Published in final edited form as:

Exp Cell Res. 2010 January 1; 316(1): 1–11. doi:10.1016/j.yexcr.2009.10.012.

Osteopontin Induces β -Catenin Signaling Through Activation of Akt In Prostate Cancer Cells

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Abstract

Secretion of Osteopontin (OPN) by cancer cells is a known mediator of tumorigenesis and cancer progression in both experimental and clinical studies. Our work demonstrates that OPN can activate Akt, an important step in cancer progression. Both ILK and PI3-K are integral proteins in the OPN/ Akt pathway, as inhibition of either kinase leads to a loss of OPN- mediated Akt activation. Subsequent to OPN-induced Akt activation, we observe inactivation of GSK3 β , a regulator of β -Catenin. Osteopontin stimulation leads to an overall increase in β -Catenin protein levels with a resultant transfer of β -Catenin to the nucleus. Through the nuclear import of β -Catenin, OPN increases both the transcription and protein levels of MMP-7 and CD44, which are known TCF/LEF transcription targets. This work describes an important aspect of cancer progression induced by OPN.

Keywords

Osteopontin; Akt; β -Catenin; TCF/LEF; ILK; PI3-kinase

Introduction

Prostate cancer is the second leading cause of cancer death in men. Bone is a common site for prostate cancer to metastasize. It has been suggested that bone-matrix associated proteins may allow these cancer cells to gain survival advantages, which eventually become androgen-independent and metastatic. Osteopontin (OPN) is an important autocrine and paracrine factor in the growth and behavior of prostate carcinoma cells [1,2]. OPN-associated androgen-independent growth of prostate cancer cells commonly occurs in advanced prostate cancer [2,3].

OPN is an extracellular matrix phosphoprotein secreted by a number of cell types and implicated in a variety of biological functions including cell adhesion, migration, immune responses, bone calcification, and tumor progression [4,5]. OPN mediates biological function through signal transduction by binding to integrins and the CD44 receptor [6-8]. A number of extracellular proteins including OPN, bone sialoprotein, and vitronectin contain a conserved

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arginine-glycine-aspartic acid (RGD) amino acid sequence required and sufficient for initiating cell signaling events upon integrin binding [9,10]. The CD44 family of receptors mediates cellular responses in a manner similar to that of integrins, including adhesion and migration of both cancerous and non-cancerous cells [11,12].

Many kinases have been implicated in cancer progression including Akt, PI3-kinase, and integrin-linked kinase (ILK); their activation induces a network of cell signaling, which ultimately leads to a proliferative advantage among cancerous cells [13-16]. Akt is a multifaceted serine-threonine kinase whose downstream targets have been shown to regulate cell growth, proliferation, glucose metabolism, angiogenesis, and resistance to apoptosis [17-19]. A key upstream component of the Akt pathway is PI3-kinase. PI3-kinase synthesizes the second messenger phosphatidylinositol 3, 4, 5-trisphosphate (PIP3) by phosphorylation of the integral membrane lipid phosphatidylinositol 4, 5-bisphosphate (PIP2). PIP3 in turn, directly binds the pleckstrin-homology (PH) domain of proteins such as, Akt and phosphoinositide-dependent kinase (PDK1). The recruitment of Akt and PDK1 to the plasma membrane occurs through the binding of their pleckstrin homology (PH) domain to PIP3 [19].

Akt has been shown to regulate many cellular processes in addition to its anti-apoptotic role. Akt regulates the activity of glycogen synthase kinase 3-beta (GSK-3 β) via phosphorylation of the serine-9 residue, resulting in its inactivation [20]. GSK-3 β has been shown to promote degradation of β -Catenin through N-terminal phosphorylation on serine-33, serine-37 and threonine-41 leading to β -Catenin ubiquitination, and subsequent targeting to the proteasome where it is degraded [21].

In prostate cancer, β -Catenin levels have been shown to correlate with disease progression [22]. β -Catenin is a multifunctional protein, which plays roles in both cadherin-mediated adhesion and the Wnt signaling cascade through its domain consisting of 12 armadillo repeats which enable multiple protein interactions including binding to TCF/LEF, APC, E-cadherin and α -Catenin [23,24]. Upon release from the cadherin complex, β -Catenin has been shown to be a transcriptional regulator [25]. β -Catenin translocates to the nucleus functioning as a cofactor for the transcription of the T-cell factor/lymphoid enhancing factor (TCF/LEF) family of genes [26,27]. The β -Catenin/TCF transcription complex has been shown to be involved in the up-regulation of many target genes, including but not limited to CD44, cyclin D1, and c-Myc [28,29].

