# The regulation of proliferation and differentiation in oligodendrocyte progenitor cells by αv integrins

**K. L. Blaschuk[1], E. E. Frost[1] and C. ffrench-Constant[1,2,*]**

[1]Wellcome/CRC Institute of Developmental Biology and Cancer, Tennis Court Road, Cambridge, CB2 1QR, UK and Department of Medical Genetics, University of Cambridge, UK
[2]Cambridge Centre for Brain Repair, University Forvie Site, Addenbrookes Hospital, Cambridge, CB2 2PY, UK
*Author for correspondence (e-mail: cfc@mole.bio.cam.ac.uk)

## SUMMARY

We have previously shown that oligodendrocyte progenitor cells exhibit developmental switching between αv-associated β integrin subunits to sequentially express αvβ1, αvβ3 and αvβ5 integrins during differentiation in vitro. To understand the role that αvβ3 integrin may play in regulating oligodendrocyte progenitor cell behaviour, cells of the rat cell line, CG-4, were genetically engineered to constitutively express αvβ3 integrin by transfection with full-length human β3 integrin subunit cDNA. Time-lapse videomicroscopy showed no effect of β3 expression on cell migration but revealed enhanced proliferation on vitronectin substrata. Comparison of mitotic indices, as measured by 5-bromo-2′-deoxyuridine incorporation, confirmed that human β3 integrin-expressing cells exhibited enhanced proliferation, as compared to both vector-only transfected, and wild-type CG-4 cells when switched to differentiation medium from growth medium,

but only in cultures grown on vitronectin and not on poly-D-lysine. The effects on proliferation were inhibited by a function-blocking antibody specifically directed against the human β3 integrin subunit. Human β3 integrin-expressing cells also exhibited reduced differentiation. This differentiation could be reduced still further by a function-blocking monoclonal antibody against αvβ5 integrin, as could differentiation in the wild-type CG-4 cells. Taken together, these results suggest that αvβ3 integrin may regulate oligodendroglial cell proliferation and that both downregulation of αvβ3 integrin expression and signalling through αvβ5 integrin may be critical to continued differentiation in vitro.

Key words: Oligodendrocyte progenitor cell, Integrin, Proliferation, Differentiation, αvβ1, αvβ3, αvβ5

## INTRODUCTION

Myelination of axon tracts in the vertebrate central nervous system (CNS) is a process critical to rapid and efficient impulse conduction. Myelin is formed by oligodendrocytes, which are widely dispersed throughout the white matter of the CNS. Oligodendrocyte progenitor cells (OPCs) arise in restricted areas of the developing CNS and migrate sometimes over considerable distances to their final destinations (Levison et al., 1993; Ono et al., 1995; Lachapelle et al., 1994; Hardy and Friedrich, 1996; Baron-Van Evercooren et al., 1996) where they differentiate into mature, myelin-forming oligodendrocytes (Hardy and Reynolds, 1991; Gansmuller et al., 1991; Levison et al., 1993; Lachapelle et al., 1994). The bulk of oligodendroglial cell development and myelination occurs after birth when neuronal pathways are already well established. Consequently, OPC interaction with other cell types such as neurons, astrocytes, radial glia and endothelial cells and the environments provided by these cells, is critical to normal development. Local cues provided by these cells such as PDGF and the neuregulins are well established as essential

regulators of such complex behaviours as OPC migration, proliferation and survival (Richardson et al., 1988; Noble et al., 1988; Armstrong et al., 1990; Bogler et al., 1990; Barres et al., 1992; Milner et al., 1997a; Calver et al., 1998). Less understood is the role that extracellular matrix (ECM) proteins may play. ECM proteins such as vitronectin (VN), laminin (LN), thrombospondin (TSP) and tenascin-C (TN-C) are known to be expressed in the developing CNS during OPC migration (McLoon et al., 1988; O'Shea et al., 1990; Sheppard et al., 1991; Bartsch et al., 1992; Laywell et al., 1992) and may contribute to regulation of OPC behaviour. Certainly, both LN and TSP have been found to promote OPC migration at least in vitro (Schmidt et al., 1997; Scott-Drew and ffrench-Constant, 1997), while TN-C has been shown to inhibit this migration (Frost et al., 1996; Kiernan et al., 1996).

Adhesion to proteins of the ECM is largely mediated by the integrin superfamily. Integrins are heterodimeric transmembrane glycoproteins that are involved in signal transduction between the extracellular and intracellular compartments (Ruoslahti et al., 1994; Hynes, 1992). Oligodendroglial cells are known to express a limited repertoire of integrins in vitro, including α6β1 integrin and

four members of the αv subfamily including αvβ1, αvβ3, αvβ5 and αvβ8 integrins. Interestingly, the patterns of expression of each of these integrins appear to be developmentally regulated, suggesting that each integrin may play a vital role during the stage of oligodendrocyte development at which it is expressed (Milner and ffrench-Constant, 1994; Milner et al., 1997b). However, while recent studies have shown that αvβ1 integrin may play a role in supporting OPC migration in vitro (Milner et al., 1996), a role consistent with its pattern of expression in which levels of αvβ1 integrin are downregulated coincidentally with loss of the migratory phenotype, roles for the remaining integrins have yet to be established.

In the present study, we have examined the roles that αvβ3 and αvβ5 integrins may play during oligodendrocyte development in vitro. Levels of αvβ5 integrin are known to be rapidly upregulated as oligodendroglial cells differentiate into myelin-forming oligodendrocytes, suggesting a possible role for this integrin during differentiation (Milner and ffrench-Constant, 1994). Conversely, levels of αvβ3 integrin have been shown to be upregulated prior to expression of αvβ5 integrin and then rapidly downregulated coincidentally with the onset of final differentiation, suggesting that αvβ3 integrin may play a role in cells which are losing migratory potential and preparing to withdraw from the cell cycle and terminally differentiate. (Milner et al., 1997b). To examine the possible roles these integrins may play, we have observed the effects on cellular behaviour when normal downregulation of αvβ3 integrin expression is prevented. Cells of an immortalised oligodendrocyte precursor cell line, CG-4, were genetically engineered to constitutively express αvβ3 integrin. We report here that constitutive expression of αvβ3 integrin both stimulates CG-4 cell proliferation in a substratum-dependent manner and inhibits differentiation. In addition, a blocking antibody against αvβ5 integrin also inhibits differentiation. Based on these and previous results from our laboratory, a model is presented in which developmental switching between αv-associated β integrin subunits may contribute to the timing of migration, proliferation and differentiation in the oligodendrocyte cell lineage.

