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Pathogenesis of Axonal Degeneration: Parallels Between Wallerian Degeneration and Vincristine Neuropathy

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Abstract. Peripheral neuropathies and Wallerian degeneration share a number of pathological features; the most prominent of which is axonal degeneration. We asked whether common pathophysiologic mechanisms are involved in these 2 disorders by directly comparing in vitro models of axonal degeneration after axotomy or exposure to the neurotoxin vincristine. Embryonic rat dorsal root ganglia (DRG) were allowed to extend neurites for 5 days in culture, and then were either axotomized or exposed to 0.01 μM vincristine. Neurites universally degenerated by 3 days after axotomy or after 6 days of vincristine exposure. The neuroprotective effects of a low calcium environment or pharmacologic inhibition of the cysteine protease calpain were compared in these 2 models of axonal degeneration. Addition of EGTA or growth in zero-calcium media provided significant protection against axonal degeneration after either axotomy or vincristine exposure. Treatment with the experimental calpain inhibitor AK295 was equally protective in both models. Chronic exposure to AK295 was not toxic to the cultures. These data suggest that common mechanisms involving calcium and calpains are involved in both axotomy-induced and vincristine-induced axonal degeneration. In addition, calpain inhibition may provide a strategy for preventing axonal degeneration and preserving neurologic function in a variety of PNS and CNS disorders.

Key Words: Axotomy; Calpain; Neuropathy; Neuroprotection; Vincristine; Wallerian degeneration.

INTRODUCTION

Axonal degeneration is the pathological substrate leading to loss of neurological function in a wide variety of acute and chronic disorders of the CNS and PNS. Diseases as disparate as stroke, spinocerebellar degenerations, and peripheral neuropathies share the common pathological finding of axonal degeneration. Even in primary demyelinating disorders such as multiple sclerosis (1) and HMSN-1 (2), axonal degeneration is the pathological finding most highly correlated with severity of clinical symptoms. The mechanisms underlying axonal degeneration in all of these disorders are unknown.

Wallerian degeneration is the simplest and most thoroughly studied model of axonal degeneration. Previous studies have demonstrated that degradation of the axonal cytoskeleton in axotomized nerve fibers is a calcium-dependent process (3–6). In experimental systems, reduction of calcium to below a critical threshold of 200 μM delays the onset of axotomy-induced axonal degeneration (5, 6). Calcium entry likely activates axonal calpains. Calpains are ubiquitous calcium-dependent cysteine proteases involved in both physiological and pathological cellular functions (for review see (7)). In experimental Wallerian degeneration, administration of calpain inhibitors is protective against axonal degeneration (5, 6).

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The pathological features of the majority of human peripheral neuropathies are similar to those seen in axotomy-induced Wallerian degeneration. This similarity has led investigators to describe many neuropathies as ''Wallerian-like'' degeneration (8). We hypothesized that similar mechanisms are involved in axonal degeneration seen in peripheral neuropathies and in Wallerian degeneration, and that strategies for protecting against axotomy-induced axonal degeneration may be protective in peripheral neuropathies. In order to investigate the roles of calcium and calpains in peripheral neuropathy, we developed an in vitro model of toxin-induced axonal degeneration using the neurotoxin vincristine, and tested the neuroprotective effects of a low calcium environment and calpain inhibition.

Vincristine is a chemotherapeutic agent used to treat leukemias and other types of human cancer (9). Patients treated with vincristine predictably develop neuropathic symptoms and signs, the most prominent of which are distal-extremity paresthesias, sensory loss, and reduction of deep tendon reflexes (10–12). Pathologically, vincristine causes length-dependent axonal degeneration that is typical of many other drug-induced, metabolic, and idiopathic peripheral neuropathies (13, 14).

Using this new in vitro model of vincristine neuropathy we demonstrate that toxin-induced axonal degeneration is a calcium-dependent, calpain mediated process, and that pharmacological inhibition of calpains is protective. These findings support the hypothesis that common mechanisms are involved in Wallerian degeneration and peripheral neuropathy, and have implications for understanding the pathogenesis of axonal degeneration in a number of neurological disorders.

