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Neurotrophins and Peripheral Neuropathy

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Neurotrophins and peripheral neuropathy

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SUMMARY

Endogenous nerve growth factor (NGF) levels were studied in patients with nerve trauma, diabetes mellitus and leprosy, the most common causes of human peripheral neuropathy. In diabetics, there was an early length-dependent dysfunction of small-diameter sensory fibres, with depletion of skin NGF and the sensory neuropeptide substance P. The NGF depletion correlated significantly with decreased skin axon-reflex vasodilatation, which is mediated by small sensory fibres at least partly via substance P release. Immunostaining showed depletion of NGF in keratinocytes in diabetic skin. In injured nerves, NGF levels were reduced when compared to intact nerve, except acutely distal to injury; NGF-immunostaining was seen in Schwann cells in distal segments, including neuromas. NGF levels were decreased in leprosy-affected skin and nerve. The role of neurotrophins in the rational treatment of human neuropathies is discussed e.g. loss of nociception and axon-reflex vasodilatation contribute to skin ulceration, a major and serious complication, for which NGF may provide prophylaxis.

1. INTRODUCTION

Neurotrophins play specific roles in the development, maintenance and regeneration of subpopulations of peripheral nerve fibres: failure of trophic interactions between the target organ and its innervation may result in nerve dysfunction, degeneration, and abnormal regeneration. The key question addressed in our studies is whether such trophic mechanisms play a role in the causation or course of human peripheral neuropathies, and in the regulation of nociception. We have described the first human neuropathy attributed to deficiency of a neurotrophic factor, nerve growth factor (NGF) (Anand *et al.* 1991*b*; Anand 1992*a*), and studied endogenous NGF levels in patients with nerve trauma (Anand *et al.* 1994*a*), diabetes mellitus (Anand *et al.* 1994*c*) and leprosy (Anand *et al.* 1994*b*), the most common causes of human peripheral neuropathy world-wide. We have also reported regional changes of neurotrophic factor levels in patients with motoneurone disease (Anand *et al.* 1995*b*). The aim of our studies is to provide a rational basis for the clinical use of neurotrophic agents in peripheral neuropathy.

Sensory and autonomic polyneuropathy is a common form of neuropathy in diabetic patients, for which no specific and effective treatment is available. It was hypothesized that NGF deprivation may determine its presentation, although metabolic or vascular abnormalities may be the cause of the neuropathy (Anand 1992*a*). Nerve injury, particularly of the brachial plexus in obstetric and road traffic accident cases, may result in lifelong disability and chronic pain, despite advances in reconstructive surgery (Birch 1992); nerve fibre degeneration and poor regeneration account for the failure of the surgery. Leprosy affects between 10 and 15 million people: the earliest reported nerve lesions in human leprosy and animal models are in unmyelinated fibres and their Schwann cells (Shetty *et al.* 1988), in accord with early loss of pain sensation and trophic changes. Previous work provided indirect evidence that NGF may be implicated in leprous neuropathy (Anand *et al.* 1983*a*).

2. DIABETIC NEUROPATHY

Diabetic neuropathy comprises of a number of clinical presentations that are likely to be caused by different mechanisms, which may coincide in the same patient (see Thomas & Tomlinson 1993). The prevalence of neuropathy rises to about 50% after 25 years of diabetes (Pirart 1978). It has been classified into symmetric and focal neuropathies. Neurotrophic mechanisms are more likely to involve symmetric polyneuropathy rather than focal neuropathy.

Our studies in insulin-dependent young adult diabetics showed an early (often sub-clinical) length-dependent symmetrical dysfunction in small-diameter sensory but not sympathetic fibres, associated with skin substance P and NGF depletion (Anand *et al.* 1994*c*). NGF depletion correlated significantly with decreased capsaicin-evoked skin axon-reflex vasodilatation. Axon-reflex vasodilatation is a test of unmyelinated afferent fibres, the flare component of Lewis' triple response in skin. Intradermal capsaicin was used to induce the flare, as it selectively activates nociceptive fibres. The increased capillary flux, as a result of vasodilation, was measured using laser-Doppler fluxmetry (see Jolliffe *et al.* 1995). NGF immunostaining was strongest in the basal keratinocyte layer in control skin, and was decreased in diabetic skin. NGF deprivation, from target organ failure and decreased axonal transport, may thus reduce chemo- and warm/heat pain sensitivity in early diabetic neuropathy, even in asymptomatic diabetics. As loss of nociception and axon-reflex vasodilatation in the feet have been shown to contribute to foot ulceration

