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Apoptotic death of neurons exhibiting peripherin aggregates is mediated by the proinflammatory cytokine tumor necrosis factor-α

Janice Robertson,[1–3] Jean-Martin Beaulieu,[1] Mohammad M. Doroudchi,[3] Heather D. Durham,[3] Jean-Pierre Julien,[1] and Walter E. Mushynski[2]

[1]Centre for Research in Neurosciences, Research Institute of the McGill University Health Centre, [2]Department of Biochemistry, and [3]Montreal Neurological Institute, McGill University, Montreal, Quebec H3A 2B4, Canada

Peripherin, a neuronal intermediate filament protein associated with axonal spheroids in amyotrophic lateral sclerosis (ALS), induces the selective degeneration of motor neurons when overexpressed in transgenic mice. To further clarify the selectivity and mechanism of peripherin-induced neuronal death, we analyzed the effects of peripherin overexpression in primary neuronal cultures. Peripherin overexpression led to the formation of cytoplasmic protein aggregates and caused the death not only of motor neurons, but also of dorsal root ganglion (DRG) neurons that were cultured from dissociated spinal cords of peripherin transgenic embryos. Apoptosis of DRG neurons containing peripherin aggregates was dependent on the proinflammatory central nervous system environment of spinal cultures, rich in activated microglia, and required TNF-α. This synergistic proapoptotic effect may contribute to neuronal selectivity in ALS.

Introduction

Accumulating evidence suggests that peripherin may be an important factor leading to motor neuron degeneration in amyotrophic lateral sclerosis (ALS).* Peripherin, an ∼57-kD neuronal intermediate filament protein, is found associated with axonal spheroids in the degenerating motor neurons of ALS patients (Corbo and Hays, 1992; Migheli et al., 1993), and with perikaryal and axonal aggregates in the motor neurons of transgenic mice overexpressing a mutant form of superoxide dismutase, a model of familial ALS (Tu et al., 1996; Beaulieu et al., 1999a). In addition, we have shown recently in transgenic mice overexpressing peripherin that there is a selective degeneration of motor neurons, characterized by the formation of presymptomatic peripherin aggregates (Beaulieu et al., 1999a, 2000).

Peripherin expression is largely restricted to the peripheral nervous system (PNS), although it is also present at low levels in defined neuronal populations of the central nervous system (CNS), particularly those that extend axonal projections towards the periphery (Parysek and Goldman, 1988; Brody et al., 1989; Escurat et al., 1990; Troy et al., 1990). After injury, peripherin expression can become increased in neurons of the PNS and CNS (Troy et al., 1990; Wong and Oblinger, 1990). This is particularly apparent in spinal motor neurons after injury to the rat sciatic nerve (Troy et al., 1990). The role of peripherin in this response to injury is unclear, but it has been suggested that peripherin may be involved in neuronal regeneration (Troy et al., 1990; Wong and Oblinger, 1990).

A dramatic proinflammatory reaction characterized by microglial activation is associated with the pathological lesions in degenerating motor neurons of ALS (Kawamata et al., 1992). The relationship between this proinflammatory response and intraneuronal proteinaceous aggregates containing peripherin remains obscure. Here we have investigated the mechanism and selectivity of neuronal death associated with increased expression of peripherin in primary neuronal cultures. We show that overexpression of peripherin leads to the formation of neuronal cytoplasmic aggregates and induces death not only of motor neurons, but also of dorsal root ganglion (DRG) neurons that were

Address correspondence to Jean-Pierre Julien, Dept. of Neurology, Montreal General Hospital Research Institute, McGill University, 1650 Cedar Ave., Montreal, Quebec H3G 1A4, Canada. Tel.: (514) 937-6011 Ext. 2361. Fax: (514) 934-8265. E-mail: mdju@musica.mcgill.ca

*Abbreviations used in this paper: ALS, amyotrophic lateral sclerosis; CNS, central nervous system; DRG, dorsal root ganglion; IL, interleukin; NF-H, neurofilament heavy subunit; NF-L, neurofilament light subunit; NF-M, neurofilament medium subunit; PNS; peripheral nervous system; TNF, tumor necrosis factor; TUNEL, TdT-mediated dUTP-biotin nick end labeling; WT, wild type.

