Osteopontin: Possible Role in Prostate Cancer Progression


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ABSTRACT

Human prostate cancer has the propensity to metastasize to the bone where reciprocal cellular interactions between prostate cancer and bone cells are known to occur. Osteopontin (OPN), a noncollagenous bone extracellular matrix, is a secreted adhesive glycoprotein with a functional RGD cell-binding domain that interacts with the αvβ3 cell surface integrin heterodimer. OPN has been associated with malignant transformation as well as being ligand to the CD44 receptor. Polyclonal antibodies to human OPN (hOPN) were prepared, and specificity was shown by preabsorption with recombinant hOPN. The stimulatory effect of hOPN protein and the inhibitory effect of hOPN antibody on human prostate cancer cell lines LNCaP and C4–2 were assessed by induction or inhibition of anchorage-independent growth, respectively. Expression of hOPN mRNA in prostate cancer cell lines and human prostate cancer tissue specimens were measured by mRNA blot analysis. Protein expression was assessed by immunohistochemistry in human prostate cancer specimens and by Western blot analysis in prostate cancer cell lines. hOPN stimulated anchorage-independent growth of the human prostate cancer cell lines LNCaP and C4–2 in vitro. Antibodies to hOPN inhibited the growth-stimulatory effect by endogenous OPN, which can be overcome by the addition of exogenous hOPN. hOPN mRNA and protein are expressed in human prostate cancer cell lines in vitro and in clinical human prostate cancer specimens. These findings taken together suggest that OPN may act as a paracrine and autocrine mediator of prostate cancer growth and progression.

INTRODUCTION

After an initial responsiveness to hormonal deprivation, advanced prostate cancer almost invariably relapses, and androgen-independent clones progress and metastasize nonrandomly to the axial skeleton. Experimental studies of human prostate cancer models have stressed the influence of the mesenchymal-epithelial interaction on the development of prostate cancer (1). In a series of studies, we observed that prostate and bone fibroblasts, in the appropriate host hormonal milieu, can induce the progression of prostate cancer cells from an androgen-dependent to an androgen-independent state, whereby cells with the latter phenotype express osseous metastatic potential (2, 3). It has been suggested that the development of osseous metastasis is dependent upon bone marrow and osteoblast-derived soluble growth factors that stimulate the growth of prostate cancer cells in a paracrine-mediated manner (4, 5).

Extracellular matrix has been demonstrated to play a critical role in tumor cell growth, adhesion, migration, and metastasis. Although the role of the extracellular matrix and its intracellular signaling through integrin heterodimers remain to be defined, interactions between bone matrix proteins and prostate cancer cells are likely to be the key determinants regulating prostate tumor cell behavior. The expression of bone matrix proteins is not restricted to bone tissue. One of these noncollagenous bone matrix proteins, OPN, has been shown to be overexpressed in human cancers (11–13), and OPN overexpression confers malignant transformation and was observed frequently in a variety of tumorigenic human cell lines (12–14). Consistent with these observations, serum levels of OPN were substantially elevated in patients with metastatic cancer (15). Ha-ras-transfected NIH 32D cells in vitro form aggregates that adhere to bone matrix proteins, OPN, and calcyclin, and migrate toward an exogenous source of OPN (4, 5).
3T3 fibroblasts, which are tumorigenic and metastatic in contrast to untransformed fibroblasts, express increased levels of OPN. When Ha-ras-transfected fibroblasts were transfected with an antisense osteopontin RNA, their tumorigenic and malignant growth were reduced significantly (16). In human esophageal cancer, ras-regulated gene products, OPN and cathepsin L, were shown to be associated with tumor invasion and metastasis (17). We previously reported, in a preliminary finding on the potential role of bone matrix proteins such as OPN, on the progression and dissemination of prostate cancer (18).

