Acceleration of Human Prostate Cancer Growth in Vivo by Factors Produced by Prostate and Bone Fibroblasts

Martin Gleave, Jer-Tsong Hsieh, Chuan Gao, Andrew C. von Eschenbach, and Leland W. K. Chung

Urology Research Laboratory, Department of Urology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

Prostate cancer, the most prevalent cancer affecting men, frequently metastasizes to the axial skeleton where it produces osteoblastic lesions with growth rates often exceeding that of the primary tumor. To evaluate the role of tumor-cell-host stromal interaction and stromal specific growth factors (GFs) in prostate cancer growth and progression, we cocultivated athymic mice with human prostate cancer cells (LNCaP) and various nontumorigenic fibroblasts. LNCaP tumor formation was most consistently induced by human bone (NS) fibroblasts (62%), followed by embryonic rat urogenital sinus mesenchymal (rUGM) cells (31%) and Noble rat prostatic fibroblasts (17%), but not by NIH-3T3, normal rat kidney, or human lung CDD1 fibroblasts. Carcinomas formed preferentially in male hosts, demonstrating in vivo androgen sensitivity. The human prostate component of these tumors was confirmed with immunohistochemical staining for prostate-specific antigen (PSA), Northern analysis for PSA expression, and Southern analysis for human repetitive Alu sequences. Elevations in serum PSA paralleled the histomorphological and biochemical findings.

LNCaP and fibroblast cell-conditioned media (CM) was used to determine whether autocrine and paracrine mitogenic pathways exist between LNCaP and fibroblast cells in vitro, and various defined GFs were tested to identify possible active factors. Mitogenic assays revealed a 200–300% bidirectional stimulation between LNCaP and bone or prostate fibroblast-derived CM. Lung, normal rat kidney, and 3T3 fibroblast CM were not mitogenic for LNCaP cells. Among the purified GFs tested basic fibroblast growth factor (bFGF) was the most potent mitogen, stimulating LNCaP growth 180% in a concentration-dependent manner. Transforming growth factor α and epidermal growth factor were both minimally mitogenic. Cocultivation of LNCaP cells with a slowly adsorbed matrix (Gelfoam) adsorbed with bFGF or dialyzed and concentrated rUGM or MS CM was also capable of inducing LNCaP tumor formation in vivo. These observations illustrate that fibroblasts differentially modulate prostate cancer growth through the release of paracrine-mediated GFs, possibly including bFGF, and that tumor-stromal cell interactions play an important role in prostate cancer growth and progression.

INTRODUCTION

Increased incidence of prostate cancer during the last decade has established prostate cancer as the most prevalent cancer and the second leading cause of cancer deaths in men (1). Most patients dying of prostate cancer experience painful and sometimes crippling osseous metastases with up to 84% having bony metastases at autopsy (2). Prostate cancer selectively spreads to the cancellous bones of the axial skeleton, where it is the only malignancy to consistently produce osteoblastic lesions (3). Frequently, these bony metastatic lesions grow at a more rapid rate than that of primary or other metastatic lesions (4), suggesting bidirectional interaction between prostate cancer and bone cells. Several factors may be responsible for this pattern of metastases. Batson (5) proposed that prostate cancer cells selectively seed the lumbar spine and pelvis via a paravertebral venous plexus through which retrograde flow from the prostate to the spine may occur at times of increased intraabdominal pressure (6). The "seed and soil" hypothesis initially described by Paget in 1889 (7) proposes that tumor cells may selectively grow in certain organs because of enhanced adhesion (8, 9), chemotaxis (10, 11), or growth (12, 13) at these sites.

The factors involved in prostatic carcinogenesis and progression with the development of nonrandom metastases remain poorly defined. A variety of GFs have been shown to enhance prostate cell growth in vitro (14–17), but the potential in vivo growth-promoting effects of these factors have not been studied. For this reason, we are using an in vivo and in vitro human prostate cancer model to assess the mechanisms involved in tumor-stromal cell interaction and the role of specific GFs in prostate cancer growth and metastasis (18, 19). In this study we compared the ability of different fibroblasts and their serum-free CM to stimulate the growth of a human prostate cancer cell line, LNCaP (20). The purposes of this study were to assess whether tissue-specific fibroblasts differentially stimulate LNCaP tumor growth in vivo and whether fibroblast GFs act in a paracrine fashion to stimulate LNCaP growth both in vitro and in vivo. The LNCaP cell line is generally considered nontumorigenic when inoculated s.c. in athymic mice, which allows us to test the inductive capabilities of specific fibroblasts. Also, the LNCaP cell line is the only prostate cell line that is PSA positive (21), a clinically important human tissue-specific antigen which would serve as a useful serum (22) and immunohistochemical (23) marker to monitor in vivo prostate cancer cell growth.