Key words: peripherin; apoptosis, ALS; neuronal culture; TNF-α

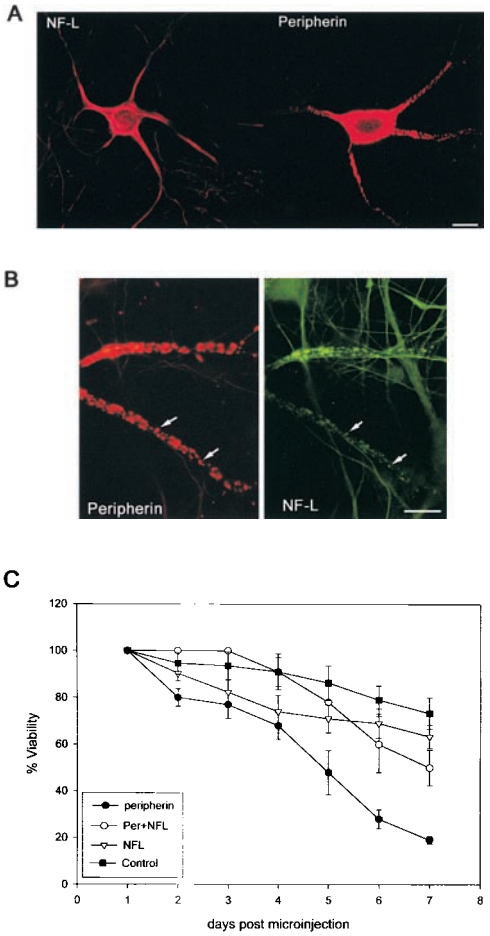


Figure 1. **Intranuclear microinjection of the peripherin gene induces death of cultured motor neurons.** (A) Mammalian expression plasmids encoding either NF-L or peripherin were microinjected into the nuclei of motor neurons in dissociated spinal cord cultures. Cultures were then labeled by indirect immunofluorescence with antibody recognizing NF-L (NR4) or peripherin (MAB1527). The results in this figure show the organization of NF-L or peripherin in microinjected motor neurons after 3 d of expression. Overexpression of NF-L led to intense immunofluorescence labeling of motor neurons, characterized by the formation of coiling loops in the perikaryon. In contrast, overexpressed peripherin did not integrate normally into the existing cytoplasmic network, but instead formed punctate aggregates that were also clearly apparent in neurites. (B) Double immunofluorescence labeling with antibodies to peripherin (MAB1527) and NF-L (AB1983) of the neurites from a motor neuron that had been microinjected with peripherin, showing the disruption, by peripherin, of the endogenous neurofilament network (arrows). (C) Motor neurons in dissociated spinal cord cultures were microinjected with expression plasmids encoding (a) peripherin; (b) NF-L; (c) NF-L and peripherin; or (d) expression plasmid alone (pRcCMV; control). The number of viable motor neurons was counted each day for 7 d, and the results from each microinjection experiment were compared. The chart shows that peripherin was extremely toxic to motor neurons. In contrast, there was no significant effect of NF-L overexpression on viability (Student's *t* test; $P < 0.05$). Indeed, comicroinjection of NF-L with peripherin offered protection from the toxic effects observed with peripherin microinjection alone. Bars: (A) 25 μm; (B) 15 μm.

cultured from dissociated spinal cords of peripherin transgenic embryos. Apoptosis of DRG neurons containing peripherin aggregates was dependent on the proinflammatory CNS environment of spinal cord cultures, rich in activated microglia, and required tumor necrosis factor (TNF)-α. These findings demonstrate a synergistic proapoptotic relationship between a proinflammatory CNS environment and neurons overexpressing peripherin, and may provide an explanation for neuronal selectivity in ALS.

Results

Peripherin forms punctate aggregates in cultured motor neurons microinjected with the mouse peripherin gene

To investigate the effect of peripherin overexpression on motor neuron viability in primary culture, the mammalian expression plasmid (pRcCMV) encoding the mouse peripherin gene was microinjected into the nuclei of motor neurons in dissociated spinal cord cultures. In a parallel experiment, the cDNA-encoding mouse neurofilament light subunit (NF-L) chain in pRcCMV was also microinjected.

In control cultures, either nonmicroinjected or microinjected with vector alone, there was minimal labeling of motor neurons with peripherin antibody (unpublished data). However, in motor neurons microinjected with the peripherin expression plasmid, there was intense peripherin immunoreactivity (Fig. 1 A). Interestingly, peripherin did not integrate normally into the existing cytoplasmic intermediate filament network, but instead formed clearly defined punctate aggregates that filled the cell body and were also present in neurites (Fig. 1 A). In contrast, the plasmid-derived NF-L protein appeared to integrate into the existing network, forming looping coils of filaments after 3 d that progressed to substantial perikaryal aggregates after 7 d of expression (Fig. 1 A). Motor neurons microinjected with the peripherin expression plasmid were double labeled with peripherin and neurofilament antibodies (Fig. 1 B; similar findings obtained with antibody to neurofilament medium subunit [NF-M]), showing that peripherin caused disruption of the existing neurofilament network.

Overexpression of peripherin induces death of cultured motor neurons

The number of viable motor neurons was counted each day for 7 d after microinjection of the peripherin or NF-L expression vectors as described in Materials and methods. In a companion experiment, both the peripherin and NF-L plasmid expression vectors were comicroinjected, and motor neuron viability assessed. A comparison of motor neuron survival in each of the different paradigms is shown in Fig. 1 C and illustrates the specificity of peripherin-induced neuronal loss. After 4 d of expression, there was an ~50% reduction in the number of viable motor neurons overexpressing peripherin and no significant reduction in the number of motor neurons overexpressing NF-L (Fig. 1 C). Interestingly, in the comicroinjection experiment, toxicity induced by peripherin could be partially precluded by increased expression of NF-L (Fig. 1 C). These findings demonstrate the relative specificity of peripherin-induced toxicity in motor neurons and confirm previous findings in peripherin transgenic mice (Beaulieu et al., 1999a, 2000).

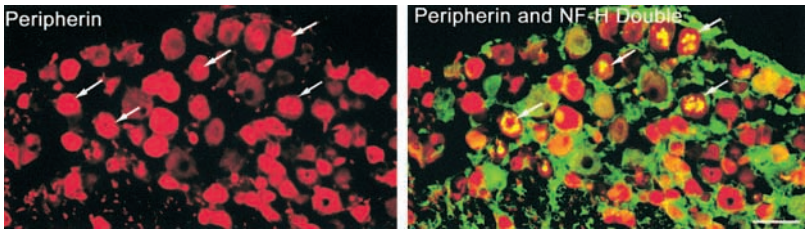


Figure 2. **Peripherin aggregates in DRG neurons of peripherin transgenic mice.** Cryostat sections from L4-L5 DRG from peripherin transgenic mice were labeled by indirect immunofluorescence with antibody to peripherin or neurofilaments (NF-H; SMI32). The panel labeled Peripherin and NF-H Double is an overlay of the images obtained with antibodies recognizing peripherin (red) and NF-H (green), and shows peripherin aggregates either alone or together with neurofilaments (arrows). Bar, 75 μm.

DRG neurons, unlike motor neurons, are spared in peripherin transgenic mice

Motor neurons in peripherin transgenic mice contain presymptomatic peripherin aggregates within their cell bodies and axons (Beaulieu et al., 1999a). We were interested to know if other neuronal cell types within peripherin transgenic mice also contained peripherin aggregates and, if so, whether there was loss of these neurons. For this study we used Per/LKO transgenic mice, in which peripherin is overexpressed in an NF-L knockout background. These mice have an accelerated phenotypic onset compared with Per transgenic mice (Beaulieu et al., 1999a), making them useful for shorter term studies. Immunofluorescence labeling of L4-L5 DRG neurons from peripherin transgenic mice showed the presence of distinctive inclusions within a subpopulation of DRG neurons expressing peripherin (Fig. 2). However, in contrast to the effect of peripherin overexpression in motor neurons, no dramatic loss of DRG neurons or subsequent behavioral phenotype due to this loss was observed in peripherin transgenic mice (Beaulieu et al., 1999a). Consequently, the mere presence of peripherin inclusions does not necessarily result in neuronal death.

