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Cell Cycle Inhibition and Retinoblastoma Protein Overexpression Prevent Purkinje Cell Death in Organotypic Slice Cultures

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ABSTRACT: **Purkinje cells are vulnerable to a number of physical, chemical, and genetic insults during development and maturity. Normal development of these cells depends on the cell-cell interactions between granule and astroglial cell populations. Apoptotic death in Purkinje neurons had been shown to be associated with cell cycle activation, and new DNA synthesis is associated with Purkinje cell death in *staggerer* and *lurcher* mutant mice. Here using an *in vitro* organotypic slice culture model from 9 (P9) and 4 days (P4) old postnatal rats we show that the cyclin dependent kinase (cdk) inhibitors (roscovitine, olomoucine, and flavopiridol) protect the Purkinje cells from cell death. The results are more pronounced in the cerebellar sections from P4 rats. Analysis of Purkinje neurons in sections from P4 rats after 1 week of culturing showed that while there were very limited calbindin positive neurons in the untreated sections the cdk inhibitor treated sections had a notably higher number. Although treatment with cdk inhibitors inhibited Purkinje cell loss significantly, the morphology of these neurons was abnormal, with stunted dendrites and axons. Since the retinoblastoma protein (Rb) is the major pocket protein involved in determining the differentiated state of neurons we examined the effect of over-expressing Rb in the organotypic cultures. Rb overexpression significantly inhibited the Purkinje cell death and these neurons maintained their normal morphology. Thus our studies show that the cell death in Purkinje neurons observed in organotypic cultures is cell cycle dependent and the optimal survival requires Rb.** Develop Neurobiol 67: 818–826, 2007

Keywords: **organotypic slice culture; Purkinje neurons; cell death; cyclin dependent kinase (cdk) inhibitor; retinoblastoma protein (Rb)**

INTRODUCTION

Programmed cell death (PCD) occurs in the nervous system during the course of normal development (Oppenheim, 1991; Burek and Oppenheim, 1996; Buss and Oppenheim, 2004). Approximately half of the neurons generated die by apoptosis, a process that is characterized by cytoplasmic blebbing, cell shrinkage, neurite degeneration, and DNA fragmentation. Although apoptosis is traditionally considered as a developmental event, cell culture models have shown that neuronal loss associated with neurodegenerative diseases can be apoptotic as well (Heintz, 1993).

Terminally differentiated neurons respond to aberrant signal transduction events by undergoing cell death (Heintz, 1993). Studies conducted in the neurological mutations of the mouse *staggerer* and *lurcher* showed that in both mutants, target-related cell death is accompanied by re-entry into cell cycle (Herrup and Busser, 1995). These mice have been extensively

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employed to study interacting cell types in cerebellar development as well as neuronal degeneration. These investigators showed that cell death in these mouse mutants was associated with new DNA synthesis and upregulation of different cell cycle markers including cyclin D, and PCNA. The involvement of cell cycle regulatory molecules in the death of neurons had also been established by conducting studies on dissociated neuronal cultures (Troy et al., 1996; Park et al., 1997a,b, 1998; Troy et al., 1997; Padmanabhan et al., 1999; Stefanis et al., 1999).

Among the cell cycle regulatory proteins, the retinoblastoma tumor suppressor protein (Rb) has been implicated in maintaining the postmitotic state of neurons (Bernards et al., 1989). Several groups have shown that the mice carrying homozygous mutations in Rb tumor suppressor gene die embryonically during mid-gestation (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). The postmitotic neurons are induced to undergo DNA synthesis and apoptosis in these mutants. Supporting this, our earlier studies on the cell death of cerebellar granule neurons (CGN) and cortical neurons had shown that these cells respond to many of the apoptotic stimuli through the activation of cell cycle (Park et al., 1997a,b; Padmanabhan et al., 1999). Since we were able to inhibit the cell death in these neurons by pharmacological cdk inhibitors as well as by overexpression of *in vivo* inhibitors of cdk or Rb (Park et al., 1997a,b, 2000; Padmanabhan et al., 1999) we decided to examine how they affect the death of Purkinje neurons using organotypic slice cultures.