Our results demonstrate that LNCaP tumors can be reproducibly induced in vivo in male (but not female) hosts by coinjecting LNCaP cells with specific fibroblasts only. We observed that human bone fibroblasts were the most effective, followed by prostate-derived fibroblasts, while LNCaP tumors were not induced by human lung, NIH-3T3, or kidney fibroblasts. Prostate- and bone-derived fibroblast CM stimulated LNCaP growth in vitro. Moreover, a novel method that delivered concentrated prostate and bone fibroblast-derived CM in vivo by adsorption of putative GFs onto a solid matrix also induced LNCaP growth in vivo. The potential significance of this model to elucidate mechanisms involved in mesenchymal-epithelial interaction in prostate cancer progression and sitespecific metastases will be discussed.

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2 To whom requests for reprints should be addressed, University of Texas M. D. Anderson Cancer Center, Urology Research Laboratory, Box 026, 1515 Holcombe Blvd., Houston, TX 77030.
MATERIALS AND METHODS

Cell Lines and Cell Culture. LNCaP cells, passage 29, were kindly supplied by Dr. Gary Miller (University of Colorado, Denver, CO) and grown in RPMI 1640 (Irvine Scientific, Santa Anna, CA) with 10% FBS. Phenotypically, the cells resembled parental lines as evidenced by the results of karyotypic analysis (performed by Dr. Gary Miller) and our androgen receptor analysis (see below).

Six nontumorigenic mesenchymal cell lines in this study include: (a) rUGM, a fetal urogenital sinus mesenchyme-derived cell line from 18-day-old Noble rat fetuses, developed in this laboratory using a procedure described previously (24). rUGM cells were maintained in DMEM (Gibco Laboratories, Grand Island, NY), 5% CS, and passages 14–16 were used. (b) A human bone fibroblast cell line, MS, derived from an osteogenic sarcoma, was established by Dr. A. Y. Wang (The University of Texas M. D. Anderson Cancer Center, Houston, TX). MS cells were maintained in T medium (80% DMEM, 20% F12K (Irvine Scientific), 3 g/liter NaHCO3, 100 units/ml penicillin G, 100 µg/ml streptomycin, 5 µg/ml insulin, 13.6 µg/ml triiodothyronine, 5 µg/ml transferrin, 0.25 µg/ml biotin, and 25 µg/ml adenine) with 5% FBS; passages 29–33 were used. (c) A rat prostatic fibroblast line, NbF-1, was established from normal Noble rat ventral prostate gland using procedures previously described (25). NbF-1 cells were maintained in DMEM and 5% CS and nontumorigenic passages 18–22 were used. (d) Normal adult human lung fibroblasts, CCD16 (American Tissue Culture Catalogue CCL 201), were kindly supplied by Dr. J. Roth (Department of Thoracic Surgery, University of Texas M. D. Anderson Cancer Center, Houston, TX), and passages 14–16 were used. (e) NIH-3T3 cells (American Type Culture Collection 6587), derived from embryonic mouse tissue, were supplied by Dr. D. Becker (University of Texas M.D. Anderson Cancer Center, Houston, TX) and maintained in DMEM with 5% CS. (f) NRK fibroblasts (American Type Culture Collection 6509) were grown in DMEM with 5% CS and passages 10–12 were used.

CM from LNCaP and all 6 fibroblast cell lines were collected and prepared as follows: Cells were cultured in 150-mm tissue culture dishes (Falcon; Becton Dickinson Laboratories, Lincoln Park, NJ) with T medium, 2% TC, a serum-free defined media supplement (Celox Co., Minnetonka, MN), and 1% FBS until 60–70% confluent, washed with PBS/EDTA, and changed to serum-free T medium containing 2% TC only. After 48 h, the CM was removed and filtered through a 0.2-µm filter (Nalge Co., Rochester, NY), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) was added. Protein concentrations in the CM were determined using a protein assay (Bio-Rad Laboratories, Richmond, CA), and ranged from 70–100% of control (T medium and 2% TC; 1.3 mg/ml). The CM was dialyzed at 4°C against distilled water containing 0.1 mM phenylmethylsulfonyl fluoride using Spectra/Por 3 dialysis membranes (Mw > 3500; PGC Sciences, Gaithersburg, MD) for 96 h, changing the water after 48 h. The samples were lyophilized to dryness and reconstituted in T medium to a 10-fold concentration, filtered, and diluted to the desired working concentration (0.1- to 2-fold) with T medium containing 2% TC.