Here, we show that OPN promotes prostate cancer cell progression processes by activating the Akt/ β -Catenin pathway. Our findings show that the OPN/Akt pathway is required for the stabilization of β -Catenin. Here, we also show that OPN induces nuclear localization of β -Catenin resulting in the activation of TCF/LEF family of transcription factors.

Materials and Methods

Materials

Horse radish peroxidase-conjugated secondary antibodies for immunoblotting were obtained from Amersham Biosciences (Pittsburgh, PA). GAPDH, actin, CD44, MMP-7, nucleoporin p62, cyclin D1, and c-Myc antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Akt inhibitor (Cat#124005) was purchased from Calbiochem (LaJolla, CA). Protein estimation reagent, molecular weight standards for proteins, and polyacrylamide solutions were purchased from Bio-Rad (Hercules, CA). Rhodamine phalloidin, DAPI, and wortmannin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). OPN antibody was purchased from Rockland Immunochemicals (Gilbertsville, PA). Complete mini protease inhibitor tablet was purchased from Roche Applied Science (Indianapolis, IN).

Phospho-Akt (serine 473), β -Catenin, Alexa Fluor® 488 conjugated β -Catenin (mouse), phospho- β -Catenin (serine 552), GSK-3 β , and phospho-GSK-3 β (serine-9) antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Purified GSK-3 β was purchased from Cell Signaling Technologies (Danvers, MA). ECL- reagent was purchased from Pierce (Rockford, IL).

Cell culture and Treatments

Prostate cancer epithelial cells (PC3, DU145, and LNCaP) were previously purchased from the ATCC (Manassas, VA). The cDNA for osteopontin (OPN) was provided by Dr. Kiefer (Chiron Corporation, Emeryville, CA). Prostate cancer epithelial cells (PC3) were transfected with OPN cDNA constructs in the pCEP4 vector and stable cells were generated as described previously [30]. PC3 cell lines were designated as PC3 (empty pCEP4 vector transfected cells), PC3/OPN (wild type OPN transfected cells). Soluble human OPN protein was purified as described previously [31].

Cancer cell lines were cultured at 37° C in Roswell Park Memorial Institute-1640 (RPMI-1640) (GIBCO-BRL, Bethesda MA) media containing 10% Fetal Bovine Serum (FBS) (GIBCO-BRL, Bethesda MA) and 1% Penicillin-Streptomycin. Upon reaching 100% confluency, cells were replated in tissue culture dishes according to the experimental plan.

Immunoprecipitation and Western Analysis

Equal concentration of lysate proteins were precleared with non-immune IgG coupled to Sepharose. The precleared supernatants were incubated with antibodies of interest for 16hr and the immune complexes were adsorbed onto protein A-Sepharose beads for 4hr. The beads were pelleted at 4000rpm for 5min in an Eppendorf 5417R desk top centrifuge at 4°C and washed three times with ice-cold PBS. The immune complexes were then boiled in sample buffer and subjected to SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane (PVDF) for Western analyses. Blots were blocked with 5% milk in PBS containing 0.5% Tween (PBS-T) for 1hr and then incubated with a primary antibody of interest (1:1000 dilutions) for 16hr. After three washes for 10min each with PBS-T, the blot was incubated with a species-specific secondary antibody conjugated to HRP (1:4000 dilutions) for 2hr at room temperature. After three washes for 10min each with PBS-T, protein bands were visualized by chemiluminescence using an ECL kit. In order to use the blot for normalization with GAPDH or Akt antibody or probe for another protein blots were stripped at 50°C for 15–30min. in a stripping buffer containing 2% SDS, 100mM beta-mercaptoethanol, and 50mM Tris-HCl, pH6.8. Blots were tested for the completion of removal of the immunodetection reagents from the previous analysis stripping prior to the second analysis with another antibody of interest.

Immunostaining

PC3 and PC3/OPN cells were cultured onto coverslips in a 12-well dish overnight at 37°C. Cells were washed three times with PBS at room temperature (RT) and fixed in 4% formaldehyde-PBS for 10min. After washing three times with PBS, cells were permeabilized with 0.5% Triton X-PBS for 10min. Cells were washed three times with PBS, followed by incubation in 5% boiled goat serum for 1 h at RT. After washing three times with PBS, cells were incubated with a 1:100 dilution of primary antibody in 5% boiled goat serum overnight at 4°C. Cells were washed three times with PBS and were then incubated for 3hr at RT with a primary antibody of interest in appropriate dilutions as per the instructions provided by the manufacturers. Cells were washed three times with PBS for 15min each and incubated with species specific secondary antibody (Cy2 or CY3 conjugated) and DAPI to stain nucleus for 15–30min in the dark. Some cover slips were stained with rhodamine phalloidin to stain actin filaments. After washing an additional three times with PBS for 5min each, the cover slip with cell side was then placed onto the drop of perma fluor mounting medium (Thermo Scientific,

Pittsburgh, PA) and allowed to set overnight in the dark. A Bio-Rad 6000 (Hercules, CA) confocal microscope was used to visualize the immunofluoresced cells. Images were stored in TIF image format and processed by the Adobe Photoshop software program (Adobe Systems, Inc., Mountain View, CA).