In this communication, we have extended our previous report on the potential role of bone matrix proteins such as OPN on the progression and dissemination of prostate cancer. We have evaluated the role of OPN in human prostate cancer progression and metastasis. We have demonstrated the value of characterizing in detail the phenotypic and genotypic alterations in LNCaP lineage-related cell models of prostate cancer progression and their utility to define clinical prostate cancer. Herein we provide the experimental evidence that OPN over-expression is associated with human prostate cancer progression. We conclude that OPN may confer selective growth and malignant potential to prostate tumor cells in situ.

MATERIALS AND METHODS

Cell Culture. LNCaP cells, passage 29 of the original line developed by Horoszewicz et al. (19), were kindly supplied by Dr. Gary Miller (University of Colorado, Denver, CO). LNCaP cells (passages 37–40), C4–2 cells (passages 20–23), and NbE 1.4 cells (passages 30–35) were grown in T-medium. DU145 and PC3 cells were purchased from American Type Culture Collection and grown in T-medium. LNCaP cells (passages 37–40), C4–2 cells (passages 20–23), and NbE 1.4 cells (passages 30–35) were grown in T-medium. Passage 25 to 33 of a human bone fibroblast cell line, MS, established from a patient with an osteosarcoma as described previously (4), were used in this study. The cell lines were tested and found free of Mycoplasma.

Purification of hOPN. hOPN was extracted and purified as published previously (21). In brief, human milk was extracted in guanidine-HCl/EDTA and was fractionated by DEAE-cellulose chromatography, eluted with 50 mM NaCl dissolved in 7 M urea (pH 4.0). A single hOPN band, migrating at Mr 60,000 and yielding a symmetrical peak on gel filtration on Sephacryl S-300, was detected.

Preparation of hOPN Antibody. hOPN purified from human milk was used as an antigen to raise hOPN polyclonal antibodies in a goat (Bethyl Laboratories, Montgomery, TX). One hundred µg of purified hOPN was injected s.c. once every 2 weeks, with the initial immunization in complete Freund’s adjuvant and subsequent immunizations in incomplete Freund’s adjuvant. Bleeds were taken prior to the first immunization and 7 days after each injection. Antibody titer was assessed using an ELISA. Goat anti-hOPN antibody was affinity-purified by using recombinant hOPN on an affinity column. Affinity-purified hOPN polyclonal antibodies were used for immunostaining.

Tissue Sampling. Radical prostatectomy specimens for routine histological examination were fixed in 4% paraformaldehyde and 5 mM MgCl₂. Six-µm, paraffin-embedded tumor sections were cut and stained with H&E. Tumors were evaluated, staged, and graded by a pathologist (R. M.). Specimens from pTURP were processed in a similar manner. Sections containing normal prostate cancer, BPH, high-grade PIN, and prostate cancer were cut and used for immunohistochemical staining. Thirty-one radical prostatectomy specimens and 8 pTURP specimens were evaluated. Tumor tissue samples for RNA blot analysis were resected and snap frozen from radical prostatectomy specimens by the pathologist. BPH and tumor tissues from TURP or pTURP were immediately snap frozen. Normal prostate tissues were obtained from organ donors according to the institutional guidelines and with approval of the University Ethical Committee. All tissues were stored at −80°C until further processing.

Immunohistochemical Staining of OPN. Immunohistochemical staining was performed on deparaffinized human prostate cancer tissue sections. Specimens were washed three times in PBS, preincubated with donkey serum 1:20 to block nonspecific activity, and then incubated with an affinity-column-purified, polyclonal, goat-specific primary antibody against hOPN. The specificity of the hOPN antibody was tested by preabsorption of column chromatographically purified hOPN before immunostaining of a positive control (human bone tissue; Fig. 3, a and b). Preimmunization serum of the same animal was used as a negative control for hOPN, and human bone tissue sections served as positive control. After 3 × PBS wash preincubation with donkey serum 1:20, the tissue specimen was incubated with the secondary antibody donkey anti-goat (alkaline phosphatase) 1:50. After three PBS washes, the alkaline phosphatase substrate (Vector Red; Vector Laboratories, Inc., Burlingame, CA) was added, incubated, and washed. Slides were then stained in filtered hematoxylin, dehydrated (80% ethanol, 95% ethanol, absolute ethanol, and xylene), and mounted with Permount (Fisher Scientific Corp., Fair Lawn, NJ). Although immunostaining for OPN in cultured prostatic cancer cell lines was uniform, multifocal OPN staining was commonly observed in clinical specimens with multifocal disease. Staining for OPN was rated as follows: −, negative staining; (+), slight staining; +, positive focal staining; and ++, strong focal staining. Only positive and strong focal staining were considered as positive staining for OPN.

