Regulation of Growth by a Nerve Growth Factor-like Protein Which Modulates Paracrine Interactions between a Neoplastic Epithelial Cell Line and Stromal Cells of the Human Prostate

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ABSTRACT

Nerve growth factor-like substance(s) were identified in both conditioned media of a human prostatic tumor epithelial cell line (TSU-prl) and a human prostatic stromal cell line (HPS) by Western blot analysis and bioassay of neurite outgrowth of PC12 cells. Nerve growth factor-β (NGF) immunofluorescence was also localized to secretory vesicles in the cytoplasm of both the TSU-prl and HPS cells. Western blot of the TSU-prl and HPS cell-secreted protein identified an M, 65,000 major protein which immunoreacted with murine NGF antibody. NGF Western blot of HPS cell-secreted protein also identified an M, 42,000 minor band under reduced and nonreduced conditions and an M, 61,000 minor band under reduced conditions. The secreted protein from the TSU-prl cells (50 μg/ml) and HPS (50 μg/ml), as well as murine NGF (50 ng/ml) or human recombinant NGF (50 ng/ml), stimulated neurite outgrowth from PC12 cells. This neurite outgrowth activity was partially inhibited by treatment with NGF antibody. Neither the serum containing growth medium nor bovine serum albumin (50 μg/ml) stimulated neurite outgrowth. The NGF-like secretory protein appeared to play a role in the paracrine regulation of prostatic growth between TSU-prl cells and HPS cells. The relative growth of TSU-prl cells, as indicated by [3H]thymidine incorporation, in response to HPS secretory protein was stimulated 2.8-fold in a dose-dependent manner. In the converse interaction, the relative growth of HPS cells in response to TSU-prl secretory protein was stimulated 1.8-fold in a dose-dependent manner. Immunoneutralization of TSU-prl and HPS secretory protein was performed with antibody against NGF, acidic fibroblast growth factor, and basic fibroblast growth factor. Removal of the NGF-like protein from the maximal stimulatory dose of TSU-prl secretory protein (100 μg/ml) with NGF antibody reduced HPS proliferation to 52% of maximal levels, and immunoneutralization of the NGF-like protein in the maximal stimulatory dose of HPS secretory protein (20 μg/ml) also reduced TSU-prl proliferation to 16% of maximal levels. Addition of normal rabbit serum or prior immunoprecipitation of either TSU-prl or HPS secretory protein with antibody against acidic fibroblast growth factor and basic fibroblast growth factor did not inhibit the proliferation of either cell type. These results suggest that TSU-prl tumor cells and HPS cells secrete NGF-like protein(s) which modulate their paracrine interactive growth in vitro.

INTRODUCTION

Prostate cancer has surpassed lung cancer as the leading cause of cancer in men (1). A better understanding of the mechanisms which regulate prostatic growth in the normal and neoplastic prostate may facilitate the clinical manipulation of aberrant prostatic growth. Since the lining epithelium of the prostatic glandular acini are surrounded by a well-developed fibromuscular stroma, cell-to-cell communication has been implicated in mediating aspects of prostatic growth. In this context, paracrine regulation of prostatic growth was first suggested by Franks et al. (2) after observing a lack of growth capacity of epithelia which had been separated from their stroma. Subsequently, Cunha (3) and Cunha et al. (4) demonstrated that fetal mesenchyme (stroma) recombined with urothelium induced prostatic epithelial morphogenesis and that this morphogenesis occurred in a cell (stroma) density-dependent manner (5). Tissue recombination studies with testicular feminized and wild-type tissues have shown that the target of androgen action is the mesenchyme (4, 6) which in turn mediates the growth of adjacent epithelial cells (7). In adult rat prostatic tissues, androgens mediate the secretion of stromal derived growth factors which stimulate epithelial proliferation (8). Rat stromal secretory proteins also modulate vectorial protein secretion from a neoplastic prostatic epithelial cell line (9). On the other hand, some tumor epithelial cell lines have a reduced requirement for exogenous growth factors (10), consistent with their reduced dependence on androgens and stromal growth factors for their proliferation. Several growth factors have been identified which may function as putative paracrine regulators of prostatic growth. In this context, epidermal growth factor-like (11) and bFGF-like (12, 13) proteins account for a considerable amount of the growth factor activity in the prostate. In addition, αFGF (14), transforming growth factor-β (15, 16), and NGF (17–19) have been identified in the prostate or prostatic tumor cells. Considerable evidence has accumulated demonstrating that NGF secreted by glial cells functions in the paracrine maintenance of neurons (20). Considering the paracrine role of NGF in neurons (20) and its presence in prostatic adenocarcinomas (19), we examined whether NGF may mediate paracrine interactions between a tumor epithelial cell line and stromal cells of the human prostate. In this report we demonstrate the presence of NGF-like proteins in these cells which mediate the paracrine regulation of growth of both the tumor epithelial cell line and the stromal cells in vitro.

