SHORT REPORT

Neurotrophin-3 is increased in skin in human diabetic neuropathy

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Abstract

Neurotrophin-3 (NT-3), a member of the neurotrophin family, has been shown to be necessary for the development of muscle spindle and Merkel cell afferent nerve fibres in animal models. The presence of NT-3 in the suprabasal epidermis, where many unmyelinated sensory fibres terminate, has been shown for the first time. As these fibres are affected in early diabetic neuropathy and a clinical trial of recombinant human NT-3 in diabetic neuropathy is in progress, the concentrations of endogenous NT-3 in skin of 24 patients at different stages of diabetic polyneuropathy have been investigated. NT-3 concentrations, measured with a specific immunoassay, were significantly higher in affected skin biopsies from patients with diabetic neuropathy than matched control skin (diabetic skin 6.32 (1.18) pg/mg *v* control skin 1.28 (0.05) (mean (SEM)); $p < 0.004$, Mann-Whitney *U* test), particularly in the later stages. The optical density of NT-3-immunostaining was also significantly greater in the epidermis in diabetic patients (diabetic epidermis 0.30 (0.06) *v* controls 0.24 (0.01); $p < 0.02$). No correlation was found between individual quantitative sensory tests and the increase of NT-3 concentration. The increase of NT-3 seems to reflect the degree of skin denervation in diabetic neuropathy, and may represent a compensatory mechanism. The concentrations of NT-3 in other peripheral targets deserve study in diabetic neuropathy.

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Diabetic neuropathy is a common complication of diabetes mellitus, for which there is no specific treatment. Nerve growth factor, brain derived neurotrophic factor, and NT-3 belong to the family of neurotrophins, are target derived neurotrophic factors, and exert their effects via specific receptors.¹ These neurotrophic factors and their receptors have been implicated in the pathogenesis of diabetic polyneuropathy.² We have previously shown that nerve growth factor immunoreactivity is

decreased in human diabetic skin, and this correlated with small sensory fibre dysfunction.² Whereas nerve growth factor is trophic to small sensory and autonomic fibres, NT-3 plays a part in development and maintenance of sympathetic and, in particular, large sensory fibres that innervate muscle spindles and Golgi tendon organs, as well as touch dome and hair follicles.³⁻⁸ In addition, we have shown the presence of NT-3 in the suprabasal epidermis of human and rodent skin, where many unmyelinated sensory fibres terminate. The application of NT-3 can attenuate experimental large fibre neuropathy in rats,⁹ and clinical trials are in progress with recombinant human neurotrophins, including NT-3, in diabetic neuropathy.^{10, 11} However, there are no data on tissue concentrations of NT-3 in human diabetic neuropathy. We have therefore measured the concentrations of NT-3 in the skin of patients with diabetes mellitus at different stages of diabetic neuropathy and compared these with matched control skin.

Patients and methods

PATIENTS

Twenty four patients with diabetes mellitus were assessed by history, examination, nerve conduction studies, and quantitative sensory and autonomic tests, and staged according to the Dyck criteria for neuropathy.¹² The mean age of subjects was 47 (SD 13) years, and duration of diabetes 16 (SD 13) years, with glycosylated haemoglobin 7.1 (SD 1.8) %.

Ethics committee approval was obtained for the study. A full thickness skin biopsy was obtained under local anaesthetic from the lateral calf of each patient. The tissue was processed in nine patients for immunocytochemical studies, and in 15 patients for NT-3 assay. Eleven control calf skin samples were obtained with consent from patients undergoing sural nerve harvesting for grafting after nerve trauma in the upper limb, and who had no neurological abnormality in the lower limbs. Four of these were processed for NT-3 assay and the remainder for immunocytochemistry. A further eight samples of normal breast skin were obtained routinely during the course of breast plastic surgery, for NT-3 assay.

QUANTITATIVE SENSORY AND AUTONOMIC TESTS

A battery of quantitative sensory and autonomic tests were performed, including thermal thresh-

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olds (instep of the right foot), vibration perception, and light touch thresholds (distal interphalangeal joint of the right big toe), sudomotor axon reflex (by injecting 0.2 ml of a 4 µg/ml nicotine solution into the skin of the right lateral calf and recording the sweat rate with an evaporimeter), and axon reflex vasodilatation (by injecting 0.01 ml of a 5 mg/ml capsaicin solution in the right lateral calf and recording the rise in blood flow using a laser Doppler). All tests have been described in detail previously.²

NERVE CONDUCTION STUDIES

Standard sural nerve sensory and common peroneal nerve motor conduction studies were carried out.

TISSUE PREPARATION

Tissue specimens for immunocytochemistry were embedded in OCT compound (R Lamb, UK) and snap frozen in melting isopentane. All specimens were stored at -70°C.

NT-3 ASSAY

NT-3-like immunoreactivity was measured with a fluorometric enzyme linked sandwich radioimmunoassay (ELISA) using recombinant human NT-3. Fresh skin biopsies were weighed and homogenised in 0.5 ml ice cold extraction buffer (50 mM Tris HCl, 600 mM NaCl, 1% bovine serum albumin (BSA), 1 mM benzamidine, 0.1 mM benzethonium chloride, 0.003 TIU aprotonin/ml, 0.2 % Triton X-100, 0.1 mM phenylmethylsulphonyl fluoride, pH 7.4) and homogenates were centrifuged at 13 000×g for 5 minutes to remove insoluble material from the extract. In addition, two control skin samples were separated into layers by immersion in E4 culture medium containing 2 mg/ml dispase-2 (Boehringer Mannheim GmbH) and incubation at 37°C for about two hours, until successful separation of the epidermis from the dermis was achieved. Extracts were stored frozen until assayed, when they were thawed and diluted 1:4 with sterile water to bring the salt and detergent concentrations in the extract into a range which was acceptable for the ELISA. Concentrations of NT-3 were determined using a two site ELISA with independent capture and reporter monoclonal antibodies. These antibodies are specific for NT-3, and do not cross react with other neurotrophin family members at up to 100 times the maximal concentration used for the standard curve. The standard curve used NT-3 spiked into homogenisation buffer, then diluted in water as the samples were. All further dilution of standards and samples necessary to bring values into the linear range of the ELISA were carried out in the assay buffer. The dynamic range of the ELISA was 40 pg/ml to 20 ng/ml.