## MATERIALS AND METHODS

### Cells and cell cultures

The CG-4 cell line was originally derived from a culture of primary OPCs isolated from rat neonatal brains by Louis et al. (1992). CG-4 cells used in this study were all passage 45 or less. CG-4 cells were grown in Sato's medium supplemented with B104 cell-conditioned medium (B104-CM) (growth medium, GM) and maintained at 37°C in 7.5% $CO_2$. Sato's medium is DMEM (Sigma, Dorset, UK) supplemented with 4 mM glutamine, 5 μg/ml bovine insulin, 50 μg/ml human holotransferrin, 100 μg/ml BSA, 6.2 ng/ml progesterone, 16 μg/ml putrescine, 5 ng/ml sodium selenite, 400 ng/ml tri-iodothyronine and 400 ng/ml thyroxine (all from Sigma). Medium conditioned by B104 cells, cells derived from a rat neuroblastoma cell line (Bottenstein and Sato, 1979), was harvested after the method of Louis et al. (1992) and routinely constituted 30% v/v of the CG-4 cell growth medium. To promote differentiation, CG-4 cells were switched to Sato's medium supplemented with 0.5% v/v fetal calf serum (differentiation medium, DM) but devoid of B104-CM. CG-4 cells were grown either on poly-D-lysine (PDL, Sigma, final concentration of 5 μg/ml) or vitronectin (VN, Sigma, final concentration of 5 μg/ml).

### Transfection of CG-4 cells

CG-4 cells were transfected with the mammalian expression vector, pcDNA3 containing a cDNA insert that encodes full-length human GPIIIa (β3 integrin subunit – a kind gift of Dr Peter J. Newman, Blood Research Institute, Milwaukee, WI (Wang et al., 1992)). Cells were transfected by the DNA-CaPO₄ co-precipitation method as previously described (Osterhout et al., 1997). Plasmid DNA samples (either vector with human β3 integrin cDNA insert or vector alone) were used at a final concentration between 5 and 12 μg DNA per transfection. Transfected cells were selected by resistance to G418 (Life Technologies Ltd., Paisley, Scotland).

### Immunoprecipitation of αvβ3 integrin

Cell surface labelling and immunoprecipitation was performed as previously described (Milner and ffrench-Constant, 1994) with the exception that the protein A-sepharose suspension (Pharmacia Biotech Ltd., Buckinghamshire, UK) was precleared with rabbit anti-mouse IgG1 antiserum (10 μl/250 μl cell lysate) (Nordic Immunological Labs, The Netherlands) prior to overnight 4°C incubation with anti-human β3 integrin antibody (1 μl) (MCA 728, Serotec, Oxford, UK). Immune complexes were then collected after Milner and ffrench-Constant (1994) and bound proteins eluted from the beads by boiling for five minutes in 45 μl of non-reducing SDS sample buffer (2% SDS, 10% glycerol, 125 mM Tris-HCl, pH 6.8). Samples of eluate were resolved by SDS/PAGE on 7.5% (w/v) acrylamide gels under non-reducing conditions and separated proteins were transferred electrophoretically onto nitrocellulose membranes (Hybond-C, Pharmacia Biotech Ltd.). Blots were blocked overnight at 4°C with 3% BSA in TBST (10 mM Tris-HCl, 0.15 mM NaCl, pH 8.0, 0.1% Tween-20) and then incubated with streptavidin-HRP in TBST (diluted 1:500) for 1 hour at room temperature. The HRP-labelled proteins were subsequently detected by enhanced chemiluminescence.

### Western blot analyses

Western blot analysis was performed as previously described by Blaschuk et al. (1997) with the exception that CG-4 cells grown in DM were solubilized in sample buffer and samples (10 μg protein) were separated as above. Blots were probed overnight at 4°C with anti-human β3 integrin antibody (MCA 728, Serotec, diluted 1:400). The secondary antibody used was peroxidase-conjugated anti-mouse IgG (Pharmacia Biotech Ltd., diluted 1:5000).

### Immunofluorescence analysis of αvβ3 integrin expression

Cell surface expression and distribution of human β3 integrin was assessed using anti-human β3 integrin monoclonal antibody (MCA 728, Serotec). Cells were plated on glass coverslips (13 mm diameter, Scientific Laboratory Supplies Ltd., Nottinghamshire, UK) coated with either PDL or VN at $1.5 \times 10^4$ cells/coverslip and maintained in GM for 2 days prior to fixation or switch to DM for developmental studies. Cells were washed once with PBS, fixed in 4% formalin in PBS (Sigma) for 10 minutes at room temperature, washed, then blocked with 3% BSA in PBS for 30 minutes at room temperature. Fixed and blocked cells were washed, incubated with either the primary antibody (diluted 1:25 in PBS) or PBS alone (negative control) for 30 minutes at room temperature, washed, incubated with biotin-conjugated anti-mouse IgG secondary antibody (diluted 1:100 in PBS) for 30 minutes at room temperature, washed, incubated with rhodamine/streptavidin (diluted 1:100 in PBS) for 30 minutes at room temperature, and finally washed with PBS (all washes were performed three times with PBS). Coverslips were then inverted and mounted on glass slides with Immunofluor (ICN Biomedicals, Hampshire, UK) for fluorescence microscopy.

### Fluorescence-activated cell sorting of human β3 integrin-expressing CG-4 cells

Cultures of human β3 integrin-expressing CG-4 cells were fluorescence-activated cell sorted (FACS) to select cells expressing high levels of β3 integrin. Primary antibody (MCA 728, Serotec) specific to

human β3 integrin was used (1:250) to label expressing cells. Secondary antibody was a FITC-conjugated anti-mouse IgG1 (diluted 1:100). Cells were sorted at a density of greater than 200,000 cells/500 µl. Fluorescence flow cytometry was performed by Dr Etienne Joly of the Babraham Institute (Cambridge, UK).

