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Protection against cisplatin induced neurotoxicity by ORG 2766: histological and electrophysiological evidence

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Abstract

Prolonged administration of the anti-tumor agent cisplatin may cause a neuropathy in patients. In an animal model, too, neurotoxicity, as evidenced by a decrease in H-related sensory nerve conduction velocity (SNCV), can be induced by repetitive injections of cisplatin. In an attempt to further the insight into the effects of cisplatin on the peripheral nervous system a combined electrophysiological and histomorphological investigation was performed on 2 groups of 6 rats, treated with cisplatin for 7.5 weeks, and a control group ($n = 6$). Concomitant administration of ORG 2766, an ACTH(4–9) analog, was previously shown to prevent cisplatin neurotoxicity in this model and more recently in patients as well. One group of rats was therefore co-treated with this peptide during the complete treatment period. A marked decrease in SNCV was observed in cisplatin/saline treated rats, but not in cisplatin/ORG 2766 treated rats. Though no statistically significant difference was seen in the total number of myelinated fibers in the sural nerves of cisplatin treated rats, a decrease in the proportion of thick myelinated fibers was present in the cisplatin/saline treated rats. This shift in fiber distribution was absent in ORG 2766 co-treated animals. Mean internodal distances and g -ratios were not affected, and signs of axonal degeneration, or de- or remyelination were not observed.

Keywords: Cisplatin; Neurotoxicity; Prevention; Melanocortin; Histology; Electrophysiology; (Rat)

1. Introduction

Beneficial effects of melanocortins (ACTH and α -MSH-like peptides) on recovery from experimentally induced peripheral nerve damage have been reported by various authors (Bär et al. 1990). ACTH, various ACTH fragments, and ORG 2766, an ACTH(4–9) analog devoid of corticotropic and melanotropic activity, enhance peripheral nerve regeneration after crush or transection, and promote collateral reinnervation following partial denervation (Strand and Smith 1986; De Koning and Gispen 1988; De Koning et al. 1989). The mechanism underlying these effects is still un-

known, but the peptide apparently influences endogenous repair processes. Other experiments have shown that ORG 2766 also prevents damage inflicted by several neurotoxins on the peripheral nervous system (De Koning et al. 1988; Gerritsen van der Hoop et al. 1988; Edwards et al. 1989; Hamers et al. 1993b).

Cisplatin is widely used as an effective anti-tumor agent in the treatment of ovarian, testicular and bladder cancer. The main side-effects accompanying its use are renal failure, sensory neuropathy and ototoxicity. The neurotoxic effects of cisplatin such as paresthesias and sensory ataxia are often dose limiting (Legha and Dimery 1985). We used an animal model developed by De Koning et al. (1987) to study the effect of putative neurotrophic or neuroprotective agents on experimental cisplatin neurotoxicity (Hamers et al. 1991b). On electrophysiological examination a decrease in the sensory nerve conduction velocity (SNCV) can be estab-

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lished, whilst the motor nerve conduction velocity remains unaffected, exactly as is seen in cisplatin neuropathy in man (Thompson et al. 1984; Gastaut and Pellissier 1985; Ongerboer de Visser and Tiessens 1985). The purpose of the present study was to describe the histological changes, if any, associated with and perhaps responsible for the decrease in SNCV, and to examine the effect of ORG 2766 on these changes.

2. Materials and methods

Animals and treatment

Female albino rats (200–220 g body weight, 12–13 weeks old) from an inbred Wistar strain (TNO, Zeist, The Netherlands) were used. They were kept on sawdust in macrolon cages, 4 animals per cage, with commercial rat chow and water available ad libitum. They were maintained on a 12-h dark/light cycle with lights on from 8.00 a.m.

A cisplatin solution containing 50 mg/100 ml solvent (Platinol[®], Bristol-Myers-Squibb, Weesp, The Netherlands) was diluted with saline to a final concentration of 0.04 mg/ml. Five ml of this solution was administered by intraperitoneal injection, twice a week. Twenty minutes in advance, a subcutaneous injection of furosemide (12.5 mg/kg, Lasix[®], Hoechst, Germany) was given to protect against nephrotoxicity.

