Localization and Expression of Ciliary Neurotrophic Factor (CNTF) in Postmortem Sciatic Nerve from Patients with Motor Neuron Disease and Diabetic Neuropathy

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Abstract. Ciliary neurotrophic factor (CNTF) is thought to play an important role in the maintenance of the mature motor system. The factor is found most abundantly in myelinating Schwann cells in the adult sciatic nerve. Lack of neuronal growth factors has been proposed as one possible etiology of amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA). Growth factor replacement therapies are currently being evaluated as a treatment for motor neuron disease. In this report we determined whether the expression of CNTF in sciatic nerve differed in patients with motor neuron disease compared to controls or patients with another form of axonopathy. We identified 8 patients (7 with ALS and 1 with SMA) with motor neuron disease and 6 patients with diabetic motor neuropathy who had autopsy material available. Immunoperoxidase staining showed reduced CNTF expression in nerves of patients with motor neuron disease but not in patients with diabetic motor neuropathy. Decreased CNTF appears to be associated with primary motor neuron disease rather than a generalized process of axon loss. This result supports suggestions that CNTF deficiency may be an important factor in the development of motor neuron disease.

Key Words: ALS; Diabetes; Growth Factor; Motor Neuron; Neurotrophins.

INTRODUCTION

Ciliary neurotrophic factor (CNTF) is a member of the interleukin family of neuronal growth factors sometimes referred to as neurokinesins. It has been shown to support survival of and neurite outgrowth from sensory, sympathetic and motor neurons when added to cells in vitro (1, 2). In vivo its effects appear to be restricted to the motor nervous system. When provided to developing chick embryos, only the spinal motor neurons demonstrated a significantly decreased rate of programmed cell death (3). In the rat, CNTF is not detectable until day 4 of life. However, if provided locally to the severed facial nerve of a 1-day-old pup, it is able to prevent the motor neuron death that results from axotomy. CNTF has also been shown to potentiate axonal sprouting in vivo (4, 5).

In mouse models of lower motor neuron degeneration, CNTF has been shown to be of benefit. When treated with CNTF, the progressive motor neuronopathy (pmn) mouse demonstrated improved motor function, prolonged life, and decreased loss of motor neurons in the facial and phrenic nerves (6). When both CNTF and brain-derived neurotrophic factor (BDNF) was given to the wobbler mouse, the motor neuron disease was arrested (7). Finally, Sedtniner and colleagues have shown that abolition of the CNTF gene by homologous recombination leads to atrophy of motor neurons in adulthood (8).

The motor neuron diseases comprise a family of neurodegenerative disorders of unknown etiology. The spinal muscular atrophies (SMA) present in childhood with degeneration of the lower motor neurons. Adult forms of motor neuron disease include amyotrophic lateral sclerosis with involvement of both upper and lower motor neurons. In other disorders there are predominant losses of lower motor neurons, upper motor neurons, or bulbar motor neurons. In 20% of familial amyotrophic lateral sclerosis (ALS), the involved gene has been localized to chromosome 21 and recent studies show that the mutations map to the Cu/Zn SOD gene (9).

Because CNTF appears to play a role in preventing motor neuron atrophy, several clinical trials of its efficacy in treating human motor neuron disease have been initiated. However, there are few studies which document expression of CNTF in patients with ALS or SMA. Amand et al (10) found significantly decreased CNTF levels in the spinal cord of ALS patients with unchanged motor cortex levels. In another study, CNTF receptor was found to be increased in spinal cord in ALS patients, possibly as a response to the decrease in substrate (11). However, since CNTF is synthesized by Schwann cells in the peripheral nerves, the significance of levels in spinal cord is unclear.

