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Low-affinity nerve growth factor receptors ($p75^{LNGFR}$) in human prostate tissue: stromal localisation

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Abstract β-Nerve growth factor (β-NGF) acts on high- and low-affinity receptors to effect sympathetic innervation. It is produced in the human prostate. This study aimed to demonstrate the presence of β-NGF receptors and the distribution of $p75^{LNGFR}$ receptor protein in the human prostate. Radioligand binding assays were performed using microsomal preparations from benign prostatic hypertrophy (BPH) tissues but no specific binding of β-NGF was demonstrated ($n = 20$). Furthermore, BPH and prostate cancer tissues were stained immunohistochemically for $p75^{LNGFR}$. Immunohistochemistry localised $p75^{LNGFR}$ to tiny areas of prostate stroma postulated to be sympathetic nerves (BPH, $n = 15$; prostate adenocarcinoma, $n = 15$). Our results suggest that β-NGF in the human prostate acts on stromal elements which, most likely, represent prostatic nerves. β-NGF may be an epithelial-stromal mediator of sympathetic nerve growth in the human prostate.

Key words Benign prostatic hypertrophy · Prostate cancer · Nerve growth factor receptors

Introduction

The protein hormone β-nerve growth factor (β-NGF) stimulates the growth and maintenance of sympathetic and some sensory neurites [8]. In neonates the administration of anti-NGF sera leads to deficient sympathetic innervation, while in adults the density of sympathetic innervation correlates with the tissue NGF concentration [23]. NGF is also a chemoattractant for sympathetic neurones [15]. By producing NGF, therefore, peripheral tissues may influence the density and distribution of their sympathetic and sensory innervation.

β-NGF has been described in the prostates of rodents, where it may be present in high concentration, and is localised by immunohistochemistry to prostate epithelium [17]. In the human gland β-NGF protein and mRNA have been demonstrated [25, 27]. Graham et al. [14] localised β-NGF immunoreactivity to prostate stromal cells. More recent results from this laboratory localise β-NGF to human prostate epithelium, in keeping with its localisation in other mammals [27].

Analysis of cellular binding of β-NGF reveals two populations of binding sites: high-affinity sites ($K_d = 10^{-11}$ M) and low-affinity sites ($K_d = 10^{-9}$ M) [34]. Two receptor molecules underlying these two groups of binding sites have been described. Klein et al. demonstrated that $p140^{trkA}$ (for tropomyosin receptor kinase) acted as a β-NGF receptor with high-affinity binding ($K_d = 10^{-11}$ M).

The other β-NGF binding protein – $p75^{LNGFR}$ – was described earlier than $p140^{trkA}$, in 1986, by Chao et al. [7]. The protein is much more widely distributed than $p140^{trkA}$ both in the central nervous system and in the periphery, where, in addition to sensory and sympathetic neurons, Schwann cells and other neural supporting cells express the protein [6, 31, 32]. In addition it was initially unclear that $p75^{LNGFR}$ had any capacity for intracellular signalling – cells expressing $p140^{trkA}$ in the absence of $p75^{LNGFR}$ continued to respond to β-NGF stimulation [9, 20]. Such findings lead to a relative neglect of the possible role of $p75^{LNGFR}$ as a β-NGF receptor; some authors proposed that $p75^{LNGFR}$ might have a role in collaborative β-NGF binding with $p140^{trkA}$ [18]. More recently, however, actions of β-NGF that are purely $p75^{LNGFR}$-mediated have been demonstrated. These have become clear by analogy with other members of a $p75^{LNGFR}$ receptor family which cause apoptotic cell death; all such receptors bear an intracellular "cell-death

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domain'' [4]. β-NGF stimulation prevents $p75^{LNGFR}$-mediated apoptosis of $p75^{LNGFR}$ transfected cells [30]. That is, the expression of β-NGF by peripheral tissues prevents constitutive cell death of $p75^{LNGFR}$-bearing cells.

