Growth Regulation of Prostatic Stromal Cells by Prostate-Specific Antigen


Background: Prostate-specific antigen (PSA) is a serine protease that can cleave insulin-like growth factor-binding protein-3 (IGFBP3), thereby decreasing its affinity for insulin-like growth factor-I (IGF-I). Dissociation of the IGF-I–IGFBP3 complex renders IGF-I available to bind to its receptor and stimulates cellular proliferation. We evaluated the potential for PSA to modulate the effects of IGF-I and IGFBP3 on the proliferation of human benign prostatic hyperplasia (BPH)-derived fibromuscular stromal cells in primary cultures. Methods: We cultured BPH-derived stromal cells for 48 hours in serum-free RPMI-1640 medium supplemented with 0.2% bovine serum albumin and studied the effects of IGF-I, IGFBP3, PSA, and ZnCl2 at varying concentrations. Differences in cell growth between control and treated cultures were evaluated by use of Dunnett’s test. Concentration-related trends were evaluated by linear regression of log-transformed concentrations of test reagents on BPH-derived stromal cell number responses. Statistical tests were two-sided. Results: We observed a concentration-dependent proliferative response of BPH-derived stromal cells to IGF-I. IGFBP3 inhibited this response in a concentration-dependent fashion. IGFBP3 alone had no effect on stromal cell proliferation. When stromal cells were incubated with PSA alone or with PSA, IGF-I, and IGFBP3, an increase in stromal cell numbers that was dependent on PSA concentration was evident in both instances. Zinc, an endogenous inhibitor of PSA enzymatic activity, was able to attenuate the stimulatory effect of PSA at intraprostatic physiologic concentrations. Conclusions: These results are consistent with the idea that PSA can modulate in vitro interactions between IGF-I and IGFBP3 and suggest that PSA may play a role in the regulation of human prostatic fibromuscular cell growth. [J Natl Cancer Inst 1999;91:1663–9]

Peptide growth factors are potent regulators of cellular proliferation and metabolic functions. Insulin-like growth factor-I (IGF-I) is synthesized in many tissues and stimulates the growth of both benign and malignant cells (1). IGF-I has been postulated to act as an autocrine or paracrine growth factor for a variety of malignant cells, including prostatic cancer cells (2–4). The mitogenic effects of IGF-I are modulated in a positive and negative manner by IGF-binding proteins (IGFBPs) (5–7). At least seven species of IGFBPs have been identified and characterized (8,9).

The exact mechanism(s) by which IGFBPs regulate the action of IGF molecules are not fully elucidated. However, evidence suggests the idea that the IGFBPs can interact with IGF, preventing the growth factor from binding to its plasma membrane receptor. Proteases are thought to regulate the biologic activity of IGFBPs (10,11).

It has been demonstrated that prostate-specific antigen (PSA) is capable of cleaving IGFBP3, lowering its affinity for IGF-I, which allows IGF-I to bind to its membrane receptor on benign prostatic hyperplasia (BPH) epithelial cells (11,12). PSA is a serine protease that possesses chymotrypsin-like enzymatic activity (13–15) produced almost exclusively by prostatic epithelia. Because of its tissue specificity and association with prostatic proliferative disorders, PSA has become a useful serum marker in the management of prostatic cancer. Numerous clinical investigations (16,17) have sought to define the importance of PSA in the early detection of prostatic cancer. In seminal plasma, PSA has been identified as the enzyme responsible for proteolysis of semenogelin, resulting in the liquefaction of the seminal gel. Seminal liquefaction is a process obligatory to the release of progressively motile spermatozoa (18). While seminal liquefaction activity has been defined for PSA, its physiologic role(s) in prostatic fluid remains undefined. The fibromuscular stromal component of the prostate and of other accessory sex organs is thought to play an inductive role in the fetal development of the genitourinary tract and the pathogenesis of BPH (19–21). Evidence obtained from prenatal animal studies supports an inductive role for the fibromuscular stroma in epithelial proliferation and phenotypic expression. Few studies have investigated the inductive capabilities of epithelial cells on fibromuscular proliferation.

The experiments described in this article were carried out to determine a possible paracrine role of PSA in the growth of human prostatic fibromuscular stromal cells.

