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Cell Cycle Arrest Induced by Ectopic Expression of p27 Is Not Sufficient to Promote Oligodendrocyte Differentiation

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Abstract Oligodendrocyte differentiation is accompanied by dramatic changes in gene expression as well as cell cycle arrest. To determine whether cell cycle arrest is sufficient to induce the changes in cell phenotype associated with differentiation, we inhibited oligodendrocyte precursor proliferation in vitro by overexpressing p27, a cyclin kinase inhibitor, using a recombinant adenovirus. Ectopic expression of p27 efficiently inhibited oligodendrocyte precursor cell division, even in the presence of exogenous mitogens, by blocking the activity of the cyclin-dependent kinase, cdk2. Although the cells had stopped dividing, they did not express galactocerebroside (GalC) or myelin basic protein (MBP), changes associated with oligodendrocyte differentiation, suggesting that they had not differentiated. After removal of exogenous mitogens, however, adenovirus-expressing oligodendrocyte precursors differentiated with a temporal profile similar to that of control, uninfected oligodendrocytes, as indicated by expression of GalC and MBP. We conclude that cell cycle arrest is not sufficient to induce differentiation of dividing oligodendrocyte precursors, and that modulation of additional, as yet unknown, signaling pathways is required for this to occur. J. Cell. Biochem. 76:270–279, 1999.

Key words: oligodendrocytes; cell cycle; differentiation; p27; cdk2; cdk5

Cell cycle arrest is a key event during the process of oligodendrocyte differentiation. Dividing oligodendrocyte progenitors arise in the subventricular zone [Goldman, 1992; LeVine and Goldman, 1988], from which they migrate out and populate the developing brain [Levinson et al., 1993]. Once they reach their final destination, the oligodendrocyte progenitors exit the cell cycle and extend multiple processes that ensheath several axons and produce myelin. These changes are associated with the activation of a program of myelin-specific gene expression.

Cell cycle progression of oligodendrocytes is regulated, at least in part, by signaling pathways mediated by aa-Platelet-Derived Growth Factor (PDGF), basic Fibroblast Growth Factor (bFGF), thyroid hormone, and axonal contacts [Barres et al., 1993, Barres and Raff, 1994; McKinnon et al., 1990; Pringle et al., 1992; Richardson et al., 1988; Hart et al., 1989]. Oligodendrocyte precursors grown in vitro in chemically defined media containing thyroid hormone and PDGF, for example, recapitulate their program of cell division and differentiation according to a schedule similar to that found in vivo [McMorris and McKinnon, 1996; Raff et al., 1983, 1988; Grinspan et al., 1990]. By con-

Abbreviations used: cdk, cyclin-dependent kinase; bFGF, basic fibroblast growth factor; PDGF; platelet-derived growth factor; cki, cyclin-dependent kinase inhibitor; GalC, galactocerebroside; MBP, myelin basic protein; TUNEL, terminal UTP nick end-labeling; MTT, 3-[4,5-dimethylthiazol-Z-yl]-2,5-diphenyltetrazolinum bromide, R-mAb, Ranscht monoclonal antibody.

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trast, the presence of bFGF and PDGF [Bogler et al., 1990; McKinnon et al., 1990] or B104-conditioned media [Louis et al., 1992] is sufficient to inhibit oligodendrocyte differentiation and to permit continued cell division. After removal of growth factors, however, oligodendrocyte precursors rapidly stop dividing and differentiate into oligodendrocytes [Bogler et al., 1990; McKinnon et al., 1990; Collarini et al., 1992; Tang et al., 1998].

The signaling pathways discussed above exert their influence on oligodendrocyte proliferation by regulating, either directly or indirectly, the activity of cyclin-dependent kinases, cdk4/6 and cdk2 [Pardee, 1989; Morgan, 1995; Peeper and Bernards, 1997], which are themselves regulated by a set of inhibitory proteins, the cyclin kinase inhibitors (cki) [Sherr and Roberts, 1995]. Cell cycle withdrawal of oligodendrocytes is associated with accumulation of the universal cyclin kinase inhibitor, p27, which is known to inhibit cdk2 activity and thus arrest cells at G1-S transition [Casaccia-Bonnefil et al., 1997; Tang et al., 1998]. Activation of additional pathways, however, must also be required for oligodendrocyte differentiation, since precursors that have accumulated p27, do not differentiate and continue to divide in the presence of mitogens [Durand et al., 1997]. In addition, oligodendrocyte precursors isolated from p27 knockout mice can stop dividing and differentiate both in vivo and in vitro [Casaccia-Bonnefil et al., 1997; Durand et al., 1998]. Because oligodendrocyte differentiation is also accompanied by an increase in cdk5 activity [Tang et al., 1998], a kinase necessary for several differentiation programs [Lew et al., 1992; Lazaro et al., 1997; Philpott et al., 1997; Hellmich et al., 1992; Lee et al., 1996; Gao et al., 1997] cdk5 activation may be one of these additional pathways.