### Time-lapse videomicroscopy

Cellular behaviour was recorded under time-lapse videomicroscopy over 4 successive days in culture. CG-4 cells (wild type, vector only- or human β3 integrin-transfected) were plated at $1.5 \times 10^4$ cells per 15 mm well on either PDL or VN in GM and maintained at 37°C with 7.5% $CO_2$ for 24 hours. Plates were then transferred to the stage of an inverted microscope housed in a closed, humidified chamber with internal temperature of 37°C and $CO_2$ levels of 5%. Cells were viewed under low illumination by videocamera set to record one frame each 30 seconds. After 24 hours, the cell medium was switched to DM. Recordings were then continued for another 72 hours. Migration was assessed by tracing the movements of individual cells under time-lapse. Windrose plots were generated by superimposing the tracings of many cells.

### Analysis of cellular proliferation

Cellular proliferation was assessed by measuring the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into cellular DNA by immunofluorescence assay (Kit 1-1296 736, Roche, East Sussex, UK). Cells were plated at $1.5 \times 10^4$ cells per glass coverslip (as above) on either PDL or VN in GM and incubated at 37°C with 7.5% $CO_2$ for 48 hours. Medium was then switched to DM and cellular proliferation was assessed after 1, 2 and 3 days in DM. Cells were incubated with 10 µM BrdU (final concentration) for 2 hours prior to fixation. The number of BrdU-positive cells was counted and expressed as a percentage of the total number of cells counted per field of view. At least five separate fields per coverslip (three coverslips per trial) were examined. A negative control in which the anti-BrdU primary antibody was omitted was also assayed with each trial.

### Analysis of differentiation

Differentiation was assessed by immunocytochemical examination of myelin basic protein (MBP) expression using monoclonal rat anti-MBP antibody (MCA 409, Serotec). Cells were plated on either PDL- or VN-coated glass coverslips (as above) at $1.5 \times 10^4$ cells per coverslip in GM and cultured in GM for 2 days prior to fixation or switch to DM for developmental studies. Cells were maintained for up to 15 days in culture with media replacements every 2 days. For detection of MBP expression, cells were washed twice with PBS, fixed in 4% formalin in PBS for 10 minutes at room temperature, washed once with PBS, permeabilised in ice-cold methanol for 5 minutes, washed three times with PBS, and blocked with 50% normal goat serum (NGS) in PBS for 30 minutes at room temperature. Following three washes with PBS, cells were incubated with either anti-MBP primary antibody (diluted 1:75 in PBS containing 1% NGS) or PBS containing 1% NGS alone (negative control) for 30 minutes at room temperature, washed three times with PBS, and incubated with FITC-conjugated anti-rat IgG secondary antibody (diluted 1:100 in PBS containing 1% NGS,) for 30 minutes at room temperature. Cells were finally washed three times with PBS. The total number of highly branched, MBP-positive cells was counted per coverslip (three coverslips per trial) to quantitate differentiation.

## RESULTS

To investigate the role that αvβ3 integrin may play during OPC development, cDNA encoding full-length human β3 integrin subunit was transfected into the rat OPC cell line, CG-4. The use of a cell line was necessary for the generation of stable transfectants. The CG-4 cell line was chosen because these cells closely resemble OPCs in primary culture (Louis et al., 1992) and have also been shown, following transplantation, to
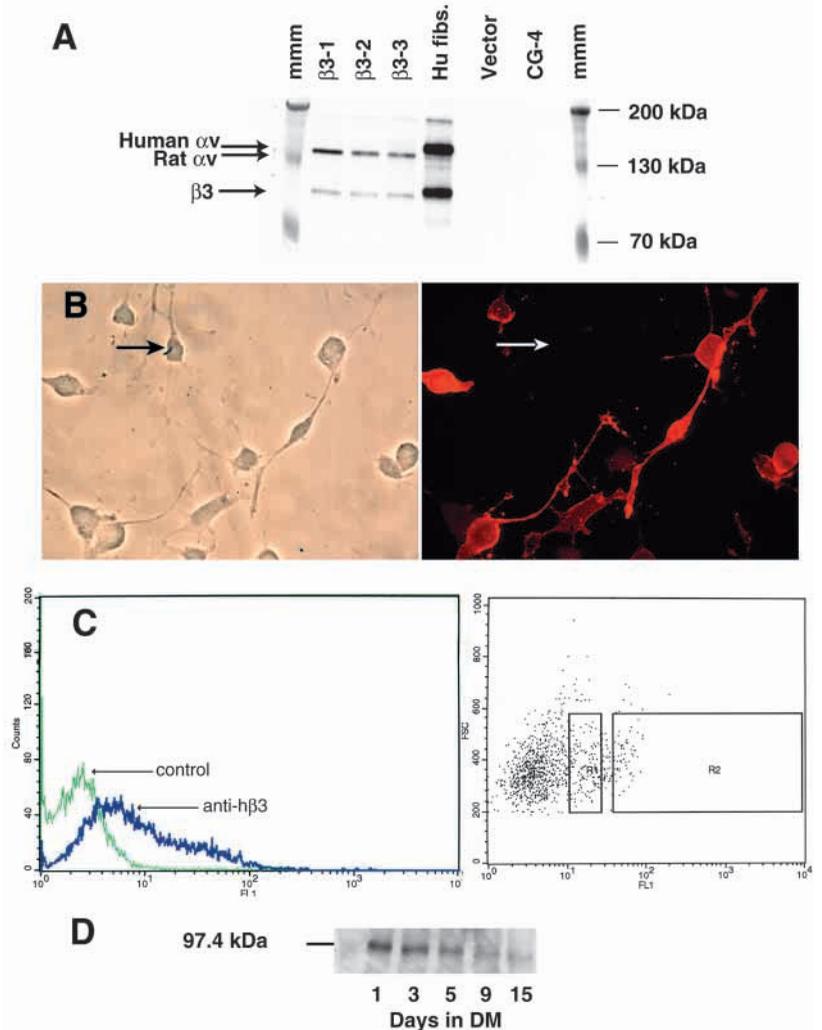