Assessment of in Vivo Tumor Growth. To determine the ability of specific fibroblasts to elicit LNCaP growth in vivo, 6- to 8-week-old athymic nude mice (BALB/c strain; Charles River Laboratory, Wilmington, MA) of both sexes were coinoculated s.c. with 1 × 106 LNCaP cells and 1 × 106 of one of the 6 fibroblast cell lines described previously. Up to 5 × 106 LNCaP cells and 2 × 106 of each of the fibroblast cell lines were injected alone as controls to assess their tumorigenicity. The cells were suspended in 0.1 ml of RPMI 1640 with 10% FBS prior to injection and inoculated via a 27-gauge needle. Tumors were measured twice weekly and their volumes were calculated by the formula L × W × H × 0.5236 (26). At the time of sacrifice, sternotomy was performed and a cardiac puncture was carried out to obtain serum for PSA analysis. Tumors were excised, weighed, and subjected to various morphological and biochemical analyses (see below).

Other experiments were performed to determine whether LNCaP tumor growth in vivo could be affected by soluble growth factors alone. LNCaP cells were injected along with a Gelfoam preparation (Upjohn, Kalamazoo, MI), adsorbed with type IV collagen (Collaborative Research, Bedford, MA), ECGF (Collaborative Research), and a 10-fold concentrated rUGM or MS CM. This novel matrix system was developed through modification of a previously described procedure (27) and serves as a reservoir for delivery of biologically active factors in vivo. ECGF was chosen as a marker of physiological response to determine whether it could retain its biological activity with this technique and whether this angiogenesis alone would be sufficient to promote tumor formation. rUGM and MS CM were used because these cells could induce LNCaP growth in vivo. bFGF (Collaborative Research) was also used because of its mitogenic effect on LNCaP cells in vitro (see below). Under sterile conditions, Gelfoam, a solid gelatin sponge, was presoaked with 100 µg/ml collagen IV for 12 h at 4°C, followed by either 1 µg/ml ECGF, bFGF, or a 10-fold concentrated stromal CM for 1 h. The Gelfoam was then miniced using a polytron to allow s.c. inoculation via an 18-gauge needle. Following s.c. injection of 0.1 ml Gelfoam, the same site was injected with 2 × 106 LNCaP cells using a 27-gauge needle. For controls, 2 × 106 LNCaP cells were inoculated with Gelfoam and collagen IV, with or without ECGF. Tumor incidence and size were monitored as described above.

Histology and Immunohistochemistry. For routine histology, specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Fixed sections, 8 µm, were cut and stained with hematoxylin and eosin. For immunohistochemistry studies, specimens were deparaffinized with xylene, rehydrated with 70% ethanol, and treated with 0.1% trypsin for 10 min at 37°C. Sections were then incubated with monoclonal antibodies prepared against cytokeratin, PSA, or PAP (Biogenex, Dublin, CA). The avidin-biotin complex method was used with all specimens using fast red AEC as chromogens (Biogenex). Slides were counterstained with aqueous hematoxylin and mounted with glycerol for visual inspection and photography.

Determination of Serum PSA Values. Animals were killed by cardiac puncture under methoxyflurane anesthesia. Blood was allowed to clot at 37°C and centrifuged, and the serum was stored at −20°C. PSA values were determined using a dual reactive enzymatic immunoassay kit with a lower limit of sensitivity of 0.4 ng/ml (Hybritech Inc., San Diego, CA).

DNA Isolation and Southern Blot Analysis. Tissue DNA was isolated from tumors according to a method previously described (28). DNA concentration was determined with a spectrophotometer. DNA specimens were applied to Zetaprobe membranes (Bio-Rad) and then baked at 80°C for 90 min prior to hybridization with a 32P-labeled human Alu-repetitive sequences probe (Oncor, Gaithersburg, MD).

RNA Isolation and Northern Blot Analysis. Total cellular RNA was prepared from frozen tissues by the 4 M guanidinium thiocyanate extraction method (29). Typical yields of total cellular RNA were about 300 µg/200 mg tissue as quantified spectrophotometrically using 40 µg RNA/450,000 unit. RNA was denatured in 50% formamide/18% formaldehyde at 55°C and fractionated by electrophoresis in a 0.9% denaturing formaldehyde agarose gel. Samples were transferred onto a Zetaprobe membrane (Bio-Rad) by the capillary method, and the membrane was then baked for 2 h at 80°C. Following this, the membrane was prehybridized in the presence of 1 M NaCl, 0.1% dextran sulfate, 1% sodium dodecyl sulfate, and 200 µg/ml salmon sperm DNA for at least 2 h at 65°C. Hybridization was carried out at 65°C overnight with a random primer-labeled probe as indicated. The complementary DNA probe for PSA was a kind gift from Dr. D. Tindall (Mayo Clinic, Rochester, MN) (30). Finally, the membrane was washed under high stringency conditions (0.5 × SSC and 1% sodium dodecyl sulfate at 65°C). Autoradiograms were prepared by exposing Kodak X-OMat AR film to the membrane at −80°C with intensifying screens.

