Dipeptidyl Peptidase Inhibits Malignant Phenotype of Prostate Cancer Cells by Blocking Basic Fibroblast Growth Factor Signaling Pathway

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Abstract

Dipeptidyl peptidase IV (DPPIV) is a serine protease with tumor suppressor function. It regulates the activities of mitogenic peptides implied in cancer development. Progression of benign prostate cancer to malignant metastasis is linked to increased production of basic fibroblast growth factor (bFGF), a powerful mitogen. In this study, using in vitro model system we show that DPPIV loss is associated with increased bFGF production in metastatic prostate cancer cells. DPPIV reexpression in prostate cancer cells blocks nuclear localization of bFGF, reduces bFGF levels, inhibits mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK)1/2 activation, and decreases levels of urokinase-type plasminogen activator, known downstream effectors of bFGF signaling pathway. These molecular changes were accompanied by induction of apoptosis, cell cycle arrest, inhibition of in vitro cell migration, and invasion. Silencing of DPPIV by small interfering RNA resulted in increased bFGF levels and restoration of mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK)1/2 activation. These results indicate that DPPIV inhibits the malignant phenotype of prostate cancer cells by blocking bFGF signaling pathway. (Cancer Res 2005; 65(4): 1325-34)

Introduction

Prostate cancer is the second leading cause of cancer deaths among men. Recent evidence suggest that the development of androgen-independent, metastatic prostate cancer is associated with increased activities of basic fibroblast growth factor (bFGF), a powerful mitogen and an angiogenic inducer (1–6). Although, the role of bFGF in cancer progression is fairly well understood, very little is known about the mechanisms that regulate bFGF signaling during the transition from benign to highly malignant prostate cancer.

Dipeptidyl peptidase IV (DPPIV), a membrane glycoprotein, regulates the activities of mitogenic growth factors and neuropeptides. Its proteolytic activity leads to inactivation or degradation of these peptides (7, 8). DPPIV is involved in diverse biological processes, including cell differentiation, adhesion, immunomodulation, and apoptosis, functions that are critical for controlling neoplastic transformation (7–13). DPPIV is expressed in normal epithelial cells and melanocytes (11–15). Its expression is lost in various cancers, suggesting loss of DPPIV as an important event during cancer progression (11, 12, 14–18). We have previously shown that reexpression of DPPIV in melanoma and lung cancers leads to reemergence of dependence on exogenous growth factors and suppresses their tumorigenic potential (11, 12).

bFGF, a multifunctional molecule regulates cell proliferation, migration, cell survival, wound healing, angiogenesis, and tumor progression (19, 20). It is expressed as a low molecular weight cytoplasmic isoform (18 kDa) and high molecular weight nuclear isoforms (21, 2.5, and 24 kDa). The high molecular weight isoforms possess the NH2-terminal nuclear localization signal and are exclusively localized to the nucleus (19–21). Recent data indicate that increased expression of bFGF promotes tumorigenic and metastatic properties of various malignancies including prostate cancer and is associated with inferior survival of prostate cancer patients (2, 3, 19, 21–26). Furthermore, loss of even one allele of bFGF in the transgenic adenocarcinoma of the mouse prostate model increases their survival by suppressing metastasis (27).

Several studies have linked bFGF signaling to activation of the mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK)1/2 pathway that promotes cancer progression (28–31). During cellular migration and angiogenesis, activation of ERK1/2 by bFGF increases the production of urokinase-type plasminogen activator (uPA), a serine protease that converts plasminogen to plasmin. Plasmin, in turn, promotes cancer metastasis by breaking down the extracellular matrix, including fibronectin and laminin (32, 33). Notably, the malignant phenotype of prostatic tumor cells correlates with high expression of both uPA and its receptor (uPAR), and plasma uPA has been identified as a biomarker for prostate cancer progression in Dunning rats and prostate cancer patients (34–36).

In this study, we investigated the mechanism by which DPPIV suppresses the malignant phenotype of prostate cancer cells. We establish a correlation between DPPIV loss, increased bFGF production, and malignant phenotype of prostate cancer cells. We show that reexpression of DPPIV in metastatic prostate cancer cells (DU-145), blocks nuclear accumulation of bFGF, decreases bFGF leading to inhibition of MAPK-ERK1/2 activation and uPA production, known downstream effectors of bFGF signaling pathway. We also show that DPPIV expression is associated with inhibition of in vitro cell migration, invasion, induction of apoptosis, and cell cycle arrest. These data identify a novel mechanism for regulation of bFGF activity by DPPIV in prostate cancer cells and provide new directions for therapeutic interventions aimed at blocking the mitogenic activities of cancer promoting growth factors.

