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MANOOCHEHR MESSRIPOUR

ABOLFAZL NAZARIAN

AZADEH MESRIPOUR

IMAN MOHAMMADI

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Nerve growth factor receptors in dementia

Manoochehr MESSRIPOUR^{1*}, Abolfazl NAZARIAN², Azadeh MESRIPOUR³, Iman MOHAMMADI⁴

¹Department of Medical Biochemistry, Islamic Azad University, Khorasgan Branch, Isfahan, Iran

²Department of Biochemistry, Isfahan University, Isfahan, Iran

³Medicinal Plant Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran

⁴Department of Oral and Maxillofacial Surgery, School of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran

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Background/aim: Nerve growth factor (NGF) promotes the survival and differentiation of sensory and sympathetic neurons. Several studies have found that certain neuropathological factors stimulate NGF receptor expression and release the truncated nerve growth factor receptor (TNGFR) to biological fluids. The aim of this pilot study was to determine urine TNGFR levels in patients with dementia and to verify whether TNGFR can be used as a biomarker of dementia.

Materials and methods: Twelve patients with dementia and 12 healthy individuals were asked to voluntarily participate in this study. Ages, sexes, and weights were matched. The first morning urine samples were collected and the concentrations of TNGFR in the urine samples were measured by fluoroimmunoassay.

Results: The mean levels of TNGFR in the urine samples of the healthy control subjects and the patients with dementia were 164 ± 23 and 341 ± 66 ng / mg creatinine respectively. A positive relationship was found between the levels of TNGFR in different ages of both control and patient subgroups. This is consistent with the previous observations that pathological condition may stimulate the NGF receptor expression.

Conclusion: These findings might be of assistance to evaluate the development of the memory loss associated with Alzheimer disease and other age-associated diseases.

Key words: Alzheimer disease, dementia, nerve growth factor, nerve growth factor receptor, fluoroimmunoassay

1. Introduction

Since the discovery of nerve growth factor (NGF) about 60 years ago, extensive studies have elaborated on the trophic relationship between a neuron and its end organ. Substantial evidence has accumulated that depicts the crucial role of neurotrophic factors in determining neuronal survival, both during development and after injury. NGF is known as a molecule that promotes the survival and differentiation of sensory and sympathetic neurons (1–3). Its roles in neural development have been characterized extensively, but recent findings point to a diversity of NGF actions and indicate that developmental effects are not the only aspect of the biology of NGF. It is thought that NGF roles begin in development and extend throughout adult life and aging, involving a surprising variety of neurons, glia, and nonneuronal cells. NGF also influences the reaction of the neuron to axotomy and subsequent axonal regeneration (4,5). Particular attention is now being given to a growing body of evidence that suggests that, among other roles, endogenous NGF signaling provides neuroprotective

and repair functions (6,7). Exogenously supplied NGF was demonstrated to prevent neuronal loss after axotomy when supplied at the site of injury (8). Furthermore, it was reported that reduction in NGF availability leads to a conditioning lesion-like effect on sympathetic neurons. Results of that study suggested that this effect might be due to the decreased ability of sympathetic neurons to accumulate NGF after axotomy (9). Considerable studies support the evidence regarding the localization of NGF within the central nervous system (CNS) and its presumed role in maintaining basal forebrain cholinergic neurons, given the original suggestion that certain human neurologic disorders may be caused by reductions in NGF in certain brain regions in Alzheimer disease (10). It was suggested that NGF may be useful in slowing the progression of Alzheimer-related cholinergic basal forebrain atrophy, perhaps by easing the cognitive deficit associated with the disorder (10,11).

