Review Article
The role of nerve growth factor in the prophylaxis and treatment of diabetic foot ulcers

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Abstract: Diabetic foot ulcers are still particularly difficult to heal. Therefore, preventing and therapeutic adjuncts are increasingly being explored. Nerve growth factor (NGF) is a promising agent exhibiting beneficial actions on both diabetic peripheral neuropathy, one of the main causes of foot ulcers, and on ulcer healing. Indeed, preclinical research in animal models of diabetes has revealed the trophic effect of NGF on small C-fibres, while phase 2 human trials have provided evidence for a favourable effect on sensory neuropathy. However, the results of a phase 3 trial were moderate and, therefore, not enough to encourage widespread use of NGF in the treatment of diabetic neuropathy. Available literature on the role of NGF on diabetic wound healing is sparse but encouraging. Exogenous supplementation of NGF or the use of alternative techniques to increase its endogenous expression could emerge as a protective and therapeutic modality for diabetic foot ulcers in addition to standard treatment and other growth factors. The present review provides an outlook on the role of NGF in the prophylaxis and treatment of diabetic foot ulcers.

Keywords: Diabetic foot, diabetic neuropathy, growth factors, nerve growth factor (NGF)

Introduction

The diabetic foot is a common complication of diabetes mellitus. Its main feature is ulceration, which is defined as full-thickness penetration of the foot dermis [1-4]. It represents a major cause of morbidity worldwide, inasmuch as the annual incidence of ulceration and amputation is as high as 2.5-10.7% and 0.25-1.8%, respectively [1-4]. Diabetic foot ulcers have a multifactorial aetiology, in which neuropathy, ischaemia and infection are of paramount importance. First, peripheral neuropathy leads to stocking-distribution loss of protective sensation and foot deformity. Thus, plantar pressures are redistributed, and certain foot areas sustain chronic high pressure, which ultimately results in ulceration. This is aggravated by the autonomic component of peripheral neuropathy: sweat secretion is reduced, facilitating dry, hyperkeratotic skin and callus formation, all taken together increasing the susceptibility to cracking. The latter creates gates of entry for bacteria, leading to potential deep-tissue infection [5,6]. A further

manifestation of neuropathy is oedema, due to the perturbed vasomotor control, increasing the risk of trauma, especially through compression and friction by footwear [6]. Secondly, wound healing may also be significantly impaired in case of concomitant lower extremity ischaemia due to peripheral arterial disease. Additionally, diabetic patients are susceptible to infection, mainly due to impaired cellular and humoural immunity [3,6]. Last but not least, the altered local bioavailability of growth factors, mainly attributed to non-enzymatic glycation owing to hyperglycaemia, may be accountable for their impaired biological actions in the healing of chronic diabetic foot ulcers [6].

Diabetic neuropathy may affect small fibres, large fibres, or both. Small fibre impairment is first manifested in the lower limbs as pain and hyperalgesia, followed by loss of thermal sensitivity, and reduced light touch and pinprick sensation. Large fibre dysfunction can be demonstrated as reduced vibration perception, loss of position sense, weakness, muscle wasting and

diminished tendon reflexes [7]. Diabetic neuropathy has a complex pathophysiology. The hyperglycaemia-induced generation of advanced glycation end products (AGEs) contributes to structural dysfunctions and lesions of nerves via non-enzymatic glycation [8]. Furthermore, increased levels of circulating immune complexes, activated T-lymphocytes, as well as auto-antibodies against nerves and ganglia, underline the role of impaired immunity on diabetic neuropathy [8]. Other proposed underlying mechanisms are myo-inositol depletion due to accumulation of sorbitol and fructose; essential fatty acid depletion with reduced availability of gamma-linolenic-acid and prostanoids; and hypoxia-induced increased free radical production and oxidative stress leading to endothelial and axonal dysfunction and structural lesions [8]. Oxidative stress through mitogen-activated protein (MAP) kinases and poly (ADP-ribose) polymerase contributes to neuropathic changes, such as motor and sensory nerve conduction deficits, decreased nerve blood flow and energy failure [11]. Other important pathogenic factors for neuropathy include increased aldose reductase activity, activation of protein kinase C [9], as well as abnormalities of the polyol pathway and of sodium and calcium channels [10]. Reduction of neurotrophic factors, such as nerve growth factor (NGF), neurotrophin-3 (NT-3), insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF), seems to further aggravate diabetic neuropathy [8,10].