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(Parkhouse & Le Quesne 1988), a major and serious complication, early and prolonged NGF treatment at an appropriate dose may provide prophylaxis. It is of interest that a targeted mutation of the gene coding for the low-affinity NGF receptor in the mouse leads to markedly decreased substance P and CGRP innervation of footpad skin, and to development of ulcers and mutilation of the feet (Lee *et al.* 1992).

NGF-related mechanisms may at least partly explain the risk factors for diabetic neuropathy, which include duration of diabetes, age, male sex, and height. The length-dependent effect may result from abnormalities of axonal transport, including retrograde transport of NGF, as observed in diabetic rats (Jacobsen *et al.* 1981; Schmidt *et al.* 1985). Ageing appears to re-capitulate these clinico-pathological changes, and exacerbates them in diabetic subjects (our observations). NGF levels appear higher in female calf skin, as do effects of exogenous NGF administration (Petty *et al.* 1994): oestrogens/proestrus upregulate NGF receptor mRNA in sensory neurons (Sohrabji *et al.* 1994), and testosterone reduces NGF mRNA in cultured fibroblasts (Siminowski *et al.* 1987).

NGF may regulate small sensory fibre sensitivity and function directly, or via changes in expression of their neuro-effector agents substance P and CGRP. In our studies, substance P, but not CGRP, was significantly depleted in skin from diabetic patients with mild neuropathy. In accord with the functional studies, there may be sparing in mild diabetic neuropathy of larger sensory fibres that contain CGRP (but not substance P), which are not dependent on NGF. Alternatively, a subset of CGRP-containing sensory fibres innervating blood vessels, particularly related to sweat glands, may have better NGF availability than fibres that contain both substance P and CGRP and take up NGF from keratinocytes. There is support for these explanations from previous immunocytochemical studies in calf skin from similar patients where variable and even increased numbers of CGRP-immunostaining fibres were reported in the dermis and related to sweat glands (Lindberger *et al.* 1989; Levy *et al.* 1992; Properzi *et al.* 1993), and from diabetic rats, where an early increase of CGRP- and VIP-immunoreactive nerves was reported in the skin (Karanth *et al.* 1990). It may be that the production of NGF by blood vessels fails at a later stage than basal keratinocytes in diabetic skin, or that the "safety factor" for NGF is better for fibres innervating blood vessels, as discussed below. If a subset of sensory fibres retracts, the remainder may survive, or even sprout, as relatively more NGF becomes available to them. Endogenous NGF has been shown to regulate collateral sprouting sensory fibres in rat skin (Diamond *et al.* 1992).

A key question is why NGF is reduced in diabetic skin. Reduced NGF retrograde transport alone, while accounting for the length-dependent effect, should not affect skin NGF levels, unless this results from a secondary failure of nerve-skin interactions. This is unlikely, as acute denervation is associated with increased NGF expression in rat skin (Mearow *et al.* 1993); in diabetic rats, NGF mRNA is reduced in skin (Fernyhough *et al.* 1994), while NGF protein but not the NGF receptor is reduced in nerve (Hellweg *et al.* 1991, 1994). There is little evidence for antibodies to NGF in diabetic neuropathy (Zanone *et al.* 1994). A simple explanation for the decrease of NGF in diabetic skin is that it results from decreased keratinocyte turnover: stratum corneum cell turnover is delayed in human diabetic and ageing skin (Yajima *et al.* 1991), and failure of an autocrine effect observed in vitro may further contribute to decreased NGF production (Di Marco *et al.* 1993). Cell culture studies show that NGF expression is highest in rapidly dividing keratinocytes (Di Marco *et al.* 1993; Anand *et al.* 1995*a*), in accord with *in situ* hybridization studies in rat skin (Mearow *et al.* 1993), and our immunostaining studies in human skin (Anand *et al.* 1994*c*). In contrast, there is proliferation of vascular smooth muscle in diabetes, which may explain the early sparing of the vascular innervation, discussed above. It has been suggested that corticosterone may decrease NGF expression in diabetes, as it reduces NGF expression in cultured fibroblasts, and may be increased in streptozotocin-treated rats (Nevue *et al.* 1992). However, the relevance of this observation to human diabetics is unclear, and there does not appear to be a significant neuropathy in diseases where corticosteroid levels are markedly elevated.

