Suppression of Death Receptor Signaling in Cerebellar Purkinje Neurons Protects Neighboring Granule Neurons from Apoptosis via an Insulin-like Growth Factor-I Dependent Mechanism

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Running Title: ΔFADD-(+) Purkinje Cells Rescue Granule Neurons via IGF-I
Neuronal apoptosis contributes to the progression of neurodegenerative disease. Primary cerebellar granule neurons are an established in vitro model for investigating neuronal death. Following removal of serum and depolarizing potassium, granule neurons undergo apoptosis via a mechanism that requires intrinsic (mitochondrial) death signals; however, the role of extrinsic (death receptor-mediated) signals is presently unclear. Here, we investigated involvement of death receptor signaling in granule neuron apoptosis by expressing adenoviral, AU1-tagged, dominant-negative Fas-associated death domain (Ad-AU1-ΔFADD). Ad-AU1-ΔFADD decreased apoptosis of granule neurons from 65±5% to 27±2% (n=7, p<0.01). Unexpectedly, immunocytochemical staining for AU1 revealed that <5% of granule neurons expressed ΔFADD. In contrast, ΔFADD was expressed in >95% of calbindin-positive Purkinje neurons (~2% of the cerebellar culture). Granule neurons in proximity to ΔFADD-expressing Purkinje cells demonstrated markedly increased survival. Both granule and Purkinje neurons expressed insulin-like growth factor-I (IGF-I) receptors and ΔFADD-mediated survival of granule neurons was inhibited by an IGF-I receptor blocking antibody. These results demonstrate that the selective suppression of death receptor signaling in Purkinje neurons is sufficient to rescue neighboring granule neurons that depend on Purkinje cell-derived IGF-I. Thus, the extrinsic death pathway has a profound, but indirect, effect on the survival of cerebellar granule neurons.
INTRODUCTION

Apoptosis is a type of programmed cell death characterized by a cascade of proteolytic events orchestrated by the caspase family of cysteine proteases (1). There are two principal pathways leading to apoptotic cell death. These include the “extrinsic” or death receptor-initiated pathway and the “intrinsic” or mitochondrial pathway (2). The extrinsic pathway originates with binding of death-promoting ligands (e.g., FasL) to their cognate death receptors (e.g., Fas) (3). Ligand binding induces oligomerization of death receptors and promotes their association with adapter molecules like Fas-associated death domain protein (FADD) (4). The receptor:FADD interaction occurs via a protein:protein binding motif known as the death domain (5). The initiator caspase, pro-caspase-8, is then recruited to the death-inducing signaling complex via binding to the death effector domain of FADD (6). The resulting proximity of multiple pro-caspase-8 molecules facilitates their autocatalytic cleavage to the active protease caspase-8 (7). The intrinsic pathway is initiated by release of cytochrome C from mitochondria and its subsequent association with apoptosis-activating factor-1 and pro-caspase-9 (8). This large protein complex (the apoptosome) promotes activation of caspase-9 (9). The intrinsic pathway is regulated by both pro- and anti-apoptotic members of the Bcl-2 family (10). Each of the above initiator caspsases, 8 and 9, cleave downstream executioner caspsases, such as caspase-3, from the pro-form to the active protease, thus resulting in the cleavage of critical cellular proteins and apoptosis (11, 12).

Neuronal apoptosis plays an essential role in the normal development of the central nervous system (13). Developmental neuronal cell death requires activation of a caspase cascade as evidenced by the substantial hyperplasia observed in many areas of the brain in caspase-3 knock-
out mice (14). Aberrant apoptotic mechanisms are thought to contribute significantly to many neurodegenerative disorders including Alzheimer’s and Parkinson’s disease (15). Therefore, elucidation of the apoptotic signaling pathways underlying neurodegeneration is critical to enhance current therapies for these disorders. Recent findings indicate that components of both the extrinsic (death receptors or their ligands) and intrinsic (Bcl-2 family members) death pathways are regulated at the level of expression during neurodegeneration or neuronal injury in vivo (16,17). Moreover, transgenic animal models or spontaneously occurring mutants of specific death receptor signaling molecules or Bcl-2 family members provide further evidence that these pathways are involved in neuronal injury (18,19).