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MATERIALS AND METHODS

Dorsal Root Ganglia (DRG) Cultures

Tissue culture dishes (Falcon) of 35 × 10 mm were precoated with rat tail collagen (type 1, Becton Dickinson, Franklin Lakes, NJ), air dried and rehydrated with DMEM (GIBCO, Rockville, MD) overnight at room temperature and then stored at 4°C. On the day of DRG culture, the dishes were washed twice with PBS buffer (pH 7.4), filled with 550 μl medium and pre-incubated at 37°C for at least 2 h. Fifteen-day-old embryos (E15) were removed from pregnant Sprague-Dawley rats (Charles River, NJ), and spinal cords with cervical and thoracic DRGs attached were dissected into L-15 medium (GIBCO). Ganglia were separated from the spinal cord, stripped of their connective tissue sheaths and roots, and then pooled and washed twice with PBS buffer (pH 7.4). DRGs were plated (4 per dish) in culture media and incubated at 37°C in 5% carbon dioxide for 4 h to allow DRGs to attach to the substrate. Medium was then added to bring the total volume to 1 ml. Standard media was MEM (GIBCO, free calcium 1.8 mM), supplemented with 1% N2 supplement (GIBCO), 7S NGF (Alomone Labs, Jerusalem, Israel) 100 ng/ml, and 1.4 mM L-glutamine (Sigma, St. Louis, MO). Calcium-free medium was prepared in the same manner, replacing MEM with S-MEM (GIBCO).

Immunostaining of Doral Root Ganglias

At the end of the treatment period (see below), DRGs were fixed for 30 min with 4% paraformaldehyde. Cultures were then rinsed with 0.1M TBS buffer, and treated sequentially with 3% H_2O_2, TBS-Triton, and 4% normal goat serum (NGS), each for 30 min at room temperature. DRGs were incubated at 4°C overnight in monoclonal antibody to MAP5 (1:500, Sigma). After washing in TBS-Triton, DRGs were incubated for 60 min in biotinylated secondary antibody, rinsed with TBS and reacted with avidin-biotin complex solution (ABC; Vector Labs, Burlingame, CA) for 1 h. Color was generated by incubation for 10 min in diaminobenzidine (DAB) solution, enhanced by addition of 0.025% cobalt chloride and 0.02% nickel ammonium sulfate. Stained tissue was rinsed, air dried, and coverslipped for microscopy with Crystal/Mount (Biomeda, Foster City, CA).

Vincristine Neuropathy

DRGs were allowed to mature for 5 days (media change on day 3) creating a lush halo of neurites. This method of allowing neuritic extension to proceed before addition of a neurotoxin tests the effect of the toxin on established neurites as opposed to the effect on primary neuritic outgrowth. Thus, the in vitro paradigm is partially comparable to the clinical situation in that an ''established'' peripheral nervous system is exposed to a toxic agent.

After day 5 of culture, the media was changed to that containing the experimental treatment. This date was defined as treatment day 0. Cultures were monitored and imaged daily using video microscopy. Vincristine sulfate salt (Sigma) was dissolved in culture medium, aliquoted, and stored at −20°C. EGTA (Sigma) was dissolved in 10 N NaOH, and diluted with ddH_2O to a stock concentration of 0.2 M. The final concentration of NaOH in DRG culture was 0.005 N, which showed no negative effects on the DRG cultures. AK 295 (Z-leu-Abu-$(CH_2)_3$-4-morpholinyl (15), gift of Dr. James Powers, Georgia Institute of Technology), was dissolved in 100% DMSO and then diluted to its final concentration with culture medium; the final concentration of DMSO was ≤0.05%. Phenylmethylsulfonyl fluoride (PMSF) was from Sigma. Addition of EGTA, AK295, or DMSO to control cultures showed no effects on neurite growth or survival (Table 1).

TABLE 1
Lack of Effect of Treatment Modalities on DRG Area and Neuritic Length

	Area (mm^2)	Length (mm)
Control (15)*	45.14 ± 4.35	5.00 ± 0.22
EGTA (2 mM) (7)	44.40 ± 2.74	4.74 ± 0.18
AK 295 (50 μM) (11)	49.18 ± 2.69	4.80 ± 0.16
DMSO (0.05%) (9)	40.80 ± 2.33	4.48 ± 0.15

Treatments were added to 5-day-old cultures. Quantitative measurements were made after 6 days of treatment. Values are mean ± SEM. $p > 0.05$, (NS) for all groups. *(n).

After 6 days of treatment (11 days in culture) immunostained DRGs were quantitated for degree of axonal degeneration. Images of the DRGs and neurites were captured onto disk using a computerized video imaging system, and analyzed using NIH Image version 1.61. DRG areas were calculated by tracing the outside circumference of the remaining culture halo. The length of the longest neurite of each DRG was measured from the center of the DRG to the distal end of the neurite, so that cultures without remaining neurites still had positive values. These quantitative data were subjected to ANOVA, with post-test correction for multiple comparisons.

