Anti-invasive, Antitumorigenic, and Antimetastatic Activities of the PHSCN Sequence in Prostate Carcinoma

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INTRODUCTION

Prostate cancer is the most common malignancy in men in the United States, causing >32,000 deaths each year (1). If localized, radical prostatectomy can be curative; however, ~60% of prostate cancers are not organ confined at the time of diagnosis (2). Metastatic disease is treated by androgen ablation therapy to induce temporary remission. However, relapse after a median of 18 months is inevitable because of the development of androgen-independent cancer (3, 4). Because androgen withdrawal is currently the only effective form of systemic therapy for metastatic prostate cancer, the appearance of androgen-independent clones of tumor cells means that there is no effective curative therapy for patients thereafter (5, 6). Thus, new therapies to treat invasive or metastatic prostate cancer are necessary.

To develop leads for new antitumorigenic and antimetastatic therapies, Copenhagen rats engrafted with metastatic MLL cells, a well-tested animal model of metanastatic prostate cancer, have been successfully used (7, 8). The Dunning (R3327) rat prostate adenocarcinoma model of prostate cancer was developed from a spontaneous adenocarcinoma of a male rat (9). The MLL subline is a fast-growing, poorly differentiated metastatic cell line that, upon injection of a million cells into the thigh of the rat, leads to animal death within ~25 days because of overwhelming primary tumor burden (10); or, if the primary tumor is removed, results in animals that die of lung metasteses ~40 days after tumor inoculation (11).

We analyzed the invasion-inducing role of a specific sequence of pFm, a soluble extracellular matrix protein present in concentrations of 0.3–0.5 mg/ml in plasma, lymph, and interstitial fluid (reviewed in Ref. 12), as well as in serum at reduced concentrations (13), by using naturally SF basement membranes (SU-ECM) as in vitro invasion substrates (14). The rationale for using naturally SF basement membranes is that they permit a precise definition of the soluble components of invasion assays. We present results showing that DU 145 cells (15) are induced to invade SU-ECM by pFm, and that this activity maps to the PHSCN sequence of the cell-binding domain. Similar concentrations of the PHSRN peptide stimulate SF DU 145 and MLL cells, as well as normal human prostate epithelial cells, to invade SU-ECM in vitro. Substitution of cysteine forms an efficient, competitive invasion inhibitor, the PHSCN peptide. Ac-PHSCN-NH₂ exhibits a substantially increased ability to block serum-induced invasion by DU 145 and MLL cells, but a scrambled peptide (Ac-HSPNC-NH₂) is inactive. The Ac-PHSCN-NH₂ peptide is a potent antitumorigenic and antimetastatic agent in rats, when treatment begins 1 day after MLL cell injection, whereas Ac-HSPNC-NH₂ is ineffective. The antitumorigenic activity of Ac-PHSCN-NH₂ is attributable to the direct inhibition of metastatic invasion in rats because the initiation of i.v. Ac-PHSCN-NH₂ treatment only after the removal of large primary MLL tumors is also very effective in reducing the number of MLL lung metastases. This result, as well as the topology of the SU-ECM invasion substrates used to define its activity, suggests that Ac-PHSCN-NH₂ can function as a potent inhibitor of basement membrane invasion in vivo, especially during extravasation by metastatic cells.

MATERIALS AND METHODS

Cell Culture and SU-ECM Invasion Assays. DU 145 cells (American Type Culture Collection, Rockville, MD), normal human prostate epithelial (prec) cells from the first or second passage (Clonetics, San Diego, CA), and MLL cells (10) were cultured according to established protocols (11, 15, 16). Cells were suspended in 0.25% trypsin/EDTA (Life Technologies, Inc., Grand Island, NY) and placed on SU-ECM invasion substrates with or without FCS according to established methods (14) for 4 h at 37°C, the time required to observe the maximal invasion percentages for normal and metastatic cells. Invasion-inducing or -inhibiting peptides or proteolytic fragments were included in the medium of the invasion assays at the appropriate concentrations. Invasion in this system is attributable to the short-term asymmetric distribution of the relevant receptors, resulting from trypsinization to suspend the cells just prior to assay.
prior to assay, which results in a gradient in signaling across the cell surface to stimulate invasion for appropriately oriented cells (Ref. 14 and data not shown). The percentages of spread and adherent cells were evaluated in each assay to check viability prior to fixation in 2% formaldehyde and scoring at 400-fold magnification in phase contrast optics. Viability ranged from 90 to 98% in all assays. The numbers of cells adhering to the surfaces of SU-ECM and their location with regard to the SU-ECM exterior surfaces were always determined for all adherent cells. Invasion percentages are the ratio of cells inside the SU-ECM invasion substrates to the sum of all cells adhering to them on their exterior and their interior surfaces. Mean invasion percentages were each the result of at least three independent determinations involving the scoring of all individual cells in contact with the invasion substrates. Fn-PCS was prepared by repeated affinity chromatography of Fn on gelatin Sepharose (Pharmacia, Piscataway, NJ) and the Fn standard (Ref. 17). The Fn depletion was confirmed by immunoblotting (18) with rabbit polyclonal anti-fibronectin antibody (13) from Life Technologies (data not shown). Proteolytic fragments of the Fn cell-binding domain were prepared on denaturing gels as described (19). The P1D6 monoclonal antibody (Ref. 20; Oncogene Research Products, Cambridge MA) was bound to DU 145 cells in SF medium with 3% BSA or in FCS-containing medium on ice for 30 min. Then, irrespective of serum content, DU 145 cells were diluted 10-fold with FCS medium prior to dropping them as cell suspensions on SU-ECM to assay invasion.

Peptide Synthesis. Peptides were synthesized using standard Fmoc/t-butylation protection strategies (21). Peptides were synthesized at 25 and 100 μmol scales on a Ranin Symphony multiple peptide synthesizer. Larger scales were synthesized on a Perkin-Elmer/Applied Biosystems model 433 peptide synthesizer or a Ranin Sonata large-scale synthesizer. Peptides were synthesized on a chlorotriyl resin, and COOH-terminally amidated peptides were synthesized on Rink resin. The completed peptides were cleaved from the resin support, and the side chain protecting groups were simultaneously removed by anhydrous TFA. The peptides were then precipitated with diethyl ether, purified by preparative high-performance liquid chromatography, and then freeze-dried. To prevent injury to the rats from TFA, a strong acid, residual TFA was removed by gel permeation chromatography on a Sephadex G-10 column equilibrated with 1 N acetic acid. Peptide structures were confirmed by mass spectrometry and amino acid analysis (21). Peptide purities, assessed by reverse phase high-performance liquid chromatography (22), were found to be 93% for PHSRN, 97% for PHSCN, 96% for Ac-PHSCN-NH₂, and 97% for Ac-HSPNC-NH₂ (not shown).

Zymography and Checkerboard Analysis of Chemotaxis. Adherent DU 145 cells in SF medium were treated with 1.4 μM PHSRN or with 1.4 μM PHSRN and 14 μM PHSCN, and their media were assayed three times by gelatin zymography (23). The effect of PHSRN on chemotaxis and chemokinase was evaluated in standard motility assays by checkerboard analysis (24). Upper and lower chambers were separated by inserts with 8-μm pores (Becton Dickinson Collaborative Biomedical Research, Bedford, MA) and coated with sterile 0.2% gelatin. DU 145 cells were preincubated in SF medium with 1.4 μM PHSRN or with 0.2% BSA for 1 h prior to suspension. Each assay contained the following combinations: 0.2% BSA in both upper and lower chambers; 1.4 μM PHSRN in the upper chamber with 0.2% BSA in the lower chamber (chemokinase control); and 0.2% BSA in the upper chamber with 1.4 μM PHSRN in the lower chamber (to assay chemotaxis). Assays also contained a well with 0.2% BSA in the upper chamber and 5% FCS in the lower chamber as a positive control. Cells migrated for 12 h before microscopic scoring of at least 10 randomly chosen fields per assay.

