HEPSIN Inhibits Cell Growth/Invasion in Prostate Cancer Cells

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Abstract

Expression of HEPSIN, a type II transmembrane serine protease in prostate cancer (CaP), has been highlighted by several studies analyzing CaP-specific gene expression alterations by cDNA microarray. Evaluations of the biological functions of HEPSIN in CaP cells are warranted for better assessment of its utility as a biomarker and/or therapeutic target. In stable clones of PC-3/HEPSIN transfectants, there was a dramatic reduction in cell growth, cell invasion, and soft agar colony formation. A higher proportion of PC-3/HEPSIN cells were in the G2-M phase of the cell cycle, and there was also an increase in the cell population undergoing apoptosis. Preliminary analysis of HEPSIN transfections into LNCaP and DU145 cells further revealed cell growth-inhibitory effects. These results underscore that exogenous HEPSIN expression negatively regulates cell growth in metastatic CaP cell lines. Although the biological consequence of HEPSIN overexpression in primary CaP remains to be determined, the negative cell growth-regulatory effects of HEPSIN in metastatic CaP cells reported here have unraveled possible cellular and molecular mechanisms underlying observations that link decreased/loss of HEPSIN expression with poor prognosis of CaP.

Introduction

CaP poses a significant clinical challenge in terms of both its prevalence and its complexity. In the year 2002, 189,000 new cases of CaP will be diagnosed in the United States, and 30,200 American males will die of this disease (1). The progression of CaP proceeds through a series of stages, including preinvasive disease defined as prostatic intraepithelial neoplasia, organ-confined invasive cancer, and metastatic disease (2). CaP has also been characterized as an androgen-dependent or androgen-independent disease, based on the response to androgen ablation or hormonal therapy, and late-stage metastatic disease often progresses to the hormone-refractory stage (3). Serum PSA is widely recognized as a highly successful CaP surrogate marker for early detection and for monitoring therapeutic responses. However, elevated serum PSA levels in men with obstructive or inflammatory uropathies often lower the specificity of PSA as a cancer marker (4). Molecular genetic studies of CaP have identified mutations, deletions, or loss of expression of tumor suppressor genes (5, 6). Oncogenes c-MYC, HER2, and BCL2 are overexpressed or amplified in subsets of CaP (5). Mutations and/or amplification of the androgen receptor have also been reported in advanced CaP (7, 8). Due to heterogeneity of CaP itself and the focal nature of oncogene/tumor suppressor gene alterations, the role of these genes in CaP onset and the diagnostic and/or prognostic value of such gene alterations (mutations or expression changes) remain uncertain. Molecular genetic alterations that are present at high frequency and in a more homogeneous manner in CaP are being discovered through unbiased genome scanning or global gene expression profiling approaches (7, 8). Some of the more common CaP-associated genetic alterations now include loss of glutathione S-transferase-π expression (9), overexpression of HEPSIN (10) and α-methylacyl-CoA racemase (11, 12), and consistent loss or gains of specific chromosomal hot spots (8).

The importance of HEPSIN in CaP has been shown by a number of independent studies analyzing consistent gene expression pattern differences between CaP and normal prostate by cDNA microarray analyses (10, 13–16). HEPSIN overexpression was generally noted in primary CaP. The association of decreased HEPSIN expression in hormone-refractory metastatic CaP and patients with elevated PSA levels has been an intriguing observation (10, 17). HEPSIN overexpression has also been reported previously in other cancers [renal cell and ovarian carcinomas (18, 19)].

HEPSIN is a type II transmembrane serine protease identified originally in the human liver as a cDNA clone (20, 21). A 1.85-kb HEPSIN mRNA is expressed in most tissues, with the highest level expressed in liver tissue, and lower levels expressed in other tissues including prostate. Subcellular localization studies in BHK-21 and HepG2 cells suggest that HEPSIN is present in the plasma membrane as type II membrane-associated protein, with its catalytic subunit at the cell surface, and the NH2 terminus facing the cytosol. Using specific antisense oligonucleotides, HEPSIN has been shown to be necessary for normal cell growth (22).

Biological functions of HEPSIN are not known in CaP. To understand the mechanisms and functional consequences of HEPSIN expression in CaP cells, we have evaluated effects of HEPSIN overexpression in widely used metastatic CaP cells with or without endogenous HEPSIN expression. This study provides new insights into the biological functions of HEPSIN in CaP cells.

