Prostate cancer exhibits a prominent zonal distribution. The peripheral zone harbors over 70% of prostate cancers, and this region of the prostate is also the primary site of high-grade prostatic intraepithelial neoplasia (HGPIN; ref. 1). In terms of tissue architecture, glands throughout the prostate are characterized by a basal cell compartment, which is believed to include potential stem cells, and a secretory compartment (2, 3). Cells within the basal cell compartment may represent the population that undergoes malignant transformation during the formation of prostate cancer (4, 5). The regional variation in prostate cancer suggests that basal cells within the peripheral zone are affected by the presence of local factors responsible for differential progression of the disease. A greater understanding of the zonal distribution of basal cells and the local factors that influence their proliferation and/or differentiation might help explain the localization of precursor lesions and prostate carcinoma.

In an attempt to identify local factors that influence cell proliferation and differentiation in the prostate, we have examined seminal vesicle fluid (SVF) as a potential source of insulin-like growth factor-I (IGF-I). Both IGF and IGF binding proteins have been identified in the semen (6–8), but there have been conflicting reports regarding this correlation (10–14). Among the possible reasons for these conflicting reports is that the serum levels of IGF-I do not accurately reflect the local concentrations of IGF-I in the prostate. Support for this concept comes from studies examining the effect of a liver specific disruption of the Igf1 gene. Mice homozygous for this disruption show a dramatic reduction in serum IGF-I levels but display normal development and achieve normal size (15). These results indicate that tissue production of IGF-I plays a greater role in tissue homeostasis than previously realized.

To determine whether tissue-specific variation in IGF-I concentrations may influence cells within the peripheral zone of the prostate, we have examined the activation status of the IGF-I receptor (IGF-IR) in this region. Staining with antibodies specific for the activated IGF-IR indicates activation of this
receptor in areas of atrophy, HGPIN, and low-grade cancer. However, normal glands in the peripheral zone contain very little activated receptor, although the protein is present at readily detectable levels. In addition, we have identified a unique basal cell lesion present in a high percentage of prostate glands within the peripheral zone. These lesions are characterized by proliferation of cells expressing p63 and cytokeratin 34βE12. These cells also display a distinctive morphology distinct from typical basal cells. In addition, we find that these lesions contain high levels of phosphorylated IGF-IR, indicating a local activation of this receptor signaling system. A potential local source of IGF-I in the peripheral zone of the prostate is the seminal vesicles. We have confirmed the presence of IGF-I in the seminal fluid, and in a separate study, we have identified spermatozoa in glands of the prostate within the peripheral zone, indicating infiltration into the prostate gland (16). Furthermore, the addition of SVF to cultures of prostate cancer lines and cell lines derived from normal prostate drives proliferation in these cells. Taken together, the results suggest that IGF-I produced in the seminal vesicles may have an effect on prostate epithelium.

**Materials and Methods**

**Tissue array production.** Human tissue was obtained from a tissue procurement facility established at the Drexel University College of Medicine by Dr. Fernando U. Garcia. The facility provides blinded samples to the Drexel College of Medicine and other institutions and serves as a repository for clinical information that cannot be traced to individual patients. Tissue procurement was done in accordance with Institutional Review Board guidelines. Antibodies to the IGF-IR and IRS-1 were obtained from Cell Signaling, Inc. An antibody to the activated IGF-IR, anti-pY1316 IGF-IRβ; rabbit polyclonal (K2895), was kindly provided by Dr. Olaf Mundigl (Roche Diagnostics, Penzberg, Germany).

The study set for the prostate tissue arrays consisted of 65 consecutively obtained radical prostatectomy specimens process in the Pathology Department, Drexel University College of Medicine that were whole-mounted, sagitally sectioned, and totally embedded. Tissue microarrays were constructed by sampling the transitional zone and peripheral zone areas in quadruplicate using 0.6-mm-diameter cores from each prostate. When sampling the transitional zone, the perirethral area was avoided as was the verumontanum and periurethral area was avoided as was the verumontanum and seminal vesicles. The H&E slides were reviewed in conjunction with the National Cancer Institute, respectively. The IBC10a cell line was kindly provided by Dr. Mark Stearns (Department of Pathology, Drexel College of Medicine; ref. 18).