Analysis of MLL Tumor Vascularization and Metastasis in Rats Whose Ac-PHSCN-NH₂ Treatment Began 1 Day after MLL Cell Injection. MLL cells (100,000) were suspended and injected s.c. into the right hind legs of 19 Copenhagen rats according to standard procedures (7). Twenty-four h later, 10 of the rats received their first Ac-PHSCN-NH₂ i.v. injection in a tail vein, whereas the other 9 rats received no i.v. treatment. Injections, at a dosage of 5 mg/kg, consisted of 1 mg of Ac-PHSCN-NH₂ peptide in 0.5 ml of normal saline and occurred three times weekly thereafter. As an in vivo sequence specificity control, four other rats were treated systemically with Ac-HSPNC-NH₂ at the same dosage and on the same dosage schedule. Three additional rats received Phospho-MLL tumor cells but received injections of the Ac-PHSCN-NH₂ peptide at the same dosage and on the same dose schedule as the treated, MLL tumor-bearing rats. After 16 days of growth and five systemic peptide treatments, the largest MLL tumor available from the Ac-PHSCN-NH₂-treated rats (1-mm diameter) was harvested for the immunohistochemical determination of vascularization and α5β1 expression, and the rat was euthanized. Normal muscle from the leg was included as a positive control for vasculature. To provide a comparable source of metastatic cells, MLL tumors were not removed from the Ac-PHSCN-NH₂-treated rats if they were smaller than about 2 cm. MLL tumors of untreated rats reached a mean diameter of 1.8 cm by days 16–18 and were removed surgically with the hind limb (7). The primary MLL tumors in the Ac-HSPNC-NH₂-treated rats reached a mean diameter of 2.1 cm on day 20 and were surgically removed at this time. Unlike the primary tumors of the Ac-HSPNC-NH₂-treated and untreated rats, the MLL tumors of all Ac-PHSCN-NH₂-treated rats grew very slowly but did reach a mean diameter of 1.7 cm 29 days after MLL tumor cell injection and were removed surgically. All rats were euthanized for metastasis analysis in their lung tissue 14–16 days after their primary injection of 9 Ac-PHSCN-NH₂-treated, 4 Ac-HSPNC-NH₂-treated, and 9 untreated MLL tumor-bearing rats were fixed in 10% formaldehyde in PBS. After fixation, lung surfaces were scored under 10-fold magnification for the numbers of lung metastases. At this time, the three Ac-PHSCN-NH₂-treated rats without MLL cells were euthanized, and their tissues were evaluated grossly and microscopically for deleterious effects of Ac-PHSCN-NH₂ treatment by veterinary personnel in the University of Michigan Department of Lab Animal Medicine.

In addition to the 1-mm tumor from the Ac-PHSCN-NH₂-treated rat, a typical 2-cm MLL tumor from an untreated rat was harvested after 16 days of growth and analyzed immunohistochemically using the mouse antirat PECAM-1 monoclonal MAB1393 antibody (Refs. 25 and 26; Chemicon, Temecula, CA). Immunohistochemistry was also performed with the anti-α5β1 integrin rabbit AB1928 antisera raised against a peptide containing the highly conserved, final 18 amino acids of the human α5 subunit (27) in the cytoplasmic domain (Chemicon). This antisera has been shown to bind the α5β1 fibronectin receptors of both rat and human fibroblasts in immunoblots (27) and has been used to immunoprecipitate rat α5β1 receptors from pheochromocytoma PC12 cells (28). Tumors frozen with liquid nitrogen in Tissue-Tek OCT Compound (VWR, Chicago, IL; Ref. 29) were cut with a cryostat in sets of 7-μm serial sections (30). One slide of each set was stained with Gill’s hematoxylin No. 3 and alcoholic eosin (Fisher Scientific, Pittsburgh, PA) (31). Adjacent sections were either fixed in acetone at −20°C for 30 s prior to incubation with mouse anti-rat PECAM-1 monoclonal antibody (Chemicon) at 1:200 in 0.5 M Tris (pH 7.6) with 2% normal horse serum overnight at 4°C, followed by incubation with biotinylated horse antirat-α5β1 secondary antibody (rat preabsorbed; Vector Laboratories, Burlingame, CA), diluted 1:200 in Tris with normal horse serum for 45 min, and quenched in methanol-H₂O₂; or they were fixed in methanol at 4°C for 20 min prior to incubation in rabbit anti-α5β1 AB1928 antisera (Chemicon) in 0.5 M Tris with 2% normal goat serum overnight at 4°C, followed by incubation with biotinylated goat antirabbit IgG (Vector) diluted 1:200 in Tris with normal goat serum for 45 min and quenching in methanol-H₂O₂. All slides were incubated in avidin-biotin complex (Vectastain ABC kit) for 1 h, then with DAB with nickel (Vector) for 12–15 min. All incubations were at room temperature unless otherwise noted. All slides were then stained in Gill’s H&E (Fisher) and DAB substrate kit (Vector) or with rabbit anti-α5β1 antisera (Chemicon), biotinylated goat antirabbit IgG secondary antibody (Vector), and DAB substrate kit (Vector).

Macros were developed for the quantitation of the total area occupied by tumor vasculature using the KS100 image analysis package (Carl Zeiss, Thornwood, NY). Macros of several images from sections of MLL tumors, which had been immunostained with antirat PECAM monoclonal antibody, were digitized and placed on a threshold. Images were chosen to represent all areas of the tumors in the sections. A number of binary operations were performed on the resultant images to differentiate DAB-stained vessels and their lumens from the surrounding tumor cells. The total area occupied by vasculature was then determined as a fraction of the total field area.

After at least 3 days of fixation, complete lower, left lobes from the lungs of each of six Ac-PHSCN-NH₂ and four Ac-HSPNC-NH₂-treated rats and from six untreated rats were embedded in paraffin and cut in the plane of the maximal area of the lobe into eight sections of 5-μm thickness (31). To sample an extensive volume of lung tissue, the eight sections were each separated by 13 sections, or 65 μm of lung tissue. Thus, a region ~0.5 mm thick of the complete lower lobe from each rat was analyzed for micrometastasis. All eight sections from each individual were stained in H&E and scored at 400-fold magnification.
magnification in their entirety for MLL micrometastases, using phase contrast optics. To be scored as a separate micrometastasis, the MLL tumor cells had to have completed extravasation and had to have been located as a cluster in the lung tissue instead of in the lumen of a blood vessel. They also had to have been surrounded by normal lung tissue, instead of by tumor cells, in the section.

Analysis of MLL Lung Metastasis in Rats Whose Ac-PHSCN-NH₂ Treatment Began 1 Day after the Surgical Removal of Large MLL Tumors. MLL (100,000) cells were suspended and injected s.c. into the right hind legs of 20 Copenhagen rats according to standard protocols (7). MLL tumors grew to a mean diameter of 1.9 cm in the rats prior to their surgical removal 20 days later. These rats received no presurgical Ac-PHSCN-NH₂ injections. Twenty-four h after surgery to remove their MLL tumors, 10 of the previously untreated rats received their first tail vein injections of 1 mg of Ac-PHSCN-NH₂ in 0.5 ml of normal saline. The rats received a total of six i.v. Ac-PHSCN-NH₂ treatments during the next 14 days. The remaining 10 rats remained untreated. All rats were euthanized for metastasis analysis in their lung tissue 14 days after surgery. The lungs of all 20 rats were fixed in 10% formaldehyde in PBS. After fixation, lung sections were scored under 10-fold magnification for lung metastases. After at least 3 days of fixation, the complete lower left lobe of lung tissue from each individual was embedded, sectioned, stained, and scored as described above for MLL micrometastasis analysis when Ac-PHSCN-NH₂ treatment began 24 h after MLL cell injection.