Organotypic slice cultures offer an excellent *in vitro* model system to study the apoptosis as well as morphology of Purkinje cells (Jaeger et al., 1988; Stoppini et al., 1991; Tanaka et al., 1994; Dusart et al., 1997; Krah and Meller, 1999; Metzger and Kapfhammer, 2000). It had been shown previously that Purkinje cells die by apoptosis in organotypic slice cultures by an age dependent process (Dusart et al., 1997; Ghoumari et al., 2000). We used explants from P9 and P4 rats to examine the effect of different cell death inhibitors on Purkinje neuronal survival. Our results showed that cdk inhibitors protect Purkinje neurons significantly in organotypic slice cultures from P9 and P4 rats; however the results from P4 were more pronounced than P9. Although there was significant protection of Purkinje neurons after inhibitor treatment, best results were obtained with overexpression of Rb. In addition to its effect on cell cycle regulation, the known anti-apoptotic properties of Rb could be contributing to the survival of Purkinje neurons in organotypic cultures (Janicke et al., 1996; Lauricella et al., 2001).

METHODS

Materials

Roscovitine and olomoucine were purchased from Alexis (Beverly, MA) or Calbiochem (La Jolla, CA) and flavopiridol was a generous gift from Dr. Peter J. Wortland (National Cancer Institute). Adenovirus expressing ΔK11 Rb mutant was a generous gift from Dr. David S. Park (University of Ottawa). Polyclonal antibody to calbindin was obtained from SWant (Switzerland) or Sigma (St. Louise, MO) and polyclonal antibody to Rb was obtained from Pharmingen. Monoclonal antibody to Rb was from Cell Signaling Technology (Beverly, MA). FITC and Rhodamine labeled secondary antibodies were purchased from Pierce chemicals. Tissue culture reagents and Alexa fluor 488 and 594 were purchased from Gibco/Invitrogen. All other chemicals unless otherwise mentioned were obtained from Sigma (St. Louise, MO).

Generation of Recombinant Adenovirus

The cDNA for WT Rb was subcloned into the shuttle vector pAdlox. Recombination of Rb with adenovirus was achieved by co-transfection of pAdlox/Rb and adenoviral backbone DNA into CRE8 cells as described previously (Hardy et al., 1997). pAdlox, parent adenovirus and CRE8 cells were generously provided by Dr. Jan Kitajewski (Columbia University). Adenovirus expressing the WT, and the phosphorylation mutant of Rb (ΔK11Rb, constitutively active) (Park et al., 2000) were used in our studies. The slices were infected with adenovirus expressing the WT, and mutant Rb as well as the control adenovirus at an MOI of 150–200 and the culture was maintained for different periods of time.

Slice Cultures

Cerebellum dissected out of postnatal day 9 and day 4 rats was placed carefully into cold PBS containing 0.6% glucose. After removal of the meninges, parasagital sections of 250–300 μm thicknesses were prepared using a McIlwain tissue chopper. The sections were gently placed in cold PBS with 0.6% glucose and individual sections were separated under a dissection microscope. Adjacent sections were plated onto membranes of Millipore tissue culture insert (Millicell, Millipore, Bedford, MA; 30 mm, pore size 0.4 μm) placed in a 6-well dish with 1.5 mL media (BME/10% serum). The media was only permitted underneath the membrane as this allowed the sections to attach very well to the membrane. After incubating the dishes containing the sections for 4 h in the CO_2 incubator, slices were treated with either the cdk inhibitors (flavopiridol, 1 μM; roscovitine, 50 μM; or olomoucine, 200 μM) or with adenovirus overexpressing WT Rb or a phosphorylation mutant of Rb at a MOI of $\sim$150–200 for the indicated times. The slices were incubated for 2 h at room temperature with the virus diluted in 2% serum media on a platform rocker. The media

was supplemented with full serum and returned to the CO_2 incubator at the end of this period. The experiment was repeated in slices from several independent animals. Sections were analyzed for Purkinje cell survival by immunostaining with calbindin antibody.

Immunostaining

Sections were fixed for 1 h at room temperature using 4% paraformaldehyde. After washing with PBS, sections were blocked and permeabilized overnight (since the sections were >200 μm thick) using 10% normal goat serum in PBS containing 0.2% Triton X-100 (blocking solution). At the end of the blocking period, primary antibody was added at a concentration of around 10 μg/mL in blocking solution, and incubated for 3 h to overnight at 4°C. After thorough washing of the sections in PBS, secondary antibody (either FITC or Rhodamine labeled) at a dilution of 1 to 100 in blocking solution or Alexa 488 or 594 at a dilution of 1 to 1000 or 1 to 4000 respectively was added and incubated for another 3 h at 4°C. At the end of the incubation period sections were washed and mounted using aquamount (VWR scientific).