In this report, we have examined CNTF expression in autopsy samples of sciatic nerves from patients with motor neuron disease. We used an immunohistochemical assay employing fixed tissue in order to evaluate
CNTF expression and determine its localization. Previous work from the laboratory has shown that in vitro CNTF production is dependent on axonal contact (12). To control for this effect, we examined tissue from patients with diabetic motor neuropathy (DM), a disease that also results in significant axon loss but which is not a primary motor neuron disorder. This study demonstrated that the percentage of cells expressing CNTF is decreased in patients with motor neuron disease but not in patients with diabetic motor neuropathy. We interpret these findings to suggest that deficiencies in neurotrophic growth factors may play a central role in the development of motor neuron disease.

MATERIALS AND METHODS

Patient Selection

Proximal sciatic nerves from the pelvis section were harvested from autopsies of patients: (a) without known neurologic disease or symptoms (Controls, 11 total samples), (b) patients with motor neuron disease (MND, 8 patients with ALS and one with Kugelberg-Wielander), and (c) patients with diabetic motor axonopathy (DM, 6 patients). Patients were selected by searching the Mayo Clinic computerized data bank of autopsy material using the diagnoses of diabetes, motor neuron disease, spinal muscular atrophy, and ALS. The clinical history, exam, EMG, and laboratory results were reevaluated to confirm the diagnoses. For the patients with motor neuron disease, the classic criteria for ALS were used: evidence of both upper and lower motor neuron signs, absence of sensory involvement and EMG changes consistent with denervation (fibrillation potentials and large motor potentials) in at least three limbs. For the patient with Kugelberg-Wielander similar criteria were used, with the exception of the lack of upper motor neuron signs. Control samples were harvested from patients with no evidence of neurologic disease and no neurologic complaints or findings at their last documented exam.

Patient Matching

Patients were matched as a population for age. In addition, the degree of neurologic involvement between the disease groups was compared using an average Neuropathy Impairment Score (NIS,13) calculated from the last neurologic exam and the results from an EMG.

Tissue Preparation and Staining

The stored tissue had been harvested within 18 hours of death and fixed in 10% formalin. After fixation, the tissue was paraffin embedded and 6-micron sections cut. The samples were encoded prior to further handling so that the following steps were performed by blind investigators. Immunohistochemical staining was carried out using the following protocol: the tissue slides were incubated at 50°C overnight followed by deparaffinization and dehydration by incubating for 5 minutes in xylene (twice), absolute ethanol (twice), and 95% ethanol (once). Endogenous peroxidases were blocked with a 30 minute incubation in 0.6% H2O2 in methanol at room temperature. The tissue was then placed in the Antigen Retrieval Solution (Citra Soln @ 4°C, 1-part Antigen Retrieval Solution to 9 parts water, BioGenex). After microwaving the samples twice for 7 minutes each time at a medium setting, they were allowed to cool to room temperature for 15 minutes, then washed with water followed by Phosphate Buffered Saline (PBS; Sigma). To block nonspecific binding, the slides were incubated in 20% normal goat serum dissolved in PBS for 30 minutes, after which the primary antibody was added.

Anti-S-100, a polyclonal rabbit antibody raised against bovine S-100 (BioGenex) was used at a 1:960 dilution. Anti-CNTF, a polyclonal rabbit antibody against rat CNTF, was a gift of the Regeneron Pharmaceutical Company and was used at a 1:200 dilution. Both antibodies were made up in PBS with 10% normal goat serum and incubated overnight at room temperature. After washing twice in PBS for 10 minutes each, the secondary antibody, Super Sensitive Biotinylated anti-Rabbit IgG (BioGenex), was applied at 1:20 dilution in PBS with 1% bovine serum albumin (BSA). After 45 minutes at room temperature, the slides were washed in PBS as above and incubated with Peroxidase-labeled Streptavidin (1:20, BioGenex) for 45 minutes at room temperature. After another PBS wash as above, the slides were stained with Diaminobenzidine (DAB) for 5 to 10 minutes and then counterstained with hematoxylin for 30 seconds, dried, and the cover slips mounted.

Controls for the immunohistochemical staining included absence of staining when primary or secondary antibodies, Streptavidin, or DAB reagents were omitted. Selective competition of CNTF staining by preincubating the primary anti-CNTF antibody with purified CNTF protein at a 1:100 dilution confirmed specificity. The antibodies have been shown to be specific for the CNTF protein by western immunostaining techniques (12).