Diabetic polyneuropathy affects nerve fibres that are both known to be dependent on NGF, and those that are not. We conclude that the presentation and subsequent development of human diabetic polyneuropathy may be related to deprivation of specific organ-derived neurotrophic factors. At the earliest stages, NGF-related sensory small fibre dysfunction generally precedes dysfunction of sympathetic, large sensory and motor fibres. At this stage, the primary vascular or metabolic causes could not, by themselves, account for the differential dysfunction of small-diameter sensory but not sympathetic fibres. Later, there is a significant decrease of CGRP levels measured by immunoassay and immunocytochemistry, and large sensory fibres are affected: other members of the neurotrophin family, via their respective specific receptors, are trophic to medium and large myelinated (proprioceptive, mechanosensitive) sensory fibres (DiStephano *et al.* 1992), and may be similarly affected. VIP normally co-exists with acetylcholine in a subgroup of post-ganglionic sympathetic fibres projecting to sweat glands. These were functionally and neurochemically unaffected in skin in our patients with mild diabetic neuropathy, but, as with CGRP, were affected in severe diabetic neuropathy. A signal, which is at present uncertain, causes increased expression of VIP in both injured and diabetic sensory and sympathetic fibres (Anand *et al.* 1988, 1990, 1991*a*): this may account for the increase of VIP-ergic innervation observed with immunocytochemistry in human and rat skin in early diabetic neuropathy.

Less than 10% of patients with diabetic neuropathy in most large series develop significant pain (see Pirart 1978; Thomas & Tomlinson 1993). Among these are unusual cases with distal hyperalgesia and allodynia, who have early or mild neuropathy, with preservation of large sensory fibre function and marked sprouting of

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unmyelinated axons. In some patients, pain may be precipitated, paradoxically, by treatment of the diabetes or improvement of its control, and the pain is usually self-limiting. It has been hypothesized (Anand 1995) that fewer (sprouting) fibres may be exposed to 'relative excess' of NGF in such cases of early painful neuropathy in humans and in rats (Courteix *et al.* 1993). Most cases, including those in our study, may have different NGF-related and unrelated nociceptive mechanisms in operation, and show no simple correlation of pain with morphological change in nerve biopsies. In such cases, chronic systemic rhNGF treatment (e.g. at doses that just produce local hyperalgesia) may 'paradoxically' both improve sensation progessively from short to long fibres, and ameliorate or prevent deafferentation pain. Sympathetic agents may increase NGF synthesis and secretion (Tuttle *et al.* 1993), with implications for NGF and its use in neuropathy with established sympathetically-driven pain.

3. TRAUMATIC NEUROPATHY

A number of different traumatic lesions may result in NGF deprivation of sensory neurones, including the removal of the target organ, cutting or crushing the nerve, and blockade of axonal transport. Crushing a peripheral nerve results in the induction of NGF synthesis by Schwann cells at the site of the lesion and the distal stump: the up-regulation of NGF synthesis after injury has been attributed to factors released by invading macrophages, including interleukin-1 (Heumann *et al.* 1987). The amount of NGF available to the injured fibres is insufficient to restore substance P levels; NGF receptor down-regulation in injured fibres may contribute to this insufficiency (Verge *et al.* 1992). However, when regeneration is complete, the target tissue is able to supply sufficient NGF to restore substance P levels and function in sensory fibres (McMahon *et al.* 1989).