Materials and Methods

Reagents

Recombinant human IGF-I for biologic experiments was produced at Lilly Research Laboratories (Indianapolis, IN). Recombinant human IGFBP3 was purchased from UBI (Lake Placid, NY) and resuspended in 10 mM acetic acid. α-Chymotrypsin was purchased from Sigma Chemical Co. (St. Louis, MO). Enzymatically active PSA was purified from human seminal plasma with the use of a three-column chromatography technique. Human semen was incubated at 37 °C for 20 minutes followed by centrifugation of the liquefied material in a Sorvall RC5C centrifuge (Kendro Laboratory Products, Newtown, CT) with the use of an SS34 rotor at 10 000 rpm (12 000g) for 15 minutes at room temperature. The resulting seminal plasma was loaded onto a Superose 6 (10/30) column (Pharmacia Biotech Inc., Piscataway, NJ) equilibrated in 100 mM ammonium bicarbonate (pH 7.8) and run at a flow rate of 0.5 mL/minute. The PSA-containing fractions were identified with the use of sodium dodecyl sulfate-
polyacrylamide gel electrophoresis (SDS-PAGE). The PSA-positive fractions were combined, adjusted to pH 5.5 with the use of 0.1 N HCl, and loaded onto a Mono S (5/5) column (Pharmacia Biotech Inc.) equilibrated in 50 mM malonic acid (pH 5.5). After a brief period of isocratic flow at initial conditions, protein was eluted from the column with a linear gradient of NaCl in 50 mM malonic acid (pH 5.5) (0–500 mM NaCl; 1.0 mL/minute; approximately 30 minutes). PSA-containing fractions were again localized with the use of SDS-PAGE, combined, and loaded onto a reversed-phase column (Aquapore RP-300; 250 × 4.6 mm; Applied Biosystems, Foster City, CA), equilibrated in 0.1% trifluoroacetic acid and 10% acetonitrile. Protein was eluted at a flow rate of 1.0 mL/minute, with the use of a linear gradient of acetonitrile increasing at 1% per minute. All columns in this purification scheme were monitored spectrophotometrically at 214 nm. The purified protein was exchanged into phosphate-buffered saline (PBS) (pH 7.4) with the use of a Sephadex G-25 column. N-terminal sequence analysis was performed with a model 470A or a model 477A protein sequencer (Applied Biosystems), and the N-terminal sequence was determined to be IVGG.

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Experimental Conditions

The concentration-dependent mitogenic activity of IGF-I (0, 1, 3, 10, 30, and 100 ng/mL) was assessed by the measurement of the in vitro proliferation of BPH-derived stromal cells with the use of the culture conditions described above. The optimal stimulatory concentration of IGF-I was determined, and the ability of IGFBP3 to modulate the mitogenic effect of IGF-I on the growth of BPH-derived stromal cells was examined. IGFBP3 (0, 30, 100, 300, and 500 ng/mL) and IGF-I (10 ng/mL) were added simultaneously to cells growing in culture, and cell numbers were determined as described previously.

After defining the maximal inhibitory concentration of IGFBP3 on IGF-I-induced stromal cell proliferation, we evaluated the ability of purified PSA (0, 3, 10, 30, 100, and 300 pg/mL) to modulate this inhibition. Similar concentrations of PSA were examined for independent effects on the cells and served as controls for the IGF-I and IGFBP3 combination experiments.

Zinc has been shown to be an endogenous inhibitor of the enzymatic activity of PSA (26). ZnCl₂ was added to stromal cell cultures containing PSA to evaluate effects on cellular responses to PSA. BPH-derived stromal cells were cultured as described above for 48 hours in the presence of 100 µg/mL PSA and various concentrations of ZnCl₂ (0, 0.1, 1.0, 10, and 100 µM). PSA and ZnCl₂ were preincubated for 24 hours at 4 °C to allow association of these two reagents before these agents were applied to the cells in culture. Similar concentrations of ZnCl₂ were examined for independent effects on the stromal cells. Levels of IGF-I, PSA, IGFBP3, and ZnCl₂ determined in the above concentration–response studies to produce marked stimulatory or antagonistic actions were tested in combination to evaluate their interaction on the proliferation of BPH-derived stromal cells.

