Human Prostatic Inhibin Suppresses Tumor Growth and Inhibits Clonogenic Cell Survival of a Model Prostatic Adenocarcinoma, the Dunning R3327G Rat Tumor

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ABSTRACT

Prostatic inhibin (PI) is a 10,700 protein found in human seminal plasma and is secreted by the prostate. Recognition of alteration of PI levels in prostatic diseases prompted us to investigate its effect on an animal prostatic adenocarcinoma model, the Dunning R3327G rat tumor. PI not only inhibited in vitro growth of tumor cells but also suppressed tumor growth in vivo. A dose-dependent inhibition of both the clonogenic cell growth and rate of proliferation (DNA synthesis) was observed in tumor cell cultures incubated with purified PI. These inhibitory activities were similar in both androgen-dependent and androgen-independent Dunning tumor cell lines. A functional decapetide of PI was also found to inhibit Dunning tumor cell colonies in a dose-dependent manner. Daily injection of purified PI into tumor-bearing rats suppressed the tumor growth. A 58% reduction in tumor weight and a 2-fold reduction in tumor growth rate were observed over a 15-day treatment period. Continued treatment with PI significantly suppressed the tumor growth rate by nearly 3-fold. These findings clearly demonstrate a potential application of PI for treating human prostatic adenocarcinoma.

INTRODUCTION

Inhibins are a class of peptide hormones that are known to control the secretion of pituitary FSH (1). Both gonadal and prostatic inhibins have been extensively characterized (2, 3). Human PI is a 10,700 basic protein composed of 94 amino acid residues (4). PI is an abundant protein in the seminal plasma (5). Immunohistochemical and metabolic labeling studies have shown that the prostate is a major source of PI (6). Although gonadal inhibin and PI are functionally similar in suppressing FSH levels in vivo, they share little structural homology (7). Furthermore, unlike gonadal inhibins, PI does not suppress FSH secretion by cultured rat pituitary cells (8).

Both the serum and urinary levels of PI increase during BPH. For example, about a 2-fold or a 5-fold increase in serum PI is detected in patients with BPH or PC, respectively (9). The increased levels of PI in sera of patients with either BPH or PC suggests a possible link between abnormal prostatic activity and PI secretion. This might be due to either a direct role of PI in the growth and differentiation of the prostate gland or the increased metabolic activity in the abnormal prostate. We reasoned that one way to understand the role of PI in such circumstances is to examine the effect of PI on a model prostatic adenocarcinoma, preferably on isolated tumor cells in vitro and on a transplantable tumor in vivo. For this reason, we investigated the possible action of PI on a model PC, the Dunning R3327G rat prostatic adenocarcinoma (DT; Ref. 10). The usefulness of DT as a preeminent animal model for the study of PC is widely documented (11).

for this investigation. It is a fast-growing, poorly differentiated, transplantable tumor. DT is also a fast-progressing tumor, changing from androgen-dependent growth to -independent growth in a few transplant generations (12). Therefore, we examined the effect of PI, not only on the growth of DT cells in vitro, but also on the tumor in vivo. Our results show that PI is cytotoxic to DT cells in vitro and it suppresses tumor growth in vivo.

MATERIALS AND METHODS

Purification of PI. PI was purified to homogeneity from pooled human seminal plasma by a 5-step procedure described elsewhere (13). Purity of the PI was confirmed by detection of a single band on a sodium dodecyl sulfate-polyacrylamide gel and as a single peak on the high performance liquid chromatography profile. The biological activity of PI was assessed on the basis of its ability to suppress circulating levels of FSH in intact adult male rats (14). A dose-related suppression of serum FSH (40% of that of untreated control) was observed at 20 μg PI/animal, without any change in the luteinizing hormone level. Lyophilized PI was dissolved in a phosphate-buffered saline (10 mM sodium phosphate, pH 7.2-7.5 mM sodium chloride) and sterilized by filtration. Sterile solutions of PI were used in all experiments.