In order to determine whether cell cycle arrest is itself sufficient to promote oligodendrocyte differentiation, we have forced expression of p27 in actively dividing precursors and have characterized both their progression through the cell cycle and their ability to differentiate. We find that oligodendrocyte precursors overexpressing p27 are arrested at the G1-S transition and cease dividing, as expected. In the presence of exogenous mitogens, however, such as those contained in B104-conditioned media, these cells fail to differentiate, although removal of these mitogens is rapidly followed by differentiation. Cell cycle arrest is thus not sufficient to induce differentiation of oligodendrocyte precursors, and the modulation of additional signaling pathways is required for this to occur.

MATERIALS AND METHODS

Adenovirus Vectors

Construction of Ad-p27 was previously described [Craig et al., 1997]. Ad-null used in this study is a replication-defective adenovirus dl312 containing no insert. Adenoviruses were propagated in 293 cells grown in monolayer, purified by two cesium chloride density centrifugations, dialyzed against a buffer containing 10% glycerol and 1 mM $MgCl_2$, titered, and stored at −70°C [Craig et al., 1997]. To control for variation between virus preparations, multiple preparations of each virus were used.

Preparation of Rat Primary Mixed Glial Cultures

Primary cortical glial cultures were established from 1-day-old Sprague-Dawley rat brain after carefully removing the meninges, as previously described [Behar et al., 1988; Tang et al., 1998; Grinspan et al., 1990]. Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) (4.5 g/L D-glucose) supplemented with gentamycin 25 μg/ml, insulin 5 μg/ml and 10% fetal calf serum (FCS) for 7–10 days in incubator at 37°C and 10% CO_2. In some experiments, mixed cultures were grown in B104-conditioned medium [Louis et al., 1992] to increase the number of oligodendrocyte precursors and decrease the proportion of astrocytes.

Preparation of Rat Immunoselected Precursors

Oligodendrocyte precursors were isolated from mixed cultures by shaking on a rotary platform (250 rpm) overnight at 37°C, followed by immunoselection. For immunopanning, 100-mm dishes were incubated overnight at 4°C with 5 μg/ml unconjugated secondary antibody in 50 mM Tris pH 9.5. The dishes were then washed with Ca- and Mg-free phosphate-buffered saline (PBS) and incubated for 2 h with primary antibody diluted 1 in 10. In order to remove microglia and macrophages, cells suspended in MEM plus 0.5% FCS were first plated on uncoated 100-mm dishes for 3 h at 37°C. Single-cell suspension in MEM containing 0.5 % FCS was passed over one dish coated with 1:10 dilution of supernatant. Unattached

cells were then passed sequentially over dishes coated with Ran-2 antibody (a kind gift of Dr. McMorris, Wistar Institute) to remove astrocytes and meningeal cells and with O1 antibody [Sommer and Schachner, 1981] (recognizes galactocerebroside, GalC) (a kind gift of Dr. McMorris) to remove mature oligodendrocytes. Finally, precursors were plated onto a dish coated with A2B5 antibody [Eisenbarth et al., 1979] and the nonadherent cells were removed by flushing. The A2B5-positive precursors were removed by trypsinization and expanded in N1 medium containing 30% B104-conditioned medium, 2 ng/ml PDGF and 10 ng/ml FGF [Louis et al., 1992].

Oligodendrocytes were differentiated in a DMEM/F12 medium (1:1) containing 50 μg/ml transferrin, 5 μg/ml putrescine, 3 ng/ml progesterone, 2.6 ng/ml selenium, 12.5 μg/ml insulin, 0.4 μg/ml T4, 0.3% glucose, 2 mM glutamine, and 10 ng/ml biotin [Casaccia-Bonnefil et al., 1997]. Precursors were plated 72 h before adenoviral infection in either 6-well plates coated with poly-D-ornithine at 5×10^5 cells per well for biochemical studies or in 24-well plates containing coverslips coated with poly-D-ornithine at 4×10^5 per well for immunocytochemistry. Precursors were infected with either recombinant or control adenovirus for 24 h, medium was changed, and cells were grown in either B104-conditioned media or differentiation medium devoid of growth factors for an additional 24–48 h.

Immunocytochemistry

Cultures were characterized by indirect immunofluorescence with stage-specific antibodies, A2B5 [Eisenbarth et al., 1979] and R-mAb [Ranscht et al., 1982], which recognizes GalC in addition to other glycolipids expressed earlier than GalC. Cells were fixed in 2% paraformaldehyde for 1 min, reacted with undiluted hybridoma supernatant for 30 min at room temperature, followed by either FITC- or rhodamine-conjugated secondary antibody diluted 1:50 for 30 min at RT, postfixed in acidic-alcohol (5%/95%) for 10 min, and stained with 4,6-diamino-2-phenolidole (DAPI) for 5 min. Expression of p27 was determined by immunofluorescence using rabbit polyclonal IgG to p27 (Santa Cruz Biotechnology, C-19). Cells were fixed in methanol at −20°C for 5 min, incubated with 1 μg/ml p27 antibody for 30 min, followed by biotinylated donkey anti-rabbit and streptavidin-fluorescein (Amersham) each diluted 1:50 for 30 min and then stained with DAPI for 5 min. Coverslips were mounted on glass slides with Vectashield; labeled cells were viewed using Leica DMR fluorescent microscope.

