Chemotaxis and Chemokinesis of Human Prostate Tumor Cell Lines in Response to Human Prostate Stromal Cell Secretory Proteins Containing a Nerve Growth Factor-like Protein

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ABSTRACT

The migration of three human prostate tumor epithelial cell lines (TSU-prl, PC-3, DU-145) in response to secreted protein from a human prostate stromal cell line was investigated by using the modified blind-well Boyden chamber assay. Migrated cells were quantified by spectrophotometrically measuring the concentration of crystal violet stain extracted from their nuclei. Cell number was correlated linearly with the concentration of extracted crystal violet stain. All three tumor cell lines showed intrinsic migratory ability in the absence of chemoattractants, such that approximately 1–7% of plated cells migrated across the filter of the Boyden chambers during a 5-h incubation period. Prostate tumor cell migration was significantly enhanced (3–13-fold) in response to stromal cell secretory protein in a dose-dependent manner, whereas bovine serum albumin had no effect on stimulating tumor cell migration. Immunoprecipitation of the stromal cell secreted protein with a nerve growth factor antibody partially and significantly reduced its stimulatory activity for tumor cell migration. A Zigmond-Hirsch matrix assay of tumor cell migration in response to various concentration gradients of stromal cell secreted protein demonstrated both chemotaxis and chemokinesis by all three cell lines. These results are consistent with the stromal cell secreted protein stimulation of chemokinetic tumor cell migration through the capsule of the prostate. Outside of the prostate gland metastasis of tumor cells may occur by chemotaxis to preferential sites containing chemoattractants similar to or related to maintenance factors that can substitute for components of stromal cell secretory protein.

INTRODUCTION

Prostate cancer has become the most commonly diagnosed form of malignant neoplasia in men throughout North America (1). Since the incidence of prostate carcinoma increases rapidly above the age of 50 years (2) and the modal adult male population is progressively aging, the incidence of prostate cancer is projected to further increase over the next several decades (3). Hence, the mechanisms which regulate prostate cancer growth and metastasis may be particularly relevant to the development of clinical strategies for the treatment and control of the disease. Prostate tumor cells have been shown to respond to soluble factors secreted from stromal cells for their growth (4–6) and protein secretion (7). A NGF-like protein has been identified as a candidate for the paracrine growth factor (5). In the human prostate the NGF-like protein localizes to the stroma in vivo, whereas a corresponding low-affinity NGFR localizes to the epithelium in vivo (8). Since low-affinity NGFR expression is partially reduced in the neoplastic prostate (in situ carcinoma and benign prostatic hyperplasia) and NGFR expression is absent in metastatic prostate tumor cell lines (TSU-prl, PC-3, DU-145, and LNCaP), loss of the NGFR expression appears to be inversely associated with the neoplastic progression of the human prostate (9). A high-affinity NGFR of the trk tyrosine kinase family of protooncogene products is also thought to occur in the epithelia of the human prostate (10). This trk-related protooncogene product may mediate the growth-promoting action of the stromal NGF-like protein on the TSU-prl metastatic prostate tumor cell line (10).

Prostate cancers which grow to a critical volume of 4 to 12 cm³ exhibit extracapsular metastases (11), which in turn are correlated with a poor prognosis for the patient. The primary path of prostate tumor cell metastasis through the nerve pedicles may be accounted for by anatomical considerations of the path of least resistance (12). However, following extracapsular migration of the tumor cells from the prostate, many metastases form at preferential sites (3), suggesting that tissue-specific factors present in the target organ may serve as chemoattractants (13, 14). In particular, prostate tumor cells migrate toward and preferentially adhere to the stroma within bone marrow (15). Indeed, conditioned media from stromal cells of bone marrow stimulate growth of the PC-3 human prostate carcinoma cell line (16).