MATERIALS AND METHODS

Culture of Cell Lines. The human neoplastic epithelial cell line (TSU-prl) derived from the prostate (21) was a kind gift from Dr. J. Isaacs (Johns Hopkins University, Baltimore, MD). These cells were grown in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with antibiotics/antimycotic (100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone; Sigma), 10% FCS (HyClone Co., Logan, UT), and 10−5 M T (Sigma). The human prostatic stromal cells were obtained from an adult male following transurethral prostatectomy at Georgetown University Hospital. The tissue was minced into small blocks (1–3 mm3) and sequentially digested in a collagenase

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The abbreviations used are: bFGF, fibroblast growth factor; aFGF, acidic fibroblast growth factor; NGF, nerve growth factor-β; FCS, fetal calf serum; T, testosterone; HPS, human prostatic stroma; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; HS, horse serum; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; BSA, bovine serum albumin; TIPS, TBS containing 0.05% Tween-20 and 0.5% BSA (Sigma); mNGF, murine NGF; hNGF, human recombinant NGF; D.I., DNA index.
enzyme solution as previously described (9). The resulting single-cell suspension and small fragments of tissue were washed in RPMI-1640 medium, resuspended in RPMI-1640 medium containing 10% FCS/T, and plated in 75-mm² flasks (Becton Dickinson Laboratories, Lincoln Park, NJ). After 3 days unattached cells were removed and the remaining cells allowed to proliferate to confluence. These cells were trypsinized and passed through four successive cycles to obtain a population of HPS free of epithelial contaminants. A seed stock of HPS was subsequently resuspended in RPMI-1640 medium containing 25% FCS/10% dimethyl sulfoxide (Sigma) and frozen at -130°C until use. A rat pheochromocytoma cell line (PC12) was obtained from the American Type Culture Collection (Rockville, MD) and cultured in Ham's F-12/DMEM medium ( Irvine Scientific, Santa Ana, CA) supplemented with 15 mM HEPES (Sigma), antibodies/antimycotic, 10% HS (Gibco BRL, Gaithersburg, MD), and 5% FCS. All cell lines were incubated at 37°C in 5% CO₂/95% air and the media replaced every second day.

Immunofluorescence. The TSU-prl cells and HPS cells cultured on glass cover slips were screened for vimentin intermediate filaments, keratin intermediate filaments, and NGF using indirect immunofluorescence as previously described (9). Methanol-fixed cells were blocked with 3% ovalbumin or 5% normal goat serum in PBS at room temperature for 60 min, or at 4°C overnight, and incubated with rabbit antimurine vimentin antibody (1:100; ICN Immunobiologicals, Lisle, IL), rabbit anti-human keratin antibody (1:25; ICN Immunobiologicals), rabbit anti-murine NGF antibody (1:100; Collaborative Research Inc., Bedford, MA) or normal rabbit serum (1:100; ICN Immunobiologicals) for 60 min. The cells were washed three times in PBS and then incubated with rhodamine-conjugated goat anti-rabbit IgG (1:400-1:1000; ICN Immunobiologicals) at room temperature for 10-30 min. Subsequently, the cells were washed three times in PBS, mounted, and viewed with a Zeiss photomicroscope fitted with an epifluorescence attachment.

Ploidy Analysis of Cell Lines. The ploidy of the TSU-prl cells, HPS cells, and murine 3T3 cells were kindly analyzed by Dr. Owen Blair (Lombardi Cancer Research Center, Georgetown University Medical Center, Washington, DC) by the method of Vindelov (22) using propidium iodide as the nuclear stain for the flow cytometry. Chicken erythrocytes and human lymphocytes were used as the internal standard cell types.