Quantification of Purkinje Cells in Slices

For the quantitative measurement of Purkinje neurons (except in the case of P9 cerebellar sections treated with Rb adenovirus), after indicated treatments the calbindin positive neurons in each lobule were counted and added to obtain the total number of cells in a slice using the fluorescent microscope. This was done for slices after different treatments and time periods. Since the experiments were done using adjacent slices, they were matched for the plane of section to the best of our knowledge. The data given here is a representative of at least three independent experiments. In the case of slices from P9 rats treated with adenovirus expressing Rb, pictures of calbindin positive sections were scanned and the fluorescence intensity of the calbindin staining was determined using NIH Image version 1.62. We also used Image J, image processing and analysis program for Macintosh OS X, to determine the number of particles per section as well as fluorescent intensity and this correlated very well with the intensity data obtained by NIH Image, 1.62. Before measurements the images were converted to 8-bit grayscale and the threshold of all the images were adjusted to the same level.

RESULTS

Cyclin-Dependent Kinase Inhibitors Prevent Purkinje Cell Death in Organotypic Slice Cultures

It had been shown that the Purkinje neurons die by apoptosis in organotypic slice cultures of rat and mouse cerebella taken between P1 and P7 (Dusart et al., 1997; Ghoumari et al., 2000). Here we show that organotypic slice cultures of cerebella taken from P9 rats show a progressive decrease in the number of Purkinje cells. Under the culture conditions there was little or no loss of the calbindin-positive cerebellar neurons within the first 24 h. However over the time period of our study Purkinje cell numbers (Fig. 1, top row) showed a time dependent decrease as analyzed by calbindin immunostaining. Since our previous work had shown that caspase

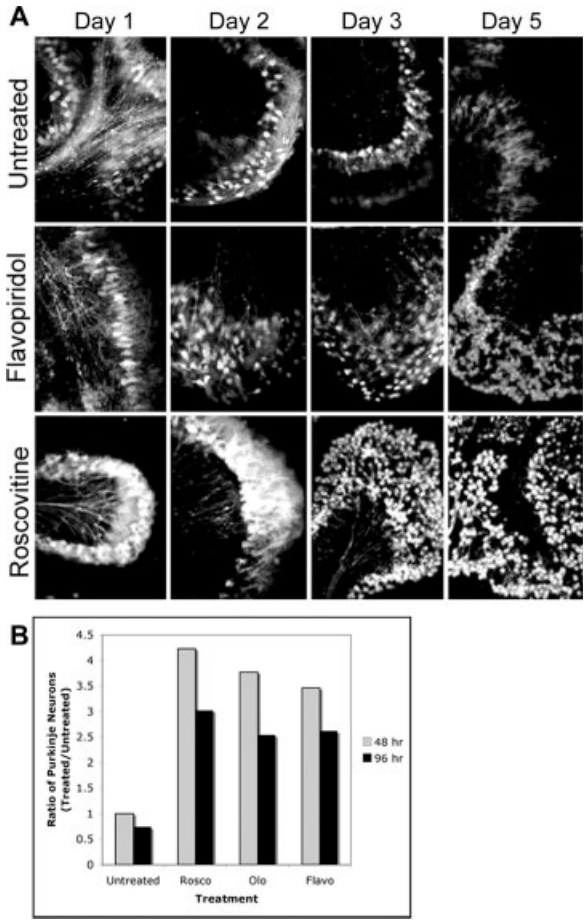

Figure 1 (A) Calbindin staining of Purkinje neurons in P9 cerebellar sections: Parasagital sections of cerebellum were left untreated or treated with flavopiridol (1 μM) or Roscovitine (50 μM) for different time periods. At the end of 1, 2, 3, and 5 days with the inhibitors, sections were fixed and stained using a polyclonal anti-calbindin primary antibody and anti-rabbit rhodamine secondary antibody. The fluorescence was visualized using a Nikon epifluorescence microscope (20× magnification). (B) Quantitative analysis of Purkinje neurons: After culturing the sections for 48 h and 96 h with or without cdk inhibitors they were fixed and stained for calbindin and the number of positive cells in all the lobules were counted. The histogram represents the ratio of the number of calbindin positive Purkinje neurons in treated sections to that in the untreated section at 48 h.

inhibitors as well as cell cycle inhibitors provide protection against apoptosis in neurons induced by different types of insults (Park et al., 1997a,b, 1998, 2000; Padmanabhan et al., 1999) we examined the effect of these inhibitors on the Purkinje cell death. Exposure of cultures to caspase inhibitors, BAF, ICE inhibitor, and DEVD, or antioxidant NAC, failed to rescue the Purkinje cells from cell death (data not shown).

