Patients Treated with Antitumor Drugs Displaying Neurological Deficits Are Characterized by a Low Circulating Level of Nerve Growth Factor

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ABSTRACT

The aim of our study was to explore whether nerve growth factor (NGF) plays any role in the development of peripheral neuropathy induced by antitumor treatment. We measured the circulating NGF levels in 23 cancer patients before and after chemotherapy. We evaluated whether the development of peripheral neurotoxicity was associated with changes in basal NGF concentrations in patients studied with a comprehensive neurological and neurophysiological examination. The results of these studies showed that the circulating levels of NGF, which are about 20 pg/ml in plasma of controls, decrease during chemotherapy and in some cases completely disappeared after prolonged treatment with antitumor agents. The decrease in NGF levels seems to be correlated with the severity of neurotoxicity.

These results clearly suggest that NGF might become a useful agent to prevent neuropathies induced by antineoplastic drugs and restore peripheral nerve dysfunction induced by these pharmacological compounds.

INTRODUCTION

Patients with malignancy exposed to chemotherapeutic agents such as Vinca alkaloids, suramin, taxanes, and cisplatin can develop PN. Neurological observations published in recent years indicate that administration of taxanes and cisplatin in patients affected by neoplasm induces nerve deficits in a dose- and time-dependent manner (1–6). Moreover, when platinum compounds and taxanes are used in combination, the patients develop more severe PNs (7). The pathophysiology of chemotherapeutic agent-induced neuropathy is still not clear, although a variety of studies have shown that taxanes interfere with axonal transport, causing axonal distal sensory-motor lesions (4), whereas platinum compounds induce sensory neuronopathy acting mainly on the neuronal cell bodies of the spinal ganglion (3). Pathological and electrophysiological studies have also indicated that neurons of the dorsal root ganglion are selectively damaged after cisplatin treatment (1, 3). It has been reported that the development of this PN can induce clinicians to interrupt therapy to prevent more severe neurological deficits (1). Because of the neurotoxic effects, much effort has been devoted to the identification of potential neuroprotective agents (8–10). It is reasonable, therefore, to hypothesize that identification of molecules, which can prevent neurotoxicity and/or promote peripheral innervation after chemotherapy, would be clinically useful. One molecule that seems to display these properties is NGF. NGF is known to play a crucial role in growth and differentiation of specific neuronal population of the peripheral nervous system (11, 12) and is able to reduce the neuronal damage induced by surgical, chemical, and physical injuries both in animal models and humans (8, 13–18). The development of neuropathies induced by antitumor drugs might be the result of impaired synthesis and/or release of endogenous NGF. As a first approach to test the validity of this hypothesis, we investigated whether there is a correlation between the PNs induced by antineoplastic chemotherapy and circulating NGF. To further understand the role of NGF, we studied the relationship between serum levels of NGF and severity of neurotoxicity in patients treated with neurotoxic drugs.

MATERIALS AND METHODS

This study (Table 1) was performed on 23 cancer patients (10 males and 13 females) hospitalized at the Regina Elena Cancer Institute (Rome, Italy) and at the S. Carlo Hospital (Potenza, Italy).

Eligibility criteria for entry in this study were as follows: (a) adult patients older than 50 years but not exceeding 70 years; (b) cytologically and/or histologically proven cancer; (c) Karnofsky performance score between 60 and 100; (d) adequate hepatic, renal, bone marrow, and cardiac function; (e) no previous cytotoxic or radiation therapy; (f) no brain metastasis, neurological disorders (including PNs), diabetes, or systemic diseases affecting the nervous system.

Study Design. This study was approved by local intramural ethics committees and was carried out following Italian law for biomedical research. An authorized blood sample was required as baseline sample before the beginning of the first
cycle of chemotherapy. Additional blood samples were collected 24 h after the end of the fourth and sixth chemotherapy courses. Chemotherapy regimens containing neurotoxic agents given alone or in combination therapy were administered at standard doses as shown in Table 1.

**Neurological and Neurophysiological Evaluations.** Patients were examined by a neurologist before treatment and after four and six courses of chemotherapy. The neurological examination consisted of a standardized history for detection of neuropathic symptoms and evaluation of pinprick and vibratory sensation, strength, and deep tendon reflex.