Overexpression of peripherin induces apoptosis of DRG neurons in dissociated spinal cord cultures

To further investigate the mechanism of peripherin-induced neurotoxicity, we prepared dissociated spinal cord cultures from peripherin transgenic mouse embryos. The major neuronal cell types within these cultures were motor and DRG neurons (Durham et al., 1997; Roy et al., 1998). Other cell types, including astrocytes, fibroblasts, and microglia, were also identified using antibodies recognizing cell specific markers (glial fibrillary acidic protein, vimentin, and Mac-2, respectively). In dissociated spinal cord cultures, peripherin was expressed almost exclusively in DRG neurons with minimal labeling of motor neurons. Peripherin labeling of wild-type (WT) cultures showed a normal intermediate filament distribution in DRG neurons (Fig. 3 A). However, in peripherin transgenic cultures (Per) the DRG neurons contained perikaryal peripherin aggregates, as revealed by their appearance and intensity of labeling with peripherin antibody (Fig. 3 A). Various types of peripherin aggregates were observed, including amorphous aggregates that filled the perikaryon, distinct, spherical perinuclear aggregates and some with more punctate-like morphologies (Fig. 3 A). There was reduced labeling of neurites in the Per cultures compared with their control counterpart, more noticeable in younger cultures, suggestive of reduced transport of peripherin into neurites (Fig. 3 A). Double immunofluorescence labeling of the cultures with antibodies recognizing neurofilaments showed their colocalization with the peripherin aggregates (Fig. 3 B; Beaulieu et al., 1999b). Microtubules, although disrupted, were not a component of the aggregates as seen by double labeling with antibody recognizing α-tubulin (unpublished data). Electron microscopic analysis of the peripherin aggregates showed that they were composed of 10-nm filaments and incorporated cytoplasmic organelles, particularly mitochondria (Fig. 3 C). Microtubules were excluded from the aggregates confirming our immunocytochemical findings. The relative expression of peripherin in the WT and Per cultures was compared by immunoblot analyses. There was an approximately sixfold increase in peripherin expression in Per cultures compared with WT, as assessed using NIH Image Software (Fig. 3 D).

By phase contrast microscopy, DRG neurons in Per cultures appeared shrunken and became detached from the substratum, characteristic features of apoptosis (Majno and Joris, 1995). To clarify if apoptosis was indeed occurring, we performed TdT-mediated dUTP-biotin nick end labeling (TUNEL) assays on Per cultures and compared the number of TUNEL-positive DRG neurons with those found in WT cultures. We found that there was a dramatic increase in TUNEL-positive DRG neurons in Per cultures. The percentage specific apoptosis of Per DRG neurons was calculated as 32.8% ± 3.5% (calculated from counts obtained from three separate litters; Fig. 4 D), and in general was found to range between 30 and 40%. After TUNEL labeling, cultures were labeled for indirect immunofluorescence with antibody to peripherin to determine if there was a correlation between the presence of peripherin aggregates and apoptosis (Fig. 4 A). Indeed, it was found that all DRG neurons in Per cultures contained peripherin aggregates.

Further confirmation of apoptosis was obtained using antibody-recognizing activated caspase-3, which labeled several DRG neurons in Per cultures (Fig. 4 B). No labeling of DRG neurons in control cultures from WT embryos was seen. In addition, disruption of mitochondrial cristae in DRG neurons containing peripherin aggregates was observed by electron microscopy (Fig. 4 C), changes that have previously been reported in DRG neurons undergoing apoptosis (Russell et al., 1999).

DRG neurons exhibiting peripherin aggregates are spared from apoptosis in pure culture

In Per-dissociated spinal cord cultures, we observed that in localized groupings of DRG neurons, very few (if any) of the neurons were TUNEL positive, despite the presence of peripherin aggregates (Fig. 5 A). To further investigate this, we compared TUNEL reactivity between pure DRG cultures and mixed spinal cord cultures derived from both WT and

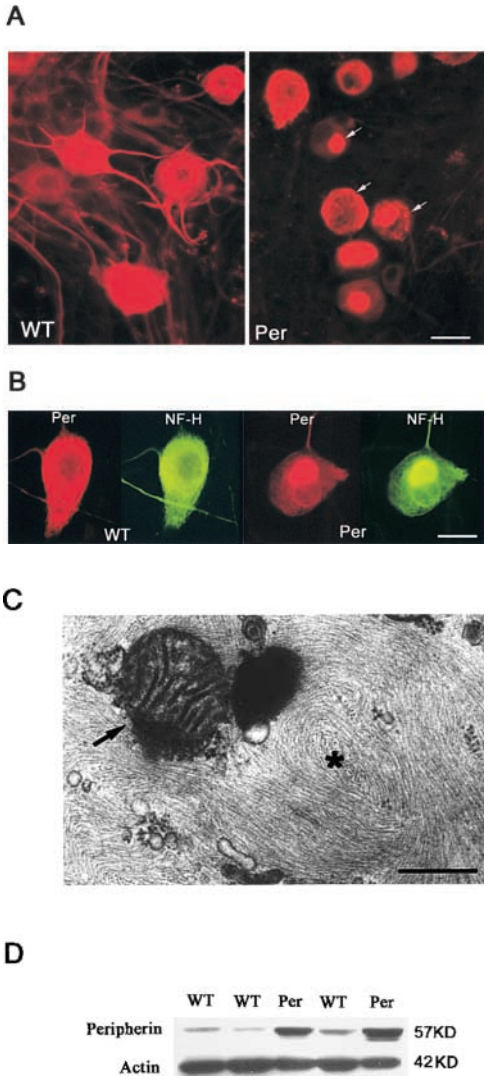
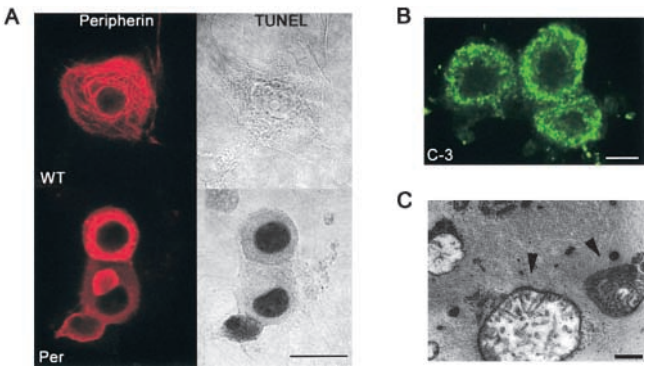