Analysis of the Data

Micrographs of the stained tissue were taken at 20, 40, and 100X magnification. The field was randomly selected by an investigator blinded to the identity of the samples. Immunostaining was considered significant if the intensity was easily discernible from the background and clearly localized to Schwann cell cytoplasm. Two methods of scoring to assess CNTF expression were employed by a second investigator, again blinded, to the origin of the micrographs. This single investigator carried out all of the assays for the sake of consistency. The micrographs were projected onto a whiteboard and the staining was rated on a 0 to 4 scale at all magnifications. At the 100X magnification, the number of nuclei was counted as well as the number of nuclei associated with immunostaining, and a percentage obtained. The advantage of the rating assay at lower magnifications was that a large area of the tissue could be examined, insuring that the results found do not reflect focal changes. The advantage of the second assay is that a greater objective quantitation is achieved. When compared, both methods gave similar results.

Four separate experiments were done with multiple samples stained from each patient’s tissue. An average value was calculated for each patient from the repetitive samples. The
number of separate data points for each population group is listed in the Results section. An analysis of variance was carried out using the mean value for each patient. If this analysis demonstrated statistical significance, a post-hoc Newman-Keuls test was performed (14). In the case of non-significant results, a power analysis was done to insure an adequate sample size.

RESULTS

Samples were collected from a total of 26 patients (Con: 11, MND: 9, DM: 6). The diabetic patients chosen had a peripheral neuropathy with predominantly motor involvement as determined by electrophysiologic studies. The ages of the three groups were 60.36±20.79 for the controls, 62.43±10.94 for the MND patients, and 62.2±15.8 for the DM group, and did not differ significantly.

The degree of neurologic involvement between the disease groups was compared in two ways. First, an average NIS (13) was calculated from the last recorded neurologic exam. Since a standard neurologic form is used at the Mayo Clinic, this can be done even with patients who have died many years ago. The NIS was 55.38±41.94 for the MND group and 52.83±35.22 for the DM group. This was not significantly different between the two groups. We also examined the compound muscle action potentials (cMAPS) from the last recorded EMG studies. The diabetic population showed a decreased cMAP for both the tibial and peroneal nerves (5.4±6.4, MND; 0.37±0.32, DM and 2.8±2.4, MND; 1.17±1.16, DM respectively) but this did not reach statistical significance.

Immunostaining for both S-100 and CNTF was considered significant if the intensity was easily discernible from the background and clearly localized to Schwann cell cytoplasm. The area of tissue surveyed was similar between sample populations. A field of approximately 300,000 µ² was examined at 20X and 12,000 µ² at 100X. The density of Schwann cells did not differ significantly (at 100X: 8.57±7 cells/field, CON; 6.8±2.7 cells/field, MND; 5.7±1.9 cells/field, DM). The total number of Schwann cells counted for the CON and MND groups were 540 and 673, respectively. Because of the decreased availability of tissue, 150 Schwann cells were assayed from the diabetic population. The above data was collected over four separate experiments.

Anti-S-100 demonstrated uniform staining of Schwann cells in controls (Fig. 1A), patients with MND (Fig. 1B), and patients with DM (Fig. 1C). The two types of evaluation were the percentage of nuclei associated with immunostain (Fig. 2A) and the global blinded examiner rating (Fig. 2B). Using the rating scale assay, there was no statistically significant difference in the S-100 staining between the groups. The percentage of nuclei-stained assay revealed a small but statistically significant decrease in the number of cells staining in the DM patients when compared to the controls or ALS populations (p<0.01).