To examine the signal transduction mechanisms underlying neuronal apoptosis, primary neuronal cultures have been extensively utilized. Primary cerebellar granule neurons isolated from early postnatal rats are a well characterized model system for investigating neuronal apoptosis (20). These cell cultures are highly homogeneous with greater than 95% of the isolated cells being granule neurons (21). The remaining cells include glial cells and other neuronal cells, such as Purkinje neurons, which demonstrate an interdependent relationship with granule neurons both in vivo and in vitro (22,23). Cerebellar granule neurons require activity-dependent signals (membrane depolarization) and serum for their survival in vitro (20, 21, 24). Following removal of serum and lowering of extracellular potassium from 25 mM to 5 mM (trophic factor withdrawal), granule neurons undergo rapid apoptotic cell death characterized by caspase activation and nuclear condensation and fragmentation (21). Cerebellar granule neuron apoptosis is attenuated by neurotrophic growth factors, including insulin-like growth factor-I (IGF-I) [20, 21, 24-26], or by inhibitors of the stress-activated protein kinases, c-Jun-NH₂ terminal kinase or p38 mitogen-activated protein kinase (27). Significantly, both neurotrophins
and stress-activated protein kinase inhibitors also rescue neurons from apoptosis \textit{in vivo}, thus validating the results obtained in cerebellar granule neurons \textit{in vitro} (28, 29).

Several studies indicate that the intrinsic death pathway plays a critical role in cerebellar granule neuron apoptosis. For example, granule neurons display a rapid translocation of the pro-apoptotic Bcl-2 family member, Bax, to mitochondria and release of cytochrome C following trophic factor withdrawal (30). Moreover, cerebellar granule neurons isolated from Bax knock-out mice exhibit a significant reduction in apoptosis in response to serum and potassium deprivation (31). In addition, expression of the BH3-only, pro-apoptotic Bcl-2 family member, Bim, is markedly increased in granule neurons during trophic factor withdrawal (32). In contrast to the above findings, relatively little is known about the involvement of the extrinsic death pathway in promoting cerebellar granule neuron apoptosis. Le-Niculescu et al. showed that FasL mRNA is induced following trophic factor withdrawal in cerebellar granule neurons, and furthermore, sequestration of FasL with FasFc attenuates granule neuron apoptosis (33). However, additional data supporting a role for the extrinsic death pathway in cerebellar granule neuron apoptosis have not been forthcoming.

In the present study, we examined involvement of death receptor signaling in cerebellar granule neuron apoptosis by expressing a dominant-negative mutant of FADD (ΔFADD) in primary cultures of cerebellar granule neurons using adenoviral vectors. Our results show that adenoviral ΔFADD was expressed almost exclusively in cerebellar Purkinje cells, yet it effectively rescued granule neurons from apoptosis. The ΔFADD-mediated survival of granule neurons was dependent on their proximity to ΔFADD-expressing Purkinje cells and required Purkinje cell-derived IGF-I. These data indicate that the extrinsic death pathway significantly, but indirectly, influences the survival of cerebellar granule neurons.
EXPERIMENTAL PROCEDURES

Materials- Adenoviral-AU1-ΔFADD (34) was kindly provided by Dr. David Brenner and the adenoviral core at the University of North Carolina (Chapel Hill, NC). Dr. Sylvia Christakos at the University of Medicine and Dentistry of New Jersey (Newark, NJ) kindly provided rabbit polyclonal antibodies raised against rat calbindin D28K (35). Monoclonal antibodies to the AU1 epitope tag were purchased from Berkley Antibody Company (Richmond, CA). Neutralizing, monoclonal antibodies against the IGF-I receptor were purchased from Oncogene (Boston, MA). FITC- and Cy3-conjugated secondary antibodies were obtained from Jackson Immunoresearch (West Grove, PA). Hoechst dye 33258 and DAPI (4,6-diamidino-2-phenylindole) were purchased from Sigma.

Cell Culture- Primary rat cerebellar granule neurons were isolated from 7-day old Sprague-Dawley rat pups as described previously (36). Briefly, cells were plated at a density of 2.0x10^6 cells/ml in basal modified Eagle’s medium containing 10% fetal bovine serum, 25mM KCl, 2mM L-glutamine, and penicillin (100units/ml)-streptomycin (100µg/ml) (Life Technologies, Inc., Grand Island, NY). Cytosine arabinoside (10µM) was added to the culture medium 24 h after plating to limit the growth of non-neuronal cells. Experiments were performed after 7 days in culture. Apoptosis was induced by removing the plating medium and replacing it with serum-free medium containing 5mM KCl.

Adenoviral AU1-ΔFADD Infection- The ΔFADD adenovirus was purified by cesium chloride gradient ultracentrifugation. The viral titer (multiplicity of infection [m.o.i.]) was determined by measuring the absorbance at 260nm (where 1.0 absorbance units=1x10^{12} particles/ml) and infectious particles were verified by plaque assay. Five days after plating,
neuronal cultures were infected with Ad-AU1-ΔFADD at a m.o.i. ranging from 5-50. After infection, cells were returned to the incubator for 48 h at 37°C and 10% CO₂. On day 7, neurons were induced to undergo apoptosis. Twenty-four hours later, the cells were fixed for quantification of apoptosis and/or immunocytochemistry as described below.