Cytoplasmic and Nuclear Protein Extraction

ProteoJET Cytoplasmic and Nuclear Extraction kit was purchased from Fermentas Life Sciences (Glen Burnie, MD) and performed as per the manufacturer recommendations. Briefly, cells were lysed with the lysis buffer (provided in the kit) containing protease inhibitor and 0.1M DTT. Protein concentration was quantified using the Bradford method and equal amount of protein was centrifuged at 500 X g to separate the nuclear fraction from the supernatant. The supernatant was centrifuged at 20,000 X g for 15min. at 4°C. The 20,000 X g supernatant and the 500 X g pellet were used as cytosolic and nuclear fractions respectively. Nuclei pellet was washed with the nuclei wash buffer provided in the kit and lysed in the nuclei lysis buffer containing protease inhibitor and DTT. Nuclear lysates was centrifuged at 20,000 X g for 5min at 4°C to obtain clear nuclear fractions. Nuclear and cytosolic fractions were subjected to protein estimation and equal amount of protein was used for immunoblotting analyses shown in Figure 5.

TCF/LEF assay

TCF/LEF Signal Reporter Assay Kit was purchased from Super Array (Frederick, MD). Experiments were performed in six well dishes; both stable cell lines and transient transfections were used. For transient transfection, equal numbers of cells were transfected with Mirus TransIt Kertinocyte transfection reagent (Madison, WI) for 72hr (see below). Stable cell lines were plated at equal densities for 48hr when performing the assay. Prior to obtaining luciferase values, the cells were lysed, the total proteins in the lysates were quantified, and equal amounts of protein lysate were added to each tube and brought to equal volumes. Reporter Assay results were obtained using the Promega Dual Luciferase Reporter Assay kit (Madison, WI) on a single tube luminometer.

Integrin- linked kinase assay In Vitro

Cells were collected, lysed and quantified using the Bradford method. 200µg of cell lysates were treated with 4µg of anti-ILK bound to protein A Sepharose. Non-phosphorylated GSK3-β (1µg) was used as an exogenous substrate in the kinase assay as described previously [32]. The levels of GSK3-β phosphorylated on serine 9 of GSK3-β and total GSK3-β were determined by immunoblotting analysis.

TUNEL apoptosis assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit was purchased from Phoenix Flow Systems (San Diego, California). Cells were plated in equal numbers and allowed to attach, Cells were kept in serum free media for 24hr prior to TUNEL assay. TUNEL assay was performed as the manufacture recommends. TUNEL results were quantified through fluorescent microscopic analysis, strictly adhering to identical exposure settings between different samples.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from PC3 and PC3/OPN cells, using RNeasy Midi kit (Qiagen, Valencia, CA). RNA was converted to cDNA using Superscript II RNase H Reverse Transcriptase kit (Invitrogen Life Sciences). Using the primer sets shown below and cDNA generated from PC3 and PC3/OPN cells, RT-PCR was performed by utilizing Accuprime DNA Taq polymerase high fidelity kit (Invitrogen, Carlsbad, CA). The primers used for amplification

were as follows: for human *GAPDH*, 5'- GAGTCAACGGATTTGGTCGT -3' and 5'- AGGGGAATTCAGTGTGGTG -3' with a product length of 1095bp, for human *OPN* 5'- TTGCAGTGATTTGCTTTTGC -3' and 5'- AACCACACTATCACCTCGGC -3' with a product length of 443bp, human *c-Myc*, 5'- AGAGAAGCTGGCCTCCTACC -3' and 5'- CGCCTCTTGACATTCTCCTC -3' with a product length of 633bp, human *Cyclin D1*, 5'- GATCAAGTGTGACCCGGA -3' and 5'- AGAGA TGGAAGGGGGAAAGA -3' with a product length of 349bp, human *MMP-7*, 5'- GAGTGCCAGATGTTGCAGAA -3' and 5'- GTGAGCATCTCCTCCGAGAC -3' with a product length of 311bp, and human *CD44*, 5'- AGCAACCAAGAGGCAAGAAA -3' and 5'- GTGTGGTTGAA ATGGTGCTG -3' with a product length of 233bp.