Materials and Methods

Antibodies and Reagents. The antibodies to bFGF, uPA, p27, and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); the polyclonal antibody against ERK1/2 from Cell Signaling Technologies...
(Beverly, MA); CY-3 goat anti-rabbit (red) secondary antibody, horseradish peroxidase–sheep anti-mouse, goat anti-rabbit IgG were from Jackson laboratories (Bar Harbor, ME). The mouse monoclonal antibody to DPPIV was purified from the culture media of S27 hybridomas obtained from American Type Culture Collection (Manassas, VA). The cyamine dimer nucleic acid stain, YOYO-1 iodide, and Oregon Green were from Molecular probes, (Eugene, OR); LipofectAMINE reagent from Invitrogen, (Grand Island, NY); 5'-(45-dimethyloxazol-2-yl)-2,5-diphenyltetrazolium bromide assay kit was from American Type Culture Collection. Cell invasion assay kit was from Chemicon International, Inc. (Temecula, CA); APOPTAG kit was from Interagen (Gaithersburg, MD); and pSilencer small interfering RNA (siRNA) expression vectors were from Ambion, Inc. (Austin, TX).

Cells. The normal prostate epithelial cells (NPrEC) were obtained from Cambrex Bio Science, Inc. (Baltimore, MD), RWPE-1, LNCaP, and DU-145 cells were obtained from the American Type Culture Collection. DU-145 and LNCaP cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 0.1 mmol/L nonessential amino acids, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. NPrEC cells were grown in media provided by the supplier. RWPE-1 cells were grown in keratinocyte medium containing bovine pituitary extract (0.05 mg/mL) and epidermal growth factor (0.5 ng/mL; Invitrogen).

Flow Cytometry. Flow cytometric analysis was done using FACSscan (Becton Dickinson, San Jose, CA). Cells were stained using the S27 monoclonal antibody (anti-DPPIV) as the primary antibody. FITC–conjugated rabbit anti-mouse IgG was used as the secondary antibody. Cells stained with secondary antibody alone were used as controls.

DPPIV Enzyme Activity. DPPIV peptidase activities were measured in presence and absence of doxycycline by colorimetric assay using Gly-Pro nitroanilide as the substrate, following previously described procedures (11).

Total RNA Isolation and Northern Blot Analysis. Total RNAs were isolated from cells using RNAeasy extraction kit (Qiagen, Valencia, CA). Total RNA (20 μg) from each cell line was size fractionated and transferred to nylon membrane. The mRNA levels of DPPIV, glyceraldehyde-3-phosphate dehydrogenase, uPA, and β-actin were assessed using the probes generated by PCR. The primers used in PCR reactions are as follows: 5’-tcctagctcctatgatt-ta-3’ and 5’-ctgccagctatcagctatc-3’ (800-bp DPPIV); 5’-acctgagggagatcagc-3’ and 5’-accatacctcacttctgtgg-3’ (275-bp glyceraldehyde-3-phosphate dehydrogenase); 5’-tccggcactatacagc-3’ and 5’-tcctcctctgtctgctgctct-3’ (387-bp uPA); 5’-ggagaacctcctattctatagc-3’ and 5’-tggctgtcctagactgctttg-3’ (275-bp β-actin). Membranes were washed and visualized by autoradiography.

Establishment of Prostate Cancer Cell Line DU-145 Reexpressing DPPIV. Tetacycline inducible expression vectors carrying full-length DPPIV (pTRE-DPPIV) was constructed as described (11). DU-145 cell line was cotransfected at 60% confluency with pTRE-DPPIV or control vector, along with the plasmid pTET-on using LipofectAMINE. Stably transfected cells were selected using G418 (600 μg/mL). To determine the growth rate, cells were plated at a density of 10^4 cells per well in triplicate in 24-well plates. Viable cells were counted daily for 10 days using trypan blue. Time of doubling was determined from a least-squares regression fit of cell number versus time during the logarithmic growth phase.