Preparation of Secretory Protein. TSU-prl cells and HPS cells were grown to confluence in RPMI-1640 medium supplemented with 10% FCS/T and antibiotics/antimycotic. At confluence the cells were washed three times and cultured in Ham's F-12/DMEM medium, with 15 mM HEPES, 2 mM glutamine (Sigma), antibodies/antimycotic, and T for 24 h. The conditioned media were collected and centrifuged at 1000 x g to remove particulates, and the supernatant was immediately frozen at -20°C. This procedure was successively alternated with a 24-h incubation in 10% FCS/T with supplements as above. Conditioned media from additional human neoplastic epithelial cell lines (PC-3, DU-145, LNCaP) were similarly prepared with a modification that T was substituted with 10⁻³ M dihydrotestosterone (Sigma) for the LNCaP cells. Conditioned media were collected/dialyzed with a hollow fiber filter cartridge of M, 10,000 exclusion limit (Cote-Parmer Instruments Co., Chicago, IL) using ice cold distilled water for dialysis. The concentrated dialyzed media were lyophilized and stored at -20°C until use.

Western Blot Analysis of NGF-like Protein. Lysylated secretory protein from the TSU-prl cells and HPS cells, as well as from the PC-3, DU-145 and LNCaP cells, were reconstituted in either reducing or nonreducing sample buffer (23), and 2.5-7.5 µg of these proteins was loaded onto each lane of a 12% polyacrylamide minigel and subjected to one-dimensional sodium dodecyl sulfate gel electrophoresis according to the method of Laemmli (23). Subsequently, the separated proteins were electrotransferred to a 0.2-µm nitrocellulose membrane at 0.9 A. The nitrocellulose was blocked with 5% non-fat milk in TBS for 1 h (24), rinsed twice with TTBS, and reacted with NGF antibody (1:1000 in 1% gelatin/TTBS) overnight. The membranes were washed in TTBS twice for 10 min, reacted with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000 in 1% gelatin/TTBS; Bio-Rad Labs, Richmond, CA) for 1-3 h, and rinsed in TTBS twice and once with TBS. The immunoreactivity was visualized by the following color development reaction. First, 4-chloro-1-naphthol was dissolved to a final concentration of 0.03% in ice cold methanol. Hydrogen peroxide was mixed with TBS at room temperature to a concentration of 0.018%. This solution was added to the 4-chloro-1-naphthol solution, and the membranes were incubated in this mixture until color developed. The reaction was stopped by replacement of the reaction mixture with distilled water.

Assays of Mitogenic Activities and Immunoneutralization Studies. HPS cells and TSU-prl cells were resuspended in RPMI-1640 medium containing 10% FCS/T, seeded at 5 x 10⁶ cells/well in Falcon 24-multiwell tissue culture plates (Becton Dickinson), and incubated in 5% CO₂/95% air. After 24 h the cells were washed three times in Ham's F-12/DMEM medium with 15 mM HEPES and further incubated in various concentrations of either HPS or TSU-prl secretory protein, respectively, reconstituted in Ham's F-12/DMEM/T medium with 15 mM HEPES. Additional wells of these cells were cultured in Ham's F-12/DMEM with or without 10% FCS/T, 1% FCS/T, and T. Some of the reconstituted TSU-prl and HPS secretory protein (1.5 ml) were mixed with 3-15 µl of polyclonal antibody against murine NGF, bovine aFGF (U.B.I. Inc., Lake Placid, NY), or bovine bFGF (R & D Systems Inc., Minneapolis, MN). A nonimmune normal rabbit serum (ICN Immunobiologicals) and a rabbit IgG (Chemicon International, Inc., Segundo, CA) were used as controls. Subsequently, the secretory protein/antibody mixture was allowed to complex for 1 h at room temperature, followed by centrifugation at 10,000 x g. The supernatant was collected and incubated with the cells as above for the nontreated secretory protein. After a 24-h incubation with the secretory protein or the various control treatments, each well of cells was supplemented with 1 µCi of [³H]thymidine (ICN Radiochemicals; Irvine, CA) and further incubated for 6 h at 37°C in 5% CO₂/95% air. Subsequently, each well was washed three times with 1 ml of ice cold PBS, the cells were fixed in 1 ml of 1% trichloroacetic acid (4°C) for 20 min, washed three times with 5% trichloroacetic acid and digested in 0.5 N NaOH (0.3 ml). This solution was neutralized with 0.5 N HCl (0.3 ml) and the incorporated radioactivity was determined by liquid scintillation spectrometry using a Beckmann scintillation counter. Relative growth of cells (25, 26) was calculated from the [³H]thymidine incorporation in the presence of secretory protein or the various culture media preparations divided by [³H]thymidine incorporation in control culture of Ham's F-12/DMEM/T.

