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Regulation of prostate stromal fibroblasts by the PIM1 protein kinase

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ABSTRACT

The PIM1 oncogene is over-expressed in human prostate cancer epithelial cells. Importantly, we observe that in human hyperplastic and cancerous prostate glands PIM1 is also markedly elevated in prostate fibroblasts, suggesting an important role for this kinase in epithelial/stromal crosstalk. The ability of PIM1 to regulate the biologic activity of stromal cells is demonstrated by the observation that expression of PIM1 kinase in human prostate fibroblasts increases the level and secretion of the extracellular matrix molecule, collagen 1A1 (COL1A1), the pro-inflammatory chemokine CCL5, and the platelet-derived growth factor receptors (PDGFR). PIM1 is found to regulate the transcription of CCL5. In co-cultivation assays where PIM1 over-expressing fibroblasts are grown with BPH1 prostate epithelial cells, PIM1 activity markedly enhances the ability of these fibroblasts to differentiate into myofibroblasts and express known markers of cancer-associated fibroblasts (CAFs). This differentiation can be reversed by the addition of small molecule PIM kinase inhibitors. Western blots demonstrate that PIM1 expression in prostate fibroblasts stimulates the phosphorylation of molecules that regulate 5′Cap driven protein translation, including 4EBP1 and eIF4B. Consistent with the hypothesis that the kinase controls translation of specific mRNAs in prostate fibroblasts, we demonstrate that PIM1 expression markedly increases the level of *COL1A1* and *PDGFRβ* mRNA bound to polysomes. Together these results point on PIM1 as a novel factor in regulation of the phenotype and differentiation of fibroblasts in prostate cancer by controlling both the transcription and translation of specific mRNAs.

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1. Introduction

Prostate cancer (PCa) tumor growth and metastasis are stimulated by "activated" fibroblasts, also known as cancer associated fibroblasts (CAFs) located in the tumor stroma. Phenotypically, CAFs closely resemble myofibroblasts exhibiting different levels of specific marker expression relative to benign fibroblasts [1–4]. Myofibroblasts secrete multiple growth factors (e.g., TGFα, TGFβ, CXCL12/SDF1, IL-6) and extracellular matrix proteins (e.g., collagens, periostin, integrins, cadherin 11) involved in regulation of angiogenesis and epithelial cell growth and migration. These in turn enable tumor growth and dissemination [2,5–8]. Specific intracellular signal transduction pathways are needed to induce the myofibroblast/CAF differentiation of normal fibroblasts and to regulate the secretion of the factors that stimulate tumor growth.

The *pim-1* gene was identified as a proviral insertion site of the Moloney murine leukemia virus in experiments designed to find new genes, which are involved in tumorigenesis [9]. Signal transduction pathways regulated by PIM serine/threonine kinases are strongly implicated in the human prostate cancer progression and growth [10]. PIM1 activity regulates the growth of the epithelial component of human PCa [11–13]. Overexpression of PIM1 has been detected in human PCa both at the mRNA and protein levels, although there is no clear definition of which cell types within the tumor were involved [12]. Moderate to strong staining of PIM1 was seen in tumors of 68% of patients with a Gleason score of seven or higher [14], and PIM staining may be helpful in differentiating benign glands from intraepithelial neoplasia [15].

Abbreviations: PIM1, Proviral Insertion site of the Moloney murine leukemia virus; CAFs, cancer associated fibroblasts; BM, bone marrow; COL1A1, collagen type I alpha 1; PDGFR, platelet-derived growth factor receptors; CCL5, chemokine (C-C motif) ligand 5; BPH, benign prostate hyperplasia; PCa, prostate cancer; CAD11, cadherin11; COL4A, collagen type IV alpha; αSMA, smooth muscle actin; Dox, doxycycline; CM, conditioning media; qT-PCR, quantitative real-time polymerase chain reaction; IHC, immunohistochemistry; TMA, tissue microarray; GFP, green fluorescein protein.

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Human PCa epithelial cells secrete a large number of factors, including IL-6 and GM-CSF, that control PIM kinase levels, and hypoxia is another stimulant known to elevate PIM levels in multiple cell types [16–18].

Using human tissue microarrays, our experiments demonstrate that PIM1 protein kinase expression levels are significantly elevated not only in the epithelial compartment, but also importantly in the prostate stromal compartment. Evaluation of prostate biopsies of human tumors demonstrates elevated levels of PIM1 mRNA in myofibroblasts/CAFs versus normal fibroblasts. This data suggests a potential role for the PIM1 protein kinase in regulation of both the differentiation state and the biologic activity of prostate fibroblasts.

2. Materials and methods

2.1. Antibodies, reagents and lentiviral and retroviral expression vectors

Antibodies, reagents and all lentiviral and retroviral expression vectors used in this study are described in the supplementary Materials and methods.

2.2. Cell culture and generation stable cell lines

Immortalized stromal fibroblasts BHPrS1 and epithelial BPH1 cells were kindly provided by Dr. Simon W. Hayward (Vanderbilt University). Both cell lines were isolated from non-tumorigenic prostate surgical samples [19–21]. The primary normal human prostate fibroblasts hPrF at passage 2 were purchased from ScienCell and grown in the recommended media (Fibroblast medium FM #2301).