Foot ulcers may be classified in terms of severity, in order to facilitate a logical approach to treatment and aid in the prediction of outcome. The most commonly used classification systems are the Meggitt-Wagner system and the University of Texas system [1,5,11,12]. Optimal management of diabetic foot ulcers is best offered by multidisciplinary foot clinics [1-4]. Revascularisation to optimise blood flow, aggressive infection control and off-loading are the cornerstones of modern treatment. Off-loading is accomplished by total contact cast or aircast walker and/or pressure-relief half shoes, in conjunction with removal of pus and debridement of non-viable tissue [1-4,6]. Other emerging therapies are human cultured dermis, human skin equivalent, topical growth factors, wound dressings and systemic hyperbaric oxygen [1-4,13]. It must also be stressed that statins constitute a further valuable treatment, since there is ever-increasing evidence that they improve ulcer healing in diabetic patients owing to their pleiotropic, non-lipid-lowering actions [14-18].

Nerve growth factor (NGF)

NGF is a soluble protein produced by and acting upon miscellaneous cells located in the nervous, endocrine and immune systems. It is released following tissue injury or inflammation from a range of cells including mast cells, macrophages, lymphocytes, fibroblasts, and keratinocytes, and it may become an important driver of pain symptoms in patients experiencing chronic pain [19]. It was the first growth factor to be identified [20] and the prototype of neurotrophins, a family of survival and differentiation factors [NGF, NT-3, NT-4, NT-5, NT-6, brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and glial-derived neurotrophic factor (GDNF)] that exert profound effects in the central and peripheral nervous system [21]. NGF has been shown to exert anti-apoptotic, nerve growth-promoting and pro-differentiative activities on neurons [20]. It plays an important role both on the embryonic development of the nervous system and on the function of nociceptors during lifetime [19] through controlling synaptic function and plasticity and sustaining neuronal cell survival, morphology and differentiation [22]. NGF is also an important regulator of P substance (PS) and calcitonin gene-related peptide (CGRP) synthesis in neurons that are implicated in diverse and widespread activities, including vasodilatation, gut motility and nociception, all of which are perturbed in diabetic neuropathy [7]. Apart from being essential for the perception of pain, it may be responsible for capsaicin-evoked hyperalgesia [19] and increase in the peripheral exocytotic activity of capsaicin-sensitive cutaneous neurons contributing to enhanced neurogenic regulation of inflammatory and wound healing processes in injured tissue [23]. Interestingly, NGF exerts various properties on cells others than neurons participating in the initiation and maintenance of inflammation in various organs [24]. It plays a critical role on fibroblasts, epithelial cells and keratinocytes involved in the control of skin homeostasis, tissue remodeling as well as wound [25-27] and ligament [28] healing by promoting re-innervation, blood flow and angiogenesis. It exerts a variety of effects in the cardiovascular system and on endothelial cells, which contribute to maintenance, survival, and function of endothelial cells by autocrine and/or

paracrine mechanisms [29].