Immunofluorescence Microscopy. For assessing bFGF, uPA, fibronectin, and ERK1/2 expression, cells grown on chamber slides for 48 hours, without or with doxycycline (1 μg/mL) were stained with the respective monoclonal antibodies and incubated with either Oregon green or CY-3 conjugated goat anti-rabbit (red) secondary antibody. Cells were counter stained with nuclear DNA-specific stain, Yo-Yo 1 iodide. Stained cells were viewed with a inverted microscope (model- ECLIPSE TE2000-U from Nikon, Melville, NY) connected to a RT Slider Spot Digital camera (Diagnostic Instruments, Sterling Heights, MI). Images were acquired and merged images were generated using the software SPOT version 3.2.

Immunoprecipitation and Western Blot Analysis. For metabolic labeling of proteins, cells were cultured in medium containing [35S] methionine for 18 hours. Total cell lysates were immunoprecipitated with the DPPIV-specific monoclonal antibody S27. The protein samples were analyzed by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the respective antibodies, over night at 4C. Signals were developed with chemiluminescence.

MTT Cell Proliferation Assay. Colorimetric MTT assays were done using MTT assay kit on days 1, 2, 4, 6, and 10. Cells (n = 5 × 10^3) per well were plated in triplicates on 96-well plates and cultured in absence or presence of doxycycline (1 μg/mL). The absorbance of released purple formazan was measured at 570 nm in a microtiter plate reader. The experiment was repeated twice and the data are presented as mean ± SD of triplicates.

Wound Induced Cell Migration Assay and Quantitative Cell Invasion Assay. Cells were plated at 60% confluence and cell monolayers were wounded 24 hours after plating, by scraping with a sterile micropipette tip. Each well was then washed and daily fed with normal medium. Cells were photographed at 2 and 24 hours after wounding using a phase contrast microscope. Additionally, a quantitative cell invasion assay was done using an invasion assay kit following the manufacturer's instructions. Absorbance values correlating with cell invasion were plotted. The experiment was repeated twice. The data are presented as the mean of triplicate plates.

In situ Apoptotic Cell Detection by Terminal Deoxynucleotidyl Transferase–Mediated Nick End Labeling Assay. For apoptosis assays, cells were grown (without or with doxycycline, 1 μg/mL) in serum-free media for 3 days. Fixed cells were washed and measurements of DNA nicks were carried out by terminal deoxynucleotidyl transferase–mediated nick end labeling assay using the APOPTAG kit. Percent apoptosis was calculated by FACScan analysis.

Cell Cycle Analysis. Equal number of DU-145, vector, and DPPIV transfected DU-145 cells (2 × 10^6) were washed, resuspended in PBS containing 2% fetal bovine serum, 5 mmol/L EDTA, and were stained with 20 mg/mL propidium iodide for 2 hours at room temperature. The cells were then examined by flow cytometry (Becton Dickinson FACS Scanner, Franklin Lakes, Nj) to determine cell cycle distribution. The percentage of cells present in G0-G1, S, and G2-M phases were determined using ModFit software package (Verity Software House, Topsham, ME).

RNA Interference Experiment. Expression of DPPIV was silenced using the vector, psiencer 3.1-H1 hygro. The 60-mer complementary oligonucleotides encoding 19-mer hairpin sequences specific to the DPPIV (NM001935) mRNA (nucleotide 1,219 to 1,237, AGTCATCGGGATAGAAGCT), a loop sequence (TTCAGAGA) separating the two complementary domains, and a polyuridyline tract (TTTTTT) to terminate transcription were synthesized and ligated into the psiencer 3.1-H1 hygro vector. Two sequential transfections (days 1 and 4) of LNCaP and DU-145 cells reexpressing DPPIV with 1 μg of siRNA plasmid were carried out using the lipofectamine reagent. Stably transfected cells were selected for further analysis by adding hygromycin (200 μg/mL) to culture media.