Synthetic Decapetide of PI. Details of the synthesis, characterization, and biological specificity of a COOH-terminal decapetide of PI have been reported elsewhere (15). This decapeptide is an analogue of carboxyl terminal 45-94 amino acid residues of PI, except that lysine is replaced by tyrosine, and cysteine is protected by an acetylamidomethyl group. The decapeptide was custom synthesized at the NIH in Bethesda, MD. The luteinizing hormone-releasing hormone nonapeptide agonist Lupron (leuprolide), was a gift from Abbott Laboratories, Chicago, IL. A sterile aqueous solution of leuprolide was injected into tumor-bearing animals.

Cells. DT cells for continuous in vitro culture were derived from cells dissociated from R3327G tumors implanted in carriers (Cop × F344 F, male rats) as described previously (12). Cells cultured from tumors in the 20th transplant generation are largely (>60%; see “Results”) androgen-dependent for their in vitro growth, although the tumor itself is a mixture of both androgen-independent and -dependent cells. Cells cultured from the 28th transplant generation are largely insensitive for androgen deprivation (>90%; see “Results”) for in vitro growth. Tumor cells were subjected to five serial passages to eliminate non tumor cell populations such as macrophages, lymphocytes, and fibroblasts before initiating any studies with PI. For reasons explained below (see “Results”), no attempt was made to clonally propagate these cells. Cells were routinely cultured in αMEM (Gibco, Grand Island, NY) fortified with fetal bovine serum (FBS, 20% [HyClone, Logan, UT] and antibiotics, complete medium [CM]) α-minimum essential medium. For clonogenic survival assays (colony assays), cells cultured in T25 flasks were treated with 0.25% trypsin-0.002% EDTA and dispersed into a single cell suspension, and 500 or 1000 viable cells were plated in triplicate 35-mm tissue culture dishes with 2 ml CM. PI was dissolved in sterile phosphate-buffered saline and added to triplicate dishes (0-40 μg ml; 0.1 ml/dish). PI was added to dishes on day 1 (24 hr after plating cells) and day 3 (72 hr). Colonies of adherent cells were fixed and stained simultaneously with a 0.1% solution of Coomassie blue in 45% methanol and 10% acetic acid on day 7.

In Vitro Selection of Steroid-dependent and Independent DT Cells. The DT cells dissociated from a tumor in the 20th transplant passage were cultured in the presence or absence of steroids. For comparison, DT cells obtained from a tumor in the 28th transplant generation, which is documented to be androgen insensitive (11), were cultured identically. Cells from both types of tumors were initially cultured in CM. After two in vitro passages, the culture medium was changed to a medium containing (20% v/v) either steroid-stripped serum...
(dextran-charcoal treated FBS; Ref. 16) or complete FBS (steroid content, 130 ng/liter). Total steroid content of the charcoal-dextran treated serum was less than the minimum detectable by available assays. To obtain steroid-insensitive cells derived from a tumor in the 20th transplant generation, dissociated tumor cells from a carrier of that generation were cultured in steroid stripped medium for 5 continuous passages in vitro.

Cell Growth Assay. Costar cluster 24-well plates were seeded with 0.5 to 1.0 × 10^3 cells/well. They were incubated with several concentrations of either PI or decapeptide on day 0. On day 3 and day 7, cells were dislodged from the wells by incubating with a solution of 0.25% trypsin and 0.02% EDTA and then counted in a hemocytometer. Dead cells identified by 0.4% trypan blue staining were excluded from the counts.

PI or decapeptide-induced inhibition of DNA synthesis was estimated by a [3H]thymidine incorporation assay. Cells were cultured as described above for 3 or 7 days. On day 3 or on day 7, culture medium in the wells was replaced with CM containing different amounts of either PI or the decapeptide for the next 24 h. DNA synthesis activity was estimated by adding [3H]thymidine (68 Ci/mmol; ICN, Irvine, Ca) to all wells at a concentration of 0.5 μCi/ml and incubated for 1 h at 37°C. Incorporated radioactivity was then estimated after precipitation with 10% trichloroacetic acid, followed by extraction with 0.5 N NaOH, mixing with a scintillation cocktail (Scintiverse, Fisher Scientific, NY) and counting.