The expression of β-NGF receptors in the prostate may, therefore, be of relevance to the growth and maintenance of the adrenergic innervation of the gland. As it is clear that α-adrenoreceptor-mediated prostatic smooth muscle tone is responsible for 40% of urethral resistance in bladder outflow obstruction in males [12], the nature and distribution of β-NGF binding sites in the human prostate are of interest. In this study we sought to demonstrate specific NGF binding to human prostate tissue microsomes in a radioligand binding assay, and to localise human $p75^{LNGFR}$ in human prostate tissue by immunohistochemistry.

Materials and methods

Tissues and cells

Benign prostatic hyperplasia (BPH) and prostate cancer transurethral resection (TURP) ''chips'' were obtained. Tissue was snap-frozen in liquid nitrogen and stored at −70 °C until analysis. Randomly selected prostate chips from each prostate adenocarcinoma specimen were evaluated to confirm the diagnosis histopathologically.

The rat phaeochromocytoma cell line PC-12 (European Collection of Animal Cell Cultures, Porton Down, Salisbury, Wiltshire, UK) bears abundant NGF receptors [11] and provided positive controls. These cells were grown in suspension to turbidity in RPMI 1640 medium with 10% horse serum, 5% fetal calf serum, 50 U penicillin/ml, 50 mg streptomycin/ml, 2.5 μg amphotericin/ml and 2 mM L-glutamine (Gibco Life Technologies, Paisley, Renfrewshire, UK) at 37 °C in humidified air with 5% CO_2.

Preparation of prostatic and PC-12 microsomes

Two grams of prostate tissue were pulverised (Mikrodismembrator, Braun Medical, Aylesbury, UK) and homogenised in 0.25 mM sucrose buffer (0.25 mM sucrose, 10 mM TRIS, 1 mM EDTA, 1 mM EGTA, 0.05 mM phenylmethylsulphonyl fluoride (PMSF) and 0.25 μg/ml of aprotonin; Rotary homogeniser, Ystrall, Dottingen, Germany.) After centrifugation at 600 *g* and 13 000 *g* at 4 °C for 30 min, the microsomal fraction was pelleted by centrifugation at 100 000 *g* for 40 min at 4 °C and resuspended in 10 mM TRIS/0.9% (w/v) NaCl (pH 7.4) [13].

To test whether endogenous NGF activity was interfering with radiolabelled NGF binding, microsomes were treated with $MgCl_2$. The microsomal pellet was resuspended in 4 M magnesium chloride, incubated at room temperature for 10 min and centrifuged (100 000 *g*, 40 min), before suspension in 0.25 mM sucrose buffer, ultracentrifugation and suspension in 10 mM TRIS/0.9% (w/v) NaCl (pH 7.4) [24].

PC-12 cells were pelleted from culture (1000 *g*, 10 min) and resuspended in phosphate-buffered saline. The mixture was added to 1 mM TRIS with 0.05 mM PMSF and 0.25 μg/ml aprotonin. The mixture was centrifuged [3000 *g*, 15 min, 4 °C). The pellets were dispersed in 0.25 mM sucrose homogenisation buffer and re-centrifuged. The supernatant was ultracentrifuged (100 000 *g*, 40 min, 4 °C). The pellets were dispersed in 500 μl of radioligand binding assay buffer [36].

The protein content of microsomal preparations was measured by Bradford assay [5].

Radioligand binding assay

The reaction mixture was TRIS 10 mM, 0.9% (w/v) NaCl, 1% borine serum albumin (BSA), 1 mM PMSF, 0.25 μg/ml aprotonin, 200 000 cpm of ^{125}I-2.5S mouse NGF (mNGF) (i.e. 1.2–1.6 ng, at 1500 Ci/mmol, NEN Research Products, Du Pont, Stevenage, UK), microsomes at 0.1 mg protein/ml and 3000, 1500, 750, 562.5, 375, 187.5 or 0 ng/ml of unlabelled 2.5S mNGF (Boehringer Mannheim Biochemica, Lewes, Sussex, UK). The reaction volume was 100 μl (10 μg microsomal protein) [13]. PC-12 mixtures were incubated at 37 °C for 1 h. Prostate assays were incubated at 4 °C or 37 °C for 30 min to 21 h.