Statistical Analysis

Unless otherwise stated, results for in vitro experiments are expressed as means ± standard error of four observations. The data shown are representative results of consistent qualitative responses observed in triplicate or more experiments. Data were analyzed for significant differences at the P<.05 level with the use of Dunnett’s test (27) and for normal distribution by the Shapiro–Wilk W test (28). Concentration-related trends were evaluated by linear regression of log-transformed concentrations of test reagents on BPH-derived stromal cell number responses. Statistical tests were two-sided.

RESULTS

IGF-I Stimulation of Proliferation of BPH-Derived Stromal Cells

IGF-I stimulated BPH-derived stromal cells in a concentration-dependent manner (Fig. 1). After 48 hours of treatment, a medium concentration of 10 ng/mL IGF-I produced a 47% increase in the numbers of BPH-derived stromal cells above control levels (P = .0015). Maximal cell numbers were observed with IGF-I concentrations equal to or greater than 30 ng/mL. Culture of BPH-derived stromal cells in defined medium containing bovine serum albumin for periods longer than 48 hours resulted in cell death.

IGFBP3 Antagonism of Proliferation of BPH-Derived Stromal Cells Induced by IGF-I

Exogenously added IGFBP3 inhibited IGF-I-induced stimulation of BPH-derived stromal cells in a concentration-
dependent fashion (Fig. 2, A). Simultaneous exposure of BPH-derived stromal cells to IGF-I (10 ng/mL) and IGFBP3 (100 ng/mL) resulted in statistically significant decreases in numbers of BPH-derived stromal cells compared with cultures treated with IGF-I (*P < .0005). The cell numbers (mean ± standard error of the mean) from IGF-I cultures inhibited by co-incubation with IGFBP3 (300 ng/mL) were not significantly different from the values for control cells in the presence of bovine serum albumin (**P < .102). The higher concentration of IGFBP3 did not produce further inhibition than that produced by 100 ng/mL IGFBP3. IGFBP3-induced reductions in cell numbers were not attributable to the binding protein per se (Fig. 2, B). Despite a slight trend toward increased stromal cell numbers (**P = .079), IGFBP3 had no statistically significant effect on the growth of BPH-derived stromal cell cultures after a 48-hour incubation.

Stimulation of Proliferation of BPH-Derived Stromal Cells by PSA

At concentrations up to 100 μg/mL, PSA stimulated modest proliferation of BPH-derived stromal cells (data not shown). In these same experiments after a 48-hour treatment, 100 μg/mL PSA stimulated statistically significant increases in numbers of BPH-derived stromal cells above control values (**P < .00004; ***P < .00001) with the use of Dunnett’s test (27). Values represent the means ± standard error of the mean of four observations from one of three representative experiments.

Fig. 1. Insulin-like growth factor-I (IGF-I) stimulates the growth of primary cultures of benign prostatic hyperplasia (BPH)-derived stromal cells in serum-free medium in a concentration-dependent fashion. The stromal cells (1 × 10⁴/well) were plated in 24-well plates. After adherence overnight (16–18 hours), the medium was changed to RPMI-1640 containing 0.2% bovine serum albumin and varying concentrations of IGF-I. The cells were detached from the culture surface after 48 hours with 0.25% trypsin and counted with the use of a Coulter counter. Significantly greater than no IGF-I control (*P < .0015; **P < .00004; ***P < .00001; ****P < .00001) with the use of Dunnett’s test (27).

Values represent the means ± standard error of the mean of four observations from one of three representative experiments.

Fig. 2. A Insulin-like growth factor-binding protein-3 (IGFBP3) inhibits insulin-like growth factor-I (IGF-I)-induced stimulation of primary cultures of benign prostatic hyperplasia (BPH)-derived stromal cells in a concentration-dependent fashion. Cells were incubated under routine culture conditions for 48 hours in the presence of 10 ng/mL IGF-I and various concentrations of IGFBP3 (0–300 ng/mL). * = significantly greater than control (no additions) (**P < .00001) and significantly less (***P < .00005; ****P < .00002; *****P < .00001) than cultures treated with only IGF-I with the use of Dunnett’s test (27). B) The same concentrations of IGFBP3 in basal medium in the absence of IGF-I had no effect on proliferation of BPH-derived stromal cells. Values represent the means ± standard error of the mean of four observations from one of three representative experiments.