Figure 3. **Peripherin aggregates in Per transgenic DRG neurons.** (A) Comparison of peripherin labeling of dissociated spinal cord cultures derived from WT and Per transgenic embryos. DRG neurons were the predominant cell type labeled by peripherin antibody in these cultures. Peripherin in DRG neurons in WT cultures displayed a normal cytoplasmic distribution, whereas DRG neurons in Per cultures formed aggregates that displayed a variety of phenotypes, including distinct spherical aggregates, punctate-like aggregates, and amorphous aggregates that filled the perikaryon (arrows). (B) Double immunofluorescence labeling of DRG neurons from WT and Per cultures with peripherin and neurofilament (N52, specific for NF-H) antibodies showed the colocalization of neurofilaments with the peripherin aggregates. (C) Electron microscopic examination of aggregates showed that they were composed of 10-nm filaments, representative of intermediate filaments (*), excluded microtubules, and contained entrapped organelles, particularly mitochondria (arrow). (D) To estimate the increase in peripherin expression in Per DRG neurons relative to WT, pure DRG neuronal cultures from WT and Per transgenic embryos were prepared, and samples of each compared by immunoblotting with antibody to peripherin. Labeling with actin antibody was used to control for protein loadings. The immunoblot shows the findings from cultures derived from five different embryos and is representative of all cultures analyzed. The increase in peripherin expression in Per DRG neurons was estimated using NIH image software and found to be approximately sixfold. Bars: (A) 25 μM; (B) 20 μM; (C) 75 μm.



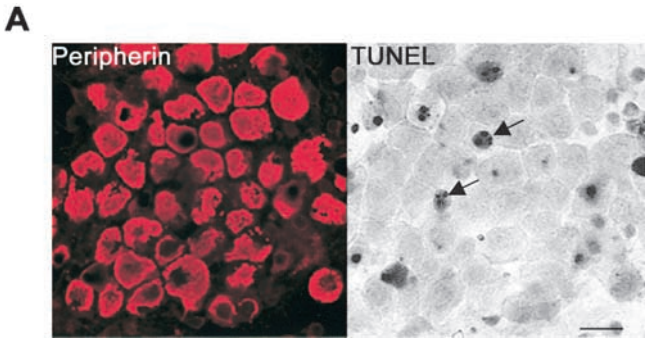
	%TUNEL +ve DRG neurons		
	Litter 1	Litter 2	Litter 3
Per	44.5 +/- 6.4 (n=3)	42.3 +/- 11.3 (n=4)	41.2 +/- 4.6 (n=4)
WT	15.5 +/- 4.9 (n=3)	14.1 +/- 6.1 (n=3)	10.5 +/- 5.4 (n=6)
Specific Apoptosis	33.7%	32.8%	34.5%

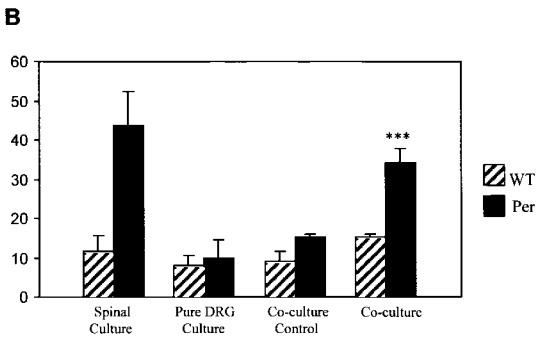
Figure 4. **Overexpression of peripherin induces apoptosis of DRG neurons.** (A) TUNEL assays were performed on WT and Per dissociated spinal cord cultures. Cultures were then labeled by indirect immunofluorescence with peripherin antibody so that TUNEL-positive DRG neurons could be correlated with the presence of peripherin aggregates. TUNEL-positive DRG neurons were shrunken and had condensed chromatin and an intact plasma membrane, consistent with the changes associated with apoptosis. (B) Dissociated spinal cord cultures were labeled by indirect immunofluorescence with antibody recognizing activated caspase-3 (C-3). A number of DRG neurons in Per cultures were labeled with C-3. No labeling of WT DRG neurons was observed. (C) Electron microscopic evaluation of apoptotic Per DRG neurons showed that there was severe disruption of mitochondrial cristae (arrowheads). (D) Comparison of specific apoptosis of Per DRG neurons in dissociated spinal cord cultures from three separate litters. The percentage of TUNEL-positive DRG neurons in WT and Per dissociated spinal cord cultures was calculated after 14 d in vitro. Specific apoptosis was calculated as the percentage of TUNEL-positive Per DRGs, minus the percentage of TUNEL-positive WT DRGs/100, minus the percentage of TUNEL-positive WT DRGs. Specific apoptosis of Per DRG neurons was usually between 30 and 40%. The results shown are from three litters and are typical of our findings. Bars: (A) 20 μM; (B) 15 μM; (C) 0.4 μM.

Per transgenic embryos. Consistent with our findings in vivo, we found that peripherin aggregates did not induce apoptosis of Per DRG neurons in pure DRG cultures. There was no significant difference between the number of TUNEL-positive DRG neurons in Per or WT DRG cultures (Fig. 5 B). A direct comparison of pure DRG cultures and spinal cultures derived from the same Per embryonic spinal cords (prepared by removing 50% of DRGs and culturing them separately from the remaining spinal cord) demonstrated that apoptosis of Per DRG neurons only occurred in the mixed cell environment of dissociated spinal cord cultures (Fig. 5 B).