Bound and free hormone were separated by the addition of 1 ml of 0.9% (w/v) NaCl to each tube and centrifugation (13 000 *g*, 4 °C, 5 min). The supernatant was aspirated and the tip of the tube containing the microsomal pellet was excised and its gamma-emissions counted (''Crystal II'', Canberra-Packard, Pangbourne, Berks., UK). Assays were carried out in duplicate and the mean of two samples calculated. A Scatchard plot was drawn from the displacement data from PC-12 microsomal preparations [33].

Immunohistochemistry for $p75^{LNGFR}$

Cryostat sections 7–8 μm thick were cut from TURP chips on to poly-L-lysine coated microscope slides (Sigma, Poole, UK). PC-12 cells were pelleted by centrifugation at 1000 *g* for 5 min and smeared on a poly-L-lysine coated slide. They were washed in tap water. Frozen sections and PC-12 smears were fixed in absolute methanol at 4 °C for 3 min.

In initial $p75^{LNGFR}$ staining a 5% albumin blocking solution was used to replicate Graham et al.'s technique [14]. Sections were incubated in: 5% BSA in TRIS-buffered saline (pH 7.6, TBS) for 30 min; mouse monoclonal anti-human nerve growth factor-receptor (Boehringer Mannheim Biochemica, Lewes, UK) diluted 1:10 in TBS with 3.5% BSA and 0.1% sodium azide (TBS/BSA/azide), 30 min; TBS wash; prediluted biotinylated goat anti-mouse IgG antibody (''Immumark Universal'' reagent kit, ICN Flow laboratories, Costa Mesa, Calif, USA), 30 min; TBS wash; prediluted streptavidin-linked alkaline phosphatase (''Immumark Universal'', ICN Flow Laboratories), 30 min; TBS wash; fuchsin chromogen solution (''New Fuchsin'', Dako, High Wycombe, Bucks, UK) 15 min; tap water (pH 9.0), 2 min; Mayer's haematoxylin, 1 min; aqueous saturated lithium carbonate, 30 s. The slides were air-dried and mounted with DPX medium and coverslips. Sections were examined for positive (red) staining for $p75^{LNGFR}$ at ×40, ×100 and ×400 magnification.

When radioligand binding assays failed to demonstrate LNGFR activity (below), the results of immunohistochemistry were re-examined. Immunohistochemistry was repeated, but 20% normal goat serum (Scottish Antibody Production Unit, Carluke, Lanarkshire, UK) in TBS/BSA/azide was used as a blocking serum in place of 5% BSA in TBS. The normal goat serum would be expected to block more fully any non-specific binding of goat secondary antibody to tissue antigens [1]. The protocol of immunohistochemistry was otherwise as above and specifically the same preparation of primary anti-$p75^{LNGFR}$ was used.

Results

Radioligand binding assay

The displacement of ^{125}I-2.5S mNGF from PC-12 microsomes by the addition of increasing amounts of unlabelled 2.5S mNGF – indicating the presence of specific NGF binding activity – was demonstrated at 37 °C

(Fig. 1). In the case of PC-12 microsomes, background non-specific binding of 60.5% of total counts was seen. A Scatchard plot, drawn from the full range of unlabelled 2.5S mNGF concentrations, showed a K_d of 2.40×10^{-8} nM. The total number of receptors was 30.5 nmol/mg protein (Fig. 2). In the case of microsomes prepared from BPH tissue, no displacement of ^{125}I-2.5S mNGF was demonstrated under these conditions (Fig. 1). Nine BPH specimens were examined but specific 2.5S mouse NGF binding was not demonstrated.