RESULTS

Invasion Stimulation by a Specific Region of Plasma Fibronec- tin. DU 145 human prostate carcinoma cells (15) were tested for invasion induction in the presence or the absence of FCS and in the presence of specific portions of the pFn molecule. SU-ECM were the most appropriate in vitro invasion substrates because they are obtained from invertebrate sea urchin embryos, which occur naturally in the SF state. They contain intact basement membrane (32) and reproduce the relative invasive behaviors of a variety of cell lines cultured from primary and metastatic tumors, as well as those of the corresponding normal epithelial or mesenchymal cells, without detectable background invasiveness (14). Fig. 1A shows the mean percentages of invaded DU 145 cells in SF, FCS, or Fn–FCS media. FCS failed to stimulate invasion by normal prec cells; however, DU 145 cells rapidly invaded in the presence of FCS. In contrast, DU 145 cells failed to invade SU-ECM under SF conditions, even if incubated for up to 24 h before fixation and scoring (not shown). This suggested that a specific component of serum induced their invasive behavior. DU 145 cells are known to express α5β1 (33). Because pFn binding by α5β1 can induce fibroblast MMP-1 gene expression in the absence of binding by the α4β1 fibronectin receptor (34), and metalloproteinases are thought to play a central role in migration through extracellular matrix, the effect of pFn on DU 145 invasion was tested. As shown in Fig. 1A, the use of Fn–FCS completely eliminated DU 145 invasion. Invasiveness could be fully restored by adding purified pFn to the Fn–FCS in amounts characteristic of whole serum (35) or of serum-containing medium. Thus, pFn was necessary for DU 145 invasion induction. Furthermore, pFn was also sufficient, because the addition of identical amounts of purified pFn in SF medium supported the full extent of DU 145 invasive behavior, as shown in Fig. 1B.

To identify the invasion-stimulating region of the pFn cell binding domain, denaturing gel-purified M₁, 120,000 chymotryptic, as well as M₅, 39,000 and M₇, 11,500 pepsin fragments, were tested in SF SU-ECM invasion assays. As shown in Fig. 1B, M₁, 120,000 fragment containing modules 2–11 was sufficient to induce DU 145 invasion in molar concentrations similar to those of pFn in serum or in serum-containing medium. Also, the invasive responses of DU 145 cells to varying concentrations of the M₁, 120,000 fragment in serum-free SU-ECM invasion assays closely resembled those of prec cells (data not shown). This is not surprising because, like other epithelial cell types and fibroblasts, prec cells express the α5β1 integrin fibronectin receptor (36). At similar molar concentrations, the M₅, 39,000 fragment, containing modules 7–9, was equally active in inducing invasion, whereas the M₇, 11,500 fragment containing module 10 with the RGD sequence and the pure GRGDSP peptide were inactive at the concentrations tested. Thus, it appeared that the constitutive invasiveness of DU 145 cells might be attributable to stimulation by a specific sequence encompassed by the M₁, 39,000 fragment.

In addition to the RGD site, the α5β1 integrin fibronectin receptor binds the PHSRN sequence of module 9 (37) and is known to be expressed by DU 145 and prec cells (33, 36) through immunostaining with the anti-α5β1 P1D6 function-blocking monoclonal antibody (Ref. 20 and data not shown). Thus, the ability of the M₅, 39,000 fragment to induce SF DU 145 invasion suggested that the α5β1 receptor might be involved. To test whether invasion induction through α5β1 occurred during substrate adhesion or at a prior step, DU 145 cells were bound to the P1D6 antibody either prior to invasion stimulation by FCS or concurrently. Although the presence of FCS was varied during antibody binding, substrate adhesion and invasion always occurred in its presence. When prebound under SF conditions, the P1D6 antibody reduced DU 145 invasion by 45% at concentrations as low as 10 μg/ml and eliminated it completely at 300 μg/ml, whereas P1D6 bound in the presence of FCS failed to reduce DU 145 invasion at concentrations as high as 300 μg/ml. Similar results were also obtained for prec cells induced to invade SU-ECM with the M₁, 120,000 pFn fragment instead of serum (data not shown). Thus, invasion induction by pFn appeared to occur through the α5β1 receptor prior to substrate adhesion and to involve the PHSRN sequence of module 9. These results were consistent with the observation that the PHSRN-containing synergy region of the ninth type III repeat of pFn blocks the binding of the P1D6 antibody to α5β1 (38) and suggested that the PHSRN sequence might be sufficient to induce SF DU 145 invasion.

Induction of Invasive Behavior by the PHSRN Peptide. The ability of the PHSRN peptide to stimulate SU-ECM invasion by SF DU 145 cells or by prec cells was evaluated. PHSRN peptide was present in the media of SU-ECM invasion assays at concentrations ranging from 14 nm to 1.4 μM (10–1000 ng/ml). The RGD peptide, GRGDSP, was also tested for its invasion-inducing activity at a concentration of 1.4 μM. As shown in Fig. 2A, PHSRN effectively stimulated invasion by DU 145 cells in a log linear fashion, whereas the RGD peptide was inactive at the highest peptide concentration tested. As expected from their expression of the α5β1 receptor (36)...
shown in Fig. 3, invasion by DU 145 cells and by prec cells was stimulated in the presence of 0–1.4 M medium containing 10% FCS, both with various concentrations of PHSRN peptide. The PHSRN sequence interacts with the third NH2-terminal domain of prec cells in 10% FCS with 1.4 M concentration (nM); Y axis, mean percentages of invaded prec cells. Bars, SD. A, prec cells in SF medium or in medium containing 10% FCS, both with various concentrations of PHSRN peptide. ■, prec cells in 10% FCS with 1.4 M GRGDSP.

and the invasive responses of other normal epithelial cells to the PHSRN peptide, very similar dose-response relationships were obtained for prec cells in SF and FCS media, as shown in Fig. 2B. Thus, the PHSRN peptide was as effective at stimulating invasion by prec cells as it was in stimulating DU 145 invasion in the absence of serum, suggesting that the dysregulation of the invasive response to the PHSRN sequence of intact pFn might contribute to the constitutive invasiveness of these α5β1-expressing invasive tumor cells.

Inhibition of PHSRN-induced Invasion by the PHSCN Peptide.

The PHSRN sequence interacts with the third NH2-terminal domain of the α5 chain, which forms a pocket that may contain a divalent cation (38). The arginine of this sequence appears to be crucial for its invasion-inducing activity in normal epithelial cells and fibroblasts because replacing it with alanine or glutamic acid eliminates its activity, even at elevated concentrations. Consistent with the importance of the PHSRN arginine in stimulating basement membrane invasion, observations published previously have shown that, although the PHSRN sequence in the context of either the 9th or the 8th fibronectin type III repeat could stimulate adherent BHK cells to de-adhere from pFn substrates, substitution of the arginine with an amino acid having a neutral or negatively charged side chain resulted in complete or near-complete loss in activity (39). Replacing the arginine with a cysteine, which has a large, electron-rich sulfhydryl group on its side chain, formed a potent competitive inhibitor of invasion induction by the PHSRN sequence for normal human epithelial cells and fibroblasts. The sulfhydryl group of the cysteine side chain is known to complex efficiently with divalent manganese and magnesium cations, either of which might occupy the PHSRN-binding pocket of the α5 chain, as well as with transition metal ions (reviewed in Refs. 40 and 41). It was hypothesized that the shorter side chain of cysteine and its ability to coordinate divalent cations might allow it to interact tightly in the binding pocket without inducing basement membrane invasion.