ORG 2766, a degradation resistant ACTH₄₋₉ analog (H-Met(O₂)-Glu-His-Phe-D-Lys-Phe-OH) devoid of corticotropic or melanotropic activity, was dissolved in 0.1% BSA/0.05 N HCl and diluted with 0.9% NaCl solution to a final concentration of 10 µg/0.5 ml solution (the peptide was generously supplied by Organon International BV, Oss, The Netherlands). ORG 2766 (10 µg/rat) or saline injections were given subcutaneously 4 times a week.

Electrophysiology

Electrophysiological examinations were performed under general anaesthesia (Hypnorm[®], Janssen Pharmaceutica BV, Tilburg, The Netherlands, containing fluanisone 10 mg/ml and fentanyl citrate 0.315 mg/ml, dose 0.8 ml/kg body weight, s.c.). A detailed description of the method used is given by De Koning et al. (1987).

Histology

Animals were killed and both sural nerves were exposed and dissected out. The nerves were stretched and fixed in a solution containing 0.1 M calcium cacodylate, 0.01 M CaCl₂ and 2% glutaraldehyde buffered at pH of 7.3 in case of the right nerve for 4 h, and the left for 20 min. Subsequently the nerves were rinsed 3 times in an isotonic solution containing 0.1 M

cacodylate, 0.01 M CaCl₂ and 3.6% glucose, pH 7.3). The tissue was post-fixed with OsO₄ (1% OsO₄, 0.1 M cacodylate, 0.01 M CaCl₂, 2.9% glucose, pH 7.3) for 2 h and dehydrated in graded series of acetone. The left sural nerve was kept in liquid, unpolymerized, cold (4°C) epoxy resin for teasing of isolated nerve fibers, whilst the right sural nerve was embedded in epoxy resin, that was allowed to polymerize.

Semithin (1 µm) transverse sections were cut using a microtome (LKB pyramitome, Bromme, Sweden) and stained with 1% *p*-phenylenediamine. Pictures were made both of transverse sections of the nerve as a whole and of 3 non-overlapping intrafascicular parts of the nerve in detail. The latter ones were printed at a final magnification of 2500 ×.

Teasing of single fibers was performed in liquid epoxy resin according to the method described by Dyck et al. (1984). At least 100 fibers of each sural nerve were isolated, collected on a slide, following which the resin was polymerized by heating.

Morphometry

Both for measuring the circumferences of the complete, monofascicular sural nerve and the outer circumferences of nerve fibers and axons a semi-automatic technique was used. Photographs were mounted on top of a graphic tablet that was connected to a PC loaded with a digitizer program. The outlines were traced with a light pen. Only the endoneurial area (without the subperineurial space) was traced in order to obtain the total sural nerve area. Diameters were calculated automatically from the manually obtained data. *g*-Ratios were calculated by dividing the individual axonal diameter by the fiber diameter. The axonal and fiber diameters, and *g*-ratios were thus obtained in more than 300 single fibers of each sural nerve studied.

Experiments and statistics

Two groups of 6 animals each were treated with cisplatin (2 mg/kg/week) for 7.5 weeks up to a cumulative dose of 15 mg/kg. One group received ORG 2766 as supplementary treatment, whilst the other group was given saline injections, both during the entire study period. A further group of 6 animals served as an age control. Electrophysiological examinations were performed at the start of the experiment and after 7.5 weeks. Immediately following this final measurement the animals were killed and the sural nerves prepared.

Differences between groups were analyzed by an analysis of variance (ANOVA) followed by Student-Newman-Keuls test. The animals received a code number before the start of the measurements. The codes were broken only after group-analysis of both the electrophysiological and the histological data had been completed.

Fig. 1. Photomicrographs of cross-sections of sural nerve (Epon, 1 μ m, myelin staining, 1000 $\times$) revealing the myelinated nerve fibers of (a) cisplatin/saline treated, (b) cisplatin/ORG 2766 treated and (c) age-control rats.

