Increased Expression of Osteopontin Contributes to the Progression of Prostate Cancer

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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 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. Osteopontin also influenced their in vitro invasive ability, and again, most strikingly in the weakly oncogenic 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 proteinaceous 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 $\alpha_\text{v}\beta_3$ 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-Fg/f8b 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 $\alpha$ (RXR$\alpha$) alleles (cRXR$\alpha$$^{-/-}$) in the prostate (13). Littermates lacking the Fg/f8b transgene or the Cre gene in the context of floxed alleles of RXR$\alpha$ 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 \( \text{Fgfb} \) and \( \text{cPen} \) 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 \( \text{cPen} \) 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, TGAAGTGCTAGTATCTTTGCA and GGACCATTGGAGT-GAAAATGT, 52°C; for human osteopontin, CATCTCGAAAGC-GAATCTCCTA and GGAAGGTCCTCAGACTAATCA, 56°C, 617 bp. To determine the linear amplification range for each primer set, 1 \( \mu \text{l} \) of cDNA was amplified for 40 cycles for mouse osteopontin, 35 cycles for human osteopontin, and 30 cycles for \( \beta \)-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 \( \text{Fgfb} \) or \( \text{cPen} \) 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% NP-40, 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 centrifuging 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. The western blot analysis for the \( \text{Fgfb} \) or \( \text{cPen} \) set was done as previously described (18), whereas osteopontin expression in the \( \text{cPen} \) set was detected with a fluorescein-conjugated secondary antibody and the Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE). To normalize sample loading, \( \beta \)-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) blot was done.

Immunohistochemistry. Prostate tissues were isolated and fixed in 10% buffered formalin. Following deparaffinization, the 5-μm tissue sections were rehydrated and subjected to antigen retrieval by microwaving in 0.1 mol/L sodium citrate (pH 6). Antigen unmasking was done 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. The western blot analysis for the \( \text{Fgfb} \) or \( \text{cPen} \) set was done as previously described (18), whereas osteopontin expression in the \( \text{cPen} \) set was detected with a fluorescein-conjugated secondary antibody and the Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE). To normalize sample loading, \( \beta \)-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) blot was done.

Infection and cell sorting. Immortalized human prostate epithelial cell lines, LNCaP and PC-3, were cultured as previously described (18). At 80% confluency, 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, \( 5 \times 10^5 \) 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 80-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).

Intravasation assay. The intravesical potential of the transfected prostate cancer cells was assessed by a PCR-based assay (20). Longitudinally incubated in a rotating incubator, chicken embryos at 9 days of gestation were selected for introducing the artificial air sac and subsequently "dropping" the chorioallantoic membrane (21). Briefly, air was suctioned through a small puncture in the side of the egg to facilitate the detachment of the chorioallantoic membrane from the shell membrane. Avoiding major blood vessels, a 1-cm² window was cut on the top surface, and the suspension of cancer cells was gently applied to the chorioallantoic membrane. Upon incubation at 37°C for 24, 48, and 72 hours, the lower chorioallantoic membrane was removed and snap frozen in liquid nitrogen. DNA was extracted using Puregene DNA extraction kit from Gentra Systems (Minneapolis, MN) following manufacturer's protocol. The samples were used in a subsequent nested PCR amplification for the GFP gene to confirm the presence of the cancer cells in the lower chorioallantoic membrane. The initial PCR products produced with GFP 1 primer set were diluted at a ratio of 1:50 and amplified with the second set (GFP 2) of primers. The experiments were repeated and confirmed with PCR amplification for \( Alu \) as previously described (20). The primer sequences, annealing temperatures, and product sizes were as follows: for GFP 1, CGAGCTAAAGGGCACAATG and GTGCTCTGATGTGTTGTGTCG, 62°C, 550 bp; for GFP 2, TACC-GAAGCTGACCTTGAAG and TGATAATGCTTTGCTTTGACTGTAG, 62°C, 343 bp; and for \( Alu \), ACCGCGTATTCCAGCAGCATT and TCGCCAGCGCTGGATTGCA, 58°C, 224 bp.

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 \( \text{Fgfb} \) 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
Significance of Osteopontin in Prostate Cancer

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

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<th>Age (mo)</th>
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| cRXR 


NOTE: Anterior prostatic lobes from Fgf8b and cRXR 


mice were also tested. The results with these tissues did not exhibit a significant differential. 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.

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 cRXR 


mice (13, 22). Accordingly, prostate tissues from cRXR 


were examined at 24 months of age, after the onset of HGPIN. Although not as remarkable as an increase as seen in the Fgf8b mice, there was also a noticeable elevation in osteopontin mRNA levels in the cRXR 


ventral and lateral prostate relative to the age-matched controls. Recognizing that invasive adenocarcinoma of the prostate would have 100% penetrance in cPten 


mice by 6 months of age, we used this age group for comparative microarray analysis (15). Compared with normal prostates, tumor-bearing prostates exhibited a 3.2-fold increase in osteopontin mRNA levels.