Neurite Outgrowth Assay. Neurite outgrowth from PC12 cells was carried out according to the method of Greene and Tischler (27). PC12 cells were resuspended in Ham's F-12/DMEM medium with 10% HS/5% FCS and seeded in 24-multiwell tissue culture plates which had been coated with poly-L-lysine (Sigma). Following 24 h incubation at 37°C in 5% CO₂/95% air, the culture medium was replaced with TSU-prl and HPS secretory protein (0-100 µg/ml) resuspended in Ham's F-12/DMEM medium with or without 10% HS/5% FCS. In control cultures PC12 cells were incubated with 0-100 µg/ml BSA, 0-100 ng/ml mNGF (Boehringer Mannheim Biochemicals, Indianapolis, MN), and 0-100 ng/ml hNGF (Genentech, South San Francisco, CA), all in the presence of either Ham's F-12/DMEM with or without 10% HS/5% FCS. Additional controls included incubation of PC12 cells in Hank's buffer, Ham's F-12/DMEM alone, or 10% HS/5% FCS in Ham's F-12/DMEM. PC12 cells incubated in each of the above treatments at 37°C in 5% CO₂/95% air were monitored every 24 h for neurite outgrowth during a period of 4 days.

Statistical Analysis. The statistical significance of differences between treatments was tested by Student's t test. The values for the SEM that are given as estimates of the dispersion in the results were calculated for each treatment from estimates of the variance between replicates.

RESULTS

Characterization of TSU-prl Cells and HPS Cells by Immunofluorescent Labeling. Fig. 1 shows phase contrast images and corresponding immunofluorescent images of keratin, vimentin,
Fig. 1. Human prostatic epithelial tumor cells (TSU-prl, A–D) and stromal cells (HPS, E–H). A and E, phase contrast images; B and F, the corresponding indirect immunofluorescence of NGF; C and G, the indirect immunofluorescence of vimentin intermediate filaments; D and H, the indirect immunofluorescence of keratin intermediate filaments immunofluorescence. Bars, 13 μm.

and NGF localization in TSU-prl cells and HPS cells. The phase contrast image of the TSU-prl cells (Fig. 1A) shows a centrally located nucleus surrounded by a cytoplasm containing numerous vesicular inclusions. Keratin immunofluorescence of the TSU-prl cells (Fig. 1D) was localized to filamentous structures throughout the cytoplasm, as was the vimentin immunofluorescence (Fig. 1C). The NGF immunofluorescent image of these cells (Fig. 1B) shows a diffuse perinuclear staining with distinct vesicular structures in the periphery of the cytoplasm. The phase contrast image of the HPS cells (Fig. 1E) shows an attenuated cytoplasm containing sparse refractile structures interspersed between an extensive network of stress fibers. HPS cells were slightly immunofluorescent for keratin intermediate filaments (Fig. 1H), whereas vimentin immunofluorescence (Fig. 1G) was intense and localized to filamentous structures throughout the cytoplasm. Based on the phase contrast images and the vimentin immunofluorescence, the HPS cell line appeared to be 100% pure, lacking epithelial contaminants. NGF immunofluorescence (Fig. 1F) of these cells was localized to punctate structures throughout the cytoplasm. At the extremities of the HPS cytoplasm adjacent to the cell borders, large vesicular bodies could be observed as well as in pseudopod-like extensions of the cytoplasm. In both the HPS and TSU-prl cells, the normal rabbit serum controls for the immunofluorescence studies exhibited negligible fluorescence (not shown).
Ploidy of TSU-prl and HPS Cells. The ploidy of the various cell lines are expressed as a D.I., where a D.I. of 1.0 (range, 0.9–1.1) is considered diploid and a D.I. <0.9 or >1.1 is considered aneuploid. Hence, the TSU-prl cells were recognized as aneuploid with a D.I. of 1.63, the HPS cells with a D.I. of 0.98 are diploid, and in comparison the murine 3T3 cells with a D.I. of 1.69 are aneuploid.