On the basis of our prior studies on KCl withdrawal-induced cell death in cerebellar granule neurons and DNA damage induced death in cortical neurons (Padmanabhan et al., 1999; Park et al., 2000), we examined the ability of cyclin dependent kinase (cdk) inhibitors, namely flavopiridol, olomoucine and roscovitine, to protect Purkinje cells from death in organotypic slice cultures. The slice cultures were treated with roscovitine (50 μM) or flavopiridol (1 μM) for the indicated times, and Purkinje cell survival was assessed after calbindin immunostaining. We found that the cdk inhibitors protected Purkinje cells from cell death observed in cultures of cerebellar slices [Fig. 1(A), middle and bottom row compared to top row], with roscovitine showing the most significant effect [Fig. 1(A), bottom row]. Olomoucine (200 μM) also gave significant protection (immunostaining data not shown). In another set of experiments, sections after 48 h and 96 h of treatment with olomoucine, roscovitine or flavopiridol were immunostained with calbindin and analyzed by counting the number of positive Purkinje cells per slice [Fig. 1(B)]. There were fewer calbindin positive neurons in untreated sections compared to the sections treated with the cell cycle inhibitors at both times. The histogram shows the ratio of number of Purkinje neurons in the cdk inhibitor treated section to that in untreated sections after 48 h.

Morphology of Purkinje Neurons in cdk Inhibitor-Treated Sections

Although the number of Purkinje neurons was significantly higher in the cdk inhibitor treated slices, their morphology was quite different. While these inhibitors protected the cell bodies very well, the neurons showed a loss of dendritic arborization, indicating that the inhibitor treatment resulted in stunted dendrites. Most of the Purkinje neurons showed shorter axons in addition to the stunted dendritic tree after cdk inhibitor treatment. The neurons that survived in the untreated sections showed very good dendritic arborization. Figure 2 shows the 4× and 60× image of part of a section showing the calbindin positive

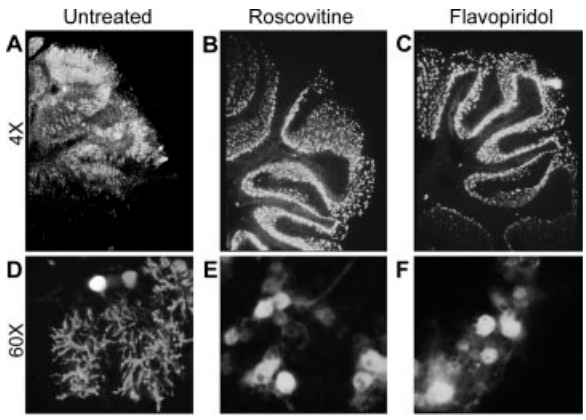

Figure 2 Calbindin stained sections showing the morphology of Purkinje neurons: P9 rat cerebellar sections were left untreated (A) or treated with roscovitine (B; 50 μM) or flavopiridol (C; 1 μM) for 1 week and fixed and stained for calbindin. The figure represents part of a section stained with calbindin and images were taken at 4× (A,B,C) and 60× (D,E,F) magnification. The 60× image clearly shows the morphology of a representative cell/cells from each slice that was left untreated (D) or treated with roscovitine (E) or flavopiridol (F). cdk inhibitor treatment resulted in loss of dendrites in Purkinje neurons (E and F compared to D).

Purkinje cells in untreated [Fig. 2(A,D)], or after roscovitine [Fig. 2(B,E)] or flavopiridol [Fig.2(C,F)] treatment for 1 week in culture. The 60× image shows a representative Purkinje neuron/s from each section. The data clearly shows that cell cycle activation is one of the major mechanisms involved in Purkinje cell death in organotypic slice cultures.

Since the cell cycle inhibitors protected the P9 rat Purkinje neurons in *in vitro* organotypic cultures, our next aim was to determine the effect of these inhibitors on P4 rat Purkinje neurons. Explants from P4 rats were cultured with or without roscovitine or olomoucine for 1 week and examined for Purkinje neuron survival using the calbindin antibody. Since it had been shown that the Purkinje neurons in explants taken from P1 to P5 rats die *in vitro* after 1 week, we decided to use P4 rats and chose the same 1 week time frame for all the experiments using P4 rat sections. The results were striking in P4 sections (Fig. 3). While the untreated sections had very few Purkinje neurons after 1 week in culture [Fig. 3 (A,D)], both olomoucine [Fig. 3(B,E)], and roscovitine [Fig. 3(C,F)] treatment resulted in significant protection of Purkinje neurons. The quantitative analysis of the data is shown in the histogram [Fig. 3(G)]. Roscovitine gave the maximum protection in P4 rat slices. This again confirmed our findings from P9 rat sections that cell cycle activation indeed leads to Purkinje cell death in explant cultures. As in the case of

