ABSTRACT
Prostate cancer is a common malignancy affecting men, which is often associated with skeletal metastases resulting in significant morbidity and mortality. In this hormone-dependent cancer, low levels of a prostate secretory protein of 94 amino acids (PSP-94) are associated with advanced disease stage. In the current study, we have examined the effect of PSP-94 on prostate cancer growth and experimental metastases to the skeleton. For these studies, MatLyLu rat prostate cancer cells were transfected with full-length cDNA encoding parathyroid hormone-related protein [PTHrP (MatLyLu-PTHrP cells)], which is known to be the major pathogenetic factor for malignancy-associated hypercalcemia. MatLyLu-PTHrP cells were inoculated s.c. into the right flank or via intracardiac route into the left ventricle of syngeneic male Copenhagen rats. Intracardiac inoculation of MatLyLu cells routinely results in the development of tumors in the lumbar vertebrae, resulting in hind-limb paralysis. Animals were infused with different doses of PSP-94 (0.1, 1.0, and 10.0 μg/kg/day) starting on the day of tumor cell inoculation. Time of hind-limb paralysis and tumor volume were determined, and comparison was made between PSP-94-treated animals and control animals receiving vehicle alone. At the end of the study, animals were sacrificed, and plasma calcium, plasma PTHrP, and tumor PTHrP levels were determined. Whereas the highest dose of PSP-94 caused a modest but statistically significant delay in the development of hind-limb paralysis, a marked dose-dependent decrease in primary tumor volume was seen in experimental animals receiving PSP-94 due to its ability to promote tumor cell apoptosis. Furthermore, whereas control animals routinely developed hypercalcemia due to PTHrP production, treatment with PSP-94 led to a near normalization of plasma calcium and a marked reduction in PTHrP production as determined by radioimmunoassay and immunohistochemistry. Collectively, these results demonstrate the ability of PSP-94 to be an effective treatment modality for prostate cancer, where decrease in plasma PTHrP and calcium levels can serve as useful biochemical markers for monitoring the efficacy of this novel antitumor agent.

INTRODUCTION
Prostate cancer is one of the most commonly diagnosed cancers in men and is the second leading cause of cancer mortality following lung cancer in men (1). A distinct feature of prostate cancer is its ability to cause osteoblastic skeletal metastases, which contributes to the high rate of morbidity and mortality associated with this hormone-dependent malignancy (2). Additionally, due to the production of PTHrP3 by tumor cells, patients with advanced prostate cancer are known to exhibit an increase in their plasma calcium levels (3, 4). Several studies have provided convincing evidence that PTHrP is indeed the major pathogenetic factor responsible for hypercalcemia of malignancy, which is observed in 15–20% of all cancer patients (5, 6).

Clinical prostate cancer can be successfully treated at its early stage, when the cancer is well confined within the prostate gland (7). However, increased production of many factors including growth factors, sex steroids, angiogenic factors, and proteases such as urokinase and matrix metalloproteinases by tumor cells and their surrounding stroma is associated with high mortality (8–11). Despite recent advances in the therapeutic modalities for organ-confined prostate cancer including surgery and radiotherapy, limited success has been obtained in treating hormone-independent metastatic prostate cancer (12).

PSP-94, a 94-amino acid, cysteine-rich, nonglycosylated protein, is one of the three predominant proteins secreted by the prostate gland and found in human seminal fluid along with prostate-specific antigen and prostatic acid phosphatase (13, 14). Alternative names used to describe PSP-94 include prostatic inhibin (β-inhibin) and β-microseminoprotein (15). One of the main biological functions of PSP-94 is the inhibition of FSH (16). Whereas the majority of FSH is produced and secreted by the pituitary gland, it has been demonstrated that the prostate gland is an extrapituitary source of FSH (17). Elevated levels of FSH, in cases of benign prostatic hyperplasia, along with the presence of FSH receptors on the prostate gland suggest an autocrine/paracrine regulation of this hormone on prostate proliferation (18, 19). Whereas decreased levels of PSP-94 in late-stage prostate cancer and better prognosis in patients with high PSP-94 have been reported, additional clinical studies are required to validate the usefulness of PSP-94 as a prognostic marker for prostate cancer (20).