3. Results

Nerve conduction velocities measured in the experiment are summarized in Table 1. In both age-control

Table 1
Motor (MNCV) and sensory (SNCV) nerve conduction velocities in m/sec in rats treated with cisplatin and saline or ORG 2766 for 7.5 weeks, and in control rats ($\pm$ SEM)

		Controls (n = 6)	Cisplatin + saline (n = 6)	Cisplatin + ORG 2766 (n = 6)
MNCV	start	51 $\pm$ 2.2	52 $\pm$ 3.3	53 $\pm$ 2.2
	end	58 $\pm$ 1.9	56 $\pm$ 1.9	59 $\pm$ 1.2
SNCV	start	64 $\pm$ 3.3	62 $\pm$ 3.6	62 $\pm$ 3.1
	end	82 $\pm$ 3.9 **	54 $\pm$ 2.5 ***	81 $\pm$ 3.7 *

* $p < 0.01$ between cisplatin/saline and cisplatin/ORG 2766 (SNK-test).

** $p < 0.01$ between cisplatin/saline and control (SNK-test).

*** $p < 0.05$ between start and end of treatment period (paired t -test).

Table 2
Total number of myelinated nerve fibers, mean nerve area and density (fibers/area) in sural nerve ($\pm$ SEM)

	Controls (n = 6)	Cisplatin + saline (n = 6)	Cisplatin + ORG 2766 (n = 6)
Sural nerve area (in μ m ²)	47726 $\pm$ 3810	49203 $\pm$ 5264	47194 $\pm$ 3108
Total number of fibers	1230 $\pm$ 35	1113 $\pm$ 37 *	1223 $\pm$ 40
Fiber density (per 1000 μ m ²)	25.8 $\pm$ 2.2	22.6 $\pm$ 2.5	25.9 $\pm$ 1.9

* Difference between control and cisplatin/saline: $t = 1.89$, $p < 0.1$, between cisplatin/ORG 2766 and cisplatin/saline: $t = 1.72$, $p < 0.1$.

animals and cisplatin/ORG 2766 treated rats an increase was seen in MNCV and SNCV, which is a normal finding considering the age of the rats (Stanley 1981). In cisplatin/saline treated rats the MNCV increased likewise, but the SNCV decreased to 54 m/sec (paired t -test: $p < 0.05$). The difference in SNCV between the first two groups and the latter one was highly significant (SNK-test, $p < 0.01$).

Microphotographs of transverse sural nerve sections are presented in Fig. 1. There was no evidence of nerve fiber degeneration in either of the treatment groups. No breakdown products of myelin were observed either. The total nerve area was slightly larger and the total number of fibers was slightly, but not significantly lower in cisplatin/saline treated rats (Table 2). In teased fiber preparations axon diameters and internodal lengths were similar in treated and untreated groups (Fig. 2). The quotients of internodal lengths and axon diameters were also comparable (136, 134, and 141 for age-control, cisplatin/saline and cisplatin/ORG 2766, respectively). Axonal degeneration, seg-

Fig. 2. The mean internodal distances in relation to axon caliber, for each of the three study groups (control, cisplatin/saline and cisplatin/ORG 2766), as measured from teased fiber preparations of rat sural nerve. The mean internodal ratios are supplied in the text. No statistical significant group differences could be demonstrated.

mental demyelination or remyelination were very infrequently (< 1%) observed in all three groups.

Data on morphometrical analysis of nerve fibers in transverse sections are summarized in Fig. 3. Nerve fibers of diameters of 10 μm and larger were present in the control group, and to a lesser extent in the cisplatin-ORG 2766 group, but very rare in the cisplatin-saline group. When nerve fibers were classified in categories, sural nerves of cisplatin/saline treated animals were found to contain less thick myelinated fibers than the other two groups (Fig. 4). The overall mean *g*-ratios (0.56, 0.62 and 0.51 for age-control, cisplatin/saline and cisplatin/ORG 2766, respectively), and the calculated regression lines through the individual *g*-ratio values were not statistically different between the three groups.