Results

DPPIV Loss Is Associated with Increased bFGF Production in Metastatic Prostate Cancer Cells (DU-145). We analyzed the expression pattern of DPPIV and bFGF in (a) NPrEC; (b) non-tumorigenic in vitro transformed RWPE-1 cells (37); (c) androgen-sensitive, LNCaP cells that grow slow and are weakly tumorigenic (38, 39), and (d) androgen-independent, tumorigenic, and moderately metastatic prostate cancer cell line DU-145 (40). Flow cytometric analysis indicated high levels of cell surface expression of DPPIV on NPrEC cells with decreased expression to moderate levels on RWPE-1 and LNCaP cells, and very low levels on DU-145 cells (Fig. 1A). Whereas DPPIV enzyme activities in NPrEC cells ranged from 180 to 220 and 100 to 120 pmol per g protein per minute in RWPE-1 and LNCaP cells, it was reduced to 20 to 40 pmol per g protein per minute in DU-145 cells (Fig. 1B). Northern blot analysis showed that decreased or loss of DPPIV expression in prostate cancer cells occurs at RNA level (Fig. 1C).

Western blot analysis showed low levels of bFGF in NPrEC, RWPE-1, and LNCaP
cells, but high levels in DU-145 cells (Fig. 1D). These observations indicate an inverse correlation between DPPIV and bFGF expression during the emergence of malignant phenotype in prostate cancer cells.

**DPPIV Blocks Nuclear Localization and Reduces Expression Levels of bFGF in Metastatic Prostate Cancer, DU-145 Cells.**

To understand the role of DPPIV in prostate cancer progression, we reexpressed DPPIV in DU-145 cells using the tetracycline inducible expression system. Two independent clones expressing DPPIV were randomly selected for further studies. Untransfected and vector-transfected DU-145 cells were used as controls. The levels of DPPIV enzyme activities in the presence of doxycycline (1 μg/mL) ranged between 180 and 225 pmol per minute per μg protein in DPPIV transfected cells and were comparable to the levels in NPPrEC (200-220 pmol per minute per μg protein; Fig. 2A). The DPPIV enzyme activities in DPPIV transfected cells ranged from 30 to 60 pmol per minute per μg protein in the absence of doxycycline, similar to control DU-145 cells (20-40 pmol per minute per g protein; Fig. 2A). Furthermore, immunoprecipitation analysis showed the expected 110-kDa protein corresponding to DPPIV with its natural post-translational glycosylation in DPPIV transfected DU-145 cells in the presence of doxycycline (Fig. 2B).

Because DPPIV and bFGF levels were inversely correlated in normal and metastatic prostate cancer cells, we investigated the effect of DPPIV on bFGF levels in DU-145 cells. Western blot analysis showed high levels of both nuclear (22, 22.5, and 24 kDa) and cytoplasmic isoforms (18 kDa) of bFGF in DU-145 and vector transfected DU-145 cells. Reexpression of DPPIV in DU-145 cells greatly decreased the levels of nuclear bFGF isoforms; the levels of low molecular weight cytoplasmic isoform were also decreased (Fig. 2C). In the absence of DPPIV induction, the same DU-145 cells showed bFGF levels comparable to control DU-145 cells, demonstrating the specific effect of DPPIV on bFGF levels (Fig. 2D).

Consistent with Western blot results, immunofluorescence staining revealed high levels of both nuclear and cytoplasmic form of bFGF in control DU-145 cells (Fig. 2E, a and d). In DPPIV reexpressing DU-145 cells, the nuclear accumulation of bFGF was greatly reduced; most of bFGF was observed in a punctate pattern around the perinuclear region (Fig. 2E, g and j). We confirmed altered nuclear localization by costaining the cells with nuclear-specific Yo-Yo 1 iodide (Fig. 2E, b, e, h, k; see also merged images in c, f, i, l). The staining patterns of bFGF in individual cells are shown in Fig. 2F. These observations indicated that DPPIV negatively regulates bFGF expression and disrupts its subcellular localization.

**DPPIV Inhibits Activation of MAPK-ERK1/2, Decreases uPA Expression, and Restores Fibronectin Matrix Assembly.**

Expression of bFGF is associated with constitutive activation of the MAPK-ERK1/2 signaling pathway. Interestingly, the levels of phosphorylated p44/42 were greatly reduced in DU-145 cells expressing DU-145 cells, compared with the control DU-145 cells (Fig. 3A). In absence of doxycycline, levels of phosphorylated p44/42 were not affected, indicating specific effects of DPPIV on ERK 1/2 kinase.
activities (Fig. 3A). The levels of total ERK1/2 remained the same in all cell lines in the presence and absence of doxycycline (Fig. 3A). Immunofluorescence staining showed the nuclear localization of phosphorylated ERK1/2 in control DU-145 cells (Fig. 3B, a and b), whereas in DPPIV-transfected cells, nuclear ERK1/2 were barely detectable in the presence of doxycycline (Fig. 3B, c and d).