CNTF staining was cytoplasmic and found in cells associated with large myelinated fibers in controls (Fig. 3A), in patients with MND (Fig. 3B) and patients with DM (Fig. 3C). There was also axonal CNTF staining noted. Since the significance was uncertain, no attempt at quantification was made. Combined data, analyzed as described in Materials and Methods from four separate experiments, is demonstrated for both the percentage assay (Fig. 4A) and the rating scale assay (Fig. 4B). There is no significant decrease in the amount of CNTF expressed between the controls and patients with diabetic motor neuropathy, even though by exam and EMG evidence these patients were more severely disabled. However, there was a 50% decrease in CNTF staining in patients with motor neuron disease that is significant (p<0.04 for both assays). As expected, the MND population is also significantly reduced when compared to the DM group (p<0.04 for both assays).

Because there were only 6 patients with diabetic neuropathy, a power analysis was performed to determine if this was an adequate sample size to detect a significant difference. The variability was calculated from the data and a 50% difference between the means was selected as a goal since that was the magnitude of change seen with the ALS population. Table 1 demonstrates the number of samples for both assays required at various power and alpha values.

As can be seen by the above calculations a sample size of 6 should have been sufficient to detect a difference of 50%.

There was no significant difference in CNTF expression when comparing the patient with SMA to the ALS population (data not shown).

DISCUSSION

Loss of CNTF has been proposed as a possible mechanism for the sporadic form of ALS (15), but there have been few studies looking at CNTF expression in patients with motor neuron disease. CNTF is

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<td>Power Analysis Determining Adequate Sample Size to Detect a 50% Variation in Staining</td>
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<tr>
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Fig. 1. Autopsy samples of human proximal sciatic nerve were stained for S-100 protein using anti-S-100 antibodies and a biotinylated secondary antibody as described in Materials and Methods. Counterstain is with hematoxylin. Micrographs are at 100X. A:CON, B:MND, C:DM. Arrows denote examples of positive cytoplasmic immunostaining. Arrowheads denote nuclei not associated with immunostain. The blue hematoxylin and the brown immunostain are readily distinguishable in the color slides used for analysis although both appear dark in the black and white micrographs shown here.
Fig. 2. Data from four separate experiments examining S-100 staining were combined as described in Materials and Methods. Means for each patient were calculated and then used in an analysis of variance. Box-and-whisker plots were generated from a post-hoc Newman-Keuls test. Panel A: Percent nuclei stained assay. Panel B: rating scale assay.

known to be synthesized by Schwann cells in the periphery (16) and presumably transported to the motor neuron via retrograde transport. Staining has been seen in axons during immunohistochemical studies. Since CNTF does not have a signal sequence and therefore is not likely to be secreted, the mechanism for CNTF release and uptake by the axons is still unclear (17). CNTF receptors are expressed on ventral horn cells (18) and are upregulated after axotomy that leads to increased transport (19).
Fig. 3. Autopsy samples of human proximal sciatic nerve were stained for CNTF using anti-CNTF antibodies and a biotinylated secondary antibody as described in Materials and Methods. Counterstain is with hematoxylin. Micrographs are at 100X. A:CON, B:MND, C:DM. Arrows denote examples of positive cytoplasmic immunostaining. Arrowheads denote nuclei not associated with immunostain. The blue hematoxylin and the brown immunostain are readily distinguishable in the color slides used for the analysis although both appear dark in the black and white micrographs shown here.
Fig. 4. Data from four separate experiments looking at CNTF levels were combined. Means were calculated for each patient and used in an analysis of variance. Box-and-whisker plots were generated from a post-hoc Newman-Keuls test. Panel A: percent nuclei stained, p<0.04. Panel B: examiner rating scale, p<0.04.

We chose to use immunohistochemical staining of fixed tissue to examine CNTF expression because of the preservation of morphology and the ability to use stored material. This allowed us to collect a reasonable sample number in a short period of time. We were also able to utilize preserved morphology to determine if there was an alteration of protein localization. Cytoplasmic staining of Schwann cells was the predominant finding. However, appreciable axonal staining was also noted. The significance of this staining is not known.
The antibodies utilized were specific for the CNTF protein as demonstrated by western immunostaining techniques (12). The staining is thought to be artifactual and due to a myelin well that forms around the axon. When a section is cut, the periaxonal cytoplasmic staining mixes with the axoplasm and is then trapped. This gives the appearance of axonal staining. This artifactual staining has been seen with other Schwann cell proteins (personal communication). Conversely, the axonal staining seen with CNTF may reflect a real observation corresponding to retrograde axonal transport of the protein. Because of this ambiguity, CNTF axonal staining was not assayed.