Wallerian Degeneration

Five-day-old cultures with extended neurites were used. Neurites were cut by excising and removing the DRG with a scalpel blade, taking care to remove all neurons within the DRG. At least 1 ganglion/dish was left unaxotomized to serve as a control for that group of ganglia. For experiments involving therapeutic interventions, the media was changed just prior to axotomy to media containing either EGTA (2 mM) or AK295 (50 μM). Cultures were observed daily for 72 h after axotomy, at which time they were fixed and immunostained.

Stained cultures were scored blindly for degree of axonal degeneration. For axotomized neurites, as opposed to those treated with vincristine, degeneration was considered an all or none phenomenon. Any fibers with interruptions along their length were scored as degenerated. The number of surviving fibers in each DRG was counted under 200× magnification, and the DRG was given a survival score of 0–3: 0 = no fibers remaining, 1 = 1–4 fibers remaining, 2 = 5–9 fibers remaining, 3 = 10 or more fibers remaining. The scores were subjected to ANOVA, with post-test correction for multiple comparisons.

RESULTS

Vincristine-Induced Axonal Degeneration

To determine the sensitivity of cultured DRG neurites to vincristine, drug concentrations of 0.01 to 4 μM were

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added to 5-day-old cultures. Axonal degeneration occurred very quickly (within 1–3 days) at concentrations $\geq$0.05 μM. At 0.01 μM, the process of axonal degeneration was relatively slow, allowing for discrimination of changes between treated and untreated cultures over time.

Signs of degeneration including axonal beading and segmentation were identified as early as 12 h after exposure to vincristine. At 24 to 48 h of exposure, the distal portions of axons showed significant pathological changes. Degeneration proceeded in a distal to proximal pattern along neuritic bundles until fibers were completely replaced by axonal debris at 5 to 6 days (Fig. 1). The neuronal cell bodies also showed changes, with the DRG becoming smaller over the 6 day time course. These changes were not quantified.

Vincristine exposure produced a progressive reduction in the area of the DRG neuritic halo (Table 2). At day 0, the DRG area was 28.98 $\pm$ 1.76 mm^2 and after 3 days exposure to vincristine was reduced by 76.6% to 6.78 $\pm$ 1.06 mm^2 ($p < 0.01$). At 4 days exposure the DRG area was 2.45 $\pm$ 0.74 mm^2, and at 6 days 0.61 $\pm$ 0.21 mm^2, representing respectively, a 91.5% and a 97.9% reduction in area. In the control group, axons continued to grow during the experimental period increasing to 33.62 $\pm$ 2.98 mm^2 on day 3, 41.94 $\pm$ 1.64 mm^2 on day 4, and 45.14 $\pm$ 4.35 mm^2 on day 6. At day 6 the area of DRG halo was 155.8% larger than at treatment day 0.

Changes in the lengths of axons reflected those seen in measurements of DRG areas (Table 2). Vincristine exposure resulted in a 57.6% reduction in length of the longest axons at day 3 (3.82 $\pm$ 0.11 mm to 1.62 $\pm$ 0.19 mm). At days 4 and 6 of vincristine exposure, axon length decreased to 0.91 $\pm$ 0.19 mm (76.2%) and 0.37 $\pm$ 0.06 mm (90.3%), respectively ($p < 0.01$ for all time points). In contrast, axonal lengths in the control group increased by 130.9% of that at day 0 during the 6-day period.

Protection Against Vincristine-Induced Axonal Degeneration

To test the role of extracellular free calcium in vincristine-induced axonal degeneration, DRGs were exposed to vincristine in standard media containing EGTA, in calcium-free media, or in calcium-free media with EGTA. These strategies provided significant protection against axonal degeneration by measures of either DRG area or axonal lengths (Table 2). EGTA at a concentration of $\leq$1 mM was ineffective (not shown), while 2 mM was as effective as calcium-free media. There was also an additive effect of using both EGTA and calcium-free media when measured at the 6 day time point (Table 2).

The neuroprotective effects of a low calcium environment led us to test whether calpains are also important in the pathogenesis of vincristine neuropathy. Addition of the experimental calpain inhibitor AK295 was effective in preventing axonal degeneration at doses of either 50 μM (Table 2) or 10 μM (not shown), and was ineffective at 1 μM (not shown). The neuroprotective effect of AK295 was equal to that of either 2 mM EGTA or calcium-free media.

The low calcium environment or treatment with AK295 did not provide complete protection against axonal degeneration. When the quantitative measures are viewed over the entire 6-day time course, a graded effect is revealed. Neurites exposed to vincristine but maintained in the neuroprotective media stopped growing (Fig. 2), and showed morphologic changes typical of axonal degeneration (Fig. 3). These changes were, however, both qualitatively and quantitatively less severe than those seen in cultures treated with vincristine alone.