During prostate cancer progression, high levels of bFGF and ERK1/2 activation are associated with increased uPA expression. Our current study shows that control DU-145 cells express high levels of uPA and reexpression of DPPIV leads to undetectable levels of uPA mRNA in these cells (Fig. 3C). Immunofluorescence staining further confirmed high levels of uPA in control DU-145 cells. (Fig. 3D, E, F).

Figure 2. DPPIV blocks nuclear localization and reduces expression levels of bFGF. A, DPPIV enzyme activity in NPrEC, DU-145 clones transfected with vector, and DPPIV in the absence or presence of doxycycline (1 μg/mL). Columns, mean values of triplicates; bars, ± 1 SD. B, immunoprecipitation analysis of DPPIV expression in DU-145 cells transfected with DPPIV and one vector in the absence and presence of doxycycline (1 μg/mL). C and D, Western blot analysis of the levels of nuclear and cytoplasmic isoforms of bFGF. DU-145 and vector control cells showed very high levels of nuclear and moderate levels of cytoplasmic isoform of bFGF in untransfected DU-145 cells and DU-145 cells transfected with either vector or DPPIV in the presence of doxycycline (1 μg/mL; C) or in the absence of doxycycline (D). E, immunofluorescence staining showing bFGF expression (red) in untransfected, vector, and DPPIV transfected DU-145 cells in the presence of doxycycline. Cells were counter stained with nuclear specific Yo-Yo 1 iodide (green). Stained cells were viewed with an inverted microscope. Merged images show either yellow (red and green) or green nucleus. (Magnification ×400). F, staining patterns of bFGF in single cells for each group are shown (Magnification ×600). Merged images (yellow) represent colocalization of bFGF (red) and nuclear-specific Yo-Yo 1 iodide (green).
cells (Fig. 3D, 1 and 2) and low levels in DPPIV reexpressing DU-145 cells. (Fig. 3D, 3 and 4). Western blot analysis showed increased levels of fibronectin proteins in DPPIV expressing DU-145 cells compared with the control DU-145 cells (Fig. 3E). Immunofluorescence staining confirmed decreased levels of fibronectin matrix on the control DU-145 cells but with increased assembly of fibronectin matrix that distributed in a fibrillar array on DU-145 cells reexpressing DPPIV (Fig. 3F).

**DPPIV Induces Contact Inhibition and Inhibits Cellular Proliferation, Migration, and Invasion of Prostate Cancer DU-145 Cells.** Restoration of DPPIV expression resulted in profound morphologic changes in DU-145 cells. DU-145 cells and vector transfected DU-145 cells showed small, rounded morphology and grew in disorganized clumps. These cells grew at higher saturation densities (Fig. 4A, 1 and 2). DU-145 cells reexpressing DPPIV in presence of doxycycline were enlarged, assumed flat epithelial morphology, and grew in an organized manner, and exhibited contact inhibition (Fig. 4A, 3 and 4). The growth pattern of DU-145 cells expressing DPPIV was comparable to NPrEC (Fig. 4A, e) with no change in absence of doxycycline (shown in insets of Fig. 4A, 3 and 4). The alteration of cell shape and growth pattern supports previous reports that DPPIV is a cell adhesion molecule and may

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**Figure 3.** DPPIV decreases activation of ERK1/2, decreases uPA expression and increases cell surface FN matrix assembly. A, Western blot analysis of the levels of total and phosphorylated ERK1/2 proteins in control- or DPPIV-transfected DU-145 cells, in the presence or absence of doxycycline. B, Immunofluorescence staining of nuclear translocation of phosphorylated ERK1/2 in DU-145- and vector-transfected DU-145 cells (a and b) and in DPPIV-transfected DU-145 cells (c and d). C, Northern blot analysis of uPA mRNA levels in untransfected, vector control-, and DPPIV-transfected DU-145 cells. The blot was reprobed with 275-bp PCR fragment of β-actin to assess for equal amount of RNA loaded from all samples. D, Immunofluorescence staining of expression of uPA protein in DU-145- (1), vector- (2), and DPPIV-transfected DU-145 cells (3 and 4). E, Western blot analysis showing the levels of fibronectin in DU-145-, vector-, and DPPIV-transfected DU-145 cells. F, Immunofluorescence staining showing very low levels of fibronectin matrix assembly on the cell surfaces of DU-145 and vector transfected DU-145 cells (1 and 2) and increased assembly of fibronectin matrix on the cell surfaces of DPPIV-transfected DU-145 cells (3 and 4). Data shown in B-F are in the presence of doxycycline.
play an important role in regulating cell motility. The doubling time for DU-145 cells reexpressing DPPIV was increased to 48 from 36 hours. DPPIV expression also inhibited cell proliferation up to 50% of the control DU-145 cells in presence of doxycycline without much change in the absence of doxycycline as indicated by MTT assay (Fig. 4B).