The actions of NGF are mediated via both transcriptional and non-transcriptional mechanisms. Responsiveness of neurons to NGF is regulated by the expression of two classes of cell surface receptor: tropomyosin-related kinase A receptor (referred to as trk or trkA or p140trk), a member of receptor tyrosine kinases family; and p75 neurotrophin receptor (referred to as p75 or p75NTR), a member of the tumour necrosis factor receptor superfamily [21,22]. After binding to the ligand, the neurotrophin-receptor complex enters the intracellular compartment and a proportion is transported retrogradely to the cell body of the sensory nerves within dorsal root ganglia, where it acts on regulators such as nuclear factor-kappa B (NF-κB) or cyclic AMP response element binding protein (CREB), thereby upregulating the expression of ion channels, such as transient receptor potential vanilloid 1 (TRPV1), voltage-gated sodium channel alpha subunit (Nav) 1.7, or Nav 1.8. It also enhances the expression of peptide transmitters, who become increasingly excitable through phosphorylation. Upregulation of BDNF might also occur in response to NGF, and it is interesting to speculate that this could contribute to central sensitisation via actions on TrkB receptors on post-synaptic neurons in the dorsal horn of the spinal cord [19]. Both receptors undergo ligand-induced dimerisation, which activates multiple signal transduction pathways [21]. The TrkA receptor controls three major signalling pathways [30]. Activation of Ras results in activation of the MAP kinase-signalling cascade, which promotes neuronal differentiation including neurite outgrowth. Activation of phosphatidyl inositol-3 (PI3) kinase through Ras or growth factor receptor bound protein 2-associated protein 1 (Gab1) promotes survival and growth of neurons and other cells. Activation of phospholipase C-g1 (PLC-g1) results in activation of Ca^{2+} and protein kinase C (PKC)-regulated pathways that promote synaptic plasticity. Each of these signalling pathways also regulates gene transcription [30]. The p75NTR receptor regulates three major signalling pathways as well [30]. NF-κB activation results in transcription of multiple genes, including several ones that promote neuronal survival. Activation of the Jun kinase pathway similarly controls activation of several genes, some of which promote neuronal apoptosis. Ligand engagement of p75NTR also regulates the activity of Rho, which controls growth cone motility [30]. Pro-apoptotic actions of p75NTR appear to require the presence of sortilin, which functions as a co-receptor for the neurotrophins [30]. In the presence of TrkA receptors, p75 can participate in the formation of high affinity binding sites and enhanced neurotrophin responsiveness, leading to a survival or differentiation signal. In the absence of TrkA receptors, p75 can generate, in specific cell populations, a death signal [31].

Based on the aforementioned properties of NGF, it has been used as a therapeutic agent for the treatment of various disorders (**Table 1**) [19-21,24,26,27,32-34]. Its application in neurodegenerative disorders [20], nerve injury [21] and neuropsychiatric diseases [32] either individually or in combination with other trophic factors, has yielded encouraging results. In addition, clinical evidence suggests that topical NGF application promotes healing without side effects on corneal and cutaneous tissue sustaining chemical, physical or surgical injury and autoimmune disorders [24,26,27,33]. On the other hand, due to its involvement in a number of painful syndromes, clinical trials are now evaluating the possible impact of NGF antagonists and monoclonal antibodies against NGF, either as monotherapy or in combination with other analgesics [19,34].

NGF for the diabetic foot: Clinical practice

a) Application of NGF in the prophylaxis of diabetic foot ulcers

As already mentioned, a cardinal causative mechanism of foot ulceration is neuropathy, and NGF application could help towards prophylaxis from neuronal impairment.

Preclinical studies

The first available evidence on the potential correlation between the deficiency of neuropeptide gene expression in diabetic sensory neurons and the reduction of NGF messenger RNA levels in target tissues was derived from experiments on rats with streptozotocin-induced diabetes [35]. PS and CGRP levels in diabetic sciatic nerves were also significantly lower compared with aged-matched controls and insulin-treated diabetic rats. Insulin treatment was able to reverse this decrease in foot skin but not in soleus muscle [35]. Further research revealed

Table 1. Diseases in which enhancement or inhibition of NGF expression could be therapeutic.

Enhancement of NGF expression

        Infections , both viral and bacterial (AIDS and meningitis)

        Neurodegenerative processes (multiple sclerosis and Alzheimer's disease)

        Nerve injury

        Neuropsychiatric diseases (dementia, depression, schizophrenia, autism, Rett syndrome, anorexia nervosa, bulimia nervosa)

        Corneal and cutaneous ulcers caused by chemical, physical, surgical injury and autoimmune disorders

        Diabetic peripheral neuropathy

Inhibition of NGF expression

        UV irradiation (sunburn)

        Osteoarthritis of the knee or hip

        Chronic low back pain

        Interstitial cystitis/painful bladder syndrome

        Bone pain in cancer

        Endometriosis

        Chronic prostatitis

        Neuropathic pain

NGF, nerve growth factor; AIDS, acquired immune deficiency syndrome; UV, ultraviolet

that mice with hypoinsulinaemic diabetes (Ins.Dd1), created by increased expression of murine major histocompatibility complex (MHC) class I antigen D^d in pancreatic β-cells regulated by the human insulin gene, developed, initially, sensory and, in progress, motor neuropathy. In a case-controlled experiment on transgenic diabetic mice, NGF treatment (1 mg/kg, 3 times per week subcutaneously) was shown to protect or restore abnormal small sensory C-fibre function, with no effect on blood glucose, but not A-fibre function [36].

