previous observation that the β8 subunit shifts up only marginally ($2\text{-}3\times10^3$ M_r) upon reduction (Moyle et al., 1991) makes β8 an unlikely candidate for the 80×10^3 M_r band. Immunoprecipitations using either of two anti-β3 antibodies, one an antiserum and the other the F11 monoclonal antibody, did pull down a pattern of two bands corresponding to αv and the 80×10^3 M_r band, but the 80×10^3 M_r band was very faint when compared with the intensity seen in the αv immunoprecipitation (Fig. 4B). We conclude therefore that oligodendrocytes do express αvβ3 but think it likely that other β subunits also contribute to the 80×10^3 M_r band.

A previous study published as this work was being completed has described α8β1 on the surface of oligodendrocytes (Malek-Hedayat and Rome, 1994). However, in our hands, experiments performed with an anti-α8 antiserum on oligodendrocytes of three different ages (2, 7 and 20 days in secondary culture) failed to immunoprecipitate any bands in this system (Fig. 5).

As reagents against α7 and α9 integrins were not available to us, we took a different approach to determine whether these or any novel β1-associated integrins were expressed on oligodendrocytes. We performed immunodepletion experiments in which cell surface extracts from oligodendrocytes were depleted of α6β1 integrins by four sequential rounds of immunoprecipitation with the G0H3 monoclonal antibody. Following these steps, immunoprecipitation with anti-β1 antiserum was performed. As shown in Fig. 6, no β1 integrins were detectable following these immunodepletion experiments while the cell lysate exposed to control immunodepletions gave a pattern identical to that seen with the normal β1 control. This result confirms that α6β1 is the major β1 integrin expressed

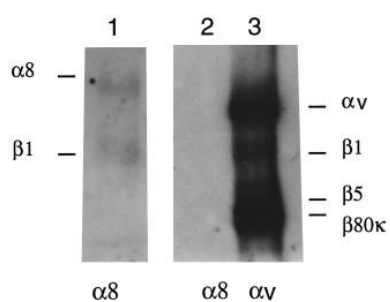

Fig. 5. Lack of α8 expression on oligodendrocytes. Lanes 2 and 3 show a biotin-labelled lysate from rat oligodendrocytes immunoprecipitated with anti-α8 and anti-αv antisera respectively. Lane 1 shows a positive control for the anti-α8 antiserum using a mouse embryonic fibroblast cell line (STO cells). The oligodendrocyte lanes are deliberately overexposed, but note that no α8 bands are seen.

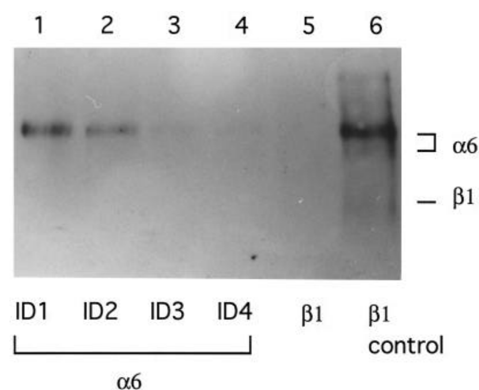

Fig. 6. α6 immunodepletion of a mouse oligodendrocyte lysate. Biotin-labelled mouse oligodendrocyte lysate was immunodepleted of α6 integrins by sequential immunoprecipitations with the monoclonal antibody GoH3 as described in materials and methods. Lanes 1-4 show the proteins removed at each round of immunoprecipitation. Lane 5 shows the subsequent immunoprecipitation with an anti-β1 antiserum while lane 6 shows the same β1 immunoprecipitation performed in the mock-depleted control. All gels were run under non-reducing conditions. Note that the α6 immunodepletion removed all detectable β1 integrins from the oligodendrocyte lysate.