In vitro cell migration and invasion are indirect measures of the in vivo metastatic potential of a transformed cell and these functions are facilitated by bFGF and ERK1/2 activation. The effect of DPPIV on prostate cancer cell migration was evaluated qualitatively by a wound induced migration assay. At 2 hours, DU-145, DPPIV expressing DU-145 cells, and control DU-145 cells showed no visible migration (Fig. 4C, 1, 2, 3, and 4). After 24 hours, DU-145 and vector control cells migrated to fill the wounded area completely, indicating their strong migratory potential (Fig. 4C, 5 and 6). However, the migratory potential of DU-145 cells

Figure 4. DPPIV expression results in contact inhibition and inhibits cellular proliferation, in vitro cell migration, and invasion of prostate cancer cells (DU-145). A, bright field photographs of morphologic changes in DU-145 cells transfected with either vector or DPPIV. Untransfected DU-145 (1) and vector-transfected DU-145 (2) grew in a disorganized array of cells with clumps indicating lack of contact inhibition. DU-145 cells transfected with DPPIV (3 and 4) showed flat epitheloid and dendritic morphology with contact inhibited growth in the presence of doxycycline, similar to normal prostate epithelial cells (5). Morphology of DPPIV transfected DU-145 cells remained similar to parental DU-145 cells in the absence of doxycycline (insets 3a and 4a). Original magnification, ×200. B, the rates of cellular proliferation of DU-145, either vector or DPPIV transfected DU-145 cells were assessed in absence (A) or presence (B) of doxycycline by MTT assay, as described in Materials and Methods. The experiment was repeated twice. Columns, absorbance expressed as mean of triplicates; bars, ±1 SD. C, wound healing assay showing migration of DU-145, vector or DPPIV transfected DU-145 cells in the presence of doxycycline, after 2 and 24 hours of scraping. (Magnification ×100). D, quantitative cell invasion assay as done using Boyden chamber cell invasion assay kit. Absorbances correlating with the number of cells invaded were read at 540 nm. Columns, mean values of triplicates; bars, ±1 SD.
reexpressing DPPIV was greatly reduced as indicated by the unfilled wounded area (Fig. 4C, 7 and 8). Furthermore, the ability of cells to invade through ECM coated filters was assessed quantitatively by the Boyden Chamber assay. The number of invading DU-145 cells reexpressing DPPIV in presence of doxycycline was greatly reduced with inhibition up to 50% to 60% when compared with control DU-145 cells (Fig. 4D). These results indicated that DPPIV inhibits in vitro invasive and migratory potential of prostate cancer cells.

**DPPIV Induces G2-M Arrest and Cell Death in Prostate Cancer Cells (DU-145).** To further understand the mechanism by which DPPIV inhibits proliferation of DU-145 cells, we did cell cycle analysis. The percentage of DU-145 cells reexpressing DPPIV in presence of doxycycline was greatly reduced with inhibition up to 50% to 60% when compared with control DU-145 cells (Fig. 5A). These results indicated that DPPIV inhibits in vitro invasive and migratory potential of prostate cancer cells.