To test oral treatment of a highly potent inducer of NGF gene expression in animal models of diabetes, CB1093 [1(S), 3(R)-Dihydroxy-20(R)-(1-ethoxy-5-ethyl-5-hydroxy-2-heptyn-1-yl)-9,10-seco-pregna-5(Z),7(E),10(19)-triene], a vitamin D(3) derivative that induces expression of endogenous NGF, was given orally to non-diabetic and streptozotocin-diabetic rats [37]. NGF, PS and CGRP depletion in sciatic nerves of diabetic rats was prevented, and soleus NGF concentrations, as well as its mRNA expression in skin, were increased by CB1093 administration. CB1093 did not affect expression of NGF receptors (trkA and p75NTR) in dorsal root ganglia in control or diabetic rats, even though p75NTR expression was reduced by diabetes. Mechanical hyperalgesia seen in diabetic rats treated with vehicle was not exacerbated by CB1093 [37].

An alternative way of raising endogenous NGF levels has been tested in mice with streptozotocin-induced diabetes through gene transfer with replication-defective genomic herpes simplex virus vectors that had been modified to express NGF [38]. This NGF-producing herpes-based vector was administered by footpad inoculation or injection into inguinal adipose tissue at two weeks after streptozotocin administration. The vector was shown to confer protection against decrease in the foot sensory nerve amplitude measured 4 weeks later [38].

The use of small molecules that enhances endogenous NGF levels was also examined in streptozotocin-diabetic rats [39]. Eight weeks of treatment with AIT-082 (4-[[3-(1,6-dihydro-6-oxo-9-purin-9-yl)-1-oxo-propyl]amino] benzoic acid) at 30mg/kg intraperitoneally prevented depletion of NGF protein in plantar foot skin and sciatic nerve of diabetic rats, while it increased NGF protein in associated skeletal muscles. These effects were accompanied by maintenance of normal nerve levels of the neuropeptides PS and CGRP. Thermal hypoalgesia and conduction slowing of large sensory fibres in diabetic rats were ameliorated. However, there was no effect on conduction slowing in large motor fibres or on reduced myelinated fibre axonal calibre [39].

Taken together, preclinical data imply that ad-

ministration of either exogenous NGF or alternative techniques that increase its endogenous expression might emerge as a therapeutic approach for the treatment of diabetic neuropathy, as shown in animal models.

Clinical studies

The role of endogenous NGF in human diabetic neuropathy was first discussed at the end of the previous century. An early length-dependent dysfunction of sensory small-diameter fibres was described. This occurred prior to sympathetic fibre dysfunction, along with depletion of skin NGF and PS. There was a significant correlation between NGF depletion in keratinocytes of diabetic skin and decreased skin axon-reflex vasodilatation, mediated by small sensory fibres partly via PS release [40]. Granted that loss of nociception and axon-reflex vasodilatation contribute to diabetic foot ulceration, early and prolonged NGF treatment at an appropriate dose was proposed to provide a rational prophylaxis for this condition [40].

To evaluate exogenous NGF administration in clinical practice, it was attempted to document any alteration in pain threshold by means of scoring systems [41,42]. Two randomised, placebo-controlled, phase 2 trials of recombinant human NGF administered to patients with neuropathy have hitherto been conducted. The first showed presented improvements in signs and symptoms of 250 patients with diabetic neuropathy after treatment with recombinant human NGF (0.1 or 0.3 µg/kg subcutaneously, 3 times per week for 6 months) [43]. The second trial demonstrated significant improvements in neuropathic pain of 270 patients with human immunodeficiency virus (HIV)-associated sensory neuropathy following treatment with recombinant human NGF (0.1 or 0.3 µg/kg, twice a week for 18 weeks) [44]. Adverse effects in these studies were self-limited injection site hyperalgesia and other pain-related syndromes [43,44].