Soft Agar Colony Formation. To determine the ability of hOPN to stimulate colony formation in LNCaP and C4–2 cells when grown in vitro, TCM, a defined serum complement (Celox Co., Minnetonka, MN, see Ref. 1) and CM from NIH 3T3 fibroblasts (negative control) were used as baseline. Cells (2.5 × 10³ LNCaP) and 1.0 × 10³ (high density) C4–2 cells were trypsinized to single-cell suspensions and incubated for 4 h either with 3T3 CM, hOPN (10 µg/ml), purified hOPN antibody (dilution 1:50), preimmunization control serum, or in combination (OPN 1 µg/ml plus OPN-antibody; dilution, 1:50) after treatment with 0.02% EDTA. The concentrations for OPN (10 µg) and OPN-antibody (dilution, 1:50) were determined in preliminary experiments. 3T3 CM was prepared as follows. Cells in 70–80% confluent culture were downshifted to serum-free T-medium containing 2% TCM, a serum-free defined medium supplement only. After 48 h, CM was removed and lyophilized, and protein concentrations in the CM were determined using a protein assay (Bio-Rad Laboratories, Richmond, CA); they ranged from 70–
cDNA (loading control) probe, having specific activity
10^8 dpm/
analysis by electrophoresis in a 0.9% agarose gel containing 2 M
mined by absorbency at 260 nm, were subjected to RNA blot
single step purification protocol. Thirty
RNAzolB method (Biotech Laboratories, Inc., Houston, TX.), a
from cells (LNCaP, C4–2, DU145, PC-3, NbE1.4, and 3T3) and
than 0.1 mm were scored 4–6 weeks after plating.

**RNA Blot Analysis.** Total cellular RNA was extracted
from cells (LNCaP, C4–2, DU145, PC-3, NbE1.4, and 3T3) and
from snap frozen, pestle-ground human tissue samples by the
RNAzolB method (Biotech Laboratories, Inc., Houston, TX.), a
single step purification protocol. Thirty µg of RNA, as
determined by absorbency at 260 nm, were subjected to RNA blot
analysis by electrophoresis in a 0.9% agarose gel containing 2 M
formaldehyde. RNAs were transferred by capillary blotting onto
Zetaprobe membrane (Bio-Rad, Richmond, Calif.) using 1×
TAE buffer. RNAs were cross-linked to the membranes by UV
exposure using a stratalinker (Stratagene, La Jolla, Calif.) at
1500 mJ and prehybridized in hybridization buffer (10% dextran
sulfate, 1% SSC, 1 M NaCl, and 20 µg/ml salmon sperm DNA
from Amersham, Inc., Arlington Hts. IL). The solution hybrid-
ization was performed by incubation at 65°C overnight by
exposing the membranes to a ^32P-labeled OPN or GAPDH
cDNA (loading control) probe, having specific activity >1×
10^9 dpm/µg. After hybridization, the membranes were washed
in 2× SSC at room temperature for 30 min, then washed under
highly stringent conditions (twice for 30 min in 2× SSC/1%
SDS, then once for 30 min in 0.5× SSC/1% SDS) at 65°C.
Autoradiograms were prepared by exposing Kodak X-Omat AR
films to the membrane at −80°C with intensifying screens.
Autoradiograms of RNA blot analysis for OPN and GAPDH
were analyzed, and OPN values were normalized to GAPDH by
means of an AMBIS Molecular Dynamics Imaging System
(Molecular Dynamics, Sunnyvale, CA).