Tumor Induction and in Vivo Tumor Growth Assays. Tumors were induced in adult CD-1 F344 male rats weighing an average of 300 g, by s.c. injection of 2.0 × 10^7 R3327G cells in the 27th transplant generation. Treatments were initiated once the tumors were palpable (50–100 mm^3). Tumor growth was estimated from three-axis microcaliper measurements approximated to an ellipsoid: length × width × height × 0.5236 (12). Tumor volume was measured every other day until necrosis occurred, at which time the tumors were excised and weighed. Tumor cell suspensions were prepared by digesting the minced tumor pieces with a trypsin/EDTA/collagenase solution as described (12). This was done to measure secretion of any PI-like material, in the absence of any externally added PI. Tumors were also fixed in formalin and embedded in paraffin, and 5-μm sections were cut for histology. Sections were stained with hematoxylin/eosin for histological examination.

RESULTS

Inhibition of in Vitro DT Cell Colony Growth by PI and the Decapeptide. The initial plating efficiency (the percentage of plated cells forming colonies of more than 50 cells) of fresh DT cell suspension was less than 1%. However, that improved to over 30% in subsequent in vitro passages and remained unchanged for the next 30 in vitro passages. The plating efficiency did not change significantly with the initial cell inoculum in the range of 100 to 1000 cells/35-mm tissue culture dish. These cells formed characteristic colonies containing solid sheets of cells. Typically, a 7-day colony of DT cells consisted of 102.3 ± 13.7 cells. As illustrated in Fig. 1, a dose-dependent inhibition of the number of colonies was observed with increasing amounts of PI added to the culture. Starting from 100 ng/ml, a significant decrease in clonogenic cell survival and cell proliferation was observed. In dishes that received 4 μg PI/ml, the number of colonies was only 10–15% of that in the control dishes; increasing the dose of PI to 10 μg/ml in one experiment resulted in almost complete (99.5%) inhibition of DT cell colony growth. Furthermore, the average size of the colonies decreased with increasing concentration of PI (data not shown).

In an attempt to examine whether the growth-suppressing activity of PI could be reproduced with the PI-decapeptide analogue, a sterile solution of the decapeptide was substituted for PI in the colony assay as shown in Fig. 2. As observed for PI-treated cultures, the colonies in decapeptide-treated dishes were fewer and smaller (mostly clusters of 4–16 cells) with increasing concentration of the peptide. A 50% reduction in the number of DT cell colonies was observed at just 50 ng decapeptide/ml and 70% inhibition at 1 μg/ml was observed. However, complete inhibition of clonogenic growth was not observed even at a dose of 5 μg decapeptide/ml. Furthermore, PI was more potent than the decapeptide on an equimolar basis.

Effect of PI on the Growth of Androgen-dependent and -independent DT Cell Colonies. R3327G tumors are composed of both androgen-dependent and -independent cells (12). The fraction of androgen-dependent cells decreases with increasing transplant generation. Therefore, the effect of PI was examined on these two cell populations cultured in vitro. As illustrated in Fig. 3A, the effect of PI was similar in all test conditions and also on both cell types. It was interesting to note that even when the DT cells were derived from the androgen-dependent tumors, only 40% of the cells required steroids for in vitro clonogenic cell proliferation. However, DT cells derived from tumor in the 28th transplant generation proliferated equally well in either medium. Regardless of their requirement for steroids for growth, they were equally sensitive to PI-induced inhibition of cell proliferation. Although the actual number of colonies appearing under each assay condition was different in these cell types (Fig. 3B), the extent of PI-induction colony inhibition was comparable in all. Four in vitro passages in steroid-stripped medium completely eliminated androgen-dependent cells in the culture. Adding complete medium after four passages did not increase the plating efficiency. The resulting colonies, however, were larger in diameter. Because the inhibitory effect of PI was comparable in both androgen-dependent and -inde-
Suppression of tumor growth by prostatic inhibin-dependent cells, all further experiments were conducted on a clonally derived androgen-independent DT cell line.

Inhibition of Cell Proliferation by PI. The inhibition of colony growth might be a consequence of either immediate cell death or a delay in the growth rate (cell cycle) due to the addition of a growth-inhibitory agent, such as PI. To distinguish between these two possibilities, cells were incubated with increasing concentrations of PI and changes in cell number were estimated. PI significantly inhibited cell doubling. For example, in control wells, cell number increased 4-fold after 3 days and 28-fold after 7 days of culture. In contrast to this, no increase (nor a decrease) in cell number was observed on day 3 while only a 5-fold increase had occurred by day 7 at 1 μg PI/ml. This inhibition of cell growth was not due to increased cell death in presence of PI, as determined by trypan blue staining of both the control and PI treated cells (data not shown), but may be due to the cytostatic effect of PI.