CNS environment induces apoptosis of DRG neurons containing peripherin aggregates

To differentiate between direct cell–cell interaction or paracrine effects, we studied DRG neuronal viability using coculture experiments in which isolated DRG neurons were separated from dissociated spinal cord cultures by a permeable membrane. The effect of diffusible molecules from the dissociated spinal cord





	Spinal Culture	Pure DRG Culture	Co-culture Control	Co-culture
WT	11.7 +/- 3.9 (n=7)	7.84 +/- 2.5 (n=5)	9.1 +/- 2.7 (n=3)	15.4 +/- 0.5 (n=3)
Per	43.6 +/- 8.7 (n=7)	9.8 +/- 4.7 (n=5)	15.4 +/- 0.5 (n=3)	34.2 +/- 3.8 (n=3)

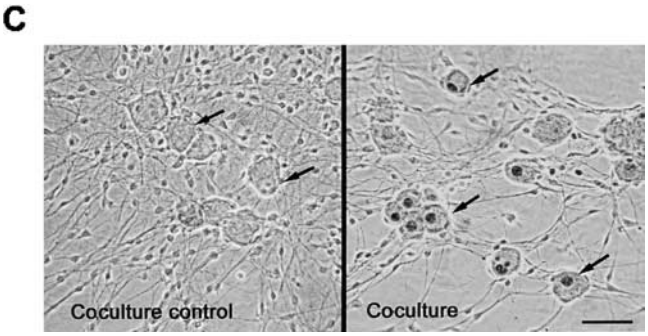


Figure 5. **Apoptosis of Per DRG neurons requires CNS cell culture environment.** (A) It was observed that localized groupings of Per DRG neurons within dissociated spinal cord cultures were largely TUNEL negative despite containing peripherin aggregates. Double labeling of such a grouping with TUNEL and with peripherin antibody shows that all the DRG neurons in the field have peripherin aggregates, but that only a few are TUNEL positive and have condensed chromatin (arrows). (B) Pure DRG neuronal cultures were prepared from both WT and Per transgenic embryos, and the number of TUNEL-positive neurons was compared with the findings from dissociated spinal cord cultures (spinal culture). The chart and conjoining table show that there was no significant increase in the number of TUNEL-positive DRG neurons in pure Per DRG cultures compared with WT DRG neurons (DRG culture). To investigate the effect of the cell culture environment on Per DRG viability, pure DRG neurons were grown for 4 d on glass coverslips and then placed in inserts in plates that had (Coculture) or had not (coculture control) been seeded with dissociated spinal cord cultures. Our findings show that when pure cultures of Per DRG neurons were cocultured with dissociated spinal cord cultures, there was a significant increase in apoptosis compared with control $P < 0.001$*** (by two-way analysis of variance). (C) A comparison of TUNEL labeling of pure cultures of Per DRGs, cocultured in the absence (coculture control) or presence of dissociated spinal cord cells (coculture). The arrows indicate DRG neurons. Note the increase in number of TUNEL-positive DRG neurons and their shrunken appearance in spinal cord coculture compared with control DRG neurons (arrows). Bars: (A) 50 μM; (C) 50 μM.

cultures on DRG neuron viability was assessed after 14 d of coculture. A comparison of the percentage of TUNEL-positive DRG neurons was made between typical dissociated spinal cord cultures (Spinal Culture), pure DRG neuronal cultures (Pure DRG Culture), pure DRG neuronal cultures grown in inserts (Coculture Control), and pure DRG neurons grown in inserts and cocultured with dissociated spinal cord cultures (Coculture) (Fig. 5 B). Using two-way analysis of variance ($P < 0.001$), there was a significant increase in the number of TUNEL-positive Per DRG neurons when they were cocultured with dissociated spinal cord cultures relative to the corresponding coculture control (34.2 ± 3.8% compared with 15.4 ± 0.5%; Fig. 5 B). The dramatic effect of the coculture environment on increasing apoptosis of Per DRG neurons is shown in Fig. 5 C.

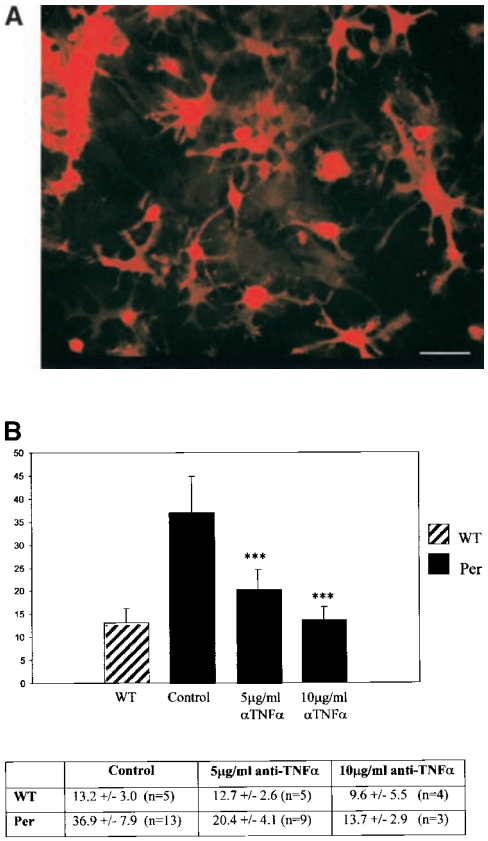
These findings suggest that the dissociated spinal cord cultures release soluble factor(s) that induce apoptosis of DRG neurons containing peripherin aggregates. The finding that Per DRG neurons by themselves do not undergo apoptosis, despite containing peripherin aggregates, provides important insights into the differences between the CNS, reflected in dissociated spinal cord cultures, and the PNS environments as they relate to neuronal viability.

Dissociated spinal cord cultures contain large numbers of activated microglia

As mentioned earlier, in addition to DRG and motor neurons, the dissociated spinal cord cultures contained a number of other cell types, including astrocytes, fibroblasts, and microglia. Microglia in culture adopt an activated phenotype characterized by hypertrophy and development of shorter, stouter processes compared with resting microglia which have smaller perikarya and are highly ramified (Davis et al., 1994; Streit et al., 1999). In addition to these morphologic changes, activated microglia express phenotypic markers reflective of their immunologic function such as major histocompatibiblty complex class I and II antigens (Gehrmann et al., 1995) and Mac-2 (Reichert and Rotshenker, 1996). There was very strong labeling of dissociated spinal cord cultures with antibody to Mac-2, demonstrating the presence of activated microglia (Fig. 6 A). In contrast, there was only minimal labeling of pure DRG neuronal cultures with Mac-2 antibody (unpublished data), most likely due to contaminating hematogenous macrophages. Although activated astrocytes, revealed by intense immunoreactivity with antibody recognizing glial fibrillary acidic protein, were also present in our cultures (unpublished data), the abundance of activated microglia in dissociated spinal cord cultures represented the most clear difference from pure DRG cultures.