**Cell culture.** Cell culture lines DU145 and CPTX were provided by the American Type Culture Collection of seminal fluid. Seminal vesicles were divided into 1-mL aliquots to minimize freeze-thawing and was thawed on ice immediately before use.

**IGF-I measurements.** IGF-I levels were measured following acid extraction using a double-sandwich coated-well ELISA obtained from DSL Laboratories. Assays were done in triplicate using 50 µL of seminal fluid from each patient.

**Immunofluorescence.** Tissue sections were washed thrice in PBS and blocked in 1% bovine serum albumin for 30 min at 37°C. Primary antibodies were incubated in PBS at 37°C for 60 min followed by secondary antibodies conjugated to FITC or rhodamine (Invitrogen Life Sciences). Sections were counterstained using 4',6-diamidino-2-phenylindole (Invitrogen Life Sciences) to visualize nuclei. The images were visualized with an inverted Nikon Eclipse TE300 fluorescence microscope equipped with a Retiga 1300 camera (Q Imaging). Series of three-dimensional images of each individual picture were deconvoluted to one two-dimensional picture and resolved by adjusting the signal cutoff to near maximal intensity to increase resolution.

**Cell culture.** Cell culture lines DU145 and CPTX were provided by the American Type Culture Collection and Dr. Susan Topalian (17) at the National Cancer Institute, respectively. The IBC10a cell line was kindly provided by Dr. Mark Stearns (Department of Pathology, Drexel College of Medicine; ref. 18).

**Proliferation assays.** Proliferation was measured by determining the percentage of cells incorporating bromodeoxyuridine in response to seminal fluid. Cultures were placed into defined, serum-free, growth factor–free medium (DMEM) for 72 h before stimulation. Cultures were stimulated with seminal fluid or FBS. Following 16 h, bromodeoxyuridine (Boehringer Mannheim, Inc.) was added to cell cultures, and incubations were continued for a further 8 h. Cultures were then fixed in cold methanol/acetone (1:1) and stained with a FITC-conjugated anti-bromodeoxyuridine antibody (Boehringer Mannheim).

In the case of IBC10a experiments, cell numbers were obtained using a green fluorescent–tagged cell population. IBC10a cells, an androgen receptor–positive cell line derived from prostate epithelium and immortalized through the introduction of the human telomerase gene (18), were seeded at 1 x 10⁴ per cm² in 12-well culture plates (Corning, Inc.). Following 48 h, cultures were changed to serum-free medium (FBS; DU145) or keratinocyte serum-free medium (CPTX) supplemented with 5% FBS.
Fig. 1. Expression and activation of IGF-IR and IRS-1 in peripheral zone prostate tissue. 
A. peripheral zone tissue microarrays were stained for total IGF-IR, IRS-1, and active, phosphorylated IGF-IR. The relative level of staining was estimated on a scale of 1 to 4 in areas of tissue sections that contained normal glands, atrophic glands, areas of PIN, or cancer. Cancers were divided into low grade (Gleason grade 3) or high grade (Gleason grade 4). Areas of atrophy, PIN, and cancer were analyzed relative to normal glands for the level of staining using two-tailed t tests. *, P > 0.05; †, P > 0.001, statistically different from normal. NS, not significantly different from normal. B. immunostaining for IGF-IR, IRS-1, and the phosphorylated IGF-IR in the peripheral zone of the prostate. Tissue array sections were stained for the presence of the IGF-IR, IRS-1, and the phosphorylated form of the IGF-IR. Representative pictures. Relative intensity of the stain in (A).
solution in Hank's buffered saline containing EDTA (Mediatech, Inc.). Cell numbers were then determined using a guava easy-cyte flow cytometer. A total of 5,000 cells from each culture were counted.

Activation of IGF-I signaling. DU145 cells were serum starved in DMEM for 72 h and then stimulated with IGF-I (20 ng/mL) or seminal fluid (1:10) for 15 min. To evaluate phosphorylation levels of IGF-IR signaling molecules, monolayer cultures were lysed for 5 min on ice with 400 μL of lysis buffer A [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 0.2 mmol/L Na-orthovanadate, and 10 μg/mL aprotinin]. Total protein extract (50 μg) was separated on a 4% to 15% gradient SDS-PAGE (Bio-Rad). The following primary antibodies were used: anti-pY1316 IGF-IR (h rabbit polyclonal [K2895, kindly provided by Dr. Olaf Mundigl]), anti-pY612 IRS-1 rabbit polyclonal (Biosource), anti-pS473 Akt and anti-pT202/Y204 extracellular signal-regulated kinase 1/2 (Cell Signaling Technology, Inc.).