Six BPH microsomal samples were assayed for hormone binding at 4 °C. No ^{125}I-2.5S mNGF displacement was shown with up to 3000 ng/ml of unlabelled 2.5S mNGF after incubations of 30 min and 1, 2, 4, 8 and 21 h. Equally, there was no evidence of specific NGF binding after endogenous NGF activity in BPH microsome preparations was abolished by previous treatment of microsomes with $MgCl_2$ (not shown).

In total, therefore, 20 BPH specimens were examined without any evidence of specific 2.5S-NGF being demonstrated.

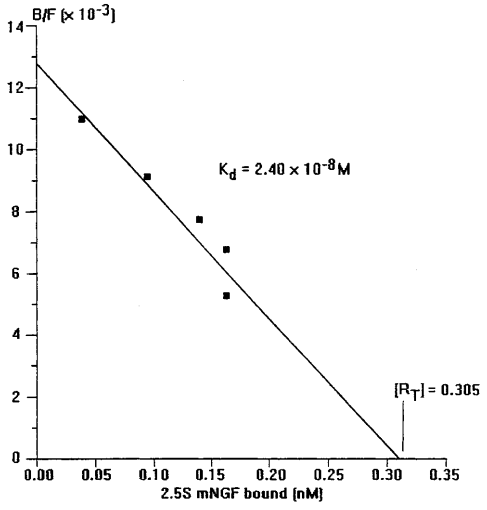


Fig. 2 Scatchard plot of the binding of ^{125}I-2.5S mNGF to PC-12 microsomes. Assay conditions were as in Fig. 1 and are further detailed in the text

p75^{LNGFR} immunohistochemistry

Immunohistochemistry was carried out on 15 samples each of prostate cancer and BPH tissues. p75^{LNGFR} immunohistochemistry with 5% BSA as the blocking solution showed profuse staining of the basal epithelial layer. There was little staining in the prostate stroma and the secretory epithelial layer did not stain [Fig. 3].

In the light of the absence of demonstrable β-NGF receptors in ligand binding assays, this finding was reviewed. When 20% goat serum was used as the blocking solution in immunohistochemistry, staining of the prostate epithelium was lost. Figures 4 and 5 show BPH and prostate cancer tissues stained in this way. The PC-12 positive control cells continued to stain for p75^{LNGFR} after goat serum blocking (Fig. 6). In spite of the lack of staining in the prostate epithelium, tiny areas (5–8 μm diameter) of stromal tissue – not related to cellular nuclei – stained positively for p75^{LNGFR}. Only 17 such areas were identified in a total of 93 tissue sections examined (Fig. 7). Such areas were noted both in BPH and prostate carcinoma tissue, and indeed in areas of BPH coexisting on sections bearing prostate carcinoma. No such staining was seen in negative control sections blocked with goat serum but omitting the primary anti-p75^{LNGFR} antibody.

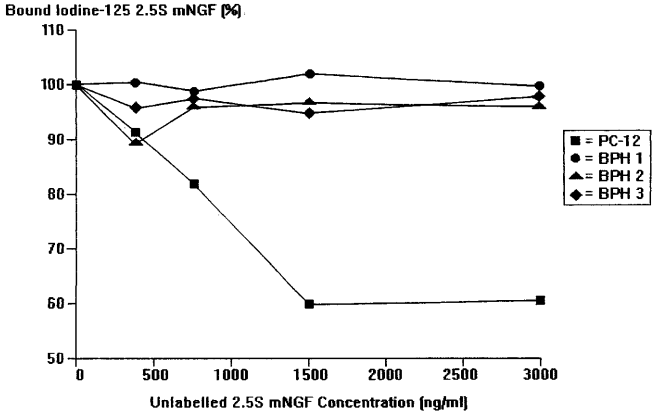
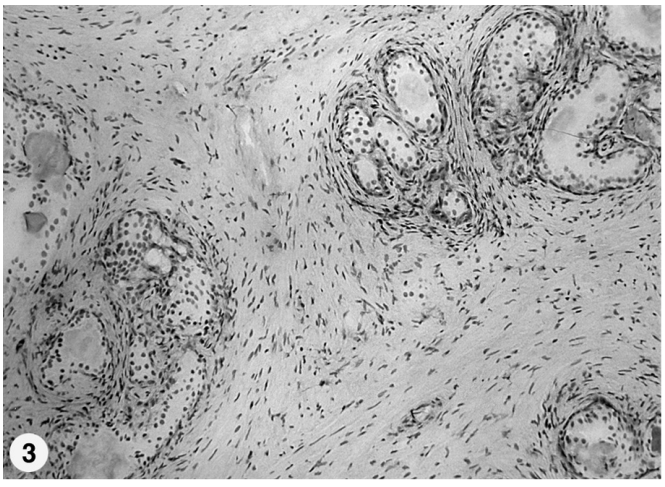


