Increased Expression of Osteopontin Contributes to the Progression of Prostate Cancer

Ani C. Khodavirdi, Zhigang Song, Shangxin Yang, Chen Zhong, Shunyou Wang, Hong Wu, Colin Pritchard, Peter S. Nelson, and Pradip Roy-Burman

Abstract

Osteopontin is a secreted glycosylated phosphoprotein known to be involved in numerous physiologic functions and associated with the late stages of various cancers. We used preneoplastic and neoplastic mouse models of prostate cancer to determine the onset of elevated expression of osteopontin in the development of this disease. Osteopontin alterations occurred early in the disease with dysregulated expression observed in lesions of low-grade prostatic intraepithelial neoplasia (PIN). Over time, osteopontin expressing dysplastic cells seemed to increase in number in high-grade PIN and increased further in adenocarcinoma, and in metastasis, almost all of the cancer cells immunohistochemically stained positive for osteopontin overexpression. We examined the biological properties of human prostate cancer cell lines LNCaP and PC-3, in which osteopontin overexpression was achieved via lentiviral gene transduction. Evidence was obtained that osteopontin could contribute to a proliferative advantage in both cell types, although more significantly in LNCaP than PC-3. Osteopontin also influenced their in vitro invasive ability, and again, most strikingly in the weakly oncocytic LNCaP. Furthermore, excess osteopontin induced the LNCaP cells to acquire a strong intravasational potential in vivo in the chicken embryo chorioallantoic membrane assay for blood vessel penetration. These results establish a correlation between an increased gradient of osteopontin expression throughout the stages of murine prostate cancer, beginning from the preneoplastic lesions to distant metastases that suggests a proliferative and invasive advantages to those prostate tumor cells overexpressing osteopontin. Together, these findings support a strategy designed to target osteopontin in the context of prostate cancer therapy. (Cancer Res 2006; 66(2): 883-8)

Introduction

Osteopontin, an arginine-glycine-aspartic acid (RGD) containing glycosylated phosphoprotein that interacts with integrins and CD44 as major receptors, is a secreted protein comprising about 2% of the noncollageneous proteins of the bone (1, 2). It is described to be present in all body fluids and in the proteaceous matrix of mineralized tissues and has multifunctional properties in cell migration, cell survival, inhibition of calcification, and cell-mediated immunity (3). In tumorigenesis, osteopontin has been implicated in tumor invasion and metastasis in prostate, colon, breast, lung, and other cancers (4–7). The finding of a strong correlation between pathologic stage and osteopontin across multiple tumor types suggests a role for osteopontin in tumor progression (6–8). In bone, this secreted adhesive protein is believed to be involved in osteoblast differentiation and bone formation and in the anchorage of osteoclasts to bone, leading to bone resorption (3, 9, 10).

Although several studies have implicated osteopontin in prostate cancer progression and metastases, the functional significance of osteopontin expression by the prostate tumor cells is only scarcely elucidated. Chemotaxis and chemoinvasion analyses with PC-3 prostate cancer cells indicated a dose-dependent increase in PC-3 cell movement induced by osteopontin, whereas cell invasion was strictly dependent on αvβ3 integrin function (11). Osteopontin is also reported to enhance cell proliferation induced by the epidermal growth factor (EGF) in prostate cancer cells (12). In this report, we describe our studies of osteopontin expression in genetically engineered mouse models for prostatic disease, which included models displaying slow, temporal development of increasingly severe preneoplastic prostatic lesions (13, 14), and a model that progresses to primary invasive adenocarcinoma of the prostate with subsequent manifestation of metastases with defined kinetics (15, 16). We present evidence that osteopontin expression, detected in preneoplastic lesions, continues to increase in adenocarcinoma, and cancer cells exhibiting high osteopontin expression seem to be enriched in the metastatic deposits. We found that all human prostate cancer cell lines tested express osteopontin. Functional studies with manipulated overexpression of osteopontin in two prostate cancer cell lines (LNCaP and PC-3) reveal that osteopontin could lead to increased proliferation, invasion, and most remarkably, to the enhanced ability to intravasate blood vessels.