Results

Activation of the IGF-IR in the peripheral zone of the prostate. Prostate tissue arrays derived from the peripheral zone of the prostate were screened to examine the expression levels of key components of the IGF-I signaling axis: the IGF-IR and IRS-1. The series of cases included in these arrays include 60 prostate samples arrayed in a grid on a single slide that can be stained simultaneously. Areas of normal prostate, PIN, cancer, and atrophy appear in these samples. Immunohistochemistry on these sections indicates that both the IGF-IR and IRS-1 are expressed in normal prostate epithelium (Fig. 1). Expression levels of the IGF-IR and IRS-1 were compared in normal glands, glands showing atrophy, HGPIN, and cancer.

The IGF-IR and IRS-1 were both increased in areas of atrophy, PIN, and cancer relative to areas of normal prostate, suggesting that the IGF-I signaling pathways are up-regulated relative to the normal prostate gland. In normal glands, there was a greater staining in both basal cells and at the basal side of luminal cells, suggesting a differential localization of IGF-IR to the basal side of the polarized luminal epithelial cells (Fig. 1). In pathologic settings (i.e., glands showing atrophy, PIN, or cancer), we observed strong staining with the antibody specific for the phosphorylated IGF-IR (Fig. 1).

Activation of the IGF-IR in areas of basal cell hyperplasia. Through a careful morphologic examination of glands from the peripheral zone versus the transitional zone, we have identified a distinct basal cell lesion that is composed of small islands of basal cells. These basal cells span from the basal compartment to the secretory compartment and display a triangular morphology with the apex directed towards the lumen of the gland (see Fig. 2, top). These basal cells seem to extend between overlying luminal cells and are positive for both p63 and 34βE12 expression, typical of prostate basal cells. This hyperplasia, which we term intermediate basal cell hyperplasia, was observed exclusively in the peripheral zone of the prostate in an analysis of 289 normal glands from the peripheral and transitional zones. Although many p63-positive basal cells in normal prostate glands displayed positive staining for the activated IGF-IR, intense staining was observed in areas of basal cell hyperplasia and coincided with p63 staining (Fig. 2, overlay).

SVF is a source of local IGF-I in the prostate. SVF from six different patients was analyzed for the presence of IGF-I. In all samples, IGF-I levels were above 300 ng/mL (Fig. 3). These high levels of IGF-I indicate that seminal fluid produces sufficient IGF-I to increase the local concentration of the growth factor in a localized region of the prostate. To verify that the IGF-I present in SVF is functional, SVF was used to stimulate prostate cell lines derived from both normal and malignant prostate tissue. In all cases, SVF produced a strong proliferative response (Fig. 4). Over 90% of prostate cells derived from either normal tissue or prostate tumors enter the S phase when stimulated with SVF. In fact, the magnitude of the response was greater than that produced by FBS (80-90% versus 70%).

Activation of IGF-I signaling in response to SVF. The activation of IGF-I signaling pathways in response to SVF was examined using DU145 cells. Stimulation of DU 145 cells with SVF resulted in phosphorylation of IRS-1, Akt-1, and extracellular signal-regulated kinase. Activation of the IGF-IR was measured through the visualization of the phosphorylated form of Akt-1 using antibodies specific to Tyr473. By this measure of activation, SVF was able to strongly activate the IGF-IR (Fig. 4). Inclusion of a small molecule inhibitor of the IGF-IR (NVP-AEW541; refs. 19, 20) inhibited phosphorylation of IRS-1, Akt-1, and extracellular signal-regulated kinase in response to both IGF-I and seminal fluid (Fig. 4, right lanes).

Proliferation of prostate epithelial cells in response to SVF. The influence of SVF on the proliferation of prostate cells was tested using cell lines derived from both normal and cancer tissue. The prostate cancer line, DU145, was strongly stimulated by SVF as was a cell line derived from normal prostate tissue, NPTX (ref. 17; Fig. 5). The response of the NPTX cells to SVF was stronger than to FBS, which is commonly used as a potent growth stimulator in many cell types. In addition, we have tested the ability of seminal fluid to stimulate growth in an androgen receptor–positive line, IBC10a. These cells are derived from an area of basal cell proliferation through the introduction of telomerase, and the cells have been documented to express the androgen receptor. In these cells, we measured proliferation using direct cell counts. Seminal fluid induced a doubling in the cell population over a 72-h period. Taken together, the results confirm that SVF contains mitogens for prostate epithelial cells.