We have studied NGF expression in patients with peripheral nerve injury (Anand *et al.* 1994*a*). In biopsies taken proximal and distal to the injured region from patients undergoing peripheral nerve repair, NGF levels were reduced when compared to intact nerve, but were generally higher acutely (less than 3 weeks) in distal when compared to proximal segments in the more complete nerve lesions. NGF staining was present in Schwann cells in distal segments, including pockets of high expression in neuromas, but not in proximal segments or control nerves. Our findings suggest that the proximal stump and cell bodies are deprived of NGF; animal models indicate that adequate NGF treatment may prevent or reverse the reduced expression of neuropeptides, NGF receptors and cytoskeletal proteins in injured nerves (Lindsay & Harmar 1989; Gold *et al.* 1991; Verge *et al.* 1992) and thereby ameliorate the degenerative changes which limit the success of surgical repair.

In patients with peripheral nerve injury, it has been hypothesized that fewer axonal sprouts with less competition for normal or even reduced NGF levels, but *relative* excess ('rkA afferent - NGF disproportion'), either in nerve trunks or the target organ, could lead to hyperalgesia (Anand 1995): all our adult plexus injury patients reported significant chronic pain, and a relative excess of NGF in the target organ may also explain the borderzone and re-innervation hyperalgesia seen in some of these patients.

There is evidence that some processes involved in determining the course of pain following nerve injury may not be related solely to NGF in the periphery. In comparing the consequences of nerve injury in human and rat neonates and adults, only the adults develop intractable neurogenic pain or autotomy; as there does not appear to be a qualitative difference in NGF changes in neonatal and adult injured nerve, the lack of chronic pain in neonates may result from spinal cord plasticity and adaptation, which may fail in adults (Anand 1992*b*).

4. LEPROSY

Schwann cells of unmyelinated fibres serve as the host for Mycobaterium leprae. The skin lesions in the early indeterminate and tuberculoid forms of leprosy, which are superficial and relatively well circumscribed, provide a unique opportunity to study the role of NGF in neuropathy. These lesions show hypoalgesia and hypopigmentation, in addition to hypohidrosis. Although anti-bacterial drugs are effective, failure of nerve regeneration, especially nociceptor sprouting within skin, leads to trophic changes, which remain a major cause of disability.

We first studied sensory neuropeptide levels in a mouse foot-pad model of leprosy; substance P levels were reduced in sciatic nerve and ipsilateral spinal cord (Anand *et al.* 1983*a*). Substance P-immunoreactive fibres were undetectable in skin biopsies from patients with leprosy: markers for the presence of nerve fibres (PGP 9.5, neurofilaments) were seen in all cases of indeterminate type, and a proportion of tuberculoid and lepromatous cases (Karanth *et al.* 1989). Our recent study shows that NGF levels are indeed decreased in leprosy-affected human skin and nerve (Anand *et al.* 1994*b*).

A study in leprosy patients showed no difference in the number of melanocytes and amount of pigment in hypopigmented lesions when compared to adjacent normal skin: it was suggested that the hypopigmentation could be caused by defective transfer of melanin into keratinocytes (Shereef 1992). Cell culture studies of melanocytes show that they express functional NGF receptors, that NGF is chemotactic to melanocytes, and that NGF increases the dendricity of melanocytes (Yaar *et al.* 1991): pigment is transfered from melanocytes to keratinocytes to determine skin colour. The evidence cited above has led to our hypothesis that nerve fibres and melanocytes are deprived of NGF in leprosy. NGF may provide a rational treatment to restore nociception in leprous neuropathy.

A number of the clinical and neurochemical changes in leprosy described above appear to be the opposite of changes in models of sunburn (Gillardon *et al.* 1994). Ultraviolet irradiation of skin produces erythema,

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hyperpigmentation and pain: it induces NGF mRNA in cultured keratinocytes, and long-term increase in CGRP levels in dorsal spinal cord. It may be speculated that sunburn is, in a sense, the 'opposite' of leprosy, with NGF expressed by basal keratinocytes driving the changes.