Materials and Methods

Tissue collection and RNA extraction. Five mice from each of three age groups (2.5, 12, and 18 months) of the preneoplastic ARR2PB-FgßRb transgenic mouse line (14) were selected for dissection and isolation of dorsolateral and ventral prostatic lobes. Similarly, ventral and lateral prostatic tissues were dissected and pooled from five 24-month-old preneoplastic model with conditional deletion of retinoid X receptor α (RXRα) alleles (cRXRα−/−) in the prostate (13). Littermates lacking the FgßRb transgene or the Cre gene in the context of floxed alleles of RXRα served as donors of the corresponding control tissues. The source of primary prostatic adenocarcinoma was the conditional Pten homozygous deletion (cPten−/−) mice (15). The whole prostates of two individual
experimental and age-matched control animals were used without differentiating the prostatic lobes for the comparative RNA analysis of the adenocarcinoma. RNA from Fgf8b and cPten−/− tissues were extracted using the Qiagen RNeasy Mini kit following the manufacturer's protocol, which included an on-column DNase I treatment for the removal of contaminating DNA (Qiagen, Valencia, CA). RNA from the cPten−/− tissues was extracted using TRIzol (Life Technologies, Rockville, MD).

Microarray analysis. Comparison of gene expression profiles of the preneoplastic or neoplastic mouse prostate tissues with littermate controls was carried out as previously described (15). Each experiment was done in duplicate with reversal of the fluorescent label to account for dye effects.

Reverse transcription and semiquantitative PCR for osteopontin. RNA samples from prostate tissues and prostate cancer cell lines were reverse transcribed using ThermoScript Reverse Transcription-PCR (RT-PCR) System following manufacturer's protocol (Life Technologies, Buffalo, NY) as described (14). The primer sequences (forward and reverse), annealing temperature, and product size were as follows: for mouse osteopontin, TGAAGTGACTGATGCTGGCA and GGAGCATGGGAGT-GAAAAGTGT, 52 °C; for human osteopontin, CATCTCAGAAGAC-GAATCTCCTCA and GGAAGATCTGACTACATCA, 56 °C, 617 bp. To determine the linear amplification range for each primer set, 1 μL of cDNA was amplified for 40 cycles for mouse osteopontin, 35 cycles for human osteopontin, and 30 cycles for β-actin. Samples were removed every three cycles, and the optimum cycle number was determined as the approximate midpoint of the linear range of amplification. The semiquantitative PCR assays were carried out using the corresponding optimum cycle number.

Western blot analysis. The dorsolateral, ventral, and anterior prostatic lobes of Fgf8b or cPten−/− mice and age-matched controls were isolated and snap frozen. The tissues were ground in liquid nitrogen with previously autoclaved mortars and pestles. The pulverized tissues were dissolved in ice-cold buffer containing 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 1 mmol/L EGTA, 150 mmol/L NaCl, 0.5% NP40, and 1% Triton X-100. To prepare the cell culture conditioned medium, 80% to 90% confluent cells cultured in T-75 flask were washed with PBS, and 10-mL serum-free medium was added. After 24 hours, medium was collected into a 15-mL tube, centrifuged to remove the cell debris, and then concentrated by centrifugation at 7,000 rpm at 4 °C for 30 minutes using a 20-mL Centrifugal Spin Concentrator (APOLLO, Continental Lab Products, San Diego, CA). Total tissue lysates or conditioned media were quantitated and fractionated by SDS-PAGE on a 10% gel and subjected to immunoblot analysis using a rabbit anti-mouse osteopontin antibody (Assay Desigs, Ann Arbor, MI) in 5% bovine serum albumin in 20 mmol/L Tris-buffered 300 mmol/L NaCl, pH 7.2. Primary antibodies were used in a ratio of 1:50 and amplified with the second set (GFP 2) of primers. The signal was detected by 3,3'-diaminobenzidine as a chromogen substrate, and the tissues were counterstained with hematoxylin as described (13–15).