The PHSCN peptide was assayed for its ability to inhibit PHSRN-induced invasion by SF DU 145 cells and by prec cells. A nonsaturating concentration of the PHSCN peptide (140 nM) was used to stimulate invasion in the presence of 0–1.4 μM PHSRN peptide. As shown in Fig. 3, invasion by DU 145 cells and by prec cells was decreased by slightly >50% in the presence of equimolar PHSRN and PHSCN. A 10-fold molar excess of the PHSCN peptide prevented invasion for both cell types. Although the other four amino acids were identical in the invasion-inducing and invasion-inhibiting peptides, they might still be very important for the productive association of the peptide with its binding pocket on the α5 chain. Furthermore, this association might even be sufficient for adhesive or migratory effects in the absence of the arginine side chain. However, these results indicate the importance of the arginine side chain, specifically for the induction of basement membrane invasion in normal, as well as in invasive, derivatives of prec cells.

The ability of the PHSCN peptide to inhibit PHSRN-induced invasion suggested that it would function as an effective invasion inhibitor in the presence of serum, which is known to contain abundant pFn (13). Thus, DU 145 cells were placed on SU-ECM in serum-containing medium with varying concentrations of the PHSCN peptide. As shown in Fig. 4A, PHSCN at 1.4 μM (1 μg/ml) inhibited the induction of DU 145 invasion by serum. To determine whether PHSCN functioned as a competitive or allosteric inhibitor of the PHSRN sequence, DU 145 cells were chilled and prebound to a fully inhibitory concentration of the PHSCN peptide (1.4 μM) in the presence of serum, prior to being rinsed thoroughly and placed on SU-ECM in the presence of serum and increasing amounts of the PHSCN peptide. PHSCN-prebound DU 145 cells failed to invade, as shown in Fig. 4A. As shown in Fig. 4B, increasing concentrations of the PHSRN peptide overcame PHSCN invasion inhibition very effectively, which would not have been expected had the PHSCN and PHSRN peptides interacted with distinct sites in the α5β1 receptor. Thus, these two peptides appeared to bind with similar affinities to the same receptor site.

This suggested that the PHSRN and PHSCN peptides might influence the expression of specific metalloproteinases functioning in cell migration through the extracellular matrix. To determine whether the PHSRN and PHSCN peptides had any detectable effect on MMP-1 or MMP-2 expression, adherent DU 145 cells in SF medium were treated with 1.4 μM PHSRN or with 1.4 μM PHSRN and 14 μM PHSCN; and their media were assayed by gelatin zymography (23). The results of these zymograms are plotted in Fig. 4C, and a representative zymogram is shown in Fig. 4D.

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program is shown in Fig. 4D. When compared with untreated cells, PHSRN-treated DU 145 cells were found to secrete a mean of 225 ± 10% more M, 70,000 MMP-1. Also, PHSCN treatment of PHSRN-stimulated DU 145 cells was found to reduce MMP-1 secretion by a mean of 50 ± 32% with respect to untreated cells. Although secreted in larger amounts, similar levels of M, 70,000 MMP-2 were expressed by DU 145 cells, irrespective of the peptides added. Thus, although the expression, localization, and function of metalloproteinases in DU 145 invasion is likely to be very complex, the effects of the PHSRN and PHSCN peptides on MMP-1 secretion were consistent with their effects on SU-ECM invasion.

Because the PHSRN peptide induced DU 145 invasion of SU-ECM basement membranes, it was likely that it would also function as a chemoattractant in gelatin-coated migration chambers (24). This was tested by prebinding DU 145 cells to 0.2% BSA or to 1.4 μM PHSRN peptide in SF medium and placing them in standard checkerboard assays in gelatin-coated migration chambers containing no PHSRN peptide or containing 1.4 μM PHSRN in either the top or the bottom chambers. As shown in Fig. 4E, the presence of 1.4 μM PHSRN in the lower chambers of 10 Boyden chamber assays stimulated the chemotactic migration of BSA-pretreated or PHSRN-pretreated DU 145 cells by means of >30-fold as compared with the presence of 0.2% BSA in the lower chambers. Because at least 3 SDs separate the means, these results are significant to >99% confidence. In contrast, the amount of chemokinesis was negligible, as shown by the observation that the presence of 1.4 μM PHSRN in the upper chambers failed to stimulate migration to the lower chambers by either PHSRN- or BSA-pretreated DU 145 cells. Also, PHSRN pretreatment alone was shown to be insufficient to stimulate the observed chemotactic migration because neither PHSRN-pretreated nor BSA-pretreated DU 145 cells migrated to the lower chambers when these chambers contained 0.2% BSA, instead of 1.4 μM PHSRN. Finally, PHSRN pre-binding was not necessary for DU 145 chemotaxis because BSA-pretreated and PHSRN-preconditioned DU 145 cells had an equally high chemotactic response to PHSRN. Thus, the ability of the PHSRN peptide to stimulate the motility of DU 145 cells, as suggested by the experiments on SU-ECM invasion substrates, was confirmed in conventional gelatin-coated chambers.

Inhibition of MATLyLu Metastatic Rat Prostate Carcinoma Cell Invasion and Metastasis by PHSCN Peptides. To test whether MLL tumor cells express the α5β1 fibronectin receptor and thus might be inhibited by PHSCN peptides, 100,000 MLL cells were injected s.c. into a rat hindlimb, and the MLL tumor was grown for 16 days. Cryostat sections of the MLL tumor were immunostained for α5β1 using a commercially available rabbit antiserum raised against a synthetic peptide derived from the highly conserved last 18 amino acids of the α5 chain. This antiserum has been demonstrated to react with rat α5β1 by both immunoblotting and immunoprecipitation (27, 28).

Fig. 5A shows a section of an untreated MLL tumor that was reacted to the rabbit anti-α5 antiserum, to biotinylated goat anti-rabbit IgG secondary antibody, and then stained with DAB. Fig. 5B shows a section of the same tumor immunostained in parallel without the primary antibody. Although the amount of anti-α5β1 immunostaining evident on the surfaces of MLL cells varied slightly because of the plane of section, all MLL cells appeared to stain with anti-α5β1 and biotinylated secondary antibody, whereas nonspecific reactivity was evident. Because the Ac-PHSCN-NH2 peptide is a competitive ligand for the α5β1 receptor, it was possible that systemic administration of it down-regulated α5β1 expression on the surfaces of the MLL tumors in the treated rats. However, as shown by the example in Fig. 5C, sections of a 1-mm MLL tumor obtained from a rat treated with five i.v. 1-mg Ac-PHSCN-NH2 injections over a period of 16 days appeared to react equally well with the rabbit anti-α5β1 antiserum as did the MLL tumors from the untreated rats. Thus, exposure to the Ac-PHSCN-NH2 peptide in vivo did not appear to affect the cell surface expression of the α5β1 receptor on the tumors.