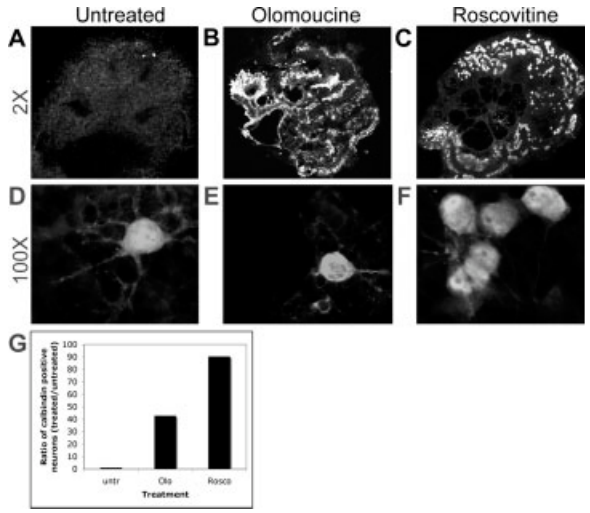

Figure 3 (A) Calbindin immunostaining in cerebellar sections from P4 rats: Explants from P4 rats were cultured for 1 week without (A) or with olomoucine (B) or roscovitine (C) and fixed and analyzed for calbindin positive Purkinje neurons. Alexa 594 was used to visualize the staining under a Nikon E 1000 fluorescent microscope. A representative slice from each treatment is shown in the figure (magnification ×2: A,B,C). D, E, and F are ×100 images of representative Purkinje neuron/s from each section left untreated (D) or treated with olomoucine (E) or roscovitine (F) for 1 week. (G) The histogram represents the ratio of number of calbindin positive Purkinje neurons in each section to that in untreated section analyzed after 1 week in culture.

explants from P9 rats, roscovitine, and olomoucine treated P4 sections also showed Purkinje neurons with dendritic loss [Fig. 3(E,F)] compared to the very few neurons from untreated sections [Fig. 3(D)] that showed good dendrites (100× magnification).

Overexpression of Rb Inhibits the Purkinje Neuronal Death and Maintains the Normal Architecture of the Neurons

Experiments described above using the pharmacological inhibitors of cyclin dependent kinases revealed that the cell cycle activation plays a significant role in the death of Purkinje neurons in *in vitro* slice cultures. This was similar to our observations in KCl withdrawal induced apoptosis in CGN. In cerebellar granule neurons, KCl withdrawal led to hyper phosphorylation of Rb followed by significant loss of this protein (Padmanabhan et al., 1999). Although we were unable to protect the granule neurons by overexpression of Rb, apoptosis induced by DNA-damage in cortical neurons was inhibited by overexpression of Rb (Park et al., 2000). These findings prompted us to study the effect of overexpression of wild-type Rb as well as a phosphorylation site mutant (constitutively active) of Rb in the slice cultures. The sections were treated with the viruses as described in the materials and methods and the Purkinje cell survival was analyzed by calbindin immunostaining. Slice cultures overexpressing WT Rb [Fig. 4(B,E)] as well as the phosphorylation mutant of Rb [Fig. 4(C,F)] showed a significantly higher number of Purkinje cells (as observed by calbindin staining) compared to the control sections expressing adenovirus alone [Fig. 4(A,D)]. The histogram [Fig. 4(G)] shows the ratio of the fluorescence intensity of the calbindin immunoreactivity in the sections analyzed by NIH Image, version 1.62 and Image J, image processing and analysis programs for Macintosh. We examined the