Cell Cycle Analysis

Cell cycle analysis was carried out by staining with propidium iodide, using the DNA test plus kit (Becton-Dickinson), according to the manufacturer's protocol. Briefly, cells were suspended in buffer containing 250 mM sucrose, 40 mM trisodium citrate, stained with propidium iodide, and then analyzed by flow cytometry.

Immunokinase Assays and Western Blot Analysis

Cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA (pH 8), 0.5% Nonidet P-40 (NP-40), 5 mM DTT, 1 mM PMSF, 10 mM NaF, 1 mM $NaVO_4$, 20 mM NaP_2O_7, 10 μg/ml each of leupeptin, aprotinin, and pepstatin) for 30 min on ice, as previously described [Tang et al., 1998]. A total of 50 μg of cell lysate were immunoprecipitated with a rabbit polyclonal IgG to cdk2 or to cdk5 followed by kinase assay with 20 μM ATP, 2 μg histone H1 (Upstate Biotechnology) as a substrate and 2 μCi of γ-^{32}P-ATP (New England Nuclear), as described [Tsai et al., 1993; Tang et al., 1998]. Phosphorylated histone H1 was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), gels were dried, and bands were visualized and quantitated by PhosphorImager (Molecular Dynamics). Steady-state levels of p27, cdk5, cdk2, and actin were determined by Western blot analysis. A total of 15–50 μg of proteins were separated by 10% SDS-PAGE, transferred to PVDF membrane, and reacted with commercially available antibodies to p27 (Santa Cruz Biotechnology, C-19), cdk5 (Santa Cruz Biotechnology, C8), cdk2 (Santa Cruz Biotechnology, M2) and actin (Santa Cruz Biotechnology, I-19) at 1:1,000 dilution, followed by horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:10,000 and developed with enhanced chemiluminescence (ECL)(Amersham) [Tang et al., 1998]. Actin was used as an internal control for accurate loading.

Cytotoxicity Analysis by MTT and TUNEL Assays

In order to assess survival, MTT assays were carried out after infection of oligodendrocyte

precursors with either 1 or 10 pfus/cell of either Ad-null or Ad-p27, using uninfected cells as a baseline for cell death. Cells were incubated in 1 mg/ml MTT at 37°C for 2 h, lysed and the solution read in a spectrophotometer at 570 nm [Yasuda et al., 1995]. Infections with 10 pfus/cell of either Ad-null or Ad-p27 induced significant cell death both at 24 and 72 h, whereas 1 pfu/cell resulted in greater cell survival both at 24 and 72 h (Fig. 1). Thus, dose-dependent cytotoxicity was detected with both control and p27 adenovirus at comparable levels (Fig. 1). Because cell death is observed with both viruses, toxicity is due to the protein load of exogenously added adenovirus. Since infection of precursors with 2 and 5 pfus/cell significantly reduced survival at 72 h, we have carried out infections with 1pfu/cell.

For TUNEL assay [Gavrieli et al., 1992; Yasuda et al., 1995], coverslips were pre-equilibrated in TdT buffer (30 mM Trizma base pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). TdT (18 U/coverslip) and biotinylated dUTP were added, and the coverslips were incubated at 37°C for 60 min. The reaction was terminated by incubating the coverslips in TB buffer (300 mM sodium chloride, 30mM sodium citrate) for 15 min at room temperature, followed by streptavidin rhodamine diluted 1:100 in PBS for 30 min at room temperature.

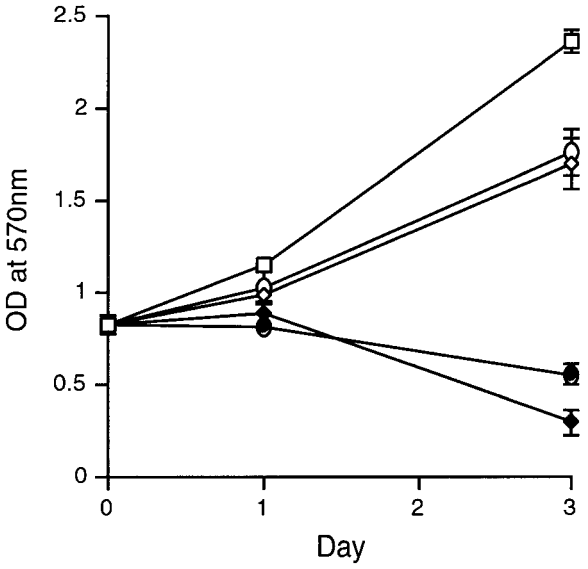


Fig. 1. Adenoviral infection affects cell viability in a dose-dependent manner. Cells were treated with no virus (squares), Ad-null virus at 1 (open diamonds), or 10 (closed diamonds) pfus/cell or Ad-p27 virus at 1 (open circles) or 10 (closed circles) pfus/cell for 24 h. Cell viability was assessed at 24 and 72 h by MTT assay. Each data point represents the mean of 4 wells ±SD.