**Western Blot Analysis.** Recombinant hOPN (20 µg)
and cell lysate samples (20 µg) were run on 10% SDS-poly-
acrylamide gels and transferred onto nitrocellulose paper by
electrophoresis. After blocking with PBST (PBS with 0.1%
Tween 20), the blots were incubated with the indicated primary
antibody (hOPN-AB) at a dilution of 1:250 at 4°C for 12 h. The
blots were then washed three times with PBST buffer and
incubated with the secondary streptavidin peroxidase-conju-
gated antibody for 1 h at room temperature. After being washed
three times, the protein bands were detected by enhanced chemi-
luminescence (Amersham, Inc.).

**Intracellular Quantification of OPN by Flow Cytom-
etry.** Cells (1 × 10^6) were harvested and washed with 1×
PBS and then fixed with 2% paraformaldehyde at 4°C for 30 min.
After fixation, cells were washed twice in ice cold permeabil-
ization buffer [PB: PBS (pH 7.4), 0.1% saponin, and 0.1% BSA]
and incubated with goat anti-OPN in PB (1:250) for 30 min on
ice. Negative control antibody consisted of normal goat serum
(Sigma) used at a dilution of 1:250. Cells were collected by
centrifugation, washed twice with cold PB buffer, and
incubated with secondary rabbit anti-goat FITC (1:500) in PB for 30 min
on ice. Cells were collected by centrifugation, washed twice
with cold PB buffer, resuspended in cold PBS, and analyzed on
a FACScan flow cytometer (Becton Dickson). Results are ex-
pressed as a ratio of mean channel fluorescence of test antibody
to that of negative control serum.

**Statistical Analysis.** For statistical analysis of soft agar
colonies and cell viability, one-sided Student’s t test with
equal variances was used (SPSS).

**RESULTS**

RNA blot analysis of several human prostate cancer epi-
theial cell lines (Fig. 1a) revealed an elevated expression of
OPN mRNA in androgen-independent and tumorogenic cell
lines C4–2, DU145, and PC-3 and to a lesser extent in the and
androgen-dependent LNCaP cell line (Fig. 1a). The normal
rat prostatic epithelial cell line NbE-1.4, derived from Nb rats, did not express OPN mRNA. By Western blot analysis, the antibody against hOPN (hOPN-AB) detected hOPN protein at M_r 60,000 (Fig. 1b). Fluorescence-activated cell sorter analysis with the hOPN antibody demonstrated that the cell lines LNCaP, C4–2, DU145, and PC3 express OPN protein on their surface (Fig. 1c). Western blot analysis confirmed these findings (data not shown).

Because conditioned media collected from bone fibroblasts stimulated human prostate cancer growth in vivo (22), we examined further whether osteopontin may support the anchorage-independent growth (soft agar colony formation of prostate epithelial cells) in vitro because this activity correlates closely with tumorigenicity of target epithelial cells in vivo (23). hOPN (10 µg/ml) stimulated the anchorage-independent growth of androgen-dependent LNCaP cells in a soft agar colony formation assay (P = 0.013; Fig. 2a). hOPN antibody did not significantly alter soft agar colony-forming activity. hOPN added in excess (200 µg/ml) could overcome hOPN antibody inhibition. The addition of CM derived from NIH3T3 cells did not significantly stimulate the anchorage-independent growth of the LNCaP cells (baseline level was defined by the addition of a serum-free supplement, 2% TCM).

Similar findings were demonstrated in the androgen-independent C4–2 cells (Fig. 2b) at low density (1.0 × 10^4 cells); hOPN (10 µg/ml) induced anchorage-independent growth (P < 0.01). Unlike LNCaP cells, hOPN antibody alone decreased the baseline number of soft agar colonies formed by C4–2 cells below baseline (P = 0.038), and again this inhibition could be overcome by exogenously added hOPN (200 µg/ml). At a higher density (5.0 × 10^4 cells), androgen-independent C4–2 cells demonstrated an elevated intrinsic anchorage-independent growth at baseline. Under these autocrine stimulatory conditions, hOPN did not significantly stimulate anchorage-independent growth, but the addition of hOPN antibody inhibited this intrinsic androgen-independent growth (data not shown). Again, the hOPN antibody inhibition could be overcome by exogenous hOPN (data not shown). The preimmune serum of the same animal did not inhibit anchorage-independent growth in preliminary experiments (data not shown).