Materials and Methods

Cell Culture and RNA Preparation. Established CaP cell lines (LNCaP, DU145, and PC-3) were obtained from American Type Culture Collection (Manassas, VA). LNCaP and PC-3 cell lines were grown in RPMI 1640 (Life Technologies, Inc.) with 10% FBS. DU145 cells were grown in MEM with 10% FBS. NIH3T3 and HT1080 cell lines were obtained from American Type Culture Collection and grown in DMEM (Life Technologies, Inc.) with 10% FBS. SV40 immortalized normal prostate pRNS-1 cells and their tumorigenic derivatives k-ras/pRNS-1 (23) and cadmium-transformed pRNS-1–2x/cadmium (24), SV40 immortalized neonatal human epithelial 267B1 cells and their tumorigenic derivatives k-ras 267B1 and X-ray-transformed 267B1 cells (25), and HPV-18 immortalized normal prostate cells (26) and their chemically transformed derivative 129NU5002 (27) were grown in keratinocyte serum-free medium (Life Technologies, Inc.) with 5 ng/ml human recombinant epidermal growth factor and 0.05 mg/ml bovine pituitary extract. Total RNA was prepared
from cells using the RNAzol B kit (Tel-Test, Friendswood, TX). Poly(A)+ RNA from different cells was prepared with a FastTrack RNA preparation kit (Invitrogen, Carlsbad, CA).

**Northern Blot Analysis.** Total RNA (20 \( \mu g \)/lane) or poly(A)+ RNA (2 \( \mu g \)/lane) prepared from different cells was used for the preparation of the blot. A cDNA probe was prepared by labeling a purified 300-bp PCR product of HEPSIN with \(^{32}P\)dCTP using a random primer labeling kit (Stratagene, La Jolla, CA). Hybridization was performed using rapid-hyb buffer (Amersham Biosciences, Piscataway, NJ). All of the blots were hybridized with a glyceraldehyde-3-phosphate dehydrogenase probe as an internal control.

**Generation of CaP Cell Lines Harboring HEPSIN Expression Vector.** A full-length HEPSIN cDNA construct encoding HEPSIN-V5 fusion protein (cloned in Genestorm expression vector, pcDNA3.1/GS; Research Genetics) was transfected into PC-3, DU145, and LNCaP cell lines using TransFast transfection reagent (Promega, Madison, WI). Before transfection, the HEPSIN cDNA clone was verified by sequencing. Zeocin-resistant colonies were selected after 3 weeks. Cells derived from individual colonies after transfection and drug selection were obtained by cloning cylinders and further expanded. Total RNA was prepared from transfectants derived from the control vector-transfected group as well as the HEPSIN expression vector group and screened by Northern blot hybridization. Protein lysates (35 \( \mu g \) of protein) were prepared from both control vector-transfected and HEPSIN expression vector-transfected cells, and HEPSIN-V5 fusion proteins were analyzed using an V5 antibody.

**Cell Proliferation and Colony Forming Assays.** To understand the effect of HEPSIN expression on cell proliferation, cells from PC-3/HEPSIN transfectants were plated in triplicate in a 6-well tissue culture plate. Cell proliferation was followed by counting cell numbers every 2 days for 6 days, and the mean values were analyzed. Colony forming assay was performed in two ways. In some experiments, cells (PC-3) were transfected with HEPSIN expression vector or control vector, and after 15 days, zeocin-resistant colonies were counted after crystal violet staining. Alternatively, clonal PC-3 or LNCaP transfectants already characterized for stable HEPSIN expression were plated at a density of 3,000 cells/T-75 flask. On day 15, cells were fixed with PBS-2% formaldehyde and stained with crystal violet for colony counting.

**Cell Invasion Assay.** Cell invasion assay was performed using the cell invasion kit (Chemicon International). Briefly, 3 \( \times 10^5 \) cells in 300 \( \mu l \) of serum-free media were placed over the inner chamber of the insert in a 24-well tissue culture plate, and 500 \( \mu l \) of media with serum were placed in the outer chamber of the insert. The plates were incubated for 48 h at 37°C. The highly invasive cell line HT1080 (human osteosarcoma) and the noninvasive cell line NIH3T3 (suggested in the kit) were used as positive controls, respectively. After 48 h, the invasive cells that migrated through on the lower surface of the membrane were stained. The stained cells were dissolved in 10% acetic acid and analyzed by an ELISA plate reader at 560 nm.

**Colony Formation on Soft Agar.** PC-3/HEPSIN transfectants were suspended in 0.3% noble agar with complete RPMI 1640, plated at a density of 1 \( \times 10^4 \) cells in a 60-mm dish coated previously with 0.5% base agar, and maintained at 37°C. On day 21, colonies > 0.2 mm in diameter were counted and analyzed.