The expression of α5β1 integrin fibronectin receptor by MLL tumors suggested that MLL cells should be stimulated to invade in serum-free medium by the PHSRN peptide. To verify that the PHSRN peptide stimulated MLL invasion in vitro, MLL cells were placed on SU-ECM invasion substrates in SF medium in the presence of 1.4 μM PHSRN peptide or in its absence, in FCS medium. The results of these invasion assays are shown in Fig. 5D. Similar to the human DU 145 cells, rat MLL cells failed to invade in SF medium but were induced to invade when 1.4 μM PHSRN peptide or FCS was added. The dose responses of DU 145 and MLL cells to PHSCN invasion inhibitors in FCS media were also evaluated. Because acetylation and amidation of the termini of the invasion-stimulating PHSRN peptide significantly increased its potency both in vitro and in vivo (4), the activities of the PHSRN and the Ac-PHSCN-NH2 peptides were compared for DU 145 and MLL cells in SU-ECM invasion assays containing FCS. As a sequence specificity control, the Ac-HSPNC-NH2 scrambled peptide was also tested. The results of these assays are shown in Fig. 6. As can be seen in Fig. 6A, the inhibitory activity of the PHSRN peptide on DU 145 cells was increased log linearly with increasing PHSRN concentration from 4.2 nM to 1.4 μM. The presence
Although the relative potencies of PHSCN and Ac-PHSCN-NH₂ were and Ac-PHSCN-NH₂ peptides also had very significant invasion-inhibitory activity on DU 145 cells in the presence of serum, an additional rat was not injected with MLL cells but received i.v. Ac-PHSCN-NH₂ peptide on the same dosage schedule as did the treated rats bearing MLL tumors. Injections of Ac-PHSCN-NH₂ occurred three times weekly. As shown in Fig. 6, after 16 days of tumor growth and a total of five i.v. Ac-PHSCN-NH₂ injections, the mean diameter of the tumors in the treated rats was <0.5 mm. In contrast, the mean diameter of the MLL tumors in the untreated rats was 1.8 cm at this time. Because spherical volume is proportional to the radius cubed, the mean volume of the MLL tumors in the treated rats was about 2000-fold less than the mean volume of the MLL tumors in the untreated rats. This suggested that systemic Ac-PHSCN-NH₂ treatment could significantly reduce the formation of blood vessels in MLL tumors. This was verified by sectioning and immunostaining the largest MLL tumor (1-mm diameter) from an Ac-PHSCN-NH₂-treated rat after 16 days of tumor growth. A typical 1.8-cm MLL tumor from an untreated rat after 16 days of growth was also sectioned and immunostained. Cryosections of the treated and the untreated MLL tumors were immunostained with rat-specific, anti-PECAM-1 monoclonal antibody (25, 26), the appropriate biotinylated secondary antibody, and DAB staining reagents. PECAM-1 is a member of the immunoglobulin superfamily, which is expressed at high levels on the cell surfaces of endothelial cells, as well as on platelets, monocytes, neutrophils, and subsets of T lymphocytes (reviewed in Ref. 42). Thus, it is a standard method for evaluating vasculization histochemically. Some of the normal muscle of the hind limb was retained in the sections of treated MLL tumor as a positive control for vasculature. The rat that was the source of the Ac-PHSCN-NH₂-treated MLL tumor sections for anti-PECAM staining was sacrificed instead of being maintained for later metastasis analysis because of the small size of the tumor removed. Scoring of 20

of 1.4 μM PHSCN peptide completely inhibited serum-induced DU 145 invasion of SU-ECM. Ac-PHSCN-NH₂ exhibited a 30-fold increased invasion-inhibitory activity, perhaps because removing charges at the NH₂ and COOH termini allowed the peptide to assume a more active conformation or increased its resistance to exoproteinases. In contrast, the Ac-HSPNC-NH₂ peptide had no detectable invasion-inhibitory activity on DU 145 cells in the presence of serum, even when present in concentrations as high as 14 μM. The histidine residue of the PHSRN peptide is replaced in rats and mice by proline (reviewed in Ref. 37). Nevertheless as shown in Fig. 6B, the PHSCN and Ac-PHSCN-NH₂ peptides also had very significant invasion-inhibitory activities in rat MLL cells on SU-ECM invasion substrates in serum-containing medium, suggesting extensive conservation of function in the other four amino acids of the sequence. As in DU 145 cells, the Ac-PHSCN-NH₂ peptide was at least 30-fold more active at inhibiting serum-induced invasion than was the unblocked peptide. Although the relative potencies of PHSCN and Ac-PHSCN-NH₂ were very similar in MLL cells and in DU 145 cells, both peptides showed slightly more invasion-inhibitory activity in the human cells, probably because they correspond to the human sequence. Also, the sequence specificity of invasion inhibition by the PHSCN sequence in rat cells was demonstrated because MLL cell invasion failed to be inhibited by concentrations as high as 14 μM of the scrambled Ac-HSPNC-NH₂ peptide.

Because the Ac-PHSCN-NH₂ peptide appeared to be a potent MLL invasion inhibitor at nanomolar concentrations, it was tested for its ability to inhibit growth and angiogenesis by injected MLL tumors in Copenhagen rats. Nineteen Copenhagen rats each received 100,000 MLL cells s.c. in the right hind leg. The following day, 10 of these rats received the first of a series of 13 i.v. injections of 1 mg of Ac-PHSCN-NH₂ peptide. To check for debilitary effects of Ac-PHSCN-NH₂ therapy, three additional rats were not injected with MLL cells but received i.v. Ac-PHSCN-NH₂ peptide on the same dosage schedule as did the treated rats bearing MLL tumors. Injections of Ac-PHSCN-NH₂ occurred three times weekly. As shown in Fig. 7A, after 16 days of tumor growth and a total of five i.v. Ac-PHSCN-NH₂ injections, the mean diameter of the tumors in the treated rats was <0.5 mm. In contrast, the mean diameter of the MLL tumors in the untreated rats was 1.8 cm at this time. Because spherical volume is proportional to the radius cubed, the mean volume of the MLL tumors in the treated rats was about 2000-fold less than the mean volume of the MLL tumors in the untreated rats. This suggested that systemic Ac-PHSCN-NH₂ treatment could significantly reduce the formation of blood vessels in MLL tumors. This was verified by sectioning and immunostaining the largest MLL tumor (1-mm diameter) from an Ac-PHSCN-NH₂-treated rat after 16 days of tumor growth. A typical 1.8-cm MLL tumor from an untreated rat after 16 days of growth was also sectioned and immunostained. Cryosections of the treated and the untreated MLL tumors were immunostained with rat-specific, anti-PECAM-1 monoclonal antibody (25, 26), the appropriate biotinylated secondary antibody, and DAB staining reagents. PECAM-1 is a member of the immunoglobulin superfamily, which is expressed at high levels on the cell surfaces of endothelial cells, as well as on platelets, monocytes, neutrophils, and subsets of T lymphocytes (reviewed in Ref. 42). Thus, it is a standard method for evaluating vasculization histochemically. Some of the normal muscle of the hind limb was retained in the sections of treated MLL tumor as a positive control for vasculature. The rat that was the source of the Ac-PHSCN-NH₂-treated MLL tumor sections for anti-PECAM staining was sacrificed instead of being maintained for later metastasis analysis because of the small size of the tumor removed. Scoring of 20
Fig. 7. Effect of Ac-PHSCN-NH₂ peptide on the growth of injected MLL tumors and on angiogenesis in them. A, MLL tumor diameter after 16 days of growth. Y axis, mean tumor diameter in cm. Bars, SD. □, MLL tumors growing in untreated rats; ▪, MLL tumors growing in Ac-PHSCN-NH₂-treated rats. More than 3 SD separate the means; thus, these data are significant to >99% confidence. B, MLL tumor tissue from a rat treated with 5 i.v. Ac-PHSCN-NH₂ injections during the 16 days of tumor growth. C, normal muscle tissue adjacent to MLL tumor tissue from the same slide as B. D, MLL tumor tissue after 16 days of growth in a rat that received no Ac-PHSCN-NH₂ injections. Tissue in figure insets was stained with anti-PECAM primary antibody, biotinylated secondary antibody, and DAB plus NiCl. Insets, adjacent sections stained with H&E and photographed under 400-fold magnification. Insets in B and in D represent identical areas of images photographed at the same magnification.