To demonstrate the presence of OPN in human tissues, we conducted immunohistochemical analysis of clinical specimens of prostate cancers obtained from radical prostatectomy and transurethral resections. As a positive control and to demonstrate the specificity of the hOPN antibody, we stained normal human bone for OPN (Fig. 3a), which exhibited distinct staining of the reversal lines and osteocytes. Preabsorption of the hOPN antibody with affinity-purified hOPN abolished the immunostaining (Fig. 3b). Immunohistochemical staining of pathologically normal human prostate showed no immunostaining or only slight staining of the luminal surface of atrophic glands, a result in agreement with the lack of OPN in a normal rat prostatic epithelial cell line, NbE-1.4. Prostate cancer showed low staining intensity in low Gleason grade prostate cancer (Fig. 3c) and more intense immunostaining in high Gleason grade (Fig. 3d) and androgen-independent prostate cancer (Fig. 3e). BPH did not stain positive for hOPN antibody (Fig. 3f), which is in contrast to the mRNA blot analysis. BPH often has an inflammatory component that is associated with macrophages, which stain strongly positive for OPN, as shown in this TURP specimen (Fig. 3g). The results of the 39 prostate tumors are summarized in Table 1.

Fifty-eight % (18 of 31) of the peripheral zone prostate cancers from radical prostatectomy specimens with a Gleason score 6 and higher stained positive for OPN, with a more intense staining in higher grade tumors. Three of four (75%) transitional zone cancers stained positively for OPN, and two of eight (25%) of the prostate cancer specimens obtained from palliative transurethral resection stained positively for OPN. High-grade PIN stained positive in 4 of 12 (33%) cases. All of the patients (n = 6) with Gleason score 9 prostate cancer showed positive staining for OPN, as did 50, 58, and 67% of patients with Gleason scores 8, 7, and 6, respectively. Fig. 3h shows representative stainings of such OPN-positive tumors.

RNA blot analysis of tumor tissues demonstrated, with few exceptions, that OPN mRNA is widely expressed by prostate...
cancer specimens (Fig. 3i). Interestingly, tumor specimens derived from patients with distant metastasis (e.g., lymph node involvement and bone metastasis) showed higher levels of OPN mRNA expression. BPH specimens demonstrated OPN mRNA expression at a lower level.

**DISCUSSION**

OPN, a noncollagenous bone matrix protein, was found to be prevalently expressed by human prostate cancer cell lines and clinical prostate cancer specimens at the levels of both mRNA and protein. OPN also stimulates anchorage-independent growth.
Role of Osteopontin in Prostate Cancer Progression

Table 1 Immunohistochemical staining of human prostate cancer specimens with the OPN antibody by tumor site

<table>
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<tr>
<th>Tumor site</th>
<th>n</th>
<th>Positive(b) (%)</th>
</tr>
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<tbody>
<tr>
<td>Peripheral zone cancers</td>
<td>31</td>
<td>18/31 (58)</td>
</tr>
<tr>
<td>Transition zone cancers</td>
<td>4</td>
<td>3/4 (75)</td>
</tr>
<tr>
<td>High-grade PIN</td>
<td>12</td>
<td>4/12 (33)</td>
</tr>
<tr>
<td>Palliative transurethral resections</td>
<td>8</td>
<td>2/8 (25)</td>
</tr>
</tbody>
</table>

\(\text{a}\), number of specimens.  
\(\text{b}\), Focal staining that is positive (+) or strongly positive (++).

In summary, OPN mRNA and protein are expressed in prostate cancer cell lines and human prostate cancer specimens. The malignant and metastatic phenotype seems to be correlated with elevated OPN expression. These findings suggest a paracrine and autocrine role of OPN in prostate cancer growth, invasion, and metastasis.

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