Construction of lentiviral vector. Human osteopontin cDNA was PCR amplified with primers containing XhoI and BsrII linkers and was inserted into the polycyoning site of the transducing lentivirus vector pSIN-GFP (17, 18). Lentivirus production was achieved with the three-plasmid system. Using Superfect reagent, human 293T cells at about 80% confluency were transfected with 7.5 μg of the vesicular stomatitis virus Env-coding plasmid, pMD.G; 15 μg of the packaging plasmid, pCMVΔ8.91; and 15 μg of either the control vector pSIN-GFP or the transgene vector pSIN-GFP-osteopontin. The media containing the pseudotyped lentiviruses were harvested daily from the 3rd to 5th day after transfection.

Infection and cell sorting. Immortalized human prostate epithelial cell lines, LNCaP and PC-3, were cultured as previously described (18). At 80% confluence, the cells were inoculated with 1 mL of the conditioned medium containing lentiviruses in the presence of 5 μg/mL polybrene for 8 hours. The cells were sorted by flow cytometry based on green fluorescent protein (GFP) fluorescence 2 days after infection.

Proliferation assay. To evaluate cellular growth, 5 × 105 GFP vector or osteopontin-GFP-transfected cells were plated in 60-mm dishes in triplicates and grown with full serum medium. The cells were counted every 2 days with the Coulter Counter (Beckman Coulter, Inc., Miami, FL). The medium was changed every 2 days.

Invasion assay. Matrigel invasion assays were done with transfected prostate cancer cells. The upper chamber of the 8.0-μm inserts with polyethylene terephthalate membrane was coated with Matrigel from BD Biosciences (Bedford, MA), and the lower chamber was filled with full serum medium. Following a 24-hour pretreatment in medium containing 0.5% serum in the presence or absence of 5 μg/mL osteopontin antibody (R&D Systems, Minneapolis, MN), the cells (105) were added to the upper chamber, correspondingly with or without 5 μg/mL osteopontin antibody, and incubated at 37 °C for 24 hours. Invasion of the cells through the membrane was detected by staining with hematoxylin and counted as previously described (18, 19).

Statistical analysis. All experiments were done in triplicates and repeated at least twice. Statistical comparisons were made using an unpaired, two-tailed t test.

Results

Analysis of osteopontin expression in prostatic lesions. Clues for consistent transcriptional alterations of osteopontin in the mouse prostatic lesions were initially obtained from the analyses of prostate gene expression profiles from three genetically engineered mouse models (Table 1). Although there was no significant increase in osteopontin gene expression in ventral or dorsolateral prostate of the Fgf8b mice relative to littermate controls at 2.5 months of age, the increase was clearly evident with the tissues obtained from the 12- and 18-month-old animals. This apparent 3- to 6-fold elevation of osteopontin RNA correlated with the temporal development of preneoplastic lesions in this transgenic model (14). Prostatic intraepithelial neoplasia (PIN) lesions, not seen at 2.5 months, were mostly low grade at 12 months and then turning to an abundant combination of low-grade PIN (LGPIN) and...
high-grade PIN (HGPIN) with further advancing of age (14, 22). When compared with the Fgf8b transgenic mice, the incidence of PIN lesions, especially HGPIN, was found to be significantly less in cRXRx−/− mice (13, 22). Accordingly, prostate tissues from cRXRx−/− mice were examined at 24 months of age, after the onset of HGPIN. Although not as remarkable of an increase as seen in the Fgf8b mice, there was also a noticeable elevation in osteopontin mRNA levels in the cRXRx−/− ventral and lateral prostate relative to the age-matched controls. Recognizing that invasive adenocarcinoma of the prostate would have 100% penetrance in mice by 6 months of age, we used this age group for comparative analyses, there was no remarkable difference in osteopontin levels between control and experimental preneoplastic tissues from controls. In contrast, but consistent with microarray and RT-PCR findings, we also examined different lobes of one to two osteopontin protein bands at around 65 kDa in the pool dorsolateral prostate; LP, lateral prostate.

NOTE: Anterior prostatic lobes from Fgf8b and cRXRx−/− mice were also tested. The results with these tissues did not exhibit a significant difference. It should be noted that the anterior prostatic lobe was found to have the lowest gene expression driven by the ARR2PB promoter (22, 27) corresponding to lowest prevalence of preneoplastic lesions in the models (13, 14).