We used a modified semiquantitative RT-PCR method (23) to first obtain a confirmation of the microarray data (data not shown). With results supporting a correlation, tissue lysates from different prostatic lobes were subjected to Western blot analysis for osteopontin protein expression. The molecular size of osteopontin protein is known to be variable ranging between 41 and 75 kDa because of alternative RNA splicing, posttranslational modifications, and proteolytic cleavages in cell type–specific manner (7). For Fgf8b, four mice at 5.5 months of age were dissected to obtain pooled dorsolateral, ventral, and anterior prostatic lobes. As illustrated in Fig. 1A, there was ~3- to 4-fold increase in the detection of one to two osteopontin protein bands at around 65 kDa in the dorsolateral and ventral prostate compared with their age-matched controls. In contrast, but consistent with microarray and RT-PCR analyses, there was no remarkable difference in osteopontin levels between control and experimental preneoplastic tissues from the anterior prostate. Similarly, prostatic lobes from the cPten 


mice between 1.6 and 14 months of age were analyzed. As shown in Fig. 1B, the development of primary prostate tumors in the cPten 


mouse was associated with progressively increased expression of osteopontin protein over an age range of 3.0 to 11.0 months. From a 13-month-old animal, we also examined different lobes for osteopontin expression. The content of osteopontin in all the lobes was elevated in the cPten 


mouse compared with the corresponding lobes of its age-matched control (Fig. 1C). The relative increase seemed to be at least 10-fold when the intensity of the protein bands detected at 65 to 70 kDa were compared with corresponding controls.

Localization of osteopontin expression in prostatic lesions. 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, cRXR 


, 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 prostatic 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 prostatic epithelium, most dysplastic cells in the LGPIN or HGPIN lesions exhibited considerably stronger osteopontin staining. The findings were similar with these preneoplastic lesions of cRXR 


mice.

The pattern of osteopontin expression was examined at various stages of prostatic tumorigenesis and metastasis in the cPten 


Figure 1. Western blot analysis of osteopontin (OPN) overexpression in preneoplastic and neoplastic prostatic tissues. A, osteopontin detection in 5.5-month-old Fgf8b transgenic and littermate control animals. Transgenics (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 


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 


mouse and its littermate control (C) were analyzed by Western blot.
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).

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

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 model. LGPIN and HGPIN lesions displayed a pattern of increased osteopontin expression similar to that found in Fgfbp and cRxRtx1−/− mice. An example of HGPIN is shown in Fig. 2C. The trend of increase in the intensity of osteopontin staining with further progression of the disease was noted in the primary adenocarcinoma. This is illustrated with a case of tumor characterized by local microinvasion (Fig. 2D). In addition, examination of metastatic deposits in the lung found elevated osteopontin expression relative to the primary prostatic lesions (Fig. 3). The prostatic origin of 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.

Figure 2. Immunohistochemical analysis of osteopontin in prostatic preneoplastic and neoplastic lesions. A, anti-osteopontin staining of a LGPIN lesion in an Fgfbp mouse illustrates that the increased intensity of osteopontin signal (arrow) is localized to the dysplastic cells compared with the minimal staining of adjacent normal epithelium (▲). Some inflammatory cells that stained positive for osteopontin were noted (*). B, anti-osteopontin immunostaining of a HGPIN lesion in the lateral prostatic lobe of an Fgfbp animal. The intensity of osteopontin signal in the dysplastic cells (arrows) is significantly higher than that of the normal cells (▲). Inset, high-power examination of osteopontin immunostaining of these dysplastic cells. C, anti-osteopontin immunostaining of the HGPIN lesion from the cPtenc−/− model. The signals are clearly shown to localize in the cytoplasm of the several atypical cells. D, anti-osteopontin immunostaining of an adenocarcinoma with local invasion in the lateral prostate of cPtenc−/− mouse. The strong signal, localized in the cytoplasm, outlined invasive cancer cells. Bar, 25 μm (A), 100 μm (B), and 10 μm (C and D).

Figure 3. Osteopontin staining of lung metastases in a cPtenc−/− mouse. A, H&E staining of a metastatic lesion displaying the localization of cancer cells to the mesenchyme of the lung tissue, adjacent to a blood vessel. Inset: these cancer cells stained positive with anti-AR antibody, confirming their prostatic origin. B, anti-osteopontin staining of metastatic cancer cells in (A). These positively stained cancer cells (arrow) can be clearly differentiated from the lung tissue. C, two other foci of osteopontin-positive cancer cells with stronger intensity in osteopontin signal. D, a lymphovascular cluster of osteopontin-positive metastatic cells. Inset: H&E staining of the cluster. Bar, 10 μm.
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 cells every 2 days using Coulter counter. Cells (10⁵) 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 Fgf8b transgenic (14) or cRXRα–/– (13) preneoplastic disease, or the cPten–/– (15) 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 was 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 cPten–/– model. When the metastatic lesions in the cPten–/– 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
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 mammary 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 apparently by accelerating the rate at which the cells access the blood vessels, as evident from the reduced time required for detectable intravasation from 48 to 24 hours of inoculation. An important question at this point is how osteopontin might be involved in facilitating tumor cell invasion. Osteopontin binds with several integrins and CD44 variants in both RGD sequence-dependent and sequence-independent manner (3). The resulting signal transduction pathways that may be activated by osteopontin are complex by nature and only poorly understood. There is, however, some relevant emerging information in this regard. For example, it has been shown that osteopontin induces activator protein (AP-1) transactivation in breast cancer cells through \( \alpha_\beta_3 \) integrin–mediated c-Src kinase activity and EGF receptor (EGFR) phosphorylation, c-Src kinase being required for osteopontin-induced EGFR phosphorylation (26). AP-1 is then linked to urokinase-type plasminogen activator (uPA) production and secretion that results in stimulation of cell motility and invasion. In other work, osteopontin has been shown to stimulate LNCaP proliferation in serum-free medium but only in the presence of EGF (12). The induced proliferation is accompanied by a sustained activation of EGFR. It is also noteworthy that previous studies using the chorioallantoic membrane model showed that breaching of the vascular wall by the cancer cells is a rate-limiting step for intravasation and that cooperation between uPA/uPA receptor (uPAR) and matrix metalloproteinases (MMP) is required to complete this step (20). Thus, crucial molecules, such as uPA, uPAR, and activated MMPs, await further studies in relation to osteopontin overexpression in prostatic cancer in the context of breaching native biological barriers preventing cancer cell metastasis.

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