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Fig. 4 illustrates the mitogenic effects of IGF-I (10 ng/mL) and PSA (100 μg/mL) on BPH-derived stromal cells (P = .00001 and P = .0036, respectively). Also shown is the inhibition of the growth of IGF-I-stimulated BPH-derived stromal cells by IGFBP3 (100 ng/mL; P = .0002). Addition of PSA (100 μg/mL) to cultures containing IGF-I and IGFBP3 restored the stimulatory effects of IGF-I on the proliferation of BPH-derived stromal cells (P < .00001). Preincubation of ZnCl₂ with PSA for 16–18 hours abolished the indirect proliferative effects of PSA (P = .00001). Preincubation of ZnCl₂ with IGF-I had no presence of ZnCl₂. Similar concentrations of CaCl₂ (10.0 μM) had no effect on the ability of PSA to cleave the substrate.

PSA and Inhibition by IGFBP3 of Cellular Proliferation Induced by IGF-I

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effect on IGF-I-induced cellular proliferation (data not shown). In addition, ZnCl₂ had no effect on IGFBP3 inhibition of IGF-I-induced stimulation of BPH-derived stromal cells (data not shown).

α-Chymotrypsin Concentration–Response Study

α-Chymotrypsin was cytotoxic to BPH-derived stromal cells at concentrations equal to or greater than 10 μg/mL (data not shown). Overall, α-chymotrypsin at a concentration of 3 μg/mL had a slight stimulatory (8.7% ± 1.2% greater than control) effect on the growth of BPH-derived stromal cells, whereas concentrations lower than 3 μg/mL had no effect. Addition of α-chymotrypsin to cultures of BPH-derived stromal cells containing IGF-I and IGFBP3 restored the stimulatory effect of IGF-I on BPH-derived stromal cells (data not shown).

DISCUSSION

Results of our study show that IGF-I stimulated in a concentration-dependent fashion the proliferation of the BPH-derived stromal cells obtained from three separate specimens grown in tissue culture. IGFBP3 inhibited IGF-I-stimulated growth of BPH-derived stromal cells, and the inhibitory effect of IGFBP3 was attenuated by the proteases PSA and α-chymotrypsin. PSA alone was also able to directly stimulate the growth of BPH-derived stromal cells in vitro by an average of 17% above control cell numbers within a 48-hour incubation. α-Chymotrypsin was only able to stimulate proliferation of BPH-derived stromal cells on an average of approximately 9% above control cell numbers within the same time period. This finding is consistent

Figure 3: A) Prostate-specific antigen (PSA) (100 μg/mL) stimulates the growth of benign prostatic hyperplasia (BPH)-derived stromal cells in culture. Intraprostatic physiologic concentrations of zinc attenuate the mitogenic effect of PSA on primary cultures of BPH-derived stromal cells. PSA (100 μg/mL) and ZnCl₂ were preincubated for 24 hours at 4 °C to allow mixing of these two reagents and applied to BPH-derived stromal cells in culture for 48 hours to evaluate the effect of zinc on PSA-induced cellular proliferation. B) Similar concentrations of ZnCl₂ had no independent effects on cellular proliferation up to 10 μM (P for trend = .088).* = significantly greater than control (no additions) (P = .0311) and significantly less than cultures treated only with PSA (***P = .0005; ****P = .0014; *****P = .0023) with the use of Dunnett’s test (27). Values represent the means ± standard error of the mean of four observations from one of three representative experiments.