**Fig. 1.** Expression of human β3 integrin subunit in rat CG-4 cells. (A) Human β3 integrin subunit immunoprecipitates of biotin-labelled cell surface proteins from three G418-resistant clones (β3-1, β3-2, β3-3), human fibroblasts (Hu fibs.), vector only-transfected cells (vector), and wild-type CG-4 cells (CG-4). All three human β3 integrin-transfected clones co-express the rat αv and human β3 subunits. mmm represents molecular mass markers. (B) Immunofluorescence assay of cell surface expression of human β3 integrin subunit. Left panel shows phase contrast of same field viewed under immunofluorescence in right panel. Not all cells express the human β3 integrin subunit (arrow). (C) Fluorescence-activated cell sorting of human β3 integrin-expressing CG-4 cells. Left panel shows FACS analysis of cells using human β3 integrin-specific antibody (anti-hβ3) and no primary antibody (control). Right panel shows two populations of cells actually sorted (boxes R1 and R2). Only cells of box R2 which exhibited high fluorescence intensity (FL1) were used for experiments. (D) Western blot analysis of human β3 integrin subunit developmental expression in human β3 integrin-transfected CG-4 cells. Human β3 integrin-transfected CG-4 cells were grown in DM and harvested every few days up to 15 days in DM.

follow similar migratory pathways to endogenous OPCs (Baron-Van Evercooren et al., 1996) and remyelinate axons within demyelinated spinal cord lesions (Franklin et al., 1995). An important advantage of this approach is that, in the absence of any well-characterised blocking antibodies against rodent αvβ3 integrin that would allow antibody blocking studies on primary cells, we could now use well-established species- and integrin-specific antibodies against human β3 in our studies.

CG-4 cell development was typically assessed following switch of cell cultures from growth medium containing B104 conditioned medium (GM) to differentiation medium containing 0.5% fetal calf serum (DM). CG-4 cells in DM usually continue to proliferate for about 3 days with fewer and fewer cells displaying the bipolar morphology of the OPC. Cells will become tripolar, then multipolar with a corresponding decrease in cell motility as the population expands. By 3 to 4 days in DM, many of the cells in culture will exhibit a highly branched, early pre-oligodendrocytic morphology with MBP expression in the periphery of the cell bodies and along the processes. By 4 to 5 days in DM, most cells will have elaborated extensively branched processes that often inter-connect to form ring-like, MBP-positive structures. CG-4 cells at this stage of development are also postmitotic and non-motile and cannot be reverted to a bipolar, proliferative, highly motile state even by subculturing in GM. By 9 days in DM, most of the CG-4 cells in culture will have reached this stage of differentiation but will progress no further along the developmental pathway. However, some of the cells do exhibit the finely branched, leaf-like processes that are characteristic of the myelin-forming mature oligodendrocyte observed in cultures of primary oligodendrocytes (data not shown).

## Expression of human β3 integrin in CG-4 cells

CG-4 cells in a bipolar, proliferative stage of growth were transfected with the mammalian expression vector pcDNA3 containing the complete coding sequence for human β3 integrin. Several stably transfected colonies were isolated, expanded and analysed for expression of human β3 integrin subunit (Fig. 1). Immunoprecipitation of the human β3 integrin subunit from membrane extracts of transfected cells also co-precipitated the rat αv integrin subunit (Fig. 1A), as previously reported for rat myoblasts transfected with human β3 integrin cDNA (Blaschuk et al., 1997). These results demonstrate that rat αv/human β3 integrin heterodimers can both form in CG-4 cells and be expressed on cell surfaces. Cell surface expression of human β3 integrin subunit was also confirmed by immunocytochemistry (Fig. 1B). Immunofluorescence assay of human β3 integrin distribution on transfected cell surfaces revealed the strongest expression of human β3 integrin to be localised to the periphery of the cell bodies and along the processes (Fig. 1B). However, transfected cultures varied with respect to levels of expression of this integrin with some cells expressing no human β3 integrin (Fig. 1B, arrow). Consequently, transfected cultures were further sorted by fluorescence flow cytometry and only cultures expressing high levels of human β3 integrin were used for experimentation (Fig. 1C). The developmental expression of human β3 integrin was also examined by western blot analysis (Fig. 1D). Rat αv/human β3 integrin was expressed at all stages of differentiation examined with high levels expressed up to 5
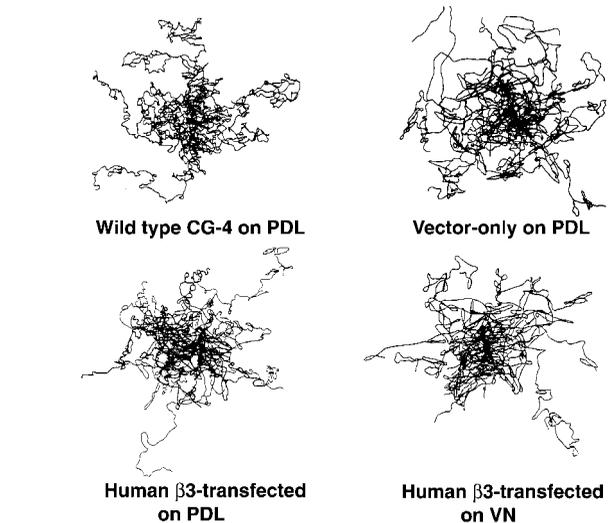


**Fig. 2.** The effects of human β3 integrin expression on CG-4 cell migration. Windrose plots of cell migration under time-lapse videomicroscopy compare wild-type CG-4 cells on PDL, vector-only transfected CG-4 cells on PDL, human β3 integrin-transfected CG-4 cells on PDL and human β3 integrin-transfected CG-4 cells on VN. Each plot was generated by tracing individual movements over a 24 hour period and superimposing the tracings of 25 cells to generate the plots as above. This figure represents 24 hours of cell movement after 1 day in DM. Cells of all three cell lines display similar patterns of migration.

days in DM. Human β3 subunit was still expressed at 15 days in DM, although levels of expression did appear to decline beyond the stages that we examined.