To further quantitate the effect of systemic Ac-PHSCN-NH₂ treatment on MLL tumor vasculature, the fraction of the total tumor area occupied by anti-PECAM-stained vessels and their lumens was determined by the use of specialized image analysis software. Images from several characteristic regions of each tumor were used in this analysis. Vasculature in the MLL tumor of an untreated rat appeared to occupy 12% of the tumor tissue, whereas vasculature in the MLL tumor of a rat that received systemic Ac-PHSCN-NH₂ treatment occupied only 1% of the tumor tissue, a 12-fold decrease in vessel density. Because this analysis was performed on the MLL tumor that grew the fastest in Ac-PHSCN-NH₂-treated rats, the vasculature of the MLL tumors in the other Ac-PHSCN-NH₂-treated rats is likely to have been even less. In contrast, the density of the vasculature in the normal muscle of the same sections as the treated MLL tumor had an apparent density of vascularization, which was 8-fold higher than that of the tumor tissue. Thus, the paucity of vasculature in the MLL tumor from the Ac-PHSCN-NH₂-treated rat was not attributable to inefficient anti-PECAM immunostaining of the sections.

Although unlikely, it was possible that Ac-PHSCN-NH₂ treatment stimulated MLL cell apoptosis, thereby slowing the growth of the primary MLL tumors in the rats. To insure that Ac-PHSCN-NH₂ treatment did not affect the MLL cell cycle or stimulate apoptosis, MLL cells were cultured for 72 h (the time required for at least two cell cycles) in serum-containing medium with 0, 1.4, or 14 μM Ac-PHSCN-NH₂. After labeling their DNA with propidium iodide, the percentages of the MLL cells in G₁, G₂, or S phase, as well as the fraction of sub-G₁ (apoptotic) cells were determined by flow cytometry (43). No sub-G₁ cells were observed, even at 14 μM, a concentration of Ac-PHSCN-NH₂ significantly higher than that to which the MLL tumors were likely exposed in vivo. Also, the percentages of MLL cells in G₁, G₂, or S phase were unaffected by the presence of Ac-PHSCN-NH₂ at 1.4 or 14 μM (not shown). Thus, the slow growth of the MLL tumors in the Ac-PHSCN-NH₂-treated rats was probably
attributable to the paucity of their vasculature, rather than to direct effects of systemic Ac-PHSCN-NH$_2$ on cell cycle or on apoptosis. This experiment, in which three times weekly Ac-PHSCN-NH$_2$ i.v. treatment commenced 1 day after the injection of MLL cells, was continued to quantitate the numbers of metastatic MLL colonies in the lungs. To control for possible nonspecific effects of Ac-PHSCN-NH$_2$ treatment, four Ac-HSPNC-NH$_2$-treated rats were also included. These four rats received 100,000 MLL cells s.c. in the right flank 24 h before the initiation of three times weekly, 1-mg Ac-HSPNC-NH$_2$ i.v. injections. Primary tumors in Ac-HSPNC-NH$_2$-treated rats reached a mean diameter of 2.0 cm prior to surgery after 17 days of growth. Because the Ac-PHSCN-NH$_2$ dosage was not increased, primary MLL tumors eventually grew in the Ac-PHSCN-NH$_2$-treated rats to a mean diameter of 1.7 cm 2 days prior to euthanization for lung metastasis analysis. Thirty-one days after MLL cell injection or 14 days after surgery, all rats were sacrificed, and the numbers and sizes of MLL colonies on the surfaces of their fixed lungs were determined by examination under 10-fold magnification. After scoring surface metastases, an entire lobe was removed from the fixed lungs for the histological analysis of MLL micrometastasis. At this time, the Ac-PHSCN-NH$_2$-treated rats that received no MLL tumor cells were also sacrificed, and necropsies were performed. Particular attention was paid to the lung tissue because, having been introduced in the tail vein, the lungs were the first organ encountered by the injected peptide. No obvious side effects, including lung infections, were observed in the rats that received Ac-PHSCN-NH$_2$ treatment without MLL tumor cells. Fig. 8 compares the numbers of lung metastases and micrometastases in the Ac-PHSCN-NH$_2$-treated, the Ac-HSPNC-NH$_2$-treated, and the untreated rats. Fig. 8A compares the mean number of MLL lung metastases per Ac-PHSCN-NH$_2$-treated rat, as well as the total number of MLL colonies in all Ac-PHSCN-NH$_2$-treated rats to the number/rat and the total number in the untreated group of rats. A total of nine metastatic colonies were observed in a total of four Ac-PHSCN-NH$_2$-treated rats. The remaining five rats in this group had no detectable MLL metastatic colonies in their lungs when the tissue was examined at 10-fold magnification. Thus, the mean number of lung metastases/rat in the Ac-PHSCN-NH$_2$-treated group was 1. In contrast, a total of 357 metastatic colonies were observed in the group of nine untreated rats, for a mean of 39.7 lung colonies/individual. The number of metastases observed per rat varied from 1 to 243. Because it does not assume a standard distribution, the significance of these results was evaluated by the nonparametric Mann-Whitney method (44). As shown in Fig. 8A, $P_0$, the probability that the incidence of MLL metastasis was the same in the Ac-PHSCN-NH$_2$-treated and in the untreated groups is $0.002$, implying a significance of $0.998%$. Fig. 8B compares the mean number of MLL lung metastases per Ac-PHSCN-NH$_2$-treated rat, and the total number of MLL colonies in the Ac-PHSCN-NH$_2$-treated group to the number/rat and the total number in the untreated group of rats. A total of nine metastatic colonies were observed in a total of four Ac-PHSCN-NH$_2$-treated rats. The remaining five rats in this group had no detectable MLL metastatic colonies in their lungs when the tissue was examined at 10-fold magnification. Thus, the mean number of lung metastases/rat in the Ac-PHSCN-NH$_2$-treated group was 1. In contrast, a total of 357 metastatic colonies were observed in the group of nine untreated rats, for a mean of 39.7 lung colonies/individual. The number of metastases observed per rat varied from 1 to 243. Because it does not assume a standard distribution, the significance of these results was evaluated by the nonparametric Mann-Whitney method (44). As shown in Fig. 8B, $P_0$, the probability that the incidence of MLL metastasis was the same in the Ac-PHSCN-NH$_2$-treated and in the untreated groups is $0.005$, implying a significance of $0.995%$. Fig. 8C compares MLL lung micrometastasis in the Ac-PHSCN-NH$_2$-treated and in the untreated rats. Fig. 8D compares the numbers of MLL lung micrometastases per Ac-PHSCN-NH$_2$-treated rat, as well as the total number of MLL colonies in all Ac-PHSCN-NH$_2$-treated rats to the number/rat and the total number in the untreated group of rats. Although nine metastatic colonies were observed in the group of nine untreated rats, for a mean of 39.7 lung colonies/individual. The number of metastases observed per rat varied from 1 to 243. Because it does not assume a standard distribution, the significance of these results was evaluated by the nonparametric Mann-Whitney method (44). As shown in Fig. 8C, $P_0$, the probability that the incidence of MLL metastasis was the same in the Ac-PHSCN-NH$_2$-treated and in the untreated groups is $0.002$, implying a significance of $0.998%$.
metastases observed in eight longitudinal sections of the complete lower left lobes from six rats treated i.v. with Ac-PHSCN-NH₂ beginning 1 day after MLL cell injection are shown. Each of these sections was separated from the next by 65 μm of lung tissue. Thus, a total thickness of about 0.5 mm was sampled. No micrometastases were detected in the sections from four of these individuals. Of the other two rats, one had a single micrometastasis and one had two micrometastases, for a metastasis frequency of 0.5/rat. In contrast, a total of 789 micrometastases were observed in eight longitudinal sections of the lower left lobes obtained from each of the six untreated rats, for a micrometastasis frequency of 131.5/rat. As indicated, *P*₀ is <0.0005, indicating a significance of >99.5%. Fig. 8E shows a typical example of sectioned and stained lung tissue from an Ac-PHSCN-NH₂-treated rat. Normal alveolar lung tissue and vasculature is seen. Fig. 8F shows a typical section of lung tissue from an untreated rat. Three micrometastases are indicated with arrows, one of which is seen to surround a blood vessel. Sections of lung tissue from the Ac-HSPNC-NH₂-treated rats had a very similar appearance (data not shown). Thus, a total of 13 Ac-PHSCN-NH₂ injections administered over a 31-day period appeared to prevent the formation of visible MLL colonies in the lungs of 55% of the treated rats and reduced the total number of MLL lung metastases in all nine treated rats by >97%, with respect to the untreated rats, and by >99% with respect to the Ac-HSPNC-NH₂-treated rats. Greater than 100-fold reductions in the total numbers of micrometastases were observed when the sections of lung tissue from the Ac-PHSCN-NH₂-treated rats were compared with those from untreated or from Ac-HSPNC-NH₂-treated animals.