RESULTS

Titrated Amounts of p27 Containing Adenovirus Resulted in High Levels of p27 Expression

Cytotoxicity associated with adenoviral infection [Craig et al., 1997] significantly reduces survival of oligodendrocyte precursors at 24 h and 72 h after infection; however, we have found that 1 pfu/cell was optimal to minimize cell death (see under Materials and Methods and Fig. 1). We chose to assess survival 24 h after infection, because it has been previously shown that cell cycle progression is arrested efficiently by this time in tumor cell lines [Craig et al., 1997] and in the oligodendrocyte progenitor cell line CG4 cells (our unpublished observations). In addition, oligodendrocyte differentiation also begins 24 h after cell cycle arrest induced by removal of mitogens [Collarini et al., 1992; Tang et al., 1998]. This transition has been associated with increased vulnerability to apoptosis [Barres et al., 1993; Yasuda et al., 1995]. We also assessed survival 72 h after infection and cell cycle arrest, as most cells will have differentiated into R-mAb-positive oligodendrocytes, after growth factor removal, by this time [Collarini et al., 1992; Tang et al., 1998].

We determined whether p27 is expressed at high levels after infection with Ad-p27 using Western blot analysis and immunocytochemistry. Precursors grown in B104-conditioned medium were infected with either Ad-null or Ad-p27 at 1 and 10 pfus/cell. Western blots of cell lysates were probed with p27 antibody. High levels of p27 expression were detected in both extracts after 24 and 72 h (Fig. 2A,B). Comparably lower levels of p27 protein were detected in Ad-null infected and uninfected precursor extracts (Fig. 2C). Thus, infection with 1 pfu/cell of p27 adenovirus induces high-level expression of p27 protein up to 72 h.

Immunocytochemical detection of p27 was carried out on cells plated on poly-D-lysine-coated coverslips 24 h after adenoviral infection by staining with a polyclonal antibody to p27. As shown in Figure 3, some endogenous expression of p27 is evident in uninfected cells (Fig. 3A); however, the nuclei of cells overexpressing p27 were clearly detected by their intense staining (Fig. 3B,C). The number of cells exhibiting nuclei brightly stained with p27 antibody was counted in 10 63× microscope fields per coverslip. The percentage of intensely stained nuclei

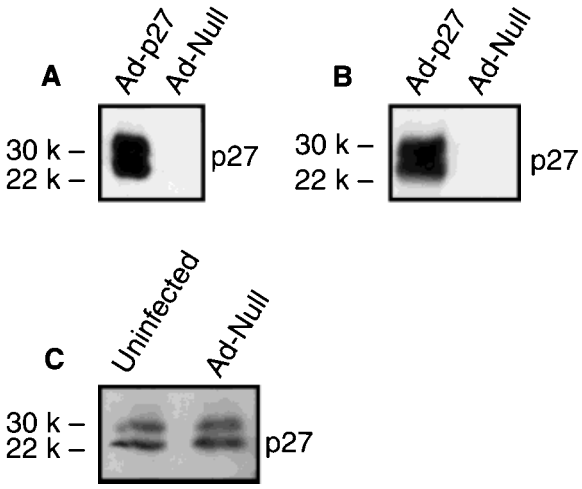


Fig. 2. Oligodendrocyte precursors infected with Ad-p27 express high levels of p27 protein. Western blot analysis demonstrating the presence of p27 in oligodendrocyte precursor extracts. Precursors were infected in the presence of exogenous mitogens with either Ad-p27 or Ad-null at either 10 pfus/cell for 24 h (**A**) or 1pfu/cell for 72 h (**B**). For Ad-p27 infected precursors, 15 µg (**A**) and 20 µg (**B**) of proteins were loaded. For Ad-null-infected (**A–C**) and uninfected precursors (**C**), 50 µg of proteins were loaded. A strong signal is detected in Ad-p27-infected oligodendrocyte extracts at both 24 and 72 h. p27 is not detected in Ad-null infected oligodendrocyte extracts (**A,B**), as blots were exposed for short time. However, in a separate blot (**C**), p27 is detected at comparable level in both infected and uninfected cell extracts.