## Comparison of cellular behaviour by time-lapse videomicroscopy

To determine whether forced expression of human β3 integrin effects cell behaviour, time-lapse videomicroscopic analysis of human β3 integrin-expressing, vector-only transfected, and wild-type CG-4 cell behaviour was performed. Cells of all three cell lines were plated on either PDL or VN and examined under time-lapse for 1 day in GM and 3 days in DM. Wind-rose plots of cell movement over each 24 hour period were compared (Fig. 2). Cells of all three cell lines moved randomly, covered similar surface areas and moved with similar speeds on both substrata. Further, the mechanics of movement including process extension and retraction were also similar for all three cell lines on both substrata, showing that αvβ3 integrin plays little role in regulating CG-4 motility. However, human β3 integrin-expressing cells switched to DM were noted to proliferate more, as compared to vector-only transfected and wild-type CG-4 cells, but only in cultures grown on VN and not on PDL. This effect was most apparent during the first day in DM.

## The effect of human β3 integrin expression on CG-4 cell proliferation

To quantitate the increase in cell proliferation observed in the time-lapse assays of migration, human β3 integrin-expressing, vector-only transfected and wild-type CG-4 cells were plated on either VN or PDL and proliferation measured by immunofluorescence assay of BrdU incorporation each of 3
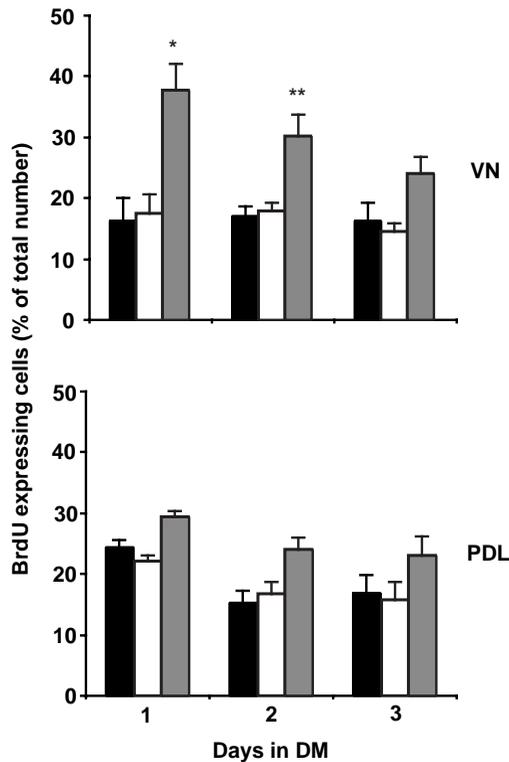
**Fig. 3.** The effects of human β3 integrin expression on CG-4 cell proliferation. Proliferation was examined in wild-type CG-4 cells (black bars), vector-only transfected CG-4 cells (white bars), and human β3 integrin-transfected CG-4 cells (grey bars) grown on either VN (top panel) or PDL (bottom panel) for 1-3 days in DM. Proliferation was assessed by immunofluorescence assay of BrdU-expressing cells. The number of cells expressing BrdU is expressed as a percentage of the total number of cells, averaged from five separate fields of view. Each bar represents the mean ± s.e.m. for three separate experiments. Each experiment is the average of three trials i.e.) 3 coverslips per cell line, per substratum, per day. Statistical test was the Student's *t*-test: *$P<0.005$; **$P<0.05$.

days following the switch from GM to DM (Fig. 3). No significant difference in the number of BrdU-expressing cells was observed between any of the three cell lines plated on PDL over the 3 days of study. In sharp contrast, cellular proliferation was markedly enhanced in human β3 integrin-expressing cells as compared to either vector-only or wild-type CG-4 cells plated on VN (Fig. 3). Consistent with the time-lapse data, the greatest effect on proliferation of human β3 integrin expression was observed after 1 day in DM for cells plated on VN. Compared to vector-only (17%) and wild-type CG-4 cells (16%), over twice the number of human β3 integrin-transfected cells (37%) were found to express BrdU after 1 day in DM when plated on VN. Proliferation by human β3 integrin-expressing CG-4 cells on VN then progressively declined over the next 2 days in DM (Fig. 3). However, proliferation remained enhanced compared to control cells even after 3 days in DM. Taken together with the time-lapse data, these results show that human β3 integrin expression stimulates CG-4 cell proliferation in a substratum-dependent manner.

To evaluate the specificity of the effect on proliferation of human β3 integrin expression, we performed a second set of experiments in which human β3 integrin-transfected CG-4

cells grown on VN were treated with an αvβ3 integrin-specific, function-blocking monoclonal antibody, LM609 (Wayner et al., 1991). Proliferation was measured 1 day after switching the cells from GM to DM as above (Fig. 4). LM609 caused a significant reduction in proliferation with the number of BrdU-expressing cells falling from 52% by nearly half to 28%. This suggests that the enhanced proliferation shown by the human β3 integrin-transfected cells is a consequence of human β3 integrin expression. Compared to the experiment shown in Fig. 3, control cells in this experiment displayed greater BrdU incorporation. This difference may reflect variations between batches of B104-CM used to supplement the GM since a new batch of B104-CM was used for cell culturing for all trials of Fig. 4.