To ascertain the safety and efficacy of recombinant human NGF in patients with diabetic neuropathy, a large multi-centre randomised, double blind, placebo-controlled, phase 3 trial with a 12-month follow-up period was then initiated [45]. A total of 1019 patients aged 18 to 74 years with either type 1 or type 2 diabetes and sensory neuropathy attributable to diabetes were evaluated after random subcutaneous

injection of either recombinant human NGF 0.1 µg/kg (n = 504) or placebo (n = 515) (3 times per week for 48 weeks) [45]. Among patients receiving recombinant human NGF, 83% completed the regimen compared with 90% who received placebo [45]. Similar to the phase 2 trials [43,44] administration of recombinant human NGF was safe, with few treatment-related adverse events apart from injection site pain/hyperalgesia and other pain syndromes [45]. Deplorably, there were no significant changes between the two groups in neuropathy assessed at baseline and week 48, as demonstrated by the Neuropathy Impairment Score for the Lower Limbs (NIS-LL) or by other clinical parameters. In contrast, the global assessment score demonstrated a significant but modest benefit from treatment with recombinant human NGF, based on the patients' subjective opinion. In addition, only pain severity in the leg and the 6-month symptoms in the feet and legs showed a modest but significant benefit of recombinant human NGF [45]. Furthermore, no beneficial effect of recombinant human NGF versus placebo was observed for the incidence of foot ulcers using Cochran-Mantel-Haenszel statistics. Overall, this phase 3 clinical trial failed to demonstrate a significant beneficial effect of recombinant human NGF on diabetic neuropathy or prevention of new ulceration [45].

b) Application of NGF in the treatment of diabetic foot ulcers

Based on the fact that NGF plays a role on skin homeostasis, tissue remodelling and wound healing, it has been examined both in animal models and diabetic patients.

Preclinical studies

Topical NGF application in diabetic animal models has been reported to augment wound closure. Topical 2.5S NGF, a biologically active subunit of the NGF polymer, was shown to promote epithelialisation and nerve regeneration in wounds of C57BL/6J-m+ Leprdb mice (db/db) in a case-controlled experiment [saline or 2.5S NGF (1 µg/day or 10 µg/day) on post-injury days 0-6] [46]. As a result, healing times in db/db mice decreased from 30 days in normal saline-treated mice to 26 days in mice treated with 1 µg/day NGF (p<0.05) and 24 days in mice treated with 10 µg/day NGF (p<0.02). Histological evaluation of inflammation in healed

wounds, however, showed no statistical difference between treatment groups [46]. The implication of NGF in diabetic wound healing is also supported by the augmentation of its levels during accelerated healing seen with the vacuum-assisted closure device applied to full-thickness diabetic mouse wounds, in parallel with the increase in dermal and epidermal nerve fibre densities and the increase in PS and CGRP [47].

Clinical studies

Patients with diabetic neuropathy exhibited reduced numbers of cutaneous nerves, which may contribute to an increased incidence of non-healing wounds [48]. Reduction of foot skin innervation showed a significant correlation with low inflammatory cell accumulation, ultimately leading to the chronicity of diabetic foot ulcers in both neuropathic (n = 8) and non-neuropathic (n = 12) patients. PS and NGF were also decreased in both groups, whereas CGRP and NT-3 were reduced mainly in neuropathic group [49]. Favourable outcomes were confirmed in three diabetic patients with peripheral neuropathy, peripheral arterial disease and chronic leg or foot ulcers unresponsive to conventional therapies. Topical NGF application (2,5µg daily for 4 weeks and twice a week for the following month) was reported to promote healing after 5-14 weeks of treatment [50]. Based on these, admittedly, very sparse observations, it may be suggested that topical NGF application should be further explored as a potential useful add-on treatment modality in recalcitrant diabetic foot ulcers.