RT-PCR reaction was carried out at an initial denaturing temperature of 94° C for 1min, followed by a touch-down protocol consisting of 12 cycles of: 25s at 94°C, 30s at 64°C with a decrease of 1°C per cycle, 30s at 68°C; then 26 cycles of: 25s at 94°C, 30s at 52°C, 30s at 68°C; finally 5min at 68°C. The reaction product from each sample (5 µl) was then subjected to electrophoresis on a 1.25 % agarose gel. Gel images were taken under Sony's Alpha Imager 2000 system.

Transient Transfection

PC3, DU145, and LNCaP cells were transiently transfected with Mirus Transit-Keratinocyte Transfection Reagent. 15µl of transfection reagent was added to 100µl of serum-free RPMI and allowed to complex for 5min; next 3µg of the appropriate plasmid was added to the complex and incubated at room temperature for 20min. DNA was added to 6-well plates and allowed to express for 48–72hr. Expression and secretion of OPN protein in the media was determined by TCA precipitation of media (see below) and western analysis with an antibody to OPN (Rockland Immunochemicals, Gilbertsville, PA).

TCA protein precipitation

TCA protein precipitation was performed by adding one volume trichloroacetic acid (TCA) to four volumes of protein sample. The contents of the microfuge tube were pelleted through centrifugation, and the protein pellet was washed three times with acetone. The protein was dissolved in 2X sample buffer and was loaded on to two gels, one for experimental purposes and one in which we stained for all proteins to ensure equal loading in the experimental gel.

PI3-kinase Assay

Elisa Assay for PI3-kinase was purchased from Echelon BioSciences. The PI3-kinase assay reactants are first mixed with a PIP3 detector protein, and next added to a PIP3-coated microplate for competitive binding. A second colorimetric detector is added in order to detect the original PIP3 detector protein binding to the PIP3 plate. The colorimetric result is inversely proportional to the amount to PIP3 produced by PI3-K.

Statistical analysis

All values presented as mean ± SEM. A value of $p < 0.05$ was considered significant. Statistical significance was determined by analysis of variance (ANOVA) with the Bonferonni corrections (Instat for IBM; Graphpad software).

Results

OPN induces Akt Activation

In order to elucidate the role of OPN signaling in Akt-mediated cell survival, stable PC3 prostate cancer cell lines which over-express OPN (referred to as PC3/OPN) or control PC3

cells transfected with empty pCEP4 vector were used. PC3 cultures were also treated with soluble OPN to corroborate the effects of PC3/OPN cells lines on Akt activation. Our results indicate that both exogenously added purified OPN and stable transfection of OPN induces Akt activation in PC3 cells, as evaluated by phosphorylation of Akt at serine 473 in Figure 1, A and B respectively. In order to show relative OPN expression in PC3 and PC3/OPN cells, total cell lysates were immunoblotted and probed for the presence of OPN protein (Figure 1C). Immunostaining analysis of p-Akt (serine 473) shows a centralized or perinuclear distribution of activated Akt in PC3 cells while PC3/OPN cells show a more diffuse staining suggesting activated Akt localization to the plasma membrane (Figure 1D). Consistent with our observations, several lines of evidence show that activated Akt is localized to the plasma membrane [19, 33].

Osteopontin induces activation of Akt through integrin linked kinase (ILK) and PI3-K

To further define the role of OPN in cancer cell survival, we analyzed the presumed downstream effectors of integrin $\alpha_v\beta_3$ and CD44. Several lines of evidence indicate that PI3-K and ILK are essential in the activation of Akt [14,16]. Therefore, an ILK inhibitor KP-392 and the PI3K inhibitor wortmannin (WM) were used to block the function of these kinases (Figure 2A and 2B). The ability of both KP-392 and WM to block Akt phosphorylation (A and B, lanes 3 and 4) suggest that both ILK and PI3-kinase are involved in this process. An increase in the phosphorylation of Akt in PC3/OPN cells suggests a possible role for ILK and PI-3-kinase in this process (lane 2 in A and B).

An *in vitro* kinase assay for ILK was performed and results showed an increase in the phosphorylation of ILK substrate GSK-3 β in PC3/OPN cells (Figure 2C, lane 2). To further investigate the role of OPN in the activation of PI3-kinase, we performed an *in vitro* PI3K activation assay (Figure 2D). The PI3K activation assay is a competitive ELISA, where the signal is inversely proportional to the amount of PIP3 produced. Thus a decrease in 450nm absorbance corresponds to an increase in overall PIP3 concentration (standard bar graph in the left). The results indicate that OPN significantly increased the activation of PI3-kinase as compared with control PC3 cells (bar graph in the right).