Quantitation of Apoptosis- Neuronal cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 30 min at room temperature, washed with PBS, and nuclei were stained with either Hoechst dye or DAPI. Cells were scored as apoptotic if their nuclei were condensed or fragmented. In general, ~500 cells from at least two fields of a 35-mm well were counted. However, some experiments were performed on glass coverslips and granule neuron apoptosis was quantified in relation to their proximity to AU1-positive, ΔFADD-expressing Purkinje cells. For these experiments, two techniques were utilized to prevent selective bias in defining the anti-apoptotic effect of ΔFADD and the spacial association between surviving granule neurons and ΔFADD-expressing Purkinje cells. First, 40X fields were randomly scanned under the Cy3 filter for AU1-positive Purkinje cells (see Immunocytochemistry below). Fields were randomly selected that contained from 0 to 4 Purkinje cells per field (where “0” Purkinje cells implied no AU1 immunoreactivity apparent in the 40X field, an area of approximately 100,000 microns²). Following identification of the above fields, the filter was changed to DAPI and granule neuron apoptosis was quantified within each given field. The second technique utilized to prevent selective counting bias was that random fields were selected by two independent researchers. Ten fields containing on average 30 granule neurons per field were counted per condition from a total of three independent experiments. Data are presented as the percentage of cells in a given treatment group which were scored as apoptotic. All experiments were performed at least in triplicate.
Immunocytochemistry- Cerebellar cultures were plated on polyethyleneimine-coated glass cover slips at a density of ~1.0x10^5 cells per cover slip. Five days after plating, cells were infected with Ad-AU1-ΔFADD and were subsequently induced to undergo apoptosis 48 h post-infection as described above. After treatment, cells were fixed with 4% paraformaldehyde and then permeabilized and blocked in PBS containing 0.2% Triton X-100 and 5% BSA. Cells were then incubated overnight at 4°C with either mouse-anti-AU1 (1:1000), rabbit-anti-calbindin (1:5000), or mouse-anti-IGF-I receptor (1 µg/ml) diluted in PBS containing 0.2% Triton X-100 and 2% BSA. The primary antibody was aspirated and cells were washed five times with PBS. Cells were then incubated with the appropriate Cy3-conjugated or FITC-conjugated secondary antibody (diluted 1:500) and DAPI for 1 h at room temperature. The cells were then washed five times with PBS and coverslips were adhered to glass slides with mounting medium (0.1% p-phenylenediamine in 75% glycerol in PBS). Fluorescence imaging was performed on a Zeiss Axioplan 2 microscope equipped with a Cooke sensicam deep-cooled CCD camera and images were analyzed and subjected to digital deconvolution using the Slidebook software program (Intelligent Imaging Innovations Inc., Denver, CO).

Data Analysis- Results shown represent the means±S.E.M. for the number (n) of independent experiments performed. Statistical differences between the means of unpaired sets of data were evaluated using one-way analysis of variance followed by a post hoc Dunnett’s test. A p value of <0.01 was considered statistically significant.
RESULTS

Adenoviral ΔFADD Attenuates Apoptosis of Cerebellar Granule Neurons Induced by Trophic Factor Withdrawal- To investigate if death receptor signaling is involved in the apoptotic cell death of primary cerebellar granule neurons subjected to trophic factor withdrawal, cerebellar cell cultures were infected with increasing titers of adenoviral, AU1-tagged, dominant-negative FADD (Ad-AU1-ΔFADD). ΔFADD is a truncated protein that lacks the death effector domain, and therefore, inhibits coupling of liganded death receptors to the initiator caspase-8 (34, 37). Forty-eight hours following infection, cells were switched from control medium containing serum and 25 mM potassium to apoptotic medium lacking serum and containing 5 mM potassium. After an additional 24 h incubation, cells were fixed and nuclei were stained with Hoechst dye. Granule neurons containing condensed and/or fragmented nuclei were scored as apoptotic. As shown in Fig. 1A, uninfected granule neurons maintained in control medium throughout the experiment demonstrated a low amount of basal apoptosis (6±1%, n=7). Apoptosis in uninfected granule neurons switched to apoptotic medium for 24 h measured 65±5% (n=7). Infection with a negative control adenovirus (Ad-CMV) at a m.o.i. of 50 had no effect on granule neuron apoptosis (69±5%, n=7), whereas infection with ΔFADD resulted in a significant reduction in granule neuron apoptosis to 27±2% (n=7, p<0.01) at a m.o.i. of 50. At the adenoviral titers used in this series of experiments (m.o.i. of from 5 to 50), there was no detectable dose-dependence of ΔFADD on granule neuron apoptosis (Fig. 1A). When granule neuron apoptosis was carefully analyzed on a field-by-field basis, it was evident that the protection observed with ΔFADD was not uniformly distributed throughout the cell culture (Fig. 1B). Like the apoptosis observed in uninfected cultures (Fig. 1B, compare upper right panel
(apoptotic) to upper left panel (control)), there were some fields in the ΔFADD-infected cultures that contained a similar high percentage of apoptotic granule neurons (Fig. 1B, Field A). However, other fields in the ΔFADD-infected cultures displayed a marked reduction in cerebellar granule neuron apoptosis (Fig. 1B, Field B). Interestingly, this localized effect of ΔFADD on granule neuron survival was invariably associated with the presence of one or more large and irregularly-shaped nuclei within the field of protected granule neurons (Fig. 1B, Field B, see the nuclei indicated by the arrows).