Western Blot of NGF-like Immunoreactivity. Fig. 2 shows the Western blots of NGF-like protein in the TSU-prl and HPS secretory protein. Under reduced and nonreduced conditions of the TSU-prl (lanes 1 and 3) and HPS (lanes 2 and 4) secretory proteins a major band of approximately M, 65,000 was identified. In the HPS secretory protein minor bands of M, 61,000 and 42,000 were observed under reduced conditions (lane 2), whereas under nonreduced conditions (lane 4) the only minor band consisted of an M, 42,000 protein. NGF antibody Western blots of secretory protein from PC-3, DU-145, and LNCaP cells confirmed the presence of NGF-like proteins in these additional neoplastic epithelial cell lines of the human prostate (not shown).

PC12 Neurite Outgrowth. Fig. 3 shows PC12 cells cultured in Ham's F-12/DMEM with 10% HS/5%FCS supplemented with secretory proteins from the TSU-prl and HPS cells, as well as BSA, mNGF, and hNGF. PC12 cells cultured with 50 μg/ml TSU-prl secretory protein (Fig. 3A) or 50 μg/ml HPS secretory protein (Fig. 3B) supplemented to Ham's F-12/DMEM with 10% HS/5%FCS exhibited neurite outgrowth in a time- and dose-dependent manner. Generally, neurites were observed after 24 h of culture. These neurites continued to extend for up to 3 days of culture without medium exchange (Fig. 3, A and B), after which further neurite outgrowth was not observed. Extension of neurites was accompanied by multiple neurite outgrowth from the same cell, branching of the same neurites, and attenuation of the cytoplasm to a squamous phenotype (Fig. 3, A and B). Negative control cultures of PC12 cells incubated in Ham's F-12/DMEM supplemented with 10% HS/5% FCS (Fig. 3C) or supplemented with 50 μg/ml BSA (Fig. 3D) did not exhibit characteristics of neurite outgrowth. Additional negative control cultures of PC12 cells incubated in Hank's buffer or Ham's F-12/DMEM alone did not exhibit neurite outgrowth. Positive control cultures of PC12 cells incubated in Ham's F-12/DMEM supplemented with 10% HS/5% FCS supplemented with 50 ng/ml mNGF (Fig. 3E) or 50 ng/ml hNGF (Fig. 3F) exhibited characteristics of neurite outgrowth comparable to that induced by the TSU-prl and HPS secretory protein. NGF antibody immunoneutralization of TSU-prl and HPS secretory proteins, as well as the mNGF and hNGF, partially inhibited neurite outgrowth from the PC12 cells (not shown).

Paracrine Influence of TSU-prl and HPS Cell Secretory Protein on Cell Growth. Fig. 4 shows the dose-dependent effect of TSU-prl epithelial cell protein on HPS relative proliferation. HPS cells were maximally stimulated 1.8-fold by TSU-prl secretory protein at a concentration of 100 μg/ml (P < 0.05). HPS relative proliferation was stimulated 10-fold by 10% FCS/T in Ham's F-12/DMEM (P <0.01) and 4-fold by 1% FCS/T in Ham's F-12/DMEM (P < 0.01). Prior immunoprecipitation of the maximal stimulatory dose of TSU-prl secretory protein (100 μg/ml) with 3 μl NGF antibody/1500 μl secretory protein solution (0.2% antisera) reduced HPS relative proliferation to 52% of the maximal levels (P < 0.01). Addition of NGF antisera to the maximal stimulatory dose of TSU-prl secretory protein equivalent to 1% of the final volume for immunneutralization resulted in a 4-fold stimulation of HPS cell relative growth. This effect appears to result from unknown components in the NGF antisera. Hence, immunoneutralization studies were performed with NGF antisera limited to 0.2% of the total sample volume. Interestingly, prior immunoprecipitation of the TSU-prl secretory protein with aFGF or bFGF antibody did not significantly influence the effect of the TSU-prl protein on the relative growth of HPS cells. Substitution of the NGF antibody with normal rabbit serum or a rabbit IgG preparation did not significantly inhibit the relative growth of HPS cells in response to TSU-prl secretory protein.