OPN induces resistance to apoptosis

In order to show the functional relevance of OPN on cell survival, we performed a TUNEL assay. The TUNEL assay labels DNA breaks to detect apoptotic cells via immunofluorescence. Please note that all images were captured at the same settings for fluorescence (Figure 3A). In order to quantitate apoptosis, total cells were counted along with cells stained for apoptosis and the percentage of apoptotic cells was then calculated (Figure 3B). Microscopic analysis revealed that more than 50% of PC3 cells were undergoing apoptosis compared to 15% in PC3/OPN cells. Our results revealed that OPN expression in PC3 cells have an anti-apoptotic advantage as compared with PC3 cells expressing the vector (Figure 3A and B).

OPN induces β -Catenin stabilization

Focusing on the role of OPN-induced Akt activation led us to investigate the downstream effects of Akt function. Previous work showed that Akt inhibits GSK-3 β activity through the phosphorylation of serine 9 on GSK-3 β [34]. Here, we show that GSK-3 β is phosphorylated more in PC3/OPN cells (Figure 4A, lane 2) when compared with PC3 control cells (lane 1). Active GSK-3 β has been shown to have a role in targeting β -Catenin for degradation [35]. Consistent with the decreased activity of GSK-3 β , we have observed an increase in the total level of β -Catenin in PC3/OPN cells (Figure 4B, lane 2).

In order to rule out the possibility that our observations were the results of clonal variation when generating our stable OPN over-expressing cell lines, we used a transient transfection

method on PC3, DU145, and LNCaP prostate cell lines (Figure 4C). TCA protein precipitation and subsequent immunoblotting analysis of the conditioned medium with an antibody to OPN demonstrated an increase in OPN expression and secretion after transfection with the OPN containing vector (Figure 4C). OPN expression induces increased β -Catenin protein levels in both PC3 and DU145 cells (5C, top panel; lanes 2 and 4), with little to no increase in LNCaP cells (lanes 5 and 6). We have yet to investigate the rationale for the OPN-induced changes in β -Catenin dynamics in highly tumorigenic PC3 and DU145 cells with little to no change in lowly tumorigenic LNCaP cells. However, our data in PC3 and DU145 cells suggests that β -Catenin may function in concert with signaling pathways induced by OPN but not in LNCaP cells. To demonstrate that equal amount of proteins in the conditioned media were used for immunoblotting analysis with an OPN antibody (Figure 4C, mid-panel), a gel was stained with Coomassie blue. Equal loading was observed (Figure 4D).

In order to further define how OPN stabilizes β -Catenin, we investigated the phosphorylation status of serine 33, serine 37, and threonine 41 on β -Catenin. These phosphorylation sites have been shown to prime β -Catenin for ubiquitination [21]. Expression of OPN in both PC3 and DU145 prostate cancer cell lines reduces the phosphorylation of β -Catenin on serine 33, serine 37, and threonine 41 (Figure 4E, lanes 2 and 4). Phosphorylation of β -Catenin on serine 33, serine 37, and threonine 41 is critical for the targeting of β -Catenin to proteasome. Mutations in these phosphorylation sites have been shown to stabilize β -Catenin protein levels in many tumor cell lines [29]. A considerable reduction in the phosphorylation state of β -Catenin provides a feasible mechanism for the observed increase in its levels and stability of β -Catenin in cells expressing OPN.

Phosphorylation of serine 552 of β -Catenin has been shown to be mediated by Akt, which targets β -Catenin for nuclear import and triggers a subsequent increase in the transcription of the TCF/LEF target genes [36]. In order to more fully explain a mechanism for OPN/ β -Catenin regulation, we used an Akt inhibitor (Figure 4F) and investigated the total β -Catenin protein levels and the phosphorylation of β -Catenin at serine 552 by immunoblotting analyses (Figure 4G and H). Here we show that OPN increases the β -Catenin protein levels (G, lane 2) and phosphorylation at Ser 552 (H, lane 2). OPN induced changes in β -Catenin dynamics appear to primarily occur through Akt since an inhibitor to Akt blocked the OPN-induced increase in protein and phosphorylation levels of β -Catenin (Figure 4G and H; lane 4). Through the use of an Akt inhibitor, we found Akt as a vital mediator of β -Catenin stabilization and phosphorylation in cells expressing OPN.