The Survival of Granule Neurons is Dependent on Their Proximity to ΔFADD-expressing Purkinje Cells- The cerebellar cell cultures utilized in this study have been extensively characterized and are deemed highly homogeneous with greater than 95% of the culture being granule neurons (21). Of the remaining cells, the most prevalent are Purkinje neurons which display an interdependent relationship with granule neurons both in vivo and in vitro (22, 23). The calcium-binding protein, calbindin, has been utilized as a marker for Purkinje cells in the cerebellum (38). Immunocytochemical staining of our cerebellar cell cultures for calbindin revealed that the large, irregularly-shaped nuclei described above in Fig. 1B (Field B) were those of Purkinje neurons (Fig. 2A). The identified Purkinje neurons demonstrated a classical morphology characterized by an expansive dendritic tree (39). Further immunocytochemical analysis demonstrated that Purkinje cells were very efficiently infected with Ad-AU1-ΔFADD as shown by their marked staining with anti-AU1 (Fig. 2B). On average, Purkinje neurons made up ~2% of the entire cerebellar cell culture, and greater than 95% of identified Purkinje cells were AU1-positive following infection with Ad-AU1-ΔFADD at a m.o.i. of from 5 to 50. The lack of concentration-dependence for ΔFADD expression in Purkinje neurons over this range of adenoviral titers correlated with the lack of dose-dependence for ΔFADD inhibition of granule
neuron apoptosis (see Fig. 1A), suggesting that the protection observed was dependent on ΔFADD expression in Purkinje neurons.

As described above, the ability of ΔFADD to rescue granule neurons from trophic factor withdrawal-induced death was unevenly distributed throughout the cell culture and the protected granule neurons were near Purkinje cells (Fig. 1B, Field B). To further examine this effect, granule neuron apoptosis was analyzed on a field-by-field basis as described in Fig. 1B; however, infected cultures were stained with both DAPI (to assess nuclear morphology) and anti-AU1 to identify ΔFADD-expressing cells. Uninfected cells displayed the expected high amount of granule neuron apoptosis following trophic factor withdrawal (Fig. 3A, compare upper right panel (apoptotic) to upper left panel (control)). Similarly, fields of infected cultures which did not contain any ΔFADD-positive Purkinje cells also exhibited significant granule neuron death following trophic factor withdrawal (Fig. 3A, Field A). In contrast, fields of infected cells that contained at least one ΔFADD-expressing Purkinje neuron displayed a marked reduction in granule neuron apoptosis (Fig. 3A, Field B). To quantitate this effect, the number of healthy versus apoptotic granule neurons was counted per 40X field in ΔFADD-infected cultures that were incubated in apoptotic medium for 24 h. The granule neuron apoptosis results were then subdivided into three data sets based on the number of ΔFADD-positive Purkinje cells (0, 1-2, or 3-4) identified per field by AU1 staining. As shown in Fig. 3B, fields of granule neurons in infected cultures which did not contain any ΔFADD-positive Purkinje cells exhibited 58±1% apoptosis (n=3 experiments, 10 fields per experiment), a value similar to that observed in uninfected cerebellar cultures. However, fields that contained either 1-2 or 3-4 ΔFADD-expressing Purkinje cells demonstrated a significant decrease in granule neuron apoptosis to 22±6% or 20±6%, respectively (n=3, p<0.01). These results indicate that the ΔFADD-mediated
protection of cerebellar granule neurons from trophic factor withdrawal-induced apoptosis depends on the proximity of granule neurons to ΔFADD-expressing Purkinje cells.