Proapoptotic effect of CNS environment on Per DRG neuron viability is blocked by TNF-α–neutralizing antibody

Activated microglia, both in a pathological in vivo setting and in culture, release a number of proinflammatory prod-



	Control	5μg/ml anti-TNFα	10μg/ml anti-TNFα
WT	13.2 +/- 3.0 (n=5)	12.7 +/- 2.6 (n=5)	9.6 +/- 5.5 (n=4)
Per	36.9 +/- 7.9 (n=13)	20.4 +/- 4.1 (n=9)	13.7 +/- 2.9 (n=3)

Figure 6. **Apoptosis of Per DRG neurons in dissociated spinal cord cultures is precluded by TNF-α–neutralizing antibody.** (A) Indirect immunofluorescence labeling with Mac-2 antibody (American Type Culture Collection) showing the abundance of activated microglia in dissociated spinal cord cultures. 30 μm. (B) The effects of TNF-α–neutralizing antibody on the percentage of TUNEL-positive DRG neurons in WT and Per dissociated spinal cord cultures was compared. Quadruplet cultures from each embryo were prepared and 5 or 10 μg/ml of TNF-α antibody added to two of the cultures, the other two acting as control. The chart and conjoining table show that inclusion of TNF-α antibody within the culture medium significantly reduced the number of TUNEL-positive DRG neurons in Per cultures ($P < 0.001$*** by two-way analysis of variance). Addition of 10 μg/ml TNF-α antibody to the culture medium enhanced the protective effect to levels similar to results found for WT. There was no significant effect of TNF-α–neutralizing antibody on WT DRG neuronal viability (unpublished data) Bar, 50 μM.

ucts including cytokines (Streit et al., 1999) and we suspected that these may be involved in the apoptosis of Per DRG neurons in dissociated spinal cord cultures. To investigate this possibility, we added neutralizing antibody to TNF-α (5 and 10 μg/ml; R&D Systems) to the medium of dissociated spinal cord cultures. After 14 d in vitro, the percentage of TUNEL-positive neurons was calculated and compared with controls lacking TNF-α–neutralizing antibody and with WT cultures. Remarkably, the level of apoptosis of Per DRG neuron in dissociated spinal cord cultures dropped to levels comparable with results obtained from WT control cultures (Fig. 6 B). This finding clearly demonstrated that the proapoptotic effect of the mixed cell environment of dissociated spinal cord cultures on Per DRG neuronal viability could be mediated by TNF-α.

Discussion

Here we have shown that overexpression of the intermediate filament protein peripherin forms cytoplasmic neuronal aggregates and induces death not only of motor neurons but also of DRG neurons in primary culture. Most importantly, we have shown that apoptotic death of peripherin transgenic DRG neurons is dependent on the CNS environment of dissociated spinal cord cultures. These cultures contain a large number of activated microglia and astrocytes that release proinflammatory products, such as cytokines and reactive oxygen species (Chao and Hu, 1994; Meda et al., 1995; Nakamura et al., 1999; Streit et al., 1999). Apoptosis of peripherin-overexpressing DRG neurons in spinal cord cultures could be precluded by the addition of TNF-α–neutralizing antibody, demonstrating the involvement of TNF-α in the mechanism of peripherin-induced DRG neuronal death. These findings, together with the fact that there is no loss of DRG neurons in peripherin transgenic mice, suggest that peripherin overexpression and aggregation is not sufficient to provoke cell death, but instead acts either by predisposing neurons to, or acting in synergy with, proapoptotic environmental signals generated by an inflammatory response. These results may be of relevance not only to ALS, but also to other neurodegenerative diseases such as Alzheimer's and Parkinson's disease, where the pathological role of protein aggregation in the disease mechanism remains elusive (Kawamata et al., 1992; McGeer and McGeer, 1995; Togo et al., 2001).

In peripherin transgenic mice, overexpression of peripherin leads to the selective degeneration of motor neurons, characterized by the formation of presymptomatic peripherin aggregates in perikarya and axons (Beaulieu et al., 1999a). Here we have provided further evidence for the neurotoxicity of peripherin overexpression in motor neurons of dissociated spinal cord cultures microinjected with the peripherin gene. Peripherin in these neurons formed small punctate aggregates that caused disruption of the endogenous neurofilament network. Interestingly, in similarly microinjected DRG neurons (unpublished data), peripherin did not form the small punctate aggregates observed in motor neurons, but instead formed larger perikaryal aggregates similar to those seen in cultured DRG neurons from peripherin transgenic embryos. This may be due to intrinsic differences between motor neurons and DRG neurons such as variations in expression of chaperonins (Bruening et al., 1999) or other associated proteins such as bullous pemphigoid antigen 1 (Leung et al., 1999). Overexpression of peripherin in cultured motor neurons resulted in a dramatic reduction in viability, with ~50% cell death after only 4 d of expression. In contrast, intranuclear microinjection of expression plasmid encoding NF-L had no such effect, despite the fact NF-L formed large perikaryal accumulations. Comicroinjection of NF-L expression plasmid together with peripherin led to an increased survival of motor neurons compared with those microinjected with peripherin alone. Although the mechanism of this protective effect of NF-L overexpression is unclear, it is reminiscent of previous findings in which increased expression of neurofilaments was shown to be neuroprotective (Wong et al., 1995; Couillard-Despres et al., 1998; Meier et al., 1999).