We developed two methods of scoring the immunohistochemical data. The rating assay was more subjective but allowed analysis of a large area of tissue. The percentage of cells stained was more objective but examined a smaller sample area. We also employed a large number of patients, pooled experiments and repeated samples to improve the accuracy.

Using these methods, we found there was approximately a 50% decrease in the percentage of Schwann cells expressing CNTF in patients with motor neuron disease (both ALS and SMA). There was no difference between the ALS patients and the patient with SMA. This reduction does not reflect a loss of Schwann cells as the S-100 staining remained fairly stable even in the face of severe axonal loss. Notably, on the percent of nuclei-stained assay, the S-100 staining was found to be lower in the DM population, which may be secondary to profound axonal loss. However, since this decrease was found in only one of the assay systems, it may also reflect variability. It is noteworthy that although the S-100 staining was decreased, CNTF staining remained equal to controls. This might represent induction of CNTF expression in normally nonproducing Schwann cells due to neurologic disease.

Because earlier work with an in vitro model system (12) had shown that CNTF expression is dependent on axonal contact, we examined patients with diabetic motor neuropathy. CNTF expression was not reduced even though there was significant loss of axons as demonstrated by electrophysiologic studies. The sample size utilized should have been sufficient to detect a significant difference as determined by power analysis. This implies that the reduction in CNTF expression is related to primary motor neuron loss and not axonal loss. Even though there were fewer cells expressing CNTF, the location of the protein did not change. This differs from the findings of Sednter et al (17) in the rat, where CNTF staining was decreased by two-thirds and much of the staining that remained was found at extracellular sites after the nerve lesion. Unless this is a response seen only in acute injury, our finding of persistent cytoplasmic staining in the ALS patients argues against the reduction of protein levels being the result of axonal loss.

Anand et al (10) have examined levels of CNTF, as measured by an ELISA assay, in postmortem samples of ventral spinal cord and motor cortex in patients with ALS. These levels were about 50% reduced in ventral spinal cord while the motor cortex showed no change. These levels correspond well with the 50% reduction we saw in sciatic nerves. Although the methods of determining CNTF expression differed between our two groups, this suggests that the cells still synthesizing CNTF may be producing normal levels. This would agree with our data that demonstrates equally intense staining in comparing the positive cells from all three populations.

Future work will be important in determining whether this decreased expression of CNTF contributes to the etiology of motor neuron disease. A causative role might be postulated if CNTF loss in the spinal cord is greater than in the periphery. These studies are currently underway. Since some of the mouse models of motor neuron disease do not show atrophy until adulthood, the decreased levels of CNTF might have to be evident for quite some time before the disease becomes detectable.

CNTF has been utilized in clinical trials for the treatment of ALS. These trials have not had much success. There may be several reasons for this lack of efficacy. Since CNTF cross-reacts with the Interleukin-6 receptor (18), there were substantial side effects which prevented higher concentrations from being given. In addition, it has been shown (20) that exogenous CNTF is removed by the liver with biphasic clearance kinetics and an initial half-life of 2.9 minutes. Therefore, the drug may not remain in the circulation long enough to get to where it is needed. Finally, the pathology of ALS must be taken into account. Since CNTF appears to have little effect on the upper motor neurons, it would not be of benefit for that aspect of the disease. In addition, supplying the growth factor late in the disease may not provide adequate support.

We have shown that the percentage of Schwann cells expressing CNTF is decreased in patients with motor neuron disease but not in patients with diabetic motor neuropathies. These results suggest that CNTF deficiency may be an important factor in the development of motor neuron disease.

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