OPN induces nuclear accumulation of β -Catenin

Stabilized cytosolic β -Catenin has been shown to have the ability to localize to the nucleus. Therefore we investigated if OPN has a role in inducing β -Catenin nuclear import. To study the effects of OPN on the distribution of β -Catenin, we isolated the nuclear and cytoplasmic fractions from the total lysate harvested from PC3 and PC3/OPN cells. Immunoblotting analysis of nuclear (Figure 5A, lanes 1 and 2) and cytoplasmic (lanes 3 and 4) fractions with a β -Catenin antibody is shown in Figure 5A. We observed that OPN induces increased levels of β -Catenin in both nuclear and cytoplasmic fractions compared to control PC3 cells (Figure 5A). Subsequently, we performed immunostaining analysis with an antibody to β -Catenin (green). Actin staining was performed with rhodamine phalloidin (red) to demonstrate the cell periphery and cell interior. Nuclei were stained with DAPI (blue). β -Catenin localizes predominantly at the cell periphery and between cell-to-cell contacts in PC3 cells transfected with empty vector. An increase in nuclear/perinuclear distribution of β -Catenin in PC3/OPN cells (Figure 5B) parallels the immunoblotting analysis shown in Figure 5A.

OPN induces activation of the TCF/LEF transcription factors

We have shown that OPN increases localization of β -Catenin in both the cytoplasm and nucleus. β -Catenin has been shown to increase cellular proliferation and metastasis in cancer through its role in activating the transcription of the TCF/LEF family of genes [28,29]. Here, we show the OPN-induced activation of TCF/LEF using a transcription reporter assay (Figure 6A). An increase of approximately 40% TCF/LEF activity was observed in PC3/OPN cells as compared with PC3 cells. As a negative control, we used a construct in which the TCF/LEF transcriptional response element (TRE) had been deleted from the firefly luciferase reporter. OPN was unable to induce TCF/LEF transcription in control cells lacking a functional TCF/LEF promoter. A Renilla luciferase construct containing a CMV promoter was cotransfected with both the negative control and TCF/LEF reporter in order to ensure equal transfection efficiency between cell types.

In order to confirm that TCF/LEF activation promotes transcription of known downstream target genes, we performed semi quantitative RT-PCR analysis. An increase in the expression of MMP-7 and CD44 expression (Figure 6B) was observed in cells expressing OPN. No changes in the expression levels of cyclin D1 or c-Myc were observed. We further investigated β -Catenin signaling induction in response to OPN by probing the protein levels of known TCF/LEF target genes (Figure 6C). OPN did not induce an observed increase in cyclin D1 or c-Myc protein levels, but promoted a marked increase in MMP-7 and CD44 protein levels (Figure 6C, lanes 6 and 8), consistent with the observed increase in transcription of these genes (Figure 6B). These observations suggest that β -Catenin-mediated, TCF-dependent transcription is activated in cells expressing OPN.

Figure 7 summarizes our results. Here we show that OPN activates Akt and that both ILK and PI3K are integral components in OPN-mediated Akt activation. Furthermore, OPN not only stimulates an accumulation of β -Catenin, but also induces the nuclear import of β -Catenin. Localization of β -Catenin in the nucleus mediates signaling that activates TCF-dependent transcription in cells expressing OPN.

Discussion

Osteopontin (OPN) expression has been shown to enhance the invasive potential of cancer cells, and plays a crucial role in cancer progression [1,3,37,38]. OPN was shown to be associated with recurrence of prostate cancer within 72 months and OPN was suggested to be a clinically useful marker for predicting biochemical recurrence [39]. Proteins such as OPN, integrin α V β 3, and CD44 have been shown to increase the metastatic potential of cancer cells [4-6,30]. OPN is secreted by tumor cells. It is considered as an autocrine motility and tumorigenic factor [3]. High OPN expression has been related to poor survival of patients. The molecular mechanisms by which OPN promotes tumor survival/metastasis are not yet clear. In order to more fully elucidate the role of OPN in cancer progression we have primarily over-expressed OPN in varied prostate cancer cell lines.

To characterize the role of OPN in prostate cancer progression, we focused on Akt signaling, investigating the signaling mechanisms downstream of OPN by inhibiting ILK and PI3-kinase activity through the use of molecular inhibitors. OPN-induced Akt activation was lost in the presence of the ILK inhibitor KP-392, showing that ILK function is required for OPN-mediated Akt activation (Figure 2A). PI3-kinase inhibition by Wortmannin illustrated the pivotal role of PI3-kinase in OPN-mediated Akt activation, as no OPN induced Akt activation was observed in the presence of wortmannin (Figure 2A). In order to demonstrate a functional role for OPN-mediated Akt activation and cell survival, we performed a TUNEL assay. Our observations indeed have demonstrated that OPN reduces apoptosis in prostate cancer cells.