*Ad-AU1-ΔFADD is Not Efficiently Expressed in Granule Neurons*- Although Purkinje neurons demonstrated marked expression of Ad-AU1-ΔFADD (Fig. 2B), most granule neurons were devoid of AU1 staining (Fig. 4A). Quantitatively, when more than 2000 granule neurons were visualized from cerebellar cell cultures infected with Ad-AU1-ΔFADD (m.o.i.=50), less than 100 stained positively for the AU1 epitope tag at 48 h post-infection (4.4±0.7%, data pooled from 3 independent experiments). These results indicate that granule neurons do not efficiently express AU1-ΔFADD following adenoviral infection. Furthermore, fields of infected cultures that contained one or more AU1-ΔFADD-positive granule neurons displayed a similar amount of apoptotic granule neuron death following trophic factor withdrawal as was observed in uninfected cultures (Fig. 4B). This latter result further demonstrates that the ability of ΔFADD to rescue granule neurons from apoptosis is not mediated by proximity to the very few granule neurons actually expressing ΔFADD. However, it is noteworthy that essentially all of the granule neurons which were AU1-ΔFADD-positive were protected from trophic factor withdrawal-induced death, regardless of their proximity to Purkinje neurons.

*ΔFADD-mediated Cerebellar Granule Neuron Survival is Dependent on IGF-I*- The above data indicate that inhibition of death receptor signaling in Purkinje cells (via infection with Ad-AU1-ΔFADD) not only maintains the Purkinje neurons during trophic factor withdrawal, but also results in the protection of closely neighboring granule neurons that require Purkinje cell-derived survival signals. One potent neurotrophin known to rescue cerebellar granule neurons from apoptosis is the growth factor IGF-I (20, 21, 24-26). *In situ* mRNA hybridization studies have shown that the principal source of IGF-I in the developing cerebellum is the Purkinje cell
(40). To investigate a potential role of IGF-I in granule neuron survival, we first analyzed the expression of IGF-I receptors in the cerebellar cell cultures. Immunocytochemical staining with an antibody to the IGF-I receptor demonstrated that cerebellar granule neurons expressed IGF-I receptors primarily at the cell membrane (Fig. 5A, left panel). Incubation with the Cy3-conjugated secondary antibody alone did not produce any positive staining (Fig. 5A, right panel). When cerebellar cultures were co-immunostained for both IGF-I receptors and calbindin, both granule neurons and calbindin-positive Purkinje neurons showed immunoreactivity for IGF-I receptors on their cell surfaces (Fig. 5B, lower right panel, see the yellow overlapping staining for co-localized IGF-I receptors and calbindin in a representative Purkinje neuron, the surrounding cells are granule neurons).

Next, the effect of an IGF-I receptor blocking antibody on the ΔFADD-mediated protection of cerebellar granule neurons was assessed (Fig. 6). Ad-AU1-ΔFADD decreased apoptosis of granule neurons from 59±5% (n=7) to 19±3% (n=7, p<0.01). Incubation with a non-immune IgG control had no effect on the ability of ΔFADD to decrease granule neuron apoptosis (19±3% apoptosis, n=7), whereas inclusion of a neutralizing antibody that binds to the extracellular domain of the IGF-I receptor significantly attenuated the protective effect of ΔFADD in a dose-dependent manner (41±5% apoptosis at an antibody concentration of 10 µg/ml, n=7, p<0.01 compared to ΔFADD alone). Finally, radioimmunoassay of serum-free media obtained from cerebellar cell cultures revealed that the concentration of IGF-I was below the limits of detection (=6ng/ml), further confirming that the protection by IGF-I was localized in nature. Collectively, these results suggest that ΔFADD-mediated survival of cerebellar granule neurons requires local secretion of Purkinje cell-derived IGF-I.
DISCUSSION

In the present study, we investigated a role for the extrinsic death pathway in the apoptotic cell death of primary cerebellar granule neurons subjected to trophic factor withdrawal. Adenoviral expression of dominant-negative FADD (ΔFADD) rescued a significant percentage of granule neurons from trophic factor withdrawal-induced death. Initially, this result suggested that death receptor signaling may play a direct role in cerebellar granule neuron apoptosis. However, immunocytochemical analysis revealed that adenoviral ΔFADD was not efficiently expressed in granule neurons, but instead showed marked expression in the small number of Purkinje neurons found in these cerebellar cell cultures. Moreover, the ability of adenoviral ΔFADD to rescue granule neurons from apoptosis was dependent on their proximity to ΔFADD-expressing Purkinje cells and required IGF-I. Two major conclusions can be drawn from the above results. First, although the data do not support a direct role for the extrinsic death pathway in cerebellar granule neuron apoptosis, the results are the first to demonstrate that death receptor signaling in Purkinje neurons indirectly influences the survival of granule neurons. Second, selective suppression of the extrinsic death pathway in Purkinje cells is sufficient to rescue neighboring granule neurons that depend on Purkinje cell-derived trophic support including IGF-I.