To ensure that the protective effect of AK295 was not a nonspecific characteristic of protease inhibitors we asked whether the serine protease inhibitor PMSF, which is not an inhibitor of calpains, could protect against axonal degeneration in this model. PMSF showed no protective effects (Table 2).

Wallerian Degeneration

As previously demonstrated (6), axotomized neurites were completely degenerated by 72 h after transection, and addition of EGTA at the time of axotomy provided significant protection against Wallerian degeneration (Fig. 4). Addition of the calpain inhibitor AK295 was as protective as EGTA. A direct comparison of the protective effects of AK295 in vincristine neuropathy and Wallerian degeneration is demonstrated in Figure 5. The preservation of axons with AK295 was not a subtle finding, and is seen easily in these low power photomicrographs.

DISCUSSION

These data demonstrate that like axotomy-induced Wallerian degeneration, axonal degeneration in vincristine neuropathy is both a calcium-dependent and calpain-mediated event. This in vitro model of vincristine neuropathy provides an experimental system with a high degree of similarity to the human condition. First, these experiments were done with primary sensory neurons and axons and not neuronal cell lines. Second, the measures of neurotoxicity and neuroprotection were on the degeneration of developed axons as opposed to inhibition of initial axon outgrowth. Third, the toxic neuropathy progressed from distal to proximal, as is seen in human neuropathies.

The pathogenesis of vincristine neuropathy is thought to be a consequence of its primary antineoplastic function as a mitotic spindle inhibitor. Experimental data suggest that vincristine alters the structure of axonal microtubules, leading to abnormalities in fast axonal transport (16–18). Dysfunctional axonal transport is a major theory for the pathogenesis of a variety of toxic neuropathies

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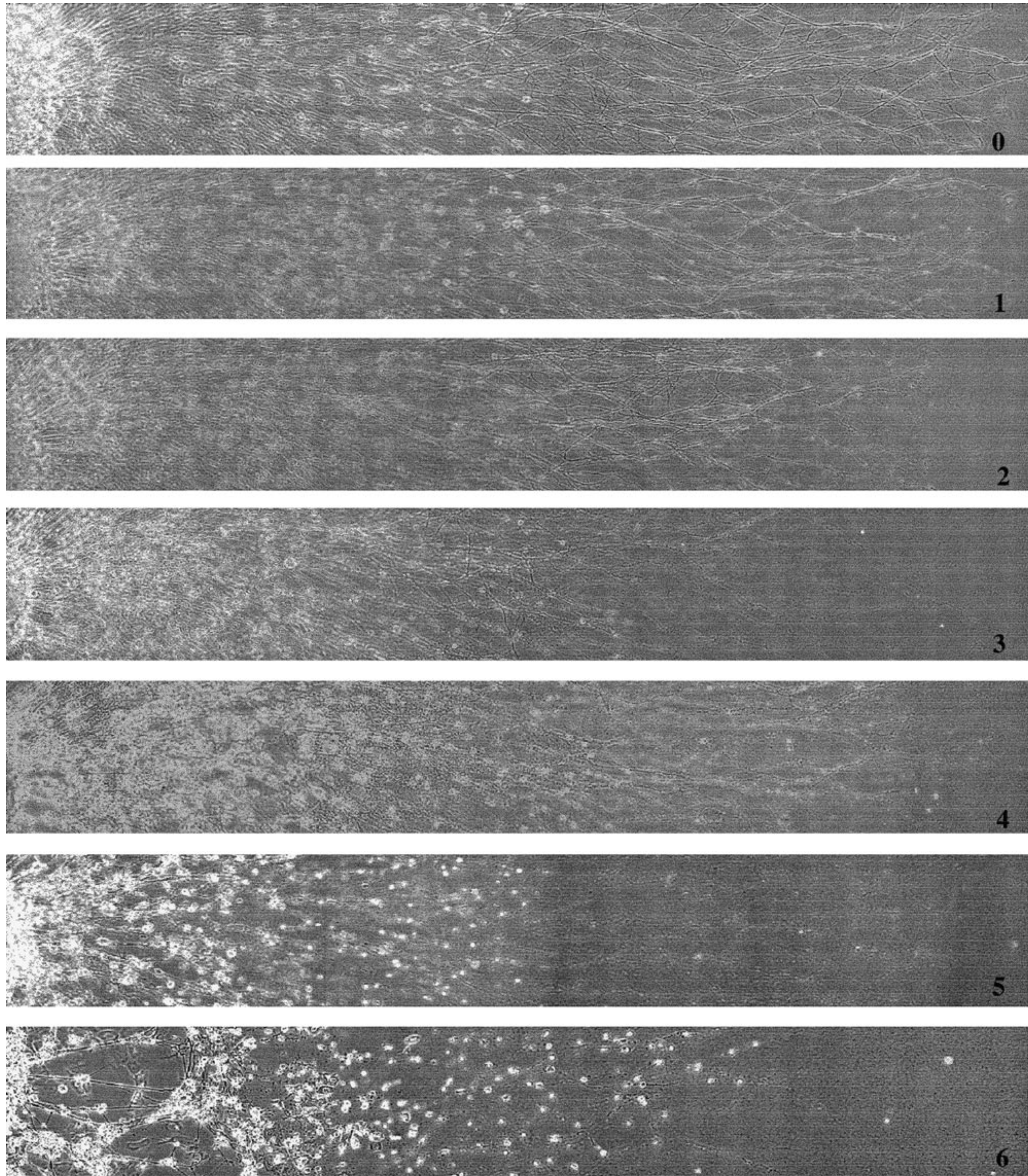

