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SHORT COMMUNICATION

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Retardation of rat sciatic nerve regeneration after local application of minute doses of vincristine

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Abstract The effect of vincristine on regeneration of rat sural and tibial nerves following a crush lesion of the sciatic nerve was studied in the pinch test. Vincristine locally applied through an osmotic minipump at the site of the lesion dose-dependently retarded regeneration of the tibial and sural nerve at a threshold dose of 5 ng/day, whereas regeneration was blocked at a dose of 200 ng/day. Regeneration of the sural nerve was more sensitive to the retarding effects of vincristine than was regeneration of the tibial nerve. Systemic weekly administration (i.p.) of 1 mg/kg of vincristine for 7 weeks had approximately the same effect as local application of 10–20 ng/day for 1 week. The differences in sensitivity between sural and tibial nerves and the large discrepancy between local and systemic administration are discussed. On the basis of the potent effects of vincristine used at low concentrations, the absence of overt effects of local vincristine on animal behavior and the short time course in which the local vincristine effects are observed, it is concluded that this paradigm is an extremely suitable model for studying vincristine-induced defects of nervous system function. This model may be used for evaluating the neuroprotective effects of neurotrophic agents against vincristine-induced neuropathies.

Key words Pinch test · Rat sciatic nerve · Vincristine neuropathy

Introduction

Vincristine is a widely used cytostatic agent, especially for the treatment of lymphomas and leukemias, but its clinical use is generally curtailed by peripheral polyneuropathies arising as a side effect of vincristine treatment. These neuropathies are characterized by clinical signs of hyporeflexia, especially a loss of deep tendon reflexes, generally followed by paresthesias in the extremities, muscle pain, and, in severe cases, weakness [2, 4, 22, 29]. Neurophysiological examination of vincristine-treated patients generally reveals reduced amplitude and conduction velocity of sensory-nerve action potentials along with mildly decreased or normal motor-nerve action potentials and occasional denervation potentials as well as an impaired interference pattern in the distal-muscle electromyogram (EMG) [2–4, 14, 15]. The vincristine-induced functional impairment does not always appear to be directly correlated with the observed neuropathologic changes reflected by nerve fiber degeneration [2, 12]. Specific high-affinity binding of vincristine to dimeric tubulin, causing its depolymerization and preventing polymerization of soluble tubulin dimers into microtubules [6, 10] is suggested to play a central role in the pathogenesis of vincristine neuropathies [13, 23, 24, 28]. Distal axonal degeneration may ensue through a disturbance of fast axoplasmic transport, which is heavily dependent on functional microtubule systems.

For the development of potential curative or preventive treatment of vincristine-induced neuropathies, good animal models are an essential prerequisite. In rat muscle fibers, degenerative changes have been described after vincristine administration [6], but in peripheral nerves only minimal histologic abnormalities have been observed [2]. The best histological model developed thus far appears to be the dose-dependent formation of paracrystalline structures in snail neurons [23, 24] caused by agglutination of tubulin molecules following vinca alkaloid treatment. In vivo studies with chronic i.v. vincristine injections in the cat led to a slowing of motor- and sensory-nerve conduction velocity and a disruption of muscle spindle functioning [11] combined with degenerative changes in peripheral nerves [6]. Weakening in electrophysiological

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parameters of peripheral nerve function has also been described for the rabbit [9] and guinea pig [2] but could not be clearly established in the rat (Ruigt and van Proosdij, unpublished data). Single s.c. vincristine injections in the catfish produced histologic and electrophysiologic changes in electroreceptor functioning [25]. In goldfish optic nerve [7] and in rat sciatic nerve [33, 34] vincristine was shown to retard regeneration of the injured nerve. In most of these functional models the effects are small and become apparent after treatment with relatively large amounts of vincristine, which affects the general condition of the animal.

Herein we report the effects on rat sciatic nerve regeneration of very small amounts of vincristine delivered by an osmotic minipump at the site of the lesion. The effects on nerve regeneration were evaluated for two major branches of the sciatic nerve, the tibial nerve (primarily motor function) and the sural nerve (a mixed sympathetic/sensory cutaneous nerve fiber). The extent of regeneration was determined using the so-called pinch test, which was originally described by Young and Medawar [36] in the rabbit and was later improved by Bisby [1] and Kanje et al. [18].