Because the primary MLL tumors of the Ac-PHSCN-NH₂-treated rats eventually reached a large size, although very few micrometastases were observed, the results of the previous experiment suggested that i.v. Ac-PHSCN-NH₂ was capable of inhibiting MLL metastasis formation directly by inhibiting invasion, as well as indirectly by inhibiting the growth of the primary tumor. The ability of Ac-PHSCN-NH₂ to inhibit MLL metastasis, independent of its antitumorogenic activity, was tested by letting the MLL tumors of 20 rats grow to a mean diameter of 1.9 cm without treatment. Twenty-four h after the surgical removal of MLL tumors, 10 rats received their first i.v. injection of 1 mg of Ac-PHSCN-NH₂, whereas the other 10 rats remained untreated. A total of six three-times-weekly i.v. injections were administered to the treated group. All rats were euthanized 14 days after the surgical removal of their 2-cm primary tumors. Subsequently, their lung tissue was examined for MLL metastasis formation and analyzed histologically for the presence of micrometastases.

As shown in Fig. 9, i.v. therapy with Ac-PHSCN-NH₂ was effective at reducing MLL metastasis and micrometastasis in rats which grew large MLL tumors and underwent surgery prior to its initiation. Fig. 9A compares the frequencies of MLL lung metastasis and the total numbers of metastases in the Ac-PHSCN-NH₂-treated and in the untreated groups. A total of eight lung metastases were observed in the 10 Ac-PHSCN-NH₂-treated rats, for a metastasis frequency of 0.8/rat. Forty % of these rats had no detectable surface metastases on their lungs, and 60% had small numbers of surface metastases, ranging from 1 to 3. In contrast, all untreated rats had surface MLL metastases on their lungs, ranging from 4 to 495 per rat. A total of 1185 metastases were observed on the lungs of the 10 untreated rats, for a metastasis frequency of 118.8/rat. As indicated in Fig. 9A, *P*₀, the chance probability of these results, is <0.0005; thus, they are significant to >99.9%. Fig. 9B compares the frequencies of MLL micrometastasis in the longitudinally sectioned left lower lobes of the Ac-PHSCN-NH₂-treated and the untreated rats. Eight sections, each separated by 65 μm, were scored for each rat. From 0 to 38 micrometastases/rat were observed in eight sections of lung tissue from each of the 10 Ac-PHSCN-NH₂-treated rats, for a total of 76 micrometastases and a mean micrometastasis frequency of 7.8/rat. In contrast, from 2 to 441 micrometastases/rat were observed in eight sections of lung tissue obtained from each of nine untreated rats, for a total of 1506 micrometastases and a mean micrometastasis frequency of 167.3/rat. As indicated in Fig. 9B, *P*₀ is 0.001, implying a significance of 99.9%. Thus, at a dosage level of 5 mg/kg i.v. Ac-PHSCN-NH₂ administered three times weekly reduced MLL metastasis by 99% and micrometastasis by 95%, although treatment did not commence until 24 h after the removal of large primary tumors.