Previous studies have implicated the intrinsic death pathway in the apoptosis of cerebellar granule neurons. For example, trophic factor withdrawal-induced death of granule neurons requires the pro-apoptotic Bcl-2 family members Bax and Bim (30-32). Bim is a BH3-only Bcl-2 family member that acts in a concerted manner with Bax to promote apoptosis (41). Similar results have also been observed in vivo in inherited animal models of cerebellar neuronal death.
The lurcher mutant mouse is characterized by a gain-of-function mutation in the delta 2 glutamate receptor that results in the death of cerebellar Purkinje cells (22). Subsequent to the loss of their target Purkinje cells, granule neurons die secondarily via an apoptotic mechanism (22). Targeted deletion of Bax has only a minor effect on Purkinje neuron death, but essentially abolishes the secondary death of granule neurons in lurcher mutant mice (42, 43). Thus, Bcl-2 family members regulate cerebellar granule neuron apoptosis both in vitro and in vivo, strongly suggesting that the intrinsic death pathway plays a principal role in granule neuron apoptosis.

However, much less is known about the involvement of extrinsic death signaling in cerebellar granule neurons. Previous work illustrated that trophic factor withdrawal induces an increase in the mRNA for FasL in primary cerebellar granule neurons, and furthermore, granule neurons isolated from FasL deficient mice (gld mice) demonstrate a significant decrease in their susceptibility to trophic factor withdrawal-induced death (33). Although these results suggest that the extrinsic pathway may be involved in granule neuron apoptosis, our data indicate that disruption of death receptor signaling in cerebellar Purkinje neurons is sufficient to significantly decrease apoptosis in neighboring granule neurons. Thus, the conclusions that were originally drawn from the gld mice may actually reflect a loss of the extrinsic death machinery in Purkinje cells that normally make up a small component of cerebellar cell cultures. Yet, it should be noted that our data do not absolutely exclude a direct role for the extrinsic death pathway in granule neuron apoptosis. In fact, we observed that the small number of granule neurons that did express ΔFADD were rescued from apoptosis, regardless of their proximity to Purkinje cells. However, recent data indicate that specific inhibitors of caspase-8 (an extrinsic initiator caspase) fail to rescue granule neurons from trophic factor withdrawal-induced death, whereas selective caspase-9 (an intrinsic initiator caspase) inhibitors block apoptosis (44). Although the relative
selectivities of synthetic caspase inhibitors are not absolute, these findings support an intrinsic-dependent, but extrinsic-independent, mode of cell death in granule neurons.

The putative role of extrinsic and intrinsic death signaling in the apoptosis of Purkinje neurons is much less well defined than in granule neurons. As described above, Bax deletion did not significantly affect Purkinje neuron survival in lurcher mutant mice (42, 43). In contrast, normal mice in which Bax is knocked out show a significant increase in Purkinje cell number in the cerebellum (45). A similar increase in the number of cerebellar Purkinje cells is also observed in mice overexpressing a human Bcl-2 transgene, suggesting that the intrinsic death pathway influences the developmental death of Purkinje neurons (46). In addition, the spontaneous apoptotic death of Purkinje cells observed in vitro in murine cerebellar organotypic cultures is reduced in tissue isolated from Bcl-2 transgenic mice (47). This latter result indicates that the intrinsic death pathway may also play a role in Purkinje neuron apoptosis induced by stress associated with in vitro culture conditions. However, a more detailed analysis of the mechanism underlying Purkinje cell death is currently lacking. Our data suggest that one important function of Purkinje neurons (ie., to provide trophic support to neighboring granule neurons) is compromised following the withdrawal of trophic factors from cerebellar cell cultures. Moreover, expression of ΔFADD, an inhibitor of extrinsic death signaling, maintains Purkinje cell function during trophic factor withdrawal. Thus, extrinsic death signals, in addition to intrinsic signals, play a significant role in Purkinje neuron loss-of-function and ultimately death.

Finally, the ability of ΔFADD-expressing Purkinje cells to rescue neighboring granule neurons from apoptosis was inhibited by an IGF-I receptor blocking antibody, suggesting involvement of IGF-I in the neuroprotection. Previous work has shown that Purkinje cells are
the principal source of IGF-I production in the developing cerebellum in vivo (40), and IGF-I is a potent neurotrophin for cerebellar granule neurons in vitro (20, 21, 24-26). In vivo studies have demonstrated that Purkinje-derived trophic support, including IGF-I, is essential for the proper development of cerebellar granule neurons (22, 48). For example, in Purkinje cell degeneration (pcd) mice, in which Purkinje cells die spontaneously prior to adulthood, cerebellar IGF-I mRNA expression decreases significantly as the Purkinje neurons degenerate (49). Following the death of Purkinje cells in pcd mice, granule neurons undergo apoptosis resulting from the loss of Purkinje cell-derived IGF-I. Our cerebellar cultures were isolated from postnatal day 7 rats and Purkinje neurons from this stage of development exhibit maximal IGF-I secretion in vivo (50). In addition, IGF-I secretion by Purkinje neurons is highly correlated with the differentiated phenotypes of both granule neurons and Purkinje cells, as is observed in our cultures (50-52). Collectively, the above findings are consistent with an important role for Purkinje cell-derived IGF-I in promoting the survival of cerebellar granule neurons.