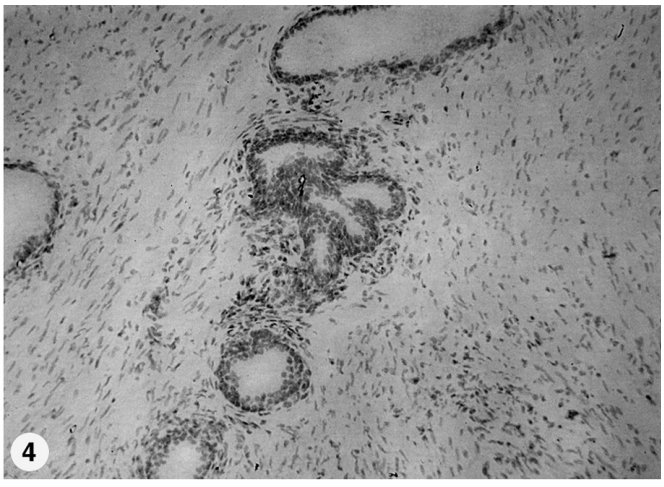
Fig. 1 Microsomal protein (0.1 mg/mL) was incubated with 200 000 cpm ^{125}I-2.5S mouse nerve growth factor (mNGF) in the presence of increasing unlabelled 2.5S mNGF concentrations and bound counts measured. Excess unlabelled 2.5S mNGF displaced ^{125}I-2.5S mNGF from PC-12 microsomes but not from benign prostatic hypertrophy (BPH) microsomes (three specimens shown). The background of non-specific binding in the case of PC-12 cells (60.5%) is comparable to that described elsewhere [31]