Several studies have demonstrated a progressive decrease in PSP-94 expression as prostate cancer progresses from a hormone-dependent to a hormone-independent state with complete lack of PSP-94 production in highly advanced metastatic prostate cancer (21). This differential expression could allow the use of PSP-94 as a prognostic marker for prostate cancer in the clinical setting (22, 23). One added advantage of PSP-94 as a prognostic marker is our ability to determine its level of production in the hormone-independent stage of the disease, which allows for a higher degree of sensitivity in tumors that have been previously exposed to androgen-ablating agents (24).

In the present study, we have evaluated the ability of PSP-94 to decrease prostate cancer tumor growth. For these studies, we have used our well-characterized syngeneic in vivo model of rat prostate cancer using the rat prostate cancer cell line Dunning R3327 MatLyLu transfected with the full-length cDNA encoding rat PTHrP (11). In this model, s.c. inoculation of tumor cells into the right flank of male Copenhagen rats routinely results in the development of primary tumors, whereas i.c. inoculation of tumor cells leads to tumor cell growth at long bones or at lumbar vertebrae, causing hind limb paralysis (10, 11, 25–28). After s.c. and i.c. inoculation of MatLyLu-PTHrP cells, the ability of different doses of PSP-94 to reduce tumor growth, experimental skeletal metastases, tumor PTHrP production, plasma calcium, and PTHrP was evaluated.

MATERIALS AND METHODS
Materials. PSP-94 was a gift from Procyon BioPharma Inc. (Montreal, Quebec, Canada) that was isolated and purified from human seminal fluid by...
ammonium sulfate fractionation, cation flow-through chromatography, and ion-exchange chromatography. Authenticity of purified protein was confirmed by mass spectrometry (29).

Cells and Cell Culture. The Dunning R3327 MatLyLu cell line was obtained from Dr. J. T. Isaacs (John Hopkins School of Medicine, Baltimore, MD) and transfected with full-length cDNA encoding rat PTHrP as described previously (11). One of the three well-characterized monoclonal cell lines, MatLyLu-PTHPitP8, was used throughout the course of these studies according to previously established methods of culture of these experimental cells (11).

Morphological analysis of control and experimental MatLyLu-PTHPitP cells treated with PSP-94 was carried out by plating 5 × 10^6 cells/well in 6-well plates (Falcon Plastics, Oxnard, CA) in the presence of 10% FBS. Cells were examined daily for any change in their morphology and photographed (30).

The effect of PSP-94 on MatLyLu-PTHPitP tumor cell invasive capacity was examined by two-compartment Boyden chamber (Transwell; Costar, Cambridge, MA) and basement membrane Matrigel (Becton Dickinson Labware, Bedford, MA) as described previously (31).

For growth curves, MatLyLu-PTHPitP cells were plated in 6-well plates (Falcon plastics) at seeding densities of 5 × 10^5 cells/well. For 4 days, cells from triplicate wells were cultured in the presence of different doses of PSP-94 (0.1, 1.0, and 10.0 μg/ml), trypsinized, resuspended, and counted in a model Z Coulter counter (Coulter Electronics, Bedfordshire, United Kingdom). Medium was changed every 2 days.

For DNA fragmentation, MatLyLu-PTHPitP cells were plated in 6-well plates (Falcon Plastics). Cells were treated with PSP-94 (10.0 μg/ml) for up to 72 h. DNA from treated cells incubated with PSP-94 and cells treated with vehicle alone was collected using a phenol:chloroform:isoamyl alcohol solution (50:48:2). Equal amounts of DNA were subjected to gel electrophoresis on a 2% agarose gel. DNA fragmentation was visualized by UV light using a transilluminator.

Animal Protocols. Inbred male Copenhagen rats weighing 200–250 g were obtained from Harlan Sprague Dawley (Indianapolis, IN). Before inoculation, MatLyLu-PTHPitP tumor cells growing in serum-containing medium were washed with Hank’s buffer, trypsinized, and collected by centrifugation at 1500 rpm for 5 min (10, 11, 30). Cell pellets (10 × 10^6 cells) were resuspended in 100 ml of saline and injected using a 1-ml insulin syringe into the left ventricle of rats anesthetized with ketamine/xylazine mixture. Animals were divided into control groups that received vehicle alone (PBS) and the left ventricle of rats anesthetized with ketamine/xylazine mixture. Animals were weighed at timed intervals throughout the duration of the study, and a statistically significant difference in animal weight was seen between control and experimental animals receiving the highest dose of PSP-94 at day 14 and 16 (Fig. 3 between control and experimental animals receiving the highest dose of PSP-94 at day 14 and 16 (Fig. 3).