Fig. 5 shows the dose-dependent effect of HPS secretory protein on TSU-prl relative proliferation. TSU-prl proliferation was maximally stimulated 2.8-fold by HPS secretory protein at a concentration of 20 μg/ml (P < 0.01). Prior immunoprecipitation of the maximal stimulatory dose of HPS secretory protein (20 μg/ml) with NGF antibody inhibited relative proliferation to 16% of the maximal proliferation of the TSU-prl cells (P < 0.01). Prior immunoprecipitation of the HPS secretory protein with aFGF or bFGF antibody did not significantly influence the effect of the HPS secretory protein on the relative growth of the TSU-prl cells. Replacement of the NGF antibody with normal rabbit serum or a rabbit IgG preparation did not inhibit the relative growth of the TSU-prl cells in response to the HPS secretory protein.

**DISCUSSION**

Vimentin intermediate filaments are generally characteristic of mesenchymal cells, whereas keratin intermediate filaments are generally characteristic of epithelial cells (28). However, some carcinoma cells have been shown to express vimentin and keratin intermediate filaments concurrently (29). Hence, expression of both keratin and vimentin intermediate filaments in the aneuploid TSU-prl epithelial tumor cells is consistent with demonstrations of vimentin expression in breast carcinomas (30), breast cancer epithelial cell lines (31), and prostatic epithelial tumor cell lines (14). Expression of vimentin in transformed epithelial cells has been correlated with the more malignant phenotype of hormone-independent cells (31).
NGF-LIKE PARACRINE REGULATION OF PROSTATIC GROWTH

Fig. 3. Neurite outgrowth from PC12 cells. PC12 cells cultured according to process described in "Materials and Methods" for 3 days in the presence of 50 μg/ml TSU-prl secretory protein (A), 50 μg/ml HPS secretory protein (B), 50 μg/ml BSA (C), no additional protein (D), 50 ng/ml mNGF (E), and 50 ng/ml hNGF (F). PC12 cells with neurite outgrowth exhibited branching of neurites (arrows) and multiple outgrowths of neurites from the same cell (arrowheads). The photographs are representative of four independent experiments. Bars, 50 μm.

Fig. 4. Dose-dependent relative growth of HPS cells in response to TSU-prl secretory protein. Immunoneutralization of the maximal stimulatory dose of TSU-prl cell secretory protein (100 μg/ml) with NGF antibody (0.2% antiserum), aFGF antibody, and bFGF antibody. Point, mean (bar, ±SEM) from four independent experiments. *P < 0.01.

Fig. 5. Dose-dependent relative growth of TSU-prl cells in response to HPS cell secretory protein. The maximal stimulatory dose of HPS cell secretory protein (20 μg/ml) was treated with NGF antibody, aFGF antibody, and bFGF antibody. Point, mean (bar, ±SEM) from seven independent experiments. *P < 0.01.

Therefore, expression of vimentin in immortalized epithelial cells may accompany epithelio-mesenchymal transition to the malignant phenotype. In contrast, the localization of vimentin and relative absence of keratin immunofluorescence in the diploid HPS cell line suggest that these cells have retained an in vitro phenotype comparable to their in vivo counterparts.