Fig. 1. Serial montage phase-contrast photomicrographs of the same DRG exposed to 0.01 μM vincristine. There is a progressive ''dying back'' of neurites from day 0 to day 6.

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(19), and is considered a causative factor in the case of vincristine. Intoxication of laboratory animals or direct exposure of nerves to vincristine has been reported to cause alterations in the structure and shape of axonal microtubules (18), shortening of microtubule lengths (16), or changes in microtubule distribution within the axon (17). Neurofilamentous axonal swellings, as is seen with hexacarbon and other intoxications, have also been reported with vincristine (20). These changes are believed to provide the pathological substrate for altered axonal transport, which may affect the delivery of nutritive substances to the axon from the cell body. Changes in axonal

TABLE 2
DRG Areas and Neuritic Lengths in Control Cultures and Treated Cultures

	Day 0		Day 3		Day 4		Day 6	
	Area	Length	Area	Length	Area	Length	Area	Length
Control	28.98 ± 1.76(13)	3.82 ± 0.11	33.62 ± 2.98(12)	4.07 ± 0.25	41.94 ± 1.64(10)	4.56 ± 0.15	45.14 ± 4.35(15)	5.00 ± 0.22
Vin			6.78 ± 1.06(17)	1.62 ± 0.19	2.45 ± 0.74(13)	0.91 ± 0.19	0.61 ± 0.21(48)	0.37 ± 0.06
Vin + AK295*			16.61 ± 1.18(18)	2.65 ± 0.11	14.77 ± 2.19(14)	2.45 ± 0.25	10.34 ± 2.14(12)	2.05 ± 0.25
Vin + EGTA*			15.05 ± 1.78#(12)	2.62 ± 0.17	12.97 ± 0.94(14)	2.44 ± 0.13	13.36 ± 1.04(14)	2.43 ± 0.07
Vin + Ca^{++}-Free*							14.90 ± 3.22(3)	2.47 ± 0.29
Vin + EGTA + Ca^{++}-Free**							21.77 ± 1.74(11)	3.08 ± 0.14
Vin + PMSF (50 μM)							0.73 ± 0.16(43) (NS)	0.55 ± 0.06(NS)

Area in mm², length in mm. Values are mean ± SEM. * $p < 0.01$, ** $p < 0.001$, # $p < 0.05$, comparison to vincristine treatment alone. Drug concentrations: Vin 0.01 μM, AK295 50 μM, EGTA 2 mM. Numbers in parentheses are the number of experiments performed.