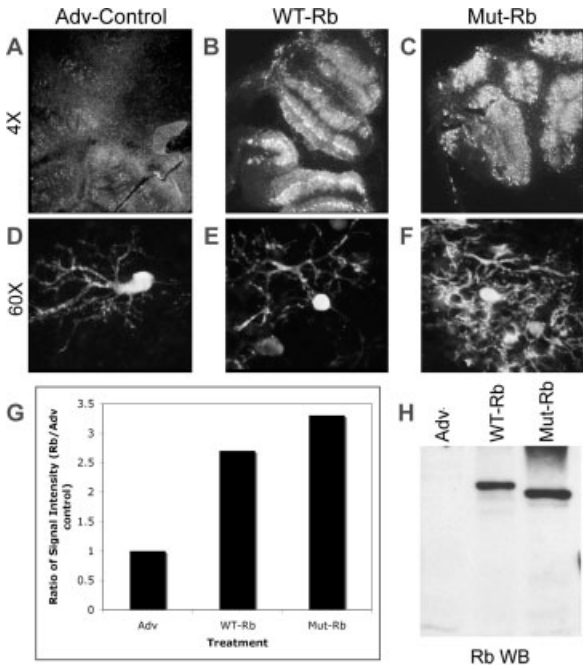

Figure 4 Protection of Purkinje neurons in cerebellar slices infected with adenovirus expressing WT or constitutively active Mut-Rb: Slice cultures were infected with control (A,D), WT Rb (B,E) or Mut- Rb (C,F) adenovirus at an MOI of 150–200 and left for a week in culture. The sections were fixed and stained using polyclonal calbindin antibodies and images were taken at 4× (A,B,C) and 60× (D,E,F) magnification. The 60× image shows the morphology of a representative calbindin positive cell/cells from each treatment. (G) Quantitative analysis of calbindin immunoreactivity in the adenovirus infected sections; the fluorescence intensity of calbindin staining was measured using NIH Image 1.62 or Image J, image processing and analysis program for Macintosh. The histogram shows the ratio of calbindin intensity in Rb expressing sections to that from the control adenovirus expressing sections. (H) Rb level in cerebellar sections: Cerebellar sections were infected with the control and Rb expressing adenovirus and cell extracts were analyzed by western-immunoblot analysis for the expression level of Rb.

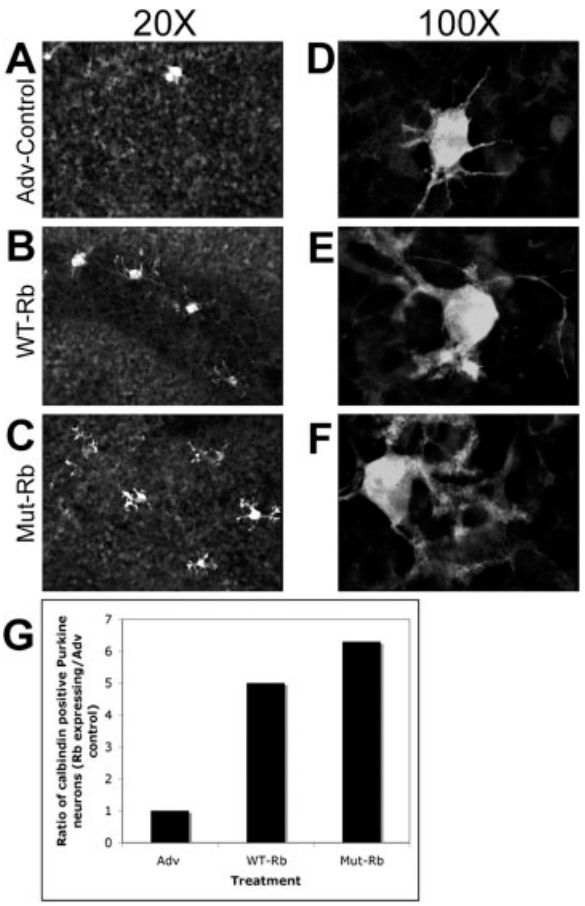

Figure 5 Protection of Purkinje neurons in cerebellar sections from P4 rats by overexpression of Rb: P4 rat sections were infected with control GFP (A,D), WT-Rb (B,E) and Mut-Rb (C,F) expressing adenovirus and cultured for 1 week. The sections were fixed and immunostained with calbindin antibodies to detect Purkinje neurons and the images were taken at ×20 (A,B,C) and ×100 (D,E,F) magnification. The ×100 magnification shows that the overexpression of Rb not only protected the Purkinje neurons but also preserved the architecture very well. (G) The Histogram shows the quantitative analysis of the data. The calbindin positive neurons from the sections were counted and the ratio of positive cells in Rb expressing sections to that in control adenovirus treated sections was plotted.

expression level of Rb in the cerebellar sections by western blot analysis and found that both the WT and mutant Rb proteins were being overexpressed [Fig. 4(H)]. The Rb overexpression not only protected the cells from undergoing apoptosis, but also preserved the morphology of the neurons to nearly normal state (Fig. 4E and F compared to D; 60× magnification shows a representative cell/cells from each section).