Mitogenic Assays. To determine the mitogenic activity of androgens (testosterone and dihydrotestosterone; Sigma) and CM prepared from various types of fibroblasts on the growth of human LNCaP cells in vitro, we used a 96-well assay based on the uptake and elution of crystal violet dye by the cells in each well (31, 32). Various defined GFs, including bFGF, TGFβ1, TGFβ3, and EGF (Collaborative Research) were also tested. Falcon 96-well plates were used and 3000 LNCaP cells, 500 MS, or 200 rUGM cells were plated per well (in T medium containing 1% charcoal stripped CS and 2% TC). Twenty-four h later, the cells were downshifted to serum-free condition (see above) with
various concentrations of androgens, GFs, or CM. To avoid stripping poorly adherent LNCaP cells with each media change, media was partially removed by gentle suction and 100 µl of fresh media was added in 50-µl aliquots. The medium was changed every 2 days; 7–10 days later the cells were fixed in 1% glutaraldehyde (Sigma) and stained with 0.5% crystal violet (Sigma). Plates were washed and air dried, and the dye was eluted with 100 µl Sorenson’s solution (9 mg trisodium citrate in 305 ml distilled H2O, 195 ml of 0.1 N HCl, and 500 ml 90% ethanol). Absorbance of each well was measured by a Titertek Multiskan at 560 nm. Control experiments demonstrated that absorbance is directly proportional to the number of cells in each well.

Androgen Receptor Assays. Whole cell androgen receptor assays were performed as described previously by Guthrie et al. with modification (33). In brief, LNCaP cells were plated in T medium plus 5% FBS in 6-well plates (Falcon) and downshifted to 0.4% charcoal-stripped CS 24 h preceding the assay. Just prior to beginning the assay, this medium was removed and cells were washed twice with PBS/EDTA, and T medium with various dilutions of [3H]R1881 (methyltrienolone 81.8 Ci/mmol; DuPont Co., Wilmington, DE) was added to appropriate wells. In some wells, unlabeled R1881 (200-fold of [3H]R1881) was added to determine the extent of nonspecific binding. Following 90-min incubation at 37°C, the media were removed, cells were washed with ice cold PBS/EDTA, and 1 ml of 100% ethanol was added to each well. A 500-µl aliquot was added to a scintillation vial and counted with a scintillation counter (Beckman Instruments, Inc., Houston, TX).

RESULTS

Effect of Coinoculated Fibroblasts on LNCaP Tumor Growth. The incidence of tumor formation in mice coinoculated with LNCaP cells and various types of fibroblasts is compared in Table 1. The observation period for all injections was 3 months. LNCaP and all fibroblast cell lines were nontumorigenic (0 of 20) with injections of up to 5 × 10⁶ or 2 × 10⁶ cells, respectively. No sex differences in tumor formation were observed in hosts coinoculated with LNCaP and rUGM cells, with an overall tumor incidence of 61% for males and 50% for females. The average latency period for measurable tumor growth was 42 days in male and 45 days in female hosts. No difference in tumor volume or latency period was observed by increasing the rUGM inoculum from 1 × 10⁶ to 1 × 10⁷ cells. Mean tumor volume was 322 ± 106 (SE) mm³. No sex differences in the incidence of tumor formation was observed in hosts coinoculated with LNCaP and 3T3 cells (67%; mean tumor volume, 420 mm³). In contrast, marked sex differences in tumor induction were observed with coinoculation of LNCaP and human bone (MS) or LNCaP and rat prostatic (NbF-1) fibroblasts, because these tumors formed only in male hosts (62% and 17%, respectively). Mean tumor volume for LNCaP/MS and LNCaP/NbF-1 tumors was 238 ± 74 and 172 ± 52 mm³, respectively. Lung CCD16 and NRK fibroblasts did not induce chimeric tumor growth in either sex. The histomorphology and relative content of LNCaP cells in the various fibroblast-induced tumors differed markedly, as characterized below.

Characterization of the Chimeric Tumors. Chimeric tumors were characterized histologically, immunohistochemically, and biochemically. A difference in histomorphology of LNCaP/rUGM chimeric tumors was noted between male and females: in males, 51% of tumors (or 31% of inoculation sites) were carcinosarcomas, with a predominantly epithelioid component separated by strips of mesenchymal cells (Fig. 1a), while 89% (16 of 18) of the tumors in females were pure sarcomas (Fig. 1b). We found MS bone fibroblasts to be the most potent inducer of LNCaP tumor formation. All tumors were carcinosomas composed of sheets of poorly differentiated epithelial cells with minimal mesenchymal cells and formed at 62% of inoculated sites in male hosts (Fig. 1e); no tumors formed in female hosts. NbF-1 cells were also capable of inducing LNCaP tumor growth in male hosts, but not as well as the MS or rUGM cells; three carcinomas formed from 18 inoculations (17%). LNCaP/3T3 tumors, however, were all sarcomas with no epithelial component. No tumors formed with coinoculation of LNCaP with human lung CCD16 or NRK fibroblasts.