Materials and methods

Male Wistar rats (Harlan, CPB, Zeist, The Netherlands) weighing 200–220 g were used. The animals were housed in groups of three and had free access to food and water with lights on between 6:00 a.m. and 6:00 p.m. Animals were anesthetized with an i.p. injection of Nembutal (60 mg/ml, 60 mg/kg). The right sciatic nerve was exposed at the midthigh level and crushed at the border of the gluteus maximus muscle for 30 s over a length of 3 mm with specially designed pliers (Crile-Wood 16923). The proximal site of the crush lesion was labeled by a 7-0 suture in the epineurium. Osmotic minipumps (model 2002 or 1007 D, Alzet; pumping rate, 12 μL/day) were filled with different concentrations of vincristine sulfate (Sigma, mol. mass, 923) in 0.9% saline or with solvent only. The pump was implanted s.c. in the abdomen of the rat. From the pump a thin silicone catheter was led under the skin to the sciatic nerve. A specially designed cuff, enclosing the crushed sciatic nerve, was fixed to the end of the thin silicone catheter. After implantation of the pump and the cuff the wound was closed and the rat was given a code number on the tail.

Regeneration was evaluated using the pinch test. At day 2 or 6 after the crush injury at day 0 the rats were anesthetized as described above and the wounds were reopened. The sural and tibial nerve branches of the sciatic nerve were exposed separately, cut distally where they disappeared into the musculature, and freed from the surrounding tissue. A fixed strain of 1 g was applied to the distal cut end of the nerves and the nerve was mechanically stimulated by gentle pinching with a pair of watchmaker's forceps, beginning distally and moving proximally toward the site of the crush lesion in 1-mm intervals. A reflex response, noted as a contraction of muscles in the thigh and in the back, marked the leading front of the regenerating nerve fibers. The distance in millimeters from the epineurial suture to this first positive pinch site was taken as the regeneration distance for the leading axons. After the regeneration distances had been determined the rats were killed.

In a separate experiment, vincristine sulfate was given not locally but systemically (i.p.) at a dose of 500 μg/kg twice per week for 7 weeks to animals weighing approx. 220–420 g at the end of the experiment. Evaluation of tibial and sural nerve regeneration in the pinch test was done at day 6 following the creation of a crush lesion at the beginning of week 7 of the vincristine treatment.

Table 1 Regeneration distances of the sural nerve as measured with the pinch test at day 2 and day 6 following an initial crush lesion of the sciatic nerve on day 0. Measurements are given in mm distal from the proximal side of the crush lesion [average values ± SEM(*n*)]. The last two columns provide the initial delay and regeneration rate as estimated from the linear regression lines through the regeneration distances on the two test days with their 95% confidence limits

Experiment	Dose of vincristine sulfate (μg/day)[a]	Day 2	Day 6	Regeneration rate expressed in mm/day (95% conf. limits)	Initial delay expressed in days (95% conf. limits)
A	20		−2.0 ± 0.0 (8)		
	0		15.7 ± 0.6 (7)		
B	2	−1.9 ± 0.1 (8)	− 2.0 ± 0.0 (8)	< 0.0 (−0.14, 0.07)	> 5.0 (−233, 126)
	0	1.5 ± 0.2 (8)	16.0 ± 0.2 (7)	3.7 (3.61, 3.83)	1.6 (1.51, 1.68)
C	0.2	−1.8 ± 0.2 (8)	−1.3 ± 0.8 (8)	0.1 (−0.26, 0.50)	> 5.0 (−24.4, 57.6)
	0	1.3 ± 0.2 (7)	14.3 ± 0.7 (6)	3.4 (2.96, 3.81)	1.7 (1.31, 2.04)
D	0.05	−1.4 ± 0.4 (8)	−0.1 ± 0.9 (7)	0.3 (−0.06, 0.74)	> 5.0 (2.66, 9.72)
	0.02	−0.9 ± 0.5 (8)	2.0 ± 1.3 (7)	0.7 (0.18, 1.29)	3.2 (1.66, 4.74)
	0	2.0 ± 0.0 (5)	15.1 ± 0.3 (7)	3.4 (2.94, 3.84)	1.5 (1.00, 1.91)
E	0.01	0.0 ± 0.5 (8)	10.9 ± 0.7 (8)	2.8 (2.44, 3.26)	2.0 (1.64, 2.42)
	0.005	1.1 ± 0.1 (7)	12.1 ± 0.7 (8)	2.9 (2.47, 3.32)	1.7 (1.20, 2.11)
	0.002	0.6 ± 0.6 (8)	15.3 ± 0.3 (7)	3.8 (3.34, 4.17)	2.0 (1.67, 2.28)
F	0.005		13.5 ± 0.4 (14)		
	0		15.5 ± 0.2 (13)		
G	500[b]		8.2 ± 0.6 (5)		
	0		12.6 ± 0.3 (8)		