## The effect of human β3 integrin expression on CG-4 cell differentiation

We next examined whether human β3 integrin-expressing cells also showed delayed differentiation. The developmental expression of MBP was examined in human β3 integrin overexpressing, vector-only transfected, and wild-type CG-4 cells induced to differentiate on either VN or PDL. By 9 days in DM, both the vector-only transfected and wild-type CG-4 cells had followed similar developmental pathways with respect to both MBP expression and process formation, with most cells having elaborated extensively branched, interconnected processes that displayed MBP expression (Figs 5, 6). In contrast, most human β3 integrin-expressing cells remained immature with respect to both MBP expression and process formation. Most human β3 integrin-expressing cells displayed a simple, multipolar morphology with few cells expressing MBP (Fig. 5). Compared to both control cell lines, about 80% fewer human β3 integrin-expressing cells were
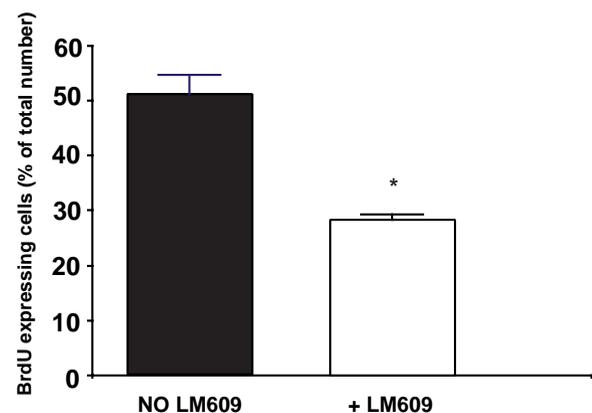


**Fig. 4.** Inhibition of proliferation by the function-blocking anti-β3 integrin antibody, LM609. Proliferation was examined in human β3 integrin-transfected CG-4 cells plated on VN after 1 day in DM in the presence or absence of the function-blocking antibody, LM609 (10 μg/ml). Proliferation was measured by immunofluorescence assay of BrdU-expressing cells. The number of cells expressing BrdU is expressed as a percentage of the total number of cells, averaged from five separate fields of view. Each bar represents the mean ± s.e.m. of three separate experiments, where each experiment represents the mean of three trials i.e. 3 coverslips per treatment. LM609 was added to cells along with the BrdU labelling reagent. BrdU incorporation was assessed 2 hours later by immunofluorescence assay. Statistical test was the Student's *t*-test: *$P<0.0005$.

**Fig. 5.** The effects of human β3 integrin expression on CG-4 cell differentiation. Differentiation was examined by immunofluorescence assay of MBP expression in wild-type CG-4 cells (left panels), vector-only transfected CG-4 cells (middle panels) and human β3 integrin-transfected CG-4 cells (right panels) plated either on PDL (this figure) or VN (not shown) and induced to differentiate in DM. Top panels show cells under phase contrast. Bottom panels show cells under immunofluorescence. This figure shows results obtained after 9 days in DM. Human β3 integrin-expressing cells are immature with respect to both MBP expression and process formation.
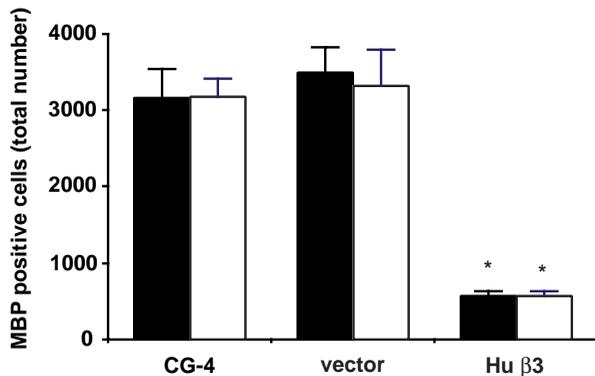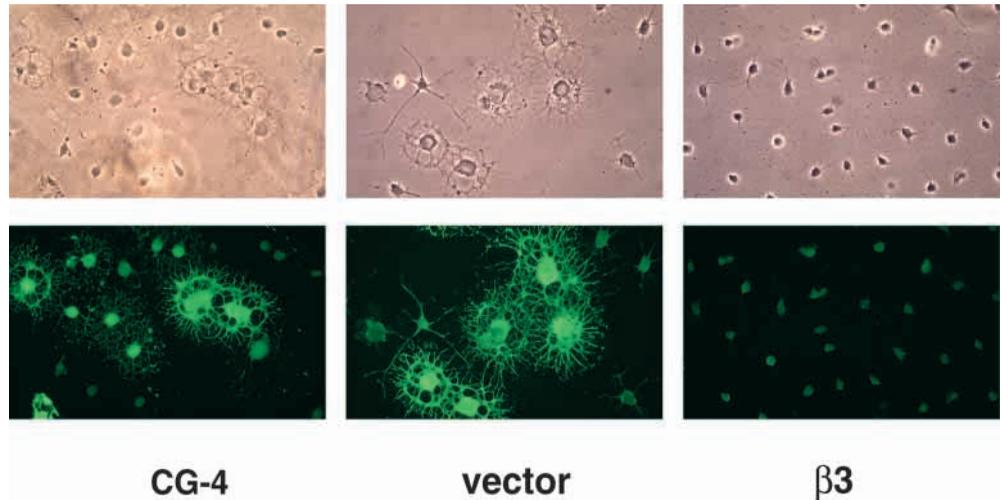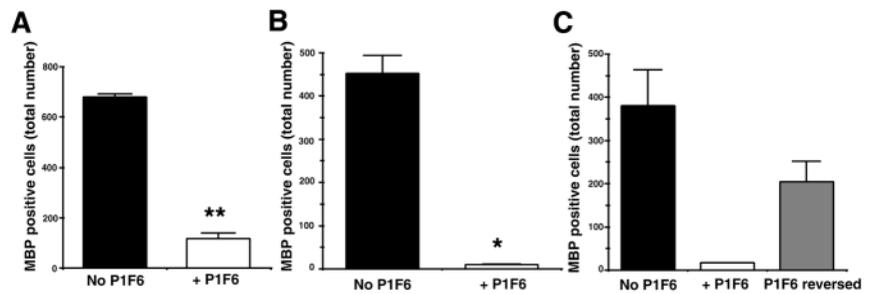


CG-4    vector    β3



**Fig. 6.** Differentiation is inhibited in human β3 integrin-expressing CG-4 cells. MBP expression was examined after 9 days in DM in wild-type CG-4 cells (CG-4), vector-only transfected CG-4 cells (vector) and human β3 integrin-transfected CG-4 cells (Hu β3) plated either on PDL (black bars) or VN (white bars). The number of cells expressing MBP is expressed as the total number of cells per coverslip. Each bar represents the mean ± s.e.m. of three separate experiments, with each experiment representing the mean of three trials i.e. 3 coverslips. Statistical test was the Student's *t*-test: *$P<0.0005$.