The prostatic origin of the epithelial cells participating in the MS-, rUGM-, and NbF-induced tumor formation in male hosts was confirmed with immunohistochemical staining procedures using monoclonal antibodies directed against PSA, PAP, and cytokeratin. The epithelial component of these tumors stained intensely positive for PSA using fast red as the chromogen (Fig. 1, d and f) with no staining of the associated stromal component. The epithelial component of these tumors also stained positively for PAP and cytokeratin (not shown) but in an irregular and scattered manner compared to PSA. In contrast, sarcomas arising from LNCaP/rUGM inoculations in females and LNCaP/3T3 inoculations in both males and females stained negatively for PSA (Fig. 1e), PAP, and cytokeratin (not shown).

Biochemical characterization using Northern and Southern hybridization techniques corroborated the histological findings to confirm the human prostatic origin of the epithelial component of the chimeric tumors (Fig. 2). The LNCaP/rUGM tumors in male hosts contained a predominantly human component as manifested by the presence of Alu sequences in 6 (2 weakly) of 7 tumors examined, compared to none in female tumors (Fig. 2a). PSA expression was more variable in these tumors and did not correlate consistently with the histomorphological and Southern dot blot analysis, likely because of different sampling from a heterogenous carcinosarcoma. All LNCaP/MS tumors were strongly positive for PSA expression and human-specific Alu sequences on Northern and Southern analysis, respectively (Fig. 2b). None of the LNCaP/3T3 tumors that formed had any human prostate component (Fig. 2b).

Table 1. Incidence of tumor induction by coinoculation of LNCaP cells with various fibroblasts in vivo

<table>
<thead>
<tr>
<th>Fibroblast</th>
<th>Host</th>
<th>Incidence of tumor formation</th>
<th>Histomorphology of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>Male</td>
<td>8/13 (62%)</td>
<td>Carcinosarcoma</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0/10 (0%)</td>
<td>Sarcoma</td>
</tr>
<tr>
<td>rUGM</td>
<td>Male</td>
<td>31/51 (61%)</td>
<td>Carcinosarcoma</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>18/36 (50%)</td>
<td>Sarcoma</td>
</tr>
<tr>
<td>NbF-1</td>
<td>Male</td>
<td>3/18 (17%)</td>
<td>Carcinosarcoma</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0/10 (0%)</td>
<td>Sarcoma</td>
</tr>
<tr>
<td>3T3</td>
<td>Male</td>
<td>8/12 (67%)</td>
<td>Carcinosarcoma</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>4/6 (67%)</td>
<td>Sarcoma</td>
</tr>
<tr>
<td>CCD16</td>
<td>Male</td>
<td>0/20 (0%)</td>
<td>Carcinosarcoma</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0/6 (0%)</td>
<td>Sarcoma</td>
</tr>
<tr>
<td>NRK</td>
<td>Male</td>
<td>0/20 (0%)</td>
<td>Carcinosarcoma</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0/10 (0%)</td>
<td>Sarcoma</td>
</tr>
</tbody>
</table>

* All carcinomas with no sarcomatous component.
PROSTATE CANCER GROWTH ACCELERATED BY BONE FIBROBLASTS

Fig. 1. Histomorphological and immunohistochemical characterization of fibroblast-induced LNCaP chimeric tumors. Hematoxylin and eosin-stained sections (a-c) reveal differences between LNCaP/rUGM tumors in male (a) and female (b) hosts, the former a carcinosarcoma, the latter a pure sarcoma with no epithelial component. LNCaP/MS tumors (c) formed only in male hosts and histologically are vascular carcinomas with a minor mesenchymal component. Immunohistochemical staining with monoclonal antibodies against PSA (d-f) demonstrates intense and generalized staining of the epithelial cells in only male LNCaP/rUGM (d) and LNCaP/MS (f) tumors but not of the sarcomatoid LNCaP/rUGM tumors (e) in female mice. LNCaP/3T3 tumors (not shown) are sarcomas histologically similar to LNCaP/rUGM tumors in females and also stained negatively for PSA.

enzyme immunoassay. Four control males in which human bladder transitional cell carcinoma cells were injected (22) had undetectable PSA levels, as anticipated, because PSA is a human prostate marker. Significant differences in median serum PSA values were observed among the different fibroblast-induced tumors as well as male and female hosts, paralleling differences in their histomorphology (Fig. 3). LNCaP/MS tumors were associated with consistently elevated serum PSA levels, ranging from 25.1 to 323 ng/ml, with a median of 68.1 ng/ml (n = 6). Similarly, male hosts bearing LNCaP/Nbf-1 tumors had elevated serum PSA (n = 4). However, nontumor-bearing females with LNCaP/MS and LNCaP/Nbf-1 injections had undetectable serum PSA levels. Serum PSA values in males with LNCaP/rUGM tumors ranged from 0.4 to 348 ng/ml with a median of 16.1 ng/ml; 11 of 12 males had detectable levels and 3 had levels >100 ng/ml. In all but one of the 8 females with LNCaP/rUGM tumors, serum PSA was undetectable. All animals with LNCaP/3T3 tumors, as well as those inoculated with LNCaP/CCD16 or LNCaP/NRK cells, had undetectable serum PSA levels.