PI3-K/Akt signaling promotes cell survival and increased cancer progression through many effectors including the inactivation of GSK-3 β by phosphorylation of the serine 9 in the pseudosubstrate domain [40]. GSK-3 β signaling pathways have been associated with the progression of androgen-independent prostate cancer [41]. Phosphorylation of GSK-3 β on serine 9 inactivates its kinase activity, resulting in high cytosolic protein levels of β -Catenin and ultimately its nuclear localization, leading to the increased transcriptional activation of the TCF/LEF family of genes. OPN induces increased phosphorylation of GSK-3 β on serine 9, and this prompted us to investigate whether OPN affects β -Catenin dynamics (Figure 4). While phosphorylation of serine 9 is the most well recognized means of inhibiting GSK-3 β , others have reported GSK-3 β inhibition in a serine 9 independent fashion, and thus we cannot rule out that OPN may be inhibiting GSK-3 β through additional mechanisms as well [42]. Previous studies have shown that β -Catenin plays a role in OPN transcription [43]. Here, we show that OPN can induce β -Catenin stabilization and subsequent translocation to the nucleus (Figures 4-6). In addition, this study also suggests that OPN-mediated signaling has the potential to modulate the activity of β -Catenin through Akt-mediated phosphorylation of β -Catenin.

The increase in β -Catenin protein levels appear to be the result of a decrease in GSK-3 β activity and hence a decrease in β -Catenin ubiquitination [44,45]. As shown by others in insulin signaling [46], we believe that OPN signaling could modulate the activity of β -Catenin through Akt-mediated regulation of GSK-3 β phosphorylation. This study found that the phosphorylation of GSK-3 β is regulated by OPN signaling (Figure 4A). Also, Akt activation seems to be an important step in OPN induced β -Catenin dynamics, as inhibition of Akt in PC3/OPN cells results in the decrease of total β -Catenin to a level comparable with the unstimulated PC3 cells.

Our investigation into OPN/ β -Catenin dynamics shows that OPN is responsible for activating the transcription of the TCF/LEF family of genes. OPN mediated increase of MMP-9 and CD44 in prostate cancer cells paired with the fact that highly tumorigenic cells often overexpress OPN provides insight to the observed increase in motility and invasiveness of metastatic cells.

Osteopontin is emerging as a major player in cancer progression consistent with its reported varied actions and observed over expression in several clinical cancer isolates [47,48]. Activation of β -Catenin signaling and subsequent localization of β -Catenin in the nucleus may increase cell proliferation via activation of transcription. Understanding of the role of OPN in neoplastic change and its regulation of survival mechanism may facilitate the development of novel therapeutic targets and approaches.

Acknowledgments

We thank Dr. Shoukat Dedhar (University of British Columbia, Vancouver, British Columbia, Canada) for the ILK inhibitor-KP-392. This work was supported by the National Institute of Health grants AR46292 (to MAC), DE18308 (F30 grant to BWR), and DE07309 (the training grant T32) from the Department of Biomedical Sciences, Dental School, University of Maryland, Baltimore).

The abbreviations used are

OPN, Osteopontin; α v β 3, vitronectin receptor; RGD, cell adhesion sequences containing arginine-glycine-Aspartic acid amino acids; SiRNA, small interfering RNA; WM, wortmannin; PDK1, phosphoinositide-dependent kinase; PC3, human epithelial prostate cancer lines; TCF/LEF, T cell factor/lymphoid enhancer factor; PTEN, Phosphatase and tensin homologue.

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FIGURE 1. OPN induces activation of Akt

A and B. Immunoblotting analyses with p-Akt (serine 473) (top panel) and Akt (bottom panel) antibodies. Cells treated with soluble OPN (+ in A; 10 μ g/ml for 30 min) or cells over-expressing OPN (PC3/OPN in B) display an increase in Akt activation (upper panel; A and B, lane 2) as compared with control cells (A and B, lane 1). Total Akt levels were used as loading controls (lower panel in A and B). C. Immunoblotting analyses of OPN expression in PC3 and PC3/OPN stable cell lines. Immunoblotting of the same blot with actin antibody after stripping serve as a loading control (lower panel). D. Confocal analysis of distribution of actin (red) and phospho-Akt (serine 473, green) in PC3 and PC3/OPN cells. Results are representative of experiments repeated at least three times.