Abbreviations: AP, anterior prostate; VP, ventral prostate; DLP, dorsolateral prostate; LP, lateral prostate.

Table 1. Mouse prostatic tissues evaluated by microarray and osteopontin expression

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Age (mo)</th>
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Table 1. Mouse prostatic tissues evaluated by microarray and osteopontin expression

Significance of Osteopontin in Prostate Cancer

Immunohistochemical staining for osteopontin was done on paraffin-embedded prostate tissues to determine the area of osteopontin signal localization. Each transgenic model was tested at different time points during tumorigenesis for the expression of osteopontin in LGPIN and HGPIN lesions in Fgf8b, cRXRx−/−, and cPten−/− mice, and primary adenocarcinoma and metastatic lesions in cPten−/− model. It was found that the osteopontin signal was mainly localized to the cytoplasm of prostate epithelial cells similar to such immunostaining observed in lung cancer cells (24).

Some reactivity was also detected in the inflammatory cells, consistent with the known expression of osteopontin in activated immune cells (3, 25). As illustrated by the representative immunostaining photomicrographs (Fig. 2) for which the H&E staining of the corresponding sections is included in the Supplementary Fig. S1A-D, osteopontin signal greater than the background level was generally associated with the development of prostatic lesions in all three transgenic mouse models. Variations in signal intensity were, however, noted among cells and lesions. The increased osteopontin signal in dysplastic epithelia of LGPIN lesions (Fig. 2A) of Fgf8b line became more prominent in HPGIN lesions (Fig. 2B). Clearly, in contrast to the adjacent normal prostate epithelium, most dysplastic cells in the LGPIN or HGPIN lesions exhibited considerably stronger osteopontin staining. The findings were similar with these preneoplastic lesions of cRXRx−/− mice.

The pattern of osteopontin expression was examined at various stages of prostatic tumorigenesis and metastasis in the cPten−/−/C0 Fgf8b of 5.5-month-old mice. Osteopontin is detected at around 65 kDa.

Figure 1. Western blot analysis of osteopontin (OPN) overexpression in prostatic and neoplastic prostatic tissues. A, osteopontin detection in 5.5-month-old Fgf8b transgenic and littermate control animals. Transgenics animals (E) and control animals (C). Osteopontin is detected at around 65 kDa. Bottom, β-actin, which served as an internal control for normalization. Overall, there was −3- to 4-fold increase in osteopontin expression in the ventral (VP) as well as dorsolateral (DLP) lobes of the transgenic mice compared with their control counterparts. B, Western blot analysis of proteins extracted from the anterior prostate (AP) of the cPten−/− (E) mice and their littermate controls (C) at different ages as indicated in months (M). C, proteins from individual lobes (dorsolateral, ventral, and anterior) from a 13-month-old cPten−/− (E) mouse and its littermate control (C) were analyzed by Western blot.
immunostaining of these dysplastic cells. The HGPIN lesion from the cell line was infected with lentivirions, which carried GFP or both.

To confirm the enhanced growth and invasive ability of the transfected cells in vivo, the intravasation assay based on the chorioallantoic membrane of the chicken egg was done. Chicken embryos at day 9 of gestation were inoculated with one million cells and incubated for 24, 48, and 72 hours. The GFP vector control LNCaP cells failed to intravasate even after 72 hours of incubation as previously reported (20). However, the presence of the osteopontin-transduced LNCaP cells in the lower chorioallantoic membrane could be readily detected by nested PCR for GFP and confirmed by PCR for Alu at time period of 48 or 72 but not 24 hours (Fig. 5C). Although both vector and osteopontin-transduced PC-3 cells were detected in the lower chorioallantoic membrane at the metastasis was verified by staining for expression of the androgen receptor. Although there were some variations in osteopontin staining intensity among individual cells, the majority of the metastasized cancer cells displayed robust immunoreactivity that set them apart from the background.