**Silencing of DPPIV by siRNA Restores bFGF Expression and Increased ERK1/2 Phosphorylation.** To confirm the direct involvement of DPPIV in regulating bFGF expression and ERK1/2 activation, we used RNA interference (siRNA) to silence DPPIV expression in LNCaP and DU-145 cells reexpressing DPPIV (DU-DPPIV-1). The siRNA plasmid transfected LNCaP and DU-DPPIV-1 cells were CHANGE analyzed for DPPIV, bFGF, ERK1/2 levels, and phenotypic changes. Flow cytometric analysis showed decreased cell surface expression of DPPIV in siRNA transfected DU-DPPIV-1 cells (Fig. 6A). Furthermore, immunoprecipitation and enzyme activity analysis confirmed the decreased levels of DPPIV protein and enzyme activities in siRNA transfected DU-DPPIV-1 cells as shown in Fig. 6B and C. The morphology and growth pattern of cells transfected with DPPIV-specific siRNA reverted to malignant phenotype and started to grow in disorganized colonies, indicating loss of contact inhibition similar to the vector transfected DU-145 cells (Fig. 6D). Knock down of DPPIV expression resulted in increased expression of bFGF (Fig. 6E) and restoration of increased levels of phosphorylated ERK1/2 MAPK (Fig. 6F). Similar results were obtained when DPPIV was silenced in LNCaP cells (Fig. 6G). These results suggested that the inhibitory effects of DPPIV on the malignant phenotype of prostate cancer cells are at least partly

**Figure 5.** DPPIV Induces G2-M cell cycle arrest and cell death in prostate cancer cells (DU-145). A, cell cycle analysis was done by flow cytometry using DPPIV transfected DU-145 cells in the presence of doxycycline (1 μg/mL). Untransfected and vector transfected DU-145 cells were analyzed in parallel. The results are depicted as a cascade plot. Inset, % cells in various stages of cell cycle. B, % apoptosis of parental-, vector-, and DPPIV-transfected DU-145 cells in serum-free condition as indicated by terminal deoxynucleotidyl transferase–mediated nick end labeling assay. Apoptosis was assessed in the absence or presence of doxycycline at days 1 and 3. Columns, mean of triplicates; bars, ±1 SD. C, Western blot analysis showing the expression of cycline D kinase inhibitor p27 (kip1) and actin in DU-145-, vector-, and DPPIV-transfected DU-145 cells in the presence and absence of doxycycline.
mediated through the negative regulation of the bFGF-MAPK signaling pathway.

Discussion

The results of our experiments show that DPPIV loss, increased bFGF production and in vitro metastatic potential of prostate cancer cells are correlated. DPPIV suppresses the malignant phenotype of prostate cancer cells by decreasing the levels and blocking nuclear localization of bFGF, thereby resulting in reduced bFGF signaling as indicated by decreased MAPK-ERK1/2 activation and uPA levels. This is the first demonstration that bFGF, a powerful mitogen and an angiogenic inducer is a target for a protease regulated growth suppressor activity in prostate cancer cells.

Progression of benign prostate cancer to fatal hormone refractory disease is associated with over expression of growth factors that mediate alternative mitogenic signaling (1–5). The cell surface protease, DPPIV controls normal cellular growth and differentiation by regulating the activity of peptide factors (7, 8).
The present studies have shown that DPPIV is expressed at high levels in NPrEC that were established from normal prostatic tissue and its expression is decreased to moderate levels in RWPE-1 and LNCaP cells, and to almost undetectable levels in DU-145 cells. The three cell lines, RWPE-1, LNCaP, and DU-145 cells vary widely in their phenotypic characteristics. The HPV-18 transformed and immortalized RWPE-1 cells represent early stage of prostate cancer progression because these cells neither grow in agar nor form tumors when injected into nude mice. Although RWPE-1 cells express cytokeratins 8 and 18, which are characteristic of luminal prostatic epithelial cells, they also coexpress basal cell cytokeratins (37). The DU-145 cell lines is AR negative and poorly differentiated, whereas the LNCaP cell line that exhibit less aggressive phenotype has functional AR, grows less rapidly, and is less tumorigenic than the DU-145 cell line. Metastasis formation by LNCaP cells is observed only when the cells were injected orthotopically into prostate glands (38, 39, 41, 42). Our data suggest that loss of DPPIV may be an important event during progression of prostate cancer cells to aggressive phenotype. This observation is supported by other studies conducted with clinical specimens, reporting loss of DPPIV expression in >50% of the metastatic and poorly differentiated advanced prostate cancer samples (16, 17).