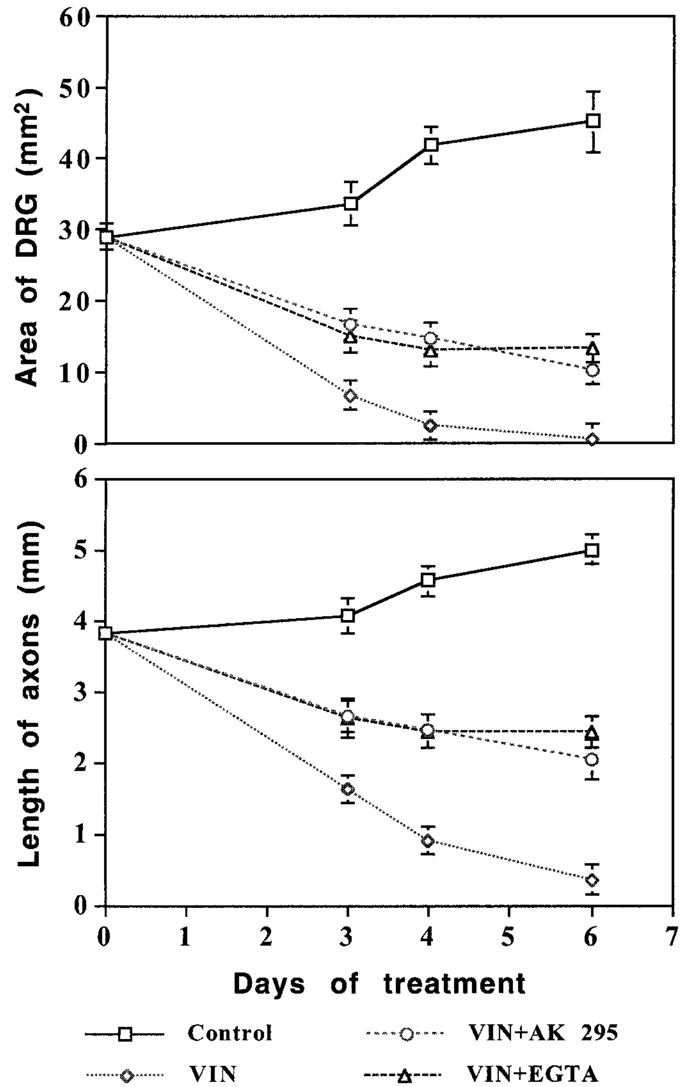

Fig. 2. Graphic representation of changes in DRG area (mm²) and neurite length (mm) with vincristine exposure alone, or with addition of 2mM EGTA or 50 μM AK295. Note the growth arrest even with addition of neuroprotective drugs.

transport profiles, including both slowing and acceleration of transport peaks, has been demonstrated in vincristine-intoxicated cats (17).

If disruption of normal axonal transport is the cause of vincristine neuropathy, it is unclear why a low calcium environment or inhibition of calpains is protective. One possibility is that the axonal transport hypothesis is incorrect. However, the cumulative evidence supporting microtubular and transport abnormalities is strong. We believe that the neuroprotective effects of low calcium and calpain inhibition reflect a final common pathway of axonal degeneration that may become active following a variety of axonal or neuronal insults. Calcium entry into neurons and axons is a common feature of experimental models of acute and chronic neurologic injury. These include Wallerian degeneration (3, 5), nerve hypoxia (21–

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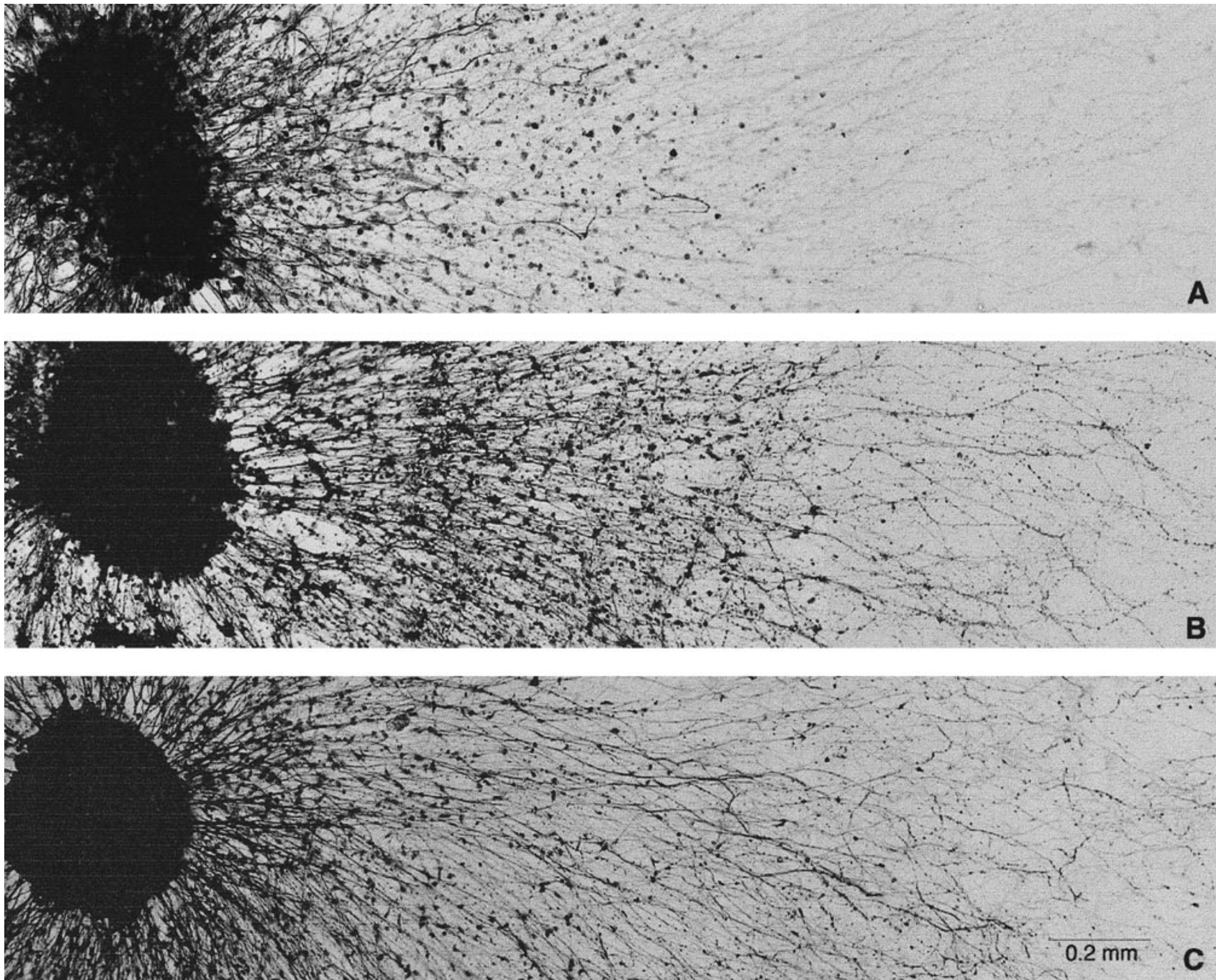