LNCaP Androgen Sensitivity and Androgen Receptor Content. To determine whether the LNCaP cell line was indeed androgen sensitive, we evaluated in vitro mitogenic effects of testosterone and dihydrotestosterone in serum-free and chemically defined medium. Peak responses were seen with 5 × 10⁻¹⁰ M testosterone and 1 × 10⁻¹⁰ M dihydrotestosterone, producing 82 and 43% increases, respectively, in cell number over 9 days when compared to controls grown in serum- and hormone-free media (Fig. 4a). Whole cell androgen receptor assays revealed the presence of a substantial number of high-affinity androgen receptors (Kd = 0.23 nM; Bmax = 332 fmol/mg protein; Fig. 4b).

Effect of Defined Growth Factors on LNCaP Cells in Vitro. To identify possible mitogens involved in LNCaP cell growth, we established a dose-response relationship between LNCaP cells and bFGF, EGF, TGFα, and TGFβ. Concentrations ranging from 0.1 to 50 ng/ml were used, and bFGF stimulated LNCaP cell growth 180% in a concentration-dependent manner compared to cells grown in serum-free media alone (Fig. 5a). Minimal increases in cell number compared to controls were seen with EGF and TGFα over a wide range of concentrations. TGFβ, at 1 ng/ml, inhibited LNCaP cell growth by 70%. Time-course studies also revealed that bFGF (50 ng/ml) stimulated LNCaP cell growth in a linear fashion during a 9-day observation period (Fig. 5b).

Effect of Fibroblast-conditioned Medium on the Growth of LNCaP Cells in Vitro. To determine whether the in vivo fibroblast specificity in inducing LNCaP growth could be explained by specific soluble GFs produced by the fibroblasts, we compared the mitogenic activity of CM from MS, rUGM, Nbf-1, 3T3, CCD16, and NRK cells on LNCaP growth in vitro. The CM from MS, rUGM, and Nbf-1 cells stimulated LNCaP cell growth up to 210% compared to controls (Fig. 6a); 3T3, CCD16, and NRK CM were ineffective. This paracrine effect is bidirectional, because LNCaP CM stimulated rUGM cell growth up to 275% (Fig. 6b) and MS CM growth 225% (data not shown). The bidirectional paracrine stimulation between LNCaP and rUGM or MS cells in vitro is CM concentration dependent. No autocrine stimulatory effect occurred by expos-
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Fig. 2. Southern and Northern analysis of fibroblast-induced LNCaP chimeric tumors. Portions of tumors removed at the time of sacrifice were processed separately for DNA and RNA isolation as described in the text. Controls consisted of human bladder cancer (+) and rUGM (−) cells. Various concentrations of DNA were loaded and probed for repetitive Alu sequences to identify human cells. RNA, 20 μg, was loaded and probed with a complementary DNA probe for PSA. a, Southern dot blot of LNCaP/rUGM tumors demonstrating variably positive Alu in 6 of 7 tumors from male hosts (lanes 1–7) and 0 of 3 tumors from female hosts (lanes 1–3). Northern analysis of corresponding tumors (a, bottom) demonstrates PSA expression only in tumors from male hosts. PSA expression did not correlate with Alu expression, likely resulting from varied tissue selection from a heterogenous tumor. b, Southern analysis of LNCaP/3T3 tumors reveals no human component in these tumors (lanes a–e), while all LNCaP/MS tumors were positive for Alu (lanes f–j). Northern analysis of LNCaP/MS tumors (b, bottom) demonstrates that all are strongly positive for PSA. Because of absence of Alu and serum PSA in LNCaP/3T3 tumors, PSA expression was not analyzed.

Effect of MS- and rUGM CM and bFGF on LNCaP Growth in Vivo. Since MS and rUGM CM and bFGF stimulated LNCaP cell growth in vitro, we tested the possible growth-promoting effect in vivo by coating these growth factor(s) onto a solid Gelfoam matrix (Fig. 7a). Control s.c. injections of Gelfoam with collagen IV plus ECGF with no coinoculated LNCaP cells induced local neovascularization at 3 weeks (Fig. 7b).

Fig. 3. Differences in serum PSA levels (ng/ml) in animals with various LNCaP chimeric tumors paralleled their differences in histomorphology. Mice bearing tumors characterized as carcinosarcomas (LNCaP/rUGM) or carcinomas (LNCaP/MS or LNCaP/NbF-1) (a) had elevated serum PSA levels (b), while mice bearing sarcomas or no tumors had undetectable serum PSA levels (<0.3 ng/ml). MS bone fibroblasts were the most reliable inducer of LNCaP carcinoma formation and resulted in the highest PSA levels, with a median of 68.1 ng/ml.