Primary cultures of human prostate stromal cells were obtained from explants of needle biopsies from the peripheral or transition zones of prostate from patients with advanced prostate cancer and from patients without evidence of neoplasia. This work was performed by J. Cerda-Infante and V.P. Montecinos, using the pathology resource at the Hospital Clínico, Pontificia Universidad Católica de Chile under IRB-approved protocols. Stromal fibroblasts were cultivated as described [22] in the DMEM supplemented with 5% fetal bovine serum (FBS) for two passages. Cells were then assayed for expression of CAFs-associated markers αSMA, vimentin, pro-collagen and calponin by qRT-PCR, immunocytochemistry and western blot analysis (data not shown) and used for measurement of PIM1 levels.

All other human cell lines, including prostate fibroblasts WPMY1, bone marrow mesenchymal cells HS-5, PC3, were supplied by American Type Culture Collection. Wild type (WT) and PIM triple knockout (TKO) mouse embryonic fibroblasts (MEFs) were established as described [23].

2.3. Cell growth, lentiviral transduction and selection of the stable cell pools

Cell growth, lentiviral transduction and selection of the stable cell pools expressing inducible PIM1 kinase were performed as described in the supplementary Materials and methods.

2.4. Co-cultivation assays

Co-cultivation of stromal fibroblasts expressing inducible PIM1 and BPH1/GFP were performed either in the absence or presence of Doxycycline (Dox) (20 ng/ml). 2×10^5 of BHPrS1 and BPH1/GFP cells were plated in 10 cm dishes, grown until confluent, trypsinized, diluted to 4×10^5 cells/plate and cultivated in a fresh media. After the third passage, 15 total days of co-cultivation, the stromal fibroblasts were separated from the epithelial cells by differential trypsinization and plated in media containing puromycin (2 μg/ml) for 48 h to remove puromycin-sensitive BPH1/GFP cells. After 48 h growth in puromycin containing media, cells were re-plated in antibiotic-free complete media and used for analysis. To isolate BPH1/GFP cells, epithelial cells attached to the plates after differential trypsinization were collected and subjected to flow cell sorting. The resultant GFP-positive epithelial cells were then assayed for cell growth and migration. To study the activity of kinase inhibitors, after the second passage of co-cultivation, cells were plated in a fresh media and after attachment overnight, PIM1 or PDGFR inhibitors were added for 5 days until cells reached confluence followed by further analysis.

2.5. CM preparation

To prepare CM, 1×10^6 prostate stromal fibroblasts grown in monoculture or after co-cultivation were first plated in complete media with or without Dox, and grown for 48 h. Cells were then washed twice with PBS and placed in serum free media. CM was collected after 48 h, centrifuged at 4000 rpm to pellet debris, and used either for the BPH1 cell migration assay (Supplementary Fig. S2C) or concentrated with an Amicon ultra 15 centrifugal unit (Millipore). The protein concentrations in each sample were determined by Bradford assay (Bio-Rad) and equal amounts of protein were used for Western blot or ELISA. Quantitation of CCL5 in the CM was measured by the Human Cytokine Array Kit (R&D Systems) or ELISA (Ray Biotech), according to the manufacturing protocols.

2.6. Tissue microarray and immunocytochemistry (IHC), cell growth and migration assays, qRT-PCR analysis, polysome profiling

Tissue microarray and immunocytochemistry (IHC), cell growth and migration assays, qRT-PCR analysis, polysome profiling were done as described in the supplementary Materials and methods.

2.7. Statistical analysis

All experiments were repeated a minimum of two times and the results of quantitative studies are reported as the mean +/− SD. Differences were analyzed by Student's t test. P values of <0.05 were regarded as significant.

3. Results

3.1. PIM1 protein levels are elevated in PCa fibroblasts

To evaluate the levels of the PIM1 protein kinase in PCa stromal cells, 28 cancer and adjacent normal samples were evaluated by IHC using the 19 F7 PIM1 mouse monoclonal antibody and samples were scored based on staining intensity (Fig. 1A and B). This antibody specifically recognizes PIM1 but not the other PIM family members and had been used by others [24]. This analysis demonstrated that PIM1 is highly and significantly over expressed in human PCa stroma when compared to those from normal prostate tissues. This observation was confirmed using an alternate prostate tissue microarray containing 32 adenocarcinomas, 26 hyperplasia and 20 normal tissues (Fig.1 C and D) with staining performed with rabbit monoclonal PIM1 antibodies (Epigenetic). Although the direct correlation between PIM1 immunostaining in PCa stroma and the Gleason grades was not found, the up-regulation of PIM1 kinase can shed light onto stromagenic response in prostate cancer.

To confirm whether the PIM1 kinase level is up-regulated in the PCa stromal fibroblasts we analyzed pooled RNA samples obtained from primary CAFs cultures isolated from the peripheral zone of primary tumor of advanced/metastatic PCa patients (CAFs, n = 7), from the benign peripheral zone (BP-BAFs, n = 9) and from the benign transition zone (TZ-BAFs, n = 6) of the prostate. As shown in Fig. 1E the level of PIM1 transcript is significantly elevated in CAFs compared to benign prostate fibroblasts. Together these results suggest a potential role of PIM1 in the activity of fibroblasts found adjacent to transformed or abnormal epithelial cells.

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