Precisely how ΔFADD expression influences Purkinje cell IGF-I production or secretion is presently unclear. It may be that ΔFADD simply blocks extrinsic death signaling in Purkinje neurons and therefore sustains their normal function of secreting trophic factors like IGF-I. Alternatively, it is possible that ΔFADD expression somehow enhances IGF-I synthesis or secretion. Further experiments will be necessary to identify the exact mechanism underlying the regulation of Purkinje cell-derived IGF-I by death receptor signaling molecules. In addition, the molecular mechanism that mediates the neuroprotective effects of IGF-I is unclear. Recently, we have shown that exogenous IGF-I rescues cerebellar granule neurons by inhibiting the intrinsic death pathway (53). Moreover, in transgenic mice overexpressing IGF-I, there was a marked increase in the expression of Bcl-2 in cerebellar Purkinje neurons (54). This finding indicates
that Purkinje-derived IGF-I may not only promote the survival of neighboring granule neurons via a paracrine mechanism, but may also support Purkinje cells directly via an autocrine mechanism, perhaps by suppressing intrinsic death signals at the mitochondria.

In conclusion, we have shown that adenoviral ΔFADD infection of cerebellar cell cultures results in the restricted expression of ΔFADD in Purkinje neurons. ΔFADD-expressing Purkinje cells rescue neighboring granule neurons from trophic factor withdrawal-induced apoptosis via secretion of IGF-I. The dependence of cerebellar granule neuron survival on Purkinje cell-derived trophic support mimics that found in vivo during cerebellar development. The results are the first to show that the extrinsic death pathway in Purkinje neurons indirectly, but significantly, influences the survival of cerebellar granule neurons.
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REFERENCES


FOOTNOTES

1The abbreviations used are: FADD, Fas-associated death domain protein; IGF-I, insulin-like growth factor-I; ΔFADD, Fas-associated death domain protein lacking a death effector domain; DAPI, 4,6-diamidino-2-phenylindole; m.o.i., multiplicity of infection; PBS, phosphate-buffered saline.
FIG. 1. Adenoviral infection with \( \Delta FADD \) attenuates cerebellar granule neuron apoptosis induced by trophic factor withdrawal: the protective effects of \( \Delta FADD \) are localized. A, Cerebellar granule neurons were infected with either a negative control adenovirus (Ad-CMV) or Ad-AU1-\( \Delta FADD \), at the m.o.i. shown, on day 5 in culture. On day 7, cells were incubated in either control (25K+Ser) or apoptotic (5K-Ser) medium for 24 h. Following incubation, granule neurons were fixed and nuclei were stained with Hoechst dye. Cells containing condensed and/or fragmented chromatin were scored as apoptotic. For each experiment, at least two fields of approximately 500 granule neurons/field were counted per condition. The results shown represent data obtained from seven independent experiments. *Statistically different from 5K-Ser alone (\( p<0.01 \)). B, Uninfected granule neurons (top panels) or granule neurons infected with \( \Delta FADD \) at a m.o.i.=50 (bottom panels) were incubated in either control (25K+Ser) or apoptotic (5K-Ser) medium for 24 h and nuclei were then stained with DAPI. Nuclei with condensed and/or fragmented chromatin were abundant in uninfected granule neurons incubated in 5K-Ser medium and in some fields of granule neurons infected with \( \Delta FADD \) (Field A). However, other fields of granule neurons infected with \( \Delta FADD \) did not display a significant number of apoptotic nuclei (Field B). These latter fields invariably contained one or more larger and irregularly-shaped nuclei (indicated by the arrows). Scale bar=10 microns.

FIG. 2. Ad-AU1-\( \Delta FADD \) is expressed in calbindin-positive Purkinje neurons. A, Cerebellar cell cultures were maintained in control (25K+Ser) medium for 7 days. Following
fixation, cells were stained with DAPI (left panel) and anti-calbindin (right panel) to identify Purkinje neurons. The small nuclei are granule neurons, whereas the large irregularly-shaped nucleus (identified by the arrow) is that of a calbindin-positive Purkinje cell. B, Cerebellar cell cultures were infected with Ad-AU1-ΔFADD at a m.o.i.=50 on day 5 in culture. On day 7, the cells were fixed and stained with DAPI (left panel) and anti-AU1 (right panel) to identify ΔFADD-infected cells. The small nuclei are granule neurons, whereas the large irregularly-shaped nucleus (identified by the arrow) is that of a ΔFADD-infected Purkinje cell. Scale bar=20 microns.