positive for MBP expression by 9 days in DM (Fig. 6). Interestingly, even by 15 days in DM, the development of human β3 integrin-expressing cells had not progressed beyond that observed for both the vector-only transfected and wild-type CG-4 cells at days 3 to 4 in DM (data not shown). These results suggest that differentiation is not merely delayed but inhibited in these cells. The substratum on which the cells were plated (PDL or VN) did not affect CG-4 cell differentiation (Fig. 6). Given that CG-4 cells probably begin synthesising their own ECM proteins after plating, cells in culture for more than 1 week may have elaborated quite a complex endogenous ECM. Consequently, whether inhibition of differentiation by αvβ3 integrin forced expression is substratum-dependent cannot be ascertained by these studies.

## Analysis of αvβ5 integrin function during differentiation

The presence of αvβ3 integrin is clearly inhibitory to CG-4 cell differentiation. However, a small amount of differentiation is still observed in human β3 integrin-expressing cells. One potential candidate that may regulate this differentiation is αvβ5 integrin, since levels of this integrin are upregulated co-ordinately with primary oligodendrocyte differentiation (Milner and ffrench-Constant, 1994). To determine whether

**Fig. 7.** Differentiation is inhibited by the function-blocking anti-β5 integrin antibody, P1F6. (A) MBP expression in human β3 integrin-expressing CG-4 cells plated on VN after seven days in DM following treatment with a function-blocking antibody directed against αvβ5 integrin, P1F6. The number of MBP-expressing cells is expressed as the total number of cells counted per coverslip. Each bar represents the mean ± s.e.m. of two separate experiments, where each experiment represents the mean of three trials, i.e. 3 coverslips per treatment. P1F6 was used at a final



concentration of 25 μg/ml. Antibody was added to cultures of human β3 integrin-expressing CG-4 cells plated on VN after 5 days in DM. (B) MBP expression was examined in wild-type CG-4 cells plated on VN after 6 days in DM following treatment. Each bar represents the mean ± s.e.m. of three separate experiments, where each experiment represents the mean of three trials i.e. 3 coverslips per treatment. Antibody was added to cultures of wild-type CG-4 plated on VN after 4 days in DM. (C) MBP expression in wild-type CG-4 cells maintained in DM for 4 days, with antibody added as above for an additional day, after which antibody was washed out and cells allowed to recover for 1 day in normal DM before analysis (P1F6 reversed). This result is compared with CG-4 cells grown for the same time in the absence of any antibody (no P1F6) or cells exposed to antibody for both of the last 2 days in culture (P1F6). Statistical test was the Student's *t*-test: *$P<0.005$; **$P<0.025$.

αvβ5 integrin plays a role during this differentiation, an αvβ5 integrin-specific, function-blocking monoclonal antibody, P1F6 (Wayner et al., 1991) was added to human β3 integrin-expressing CG-4 cells in an attempt to further inhibit differentiation (Fig. 7A). As the results of Fig. 7A show, treatment with P1F6 did further inhibit this differentiation. Similarly, differentiation of wild-type CG-4 cells could be almost completely blocked by treatment with the P1F6 antibody (Fig. 7B). Interestingly, the effects of P1F6 were reversible since CG-4 cells showed a rapid increase in MBP expression only 1 day following removal of the P1F6 antibody (Fig. 7C). This latter result excludes antibody toxicity as the cause for the observed block on differentiation by P1F6 antibody and further suggests that the effect of αvβ5 integrin on differentiation may be specific to signalling through this integrin. Taken together, the results suggest that both integrins may contribute to regulation of differentiation in that levels of αvβ3 integrin must be downregulated before signalling through αvβ5 integrin initiates final differentiation.

## DISCUSSION

Studies describing a switch in expression between αv-associated β integrin subunits have suggested that αvβ1, αvβ3 and αvβ5 integrins may be important regulators of OPC developmental behaviour in vitro (Milner and ffrench-Constant, 1994; Milner et al., 1996, 1997b). This hypothesis predicts that each αv integrin will function uniquely and appropriately to the stage of development at which it is maximally expressed. In support of this hypothesis, recent evidence has suggested a role for αvβ1 integrin during OPC migration in vitro, a role that is consistent with its pattern of expression in which levels of αvβ1 integrin are downregulated with loss of the migratory phenotype (Milner et al., 1996). In the present study, we have examined the possible roles that αvβ3 and αvβ5 integrins may play during OPC development in vitro. Levels of αvβ5 integrin are upregulated coincidentally with the onset of terminal differentiation suggesting a role for this integrin during differentiation. Less clear is the possible role that αvβ3 integrin may play since levels of this integrin are transiently expressed just prior to the onset of final differentiation (Milner and ffrench-Constant, 1994; Milner et al., 1996).

To examine the possible roles that αvβ3 integrin may play during OPC development in vitro, we have genetically engineered cells of the CG-4 cell lineage to express human β3 integrin so generating expression of αvβ3 integrin at times during which this integrin is not normally present. This expression of αvβ3 integrin was found to both stimulate CG-4 cell proliferation and inhibit their differentiation. This suggests that signalling through αvβ3 integrin may regulate OPC proliferation and that downregulation of αvβ3 integrin expression may be an important step controlling the timing of the final stages of OPC differentiation in vitro. αvβ3 integrin has been shown to regulate proliferation in other developmental systems in vitro (Vuori and Ruoslahti, 1994; Yokosaki et al., 1996; Schneller et al., 1997) and in both human pancreatic carcinoma cells (Vuori and Ruoslahti, 1994) and human colon carcinoma cells (Yokosaki et al., 1996) genetically engineered to constitutively express αvβ3 integrin.