We found that, in contrast to motor neurons, DRG neurons in peripherin transgenic mice were able to tolerate the presence of peripherin aggregates (Beaulieu et al., 1999a). A number of reasons could account for this selective vulnerability of motor neurons as compared with DRG neurons in vivo, including differences in the content of chaperonins (Bruening et al., 1999), neurofilaments (Kawamura et al., 1981; Sobue et al., 1987), and calcium-binding proteins (Ince et al., 1993; Alexianu et al., 1994). Furthermore, DRG neurons are packed closely together in DRG, and as we observed in peripherin transgenic spinal cord cultures (Fig. 4), potential paracrine effects may be neuroprotective for DRG neurons. Moreover, as we have shown here, differences between the PNS and CNS environments, such as the presence of activated microglia, may account for the neuronal selectivity in peripherin-mediated neurodegeneration. In contrast to our findings in vivo, there was apoptosis of DRG neurons in spinal cord cultures derived from peripherin transgenic embryos. As in DRG neurons in vivo, DRG neurons in culture exhibited cytoplasmic peripherin aggregates, the presence of which correlated with the incidence of cell death. Most importantly, by comparison with pure DRG cultures, we found that apoptosis of Per DRG neurons in spinal cultures was dependent on soluble factor(s) derived from the spinal cord milieu. Spinal cultures contained a large number of activated microglia that were not present in pure DRG cultures, reflective of the differences between CNS and PNS culture environments. Microglial activation, as part of the inflammatory response, is associated with the pathological lesions in a number of major neurodegenerative diseases, including ALS (Kawamata et al., 1992; Banati et al., 1995), Alzheimer's (McGeer and McGeer, 1995; Sheng et al., 1997; Uchihara et al., 1997), and Parkinson's disease (Togo et al., 2001). However, this activation and the relationship with neuronal protein aggregates remains unclear. Activated microglia, both in vivo and in culture, release a number of proinflammatory cytokines, such as TNF-α and interleukin (IL)-1β, and other proinflammatory products such as reactive oxygen species and nitric oxide (Perry et al., 1993; Chao and Hu, 1994; Banati et al., 1995; Streit et al., 1999). Although astrocytes, oligodendrocytes, and neurons can release proinflammatory species, the primary source after injury to the CNS is activated microglia (Perry et al., 1993; Hopkins and Rothwell, 1995; Bartholdi and Schwab, 1997). In primary culture, microglia are activated by the presence of serum and tissue debris, generated during the process of preparing cultures (Hurley et al., 1999; Nakamura et al., 1999; Streit et al., 1999). These microglia are considered to be "superactivated," as they release increased amounts of proinflammatory cytokines compared with their counterparts in vivo, mimicking in part the situation that occurs in neurodegenerative disease (Hurley et al., 1999; Streit et al., 1999).

The major proinflammatory cytokine released by activated microglia is TNF-α. TNF-α levels are elevated in the CSF and affected brain tissue of patients with Alzheimer's (Fillit et al., 1991; Dickson et al., 1993) and Parkinson's disease (Boka et al., 1994; Mogi et al., 1994); TNF-α levels are elevated in the blood of patients with ALS (Poloni et al., 2000).We show here that apoptosis of DRG neurons containing peripherin aggregates requires TNF-α. It is presently unclear whether TNF-α is acting directly on DRG neurons or mediating its proapoptotic effects through other species such as IL-1β (Venters et al., 2000) or nitric oxide (Galea et al., 1992; Murphy et al., 1993; Combs et al., 2001). There is conflicting literature on the neuroprotective as opposed to the neurotoxic effects of TNF-α both in vivo (Bruce et al., 1996; Barone et al., 1997; Gary et al., 1998; Lavine et al., 1998) and in vitro (Schwartz et al., 1991; Piani et al., 1992; Cheng et al., 1994; Barger et al., 1995). Administration of TNF-α to neurons in vivo (Liu et al., 1994) or in primary neuronal culture does not appear to directly cause neurotoxicity (Loddick and Rothwell, 1999). Instead, TNF-α can augment the effects of other neuronal insults such as after focal ischemia (Barone et al., 1997) or glutamate toxicity (Chao and Hu, 1994), or it can act synergistically with other proinflammatory cytokines such as IL-1β (Chao et al., 1995; Combs et al., 2001). In our culture model, TNF-α appears to act synergistically with peripherin overexpression, characterized by the presence of peripherin aggregates, to induce apoptosis of DRG neurons. This model, in which the downstream effects of neurotoxicity include mitochondrial defects and caspase-3 activation (Fig. 3), should allow elucidation of the precise mechanism of neuronal degeneration induced by the interdependent relationship between an inflammatory environment and neurons exhibiting peripherin aggregates.

The evidence presented here, combined with previous studies in transgenic mice, suggests that peripherin has specific properties that lead to neuronal degeneration. In transgenic mouse models of ALS, peripherin is the only WT neuronal intermediate filament protein that, when overexpressed, has caused the dramatic and selective degeneration of motor neurons (Beaulieu et al., 2000). Compared with other neuronal intermediate filament proteins, peripherin is unusual in that it can be phosphorylated on tyrosine residues (Angelastro et al., 1998). The function of peripherin tyrosine is unknown, and although it appears to occur normally on at least one tyrosine residue (Angelastro et al., 1998), it is possible that TNF-α may induce a change in peripherin tyrosine phosphorylation by signaling through nonreceptor protein-tyrosine kinases. For example, it has recently been shown that TNF-α can induce reorganization of the actin network by a mechanism involving tyrosine phosphorylation of paxillin and focal adhesion kinase (Koukouritaki et al., 1999). It is also possible that peripherin tyrosine residues may be a target for nitration by peroxynitrite, which is formed by the reaction between nitric oxide and superoxide radicals, as part of the inflammatory response leading to neuronal injury (Beckman, 1994). Although evidence for such modifications of neuronal proteins in ALS is somewhat tenuous (Bruijn et al., 1997; Strong et al., 1998), peripherin has not yet been studied as a potential target.

We have provided direct evidence that signaling between a proinflammatory environment containing activated microglia and neurons exhibiting peripherin aggregates leads to neuronal death. Microglial activation in the CNS occurs as a consequence of aging (Morgan et al., 1999; Sloane et al., 1999; Streit et al., 1999; Felzien et al., 2001), and it is pertinent that the phenotypic degeneration of motor neurons in peripherin transgenic mice occurs in aged mice (18 mo to 2 yr; Beaulieu

et al., 1999a). Most importantly, we have demonstrated a synergistic proapoptotic relationship between TNF-α and DRG neurons exhibiting peripherin aggregates. These findings reflect differences between CNS and PNS environments, and may provide an explanation for neuronal selectivity in ALS. Our findings suggest that treatments aimed at attenuating the proinflammatory response associated with the pathological lesions in ALS could have therapeutic potential.