Fig. 3. Direct comparisons of morphology on day 3 of cultures exposed to vincristine alone (A), or with addition of AK295 (B) or EGTA (C). Axonal degeneration is apparent even in the treated cultures, but is noticeably less than in the untreated culture. DRGs stained with MAP-5 antibody.

23), glutamate-induced excitotoxicity (24), and other toxic neuropathies (25–27). Calpain activation has also been implicated in the pathogenesis of a wide variety of neurologic disorders such as stroke (28), head and spinal cord trauma (29, 30), and even Alzheimer disease (31). A current hypothesis is that elevated intracellular calcium in injured cells leads to pathologic activation of calpains, and results in neuronal/axonal degeneration (for review see (32)). In the case of vincristine neuropathy, altered axonal transport may lead to elevations of intracellular calcium and activation of calpains through metabolic compromise, alteration of calcium conductance, or loss of membrane integrity.

The therapeutic potential of calpain inhibitors will be based on their effectiveness in preventing axonal degeneration, but will be limited by their inherent cellular toxicity. Calpains are ubiquitous cytosolic enzymes that are putatively involved in a number of normal cellular functions. Certainly, chronic calpain inhibition has the potential for interfering with these functions. Previous uses of AK295 have been in acute neurologic injuries, where drug exposure has been limited to single or short term dosing (33, 34). In our DRG cultures we found that chronic calpain inhibition was neither toxic to neurites, nor affected their normal growth, providing encouraging data for further study regarding its long term use in animals or humans.

The results of these studies not only provide new insight into the pathogenesis of vincristine neuropathy, but also have implications for understanding the general mechanisms underlying axonal degeneration. We have shown that a common pathway involving calcium and calpains leads to axonal degeneration in 2 different models of axonal injury, Wallerian degeneration and

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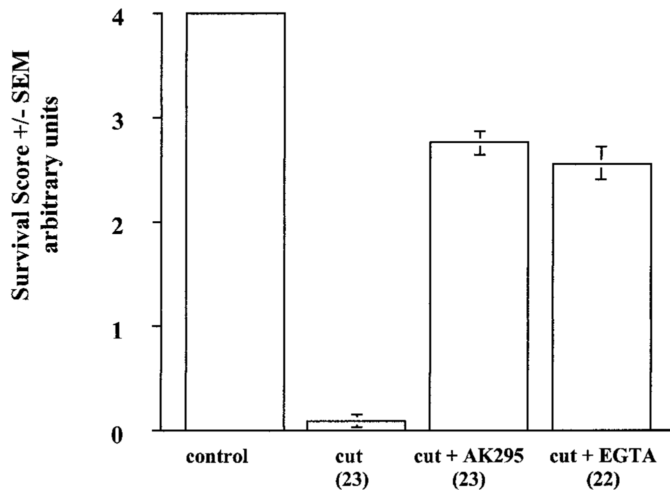