Additionally, recent studies have suggested that αvβ3 integrin may directly interact with PDGF-receptor intracellular signalling pathways to potentiate the mitotic effects of PDGF on mouse fibroblasts in vitro (Schneller et al., 1997). Given the well-established role of PDGF in regulating OPC proliferation both in vitro and in vivo (Richardson et al., 1988; Calver et al., 1998), it is tempting to speculate that signals similarly transduced through αvβ3 integrin and PDGF-receptor signalling pathways may converge to synergistically regulate OPC proliferation. Clearly, further work on a possible cooperation between this integrin and PDGF and other known mitogens such as bFGF and GGF-2 (Bogler et al., 1990; Canoll et al., 1996) is required.

A role for αvβ3 integrin in regulating OPC proliferation is not readily predicted from its pattern of expression in vitro. Levels of this integrin are upregulated in OPCs which are maturing beyond the bipolar morphology associated with a highly migratory, rapidly proliferative phenotype following the withdrawal of mitogens (Milner et al., 1997b). However, by constitutively expressing this integrin in CG-4 cells, we were able to demonstrate a role for αvβ3 integrin in regulation of OPC proliferation in vitro. One interpretation of these results is that signalling through αvβ3 integrin regulates a later phase of proliferation distinct from that associated with the migratory, bipolar phenotype. This concept of distinct phases of proliferation is consistent with data from in vivo studies. OPCs arising in the subventricular zone of the developing CNS proliferate while travelling along their migratory routes. As the OPCs reach their final axonal targets, they lose migratory potential and begin to elaborate processes in preparation for final maturation into myelin-forming oligodendrocytes. However these post-migratory OPCs exhibit a further and final stage of proliferation, which is believed to ensure that sufficient cells are available for subsequent differentiation and myelination of axon tracts (Hardy and Reynolds, 1991; Gansmuller et al., 1991; Levison et al., 1993; Lachapelle et al., 1994; Miller, 1996). This final burst of proliferation could be regulated through αvβ3 integrin. The hypothesis that distinct cues regulate separate phases of OPC proliferation is supported by previous work on the neuregulin, GGF-2. This is a mitogen for OPCs (Canoll et al., 1996) which is thought to be associated with the axon surface (Morrissey et al., 1995.) and unlike PDGF, has no effect on OPC migration (Milner et al., 1997b). Based on these observations, GGF was proposed to play an important role in the proliferation that occurs only when the OPCs have reached their axonal targets and further migration is not required (Milner et al., 1997b).

Constitutive expression of αvβ3 integrin was also found to inhibit CG-4 cell differentiation. Since levels of αvβ3 integrin expression are normally downregulated coincidentally with the onset of final OPC differentiation in vitro (Milner and ffrench-Constant, 1994), these findings suggest that downregulation of αvβ3 integrin expression may be required for OPC differentiation to proceed. Blaschuk et al. (1997) have similarly suggested that downregulation of αvβ3 integrin expression may be an essential feature of myogenic terminal differentiation, since forced expression of αvβ3 integrin was found to maintain skeletal myoblasts in an undifferentiated state. Interestingly, we have also observed that treatment with function-blocking antibodies directed against αvβ5 integrin can both further inhibit differentiation in human β3 integrin-

expressing CG-4 cells and inhibit differentiation in wild-type CG-4 cells. This suggests that αvβ5 integrin may play a role in regulating OPC differentiation, a role that is consistent with its pattern of expression, in which levels of αvβ5 integrin are upregulated during the final stages of differentiation in vitro (Milner and ffrench-Constant, 1994). These results are also consistent with the possibility that αvβ3 integrin expression may regulate the function of αvβ5 integrin, with downregulation of αvβ3 integrin expression required to allow αvβ5 integrin to stimulate differentiation. αvβ3 integrin could inhibit the function of αvβ5 integrin either by competing with αvβ5 integrin for binding to the same ligand or by inhibiting upregulation of αvβ5 integrin expression. Further studies are required to distinguish between these possibilities although the present studies suggest that a switch in expression from the β3 to the β5 integrin subunit may control the timing of the onset of final OPC differentiation in vitro.

## A model for the control of oligodendrocyte behaviour by developmental switching between αvβ1, αvβ3 and αvβ5 integrins

Distinct roles for αvβ1, αvβ3 and αvβ5 integrins during oligodendrocyte development in vitro can now be ascribed, lending support to the hypothesis that developmental switching between the αv-associated β subunits may control oligodendrocyte behaviour (Milner and ffrench-Constant, 1994). Based on previously published and currently presented observations, the following model is proposed. During the early stages of oligodendrocyte development, OPCs are mitotic and highly migratory. OPC migration is mediated at least in part through αvβ1 integrin, levels of which are upregulated at this time. Levels of αvβ1 integrin are subsequently downregulated as OPCs lose their migratory potential. A switch in expression from αvβ1 to αvβ3 integrin occurs as cells prepare for final differentiation. Signalling through αvβ3 integrin contributes to a final burst of proliferation. Levels of αvβ3 integrin are subsequently downregulated to allow differentiation to proceed. A switch in expression from αvβ3 to αvβ5 integrin then regulates the onset of final differentiation, with levels of αvβ5 integrin upregulated co-ordinately with final differentiation.

In this model, developmental switching between αvβ1, αvβ3 and αvβ5 integrins instructs the transition between successive stages of oligodendrocyte development in vitro. This provides a novel mechanism that would form part of the controls of oligodendrocyte differentiation. The model also significantly extends the proposed roles of integrins in the control of development. Previous studies have shown how changes in integrin expression and activation can regulate sequential steps within developmental processes, including keratinocyte differentiation, trophoblast invasion and lens cell differentiation (Watt and Jones, 1993, Sutherland et al., 1993, Damsky et al., 1994, Hotchin et al., 1995, Walker and Menko, 1999). Our work now shows how sequential switching between three different αv integrins within a single cell type may control the timing of proliferation and differentiation, in addition to migration. Future studies are aimed at dissecting each of the intracellular signalling pathways through which each integrin regulates oligodendrocyte behaviour. The use of transgenic animals lacking individual β integrin subunits and the expression of chimeric integrins in which the different

cytoplasmic domains are interchanged should prove useful to these studies.

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