PI-induced Inhibition of [3H]Thymidine Incorporation. The effect of PI on DNA synthesis in DT cells was measured by pulse labeling the cells with [3H]thymidine. The results, represented in Fig. 4, indicate that at 1 μg PI/ml, the thymidine incorporation rate was only 20% of that of the control. The decapeptide of PI also caused inhibition of DT cell proliferation. Unlike the PI-induced inhibition of DNA synthesis, the maximum inhibition with decapeptide was only 40% of the rate of DNA synthesis in control cells (Fig. 4B).

PI-induced Suppression of in Vivo Tumor Growth. A daily injection of PI (5 μg/kg body weight) in tumor-bearing rats (tumor volume ≤ 100 mm³) during the exponential growth of the tumor resulted in a dramatic slowing of tumor growth as revealed by daily measurement of the tumor volume (Fig. 5). However, tumors in the saline-injected control group grew rapidly and turned necrotic within 10 days (Fig. 6). The corresponding tumor weights for PI-treated and control groups on day 10 were 2.66 ± 0.48 and 6.44 ± 1.19 g, respectively. Thus, a 58% mean reduction in tumor weight was observed after 10 days of treatment. Sections of control and PI-treated tumors were also examined for gross histological differences. Little difference was found between the two groups. Both showed poorly differentiated morphology (data not shown).

The extent of tumor growth delay was reexamined in an extended study. In addition, the androgen insensitivity of the tumor was confirmed by treating a group of tumor-bearing animals with leuprolide in this extended study. Instead of terminating the treatment with PI when tumors in the control group reached 10 cc, daily injection with PI was continued until the tumors in PI-treated animals turned necrotic. As illustrated in Fig. 7, once again there was a significant initial delay on the tumor growth rate in animals given PI injections. In the control
group or leuprolide-treated group, however, tumors reached a size of 10 cc in 13 days, similar to the earlier observation. Tumor size continued to increase very slowly in the PI-treated group. After 30 days of treatment the tumors turned necrotic, and volumes averaged 8.28 ± 1.2 cc. Thus, a 2-fold delay in tumor growth rate by daily injection of PI was observed. Previous work has shown that daily leuprolide injection results in significant reductions in serum testosterone levels approaching that achieved by orchietomy (17). However, as shown in Fig. 7, on a G27 tumor, a hormonally mediated steroid suppressive drug (leuprolide) had no effect on the growth of the tumors of this transplant generation.

**Oversecretion of a PI-like Substance by PI-treated DT Cells.** Tumor cells dissociated from the control group and from PI-treated groups were plated separately and cultured for 24 h to remove residual PI (if any) present in the cells. The culture medium in the flasks was exchanged for fresh medium and the flasks were incubated for an additional 3 days. Spent culture medium was collected from each flask. The presence of a PI-like substance in the spent culture medium was estimated by a specific radioimmunoassay for PI (18). PI or immunocompetent PI-like substance was not detectable in fresh culture medium within the detection limit of the assay (0.1 ng/ml), but the spent culture medium from untreated and PI-treated tumor cell cultures contained detectable amounts of a PI-like substance. This PI-like material in the spent medium of untreated DT cell cultures was 0.68 ± 0.2 ng/ml and that in PI-treated tumor cell cultures was 8.0 ± 1.2 ng/ml, about 12-fold higher. The enhanced secretion of the PI-like substance into the culture medium continued for two in vitro passages of cells cultured from both PI-treated and control tumors. PI secretion decreased rapidly thereafter. PI was not detectable in the spent medium of cells after the fifth passage.