Fig. 4. Quantitative measure of axonal survival with EGTA and AK295 in axotomized neurites. Unaxotomized control cultures are arbitrarily given a score of 4 to separate them from the experimental axotomized cultures. Numbers in parentheses are the number of experiments performed.

vincristine exposure. These findings secure the link between Wallerian and Wallerian-like degeneration by demonstrating common pathophysiology. We believe that the neuroprotective effects of calpain inhibition in our model of vincristine neuropathy are pertinent for a wide variety of neurological disorders where axonal degeneration is prominent, including disorders of the PNS and CNS (35). Calpain inhibition may be a reasonable strategy for preventing axonal degeneration and preserving neurologic function in acute injuries such as stroke and trauma, or in chronic disorders such as diabetes mellitus, hereditary neuropathies, or multiple sclerosis.

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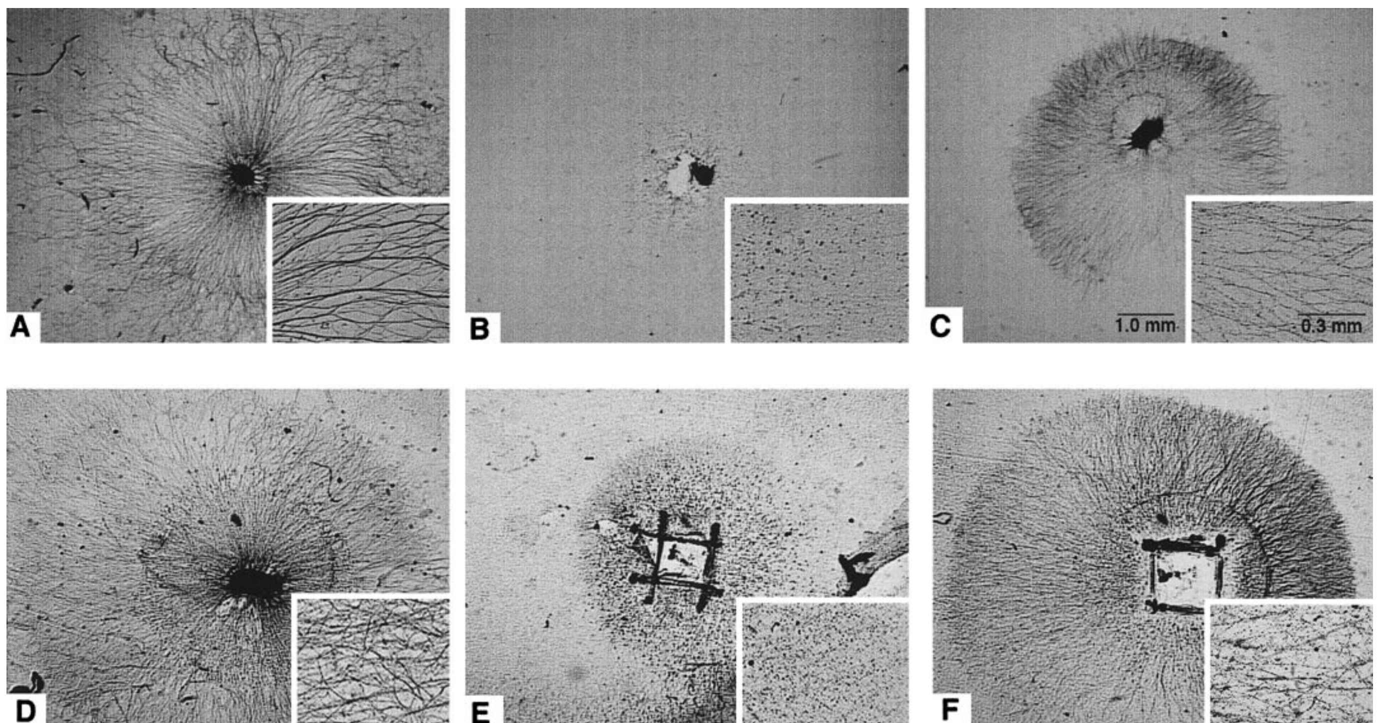

Fig. 5. Comparison of protective effects of AK295 in vincristine neuropathy (A–C) and Wallerian degeneration (D–F). (A and D) are unmanipulated controls (11 days in culture), (B) and (E) are untreated cultures exposed to vincristine for 6 days (B) or axotomized for 3 days (E). (C and F) are the same respective injuries in media containing 50 μM AK 295. Magnification is the same for all images as shown by the scale bars in (C).

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