Biological effect of osteopontin overexpression in human prostate cancer cells. The expression of osteopontin was assessed in five human prostate cancer cell lines (PC-3, PC-3M, DU145, LNCaP, and CWR22R) and one nonneoplastic prostatic epithelial cell line (BPH-1) by semiquantitative RT-PCR. All of these cell lines expressed variable levels of osteopontin (Supplementary Fig. S2A). For the detection of secreted osteopontin protein in the cell culture medium, we used conditioned medium from some of the cell lines (BPH-1, PC-3, DU145, and LNCaP) for Western blot analyses. As shown in Fig. 4A, whereas all of the tested cell lines were found to produce osteopontin, the levels of mRNA and secreted protein, however, did not exhibit a strong correlation. Because it seemed that compared with the LNCaP cells, PC-3 cells expressed a higher level of protein for osteopontin, LNCaP and PC-3 were selected for studies of the biological effect of osteopontin overexpression. Each cell line was infected with lentivirions, which carried GFP or both osteopontin- and GFP-transducing genes. As previously described (17, 18), the transfected cells were sorted by fluorescence-activated cell sorting based on GFP fluorescence. The newly established cell lines were examined for osteopontin overexpression by RT-PCR (Supplementary Fig. S2B) with RNA prepared from cell extracts as well as by Western blot analysis of conditioned media (Fig. 4B).

The effect of osteopontin overexpression on cellular growth was assessed by a proliferation assay. Transfected LNCaP cells were grown in the presence of full serum over a course of 8 days. Compared with the GFP control, osteopontin-transduced LNCaP cells exhibited a strong proliferative advantage (Fig. 4C). The effect on proliferation was much less pronounced on the PC-3 cells (Fig. 4D), which already contained a higher endogenous osteopontin expression relative to the LNCaP cells. LNCaP and PC-3 cells with overexpression of osteopontin were also examined in a Matrigel invasion assay. The results showed a drastic enhancement of the invasion ability for LNCaP and a less pronounced but still significant effect on PC-3 when the cells were manipulated to express higher osteopontin levels. Furthermore, although the LNCaP and PC-3 cell lines are of different origin, it was remarkable to find that the response to osteopontin overexpression of each could be significantly suppressed by the presence of anti-osteopontin antibodies in the invasion assays (Fig. 5A and B).
Figure 4. Detection, overexpression, and functional effects of osteopontin in prostate tumor cells. A, conditioned medium from some of the cell lines was examined for osteopontin protein expression by Western blots. B, Western blots of osteopontin with conditioned media from LNCaP/GFP (lane 1) and LNCaP/osteopontin (lane 2). C, proliferation of LNCaP/GFP and LNCaP/osteopontin cells in full serum medium was determined by counting the cell every 2 days using Coulter counter. Cells (10^5) were plated in each well in triplicates. D, analysis of growth of PC-3/GFP and PC-3/osteopontin cells as in (C). * P < 0.001, differences in the growth between control and osteopontin-overexpressing cells were significant in both (C) and (D).

Discussion

While conducting cDNA microarray assays for differentially expressed genes in the prostatic lesions of genetically engineered mouse models, we identified osteopontin as a gene of interest. It is particularly noteworthy that in these models, whether Fgfb trangenic (14) or cR xR^-/- (13) preneoplastic disease, or the Cten^-/- neoplastic disease system, we found significant up-regulation of osteopontin RNA and protein levels in all, relative to the corresponding littermate controls. We attempted to localize the overexpression of osteopontin in the prostatic lesions by immunohistochemistry. The increased intensity of osteopontin staining readily visible in many of the dysplastic epithelial cells of LGPIN lesions seemed to become more prominent in HGPIN. Relative to these preneoplastic lesions of all three models, significantly higher staining was observed in the primary adenocarcinoma that developed in the Cten^-/- model. When the metastatic lesions in the Cten^-/- mice were examined, the intensity of staining seemed to be even higher. Together, the results imply that up-regulation of osteopontin expression in prostatic lesions is consistent in all three models and independent of how the models were generated.