Fig. 3. Fiber distribution of myelinated fibers in the sural nerve in the three study groups as divided in categories, according to fiber diameter.

Fig. 4. Fiber distribution of myelinated fibers, divided in three large categories (< 6 μm, 6–7 μm, and >= 8 μm). There were significant differences in the category of thick myelinated fibers only (ANOVA: $F_{2,15} = 6.22$, $p < 0.02$) and Student-Newman-Keuls test indicated that the cisplatin/saline group differed significantly ($p < 0.05$) from both the control group, and the cisplatin/ORG 2766 group.

4. Discussion

The present study shows that intoxication of rats with cisplatin may induce a subclinical sensory neuropathy, expressed in two related phenomena, slowing of sensory nerve conduction velocity and a shift in the distribution of sensory nerve fiber diameters towards smaller diameters. The development of this neuropathy is prevented or postponed by subcutaneous treatment with ORG 2766.

These results are in agreement with earlier observations. Intoxication of rats with cisplatin at a dose of 2 mg/kg/week was previously shown to induce a slowing of the SNCV, whereas no effects were seen on the MNCV (De Koning et al. 1987). Treatment with ORG 2766 was shown to be of benefit when given from the start of cisplatin treatment (De Koning et al. 1988; Hamers et al. 1991a, 1993a).

Although it could be argued that the differences in SNCV between cisplatin-treated animals and age-control animals are due to a cisplatin-induced block in sensory nerve maturation such a block can not explain the decrease in SNCV during the treatment period. Moreover, we have recently shown that cisplatin administration to full-grown rats also results in a decrease of SNCV, thus providing undeniable evidence of the neuropathic effect of cisplatin in the rat (Hamers et al. 1993a). In patients treated with cisplatin based chemotherapy a slowing of the SNCV is also found, whereas the motor conduction is not affected (Thompson et al. 1984; Gastaut and Pellissier 1985; Ongerboer de Visser and Tiessens 1985). Therefore this animal model seems appropriate for the study of cisplatin neurotoxicity. The predictive value of this animal model

is underscored by the observation in a double blind, placebo controlled trial with ORG 2766 in ovarian cancer patients treated with cisplatin based chemotherapy which showed that ORG 2766 counteracts neurotoxicity also in man (Gerritsen van der Hoop et al. 1990).

Little is known of cisplatin neurotoxicity on the histological level. In man a decrease in the number of thick myelinated fibers in sural nerve biopsies was reported whereas the number of fibers on the whole was unchanged or slightly diminished. Teased fiber preparations showed some axonal degeneration and secondary demyelination (Gastaut and Pellisier 1985). Post-mortem studies reported moreover changes in the dorsal columns and optic nerve atrophy (Walsh et al. 1982; Thompson et al. 1984). On the basis of post-mortem cisplatin concentration measurements it is probable that cisplatin affects sensory neurons by an action on the level of the dorsal root ganglion, because cisplatin concentrations in peripheral nerve and dorsal root ganglion were similar to those in tumour tissue, whereas in brain and spinal cord cisplatin concentrations were five to twenty times lower (Thompson et al. 1984). Observations in the rat model closely correlate with these findings in man (Terheggen et al. 1989). Signs of nerve degeneration in the peripheral nerves of rats treated with cisplatin were reported by Clark (1980), but no quantification was presented. In another, morphometric, study of dorsal root ganglia in rats changes were observed in the nucleus-cell and nucleolus-nucleus size ratio but no data on fiber distribution in the peripheral nerve were obtained (Tomiwa et al. 1986).