Figure 4: Prostate-specific antigen (PSA) antagonizes insulin-like growth factor-binding protein-3 (IGFBP3)-induced inhibition of insulin-like growth factor-I (IGF-I) stimulation. IGF-I (10 ng/mL) stimulated growth of benign prostatic hyperplasia (BPH)-derived stromal cells as indicated by the increase in cell number after 48 hours. Simultaneous addition of IGF-I (10 ng/mL) and IGFBP3 (100 ng/mL) did not produce a substantial increase in cell number. Addition of PSA (100 μg/mL) to the culture medium attenuated the effect of IGFBP3, restoring the mitogenic properties of IGF-I. ZnCl₂ (1 μM) abolished the indirect proliferative effect of PSA. *P = .0036 and **P = .00001 were significantly greater than control (no additions); *** significantly (P < .009) less than IGF-I-stimulated cells; **** significantly (P = .0001) greater than IGF-I + IGFBP3-treated cells; ***** significantly (P = .00001) less than IGF-I + IGFBP3 + PSA-treated cells, with the use of Dunnett’s test (27). Values represent the means ± standard error of the mean of four observations from one of three representative experiments.

Table 1: Effect of ZnCl₂ on ability of prostate-specific antigen (PSA) to cleave a 10-amino-acid substrate between two tyrosine residues*

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Peak area†</th>
<th>% inhibition of PSA enzymatic activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>92,812 ± 1387</td>
<td>0</td>
</tr>
<tr>
<td>0.1 μM ZnCl₂</td>
<td>90,498 ± 1715</td>
<td>4.25 ± 1.1</td>
</tr>
<tr>
<td>1.0 μM ZnCl₂</td>
<td>73,660 ± 806</td>
<td>20.4 ± 1.2</td>
</tr>
<tr>
<td>10 μM ZnCl₂</td>
<td>67,889 ± 861</td>
<td>27.2 ± 1.4</td>
</tr>
<tr>
<td>10 μM CaCl₂</td>
<td>92,212 ± 1804</td>
<td>0</td>
</tr>
</tbody>
</table>

*PSA was incubated with substrate (SGAWYYVPLG) for 2 hours at 37 °C in the presence of various concentrations of ZnCl₂. CaCl₂ served as a nonspecific control with no effect on PSA enzymatic activity.

†Values (absorbance units) represent the means ± standard error of the mean of five observations from one of two representative experiments.
with the hypothesis that the serine protease activity associated with both enzymes may only be a part of the stimulatory function of PSA in the proliferation of BPH-derived stromal cells. While the magnitude of PSA-induced cellular proliferation observed in our assays is relatively modest when compared with the IGF-I responses, the protease stimulated concentration-dependent increases in cell numbers. Prostatic stromal cells make insulin-like growth factors and IGFBP3 (29). Exogenous PSA may be proteolyzing the stromal cell-derived IGF-I–IGFBP3 complex, producing apparent direct stimulatory responses. Whatever the mechanism of PSA-induced stromal proliferation, the magnitude of the response is consistent with the relatively low rates of cellular proliferation in benign and malignant prostatic neoplasia. In addition, human prostatic stromal cells will not be exposed to α-chymotrypsin, since this protease is localized to the gut. α-Chymotrypsin is also cytotoxic to prostatic stromal cells at concentrations much lower than the concentration of PSA used to stimulate proliferation of BPH-derived stromal cells. These observations support the idea that PSA may play a role in selectively regulating prostatic fibromuscular proliferation.

Epidemiologic observations document that BPH and prostatic cancer are major factors affecting the health of the male population in the United States (30). Despite the importance of these diseases, our current perceptions of the cellular aberrations responsible for the development of BPH and prostatic cancer remain primitive. BPH results from the proliferation of prostatic acinar, ductal, and stromal elements (31). Several investigators (20,21) have suggested that the cellular inductive interactions by stromal cells on epithelial tissue elements play a dynamic role in modulating the normal and neoplastic growth of the prostate. The stromal–epithelial inductive interactions are likely multifaceted events, which involve cell–cell contact, extracellular matrix elements, and the production of soluble mediators (32–34). Our data provide evidence for a potential stimulatory induction of stromal cell proliferation by the paracrine actions of PSA, an epithelial, androgen-dependent cellular secretory protein.

PSA is a serine protease belonging to the family of glandular kallikreins (15). PSA is a glycoprotein regulated by androgens and produced almost exclusively by prostatic epithelium (13,35,36). The observations by Cohen et al. (12) that IGFBP3 is an in vivo substrate for PSA provide evidence supporting a paracrine role for PSA on prostatic stromal cells. The clinical utilities of PSA for monitoring the progression of prostatic cancer and responses to therapy have been well documented. However, PSA serum levels may also be elevated in BPH (16). In addition, several investigators (37–40) have shown that the concentrations of intraprostatic PSA are significantly higher in patients with BPH than in patients with prostatic cancer. These published observations provide evidence that the prostatic fibromuscular stroma may proliferate when exposed to intraprostatic concentrations of PSA.