A neurophysiological examination was performed in each patient by analyzing nerve conduction of motor and sensory median, sural, and peroneal nerves using surface electrodes, according to standard methods as described previously (6). Conduction velocities and amplitudes of motor and sensory potentials were recorded at baseline and after four and six courses of therapy.

Neurotoxicity score was assigned to each patient on the basis of neuropathic signs and symptoms and neurophysiological changes (6, 7). The severity of neurotoxicity was graded on the basis of the obtained score as follows: mild neurotoxicity, total score of 1–4; moderate neurotoxicity, total score of 5–10; severe neurotoxicity, total score of more than 10 (corresponding to WHO the neurotoxicity scale, grades 1, 2, and 3–4, respectively).

**Sample Collection.** Samples were collected from the forearm vein between 8:00 and 10:00 a.m. and immediately centrifuged at 8500 \( \times g \) for 20 min at 4°C to remove cells and debris, and the supernatant was carefully removed and stored at \(-70°C\) until NGF determination. For controls, we used blood from 10 age-matched healthy subjects (5 males and 5 females).

**NGF Determination.** The levels of NGF were measured by a highly sensitive two-site immunoenzymatic assay (19) that recognizes human and murine NGF and does not cross-react with brain-derived neurotrophic factor (20, 21). Briefly, poly- styrene 96-well immunoplates (Nunc) were coated with monoclonal anti-NGF antibody (Roche Molecular Biochemicals clone 27/21, Mannheim, Germany), which does not cross-react with brain-derived neurotrophic factor diluted in 0.05 M carbonate buffer (pH 9.6). Parallel wells were coated with purified goat IgG (Zymed, San Francisco, CA) for evaluation of the nonspecific signal. After an overnight incubation at room temperature and 2 h of incubation with a blocking buffer (0.05 M carbonate buffer, pH 9.5, 1% BSA), plates were washed three times with 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.5% gelatin, 0.1% Triton X-100. After extensive washing of the plates, the samples and the NGF standard solutions were diluted with sample buffer (0.1% Triton X-100, 100 mM Tris-HCl, pH 7.4, 400 mM NaCl, 4 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM benzethonium chloride, 2 mM benzamidine, 40 units/ml aprotinin, 0.05% sodium azide, 2% BSA, and 0.5% gelatin), distributed into the wells, and left at room temperature overnight. The plates were then washed three times and incubated with 4 milliunits/well anti-b-NGF-galactosidase (Roche Molecular Biochemicals) plus substrate buffer (100 mM HEPES, 150 mM NaCl, 2 mM MgCl₂, 0.1% sodium azide and 1% BSA) for 2 h. After washing, the plates were read at 405 nm.

**Table 1 Clinical and laboratory data of cancer patients**

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<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Histology</th>
<th>Therapy (mg/sm)</th>
<th>No. of cyclesa</th>
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<th>NGF (pg/ml), cycle 4</th>
<th>NGF (pg/ml), cycle 6</th>
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<td>M</td>
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a If not otherwise specified, each drug was given for the indicated number of cycles.

b BC, breast cancer; NA, not available; OC, ovarian cancer; NSCLC, non-small cell lung cancer; GC, gastric cancer; UPSC, unknown primitive site cancer; ADM, Adriamycin; Epi, epirubicin; DDP, cisplatin; CBDCA, carboplatin; GEM, gemcitabine; VNR, vinorelbine; FU, fluorouracil; AF, leucovorin; VP16, Vepesid.
at 37°C, the absorbance was measured at 575 nm using an ELISA reader (Dynatech, MR 5000, PBI International, Stuttgart, Germany), and the values of standards and samples were corrected by taking into consideration the nonspecific binding. The recovery of NGF during assay procedure was estimated by adding a known amount of highly purified NGF to the samples or to the homogenization buffer as an internal control. The yield of the exogenous NGF was calculated by subtracting the amount of endogenous NGF from the value of endogenous plus exogenous values. Under these conditions, the NGF recovery was over 90%. Data are represented as pg/ml of serum, and all assays were performed in triplicate.