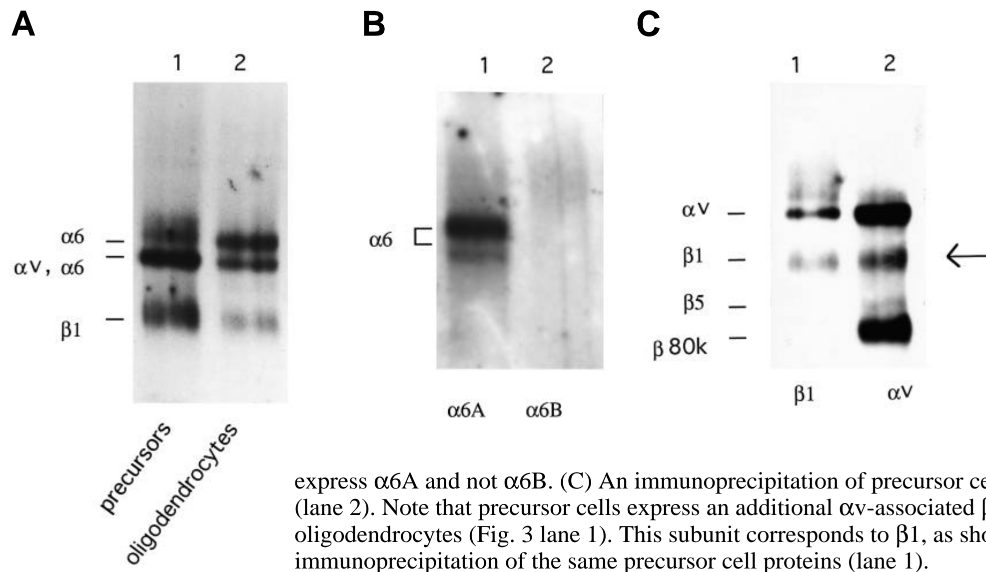

Fig. 7. Integrin expression by oligodendrocyte precursors. (A) β1 immunoprecipitates of biotin-labelled cell surface proteins from oligodendrocyte precursors (lane 1) and mature oligodendrocytes (lane 2) run under non-reducing conditions. The pattern of bands is identical, but note that the relative intensity of the upper two bands is different. (B) Immunoprecipitations of precursor cell proteins with the splice-specific α6A and α6B antisera run under the same conditions as A. Note that, just as in the oligodendrocytes, precursors express α6A and not α6B. (C) An immunoprecipitation of precursor cell proteins with the anti-αv antiserum (lane 2). Note that precursor cells express an additional αv-associated β subunit (arrow) as compared to the oligodendrocytes (Fig. 3 lane 1). This subunit corresponds to β1, as shown by the neighbouring β1 immunoprecipitation of the same precursor cell proteins (lane 1).

on oligodendrocytes and suggests that no other β1 integrins are expressed at a significant level.

Integrin expression on oligodendrocyte precursors (0-2A progenitor cells)

These results show that oligodendrocytes express at least three integrins α6β1, αvβ3 and αvβ5. As one of the major goals of this study was to determine the role of integrins in the differentiation of oligodendrocyte precursors cells, we next analysed integrins on oligodendrocyte precursors. This analysis was performed on secondary oligodendroglia cultures soon after shaking, at which time less than 10% of the cells were GalC-positive oligodendrocytes and the vast majority of the cells were therefore oligodendrocyte precursors.

As with the oligodendrocytes, we found precursors expressed α6 and αv integrins (Fig. 7) but were negative for α1, α2, α3, α4, α5 and α8. The expression pattern of α6 integrin on precursors was similar to that of oligodendrocytes with two bands present. Previous work examining α6A and α6B has suggested that these forms are developmentally regulated (Cooper et al., 1991; Hierck et al., 1993). In order to determine whether a similar regulation occurs during oligodendrocyte differentiation, we performed immunoprecipitation analysis of precursors or differentiated oligodendrocytes using α6A and α6B antisera. As shown in Fig. 7B, we only observed α6A forms in precursors, just as we had found in oligodendrocytes. α6B forms were never observed in the oligodendroglial lineage cells examined in this study.