Since a NGF-like protein secreted by prostate stromal cells and its corresponding receptor system on epithelia have been implicated in human prostate neoplasia (5, 8, 9), and a high-molecular-weight NGF has been shown to stimulate embryonal carcinoma cell migration (17), we undertook to investigate whether human prostate stromal cell-derived NGF-like protein may facilitate prostate tumor cell migration. For these studies we utilized a modification of the quantitative Boyden chamber chemoinvasion assay (14) which has been shown to correlate with the capacity for metastatic dissemination in animal models (18, 19). Specifically, we used a modified blind-well Boyden chamber chemoinvasion assay (20) in which a permissive coat of reconstituted basement membrane (Matrigel) has been substituted for collagen. In this context, cell migration in response to growth factors is thought to play an important role in cancer cell dissemination and metastasis (21).

MATERIALS AND METHODS

Culture of Human Prostate Cell Lines. PC-3 and DU-145 prostate epithelial tumor cell lines were obtained from the American Type Culture Collection (Rockville, MD). The TSU-prl prostate epithelial tumor cell line was a generous gift from Dr. John Isaacs (Johns Hopkins University, Baltimore, MD). The hPS cell line was established from an adult male undergoing transurethral prostate resection at Georgetown University Hospital (5). The PC-3, DU-145, TSU-prl, and hPS cell lines were cultured as previously described (9).

In brief, the four cell lines were maintained in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with antibiotics/antimycotic (100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone; Sigma Chemical Co.), 10% fetal bovine serum (Sigma Chemical Co.), 10% fetal bovine serum (Sigma Chemical Co.), and 0.1 µM testosterone (Sigma Chemical Co.). All cell cultures were incubated at 37°C in 5% CO2/95% air, and the medium was replaced every second day.

Preparation of hPS Secretory Protein. After subculture of the hPS cells in 10% fetal bovine serum containing medium as described above, the medium was replaced with fresh RPMI 1640 supplemented with 0.1 µM testosterone. Secretory protein from the hPS cells was collected and concentrated as previously described (5). The hPS cell secretory protein was subsequently recon-
stimated in RPMI 1640 supplemented with 0.1 μM testosterone. In some cases the hPS cell secretory protein was immunoprecipitated with anti-NGF antibody-conjugated protein A-Sepharose beads (22). For the preparation of the NGF antibody/protein A-Sepharose complex, either a monoclonal (Boehringer Mannheim Biochemica, Indianapolis, IN) or polyclonal (Collaborative Research, Inc., Bedford, MA) antibody was used. As an antibody control normal rabbit serum (Sigma Chemical Co.) was substituted for the antibody in the conjugate preparations.

Migration Assay of Tumor Cell Lines. Tumor cell migration in blind-well Boyden Chambers (Neuro Probe, Inc., Cabin John, MD) was carried out as previously described (14, 18) with recent modifications. Polyvinylpyrrolidone-free polycarbonate filters of 12-μm pore size (Poretics Corp., Livermore, CA) were coated with 10 μg/50 μl of reconstituted basement membrane Matrigel (Collaborative Research, Lexington, MA) and allowed to air dry in a flow hood overnight. A coating of 10 μg/filter of Matrigel was found previously not to discriminate between the migration of noninvasive and highly metastatic K1675 murine melanoma cells4 and appeared not to constitute a basement membrane-like barrier. Accordingly, the Boyden chamber assays used in this study are referred to as assays of chemomigration rather than chemoinvasion.

For the assay of tumor cell migration in response to various concentrations of hPS secretory protein, Boyden chambers of 200 μl lower compartment volume and 800 μl upper compartment volume (large chambers) were utilized (Neuro Probe, Inc.,). In the lower compartment we placed 220 μl of hPS secretory protein at a concentration of 0–100 μg/ml. As a protein control the hPS secretory protein was substituted with a similar concentration of BSA. Matrigel coated filters were soaked in RPMI 1640/0.1% BSA/testosterone and placed over the sample solution (usually hPS secretory protein) in the lower compartment, taking care to exclude the formation of air bubbles between the filter and sample solution. The upper compartment was mounted on the filter, after which a single cell suspension of prostate tumor cells (3 × 106 cells) in 800 μl of RPMI 1640/0.1% BSA/testosterone was placed in the upper well.