The cerebellar sections from P4 rats were also analyzed for Purkinje cell protection using adenoviral overexpression of Rb. Similar to the P9 rat sections, P4 also showed protection of Purkinje neurons upon overexpression of Rb, with the constitutively active mutant Rb showing slightly more protection than WT [Fig. 5(B,C) compared to (A)]. The number of Purkinje neurons in Rb overexpressing sections was much lower than that observed in roscovitine and olomoucine treated sections. This may suggest that the protection of Purkinje neurons by pharmacological agents is attained by not only maintaining Rb in its active non-phosphorylated state, but also through some other additional mechanisms as well. The histogram in Figure 5(G) shows the quantitative data from the experiments done in P4 sections. The number of calbindin positive neurons from different sections were counted and compared. The Purkinje neurons in sections overexpressing WT and mutant Rb showed a morphology similar to normal ones that survived in the untreated sections [Fig. 5(E,F) compared to (D), 100× magnification] suggesting that Rb plays a significant role in protection of differentiated neurons.

In order to verify the overexpression of Rb in Purkinje neurons, we co-immunostained the P4 sections with a monoclonal Rb antibody [Fig. 6(A,D,G)] and the polyclonal calbindin antibody [Fig. 6(B,E,H)]. The immunostaining analysis showed overexpression of Rb in the sections, and cells including Purkinje neurons were expressing higher levels of Rb after adenoviral infection [Fig. 6.(D,G) compared to (A)]. The composite image in Figure 6(C,F,I) shows the co-localization of Rb and calbindin in Purkinje neurons.

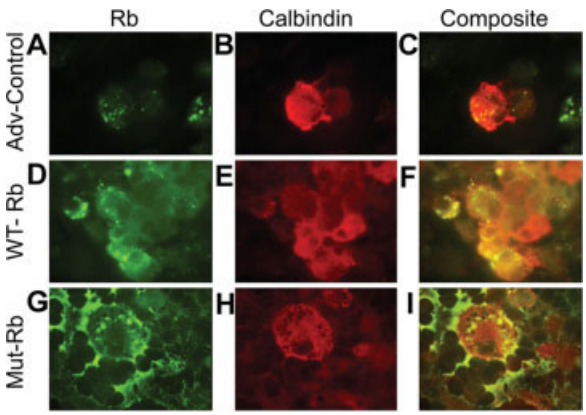

Figure 6 Rb and calbindin co-immunostaining in P4 cerebellar sections: Cerebellar sections were infected with control adenovirus (A,B,C) and WT-Rb (D,E,F) and mutant-Rb (G,H,I) adenovirus and cultured for 1 week. The sections were fixed and stained with monoclonal Rb (A,D,G) and polyclonal calbindin antibodies (B,E,H). C, F, and I are the composite image of sections showing localization of Rb and calbindin in Purkinje neurons.

In some of the Purkinje neurons the Rb staining was visible even in the dendrites [Fig. 6.(G), Mut-Rb].

DISCUSSION

Many extracellular signals can stimulate multiple pathways that converge upon a common cell death mechanism in neurons. Some of the well-studied pathways include oxidative stress and caspase activation-induced neuronal cell death. Recently, cell cycle activation had gained a lot of attention as a mediator of neuronal apoptosis. Here, using an *in vitro* slice culture model, we demonstrate that cell cycle activation is one of the major causes of Purkinje cell death in organotypic slice culture model. Three pharmacological inhibitors of cyclin dependent kinases, namely, roscovitine, olomoucine and flavopiridol, enhanced Purkinje cell survival in the *in vitro* slice cultures significantly. In contrast, the caspase inhibitors we tested did not have any significant effect on survival in this system.

While cdk inhibitors protected the Purkinje cells from death, the accompanying morphology change was puzzling. A gradual loss of dendrites was observed over the time period of the treatment (Fig. 1), suggesting that the cdk inhibitors were directly or indirectly affecting the dendrites while protecting the Purkinje cell body. If the inhibitors were not having any specific effect on the development of the dendrites, we should not see any loss over the period of treatment and the dendrites that were already formed should be preserved until the end of the treatment period. Although the actual mechanism involved in dendritic loss is not clear at this time, we speculate that it could be due to one of the following; (a) a toxic effect of cell cycle inhibitors on dendrites, (b) an inhibition of cell division in some of the supporting cells, or (c) due to loss of other cell types during treatment thus affecting the cell-cell interaction and the extracellular support required for the normal development of these neurons. Our earlier studies in *in vitro* cultured CGN (Padmanabhan et al., 1999) have shown that the cdk inhibitors protect these cells from KCl-withdrawal-induced apoptosis, while the current studies in slice cultures show protection of mainly Purkinje neurons. The possible explanation for this could be that the cdk inhibitors protect neurons only when they exist in their fully differentiated state. In the organotypic cultures, we believe that the granule neurons are still immature (Canudas et al., 2004) and upon cdk inhibitor treatment they undergo cell cycle arrest and or even apoptosis; the Purkinje neurons being fully differentiated (Altman and Bayer, 1978; Dusart et al., 1997) survive the inhibitor treatment. If the cdk inhibitors induce apoptosis or premature growth arrest in CGN, this could have a profound effect on Purkinje cell development since the Purkinje cell dendritic elongation and synaptogenesis continues until the end of first postnatal month.