**Cell Cycle Analysis.** Cells from PC-3/HEPSIN transfectants were trypsinized, washed in PBS, and then fixed in cold 70% ethanol overnight. The cells were then washed twice in ice-cold PBS and stained with 50 \( \mu g/ml \) propidium iodide (Boehringer Mannheim, Indianapolis, IN). The samples were analyzed using an EPICS ELITE ESP (Beckman Coulter, Miami, FL) flow cytometer. DNA histograms were analyzed using Modfit LT software (Verity Software House, Topsham, ME).

**Results**

**Evaluation of HEPSIN Expression in CaP Cell Lines and Generation of Stably Transfected PC-3/HEPSIN and LNCaP/HEPSIN Cells.** HEPSIN expression was evaluated by Northern blot hybridization. Among the established cell lines (LNCaP, DU145, and PC-3), as described previously, HEPSIN expression was detected only in LNCaP cells. HEPSIN expression was also not detected in SV40 immortalized normal prostate cells (pRNS-1).
or in HPV-16E6E7 immortalized CaP cells (CPDR-1; data not shown). Furthermore, HEPSIN expression was not detected in two tumorigenic derivatives of pRNS-1, ras-transformed pRNS-1 (23) and a cadmium-transformed pRNS-1 (Ref. 24; data not shown). We also tested HEPSIN expression in two cell culture models of CaP progression: (a) SV40 immortalized neonatal human epithelial 267B1 cells (25) and their tumorigenic derivatives, ras-transformed and X-ray-transformed 267B1 cells (25); and (b) HPV-18 immortalized normal prostate cells (26) and their chemically transformed derivatives, 129Nu 5002-1 (27). None of these cell lines expressed HEPSIN (data not shown).

To study the biological function of HEPSIN in CaP cells, we transfected HEPSIN-negative PC-3 and HEPSIN-positive LNCaP cells with a full-length HEPSIN cDNA-V5 fusion expression construct. Total RNA from mass population of transfected cells and clonal cells were screened by Northern hybridization. Clonal PC-3/HEPSIN-10 and PC-3/HEPSIN-2 cells showed strong HEPSIN expression. As expected, clonal PC-3/GS2 cells derived from control vector-transfected PC-3 cells did not have detectable HEPSIN RNA (Fig. 1A). Furthermore, HEPSIN expression in PC-3/HEPSIN transfectants was also confirmed by detection of Hepsin-V5 fusion protein, using the anti-V5 tag antibody (Fig. 1B). Similarly, LNCaP/HEPSIN-1 clonal cells showed overexpression of HEPSIN compared with the low basal level of endogenous HEPSIN in untransfected LNCaP cells and control vector-transfected LNCaP/GS5-5 clones (Fig. 1C and D).

Cell Growth-inhibitory Effect of HEPSIN in PC-3 Cells and LNCaP Cells. The effect of HEPSIN expression on cell proliferation was analyzed in PC-3/HEPSIN-10 and PC-3/HEPSIN-2 cells. In three independent growth assays, PC-3/HEPSIN-10 and PC-3/HEPSIN-2 cells showed dramatically reduced cell numbers (~75% inhibition) compared with the vector (Fig. 2A). These preliminary results suggested that HEPSIN negatively regulated cell growth in PC-3 cells. PC-3 HEPSIN-10 and HEPSIN-2 cells were similarly inhibited for colony formation in comparison with control PC-3/GS2 cells (Fig. 2B). Similar growth inhibition was observed in LNCaP HEPSIN transfectants also (Fig. 2C). Cell growth-inhibitory effects of HEPSIN expression vector on PC-3 and DU145 cells were also noted in the colony forming assays after primary transfections (data not shown).

Increased Accumulation of PC-3/HEPSIN Cells in G2 Phase of Cell Cycle and Apoptotic Phase. To investigate the mechanism of reduced cell proliferation in PC-3/HEPSIN cells, cell cycle distributions of PC-3/HEPSIN cells and PC-3/GS2 control cells were compared (Fig. 3A). In comparison with the PC-3/GS2 control with 68.21% of cells in G1, and 4.21% of cells in G2, PC-3/HEPSIN-10 cells had 53.88% of cells in G1 and 12.55% of cells in G2, and PC-3/HEPSIN-2 cells had 58.11% of cells in G1 and 18.98% of cells in G2. Furthermore, there was a prolonged S phase in PC-3/HEPSIN-10 cells compared with the control. These observations suggest that PC-3/HEPSIN cells may be blocked in the G2 phase of the cell cycle, which could suggest overall negatively regulated cell growth. In addition to cell cycle alterations, a significant proportion of cells were found to be apoptotic in the PC-3 HEPSIN-2 clone (18.4%) compared with 2.63% of cells in the PC-3 GS2 vector control transfectant. This is being confirmed by other assays such as DNA fragmentation analysis and annexin V analysis.