For the Zigmond-Hirsch assay (23) of tumor cell chemotaxis in response to various concentration gradients of hPS secretory protein, small Boyden chambers (25 μl lower compartment volume and 200 μl upper compartment volume; Neuro Probe, Inc.) were utilized. Within the lower compartment was placed 0–50 μg/ml hPS secretory protein. The Matrigel-coated filter was placed over the lower compartment solution, the upper compartment was mounted in place, and 170 μl of cell suspension (7 × 106 cells) with 0–50 μg/ml hPS secretory protein were placed in the upper compartment. Both the small and large Boyden chambers were incubated at 37°C in 5% CO2/95% air for 5 h.

Quantitation of Cells Migrated across the Filter of the Boyden Chambers. Cells which had migrated through the filter of the large Boyden chambers in response to 0–60 μg/ml hPS were quantitated by the crystal violet staining technique of Gillies et al. (24), as recently adapted for use with Boyden chamber filters (25). At the termination of the incubation period the filters were removed from the Boyden chambers, inverted, and pinned to a wax plate with the migratory cells which had traversed the filter facing upward. The nuclei of these cells (Fig. 1A–C). This correlation was statistically significant (r = 0.94, P < 0.05), and the DU-145 cells (r = 0.98, P < 0.05).

The migrated cells on some filters were restained with hematoxylin and thiazole (Diffquick Kit, American Scientific Products, McGaw Park, IL) for quantitation of cell numbers. The restained cells which had traversed the filter were electronically quantitated with a Zeiss IBAS 2000 image analysis system with a Kontron Alog processor interfaced with a Zeiss Axiopt microscope equipped with an automated stage (26).

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 duplicate replicates obtained from four independent experiments.

RESULTS

Spectrophotometric Quantitation of Migrated Tumor Cells in the Boyden Chambers. The number of tumor cells which had migrated through the filter of the Boyden chambers was linearly correlated with the concentration of the crystal violet stain extracted from the nuclei of these cells (Fig. 1, A–C). This correlation was statistically significant for the TSU-prl cells (r = 0.93, P < 0.05), the PC-3 cells (r = 0.94, P < 0.05), and the DU-145 cells (r = 0.98, P < 0.05). Fig. 1D shows the number of cells per filter that migrated through the filter in response to various concentrations of hPS secretory protein. Interestingly, in the absence of chemotactant, the TSU-prl cells possessed an intrinsic migratory ability approximately 7-fold greater than that of either the DU-145 or PC-3 cells (Fig. 1D). In order of magnitude the TSU-prl cells migrated to the greatest degree in response to the stromal protein, followed by the DU-145 cells and lastly the PC-3 cells (Fig. 1D).

Dose-dependent Tumor Cell Migration in Response to Stromal Cell Secretory Protein. Fig. 2 (A–C) shows the dose-dependent migration of TSU-prl, DU-145, and PC-3 cells, respectively, through the Boyden chamber filters in response to 0–100 μg/ml hPS secretory protein or BSA. hPS secretory-protein significantly stimulated cell migration of all three tumor cell types (P < 0.01) in a dose-dependent manner up to a maximum stimulatory concentration of approximately 50 μg/ml hPS secretory protein (Fig. 2, A–C). The migration of the TSU-prl (DU-145, and PC-3 cells was maximally stimulated 2.6-fold, 13.3-fold, and 5-fold, respectively, by stromal cell secretory protein (Fig. 1D). In contrast, BSA had no effect (P > 0.05) on stimulating tumor cell migration at comparable concentrations of protein (Fig. 2, A–C). The insets to Fig. 2 (A–C) showed representative stained tumor cells which have migrated through the filters in response to 0–100 μg/ml hPS secretory protein. It is apparent that the density of the stained cells on the filters increases in concordance with the concentration of the crystal violet stain extracted from the migratory cells on the filter.