**Statistical Analysis.** ANOVA was used to analyze NGF levels and neurophysiological parameters (velocity and amplitude of potentials of motor and median sensory, sural, and peroneal nerves). Post-hoc comparisons were carried out using Fisher’s test. Neurotoxicity scores were analyzed by the $\chi^2$ test. Correlations between serum NGF levels and neurotoxicity score were analyzed by using the Wilcoxon signed rank test.

**RESULTS**

Polychemotherapy was applied for 23 cancer patients which were considered fully eligible for evaluation (see in “Patients and Methods”). Nineteen patients completed four courses of therapy, and 9 patients completed six courses of chemotherapy. Patients who did not conclude the therapy (14 cases) had severe progression of disease, death, or marked neurotoxicity. The mean cumulative doses were 340 mg/m² for cisplatin, 3175 mg/m² for carboplatin, 1115 mg/m² for Taxol, and 351 mg/m² for docetaxel (see Table 1). No significant differences were observed between male and female patients in all analyzed parameters, and for this reason, sex was not considered in the statistic evaluation.

**Neurological Evaluation.** Before the beginning of the treatment, neurological examination showed normal values in all patient. After four courses of therapy, 17 of 19 evaluable patients had clinical and/or electrophysiological signs of PN; at the end of chemotherapy, 8 of the 9 patients still evaluable showed clinical symptoms and electrophysiological signs of PN (in 14 patients, treatment was discontinued due to progression of disease or death; in 1 patient, it was discontinued due to severe neurotoxicity after four cycles).

The most common clinical symptoms complained by patients was burning distal paresthesia prevalently in the lower limbs. Neurological examination performed after four cycles of therapy showed hypo-areflexia in 12 of 19 patients (63%) and distal hypoesthesia and hypopallesthesia in 13 (68%). Of the nine patients evaluable at the end of therapy, eight showed reduction or absence of tendon reflexes, and seven showed distal hypoesthesia and hypopallesthesia.

Neurophysiological evaluation (Fig. 1) after four and six courses of chemotherapy revealed a reduction of mean amplitude of sensory action potential on sural and median sensory nerves ($P < 0.05$). Even mean amplitude of motor potentials measured on peroneal nerve decreased following chemotherapeutic treatment ($P < 0.05$). The amplitude decrease was evident in sural and median sensitive nerves, mainly at the end of the therapy, six cycles after the beginning of the therapy ($P < 0.05$ in post-hoc comparisons). In the peroneal nerve, differences were revealed between pretreatment and four courses of therapy. Distal latencies and motor and sensory conduction velocities were normal before and after chemotherapy.

The neurotoxicity score (Fig. 2A) assigned to each patients on the basis of clinical and neurophysiological findings showed, after four courses of treatment, a severe peripheral neurotoxicity (score of >10) in four patients who complained of balance impairment, sensory ataxia, and burning distal paresthesia. It was also revealed a moderate neurotoxicity (score of 5–10) in 3
patients, mild neurotoxicity (score of <5) in 10, and no signs of neurotoxicity in 2 ($\chi^2 = 352.42; P < 0.01$).

After six cycles of therapy, severe neurotoxicity was also present, as revealed by statistical analysis ($\chi^2 = 126.88; P < 0.01$).

**NGF Evaluation.** The mean NGF serum levels of 10 healthy age-matched blood donors were 19.23 ± 2.95 pg/ml (Fig. 2B). Fig. 2 also shows the NGF serum levels of cancer patients measured at the beginning of the therapy, four and six cycles after the onset of the chemotherapy. The NGF serum levels of cancer patients at the beginning of the therapy were highly variable but biologically active, as revealed by a bioassay (22). NGF measured after four or six cycles of chemotherapy was comparable between patients. ANOVA showed a main effect of chemotherapy ($P < 0.05$). In fact, post-hoc comparisons between the beginning of therapy and six cycles of antineoplastic compounds revealed a decrease ($P < 0.05$) in the NGF levels. Very interestingly, ANOVA did not reveal differences between the NGF levels of the 10 healthy subjects and the 23 cancer patients at the beginning of the therapy.