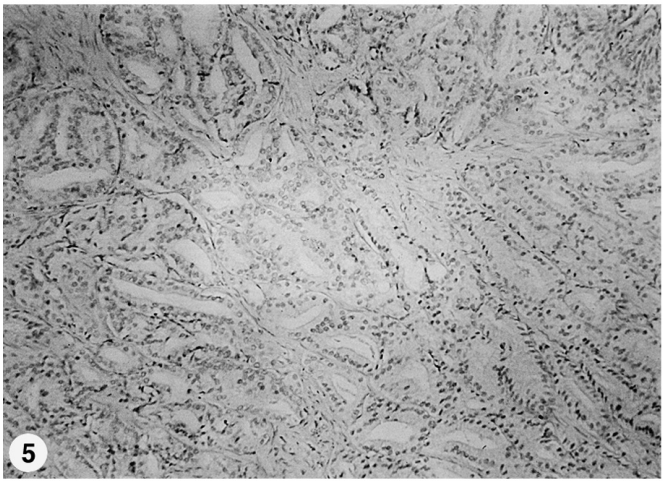
Discussion

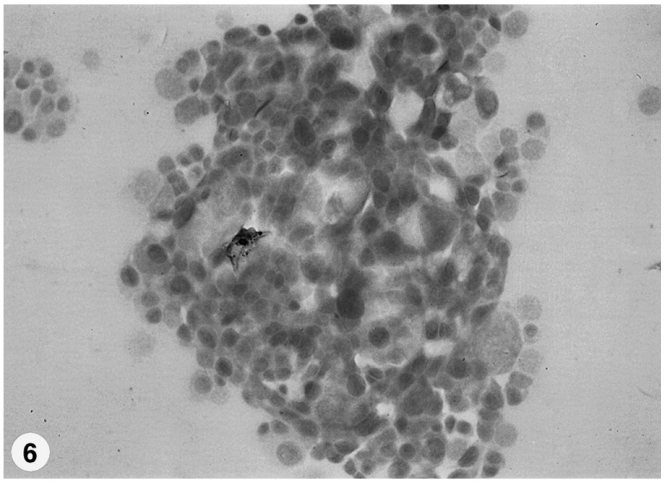
p75^{LNGFR} is a plasma membrane receptor and was, therefore, sought in microsomal preparations that are simply prepared and allow enrichment of the plasmalemma fraction as mitochondrial and nuclear membranes are excluded. The demonstration of specific NGF binding to PC-12 microsomes was straightforward. An initial experiment not reported here showed NGF binding to whole PC-12 cells at 37 °C for 1 h. These conditions were used for microsomal binding, therefore, and are thought to explain the high K_d found (10^{-8} M); other authors have found K_d values in the region of 10^{-9} M with microsomal preparations at 4 °C. Here, background non-specific binding of 60.5% of total counts to PC-12 microsomes was seen. Other authors have described non-specific backgrounds of around 50% for LNGFR assays on PC-12 membrane preparations [31].

Specific binding of NGF to BPH microsomes was not demonstrated, in spite of the use of a variety of conditions and the abolition of any endogenous NGF activity.









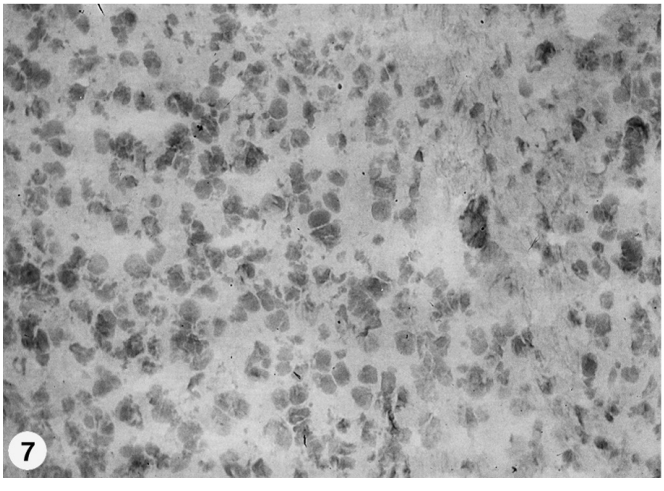


Fig. 3 BPH tissue stained for $p75^{LNGFR}$ using a blocking solution of 5% borine serum albumin (BSA) (original magnification × 32). The basal layer of prostate epithelium is positively stained while the prostate stroma and luminal epithelium is unstained

Fig. 4 BPH tissue stained for $p75^{LNGFR}$ using a blocking solution of 20% goat serum (original magnification × 32.) Neither any of the glands in the middle of the field, nor the surrounding stroma, has stained for $p75^{LNGFR}$

Fig. 5 Well-differentiated prostate adenocarcinoma tissue stained for $p75^{LNGFR}$ with the 20% goat blocking serum. No malignant gland, nor the small amount of intervening stroma, has stained positively for the receptor (original magnification × 32)

Fig. 6 A smear of PC-12 cells stained for $p75^{LNGFR}$ after blocking with 20% goat serum as in Figs. 4 and 5. Positive staining is dark. The cytoplasm of the PC-12 cells is clearly stained (original magnification × 80)

Fig. 7 Poorly differentiated prostate adenocarcinoma tissue stained as Figs. 4–6 (original magnification × 128). Punctate and fibrillary $p75^{LNGFR}$ staining is surrounded by adenocarcinoma cells

In the light of the results of our initial $p75^{LNGFR}$ staining, and those of other authors, this was a surprising result. Using albumin blocking solutions, immunohistochemistry reported here and by Graham et al. [14] suggests the presence of profuse $p75^{LNGFR}$ in prostate epithelium. MacGrogan et al. [25] report similar staining in the human prostate but the exact protocol of immunohistochemistry they used is not detailed.