Partial Reduction of Tumor Cell Migration following Immunoprecipitation of Stromal Cell Secretory Protein with NGF Antibody. Table 1 shows the relative migration of the human prostate tumor cell lines TSU-prl, PC-3, and DU-145, in response to 100 μg/ml of hPS secretory protein that has been immunoprecipitated with or without a monoclonal or polyclonal antibody against NGF. After immunoprecipitation of hPS secretory protein with the NGF antibodies, the migration of prostate tumor cells was significantly reduced by 15%–64% relative to intact hPS secretory protein. The TSU-prl cells exhibited the greatest response and the DU-145 cell line exhibited the least response to immunoprecipitation of hPS secretory protein with NGF antibody. Substitution of the NGF antibodies with normal rabbit serum during immunoprecipitation of hPS secretory protein eliminated the reduction in tumor cell migration. Hence, a NGF-like protein in hPS secretory protein partially, but not completely modulates migration of the TSU-prl, DU-145, and PC-3 prostate tumor cell lines.

Chemotaxis and Chemokinesis of Tumor Cells in Response to Concentration Gradients of hPS Secretory Protein. Fig. 3 (A–C) shows the effect of varying concentration gradients of hPS secretory protein on the migration of TSU-prl, DU-145, and PC-3 cells, respectively. All three tumor cells migrated in a dose-dependent manner. When there was no hPS secretory protein in the upper compartment and the highest amount of hPS secretory protein was in the lower compartment (50 μg/ml) beneath the filter, tumor cells most actively

4 E. W. Thompson, unpublished data.
migrated ($P < 0.01$) consistent with the chemotaxis of these cells in response to directional chemical stimuli. Under these conditions the cell lines TSU-prl, DU-145, and PC-3 exhibited increases in chemotaxis of 47%, 79%, and 70% respectively, in response to 50 ng/ml hPS (Fig. 3, A–C). When a negative concentration gradient of hPS secretory protein was established (data above the diagonal), no significant ($P > 0.05$) chemotaxis was observed; however, some background random chemokinesis was evident at all concentrations of hPS secretory protein. Hence, hPS secretory protein contains both chemokinetic agents and chemotactic agents which can stimulate the directional migration of the prostate tumor cell lines TSU-prl, DU-145, and PC-3, respectively.

**DISCUSSION**

Spectrophotometric absorption measurements of crystal violet extracted from cell nuclei has been successfully utilized to estimate the cell number of BALB/c 3T3 mouse embryo fibroblasts (24) and MDA-MB-231 BAG human breast cancer cells (25). In this report we extend the utility of this technique to three human prostate tumor cell lines, TSU-prl, DU-145, and PC-3. The results demonstrate a positive correlation between cell number and concentration (absorbance) of extracted crystal violet for the three prostate tumor cell lines. However, since the slope and absolute magnitude of this relationship varied between the three cell lines, it is clear that for each new cell type under consideration a calibration curve characterizing the relationship between cell number and absorbance must be established. Nevertheless, the crystal violet technique of estimating cell numbers in conjunction with the Boyden chamber assay provides a simple, rapid (5 h), reproducible, and quantitative method of evaluating cell migration in response to chemoattractants.

Cell migration has been shown to be influenced by motility-stimulating proteins, cell adhesion molecules, substrate-adhesion molecules, metalloproteinases, and peptide growth factors (27). Cell migration may occur in response to either an immobilized substrate such as extracellular matrix (haptotaxis) or soluble factors which may induce random motility (chemokinesis) and directed motility along a positive concentration gradient of chemoattractant (chemotaxis). Several growth factors have been identified as chemoattractants (reviewed in Ref. 21). These chemoattractant growth factors include nerve growth factor (17), epidermal growth factor/transforming growth factor α (28, 29), the insulin family of polypeptides (30, 31), transforming growth factor β1 (32), the c-kit ligand (33, 34), platelet-derived growth factor (35), and basic fibroblast growth factor (36, 37). Enhanced levels of basic fibroblast growth factor in motile cells are coincident with the induction of urokinase plasminogen activator (38) and metalloproteinases (39), which are thought to facilitate the invasive migration of cells. Many of these chemoattractant growth factors also stimulate the growth of tumor cells (34) and have been termed

| Table 1 Migration of prostate tumor cell lines following immunoprecipitation of hPS secreted protein (100 µg/ml) with NGF antibody* |
|------------------------|-----------------|-----------------|
|                       | Poly            | Mono            |
| TSU-prl                | 36.4 ± 5.3      | 43.3 ± 4.9      |
| PC-3                   | 66.1 ± 6.7      | 53.0 ± 7.8      |
| DU-145                 | 95.9 ± 21.3     | 95.9 ± 21.3     |