FIG. 3. Granule neurons in proximity to ΔFADD-expressing Purkinje cells demonstrate increased survival. A, Uninfected granule neurons (top panels) or granule neurons infected with Ad-AU1-ΔFADD at a m.o.i.=50 (bottom panels) were incubated in either control (25K+Ser) or apoptotic (5K-Ser) medium for 24 h and were then stained with DAPI and anti-AU1. Condensed and/or fragmented nuclei were abundant in uninfected granule neurons incubated in 5K-Ser medium. Apoptotic granule neurons were also plentiful in fields infected with ΔFADD which did not contain any ΔFADD-expressing Purkinje cells (Field A). In contrast, fields infected with ΔFADD that contained one or more ΔFADD-positive Purkinje cells displayed a significant decrease in the number of apoptotic granule neurons following trophic factor withdrawal (Field B). Scale bar=20 microns. B, ΔFADD-infected cerebellar cell cultures were incubated in apoptotic medium and stained for DAPI and AU1, as described in (A). Following staining, the number of healthy vs. apoptotic granule neurons were counted in fields containing either 0, 1-2, or 3-4 ΔFADD-expressing Purkinje cells per field. Ten fields containing an average of 30 granule neurons/field were counted per condition. The results
shown represent data obtained from three independent experiments. *Statistically different from “0” ΔFADD-(+) Purkinje cells per field (p<0.01).

FIG. 4. Cerebellar granule neurons do not efficiently express Ad-AU1-ΔFADD. A, Cerebellar cell cultures were infected on day 5 with Ad-AU1-ΔFADD at a m.o.i.=50. On day 7, cells were fixed and stained with DAPI (left panel) and anti-AU1 (right panel). AU1 staining revealed that less than 5% of granule neurons expressed ΔFADD (identified by the arrows). B, Uninfected granule neurons (top panels) or AU1-ΔFADD-infected granule neurons (bottom panels) were incubated in apoptotic (5K-Ser) medium for 24 h, fixed, and stained with DAPI (left panels) and AU1 (right panels). Trophic factor withdrawal induced a similar apoptotic response in uninfected cells and infected cells in close association with granule neurons expressing AU1-ΔFADD (indicated by the arrow). Scale bar=20 microns.

FIG. 5. Cerebellar granule neurons and Purkinje neurons express IGF-I receptors on their cell surfaces. A, Cerebellar granule neurons were fixed and stained with a monoclonal antibody that recognizes the IGF-I receptor (IGF-IR). Following incubation with a Cy3-conjugated secondary antibody, IGF-I receptors were identified by fluorescence microscopy. IGF-I receptors were expressed primarily at the cell membrane (left panel). Incubation with the secondary antibody alone (2° only) did not produce any positive staining (right panel). B, Cerebellar cell cultures were stained as described in (A), but in addition calbindin-positive Purkinje cells were identified using a polyclonal antibody to calbindin D28K and a FITC-conjugated secondary. Both granule neurons and calbindin-positive Purkinje neurons showed immunoreactivity for IGF-I receptors. Note the overlapping yellow staining indicative of IGF-I
receptor and calbindin colocalization in Purkinje cells (*lower right panel*). Scale bar=20 microns.

**FIG. 6.** ΔFADD-mediated granule neuron survival is inhibited by an IGF-I receptor blocking antibody. Cerebellar cell cultures were either uninfected or infected with Ad-AU1-ΔFADD (m.o.i.=50) on day 5. On day 7, cells were incubated in either control (25K+Ser) or apoptotic (5K-Ser) medium alone or containing either an anti-IGF-I receptor blocking antibody (αIGF-IR, 1 or 10 µg/ml) or a non-immune (N.I.) control IgG (10 µg/ml) for 24 h. Following incubation, cells were fixed and nuclei stained with Hoechst dye. Cells containing condensed and/or fragmented nuclei were scored as apoptotic. For each experiment, at least two fields of approximately 500 granule neurons/field were counted per condition. The results shown represent data obtained from seven independent experiments. *Statistically different from ΔFADD alone (p<0.01).*
FIG. 1. 

A 

% APOPTOTIC

25K+Ser

CMV (50mol)

5

10

25

50

\( \Delta FADD \) (mol)

5K-Ser

B

25K+Ser

5K-Ser

\( \Delta FADD \)

5K-Ser

Field A

Field B
FIG. 2.
FIG. 4.
Suppression of death receptor signaling in cerebellar purkinje neurons protects neighboring granule neurons from apoptosis via an insulin-like growth factor-I dependent mechanism
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