Since the early work of Harper et al. (17) demonstrating the presence of NGF-like protein in the prostate gland of the guinea pig, several investigators have found NGF-like proteins throughout the male reproductive system including the seminal
vesicles (20), testis (32, 33), epididymis (33), and spermatozoa (34). Of particular interest is the observation that nonmalignant prostates (35) and prostatic adenocarcinomas (19) are immunoreactive for a NGF-like protein. Our demonstration of the secretion of NGF-like protein(s) by both a human prostatic epithelial tumor cell line (TSU-prl) and a prostatic stromal cell line (HPS) along with the ability of these proteins to modify the growth of these cells is consistent with a paracrine function for the NGF-like protein(s). Clearly, growth of the epithelial tumor cells appears to be sensitive to the NGF-like protein since immunoneutralization of the HPS secretory protein reduced epithelial tumor cell growth to 16% of that observed with the intact HPS secretory protein. Similarly, growth of the HPS cells appears sensitive to the NGF-like protein since immunoneutralization of the TSU-prl secretory protein reduced HPS cell growth to 52% of that observed with the intact TSU-prl protein. Hence, it is clear that TSU-prl epithelial tumor cells and HPS cells each regulate the growth of the other via a paracrine mechanism mediated by NGF-like protein(s). Interestingly, immunoneutralization of both aFGF and bFGF from the secretory proteins of the TSU-prl cells and the HPS cells did not influence the paracrine regulation of growth of these two cell types. The prostate has been shown to be a rich source of aFGF (14) and bFGF (12, 13). However, since aFGF and bFGF lack a hydrophobic leader sequence for their secretion (36), it seems more likely that these FGF proteins may participate in autocrine-regulated growth, as recently suggested by Story et al. (13).

Our observations of NGF-like protein(s) in prostatic cell lines are consistent with previous observations of this factor in the prostate gland (17, 18, 20). Whole antiserum and antibodies purified by affinity chromatography against murine submaxillary gland NGF (37) have been shown to completely inhibit the biological activity of prostatic extracts with regard to promotion of nerve fiber outgrowth from dorsal root ganglia in vitro (17). The immunofluorescent localization of NGF-like protein to vesicular structures within HPS and TSU-prl cells indicates that the protein is present in the secretory pathway, which is a prerequisite for its role as a paracrine growth factor. Furthermore, Western blot analysis and immunoneutralization of the NGF-like protein from conditioned media of both cell types provide additional evidence for its secretion. Western blot of the TSU-prl and HPS secretory protein for NGF-like protein identified a major band of approximately $M_\text{r}$ 65,000 in both cell types. Since the mature form of murine submaxillary gland NGF is a homodimer (38) consisting of $M_\text{r}$ 13,000 subunits (39), which are generated by proteolytic cleavage of a large precursor protein (40), the $M_\text{r}$ 65,000 prostatic NGF-like protein recognized in the Western blots either may represent a prostatic precursor form of the NGF protein or may be a distinct protein which belongs to the NGF family of proteins. With regard to the first possibility, precedents for high molecular weight proteins corresponding to putative NGF precursor proteins or partial cleavage products have been reported for the murine submaxillary gland (41, 42) and the rat thyroid (43). The processing of many secretory proteins appears to involve multiple specific proteolytic events (25, 44, 45). Since the Western blots also identified minor bands of slightly lower molecular weight of the NGF-like protein secreted by the HPS cell line, these minor bands may represent partial cleavage products of the $M_\text{r}$ 65,000 form. With regard to the NGF family of proteins, two additional genes for neurotrophic proteins have been cloned recently. Brain-derived neurotrophic factor (46) and neurotrphin-3 (47, 48) are polypeptides of similar molecular weights and with isoelectric points to NGF and share nearly 50% homology of amino acid residues. Although these proteins exhibit unique patterns of biological activity for different neurons and distribution patterns of mRNA in the brain (49), it is possible that polyclonal antibodies to NGF may cross-react with these similar molecules. The NGF-like protein(s) that we describe may be the product of one of these neurotrophin genes or of an additional member of this gene family.

In addition to the immunological identity of NGF-like proteins in the TSU-prl cells and HPS cells by immunocytochemistry, Western blot, and immunoneutralization, the functional assay of NGF-like activity by PC12 neurite outgrowth in response to secretory proteins from the TSU-prl cells and HPS cells provides strong corroborative evidence for the expression of NGF-like protein in these prostatic cells. The PC12 cells can differentiate along the lines of either chromaffin cells or, when grown in the presence of NGF, can differentiate into neuronal like cells (50) as indicated by the phenotypic outgrowth of neurites (27), the development of small vesicles, and the ability to become electrically excitable (51). Hence, the ability of prostatic secretory proteins from TSU-prl cells and HPS cells to promote PC12 differentiation to a neuronal phenotype is consistent with the secretion of high molecular weight prostate-specific forms of NGF-like protein.

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