* Relative migration of prostate tumor cell lines (TSU-prl, PC-3, DU-145) in response to a maximum stimulatory dose of hPS secreted protein (100 µg/ml) which has been immunoprecipitated with a polyclonal antibody (Poly), or monoclonal antibody (Mono) against NGF-β, or normal rabbit serum (NRS). Results are expressed as percentage migration of nontreated hPS secreted protein (100 µg/ml).

a $P < 0.01$.

b $P < 0.05$.

c Not significant.
Cell modulation of tumor cell chemotaxis and chemokinesis

The NGF-like protein, also contribute to chemoattractant activity is consistent with the observation that some growth factor chemoattractants work in concert with other soluble cofactors to maximally stimulate cell migration (40). Indeed, the chemoattractant activity of the NGF-like

mitoattractants (28), although in some cases growth factor-mediated cell migration and proliferation operate by completely independent mechanisms (40). The NGF-like factor secreted by human prostate stromal cells appears to be a mitoattractant, since it participates in the proliferation of a prostate tumor cell line (5) and it constitutes one component of the chemoattractant activity in the stromal cell secretory protein for three prostate tumor cell lines. The observation that components of the stromal cell secretory protein, other than the NGF-like

Fig. 2. Dose-dependent migration of TSU-pr1 (A), DU-145 (B), and PC-3 (C) prostate tumor epithelial cells through Matrigel-coated filters in Boyden chambers in response to various concentrations (0–100 μg/ml) of hPS cell secretory protein or BSA. Prostate tumor cells were quantitated by crystal violet staining. Inset, representative stained tumor cells on filters that have migrated across the filter in response to the various concentrations of hPS secretory protein. Points, mean from four independent experiments; bars, SEM.

Fig. 3. Chemotaxis of TSU-pr1 (A), DU-145 (B), and PC-3 (C) prostate tumor epithelial cells through Matrigel-coated filters in Boyden chambers in response to concentration gradients of hPS secretory protein. Chemotactic prostate tumor cells were quantitated by staining with crystal violet, and the concentration of the extracted dye was measured at an absorbance of 540 nm. Each data point represents the mean (± SEM) of absorbance at 540 nm from four independent experiments.
protein varied between the three cell lines. Since the DU-145 cell line showed the greatest stimulation of chemomigration in response to hPS but the lowest reduction of chemomigration following immunoprecipitation of hPS with the NGF antibodies, other growth factors may predominate as chemoattractants for the DU-145 cell line.

The three prostate tumor cells examined exhibited some degree of chemokinesis and chemotaxis in response to stromal cell secretory protein. Since epithelia within the prostate organ are surrounded and continually bathed in stromal cell secretory protein, the establishment of chemoattractant concentration gradients is unlikely to occur over a significant distance. Hence, migration of tumor cells within the capsule of the prostate may occur more as a function of chemokinesis along anatomical paths of least resistance, predominantly by perineural (12) and ejaculatory duct (41) invasion. Nevertheless, following extracapsular invasion prostate tumor cells preferentially metastasize to specific anatomical sites, perhaps in response to organ-derived chemoattractants (13). Hence, the capacity for chemotaxis of the three prostate tumor cell lines examined in this study is consistent with extracapsular migration toward preferential sites which most likely contain the best available combination of maintenance factors that will support the survival and growth of the tumor cells. Many chemoattractants are relatively specific for the migratory responder cell type (28). Hence, the maintenance factors which occur at preferential sites of metastasis may include chemoattractants that are capable of substituting for components of stromal cell secretory protein or are related to the paracrine growth factors therein, such as the NGF-like protein, which promotes the growth of epithelial cells.

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