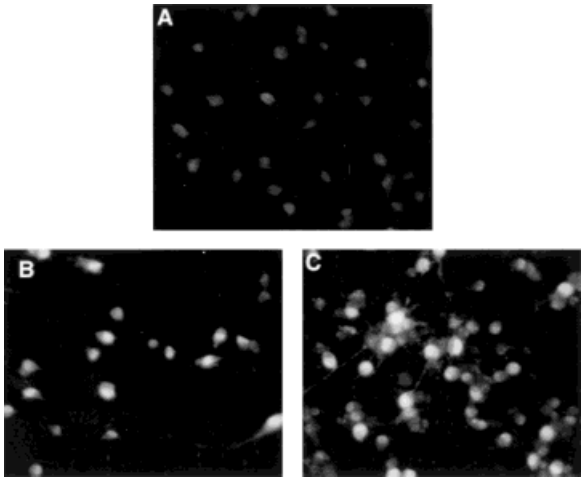


Fig. 3. p27 is expressed in the nucleus of oligodendrocyte precursors infected with Ad-p27. Oligodendrocyte precursors were infected with Ad-null virus (**A**) or with Ad-p27 virus at 1 (**B**) or 10 (**C**) pfus/cell and maintained in B104 conditioned medium for 24 h. Labeling with antibody to p27 (Santa Cruz Biotechnology, CA, C-19) was visualized by immunofluorescence microscopy, as described under Materials and Methods.

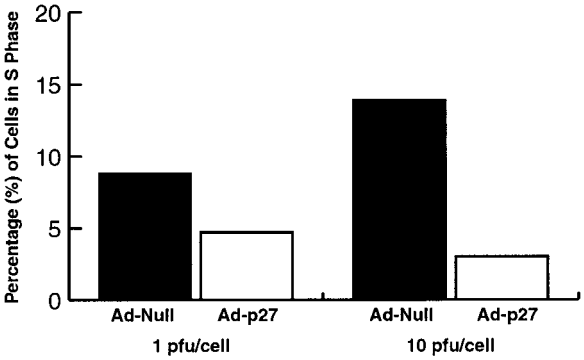


Fig. 4. Forced expression of p27 leads to significant decrease in the number of oligodendrocyte precursors traversing S phase. Representative cell cycle analysis of oligodendrocyte precursors infected with 1 or 10 pfu/cell of either Ad-p27 or Ad-null. Infection with 1 and 10 pfu/cell of Ad-p27 reduced the percentage of cells synthesizing DNA of 50% and 80% compared with Ad-null infected oligodendrocytes, respectively.

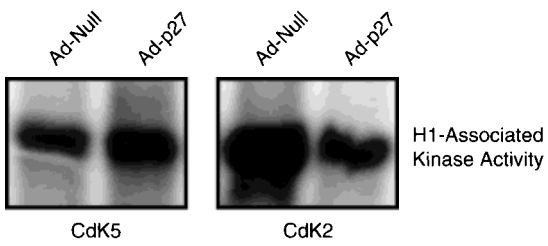
averaged 35% and 49 % of the total number of nuclei in cells infected with 1 and 10 pfus/cell, respectively.

Overexpression of p27 Induces Cell Cycle Arrest in G1 and Inhibits cdk2 Activity of Oligodendrocyte Precursors

Cell cycle arrest was determined by flow cytometry with propidium iodide at 24 h after adenoviral infection. After infection of precursors with 1 and 10 pfus/cell of Ad-p27, the number of cells traversing S phase was found to be respectively 50 and 80% lower than in precursors infected with control adenovirus (Fig. 4). No sub-G1 peak or hypodiploid peak was identified, suggesting that apoptosis does not occur at this time point. Infection with Ad-null did not have an effect on cell cycle distribution, as compared with uninfected precursors (data not shown).

We then determined whether the arrest of cell cycle progression at G1-S transition results from inhibition of cdk2 activity. H1-associated cdk2 kinase activity was assayed in extracts of precursors at 24 and 72 h after adenoviral infection with 1 pfu/cell. Cdk2 kinase activity in oligodendrocytes overexpressing p27 was 50% lower than that detected in precursors infected with Ad-null at both 24 h and 72 h after infection (Fig. 5A,B). The fall in cdk2 activity was of the same order of magnitude as the decrease in number of cells traversing S phase, suggesting that cell cycle arrest induced by p27 at G1-S transition results from inhibition of cdk2 activity by p27. In support of such a mechanism, is the finding that the level of cdk2 protein detected by Western blot analysis in Ad-p27 in-

A



B

Time in culture after Infection with Ad-p27	Cdk2 activity	Cdk5 activity
24 hours	54% ± 17	170% ± 38
72 hours	47% ± 7	112% ± 29