RESULTS

Effect of PSP-94 on MatLyLu-PTHPitP Cell Growth, Morphology, and Invasion. MatLyLu-PTHPitP cells were grown in the presence of 0.1, 1.0, and 10.0 μg/ml PSP-94 or vehicle alone for up to 4 days, and the ability of PSP-94 to alter cell doubling time was evaluated daily. Comparison was made with doubling time of wild-type untransfected MatLyLu cells. Transfection of MatLyLu cells with PTHPitP cDNA resulted in reduced doubling time and increase in tumor cell growth due to the growth-promoting effects of PTHPitP. A significant decrease in MatLyLu-PTHPitP cell growth was seen after treatment with 10.0 μg/ml PSP-94 for 4 days (Fig. 1). Lower doses of PSP-94 (0.1 and 1.0 μg/ml) failed to exhibit any significant effect on tumor cell growth (data not shown). Treatment of MatLyLu-PTHPitP cells with 10.0 μg/ml PSP-94 for 4 days resulted in a noticeable change in tumor cell morphology, in which tumor cells were found to change their normal spindle-like shape to a more rounded and condensed appearance (data not shown). Using a Boyden chamber Matrigel invasion assay, all doses of PSP-94 failed to alter the invasive capacity of MatLyLu-PTHPitP cells (data not shown).

Effect of PSP-94 on MatLyLu-PTHPitP Tumor Growth. Male Copenhagen rats were inoculated with MatLyLu-PTHPitP cells via s.c. route of injection into the right flank. Starting on the day of tumor cell inoculation animals were weighed via s.c. route, below the tumor cell inoculation site, with different doses of PSP-94 (0.1–1.0 μg/kg/day) for up to 15 days. The effect of PSP-94 on reducing tumor growth was evaluated by daily determination of tumor volume, with comparison to control tumor-bearing animals receiving vehicle alone. Control animals showed a progressive increase in tumor volume throughout the duration of the study. In contrast to this, experimental animals receiving PSP-94 showed a marked dose-dependent reduction in tumor volume throughout the course of this study (Fig. 2). Both control and experimental animals were monitored for any noticeable side effects and cachexia resulting in weight loss. All animals were weighed at timed intervals throughout the duration of the study, and a statistically significant difference in animal weight was seen between control and experimental animals receiving the highest dose of PSP-94 at day 14 and 16 (Fig. 3A). However once the tumor weight
Effect of PSP-94 on MatLyLu-PTHrP Tumor Weight. To determine the effect of PSP-94 on tumor weight, animals inoculated with MatLyLu-PTHrP via s.c. route of injection were sacrificed at the end of the study (day 16), and their tumors were excised and weighed. Control animals receiving vehicle alone exhibited large tumors, whereas treatment with different doses of PSP-94 (0.1–10.0 μg/kg/day) resulted in a significant dose-dependent decrease in tumor weight (Fig. 4).

Effect of PSP-94 on the Development of Experimental Skeletal Metastases. MatLyLu-PTHrP cells were inoculated into male Copenhagen rats via i.c. injection into the left ventricle. Starting on the day of tumor cell inoculation (day 0), animals were given different doses of PSP-94 (0.1–10.0 μg/kg/day) via i.p. route. The effect of PSP-94 on delaying the development of experimental skeletal metastases was evaluated by daily monitoring of the animals for the development of hind-limb paralysis. All (100%) control animals inoculated with MatLyLu-PTHrP cells and receiving vehicle alone developed hind-limb paralysis by day 13. Whereas 0.1 and 1.0 μg/kg/day PSP-94 had no significant effect on delaying the time of hind-limb paralysis (data not shown), treatment with 10.0 μg/kg/day PSP-94 resulted in a modest yet statistically significant delay in the number of animals developing...
hind-limb paralysis. The percentage of the total number of animals not developing hind-limb paralysis at different days is shown in Fig. 5.