PSA enzymatic activity is regulated by several factors. In the serum of healthy males, 85%–95% of the circulating PSA is bound to either α1-antichymotrypsin or α2-macroglobulin (41). Changes in the ratio of bound PSA to free PSA occur in the serum of patients with BPH and prostatic cancer. PSA is bound to α1-antichymotrypsin at a higher proportion in the circulation of untreated prostatic cancer patients relative to BPH patients. The association of PSA in the circulation with α1-antichymotrypsin and α2-macroglobulin requires the enzymatic activity of PSA on these carrier proteins. Our findings are consistent with the possibility that the prostatic stroma may be exposed to increased levels of biologically active PSA before it enters the circulation. Evidence also exists for an association between PSA levels and levels of IGFBPs in the serum of patients with prostatic cancer (42). Elevated levels of serum PSA are associated with high levels of serum IGFBP2 and reduced levels of serum IGFBP3 compared with levels in healthy male control subjects. This observation supports the observation by Cohen et al. that IGFBP3 is an in vivo substrate for PSA. In addition, it has recently been shown that increased levels of IGF-I are associated with an increased risk of prostatic cancer (43).

Free zinc has been identified as the factor responsible for the antibacterial activity of normal prostatic fluid (44). Zinc has been shown to serve as an in vivo defense mechanism against prostatic invasion and subsequent urinary tract infections in men (45). In addition to the antibacterial role of zinc in prostatic tissues, we have provided evidence for a role of zinc in regulatory PSA enzyme activity. Reductions in tissue zinc concentrations have been reported in prostatic cancer patients (46,47). In addition, the concentration of zinc is significantly lower in stromal than in epithelial preparations from BPH specimens (48). These lower concentrations of zinc in prostatic cancer patients and stromal tissue may increase the local availability of enzymatically active PSA to enhance cellular proliferation. These findings provide further clinically relevant associations supporting regulation of enzymatically active PSA, which may be involved in prostatic cellular proliferation. Before entering the circulation, PSA is exposed to the surrounding prostatic stroma in BPH and cancer tissues. This transient exposure may induce prostatic fibromuscular stromal proliferation by at least two different mechanisms. The first mechanism for PSA induction of cellular growth can be derived from its action in seminal fluid as described previously by Cohen et al. (12). Intraprostatic PSA may alter the affinity of IGFBP3 for IGF-I found in the surrounding growth milieu, releasing the IGF-I and allowing it to bind to its plasma membrane receptor on fibromuscular cells to stimulate cellular proliferation. An alternative proliferative mechanism might result from PSA binding to a specific cell surface receptor. The proteases, thrombin, and nerve growth factor have been demonstrated to exert some of their biologic activity through specific cell surface receptor interactions (49–51). This hypothesis is currently under investigation in our laboratory. PSA may also work as an autocrine growth factor on the surrounding responsive prostatic epithelia. Findings of Cohen et al. (52) and our laboratory (53) demonstrate both direct and indirect in vitro growth responses of normal and neoplastic prostatic epithelial cells to PSA.

From the above evidence, we conclude that PSA may play both a direct and an indirect role in stimulating prostatic fibromuscular stromal cells. These observations are consistent with the concept that PSA may play a physiologic role in the regulation of prostatic cell growth. However, many issues remain to be addressed before a definitive conclusion regarding this possibility can be reached. Additional experimentation will be necessary to determine the role of PSA in stimulating normal and neoplastic accessory sex organ growth in vivo.

**REFERENCES**


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NOTES

At the time that these studies were conducted, D. M. Sutkowski, R. L. Goode, C. Teater, A. M. McNultt, H. M. Hsiung, G. W. Becker, and B. L. Neubauer were employees of Eli Lilly and Company. They own that company’s stock as part of their compensation and retirement plan participation. P. Cohen is a member of the speaker’s bureau for Eli Lilly and Company.

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