[a] Local application by osmotic minipump at the site of the crush lesion
[b] 500 μg/kg given i.p. twice per week for 7 weeks

Results

A summary of the results of seven separate experiments performed with different doses of vincristine is given in Table 1 for the sural nerve and in Table 2 for the tibial nerve. In all but one experiment a placebo control group was included. The regeneration rates for these placebo groups varied between 3.8 and 4.2 mm/day for the tibial nerve and between 3.4 and 3.7 mm/day for the sural nerve, which is in line with earlier observations that regeneration of the tibial nerve takes place faster than that of the sural nerve [8, 20, 26].

Local application of vincristine at doses of 0.2 μg/day or higher completely blocked the regeneration of the tibial and sural nerve except on day 6 after dosing with 0.2 μg/day of vincristine, at which time a slight regeneration of the tibial nerve could be observed. Local application of a dose of 2 ng/day of vincristine (a factor of 100 less than the dose at which regeneration is completely blocked) resulted in tibial- and sural-nerve regeneration rates that fell within the range of control values for both nerve branches. The lowest dose at which a retardation of tibial and sural nerve regeneration could be observed was 5 ng/day of vincristine locally applied by minipump at the site of the crush lesion.

Regeneration of the sural nerve appeared to be more sensitive to vincristine than did regeneration of the tibial nerve (Fig. 1). The regeneration rate for the tibial nerve was 69% and 40% of the control rate at 20 and 50 ng/day of vincristine, respectively, whereas the respective values were 21% and 9% for the sural nerve. The same held true for the initial delay values, which at the doses of 20 and 50 ng/day of vincristine were longer for the sural nerve as compared with the tibial nerve.

To compare the effect of local application of vincristine versus systemic administration a single experiment was done at a dose of 500 μg/kg (given i.p. twice per week for 7 weeks). The effect of the latter vincristine treatment roughly corresponded to local application of 10–20 ng/day of vincristine for 1 week.

Local application of vincristine at a dose of 2 μg/day and higher affected the condition of the animals and/or the tissue surrounding the minipump. At 200 μg/day of vincristine, half of the animals died. At 2 and 20 μg/day, no general effect on animal behavior was observed, but when the nerve was exposed for the pinch test at day 6 following crush and implantation of the vincristine pump, the tissue around the osmotic minipump was hard, bloodless, and swollen, suggesting local tissue degeneration and formation of scar tissue. Such morphological changes were not observed at doses below 2 μg/day.

Discussion

The main finding of the present experiments is that by local application of vincristine to a nerve a clear neuropathic effect of the cytostatic can be obtained without jeopardizing the general condition of the animal,

Table 2 Regeneration distances of the tibial nerve as measured with the pinch test at day 2 and day 6 following an initial crush lesion of the sciatic nerve on day 0. Measurements are given in mm distal from the proximal side of the crush lesion [average values ± SEM(*n*)]. The last two columns provide the initial delay and regeneration rate as estimated from the linear regression lines through the regeneration distances on the two test days with their 95% confidence limits