At present, the Alzheimer's Association uses a checklist of 10 warning signs for the disease: "1 - memory loss,

* Correspondence: m_messripour@yahoo.com

2 - difficulty performing familiar tasks, 3 - problems with language, 4 - disorientation to time and place, 5 - poor or decreased judgment, 6 - problems with abstract thinking, 7 - misplacing things, 8 - changes in mood or behavior, 9 - changes in personality, 10 - loss of initiative" (http://www.alz.org/alzheimers_disease_10_signs_of_alzheimers.asp). In addition, it is suggested that the use of the Patient Behavior Triggers for Clinical Staff and family questionnaires would identify many individuals with possible dementia. These measures, however, lack pathological specificity, as some of the criteria might be applicable to other dementing illnesses. In order to deal with this difficulty, several studies have focused on the detection of neuronal components in biological fluids as biomarkers of the brain. These studies are based on the concept that the release of these components into the surrounding extracellular gap might be detectable in bodily fluids. The specific biological functions of NGF actions are modulated via the specific NGF receptors (NGFRs) of the target cells. It has been shown that the common NGFR (p 75 neurotrophin receptor, p75NTR) is highly expressed in the CNS during embryonic development and downregulated to a detectable trace level during the postmaturation phase, but pathological factors stimulate NGFR synthesis and release the extracellular parts of the receptors as the truncated soluble form (TNGFR) to biological fluids (12–15). The objective of this pilot study was to use an indirect quenching fluoroimmunoassay to determine urine TNGFR levels in patients with dementia and to verify whether TNGFR has the potential of being a marker of Alzheimer disease progression.

2. Materials and methods

2.1. Chemicals

Nerve growth factor (purified 75 NGF), fluorescein isothiocyanate isomer 1 (FITC), Freund's adjuvant (complete and incomplete), Sephadex G-25, and agarose were purchased from Sigma (Poole, UK). Unless stated otherwise, all reagents were of the highest grade and made up in double glass-distilled water.

2.2. Patients and samples

Twelve patients with dementia were asked to participate in this study voluntarily. The clinical presentation and diagnostic investigations were confirmed by a clinical specialist. The patients were divided into 3 age groups according their ages: group 1 (52–63 years), group 2 (63–77 years), and group 3 (77–87 years). Twelve healthy volunteers with no apparent signs of dementia were included as a control population. Adjustments for age, sex, and weight were made in the selection of the control group. The first morning urine samples were collected and urine TNGFR was assayed the same day.

2.3. Fluorescein-labeled NGF

Fluorescein-labeled NGF was prepared as described by Messripour and Moein (16). Equal volumes of FITC solution (1 mg/mL) and purified NGF solution (about 0.25 mg protein/mL) were mixed and stirred overnight at 4 °C. The labeled NGF was separated from unconjugated FITC using Sephadex G-25 columns (1.2 × 20 cm).

2.4. Antifluorescein antibody

Four male albino rabbits (1.5–1.7 kg) were immunized with fluorescein-labeled NGF as recommended by London and Moffat (17). For determination of antifluorescein antibody, a double-dilution serum was made in Tris-HCl buffer (pH 7.4); 100 µL of fluorescein-labeled NGF solution (2.5 µg protein) was added to 100 µL of each dilution and mixed. After 15 min of incubation at room temperature, the volume was increased up to 2 mL by addition of the buffer. The fluorescence intensities of the mixture were measured using a PerkinElmer (Norwalk, CT, USA) LSE spectrophotofluorometer with excitation wavelength of 495 nm and emission wavelength of 540 nm fluorescence, and antifluorescein titer was measured by the ability to quench fluorescein-labeled NGF.

2.5. Determination of TNGFR

The concentrations of TNGFR were measured by indirect quenching fluoroimmunoassay essentially as described by Messripour and Moein (16). Briefly, fluorescein-labeled NGF (100 µL) was added to triplicate tubes containing 100 µL of urine samples. After 5 min, 100 µL of a 1:100 diluted antifluorescein serum (rabbit) in saline was added, and after 15 min of incubation at room temperature, the volume was increased up to 2 mL by addition of Tris buffer and fluorescence intensities of the mixture were measured spectrofluorometrically as described above. In all experiments, a correction was made for the background signal contributed by reactions other than that of the fluorescein-labeled NGF. The amount of TNGFR is expressed as ng/mL of fluorescein-labeled NGF that remained fluorescent. All samples were run in duplicate, and the average value is reported.

2.6. Gel diffusion method

Agarose gel diffusion of NGF against urine samples was carried out as described by Ouchterlong (18). Agarose (1%) in buffer containing NGF was layered on a plastic plate. The urine samples taken from diagnosed patients and apparently normal subjects were pipetted into the gel wells. The plates were placed in the cold room for 72 h and a radial band around the wells was considered as positive.