In contrast to the situation with α6, we observed a different pattern of integrin expression when αv immunoprecipitation experiments were performed on oligodendrocyte precursors. αv was expressed on precursor cells as well as oligodendrocytes, but the pattern of associated β subunits was different. The 90×10^3 M_r β5 band was barely visible, while a new band was seen at 110×10^3 M_r in the precursor cells. Two lines of evidence suggest that this band represents β1 associated with αv. First, this band co-migrated with the β1 band observed in a β1 immunoprecipitation of precursor cells (Fig. 7C). Second, in β1 immunoprecipitation experiments, we consistently observed an increase in the intensity of the lower α band in precursor cells as compared with differentiated oligodendrocytes. This band is formed by both α6 (lower) and αv which co-migrate (Fig. 7A). An increase in intensity of this band would therefore be predicted in the β1 immunoprecipitations of precursor cells if αv as well as α6 was now being immunoprecipitated in association with β1.

To investigate these changes in the β subunits associated with αv further, we analysed a developmental time course experiment in which oligodendrocyte precursor cultures were left for up to 15 days in culture. As shown in Fig. 8, the levels of the 80×10^3 M_r band containing β3 remained at a high level throughout this experiment. The levels of β1 and β5 changed as expected; at day 1 αvβ1 expression is high while αvβ5 is barely detectable, by day 4 αvβ1 is downregulated and by day

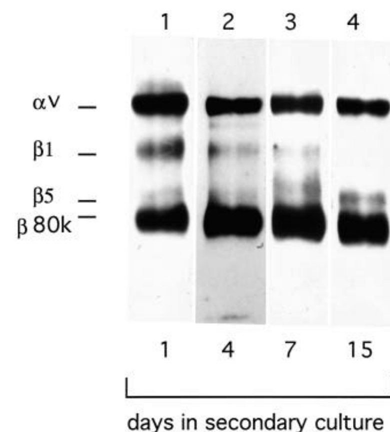

Fig. 8. A developmental time course of αv integrins on differentiating oligodendrocytes. Immunoprecipitations using the anti-αv antiserum were performed on biotin-labelled cell surface proteins after the precursor cells had been left for 1-15 days in secondary culture and then run under non-reducing conditions. Note that the αv-associated β1 is lost from differentiating oligodendroglia between 7 and 15 days, while αv-associated β5 increases in amount over the time period examined. β80kDa, in contrast, shows no change in intensity.

7 when greater than 80% of the cells have differentiated into oligodendrocytes αvβ5 predominates over αvβ1. By day 15, αvβ1 has virtually disappeared while αvβ5 levels remain elevated. This reciprocal change in β1 and β5 was observed in 5 consecutive experiments left for 7 days in secondary culture. The β1 band was reduced in intensity by a factor of 2.06 ± 1.2 while the β5 band was increased by a factor of 3.27 ± 2.27, giving an estimated 6-fold increase in the β5/β1 ratio. In cultures left for 12 days or longer, the reduction in β1 becomes progressively more marked, with reductions by factors of 3 (12 days), 13 (15 days) and 21 (26 days) obtained in three separate experiments. As a result, the β5/β1 ratio increased at least 24-fold by day 15. These results show that the downregulation of αvβ1 and increase in αvβ5 occurs as the precursor cells differentiate and change their migratory, proliferative phenotype for a non-motile, postmitotic phenotype.

DISCUSSION

Two major conclusions emerge from this study of integrins on oligodendroglial cells. First, oligodendroglial cells express a limited repertoire of integrins comprising α6β1 and αv-associated integrins. Second, the expression of integrins is developmentally regulated; αvβ1 is downregulated and αvβ5 upregulated in association with differentiation in this lineage.