**Effect of PSP-94 on Plasma PTHrP and Calcium Levels and Tumor PTHrP Production.** To determine the effect of PSP-94 on plasma PTHrP and calcium levels, animals inoculated with MatLyLu-PTHrP cells via s.c. route were sacrificed at the end of the study, plasma was collected, and PTHrP levels were analyzed using a radioimmunoassay. Comparison was made between plasma collected from normal, non-tumor-bearing animals, control tumor-bearing animals receiving vehicle alone, and experimental animals receiving different doses of PSP-94 (0.1–10.0 μg/kg/day). Normal non-tumor-bearing animals showed basal levels of plasma PTHrP, whereas animals inoculated with MatLyLu-PTHrP cells and receiving vehicle alone showed marked elevated levels of immunoreactive plasma PTHrP. Treatment of tumor-bearing animals with PSP-94 resulted in a dose-dependent decrease in plasma PTHrP levels (Fig. 6A). Analysis of plasma collected from normal non-tumor-bearing animals and tumor-bearing animals receiving vehicle alone revealed a marked increase in plasma calcium levels of control tumor-bearing animals at the time of sacrifice on day 16 after tumor cell inoculation. In contrast, experimental groups treated with different doses of PSP-94 for 15 consecutive days as described in “Materials and Methods.” All animals were sacrificed at the end of the study (day 16), and plasma was collected from control (CTL), vehicle-treated animals and PSP-94-treated animals and analyzed for immunoreactive plasma PTHrP (iPTHrP) levels using a standard radioimmunoassay as described in “Materials and Methods.” Plasma PTHrP levels in normal non-tumor-bearing animals are also shown (N). B, male Copenhagen rats were inoculated s.c. with 1 × 10^6 MatLyLu-PTHrP cells. Starting at the time of tumor cell inoculation, animals were infused with different doses of PSP-94 for 15 consecutive days as described in “Materials and Methods.” All animals were sacrificed at the end of the study (day 16), and plasma was collected from vehicle-treated control (CTL) and PSP-94-treated animals for analysis of plasma calcium levels as described in “Materials and Methods.” Plasma calcium levels from normal, non-tumor-bearing animals are also shown (N). Results represent the mean ± SE of five animals in each group in three different experiments. Significant differences from control tumor-bearing animals receiving vehicle alone are represented by asterisks (*P < 0.05).

**Effect of PSP-94 on Experimental Spinal Metastases.** Male Copenhagen rats were inoculated via i.c. route into the left ventricle with 1 × 10^6 MatLyLu-PTHrP cells. Starting on the time of tumor cell inoculation (day 0), animals were infused with different doses of PSP-94 (0.1–10.0 μg/kg/day) until the day of hind-limb paralysis development as described in “Materials and Methods.” Animals receiving vehicle alone as control (CTL) or PSP-94 were monitored daily for the development of hind-limb paralysis, and the percentage of animals not paralyzed at different time points in each group was calculated. Results represent the mean ± SE of five animals in each group in three different experiments. Significant differences in the percentage of nonparalyzed animals from control tumor-bearing animals receiving vehicle alone are represented by asterisks (*P < 0.05).

**Effect of PSP-94 on MatLyLu-PTHrP Tumor Cell Apoptosis in Vitro and in Vivo.** To investigate the underlying molecular mechanism of action of PSP-94 in reducing tumor growth, MatLyLu-PTHrP cells were cultured in the presence of PSP-94 (10.0 μg/ml) or vehicle alone for different time intervals. Genomic DNA was collected from

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**Fig. 4.** Effect of PSP-94 on MatLyLu-PTHrP tumor weight. Male Copenhagen rats were inoculated with 1 × 10^6 MatLyLu-PTHrP cells via s.c. injection into the right flank. Starting on the day of tumor cell inoculation, animals were given different doses of PSP-94 for 15 consecutive days as described in “Materials and Methods.” At the end of the study, tumors from control (CTL), vehicle-treated animals and PSP-94-treated animals were excised and weighed. Results represent the mean ± SE of five animals in each group in three different experiments. Significant differences from control tumor-bearing animals receiving vehicle alone are represented by asterisks (*P < 0.05).