Fig. 5. Analysis of cdk2 and cdk5 H1-associated kinase activity of oligodendrocyte precursors infected with p27 containing adenovirus. Oligodendrocyte precursors infected with either Ad-null or Ad-p27 were cultured in B104 conditioned medium for 24 and 72 h. Cdk2 and cdk5 proteins were immunoprecipitated with commercial antibodies (Santa Cruz Biotechnology, CA) from 50 μg of cell extracts. Kinase assays were carried out as described under Materials and Methods. **A:** Representative cdk2 and cdk5 kinase activities in oligodendrocytes 24 h after infection with 10 pfus/cell of either Ad-p27 or Ad-null. Cdk2 activity is greatly reduced by Ad-p27 infection, whereas a modest increase in cdk5 activity is observed. **B:** Percentage changes of cdk2 and cdk5 kinase activities of oligodendrocytes infected with 1 pfu/cell of Ad-p27. Cdk2 and cdk5 kinase activities are expressed as percentage of the activities detected in Ad-null-infected oligodendrocytes, which are taken as 100%. The data represent the mean of three independent experiments ± SE. Cdk2 activity is decreased to approximately 50% of the control values, at both 24 and 72 h. An increase in cdk5 activity is detected at 24 h, although, at 72 h, cdk5 activity is approximately equivalent to that of control cells.

fected precursors is similar to that in Ad-null infected precursors (data not shown).

Oligodendrocyte Precursors Overexpressing p27 Fail to Undergo Differentiation and Do Not Activate cdk5

In an effort to determine whether oligodendrocyte precursors would undergo differentiation after cell cycle arrest in G1, untreated precursors and precursors infected with Ad-p27 or Ad-null were maintained in B104-conditioned medium, and the number of immature and mature cells was determined by immunofluorescence microscopy. The A2B5 antibody was used to identify precursors and R-mAb was used to identify differentiated oligodendrocytes. These experiments were performed using 1 pfu/cell of virus.

The percentage of R-mAb positive cells (Fig. 6, panel 1, A,B, and panel 2) in precursors infected with Ad-p27 was similar to that of precursors infected with control virus. We have found that the numbers of R-mAb positive cells are slightly higher in both precursors infected with Ad-p27 and Ad-null than in uninfected oligodendrocytes (Fig. 6, panel 2). However, the increase was not statistically significant (see *P*-values in legend Fig. 6). In addition, since this phenomenon is observed with both Ad-p27

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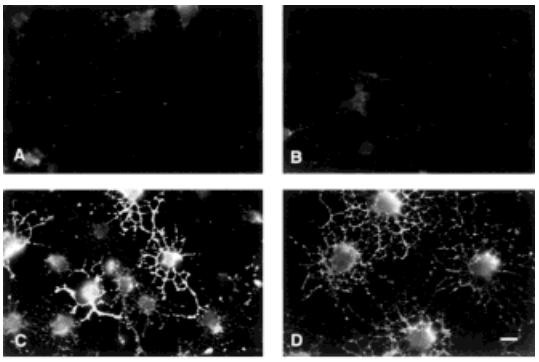

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% GalC+ cells	48hrs	72hrs
In conditioned medium:		
Uninfected	5 ± 2.1	10 ± 0.5
Ad-null	9 ± 2.8	19 ± 4
Ad-p27	15 ± 4.3	28 ± 5
In differentiation medium:		
Uninfected	59 ± 27.8	83 ± 4.2
Ad-null	52 ± 17	86 ± 0.7
Ad-p27	56 ± 15.7	88 ± 4.2

Fig. 6. Oligodendrocytes infected with Ad-p27 differentiate on the same schedule as control oligodendrocytes. **1:** R-mAb labeling of oligodendrocyte cultures infected with either Ad-null (**A,C**) or Ad-p27 (**B,D**). Oligodendrocyte precursors were infected with virus and maintained in B104 conditioned medium for 24 h. Some coverslips remained in conditioned medium for another 48 h, followed by staining with the R-mAb antibody (A,B). Few R-mAb + cells were detected. Other coverslips were switched to differentiation medium for 48 h, followed by staining with R-mAb (**C,D**). Most cells extended processes and were labeled with the antibody. Scale bar = 10 μm. **2:** Percentages of R-mAb + cells in oligodendrocyte precursors treated with no virus, Ad-null, or Ad-p27 at 1 pfu/cell. Cells were maintained in either B104 conditioned medium or differentiation medium for 24 or 72 h. Each data point represents the mean ± SE of three independent experiments. Analysis of variance indicates that the differences between uninfected, Ad-null, and Ad-p27 at 48 and 72 h in conditioned medium are not statistically significant ($P = 0.11$; $P = 0.19$, respectively, using the exact Monte Carlo methods for the Kruskal-Wallis test).

and Ad-null virus, it most likely represents a nonspecific effect of the adenoviral infection per se. Precursors may be more sensitive to cytotoxicity caused by the adenoviral infection than are early oligodendrocytes, resulting in preferential survival of R-mAb-positive cells.