**Correlation between NGF and Neurotoxicity Score.** To determine whether the decrease in circulating NGF levels in patients treated with chemotherapeutic drugs was associated with increased neurotoxicological score after four and six cycles of therapy, we used the Wilcoxon test. Significant correlation was found four cycles after therapy ($P < 0.01$), whereas after six cycles of chemotherapy, the correlation was not fully statistically significant. The apparent discrepancy in these data can be explained by the fact that 19 patients were used for the analysis after four drug cycles, and only 9 patients were used for the analysis after the end of the therapy (six cycles; see in “Patients and Methods”).

**DISCUSSION**

It is well known that PN induced by neurotoxic drug has become one of the principal dose-limiting side effect of anti tumor chemotherapy. A possible cumulative neurotoxicity has been suggested using combination treatment with platinum compounds and taxanes, resulting in more severe and disabling neuropathy. This peripheral deficit very frequently represents a serious limitation to the use and efficacy of chemotherapy in humans (1–6, 23, 24). With the growing clinical utilization of combined platinum and taxane schedules, these undesired neurological effects have become of great concern for oncologist. To reduce or overcome these effects, scientists have recently focused their attention on the potential benefits of neuroprotective drugs, such as the radioprotective agents WR 2721 (25) and adrenocorticotropic hormone analogue (9, 10). Because NGF exerts a pivotal role on the growth, differentiation, and functions of peripheral nerve cells (11), we investigated whether the human blood levels of NGF were affected by chemotherapeutic treatment.

The results of our study showed that no significant difference was found between the circulating NGF levels of healthy subjects and those of patients before chemotherapy. However, the amount of NGF in serum patients who received chemotherapy was lower than the NGF levels of both untreated patients and healthy subjects, and this decrease was associated with the peripheral neurological deficits.

These observations suggest a possible functional link between the low presence of NGF and the development of peripheral neurotoxicity. Our working hypothesis is that NGF-producing cells do not synthesize and/or release the amount of NGF necessary to promote recovery of damaged tissues.

This hypothesis is consistent with other basic and clinical findings demonstrating that NGF promotes recovery in damaged nerve cells (14, 26) or that endogenous NGF deprivation through administration of NGF antibodies causes sensory and sympathetic deficits (15, 27). Indeed, both in animal models and in humans, a low level of circulating NGF is associated with sensory and/or sympathetic neuronal deficits and even cell death. However, administration of NGF is able to prevent peripheral neuronal death (28–30) and diabetic neuropathies (26, 31, 32).

Although the mechanisms through which antitumor drugs induce neurotoxicity are at present not fully known and need to be further evaluated, our findings indicate that the decrease in circulating NGF might be one of the mechanisms through which these drugs induce PNs. Because systemic investigation, including biochemical and structural studies of the peripheral nerves, are not feasible in humans, we used laboratory animals to further explore the role of cisplatin and Taxol on NGF synthesis and release. We injected cisplatin and Taxol also in laboratory animals and monitored the alteration of NGF levels in NGF-producing tissues. The results thus far obtained clearly indicate that cisplatin lowers...
the constitutive levels of NGF in the paws, spleen, intestine, and bladder. These studies also revealed that exogenous administration of NGF promotes peripheral functional recovery. These observations, although they support the hypothesis that these chemotherapeutic drugs lower the constitutive amount of circulating NGF levels, also suggest that NGF may prevent the neurotoxic effects.\(^4\) However, whether this occurs by reducing the synthesis or enhancing the uptake by NGF-responsive cells is at present not clearly known.

The potential clinical use of NGF is also suggested by basic and ongoing clinical studies indicating that NGF promotes nerve regeneration in neuropathies (30–33), including those induced by chemotherapeutic agents (8, 34), ocular hypertension (16), corneal ulcer (35), and leprosy (29). This type of study is encouraged by several other factors showing that peripheral nerves are accessible to protein given systematically and that NGF and other neurotrophic factors have entered in preclinical trials (35–36). Cumulatively, our observations in human blood samples and findings in animal models suggest the hypothesis that exogenous administration of NGF can prevent peripheral neurotoxicity and most likely promotes regeneration of peripheral nerve damages.

REFERENCES

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