Evaluation of PC-3/HEPSIN Transfectants for Cell Invasion Properties. A cell invasion assay was performed using the invasion chamber as described in “Materials and Methods.” Cells with invasive properties migrating on the lower surface of the membrane were stained and analyzed in an ELISA plate reader at 560 nm (Fig. 3B). There was about a 50% reduction in the PC-3/HEPSIN-10 cell migration through the Matrigel layer compared with the controls. These
results suggest that HEPSIN overexpression in PC-3 cells inhibits cell motility and invasiveness.

**Inhibition of Colony Formation in Soft Agar by PC-3/HEPSIN Cells.** Anchorage-independent growth is considered an in vitro marker for tumorigenesis. Therefore, PC-3 HEPSIN transfectants were examined for anchorage-independent growth in a semisolid agar medium. As shown in Table 1, both PC-3 HEPSIN-10 and HEPSIN-2 cells formed a reduced number of colonies compared with the control vector PC-3/GS2 cells. Furthermore, the majority of the colonies in the PC-3/HEPSIN cells were smaller than those in the control. These results suggest that HEPSIN may inhibit the tumorigenic phenotype.

**Discussion**

Recent reports of consistent alterations of HEPSIN expression in CaP led us to investigate the biological functions of HEPSIN in CaP cells. Although previous reports have shown that HEPSIN may be involved in cell growth, its function in cancer biology, if any, remains to be defined. The biological basis of observations showing that HEPSIN expression is generally increased in primary CaP and that decreased HEPSIN expression correlates to poor prognosis remains to be understood. In this report, we have shown that overexpression of HEPSIN inhibited cell proliferation and colony formation in HEPSIN-negative or HEPSIN-positive metastatic CaP cells. The increased fraction of PC-3/HEPSIN cells in the G2 phase of the cell cycle and apoptosis may provide possible mechanisms for negative regulation of cell growth by HEPSIN. Reduction in the

<table>
<thead>
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<th>Cell line</th>
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<tr>
<td>PC-3 GS2</td>
<td>195</td>
</tr>
<tr>
<td>PC-3 HEPSIN-10</td>
<td>89</td>
</tr>
<tr>
<td>PC-3 HEPSIN-2</td>
<td>105</td>
</tr>
</tbody>
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Table 1 Colony formation on soft agar by PC-3/HEPSIN cells

Cells from PC-3/HEPSIN transfectants were suspended in 0.36% agar with complete RPMI 1640 and then layered on top of 0.5% bottom agar (10⁴ cells/dish). Colonies were counted after 3 weeks. The numbers represent the mean number of colonies in each group (P < 0.0001).
number of PC-3/HEPSIN cell colonies in soft agar and inhibition of migration of PC-3/HEPSIN cells through Matrigel further suggest that HEPSIN expression may protect tumor cells from acquiring more invasive or metastatic phenotypes.

Recently, a number of type II transmembrane serine proteases have been identified. Some of these, including TMPRSS2, TMPRSS4, and Matriptase, have been shown to be overexpressed in different cancers (28). Similarly, HEPSIN has been shown to be overexpressed in CaP by different investigators (10, 17, 29). On the basis of known biological and biochemical functions, it is not clear whether HEPSIN has any function in the neoplastic process. The role of HEPSIN in the blood coagulation pathway has been studied in baby hamster kidney cells transfected with HEPSIN (30). HEPSIN-transfected cells proteolytically activate factor VII in a time- and calcium-dependent manner and initiate the blood coagulation pathway on the cell surface, leading to thrombin formation. The biological functions of HEPSIN have been studied in HEPSIN knockout mice (31–33); however, no phenotype related to the tumorigenesis process was noted. Our report provides new insights into the biological functions of HEPSIN in cancer cells in general and in metastatic CaP cells in particular. All of the results reported here suggest that HEPSIN has antitumorigenic effects on the CaP cells studied, which in fact supports the correlative observations reported here suggest that HEPSIN has antitumorigenic effects on the CaP cells studied, which in fact supports the correlative observations.
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