FIGURE 2. PI3-kinase and integrin-linked kinase (ILK) are required for OPN-induced Akt activation

A and B. PC3 cells stably expressing empty vector or wild type OPN were treated with and without ILK inhibitor (KP-392, 100μM for 16hr; panel A, lanes 3 and 4) or PI3-K inhibitor (Wortmannin, 200nM for 1hr; panel B, lanes 3 and 4). The effects of inhibitors on Akt activation were determined by immunoblotting analysis (upper panels, A and B). Akt levels were used as loading controls (lower panels in A and B). C. In vitro kinase assays for ILK (panel C) and PI-3kinase (panel D, right panel) were performed using lysates from PC3 and PC3/OPN cell lysates. A standard graph was plotted using the ELISA. A decrease in 450nm absorbance corresponds to an increase in overall PIP3 concentration (standard bar graph in the left). PI3-kinase assay was performed in lysates made from PC3 and PC3/OPN cells (bar graph in the right); ***p<0.05 vs. PC3 cells. Results shown are representative of experiments repeated at least three times.

FIGURE 3. OPN induces a decrease in apoptosis

A. PC3 cells stably expressing empty vector or OPN (PC3/OPN) were treated with serum free media for 24hr and the TUNEL assay was performed in these cell lines. (B) Results were quantified as the percentage of cells undergoing apoptosis. A significant increase in the apoptosis was observed in PC3 cells as compared with PC3/OPN cells; *** $p < 0.001$ vs. PC3/OPN cells. Results shown are representative of three experiments.

FIGURE 4. OPN induces β -Catenin stabilization

A and B. Equal amount of proteins in lysates made from PC3 cells stably expressing empty vector (PC3) or OPN (PC3/OPN) were probed for phospho-GSK-3 β (serine 9; panel A) and β -Catenin (panel B) proteins. C and D. PC3, DU145, and LNCaP cell lines were transiently transfected with either empty vector or OPN. Immunoblotting analyses with antibodies to β -Catenin (panel C, top), osteopontin C, middle), and GAPDH (C, bottom), are shown. Coomassie blue staining of TCA precipitated media fractions were used as loading control for immunoblotting analysis with an OPN antibody (panel D). E. Immunoblotting analysis with a β -Catenin phospho-specific antibody towards serine 33, serine 37 and threonine 41 in PC3 and DU145 cells expressing vector and OPN. F-H. PC3 cells or PC3/OPN were treated with Akt inhibitor and equal amount of lysates from these cell lines were immunoblotted with a β -Catenin and phospho-specific antibody to β -Catenin towards serine 552. GAPDH (bottom panels in A-C, E, G, and H) and total Akt (F) levels were used as loading controls. Results shown are representative of three experiments.

FIGURE 5. OPN induces the nuclear localization of β-Catenin

(A) Immunoblotting analysis of β-Catenin in the nuclear (lanes 1 and 2) and cytoplasmic (lanes 3 and 4) fractions of stable PC3 and PC3/OPN cells. Immunoblotting with an antibody to nucleoporin p62 was used as a loading control (A, bottom panel in lanes 2 and 3) in the nuclear fraction. Immunoblotting with a GAPDH was used as a loading control for the cytoplasmic fraction (bottom panel in lanes 3 and 4) and total cellular lysates of PC3 and PC3/OPN cells (lanes 5 and 6). (B) Confocal fluorescent microscopy analysis of the distribution of β-Catenin (green) and actin (red) in PC3 and PC3/OPN cells. DAPI (blue) staining allows visualization of the nucleus. Results shown are representative of three experiments.

FIGURE 6. OPN induces TCF/LEF gene family expression

A. TCF/LEF reporter assay was performed with lysates prepared from PC3 and PC3/OPN cells. The results presented are mean $\pm$ S.E. for three experiments, ** indicates that the results are statistically significant, the p value was <0.014 when comparing PC3/OPN and PC3 cells expressing vector pCEP4. B. RT-PCR analysis in PC3 and PC3/OPN cells. Analyses were performed for OPN, c-Myc, cyclin D1, MMP-7, and CD44 genes. GAPDH was used as a loading control. C. Immunoblotting analyses with the following antibodies using lysates from PC3 and PC3/OPN cells. cyclin D1 (lanes 1 and 2), c-Myc (lanes 3 and 4), MMP-7 (lanes 5 and 6), and CD44 (lanes 7 and 8). GAPDH immunoblots were used as loading controls. Results shown are representative of experiments repeated at least three times.

FIGURE 7. A schematic representation of OPN signaling in the regulation of cell survival in prostate cancer cells

Osteopontin (OPN) via ILK and PI3-K activates Akt. GSK-3 β and β -Catenin function downstream of Akt activation in PC3 cells in response to OPN signaling. OPN-induced nuclear import of β -Catenin and the expression of the TCF/LEF family of genes occur as a result of Akt activation.