**Fig. 5.** Effect of PSP-94 on experimental spinal metastases. Male Copenhagen rats were inoculated via i.c. route into the left ventricle with 10 × 10^5 MatLyLu-PTHrP cells. Starting on the time of tumor cell inoculation, animals were given different doses of PSP-94 for 15 consecutive days as described in “Materials and Methods.” Animals receiving vehicle alone as control (CTL) or PSP-94 were monitored daily for the development of hind-limb paralysis, and the percentage of animals not paralyzed at different time points in each group was calculated. Results represent the mean ± SE of five animals in each group in three different experiments. Significant differences in the percentage of nonparalyzed animals from control tumor-bearing animals receiving vehicle alone are represented by asterisks (*P < 0.05).
cells cultured in the presence of vehicle alone or PSP-94. Equal quantities of DNA were subjected to electrophoresis on a 2% agarose gel and assessed for the degree of DNA fragmentation. Control MatLyLu-PTHrP cells cultured in the presence of vehicle alone exhibited no signs of DNA fragmentation. However, experimental MatLyLu-PTHrP cells cultured in the presence of PSP-94 (10.0 μg/ml) exhibited marked DNA fragmentation after 72 h of treatment (Fig. 8A). The degree of DNA fragmentation was also analyzed in vivo using TUNEL assay, which can serve as a marker for apoptosis. Tumor sections treated with PSP-94 (10.0 μg/kg/day) were significantly more TUNEL positive as compared with vehicle-treated control tumors (Fig. 8B). Counterstaining with Hoechst reagent revealed the presence of apoptotic bodies in tissue sections from animals treated with PSP-94. Furthermore, condensed chromatin, characteristic of apoptotic cells, was observed in PSP-94-treated tumors. Control, vehicle-treated tumors exhibited normal DNA staining patterns (Fig. 8B). These in vitro and in vivo findings demonstrate that reduction in tumor volume after treatment with PSP-94 is indeed due to its ability to promote tumor cell apoptosis.

**DISCUSSION**

In this study, we have used a syngeneic model of rat prostate cancer to demonstrate the ability of PSP-94 to reduce prostate cancer growth and experimental skeletal metastases (10, 25). Amino acid sequence homology between human and rat prostate secretory protein and similarity in their tertiary structures as determined by highly conserved cysteine residues have allowed the use of human PSP-94 for these studies (35). Use of this homologous model for prostate cancer allows for full interaction between the host environment and growth factors [epidermal growth factor and transforming growth factor β (36)] and proteases [urokinase and matrix metalloproteinases (32, 37)] secreted by tumor cells. To evaluate the efficacy of PSP-94, MatLyLu cells transfected with full-length cDNA encoding PTHrP (MatLyLu-PTHrP cells) were used. These prostate cancer cells are hormone independent, allowing for the evaluation of the effect of PSP-94 on late-stage prostate cancer. Due to the high levels of PTHrP production, these animals routinely develop hypercalcemia, a common complication in patients suffering from advanced prostate cancer (3, 4).

Inoculation of male Copenhagen rats with MatLyLu-PTHrP cells into the right flank via s.c. injections resulted in the development of primary tumors. Whereas control, vehicle-treated animals developed...
large primary tumors, treatment with different doses of PSP-94 resulted in a dose-dependent decrease in their tumor mass. These antitumor effects were not associated with any noticeable side effects or weight loss of experimental animals. TUNEL analysis carried out on tumor sections from control and experimental animals revealed that PSP-94-treated tumors were more TUNEL positive than control tumors, indicating a higher degree of apoptosis in PSP-94-treated animals. In addition to this, counterstaining with Hoechst reagent revealed condensed, apoptotic chromatin in PSP-94-treated tumors, whereas control tumors exhibited normal DNA staining. Upon sacrifice of the animals, plasma was collected and analyzed for plasma PTHrP levels. These findings allow us to speculate that PSP-94 may reduce plasma PTHrP levels. In addition, the same dose-dependent decrease was observed in tumor PTHrP production when tumor samples from control, vehicle-treated, and PSP-94-treated animals were subjected to immunohistochemical analysis. Being the major pathogenetic factor of hypercalcemia of malignancy, plasma calcium levels correlated with that of plasma PTHrP levels (3–5). Inoculation of MatLyLu-PTHrP cells into the animals resulted in a marked increase in their plasma PTHrP levels. In contrast to this, treatment with different doses of PSP-94 resulted in a dose-dependent decrease in plasma PTHrP levels. In addition, the same dose-dependent decrease was observed in tumor PTHrP production when tumor samples from control, vehicle-treated, and PSP-94-treated animals were subjected to electrophoresis on a 2% agarose gel and visualized under UV light. A representative photograph of three such experiments is shown. B, male Copenhagen rats were inoculated with 1 × 10⁶ MatLyLu-PTHrP cells. Starting at the time of tumor cell inoculation, animals were infused with different doses of PSP-94 for 15 consecutive days as described in “Materials and Methods.” All animals were sacrificed at the end of the study, and their primary tumors removed, embedded in paraffin, sectioned, and processed by TUNEL assay as described in “Materials and Methods” (top panel). After the TUNEL assay, they were counterstained with Hoechst reagent (bottom panel). Three animals were present in each group, and three sections were analyzed for each animal. At least 10 random fields of observation were evaluated. A representative photomicrograph for three such experiments in each group is shown. Magnification, ×200.