Experiment	Dose of vincristine sulfate (μg/day)[a]	Day 2	Day 6	Regeneration rate expressed in mm/day (95% conf. limits)	Initial delay expressed in days (95% conf. limits)
A	20		−2.0 ± 0.0 (8)		
	0		19.1 ± 0.4 (8)		
B	2	−1.1 ± 0.2 (8)	−1.9 ± 0.1 (8)	< 0.0 (−0.32, −0.06)	> 5.0 (−9.20, 1.59)
	0	1.9 ± 0.1 (8)	18.1 ± 0.1 (7)	4.2 (4.04, 4.31)	1.5 (1.45, 1.64)
C	0.2	−0.9 ± 0.1 (8)	0.5 ± 1.2 (8)	0.3 (−0.18, 0.86)	4.5 (1.46, 7.60)
	0	1.9 ± 0.1 (8)	16.7 ± 0.6 (7)	3.8 (3.30, 4.38)	1.6 (1.14, 1.99)
D	0.05	0.0 ± 0.3 (8)	6.6 ± 2.1 (7)	1.7 (1.00, 2.48)	2.1 (0.97, 3.20)
	0.02	0.8 ± 0.3 (8)	11.9 ± 0.5 (8)	2.9 (2.58, 3.13)	1.8 (1.49, 2.03)
	0	2.0 ± 0.0 (6)	18.1 ± 0.4 (8)	4.2 (3.40, 4.96)	1.6 (0.96, 2.19)
E	0.01	1.3 ± 0.2 (8)	14.3 ± 0.6 (7)	3.4 (3.05, 3.78)	1.7 (1.36, 1.97)
	0.005	1.9 ± 0.1 (7)	16.0 ± 0.7 (8)	3.7 (3.35, 4.08)	1.5 (1.23, 1.85)
	0.002	2.1 ± 0.1 (8)	17.6 ± 0.5 (8)	4.0 (3.70, 4.25)	1.6 (1.39, 1.83)
F	0.005		15.8 ± 0.5 (16)		
	0		17.7 ± 0.3 (15)		
G	500[b]		9.2 ± 0.3 (5)		
	0		15.0 ± 0.3 (8)		

[a] Local application by osmotic minipump at the site of the crush lesion
[b] 500 μg/kg given i.p. twice per week for 7 weeks

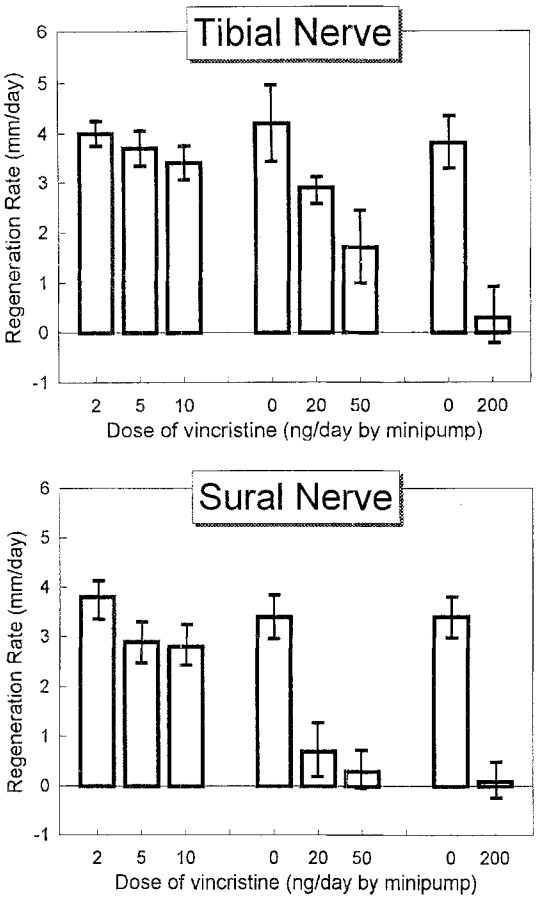

Fig. 1 Effect of different doses of locally applied vincristine (given by osmotic minipump at the site of the sciatic nerve crush lesion) on the regeneration rate of the tibial and sural nerve. *Bars* indicate the estimated regeneration rates together with their 95% confidence limits

a problem that is always encountered in experiments where vincristine is given systemically. Another important finding is that the local doses of vincristine at which a retarding effect on nerve regeneration is observed are as low as 5 ng/day for 6 days. This corresponds to a maximal concentration of approx. 0.4 μg/ml in the fluid surrounding the nerve, which would be attained only if there would be no fluid transport in or out of the cuff. The cuff was open-ended and fitted loosely around the nerve, enabling easy exchange of fluids and making it difficult to estimate how much lower the vincristine concentration around the nerve actually was. The actual concentration of vincristine inside the nerve at the site of regeneration would be even lower. In a recent in vitro study of the effects of vincristine on neuritogenesis in isolated embryonic-rat dorsal root ganglia, vincristine was found to be the most potent inhibitor of several cytostatics on neurite outgrowth [19a]. The threshold concentration at which an effect was observed in this system was 0.4 ng/ml in a volume of 100 μl for 2 days.