Despite cell cycle withdrawal, cells continued to be stained with A2B5 antibody and maintained a bipolar morphology typical of precursors. Finally, we have not found an increase in apoptotic nuclei, by nuclear DNA fragmentation with either DAPI staining or TUNEL technique (data not shown), in p27 overexpressing precursors 72 h after infection.

We have previously reported that an increase in cdk5 activity accompanies oligodendrocyte differentiation 72 h after removal of growth factors [Tang et al., 1998]. In order to elucidate whether cdk5 activity rises as part of oligodendrocyte differentiation, we have assayed cdk5 activity in p27 overexpressing precursors. These precursors stop dividing in the presence of growth factors, however they fail to differentiate (see above). Thus, cdk5 activity would not be expected to increase in these cells, in the event that cdk5 activation and differentiation are coupled. At 24 h postinfection, when precursors are stopped in G1-S transition, H1-associated cdk5 activity was found to be increased, approximately 1.5-fold higher than that of cells infected with Ad-null. However, at 72 h postinfection, H1-associated cdk5 kinase activity in extracts of precursors infected with Ad-p27, which have not differentiated, was found to be similar to that of control oligodendrocytes infected with Ad-null (Fig. 5B). Thus, we interpret these data as suggesting that an increase in cdk5 activity may be coupled with oligodendrocyte differentiation.

Precursors Overexpressing p27 Differentiate Normally After Removal of Growth Factors

In order to determine whether oligodendrocyte precursors overexpressing p27 and arrested in G1 are able to differentiate, growth factors were removed at 24 h after adenoviral infection, and the cells were allowed to differentiate. They were then analyzed by immunofluorescence microscopy and the number of R-mAb-positive cells was counted as a measure of the number of differentiated oligodendrocytes. The results of this experiment, shown in Figure 6, demonstrate that precursors infected with either Ad-p27 or Ad-null differentiate into mature oligodendrocytes in a similar temporal schedule after removal of growth factors. A comparable increase in the percentage of R-mAb-positive cells at 24 and 48 h was found in both Ad-null and Ad-p27 infected oligodendrocytes (Fig. 6, panel 2, C,D). Finally, MBP, a marker of mature myelinating oligodendrocytes usually expressed 4–5 days after removal of mitogens, was detected in similar numbers in either Ad-null or Ad-p27-infected cells (data not shown). In addition, we have observed that oligodendrocytes overexpressing p27 are more highly branched than control virus-infected cells.

DISCUSSION

Oligodendrocyte differentiation is a multistep, temporally regulated process that includes both a dramatic change in gene expression and the arrest of cell proliferation. In the in vitro setting, bFGF [McKinnon et al., 1990; Bogler et al., 1990; Louis et al., 1992] can reversibly inhibit oligodendrocyte differentiation as well as activate progression through the cell cycle, suggesting that these events are linked. In this article, we show that inhibition of cdk2 in the presence of bFGF or B104-conditioned media can produce cessation of proliferation without oligodendrocyte differentiation, demonstrating that these processes can be uncoupled from one another. Using different experimental conditions, Tikoo et al. [1998] have reported results similar to ours. Interestingly, although these investigators used 100 pfus/cell, only 50% of cells were infected, similar to our results using only 1 pfu/cell. In addition to previously reported results, we show that oligodendrocytes overexpressing p27 differentiate with a temporal profile similar to that of control oligodendrocytes after the removal of mitogens.

Oligodendrocyte differentiation is thus accompanied by the coordinate regulation of two separate processes: one that inactivates cdk2 and thus cell cycle progression, and a second that modulates the pattern of oligodendrocyte gene expression and activates a set of myelin-specific genes. Consistent with the above interpretation, during the early stages of oligodendrocyte differentiation there is both a decrease in cell proliferation and an increase in apoptosis [Barres et al., 1993, Barres and Raff, 1994]. In oligodendrocyte precursors overexpressing p27, however, which do not differentiate, we have found that apoptosis does not increase, even though cell proliferation has stopped. The onset

of apoptosis may thus depend on the activation of signaling pathways associated with cell differentiation, and not on those associated with cell cycle arrest, again suggesting that these two processes are regulated separately.

Although the cyclin kinase inhibitor, p27, has been implicated in the regulation of oligodendrocyte proliferation [Casaccia-Bonnefil et al., 1997; Durand et al., 1997] and can inactivate cdk2, it is not necessary for the regulation of oligodendrocyte proliferation or for differentiation. Dividing oligodendrocyte precursors isolated from p27 knockout mice, for example, cease to proliferate and differentiate in the absence of p27, suggesting that other mechanisms to inhibit cdk2 and cell cycle progression must exist [Fero et al., 1996; Nakayama et al., 1996; Kiyokawa et al., 1996; Casaccia-Bonnefil et al., 1997]. Since oligodendrocyte precursors from p27−/− mice divide a greater number of times before differentiation than those from wild-type mice [Durand et al., 1998], Raff and co-workers have postulated that the role of p27 is to limit the number of oligodendrocyte precursor cell divisions in response to extracellular influences, such as growth factors. Additional mechanisms to stop cell cycle probably involve the cell cycle inhibitors, p21 and p16, which are also expressed in oligodendrocytes [Casaccia-Bonnefil et al., 1997]. Furthermore, p57 may also be important [Sherr and Roberts, 1995], although its expression in oligodendrocytes has not been characterized.