2.7. Statistical analysis

The obtained data were subjected to statistical analysis using SPSS 18. In all cases, one-way analysis of variance (ANOVA) was used to compare the mean of each group with that of the control group. The least significant

difference complementary test was conducted to determine exact differences at P-values of lower than 0.05. Data are presented as mean \pm SD for all cases.

3. Results

The quality of the antibody and its application for the assay was evaluated and is summarized in Table 1. Addition of the antifuorescein serum (1:100 dilution) with or without healthy urine samples caused a marked decrease in fluorescence of the fluorescein-labeled NGF from 89 to 26 units, whereas the additions of 100 μ L of rabbit control serum (1:100 dilution) or healthy urine sample (100 μ L) in the buffer (final volume: 2 mL) did not change the fluorescence intensity significantly. Conversely, the addition of a patient's urine (100 μ L) to the mixture of the fluorescein-labeled NGF and antifuorescein serum resulted in the enhancement of the fluorescence intensity to about the unit value of the fluorescein-labeled NGF in the buffer. These results indicate that the binding of fluorescein residues by antifuorescein serum caused efficient quenching of fluorescence, whereas the enhancement of the fluorescence intensity in the presence of TNGFR is best explained in terms of inhibition of quenching of the labeled NGF (Figure 1). It appears that when the NGF moiety becomes interlocked with the combining site of the TNGFR, because of strict hindrance selectivity, antifuorescein antibody cannot quench the fluorescence intensity of the labeled NGF (Figure 1). This is in good agreement with reports of other investigators (16).

This method was used to determine TNGFR in the urine of patients with dementia (Table 2). The mean level of TNGFR in the urine samples of healthy control

subjects and the patients with dementia was 164 ± 23 and 341 ± 66 ng/mg creatinine, respectively. The value for the patients was about 2-fold greater than that recorded for the healthy control subjects. The differences were statistically significant ($P < 0.05$). Table 2 shows comparative studies of urinary TNGFR as assayed by both fluoroimmunoassay and agarose gel diffusion in different subgroups of both patients and healthy subjects (Figure 2). As can be seen in Table 2, there is a relationship between the levels of NGFR in different ages of both control and patient subgroups.

4. Discussion

The results obtained in this work showed that the abnormal levels of TNGFR are higher in the urine of patients with dementia as compared to that of healthy subjects. This supports the results of Lindner et al. (19), who reported that urine TNGFR levels were elevated in mildly demented patients relative to nondemented controls. NGF and its low-affinity receptor are abundantly present within the dementing brain, although this does not rule out an NGF-related mechanism in the degeneration of basal forebrain neurons, nor does it eliminate the possibility that exogenous NGF may be successfully used to treat Alzheimer disease (10,11). TNGFR has been found in newborn infant urine, but declines to low detectable levels in adult urine (12). Importantly, TNGFR was found in urine of rats following sciatic nerve injury, but it is hardly detectable in normal adult rat urine (12). These studies suggest that the extracellular fragment of the receptor is detached after injury and excreted in the urine. The higher levels of TNGFR observed in the patients may be interpreted as being consistent with the upregulation of NGF receptors in the brains of demented patients to represent an adaptive

Table 1. Quenching effect of TNGFR on the mixture of fluorescein-labeled NGF and antifuorescein antibody.

Reagents	Fluorescence intensities
Buffer	00 \pm 0
Buffer + fNGF	89 \pm 3
Buffer + fNGF + rabbit control serum	83 \pm 4
Buffer + fNGF + rabbit antifuorescein	26 \pm 2
Buffer + fNGF + patient urine sample	91 \pm 5
Buffer + fNGF + healthy urine sample	89 \pm 5
Buffer + fNGF + antifuorescein + control urine	29 \pm 2
Buffer + fNGF + antifuorescein + patient urine	87 \pm 3

Fluorescein-labeled NGF (fNGF) was incubated with the indicated reagent (100 μ L) in a total volume of 2 mL and fluorescence intensities (arbitrary units) were measured as described in Section 2. The results are means and standard deviations of 20 separate determinations.