Materials and methods

Transgenic mice

The mice used in this study have been characterized previously, and include C57BL6 (WT) and hemizygous transgenic mice overexpressing the WT mouse peripherin gene under the control of its own promoter elements (Per) (Beaulieu et al., 1999a). Primary neuronal cultures were prepared from E13 mouse embryos that were derived from breeding Per × C57BL6 mice. These breedings produced both WT and peripherin transgenic littermates, thereby providing internal controls for comparison. Embryos carrying the peripherin transgene were identified by Southern blot analysis.

Dissociated spinal cord cultures

Primary cultures of dissociated spinal cord and DRGs were prepared as described by Roy et al. (1998). In brief, spinal cords and associated ganglia were dissected from embryos, dissociated with trypsin, and plated on 12-mm coverslips precoated with poly-D-lysine and extracellular matrix (Sigma-Aldrich) at a density of 2.5×10^5 cells per well of a four-well plate (Nunclon). Approximately $1–2 \times 10^6$ cells were obtained from each spinal cord, each cord being processed and plated separately. For microinjection studies, cultures were prepared from cytoplasmic dynein 1 mouse embryos and plated at a density of 6.5×10^5 per well in 12-well dishes (Roy et al., 1998). All cells were plated in modified N3 medium as described in Roy et al. (1998). On days 4 and 5, cultures were treated with 1 μM cytosine arabinoside for 1–2 d to limit growth of nonneuronal cells, and were maintained in modified N3 medium at 37°C in 5% CO_2. Cultures were used for analyses after 14 d in vitro for transgenic DRG studies and after 4–6 wk for microinjection studies.

DRG neuron–dissociated spinal cord cocultures

DRG cultures were prepared as described in O'Ferrall et al. (2000) with the following modifications. The medium for plating and general maintenance was as for the dissociated spinal cord cultures described above. DRG neurons were plated at 12–15 dissociated DRGs per well of a four-well plate containing coverslips precoated as above.

For coculture experiments, Falcon cell culture inserts (0.4 μM polyethylene terephthalate track etched membrane, six-well format; Becton Dickinson) were placed in six-well insert companion plates that contained medium only, or that had been preplated with dissociated spinal cord cultures at a density of 10^6 cells per well. DRG neurons were plated on glass coverslips as described above and allowed to establish for 4 d. Coverslips were then transferred to Falcon cell culture inserts and cocultured with the dissociated spinal cord cultures or with medium only for 10–14 d. After this time, coverslips were removed and labeled using the TUNEL assay as a marker of apoptosis.

Immunocytochemistry

Immunocytochemistry was performed as in Roy et al. (1998) using antibodies from Chemicon (peripherin, monoclonal MAB1527, and polyclonal AB1515; poylclonal neurofilament antibodies to NF-L, AB1983; NF-M, AB1981; and neurofilament heavy subunit [NF-H], AB1982; all 1:1,000), Sigma-Aldrich (monoclonal antibodies to neurofilaments NF-L, NR4; NF-M, NN18; NF-H, N52; and α-tubulin, DM1A; all 1:1,000), and nuclear envelope breakdown (polyclonal antibody to activated caspase-3, 1:100; following supplier recommendations). Antibody distribution was visualized by epifluorescence/confocal microscopy after incubation with the appropriate secondary antibody (Alexa Fluor–labeled secondary antibody; 1:100; Molecular Probes).

For electron microscopy and immunohistochemical analysis of transgenic mouse tissue sections, the method of Beaulieu et al. (1999) was used.

Immunoblotting

Cells were harvested in 7 mM Tris, pH 6.75, containing 2% SDS and 10% glycerol, and assayed for total protein using the bicinchoninic acid assay. Loadings of 10–15 μg of protein were routinely analyzed on 6–12% gradient SDS–polyacrylamide gels and then blotted to polyvinyldifluoride membrane. For immunoblotting, membranes were incubated with monoclonal antibodies recognizing peripherin (MAB1527, 1:5,000; Chemicon) or actin (MAB1501,1:10,000; Chemicon), and antibody binding was revealed using the ECL detection system (NEN Life Sciences).

TUNEL Assays

The In Situ Cell Death Detection Kit, POD, from Roche Molecular Diagnostics (Laval, QC) was used for TUNEL assays, with DAB as the substrate (Gavrieli et al., 1992). Fluorescent double labeling of cultures with antibody to peripherin was performed in conjunction with the TUNEL assay to enable correlation of TUNEL-positive cells with the presence of peripherin aggregates. TUNEL labeling in itself is not indicative of apoptosis, and confirmatory evidence of apoptosis was obtained from morphological criteria such as cell shrinkage and maintenance of an intact plasma membrane, chromatin condensation, clearly observed with DAB-TUNEL labeling and labeling with antibody recognizing activated caspase-3 (Wyllie, 1980; Majno and Joris, 1995; Thornberry and Lazebnik, 1998; Nijhawan et al., 2000). TUNEL-positive DRG neurons from dissociated spinal cord cultures were counted after 14 and 21 d in culture. To calculate the percentage of TUNEL-positive DRG neurons, cell cultures were counted using the 25× objective covering ten fields in the vertical axis and ten in the horizontal axis. Individual cultures were counted a minimum of three times and each time no less than 100 DRG neurons were counted. The percentage specific apoptosis (% experimental apoptosis − % spontaneous apoptosis/100 − % spontaneous apoptosis) was calculated using the averages of the total counts from Per and WT cultures from the same litter. This enabled a direct comparison between different culturing experiments.

Intranuclear microinjection of cultured motor neurons

The mouse peripherin gene and the cDNA-encoding mouse NF-L were subcloned into the HindIII cloning site of the mammalian expression vector pRcCMV (Invitrogen). The pRcCMV expression vectors (100 ng/ml) incorporating either peripherin or NF-L were microinjected into motor neuronal nuclei along with the fluorescent marker, 70-kD dextran-FITC (15 mg/ml; Molecular Probes) according to the method of Durham et al. (1997). Injected motor neurons were identified by the presence of dextran-FITC visualized by epifluorescence microscopy; morphology was evaluated by phase microscopy. Viability was assessed daily by counting the number of motor neurons containing the marker. The number of viable neurons counted on each day was normalized to the number present on day 1 following microinjection. Experiments were performed in triplicate cultures, with 25–50 motor neurons on each coverslip surviving the injection.

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