**DISCUSSION**

Our aim in this study was to examine the role of PI in a model prostate adenocarcinoma. Since PI isolated from human seminal plasma shows biological activity on rat systems, the Dunning tumor is a suitable model system for such a study. We found that PI, a protein hormone secreted by human prostate, inhibits the growth of this tumor both in vivo and in vitro. This study also provided compelling evidence that PI is a growth-inhibitory protein effective on rat prostatic tumors. In addition, results presented here also showed that PI is equally effective on inhibiting the growth of both androgen-responsive and -unresponsive DT cell populations. The latter indicates that the mechanism of action of PI as a growth inhibitor is probably independent of steroid stimulated growth pathways in prostatic tumors. Although PI is shown to be an inhibitor of testosterone metabolism in normal rat prostate tissue (19), its activity on DT did not depend on the steroid requirement of DT cells. PI was equally effective on both androgen-dependent and -independent cultures.

The mechanism of action of PI on DT cells is unknown at present. We only found a growth delay during the early phase of colony growth and a PI-induced inhibition of DNA synthesis. However, the action of PI is clearly mediated through its cell-surface receptor(s) as shown by binding studies with a plasma membrane preparation of human prostate tissue (20).

On the basis of our observation that a significant but partial inhibition of DT cell growth by PI occurs, we offer the hypothesis that this might be the result of two independent mechanisms: (a) if we assume that PI is involved in the regulation of normal growth and differentiation of the prostate, at above-normal PI concentrations (as used in this study), cell-surface PI receptors might be down-regulated, as a result of which further activity of PI is reduced; (b) PI might also down-regulate the mitogenic activity of some yet unknown growth factors that may influence DT cells. It is not clear at present, however, whether the known physiological functions of PI, i.e., in vivo suppression of FSH in adult rats (3) and inhibition of testosterone metabolism (19) have any role in our finding. For example, PI and its active peptide are devoid of any FSH-suppressing activity in vitro as shown by a rat pituitary cell culture assay (8). Furthermore, the findings in this study that PI was equally effective in suppressing the clonogenic growth of DT cells in vitro and tumor growth in vivo indicate that the tumor-suppressing activity of PI may not be coupled to its FSH-suppressing activity. However, it is worth noting here that although androgens are the primary hormones responsible for growth and differentiation of prostate, pituitary hormones such as FSH, prolactin, and adrenocorticotrophic hormone are also implicated in prostatic growth (21, 22). It was also recently reported that human prostate itself secretes an FSH-like protein (23).
Earlier studies have shown that normal prostatic epithelial cells synthesize and secrete low amounts of PI (24), and increased secretion is associated with the development of benign and malignant prostatic conditions (9). Transplantable tumors such as the DT however, secrete very small amounts of PI-like material (<1 ng/ml serum). Therefore, as found in this study, growth inhibition at a 10X higher concentration than that reported in the sera of patients with PC might suggest a bimodal action of PI on prostate growth. Compared to its level in normal rat serum (<1 ng/ml), the 50% inhibitory concentration determined in this study is 100-fold higher (~1 μg/ml). This may mean that PI is stimulatory at very low concentrations (<1 ng/ml) but growth inhibitory at significantly higher concentrations. Furthermore, the effective dose for significant suppression of colony growth was 1 μg/ml, compared to only 0.1–0.2 ng/ml serum levels in BPH patients. Even at 4 μg PI/ml, clonogenic growth of DT cells was inhibited up to 90% but not 100%. Therefore, PI is probably a tumor growth suppressor.

Another significant finding of this study is that a synthetic peptide of PI also inhibited the clonogenic growth of DT cells. This finding not only proves the specificity of PI action on DT cells but also provides a possibility to synthesize smaller and more potent peptide analogues of PI. The reduced potency of the decapeptide may be due to its instability in culture medium or its lower affinity to cell-surface receptors (15).

We found that tumor cells pretreated with PI in vivo continue to secrete PI-like material in vitro. Thus, the excess production/secretion of PI might be detrimental to the survival of DT cells. Excess secretion of certain growth factor(s) or overexpression of growth factor receptor(s) causes the same growth factor to be cytotoxic to some carcinoma cells. For example, the EGF is cytotoxic to A431 cells, an epidermoid carcinoma cell line that overexpresses EGF receptor (25). Similarly, EGF inhibits mitogenesis of a cervical carcinoma cell line (26). Therefore, if indeed PI is a growth regulator of normal prostatic epithelium, then an excess dosage might inhibit the malignant growth of the same cells. In summary, our present data support the hypothesis that an autocrine/paracrine regulation of prostate tumor growth exists in a model prostate tumor.

4 Unpublished observation.

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