In the present study, we examined a pure sensory nerve to find out whether structural changes are related to the decrease in sensory conduction velocity obtained from the mixed sciatic nerve. The total number of fibers present in the sural nerve of cisplatin/saline treated rats appeared to be slightly diminished. No clear evidence of axonal degeneration was found, however, either in teased fiber preparations or cross-sections. Slight, but not significant, enlargement of total sural nerve area was observed in cisplatin/saline treated rats. Nor were mean internodal length or *g*-ratios affected. The latter excludes processes that change the axon/myelin ratio. A decrease, however, was observed in the number of thick myelinated fibers. In all, the only objective difference between control animals and cisplatin/saline treated rats was a shift towards the left of the spectrum, i.e. towards smaller fibers, in the fiber distribution of the latter group. In rats co-treated with ORG 2766 no decrease in the number of fibers and only a marginal shift in fiber distribution was noted.

Numerous morphological factors can be responsible for a decrease in nerve conduction velocity. A decrease

in internodal length can seriously affect nerve conduction velocity (Waxman 1980). Such a change was not observed in this study. The most important morphological factor, however, determining nerve conduction velocity is the fiber diameter. In empirical studies it was demonstrated that an almost linear relationship exists between diameter and velocity. The fast component of nerve conduction is determined by thick myelinated fibers and thus loss of thick fibers can lead to a decrease in NCV (Waxman 1980). In our study sural nerves of cisplatin/saline treated rats contained less thick myelinated fibers than either untreated controls or cisplatin/ORG 2766 treated animals. This shift in fiber distribution explains the observed changes in nerve conduction velocities. In fact, in the only human study in which fiber distribution was reported, a substantial decrease in the number of thick myelinated fibers was noted too and accompanied by a decrease of SNCV (Gastaut and Pellisier 1985). The relative thickness of myelin, as indicated by the *g*-ratio (the quotient of axon diameter and fiber diameter) is yet another factor that influences NCV, but various studies have shown that changes in *g*-ratio values between 0.47 and 0.74 can not be responsible for more than a 5% change in NCV (Rushton 1951; Waxman 1980). In the present study *g*-ratios fall well within these limits. Thus it is unlikely that a change in myelin thickness was responsible for the decrease in NCV.

With respect to the mechanism of action underlying cisplatin neurotoxicity, little is known. The anti-tumor activity of cisplatin results from its affinity to form adducts with guanine and cytosine in DNA. This can lead to mispairing during replication (in dividing cells), but also negatively affects transcription (Corda et al. 1992). This ultimately decreases protein synthesis. Changes that affect membrane potential, such as decrease in axonal sodium channel density or conductance and diminished Na^+ , K^+ -ATPase activity can also lead to disturbances in action potential propagation and thus to diminished NCV, even before morphological changes occur. Cisplatin has also been reported to diminish ATPase activity. Brady and co-workers (1990) showed the diminished ATPase activity in the renal proximal tubule cell to be due to mitochondrial damage caused by cisplatin. Both protein synthesis and oxidative phosphorylation are important for neurons, and impairment of either of them would be enough to induce disease.

The beneficial effects of melanocortins (ACTH, α -MSH, and related peptides) on peripheral nerve regeneration and collateral sprouting are documented by behavioral, neurophysiological and histological studies (for reviews see De Wied and Jolles 1982; De Koning and Gispen 1988; Bär et al. 1990; Strand et al. 1991). The analog ORG 2766 has also been shown to support nerve function in metabolic, iatrogenic, or toxic in-

duced neurotoxicity, in which the function of the peripheral nervous system is hampered, as can be seen in models of diabetes mellitus, (streptozotocin-induced or genetically determined (BB/Wor rat)) (Van der Zee et al. 1989; Bravenboer et al. 1992), demyelinating disease (Duckers et al. 1993), and after the repeated administration of cisplatin (De Koning et al. 1988; Hamers et al. 1991a, 1993a) or acrylamide (Edwards et al. 1989). Access to the melanocortin receptor family (Gantz et al. 1993ab; Chhajlani et al. 1993) will undoubtedly lead to a better understanding of melanocortin-like peptides in nerve regeneration at the molecular level. Nonetheless, the present study shows that the protective effect of ORG 2766 in cisplatin neurotoxicity in rats is expressed on both the electrophysiological and the histological level.

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