Fig. 4. LNCaP cells are androgen sensitive in vitro, a, LNCaP cells were stimulated in vitro by androgens with a 182 and 142% increase in cell growth with 1.0 nM testosterone (T) and 0.1 nM dihydrotestosterone (DHT), respectively. No mitogenic response was observed for rUGM cells using androgens in concentrations ranging from 0.1 to 100 nM. b, androgen receptor assays demonstrated the presence of a substantial amount (Bmax = 332 fmol/mg protein) of high-affinity (Kd = 0.23 nM) androgen receptors in LNCaP cells. Points, averages of 6 replicated determinations from 3 separate experiments; bars, SE ranging from 3–9%.

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growth and function (35). These interactions also help to regulate hormonal responsiveness (36) and likely play an important inductive and/or permissive role in the pathogenesis of tumor growth (37–39) and metastases (40, 41). The growth of a number of epithelial malignancies are influenced by their surrounding stroma, including the urinary bladder (18, 39), prostate (18, 42), colon (43), and breast (44). Recent investigations of prostate tumorigenesis in this laboratory have demonstrated that mesenchymal and epithelial cells interact in vivo in a bidirectional stimulatory manner (18, 19).

In this study, we examined whether fibroblast specificity exists in affecting the growth of a human prostate cancer cell line, LNCaP. This prostate cancer cell line was chosen for several reasons. (a) LNCaP cells are nontumorigenic when injected s.c. in athymic mice with <4 × 10^6 cells/inoculum (20) and in our experience are nontumorigenic even at higher doses; (b) the LNCaP cell line is the only prostate cell line that produces PSA (21), a human tissue-specific tumor marker of significant clinical importance (22, 23); (c) LNCaP cells are

![Fig. 5. Effect of defined GFs on LNCaP cell growth in vitro. The growth of LNCaP cells are stimulated in vitro by bFGF in a concentration-dependent manner (a), producing a 180% increase in cell number over 9 days. Both TGFα and EGF had no significant effect on LNCaP growth in vitro using concentrations from 0.1 to 50 ng/ml. A 50% reduction in LNCaP cell growth was produced by 0.1 ng/ml TGFβ (b). Points, averages of 6 replicated determinations from 3 separate experiments; bars, SE ranging from 3–9%.

Fig. 6. Stimulation of LNCaP cell growth in vitro by prostate- and bone-derived CM. a, LNCaP cells are stimulated up to 210% in a concentration-dependent manner from 0.1- to 1.0-fold by rUGM CM and are also stimulated by NbF-1 and MS CM, but not by 3T3, CCD16, or NRK CM. b, a bidirectional paracrine-mediated stimulatory pathway exists between LNCaP cells and rUGM or MS fibroblasts. LNCaP CM is stimulated 400% in a concentration-dependent manner from 0.1- to 2-fold by LNCaP CM and also less so by NbF-1, MS, 3T3, and CCD16 CM. MS cells are stimulated 225% by LNCaP CM (data not shown). No autocrine growth loop was demonstrated as evidenced by lack of stimulation of LNCaP CM on LNCaP cells or rUGM CM on rUGM cells. Columns, averages of 6 replicated determinations from 3 separate experiments; bars, SE ranging from 2–7%.

DISCUSSION

Cellular interactions between mesenchymal and epithelial cells are an integral part of embryonic development (34), continuing through adulthood by maintaining differentiated organ...
androgen responsive both in vivo (45) and in vitro (17), which would allow us to characterize sex-dependent differences in chimeric tumor growth; (d) of the androgen-responsive human prostate cancer models available, including PC82, HONDA, and LNCaP cell lines, only the LNCaP can be consistently grown in vitro (46), allowing us to investigate cell-cell interaction both in vivo and in vitro.

Our results demonstrate that fibroblasts induce LNCaP tumor growth in vivo in a cell-type specific and androgen-dependent manner. Of the 6 fibroblast cell lines tested, bone fibroblasts, followed by the prostate-derived fibroblasts, were the most effective in stimulating LNCaP cell growth both in vivo and in vitro. The presence of bidirectional paracrine pathways between LNCaP and fibroblast cells is illustrated in vitro by the development of sarcomas with the coinoculation of LNCaP cells and nontumorigenic rUGM and 3T3 fibroblasts and in vitro by LNCaP and rUGM CM producing a bidirectional increase in growth in a paracrine-mediated, but not autocrine-mediated, fashion. These observations suggest that LNCaP and fibroblast cells secrete factors that produce a more favorable microenvironment for tumorigenesis by reciprocally promoting growth, adherence, or angiogenesis. LNCaP cells participated in chimeric tumor formation preferentially in males, demonstrating in vivo androgen-sensitive growth. These results, along with those with their in vitro androgen sensitivity, further support that the growth of LNCaP cells in vivo may be androgen responsive, as demonstrated previously by Sonnenschein et al. (45) with 1 x 10^5 LNCaP cells/inoculum.