During our investigation, we observed that oligodendrocytes overexpressing p27 have more complex, highly branched processes than are found in oligodendrocytes infected with control virus grown under the same culture conditions. Although the significance of these morphological changes remains to be elucidated, ectopic expression of the cyclin kinase inhibitor p21 in the neuronal cell line, PC12, can also cause morphological changes similar to those seen with differentiation, such as neurite extension [Erhart and Pittman, 1998]. Perhaps, p27-induced arrest of proliferation also produces an acceleration of the temporal sequence of events that regulate the morphological changes of differentiation, but without activating the known program of myelin-specific gene expression.

How might oligodendrocyte differentiation and cell cycle progression be regulated coordinately? One possibility, as in muscle cells, is through a key regulatory molecule, such as MyoD, which can participate directly in the regulation of both differentiation and cell cycle progression. MyoD a member of the bHLH transcription factor family, effects differentiation by activating muscle-specific gene expression, and effects cell cycle progression by activating the gene encoding the cyclin kinase inhibitor, p21 [Li and Olson, 1992; Olson, 1992; Lassar et al., 1994]. In addition, MyoD activity can be inactivated by mitogens through phosphorylation, which can also block muscle differentiation [Lassar et al., 1994]. Since MyoD phosphorylation is mediated, at least in part, by activated cdks [Skapek et al., 1995], overexpression of p21 in muscle cells, unlike p27 in oligodendrocytes, can produce both cell cycle arrest and differentiation [Skapek et al., 1995; Parker et al., 1995; Halevy et al., 1995].

Regulatory molecules necessary for oligodendrocyte differentiation, like MyoD in muscle, have not yet been identified. One candidate regulator, Gtx, a homeodomain transcription factor, is uniquely expressed in differentiated oligodendrocytes in postnatal brain [Awatramani et al., 1998], but it has not been shown to be required for differentiation. Interestingly, Kawabe et al. [1997] recently showed that Hox11, homeodomain protein necessary for spleen development, can also interact with protein phosphatases PP2A and PP1, thereby regulating the G2/M cell cycle checkpoint. Like Hox11, Gtx could have two functions. One, a differentiation function, regulating myelin gene expression, and the second, a proliferation function, regulating the cell cycle. In addition, both functions could be further regulated by mitogens. Future experiments will be necessary to test this hypothesis.

Although we do not know how oligodendrocyte differentiation and proliferation are coordinately regulated, modulation of the activity of the cyclin-dependent kinase, cdk5, may be implicated in this process. An increase in cdk5 activity accompanies oligodendrocyte differentiation in vitro 72 h after removal of mitogens [Tang et al., 1998]. In addition, cdk5 is redistributed from the nucleus to the cytoplasm when oligodendrocytes differentiate (our unpublished observations). Oligodendrocytes overexpressing p27 do not increase cdk5 and do not differentiate, at 72 h after adenoviral infection. An increase in cdk5 activity occurs at the time of G1-S arrest, but it is not detected later when oligodendrocytes fail to differentiate. These findings raise the possibility that the function of

Cdk5 in oligodendrocyte development and differentiation may be a complex one, as shown in other cell types. Cdk5 has been found to be required for differentiation and survival of neurons [Ohshima et al., 1996; Nikolic et al., 1996] and for muscle differentiation and patterning [Philpott et al., 1997]. Finally, cdk5 is found in the nuclei of muscle cells [Philpott et al., 1997] and is re-distributed between the nucleus and the cytoplasm when myoblasts differentiate [Lazaro et al., 1997].

The mechanisms whereby cdk5 influences differentiation remain to be elucidated. It is possible that cdk5 phosphorylates cell-specific targets depending on subcellular locations [Lazaro et al., 1997]. In the nucleus, targets of cdk5 are the retinoblastoma protein [Lee et al., 1997], D1 cyclin [Lee et al., 1996], and cytoskeletal proteins [Inagaki et al., 1996], possibly involved in nuclear matrix organization. Phosphorylation of these substrates may result in both positive and negative effects on transcriptional regulation of genes involved in differentiation and irreversible growth arrest of oligodendrocytes.

In conclusion, our results support the hypothesis that a rate-limiting signal, generated in vitro by removal of growth factors, is necessary for oligodendrocyte differentiation, and that activation of additional pathways is required for differentiation to occur. Future studies will be aimed at characterizing the components of this signaling pathway and the nuclear targets that participate in the control of oligodendrocyte differentiation.

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