**DISCUSSION**

An unregulated invasive response to the PHSRN synergy sequence found in intact pFn may contribute significantly to both the growth and the metastasis of prostate cancers. Results presented here show that serum is required for DU 145 cells to invade an extracellular matrix containing a basement membrane in vitro. Furthermore, in invasion assays involving selective depletion of pFn from serum and its re-addition as a purified component, pFn appears to be the only serum component required for invasion. A purified fragment of the pFn cell-binding domain containing the PHSRN but not the RGD sequence is sufficient to induce invasion by serum-free DU 145 cells in vitro, whereas a fragment containing RGD without PHSRN is not. Consistent with these results, the PHSRN peptide is sufficient to induce invasion both by DU 145 cells and by normal prostate epithelial cells, with very similar dose responses. These results suggest that a significant portion of the constitutive invasiveness of DU 145 cells in the presence of serum may be explained by their response to the PHSRN sequence of intact pFn, and that, although precells do not respond to the PHSRN sequence of intact pFn by invading, they invade when the PHSRN sequence is presented as a peptide.
Replacing the arginine of the PHSRN peptide with cysteine forms a competitive inhibitor of basement membrane invasion in vitro. This PHSCN inhibitor blocks PHSRN-induced invasion both by SF DU 145 cells and by prec cells with similar activities. The PHSCN peptide also inhibits the serum-induced invasion of DU 145 cells. This suggested that the PHSCN peptide might be an effective inhibitor of metastatic tumor cell invasion in vivo. Metastatic rat MLL prostate carcinoma cells also require serum for in vitro invasiveness, and the PHSRN peptide is sufficient to induce their invasive behavior under SF conditions. Blocking the ends of the PHSCN peptide by acetylation and amidation increases its invasion inhibitory activity in serum-containing medium by 30-fold for both DU 145 and MLL cells, perhaps because removal of the charges at the NH₂ and COOH termini allows the peptide to assume a more active conformation or results in increased stability. Furthermore, the invasion-inhibitory activity of Ac-PHSCN-NH₂ is shown to be attributable to the specific spatial arrangement of its amino acid side chains because the scrambled peptide Ac-HSPNC-NH₂ fails to inhibit serum-induced invasion by either DU 145 or MLL cells. The Ac-PHSCN-NH₂ peptide appears to function as an effective, nontoxic, antitumorigenic agent in Copenhagen rats bearing metastatic MLL tumors because its systemic administration reduces the growth of primary MLL tumors by 30-fold and the density of their vasculature by 10-fold during the first 16 days of growth in rats. As shown by comparing sections of rat anti-PECAM-stained tumors from Ac-PHSCN-NH₂-treated and from untreated rats and quantitating the relative volumes occupied by the vasculature in their tumors, as well as by the inability of Ac-PHSCN-NH₂ to affect the MLL cell cycle or to stimulate apoptosis in vitro at similar or higher concentrations, its pronounced antitumorigenic effects are likely to be attributable to the inhibition of neovascularization. The appearance of MLL cells in rats treated systemically with Ac-PHSCN-NH₂ is also consistent with its ability to inhibit nourishment of the tumor by new blood vessels. The effects of systemic treatment with the Ac-PHSCN-NH₂ peptide could be attributable to a direct anti-invasive effect on the α5β1 receptors known to be expressed by rat endothelial cells (45). Consistent with this possibility, we have observed that the Ac-PHSPRN-NH₂ peptide can stimulate invasion in vitro by normal human microvascular cells, which can be inhibited by Ac-PHSCN-NH₂ (data not shown). Also, the presence of Ac-PHSCN-NH₂ in the circulation could have inhibited proteinase secretion by MLL tumor cells and hence reduced the fragmentation of host pFn by secreted MLL tumor proteinases. Thus, the potential effect on endothelial α5β1 receptors might have also been mediated, in part, through the inhibition of PHSRN sequence binding by the α5β1 receptors shown to be present on the surfaces of MLL tumor cells. Invasion by host endothelial cells in response to fibronectin fragments produced in wounds is believed to be crucial to the angiogenic response necessary for migration through the extracellular matrix during wound healing. The constitutive activation of this pathway by an aggressive tumor such as MATLyLu could thus result in neovascularization and rapid tumor growth, as well as in tumor invasion and metastasis. Systemic Ac-PHSCN-NH₂ peptide has a very significant and sequence-specific antimetastatic effect on rats bearing metastatic MLL tumors, irrespective of whether systemic treatment is initiated 1 day after MLL cell injection or not until 1 day after the surgical removal of large MLL tumors. I.e., injections of Ac-PHSCN-NH₂, beginning 1 day after MLL tumor cell injection and continuing for the following month, prevent the formation of detectable lung metastases or micrometastases in the majority of treated rats and reduce the mean number of lung metastatic colonies or micrometastases/rat in the treated cohort by 40–200-fold, with respect to untreated, MLL-bearing rats. Consistent with its lack of invasion inhibitory activity in vitro, the Ac-HSPNC-NH₂ peptide also lacks antitumorigenic or antimetastatic activity in vivo. The antimetastatic effects of systemic Ac-PHSCN-NH₂ are nearly as pronounced in treated rats that do not begin systemic therapy until 1 day after their 2-cm MLL tumors are removed, as they are in rats that begin to receive treatment 1 day after MLL cell injection. Because metastases are scored under conditions that permit the detection of single MLL cells in lung tissue, and because Ac-PHSCN-NH₂ does not appear to affect MLL cell cycle progression or to stimulate apoptosis at the concentrations used, it is very likely that the inhibition of postsurgical MLL metastasis in rats is a consequence of the invasion-inhibitory activity of Ac-PHSCN-NH₂ on MLL cells in vivo. Because neither the sensitivity of the assay nor the growth of metastases is likely to have limited their detection, the paucity of MLL metastases observed in these rats suggests that the metastatic potential of individual MLL cells remaining in a rat increases significantly after surgery. This could be because some MLL cells are released into the blood or lymph during surgery, thus reducing the requirement for invasation in this system. It is interesting to note that specific proteinases may be required for invasation, a process that requires invading cells to traverse the basement membrane in an orientation opposite from that required during extravasation (47). If MLL cells were less adept at invasation than at extravasation because of their proteinase expression pattern, then perhaps Ac-PHSCN-NH₂ may be functioning primarily as an extravasation inhibitor. This implies that a slow rate of MLL invasation may permit the pronounced antimetastatic activity of systemic Ac-PHSCN-NH₂ therapy to be observed, even when initiated only after the surgical removal of a large tumor. In this context, it is interesting to note the topology of the SU-ECM in vitro invasion substrates used to evaluate the activity of Ac-PHSCN-NH₂. In this assay, invading cells are placed on the extracellular matrix that surrounded ectodermal cells located on the apical side of the basement membrane. Thus, cells encounter the extracellular matrix of the ectoderm and the underlying lamina lucida prior to encountering the lamina densa (32), as they do when crossing the endothelial cell layer and its underlying basement membrane during extravasation. Therefore, the SU-ECM invasion assay may be especially well suited for defining effective inhibitors of extravasation. Proteolytic fragments of pFn containing the cell-binding domain are present in wounds and have been shown to stimulate monocyte extravasation (48) and fibroblast chemotaxis through the extracellular matrix (49). The constitutive induction of invasive behavior by this intact and prevalent serum protein is a possible mechanism for stimulating tumor cell invasion and metastasis, as well as angiogenesis. The ability to stimulate prec cell invasion in vitro by the same PHSRN pFn sequence and to inhibit it by the PHSCN competitive inhibitor suggests that metastatic prostate carcinoma cells may constitutively express an invasive behavior latent in normal prec cells and necessary for migration through the extracellular matrix during wound healing. This hypothesis is consistent with the fibronectin receptor expression patterns of these two cell types. Both DU 145 and prec cells express the α5β1 integrin fibronectin receptor (33, 36), which is known to bind the PHSRN sequence of the cell-binding domain (39). It has been shown that the interaction of fibroblast α5β1 with the pFn cell-binding domain induces interstitial collagenase MMP expression in the absence of α4β1 binding (34). Loss of the α4β1 receptor, which is present on the surfaces of prostate epithelial cells, from the cell membranes of prostate cancer cells (Refs. 33 and 36 and data not shown) is a possible mechanism for generating constitutive invasive responses. The indistinguishable in vitro invasive behaviors of prostate epithelial and DU 145 cells in the presence of the PHSRN sequence of the pFn cell-binding domain are consistent with this hypothesis.
Specific therapeutic strategies for the treatment of androgen-resistant prostate carcinomas in Copenhagen rats have been devised by a number of labs to control the growth of the primary tumors or to limit metastasis without necessarily limiting tumor growth. For example, systemic treatments with a peptide growth hormone-releasing hormone antagonist (50), with interleukin-2 and radiation (51), with somatostatin analogue (RC-160) and the bombesin/gastrin-releasing-peptide antagonist (RC-3095; Ref. 52), or with the cytotoxic drug methotrexate coupled to a luteinizing hormone-releasing hormone receptor ligand (53, 54) all displayed some effectiveness in slowing the growth of AT-1 tumors in rats. Paclitaxel and quinacrine have also been used in combination to inhibit microtubule function in DU 145 prostate carcinoma cells implanted in athymic mice. DU 145 tumors in the treated nude mice were shown to be reduced in volume by ~2.5-fold relative to the untreated tumors during 2 weeks of treatment (55). In contrast to these results, the systemic therapy with the Ac-PHSCN-NH₂ peptide reduces tumor volume by >2000-fold during the first 16 days of growth.

In a strictly antimetastatic therapy, modified citrus pectin, which inhibits the adhesive interactions of metastatic cells with endothelium by competing with the natural ligand(s) for binding to tumor cell surface galectins, was tested in Copenhagen rats bearing MLL primary s.c. tumors. Oral administration of this agent resulted in a 40–50-fold reduction in the mean number of metastases/lung without affecting the rate of growth of the primary tumor (7). Thus, the antimetastatic effects of this agent were comparable with those of Ac-PHSCN-NH₂ treatment; however, therapy with the Ac-PHSCN-NH₂ peptide was uniquely effective in that it also had significantly more pronounced effects on the growth of the primary tumors than did the other antitumorigenic therapies reported.

Although the role of Ac-PHSCN-NH₂ as an antimetastatic agent, i.e., as an inhibitor of the induction of a constitutive invasive response in tumor cells by a ligand of the α5β1 receptor, is distinct from the competitive inhibition of the adhesive interaction between tumor cells and the extracellular matrix by an excess of the matrix adhesion sequence, the adhesion of tumor cells to the extracellular matrix plays a very significant role in metastasis formation. This is shown by a number of in vitro and in vivo experiments using peptides as competitive inhibitors. For example, peptides containing the RGD sequence of the cell-binding domain in fibronectin, which binds to the α5β1 integrin, or the Tyr-Ile-Gly-Ser-Arg (YIGSR) sequence of laminin, which binds to a high-affinity M₆ 67,000 glycoprotein receptor, showed marked inhibition of lung metastasis when conjuncted with melanoma cells and of invasion through reconstituted basement membrane in vitro (56, 57). In a model of spontaneous lung metastasis, in which tumors of B16-BL6 melanoma were allowed to grow 21 days prior to treatment, repeated administration of the poly(RGD) or poly(RGD) polypeptides before or after surgical excision of the primary tumor resulted in a 5-fold reduction of metastatic lung colonies without affecting primary tumor growth, thus substantially prolonging the survival time of mice (58). However, the significantly more pronounced antimetastatic activity of systemic Ac-PHSCN-NH₂ therapy, even when begun after the removal of a large primary tumor, suggests that therapeutic intervention in the dysregulation of an integrin-mediated invasive response may be a promising avenue for treating androgen-resistant prostate neoplasms.

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