Since CGN and Purkinje cells develop symbiotically, conditions that alter the viability or morphology of either cell type can have profound effect on the other cell type (Wetts and Herrup, 1982, 1983; Herrup et al., 1984; Vogel et al., 1989, 1991; Smeyne and Goldowitz, 1990; Herrup and Busser, 1995; Smeyne et al., 1995). The available data on requirement of granule neurons and other supporting cells for normal development of Purkinje neurons is diverse. *In vitro* studies have shown that for the proper development and differentiation of Purkinje neurons granule cells are necessary (Baptista et al., 1994). Although BDNF is required for normal development of cerebellar cortex and has been shown to enhance dendritic outgrowth (Schwartz et al., 1997, 1998; Morrison and Mason, 1998; Shimada et al., 1998), we did not observe any significant change in the morphology of Purkinje cell after addition of BDNF to the P9 explant culture (data not shown). Experiments with NT3 also produced similar results (data not shown); the morphology of Purkinje cells remained the same irrespective of the presence of exogenous neurotrophins. This is similar to the observations made by Dusart et al. (Dusart et al., 2005) where the Purkinje neurons completely failed to regenerate their axons suggesting that the regeneration of axons in Purkinje cells is more complex. Although we believe that the loss of granule neurons could be the reason for abnormal development of dendrites it has been shown that the Purkinje neurons preserve the dendritic arborization in the absence of signals from granule neurons (Kapfhammer, 2004). Thus without further experiments, it is difficult to explain the exact mechanisms involved in the morphology changes associated with cdk inhibitor treatment.

Among the pharmacological inhibitors, we found that roscovitine is the most effective, with least toxicity followed by olomoucine and finally flavopiridol in protecting Purkinje neurons. While flavopiridol is more specific for cyclin D associated kinases cdk 2, 4, and 6, roscovitine and olomoucine are potent inhibitors of cdk 1, 2, and 5. Roscovitine is a much more potent inhibitor of these kinases compared to olomoucine, and the differential effect we see with these pharmacological agents could be due to their difference in potencies. Recently it has been shown that the early stages of neuronal apoptosis are associated with cdk 5 induced phosphorylation of Rb (Hamdane et al., 2005). Thus we believe that the inhibition of

cdks, including cdk5, by roscovitine and olomoucine prevent the Rb phosphorylation and inactivation thus preventing the cell cycle activation and Purkinje neuronal death.

Cell cycle activation is associated with phosphorylation and inactivation of Rb, and transcriptional activation or derepression of genes (Liu et al., 2004; Liu and Greene, 2001). Studies conducted in mice expressing different mutants of SV40 large T-Antigen have shown the significance of Rb in Purkinje cell survival (Feddersen et al., 1992, 1995, 1997) and this agrees with our current results. Overexpression of both the WT and constitutively active Rb not only protected the Purkinje neurons from cell death but retained their morphology to a normal state. Rb, in addition to having a significant role in cell cycle regulation is known to have anti-apoptotic activities (Janicke et al., 1996; Lauricella et al., 2001) and this could be one of the reasons why the Purkinje cell morphology was preserved in the sections overexpressing Rb. Thus Rb function appears to be critical when the neurons become postmitotic; in the absence of functional Rb, this postmitotic state may not be sustainable and neurons may undergo cell death.

In conclusion, our experiments using cdk inhibitors and Rb specifically show the importance of involvement of cell cycle regulatory molecules in neuronal fate determination. These findings underline the importance of the major cell cycle regulatory proteins like Rb in the survival and apoptosis of neurons, which are post-mitotic. It also raises the possibility that cell cycle regulators may be good targets for developing therapeutic agents to combat neurodegenerative diseases associated with cerebellar degeneration.

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