IMMUNOCYTOCHEMISTRY

Cryostat sections of 10 µm were thaw mounted on to double coated poly-L-lysine glass slides and fixed for 30 minutes in 4% w/v paraformaldehyde in 0.1 M phosphate buffered 0.9 % w/v saline (PBS, pH 7.2). The slides were rinsed for 5 minutes in PBS twice, dehydrated through a

Figure 1 Concentrations of NT-3 in lateral calf skin (if not indicated otherwise) in diabetic patients in different stages of diabetic neuropathy. Horizontal bars show mean values.

methanol series, and immersed in pure methanol with 0.05% hydrogen peroxide (H₂O₂) for 30 minutes, to inhibit endogenous peroxidase. After rehydration in PBS, the sections were incubated with goat serum (1:30) in PBS for 30 minutes to block non-specific background activity before incubation with primary rabbit antibodies to rhNT-3 (ref 8C/845 No 883, Amgen, USA 1:80 000 dilution), which were left to incubate overnight at room temperature. Sections were rinsed in PBS and immunoreactive binding sites were detected using a nickel enhanced avidin-biotin peroxidase (ABC) method (Vector Labs, Ltd), as described by Shu *et al.*¹³ Slides were dehydrated and cover slips applied with DPX (H & E Ltd, UK). Negative controls included successful preabsorption of immunostaining with NT-3 antigen at concentrations of 0.002–0.2 nmol/ml. Preabsorption studies of anti-NT-3 with rhNT-3 prevented immunostaining (see fig 2B) and replacement of primary antibody with normal rabbit serum gave no staining. Diabetic and control optical density values of NT-3 immunostaining were obtained by measuring fields (See-scan, UK) along the length of the suprabasal epidermal layer of the skin.

Results

NT-3 ASSAY

The concentrations of NT-3 in skin extracts are shown in fig 1. Concentrations of NT-3 were clearly increased in diabetic patients (6.3 (SEM 1.2) pg/mg, n=15; p<0.004, Mann-Whitney U test *v* control calf skin (1.3 (SEM 0.05), n=4), particularly at later stages of neuropathy. Normal breast skin samples gave values that were similar to control calf skin (1.4 (SEM 0.3) pg/mg, n=5). The samples of separated layers showed higher NT-3 concentrations in the epidermis (3.65 and 2 pg/mg) than the dermis (below detection limit and 1 pg/mg respectively).

NT-3 IMMUNOHISTOCHEMISTRY

NT-3-like immunoreactivity was found in keratinocytes in the suprabasal layer of the epidermis in all samples (fig 2). Optical density measurements showed the NT-3 immunostaining to be significantly more dense in the skin of diabetic patients (0.30 (SEM 0.06) (n=7)) *v* controls (0.24 (SEM 0.01) (n=8); p<0.02,

Figure 2 NT-3-like immunoreactivity in suprabasal keratinocytes of calf skin in (A) a control subject, and (B) a patient with diabetic neuropathy. The immunoreactivity in the suprabasal epidermis was increased in diabetic patients compared with controls. Black arrow head=basal membrane of epidermis; white arrowhead =NT-3 staining in suprabasal layers of epidermis

Mann-Whitney *U* test). As with the assay results, there seemed to be denser staining with advanced diabetic neuropathy.

Although all quantitative tests and nerve conduction studies were significantly abnormal in diabetic patients, no correlation was found between individual quantitative tests and NT-3 concentrations.

Discussion

We have shown, for the first time, increased concentrations and suprabasal keratinocyte staining of NT-3-immunoreactivity in the skin of patients with diabetic neuropathy. This increase seems to correlate with severity of diabetic neuropathy, and not with individual quantitative sensory tests. By contrast, we have previously shown that nerve growth factor immunoreactivity is decreased in the basal keratinocytes in the skin of patients with early mild diabetic neuropathy,² and that this correlated with decreased axon reflex vasodilatation. Diabetic polyneuropathy particularly affects sensory neurons, often with initial loss of small sensory fibre function, followed by loss of large fibre function. Increased NT-3 in skin could reflect reduced uptake by nerve fibres, as Rodriguez-Pena *et al*¹⁴ have reported that mRNA for the NT-3 high affinity receptor trk C is reduced in the sciatic nerve in streptozotocin diabetic rats. Alternatively, fibre loss may be initiated by another mechanism and followed by a compensatory overexpression of NT-3, which then cannot be taken up by the missing fibres. Skin keratinocytes themselves may take up the NT-3 via an autocrine loop, and we have shown trk C receptor expression to be increased in keratinocytes in human diabetic skin.¹⁵

The concentrations of NT-3 in other peripheral targets deserve study in human diabetic neuropathy. NT-3 supports large sensory peripheral neurons in mice and particularly supports Ia muscle afferents,^{3,4,16} touch dome, and hair follicle innervation.⁵⁻⁸ Ihara *et al*¹⁷ and Fernyhough *et al*¹⁸ have found a decreased NT-3 mRNA in muscle tissue of streptozotocin diabetic rats. Similar data in human diabetic muscle and other large sensory fibre targets will be of importance to establish end points for clinical trials with rhNT-3.

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