Although osteopontin is described to be a marker for the late stages of progression of various cancers (6, 7), our results which were not conflicting, do however, point to osteopontin dysregulation beginning at a much earlier time point (e.g., at LGPINs). With advancing time, osteopontin levels seem to continue increasing with progression from LGPIN to HGPIN to adenocarcinoma, and most remarkably, the cancer cells expressing the highest levels of osteopontin seem to be selected during metastatic progression.

Our results indicate that osteopontin contributes to several steps in the process of prostate carcinogenesis and metastasis. Osteopontin seems to modulate cell proliferation and potentially the survival of the dysplastic and neoplastic prostatic cells, thus providing a selective advantage in early-stage lesions. The findings with manipulated overexpression in human prostate cancer cells as well as those of other published reports (11, 12) lend support to an autocrine effect of osteopontin overproduction on cell proliferation. This is shown with the LNCaP cells transduced with osteopontin expressing lentivirus vector. This effect was less pronounced on the PC-3 cells. PC-3 cells, however, are by nature, highly proliferative. We also used in vitro Matrigel invasion assay to assess the invasiveness of the cells. Although the control LNCaP cells were completely incapable of penetrating the membrane, there was a drastic enhancement in the invasive ability when osteopontin was overexpressed. A similar pattern, albeit relatively less pronounced, was produced by osteopontin overexpression in

Figure 5. Matrigel and chorioallantoic membrane assays with osteopontin (OPN)–transduced LNCaP and PC-3 cells. A, in the Matrigel invasion assay, while LNCaP/GFP failed to penetrate the matrix, LNCaP/osteopontin displayed a strong invasiveness. This invasiveness could be significantly blocked by anti-osteopontin antibodies. B, PC-3/osteopontin demonstrated a 2-fold increase in invasive ability compared with PC-3/GFP. This bioactivity was also suppressed significantly by anti-osteopontin antibodies. C, nested PCR amplification for GFP using DNA extracts from the isolated lower chorioallantoic membrane. The experiment was repeated at least five times. The reproducibility of the assay is shown in a second experiment (Exp. 2) followed by a subsequent PCR amplification for Alu for confirmation of the data obtained from the GFP PCR. D, analysis of invasiveness in transduced PC-3 cells as in (C).
PC-3 cells in which, besides osteopontin, multiple other factors may be contributing to its naturally highly invasive character. The fact that osteopontin could be an important player is further shown by the ability of anti-osteopontin antibodies to significantly neutralize this biological response induced by osteopontin overexpression.

Considering that intravasation is an early required event for the multistep process leading to metastasis, we also checked a potential role of osteopontin in intravasation in vivo. A model system, first developed by Kim et al. (20) and based on blood vessel penetration of xenotransplanted mammalian cancer cells on the chicken embryo chorioallantoic membrane assay, was used. Consistent with published work (20), PC-3 cells but not LNCaP cells were determined to be capable of intravasation in the chorioallantoic membrane model using qualitative PCR-based assays. Importantly, analysis of osteopontin-transduced LNCaP cells revealed that overexpression of osteopontin alone was sufficient to induce the ability to intravasate. The change in invasive ability in vivo, potentiated by the excess osteopontin production, was less pronounced in PC-3 cells compared with LNCaP. In PC-3 cells, however, higher osteopontin expression seems to affect the kinetics of intravasation. The change in invasive ability overexpression of osteopontin alone was sufficient to induce the ability to intravasate. The change in invasive ability in vivo, potentiated by the excess osteopontin production, was less pronounced in PC-3 cells compared with LNCaP. In PC-3 cells, however, higher osteopontin expression seems to affect the kinetics of intravasation overexpression of osteopontin alone was sufficient to induce the ability to intravasate. The change in invasive ability in vivo, potentiated by the excess osteopontin production, was less pronounced in PC-3 cells compared with LNCaP. In PC-3 cells, however, higher osteopontin expression seems to affect the kinetics of intravasation overexpression of osteopontin alone was sufficient to induce the ability to intravasate. The change in invasive ability in vivo, potentiated by the excess osteopontin production, was less pronounced in PC-3 cells compared with LNCaP. In PC-3 cells, however, higher osteopontin expression seems to affect the kinetics of intravasation.
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