Patterns of integrin expression on oligodendroglial cells

Oligodendrocytes and astrocytes form the two major classes of glial cells within the vertebrate CNS. Previous work has shown that astrocytes express α6β1 (Tawil et al., 1993). Our finding that oligodendroglia also express α6β1 suggests that there is wide-spread expression of this α subunit in the CNS. The absence of α1-5 in oligodendrocytes is in contrast with astrocytes where we detected α1, α3 and α5. Interestingly, however, oligodendrocytes do resemble Schwann cells, the myelinating cell of the peripheral nervous system, in their expression of α6 as the dominant β1 integrin (Einheber et al., 1993). The similar pattern of integrins in the two myelinating cells is of interest as the cells have otherwise very different phenotypes – oligodendrocytes are multipolar cells that form many myelin sheaths and lack a basal lamina while Schwann cells form only a single myelin sheath as well as a basal lamina. In addition to α6β1, myelinating Schwann cells also express α6β4 (Einheber et al., 1993; Feltri et al., 1994; Niessen et al., 1994). We found no evidence for α6β4 in this study; however our purified oligodendrocyte cultures are neuron-free and so myelin sheath formation would not be expected.

Previous studies on the α6 subunit show alternative splicing in the cytoplasmic domain producing two different forms, α6A and α6B (Hogervorst et al., 1991; Cooper et al., 1991). These two isoforms are regulated both in a temporal and spatial fashion. α6B is the only form expressed in blastocyst-stage mouse embryos (Hierck et al., 1993), while adult tissues express both α6A and α6B in cell-type-specific patterns (Tamura et al., 1991; Hogervorst et al., 1993). This observation suggests that differentiation is associated with an increase in α6A. In support of this, blastocyst or ES cells allowed to differentiate in vitro upregulate α6A so as to coexpress both α6A and α6B (Cooper et al., 1991; Hierck et al., 1993). These observations prompted us to analyse the α6A-α6B expression pattern in oligodendroglia at different stages of development in order to determine whether there was any evidence for developmental control. We found that both precursors and differentiated oligodendrocytes expressed the α6A but not the α6B form. This does not preclude the possibility that α6B is expressed earlier in the oligodendroglial lineage, prior to the developmental point at which we were able to obtain large numbers of oligodendrocyte precursors. It does, however, argue against a relationship between α6 isoform switching and the migratory and proliferative phenotype of the cell, as both the migratory, proliferating oligodendrocyte precursor and the postmigratory, postmitotic oligodendrocyte express α6A and not α6B.

The α6 immunoprecipitations from oligodendrocytes consistently gave a pattern of two α6 bands. Western blotting of proteins from cells rapidly lyzed in SDS sample buffer also showed the same pattern, arguing against handling degradation as an explanation for these two bands. Labelling of an intracellular α6 precursor seems unlikely due to the impermeant nature of the biotin label. Another possibility is that the lower band is contributed by contaminating type-1 astrocytes in the culture, as we found that the α6 band expressed by astrocytes co-migrated with the lower oligodendroglial α6 band. This seems unlikely for two reasons. First, there are negligible levels of type-1 astrocytes seen in these cultures, as determined by their fibroblast morphology and immunolabelling by an antiserum against glial fibrillary acidic protein (GFAP). Second, experiments with an oligodendroglial cell line CG4 (Louis et al., 1992, kindly provided by Dr J. C. Louis) also show expression of the lower α6 band in the cultures using this cell line (R. M. and C. ff-C., unpublished observations). Therefore we conclude that oligodendroglia express two forms of α6 and that there is a further level of heterogeneity within the α6 subunit in addition to the alternative splicing responsible for α6A and α6B.