To determine whether certain fibroblast GFs are responsible for in vivo LNCaP tumor growth, we developed a method to test the effect of fibroblast CM in vivo. We demonstrated that certain nondialyzable factor(s) derived from rUGM and MS CM treated Gelfoam was injected alone to detect angiogenesis. Angiogenesis was visible after 3 weeks when Gelfoam plus ECGF-treated Gelfoam was injected alone to detect angiogenesis. Angiogenesis was visible after 3 weeks when Gelfoam plus collagen IV adsorbed with ECGF was injected (b). At RUGM CM-treated sites, 5 of 10 (50%) tumors formed by 10 weeks (mean tumor volume, 278 mm^3). With MS CM-treated Gelfoam, 3 tumors formed at 8 sites (38%). bFGF was also tested because of its in vitro mitogenic activity and induced tumor formation at 3 of 5 sites (60%). All tumors were histologically carcinomas and stained intensely and uniformly for PSA (c). Southern blot analysis for LNCaP and corresponding Northern analysis for PSA expression are both positive (d).

The finding that bone fibroblasts are the most potent inducer of LNCaP tumor growth in vivo allows us to speculate concerning the possible role of stromal-epithelial interactions in site-specific metastases. Batson (5) attributed the nonrandom distribution of bony metastases in patients with prostate cancer to retrograde flow of cancer cells through paravertebral venous channels. However, most tumor cells in the venous circulation pass through the lungs (54) and yet the incidence of clinically apparent lung metastases in patients dying of prostate cancer is low (55, 56). Also, kinetic distribution studies in which radiolabeled tumor cells are used do not show a correlation between organ seeding and subsequent metastatic formation (57, 58), suggesting that factors other than the simple mechanical arrest of tumor cells are responsible for the development of prostate cancer bony metastasis.

The “seed and soil” hypothesis attempts to explain the nonrandom distribution of metastases with certain tumors (seed) that preferentially grow at particular sites (soil) (7, 13). Several interrelated factors contributing to the development of site-specific metastases have been identified and include (a) differential adhesion between microvessel endothelial cells or basement membrane collagen IV from one organ to another (8, 9, 59) or through alterations in integrin expression or binding (60); (b) organ-specific chemotactic factors capable of inducing migration of tumor cells from vascular to interstitial spaces (10, 11); and (c) organ-specific growth factors (or inhibitors) capable of accelerating (or preventing) the growth of tumor cell deposits (12, 13). These factors likely all contribute to the development of bony metastases (40, 59, 61). Skeletal tissues produce various GFs (62–66) including bFGF (65). Osteoblasts are the principal...
source of synthesis and deposition of bone matrix, the site where bFGF is stored, and mediates its mitogenic activity (65, 66). bFGF may act in a paracrine fashion to stimulate metastatic cancer cell growth because a number of tumor lines are stimulated by bFGF (16, 48) including LNCaP cells. Chackel-Roy et al. (40) reported that bone marrow CM from human, bovine, and rat bones stimulated the growth of human prostate cancer cell lines DU145 and PC3 in vitro but were unable to duplicate this response with various defined GFs. Our findings broadly concur with the findings of Chackel-Roy et al. (40) that bone marrow CM from human, bovine, and rat bones stimulated the growth of human prostate cancer cell lines DU145 and PC3 in vitro but were unable to duplicate this response with various defined GFs. Using the bidirectional in vitro and in vivo models of LNCaP/ rUGM and LNCaP/MS tumor growth, as well as the Gelfoam/CM model, we are in the process of identifying GFs and extracellular matrix involved in LNCaP tumor growth. Work thus far has demonstrated strong bidirectional paracrine growth loops in each model but no autocrine loop. The production of extracellular matrix (collagen and fibronectin) and bFGF by bone- and prostate-derived fibroblasts has been demonstrated using Northern analysis, but other uncharacterized GFs may also be involved.

In summary, we have demonstrated fibroblast specificity in the induction of LNCaP tumor growth in vivo, with human bone- and prostate-derived fibroblasts being the most effective in inducing carcinomas from otherwise nontumorigenic LNCaP cells. LNCaP tumor growth in vivo appears to be androgen responsive, and the cellular interactions between cocultured LNCaP cells and fibroblasts are bidirectional based on both in vitro and in vivo analyses. Prostate and bone fibroblast CM, as well as bFGF, induced LNCaP tumor growth in vivo, suggesting that soluble fibroblast GFs may play a role in prostate cancer growth and metastasis.

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Acceleration of Human Prostate Cancer Growth \textit{in Vivo} by Factors Produced by Prostate and Bone Fibroblasts

Martin Gleave, Jer-Tsong Hsieh, Chuan Gao, et al.


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