5. NGF AND NOCICEPTION

There is increasing evidence that NGF may regulate nociception in human neuropathies, with potential implications for clinical trials (Anand 1995). Exogenous NGF may reverse hypoalgesia and in excess may produce hyperalgesia by: (i) directly sensitizing nociceptors; (ii) increasing levels of substance P and CGRP, which may play a role in central sensitization and neurogenic inflammation; and (iii) local effects, such as release from mast cells (see Lewin & Mendell 1993; McMahon *et al.* 1995). Although largely speculative at this stage, human conditions may present with pain related to alterations of NGF activity: these may provide suitable models for the study of mechanisms of NGF and pain in man, and new NGF-related prophylaxis and therapies (see Anand 1995). The 'hyperalgesic' conditions include arthritis, some small fibre neuropathies (including erythromelalgia), painful hypertrophic scars, sunburn, urinary bladder pain, migraine and vascular head pain. The 'hypoalgesic' group, with decreased NGF activity, includes leprosy neuropathy. Other conditions, including the major neuropathies that follow trauma and diabetes, are more complicated, as discussed above, and may display different NGF-related features during their development.

A recent study provided the first demonstration that systemically administered NGF in pharmacological doses may produce hyperalgesia in healthy humans (Petty *et al.* 1994). The subjects were given either intravenous (i.v.) or subcutaneous (s.c.) recombinant human NGF (rhNGF). The systemic effects, which were dose-dependent, and more apparent in the i.v. group and in women, comprised of mild to moderate pain with swallowing, pain in the masseter muscles increased by chewing, sore throat, and pain with eye movements. The myalgias were less prominent in distal abdominal and limb muscles. Given that these effects began in the i.v. group about 60 to 90 minutes after administration, it is likely that the myalgic effects of rhNGF may be mediated via local inflammatory cells, such as mast cells, in these regions, and consequent sensitization of muscle afferents. The description of these effects of rhNGF is reminiscent of the clinical and neurophysiological effects of inflammation in muscle, where lowered sensory thresholds have been invoked to explain the tenderness of inflamed muscle and movement-related pain (Mense 1993). In the s.c. group, rhNGF produced an injection-site hyperalgesia lasting from 1 to 49 days. The local hyperalgesia following s.c rhNGF is likely to be mediated by sensitized unmyelinated afferents expressing trkA receptors, and, in addition, central effects. It is similar to mechanical and thermal (heat) hyperalgesia after topical application of capsaicin, soon after local redness and spontaneous pain have subsided (Anand *et al.* 1983*b*). NGF may particularly regulate chemical and heat nociception in humans. The author and colleagues have described a patient with autonomic failure, who was demonstrated to have complete loss of adrenergic sympathetic function and of sensory neuropeptides substance P and CGRP, with undetectable NGF in skin and sural nerve. Skin capsaicin and histamine-induced axon reflex vasodilatation and pain were markedly diminished, and heat pain threshold was elevated (Anand *et al.* 1991*b*).

NGF is increased in inflammatory conditions (Donnerer *et al.* 1992, Anand 1995); re-innervation of pockets of relatively high NGF expression, disproportionately few regenerating fibres exposed to normal levels of NGF (with a relatively high quantity of NGF taken up by each fibre), or increased susceptibility of nerve sprouts to NGF, could also lead to hyperalgesia. This hypothesis unifies the role of NGF in inflammatory and neurogenic pain. In such conditions, anti-NGF treatments may reduce hyperalgesia.

However, NGF may play a different role in the development of chronic pain that results from cell death or atrophy, and failure of adaptation in the spinal cord, for example in de-afferentation pain. Changes of NGF expression may, at the early stages of nerve injury or disease, form part of an adaptive response. Failure of this response, or of secondary adaptation in the dorsal spinal cord, may contribute to the development of chronic pain. NGF may thus, administered appropriately, provide prophylaxis and treatment in these conditions.

6. CONCLUSIONS

Our initial studies of endogenous NGF in the most common human peripheral neuropathies provide a model for future investigations of neurotrophic factors and their receptors in the full range of human peripheral neuropathy, from inherited to toxic neuropathy. Whether or not neurotrophic mechanisms are implicated in the causation or course of a neuropathy, neurotrophic agents may possess the potential for its prevention and treatment.

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