Oligodendrocytes also express another major class of integrins, the αv family. αv has been described in association with a wide range of β subunits (β1, β3, β5, β6 and β8) (Bodary and McLean, 1990; Vogel et al., 1990; Cheresh and Spiro, 1987; Cheresh et al., 1989; Ramaswamy and Hemler, 1990; Busk et al., 1992; Moyle et al., 1991) and this range of subunits makes αv unique amongst integrin α chains. Interestingly, we found that the β subunit association of αv in oligodendroglia changes with differentiation. Precursor cells express αvβ1 and low levels of αvβ5 whereas oligodendrocytes express much higher levels of αvβ5 while αvβ1 is barely detectable. The developmental time course that we performed shows this change to occur over 26 days in secondary culture, with the increase in αvβ5 seen by 7 days while αvβ1 decreases over the entire time in culture. The low levels of αvβ5 observed in the precursor cell cultures might reflect either expression by the precursors of low levels of αvβ5 or alternatively αvβ5 on the small contaminating population of oligodendrocytes. Either interpretation is consistent with our conclusion from these experiments that differentiation is associated with a switch of αv-associated β subunits from β1 to β5. In addition to αvβ1 and αvβ5 a third αv integrin is expressed on both oligodendrocyte precursors and differentiated oligodendrocytes. This integrin was present at higher levels than either αvβ1 or αvβ5. As explained in the results,

we have shown that αvβ3 is present on oligodendroglial cells and represents a likely candidate for an extra αv integrin. However in our hands two anti-β3 antibodies produced only faint bands raising the possibility that another novel integrin β-subunit is also expressed on oligodendrocytes.

Our results contradict those of Malek-Hedayat and Rome who examined integrins on oligodendroglia by I^{125} surface-labelling prior to immunoprecipitation experiments. They found no evidence for αv integrins and did not examine α6 integrins. They did, however, find β1 associated with the integrin α8 subunit. In our hands, an α8 antiserum did not immunoprecipitate any bands and an immunodepletion experiment with α6 removed most, if not all, β1-associated integrins from oligodendrocytes. This makes it unlikely that significant levels of other β1 integrins are present on oligodendrocytes. We cannot explain this discrepancy; however the oligodendrocytes used in the two studies were grown using different culture conditions. Malek-Hedayat and Rome used 5% calf serum to grow their oligodendrocytes while we used low serum (0.5% FCS) media for our secondary culture. It is possible that the growth factors present in serum alter the integrin expression of oligodendroglial cells in secondary culture.

Potential ECM ligands for oligodendroglial cells

The definition of the pattern of integrin expression on oligodendroglial cells allows predictions to be made as to the extracellular matrix ligands likely to interact with these receptors. α6 integrins are receptors for the laminin family of molecules (Sonnenberg et al., 1988). αv integrins are more promiscuous, with fibronectin (αvβ1, αvβ6), thrombospondin (αvβ3), tenascin (αvβ3) and vitronectin (αvβ1, αvβ3, αvβ5) identified among other ligands (Vogel et al., 1990; Busk et al., 1992; Lawler and Hynes, 1989; Prieto et al., 1993; Sriramarao et al., 1993; Bodary and McLean, 1990; Cheresh and Spiro, 1987; Cheresh et al., 1989). As we would have predicted, we have found that oligodendrocyte precursors bind to and extend processes on both vitronectin and laminin (R. M. and C. ff-C., unpublished observations). Interestingly, a previous study found that oligodendrocyte precursors, but not oligodendrocytes also bind to fibronectin (Payne and Lemmon, 1993). Given that αvβ1 is a fibronectin receptor, this result would be predicted from our data – αvβ1 being lost on oligodendrocytes as differentiation proceeds. The functional significance of this is unclear, as fibronectin is expressed only transiently in the central nervous system and appears to be absent from areas at the time of myelination later in development (Sheppard et al., 1991). In contrast, laminins, thrombospondin, tenascin and vitronectin have all been identified in the developing CNS in regions that will develop into myelinated tracks. Vitronectin is present in the early stages of development (E5-6) in chick optic nerve and retina (Neugebauer et al., 1991), although the pattern of vitronectin expression later in development has not yet been addressed. Thrombospondin and tenascin are present in developing white matter within the cerebellum (O'Shea et al., 1990, Bartsch et al., 1992). Laminin is present in the early developing central nervous system (Liesi, 1985) and has also been described at later stages within the optic nerve during the phase of oligodendroglial cell migration (McLoon et al., 1988). The laminins constitute an extensive family of extracellular matrix molecules and the recent observation that one member of this family, merosin, is present in developing white matter tracts in the chick (N. Morissette and S. Carbonetto, Society for Neuroscience Abstracts, 1993) suggest that this form of laminin may be an important ligand for α6β1 on oligodendroglial cells. The function of the extracellular matrix in CNS development is poorly understood; however, our evidence that oligodendroglial cells express integrin receptors for at least four classes of extracellular matrix molecules present in the central nervous system does strengthen the evidence that cell-ECM interactions play a significant role in the formation of the CNS.