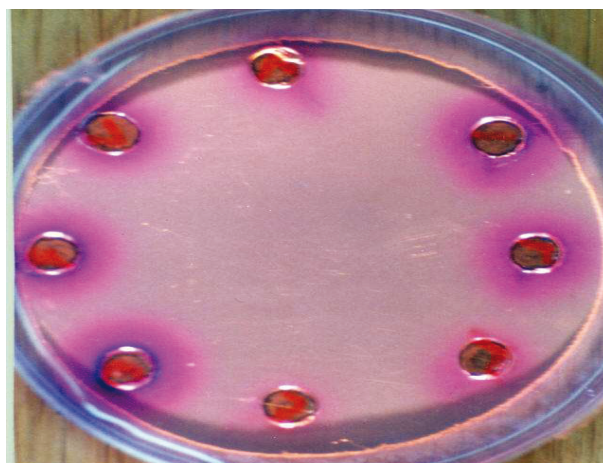
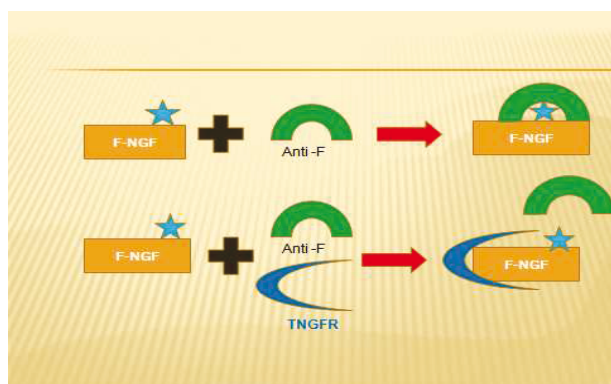


Figure 1. The competitive selectivity of anti-fluorescein (anti-F) and TNGFR for binding to fluorescein-labeled NGF (F-NGF). The amount of TNGFR is expressed as ng/mL of F-NGF that remained fluorescent.

Figure 2. Agarose gel diffusion of urine samples against fluorescein-labeled NGF. Agarose in buffer containing NGF was layered on a plastic plate and urine samples taken from patients and control subjects were pipetted into the gel wells. The radial band around the wells was considered as positive.

Table 2. Comparison of TNGFR levels in urine of patients with dementia and healthy individuals.

Samples	Age (years)	ng TNGFR/mg creatinine	Gel diffusion
Patients			
Group 1	52–63	276 ± 47 [4]	UN [3], + [1]
Group 2	64–77	353 ± 71 [4]	+ [2], ++ [2]
Group 3	78–87	388 ± 64 [4]	+++ [4]
Controls			
Group 1	52–63	106 ± 18 [4]	UD [4]
Group 2	64–77	173 ± 12 [4]	UD [3], WP [1]
Group 3	78–87	202 ± 37 [4]	UD [3], + [1]

NGFR was assayed either by fluoroimmunoassay or agarose gel diffusion in urine samples from patients with dementia and healthy individuals. Ages were matched and are shown as different subgroups. Numbers in each subgroup are given in brackets. SD = Standard deviation, UD = undetectable.

response to the reduction of NGF (14). However, further studies of the degree and distribution of NGF within the human brain in normal aging and in Alzheimer disease, and of the possible relationship between target NGF levels and the status of basal forebrain neurons *in vivo*, are necessary before engaging in clinical trials.

Biomarkers that reflect the progression of dementia will improve the survey of clinical assessments and permit for more rapid screening of the population with smaller numbers of patients for identifying demented patients earlier and improving the effectiveness of treatment. Clinical utility of objective biomarkers may require a combination of physiological and biochemical

methodologies. The immunological reaction and the high degree of fluorescence sensitivity indicate that indirect quenching fluoroimmunoassay is accurate and sensitive enough for the screening of a large number of urine samples for the measurement of TNGFR for identification of people who are at risk of Alzheimer disease.

It is concluded that the present method for urine samples may provide a suitable measure of dementia-related neuropathological changes, but further study is needed to determine the source and potential clinical utility of increased TNGFR levels in the urine of demented patients.

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