The potent effects of vincristine at low concentrations, the absence of overt effects on animal behavior following this treatment, and the short time course in which the vincristine effects are observed make this paradigm an extremely suitable model for studying vincristine-induced defects of nervous system function and for examining the potential neuroprotective effects of neurotrophic factors against vincristine-induced neuropathies. The main limitation of the model is that in this paradigm, vincristine's effects are studied on a nerve that has been damaged, in contrast to the clinical condition where vincristine neuropathies occur in an intact nervous system. However, both the retardation of nerve regeneration reported herein and the observed neuropathologic signs following vincristine treatment in the clinic are thought to be direct effects of the depolymerization of microtubules and the resulting disturbance of fast axoplasmic transport induced by vincristine [13, 23, 28, 32–35].

The sural nerve appears to be more sensitive than the tibial nerve to the action of vincristine. Furthermore, the normal regeneration rate for the sural nerve is also slower than that of the tibial nerve. That the sural nerve contains more sensory fibers than the tibial nerve cannot explain this difference in sensitivity, since the pinch test reflects only the regeneration of sensory fibers. The characteristics of these sensory fibers in the tibial and sural nerve, such as fiber diameter, have not yet been described but may be relevant. What is known is that the fiber diameter of myelinated fibers in the sural nerve is smaller than that of the tibial nerve [16, 17, 27] and that the DRG neuron somata from which the cutaneous sensory-nerve fibers in the sural nerve are derived are much smaller than the DRG neurons supplying sensory fibers to the tibial nerve [36]. This could be taken to suggest that sural nerve fibers may be smaller and may have fewer microtubules than tibial nerve fibers and that they are therefore more sensitive to the microtubule-depolymerizing action of vincristine.

The retarding effects of vincristine on nerve regeneration in the rat have previously been described by Shiraishi and co-workers [33–35]. Using systemic vincristine (20–200 μg/kg given weekly i.p.) application and studying electrophysiologically the recovery of nerve function after a sciatic nerve crush, they observed a delay in functional recovery at a threshold dose of 50 μg/kg per week, whereas 200 μg/kg per week completely blocked reinnervation of the foot musculature without affecting electrophysiological parameters on the control side [33]. Subsequent histologic evaluations revealed a dose-dependent reduction in nerve fiber size and microtubule concentration, a reduction in the number of unmyelinated axons, and a failure of removal of myelin debris, whereas the nerve on the noncrushed control side appeared normal [33]. In one experiment we studied tibial and sural nerve regeneration in the pinch test following 7 weeks of i.p.

administration of 500 μg/kg vincristine twice per week and observed an effect that compared well with that of local application of 10–20 ng/day for 1 week.

The difficulty of establishing animal vincristine-neuropathy models as well as the large differences between local and parenteral doses of vincristine giving neurotoxicity may partly be due to the observation that vincristine does not cross the blood-brain barrier or, possibly, the blood-nerve barrier [5, 21, 34]. The most prominent neurotoxic effects of vincristine are obtained either after local application to a damaged nerve in which such a barrier has been damaged, as done in the present experiments, or after direct injection into nerve [30], CSF [31], or defined brain areas [10, 19]. Direct injection of vincristine into the rat peripheral nerve resulted in the formation of axonal paracrystalline structures, an increase in neurofilaments, and a loss of microtubules [30]. Similar changes were observed after intrathecal administration of vincristine in rabbits [31].

It would be very interesting to study the effects of compounds with a potential neuroprotective action in this model of vincristine neurotoxicity. It would be a challenge to study the effects of nerve growth factor (NGF) or of compounds that are thought to increase endogenous levels of NGF on the delayed regeneration observed after vincristine administration, since it has been found that NGF counteracts the inhibitory effects of vincristine on neurite formation in rat dorsal root ganglia in vitro, in which the cell bodies of a large proportion of the sural nerve fibers are located [19a].

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