What is the role of integrins in oligodendrocyte differentiation and myelin formation?

Differentiation of oligodendroglial cells involves three different events: cessation of migration, ending of proliferation and subsequent expression of myelin-specific gene products, associated with the formation of the myelin sheath. We found that migratory, proliferating precursor cells express αvβ1 whereas postmigratory, postmitotic differentiated oligodendrocytes upregulate αvβ5 while αvβ1 disappears. In addition, both precursor cells and differentiated oligodendrocytes express α6β1 and other αv integrins including αvβ3. Based on the work of others examining the functional consequences of the expression of different αv integrins, we propose that the switch from αvβ1 to αvβ5 is an essential part of the molecular mechanism responsible for the differentiation of oligodendrocyte precursor cells. By constructing β1 chimeric integrins expressing either β1 (wild type) or β5 cytoplasmic domains, Pasqualini and Hemler (1994) showed that CHO cells expressing α5β1 showed increased proliferation on fibronectin substrates, while cells expressing α5β1/5 failed to do so. This suggests that β1 but not β5 cytoplasmic domains support increased cell proliferation. Both integrin subunits supported cell adhesion equally, suggesting that the different cytoplasmic domains play an instructive role in controlling cell proliferation by stimulating different signal transduction pathways within the cells. The observed switch from αvβ1 to αvβ5 in oligodendrocyte precursors might therefore represent a critical step in the change to a postproliferative phenotype. If oligodendroglia behave in the same manner as the CHO cells analysed by Pasqualini and Hemler (1994), then the upregulation of αvβ5 would be expected to reduce proliferation. This would in turn induce differentiation in the precursor cell, as tissue culture studies have illustrated that oligodendrocyte precursors constitutively differentiate into oligodendrocytes once proliferation has ceased (Temple and Raff, 1985, for review see Barres and Raff, 1994). In such a model, integrins will therefore play a key instructive role in the control of oligodendrocyte differentiation.

The observations of Rome and colleagues that RGD peptides inhibit the expression of myelin-specific genes by oligodendrocytes (Cardwell and Rome, 1988b, Malek-Hedayat and Rome, 1994) suggests a further role for integrins in myelination. Our results suggest a molecular mechanism for this observation, as RGD peptides block all the αv integrins that we have characterized on oligodendroglial cells (Koivunen et al., 1993). We propose, therefore, that once differentiation has occurred the interaction of oligodendrocytes with the extracellular matrix via αv integrins is essential for the formation of myelin.

Signal transduction of integrins is subunit specific, such that different β subunits associated with αv and binding the same

ligand (vitronectin) can transduce different signals to the inside of the cell (Leavesley et al., 1992). This evidence suggests that the switching of β subunits that we have observed during the differentiation of oligodendroglial cells may play important instructive roles in the regulation of cell behaviour. Such switching of β subunits may be an important general mechanism for the regulation of cell behaviour during development in a wide range of cell types. Our results have enabled us to generate predictions as to the effects of switching these different β subunits. We are now in a position to take advantage of the ability to analyse oligodendroglial cell behaviour either in vitro or in vivo following transplantation so as to test these predictions

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