Discussion

The presence of IGF-I in semen has been documented in previous reports, but the reported levels of IGF-I were rather low, in the range of 20 to 40 ng/mL (8, 21). The levels of IGF-I in the seminal fluid, is in direct proximity to the prostate, seem to be higher than in semen (~300 ng/mL). This concentration is similar to that found in human serum and suggests that seminal fluid may drive the proliferation of prostate cells. In fact, SVF was able to drive prostate cells into the S phase more effectively than FBS in vitro. This is an unexpected result when one considers the high concentrations of multiple growth factors in FBS. These results suggest that penetration of seminal fluid may have important consequences in the prostate, and documentation for penetration of seminal fluid into the prostate has been provided by the presence of spermatozoa preferentially within the peripheral zone of the prostate (16). Taken together, these results indicate that the region of the prostate near the distal end of the seminal ducts is chronically exposed to a strong proliferative signal.

In terms of the IGF-I axis in the peripheral zone, the expression of the IGF-IR and IRS-1 was increased in the tissue array analysis in areas of atrophy, PIN, or cancer. Levels of the activated IGF-IR were also increased in all of these conditions. The activation of the IGF-IR in areas of atrophy suggests that local factors activate the receptor during tissue response to denudation of the luminal epithelium perhaps to facilitate reestablishment of the normal epithelial layer. Staining for both the IGF-IR and IRS-1 in normal areas suggest a preferential localization to the basal side of the luminal epithelial cells and a stronger staining in basal epithelial cells. This suggests that there may be a differential subcellular localization of these proteins within the prostate epithelium, although more detailed studies are required to confirm this observation.
Interestingly, there was a trend for decreased activation of the IGF-IR in areas of higher-grade cancer (Gleason grade 4). In addition, several sections of clear cell carcinoma showed very low levels of the activated IGF-IR, whereas neighboring areas of PIN or atrophy showed relatively high levels (sections of clear cell carcinoma are included in Supplementary Material). The results suggest that the IGF-IR is activated in the early stages of prostate cancer and in areas of PIN or atrophy, but that as areas of cancer progress toward Gleason grade 4, there is a decrease in the activation of the IGF-IR. It must be emphasized, however, that there were relatively few areas of Gleason grade 4 in the sections examined (an average of 10 areas of grade 4 and only sporadic areas of clear cell cancer). Although caution must be used in the interpretation of a small number of cases, the results suggest that the areas of higher-grade cancer are not exposed to IGF-I and raise the possibility that a compensating mutation has occurred that circumvents the need for IGF-IR activation for growth.

A significantly greater number of glands contain intraepithelial basal cell hyperplasia in the peripheral zone than other regions of the prostate. We postulate that it is the chronic presence of IGF-I in the peripheral zone that may drive the development of these lesions. This possibility is supported by the preferential activation of the IGF-IR in these lesions. The penetration of factors such as IGF-I into these areas may signify a local loss of epithelial barrier function, and it has been suggested that a local breach in the epithelial barrier may be an early event in tumor formation (22). We have shown that oncogenic conversion of epithelial cells produces profound changes in epithelial barrier function (23), and it has been reported that seminal fluid can alter epithelial barrier function in vitro (24, 25). This supports the idea that factors from the seminal fluid enter the basal compartment in the prostate where these factors can influence basal cell proliferation.

The results of this study are of particular interest when related to the recent reports that sexual activity and specifically ejaculation frequency is inversely related to the risk for prostate cancer (26, 27). These results have lead to the suggestion that reduced or abnormal ejaculation may be related to an increased risk for prostate cancer (28).

In summary, the results presented here suggest that infiltration of seminal fluid into the peripheral zone of the prostate may be a mediator of basal cell proliferation in this region of the prostate. IGF-I produced by the seminal vesicles may be one mediator of this basal cell proliferation.

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References

Insulin-Like Growth Factor-I Produced by Seminal Vesicles: Relationship to Intraepithelial Basal Cell Hyperplasia in the Prostate

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