Because the major cause of prostate cancer-related mortality is the development of metastases, evaluation of the effect of PSP-94 on delaying the development of experimental skeletal metastases was carried out by inoculating male Copenhagen rats with MatLyLu-PTHrP cells via i.c. route into the left ventricle. In this experimental model of skeletal metastases, injection of MatLyLu cells into the left ventricle results in tumor cell growth at lumbar vertebra, causing compression of the spinal cord and leading to hind-limb paralysis (10, 11, 25, 26). Whereas all control, vehicle-treated animals developed hind-limb paralysis by day 13, administration of the highest dose of PSP-94 starting from the time of tumor cell inoculation resulted in a modest delay in the development of tumors in the lumbar vertebra. Such a modest response on blocking tumor growth in the skeleton after PSP-94 treatment could be due to several factors including the possibility of low bioavailability of PSP-94 to the skeleton, a common drawback associated with developing effective therapeutic agents for skeletal metastases (38).

Using this model, we were not only able to demonstrate the antitumor effects of PSP-94 by reduction in tumor volume and weight, but biochemical parameters such as plasma calcium and PTHrP levels also showed a marked decrease after therapy. A significant finding in these studies was that whereas decrease in tumor volume was dose dependent, 10.0 μg/kg/day PSP-94 did not show a marked decrease in tumor volume as compared with 1.0 μg/kg/day PSP-94. In contrast, the ability of PSP-94 to reduce plasma calcium, plasma PTHrP, and tumor PTHrP continued to show a dose-dependent effect, with 10.0 μg/kg/day PSP-94 causing near normalization of plasma calcium and PTHrP levels. These findings allow us to speculate that PSP-94 may
also have additional effects including its ability to regulate PTHrP production by tumor cells or alter calcium homeostasis. Indeed PSP-94 has been shown to suppress FSH, which is known to regulate intracellular calcium (39). Suppression of FSH by PSP-94 may serve as an additional mechanism to cause antitumor effects due to the growth-promoting effects of FSH in prostate cancer (18). Furthermore, although cloning and characterization of a putative PSP-94 receptor has not been performed, several studies have provided evidence for the existence of PSP-94-binding proteins on prostate cancer cells that could allow PSP-94 binding to initiate a signaling cascade that results in the observed antitumor effects (15, 40).

Collectively, the results of this study demonstrate PSP-94 to be an effective inhibitor of hormone-independent, late-stage prostate cancer growth and its associated hypercalcemia of malignancy without manifesting any noticeable cytotoxic effects. Additional studies will define the minimum sequence requirement to obtain maximum level of efficacy. These synthetic peptides and their peptidomimetic analogues alone or in combination with currently available chemotherapeutic agents will provide unique opportunities to block prostate cancer progression with highly effective nontoxic biotherapeutic agents that can be delivered over a long period of time without any drug-associated side effects. These approaches will go a long way in reducing prostate cancer-associated morbidity and mortality.

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Prostate Secretory Protein PSP-94 Decreases Tumor Growth and Hypercalcemia of Malignancy in a Syngenic in Vivo Model of Prostate Cancer

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