When immunohistochemistry was repeated using a blocking serum derived from the species in which the secondary antibody was raised, the distribution of staining found was more consistent with ligand-binding results. That is, in the presence of specific staining of positive control cells, positive staining of benign hyperplastic and malignant prostate epithelium was lost in the 30 specimens examined. The only staining that persisted was in very small and sparse areas of prostate stroma where staining was not obviously related to prostate stromal cells. It is believed that these areas represent staining of $p75^{LNGFR}$ in sympathetic or sensory neural processes, where $p75^{LNGFR}$ activity is well described [22]. The infrequency of these areas, and hence the low numbers of $p75^{LNGFR}$ molecules present, is consistent with the failure of radioligand binding to demonstrate LNGFR activity. It should be stressed that the same primary antibody preparation was used in both immunohistochemistry techniques here and that this was the same antibody preparation, from the same manufacturer, as that used by Graham et al. [14].

Djakiew and co-workers have reported studies of low- and high-affinity NGF receptors in the human prostate. Their immunohistochemical studies of human prostate tissue, whereby $p75^{LNGFR}$ localised to epithelial cells, have already been cited [14]. Djakiew et al. [10] have also described an increase in prostate epithelial growth in vitro in response to NGF, and have stained primary cultures of normal prostate epithelial cells and PC-3, DU145, LNCaP and TSU-pr1 prostate cancer cell lines. They found that normal epithelial cells stained positively for $p75^{LNGFR}$ while the malignant cell lines did not. In immunoblotting, primary cultures of normal epithelial cells expressed $p75^{LNGFR}$ while BPH and adenocarcinoma cells showed reduced or absent expression [29]. More recently they have demonstrated $p140^{trkA}$ immunohistochemical staining in epithelium of hyperplastic, adenocarcinomatous and normal prostate tissue. Malignant TSU-pr1 cells in culture showed high-affinity NGF binding only in ligand binding assay, while primary cultures of normal cells showed high- and low-affinity binding [28]. In addition to these immunochemical results Djakiew's group has been able to demonstrate specific binding of β-NGF in prostate cells, in contrast to the results reported here [28, 29]. It should be noted, however, that specific β-NGF binding was only demonstrated in primary cultures of prostatic cells rather than in membrane preparations from prostatic tissue as reported here. There is little doubt that the phenotype of prostate cells alters significantly in primary culture, and we suspect that this explains the disparity in these results [16]. On the basis of their results, Djakiew et al. have stressed the possibility that NGF or NGF-like proteins in the human prostate are stromally produced paracrine determinants of epithelial growth.

The results reported here would support the suggestion that $p75^{LNGFR}$ expression is low in BPH – it was not possible to demonstrate specific NGF binding in BPH tissue. These immunohistochemistry results, however, would suggest a different interpretation of the localisation of $p75^{LNGFR}$ from that previously reported. In association with the epithelial localisation of NGF reported from this laboratory [27] these data would suggest that β-NGF in BPH tissue in the human is an epithelial product whose receptors are localised to putative nerve endings in the prostate stroma.

In this view, β-NGF in the prostate would have a role similar to that in other peripheral tissues – namely the control of the density and distribution of sympathetic innervation [15, 23]. Recently it has been shown not only that β-NGF has the capacity to rescue susceptible nerve fibres from naturally occurring cell death in peri-natal life [2], but also that it can promote the new growth of adrenergic neurones in the adult [35]. Further it is clear that the catecholamines released by sympathetic nerves themselves act in turn as trophic factors for smooth muscle cells [3, 19].

Therefore it can be postulated that β-NGF production by growing hyperplastic prostatic epithelium may be a means whereby the sympathetic innervation and the smooth muscle elements of the mature BPH nodule are recruited [26].

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