4. Discussion and conclusions

Given the magnitude of the health concern associated with the diabetic foot worldwide, alternative and/or add-on therapies are essential [1-4]. Clinical priorities include improved treatment options for chronic, non-healing foot ulcers, but also amelioration of peripheral neuropathy, leading to lower incidence of ulceration. There are very few drugs available to directly treat diabetic neuropathy. Those that are clinically indicated provide symptomatic relief, but do not repair or reverse the underlying nerve pathology. However, some agents are in clinical development that may support adult neurons and direct reparative processes after injury [51]. It is a sad fact that this is also the case for the treatment of diabetic foot ulcers: little progress towards 50% reduction of amputations has been achieved since 1990 [52]. In this context, growth factors represent important promising new agents. At present, only topical application of recombinant human platelet-derived growth factor (PDGF) has been approved by U.S. Food and Drug Administration and European authorities for the management of diabetic neuropathic ulcers with adequate arterial supply [6]. Its combination with other growth factors to enhance healing is an attractive notion justified by their interaction during the healing cascade [53]. NGF could be a valuable add-on treatment modality for the diabetic foot in view of its implication both in diabetic neuropathy and in ulcer healing. Because innervation has been shown to be essential for normal wound healing, the stimulatory effect of NGF on the wound repair process is likely to be at least partially due to its effect on nerves [24]. Obviously, this might be of particular importance in neuropathic patients, in whom wound healing is known to be impaired [24].

NGF is depleted in the foot skin of patients with early diabetic neuropathy, along with nociceptor fibre dysfunction [54]. Data from animal models of diabetes with administration of either exogenous NGF or alternative techniques that increase its endogenous expression have been promising. Such favourable actions were, initially, confirmed in phase 2 human studies [43,44]. However, a phase 3 clinical trial [45], failed to achieve the expected efficacy, possibly related to the difficulty in delivering adequate doses and the need for multiple trophic factors [54,55]. Preclinical animal studies used NGF at doses of 3-5 mg/kg, compared with the dose of 0.1 g/kg used in the human trial. Given the short serum half-life of NGF, these intermittent low-dose bolus injections gave a rather disappointing therapeutic effect [38]. Among the explanations offered for the discrepancy between the two sets of trials was the strong placebo effect, the different study populations and the changes to the formulation of recombinant human NGF in the phase 3 trial [54]. This is the reason why various means of augmentation of NGF endogenous expression, such as orally administered inducers of its expression or gene transfer with virus vectors, should be further considered as alternative methods.

In wound healing, NGF is pro-angiogenic, facilitates cutaneous wound repair [56] and promotes epithelialisation along with nerve regeneration in wounds [46], as shown in animal

studies. Although data in diabetic patients with foot ulcers are extremely sparse, favourable experience gained from other kind of ulcers, such as severe pressure ulcers [57], corneal ulcers [26], vasculitic ulcers [27] or even myocardial infarction [58] indicate that topical application of NGF may be an effective therapy.

NGF administration, either by exogenous supplementation or by alternative techniques aiming to increase its endogenous expression seems promising in diabetic patients. This growth factor could improve prophylaxis for and treatment of diabetic neuropathy [59,60] and represent a valuable treatment option for diabetic foot ulcers. At the same time, there is some indication for further advantageous effects in concomitant complications, such as diabetic retinopathy [61] nephropathy [62] and urodynamically abnormal bladder function [63]. Combination with other growth factors could enhance its therapeutic utility [6,64].

In conclusion, NGF may be described as a promising agent for the management of refractory foot ulcers. Obviously, there is a very long way to go before its implementation in the everyday foot clinic. Nonetheless, as long as the diabetic foot remains a global threat [65], continuous enquiry into its improved treatment will not only be justified but represent a constant medical and societal priority.

Conflicts of interest

Dr. N. Papanas has been an advisory board member of Miro; has participated in sponsored studies by Novo Nordisk and Novartis; received honoraria as a speaker for Novo Nordisk and Pfizer; and attended conferences sponsored by Miro, Novo Nordisk, Sanofi-Aventis and Pfizer. Professor E. Maltezos has participated in sponsored studies by Novo Nordisk and Novartis and attended conferences sponsored by Wyeth, Pfizer and Bayer.

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