Several studies have also shown that bFGF is up-regulated in prostate cancer cell lines and tumors tissues with increased metastatic and invasive potential (1–6). Thus, the mutually exclusive expression patterns of DPPIV and bFGF in metastatic cell lines probably reflect their role in dedifferentiation from an epithelial to a malignant phenotype. Supporting this notion, our data show that reexpression of DPPIV in DU-145 cells induces the normal epithelial phenotype, cell-cell contact, decreased cell proliferation rate, and reduced anchorage-independent growth ability. We have previously shown that DPPIV induces similar effects and abrogates the tumorigenic potential of melanomas and lung cancer cells (11, 12). These data support the idea that DPPIV is associated with nonproliferative phenotype of normal prostate cells.

Although elevated levels of bFGF are often associated with prostate cancer progression, the underlying mechanism leading to dysregulated bFGF signaling is not known. Our studies suggest that this dysregulation could be in part due to loss of DPPIV leading to high levels of bFGF in the nucleus that disrupts the normal cellular transcriptional program. We speculate that in normal cells DPPIV cleaves the NH2 terminal nuclear localization signal thereby confining bFGF to the perinuclear region. This initial cleavage may be the first step in the degradation of bFGF. Alternatively, it is possible that DPPIV directly associates with bFGF and interferes with post-translational modifications of bFGF such as NHex-terminal methylation shown to affect its nuclear accumulation (19–21). DPPIV may also affect the stability of bFGF resulting in its reduced levels. Indeed, high levels of bFGF are shown to be required for promoting malignant transformation (1–3, 27).

Loss of expression of other membrane associated peptidases such as CD10/NEP, CD13/APN, and BP-1/6C3/APA has been reported in several types of human cancers (32–35). Elevated levels of uPA are found in patients with advanced stages of prostate cancer and it is a strong indicator of poor prognosis (34–36). It is interesting to note that uPA activates plasminogen to generate plasmin that degrades extracellular matrix components including fibronectin, a required event for malignant cell metastasis. We have shown that DPPIV dramatically decreases uPA mRNA production and increases the fibronectin assembly on the cell surface. Thus, increase in uPA expression seen in DU-145 cells, may result from the cooperative action of signaling provided by bFGF. Disruption of these signaling events by DPPIV is probably involved in the assembly of fibronectin matrix and increase in cell adhesion that serves as a barrier for cell motility. This possibility is supported by data showing DPPIV binds to fibronectin and functions as an adhesion molecule (10), and that fibronectin on the cell surface disappears upon oncogenic transformation. Furthermore, overexpression of fibronectin leads to suppression of the transformed phenotype (49, 50). Thus, loss of several studies have linked bFGF to activation of the MAPK-ERK1/2 signaling pathway that plays a critical role in survival and migration of tumor cells (28, 29). Indeed, bFGF-deficient endothelial cells lack ERK1/2 activation and loose their migratory potential (28). Activation of ERK1/2 is shown to be associated with progression of prostate cancer to the lethal androgen-independent state (30, 31). However, the causal role of these downstream kinases in androgen-independent prostate cancer is not completely understood. Our data show that DPPIV expression leads to decreased levels of phosphorylated ERK1/2 resulting in inhibition of migratory and invasive potential of prostate cancer cells (DU-145). This effect was abrogated upon DPPIV silencing by siRNA treatment of the same cells demonstrating the specific effects of DPPIV on bFGF mediated ERK1/2 activation. Interestingly, silencing of DPPIV in LNCaP cells also resulted in increased bFGF expression and activation of ERK1/2. This indicates that regardless of androgen receptor expression, DPPIV regulates bFGF and ERK1/2 signaling in prostate cancer cells. This observation is of importance because most clinical samples of hormone refractory disease still express androgen receptor and continue to survive and proliferate probably through activating alternative survival and proliferative signaling pathways (1–5). There is evidence to show that, among other factors, polypeptide growth factors that are associated with activation of MAP kinase signaling pathways play a critical role in this phenomenon (4). Our present studies link DPPIV with blocking of bFGF-mediated signaling pathway implicated in prostate cancer progression.

DPPIV expression in DU-145 cells lead to an increase in the expression of a cyclin D kinase inhibitor, P27 (kip1) leading to an arrest in the G2-M phase, and induced apoptosis upon serum withdrawal, indicating the emergence of dependence on exogenous growth factors for their survival. It is shown that bFGF promotes cell proliferation and protection from apoptosis by decreasing p27 expression (46, 47). Also, disruption of FGF signaling by the dominant-negative FGFRI is shown to result in cell cycle arrest of prostate cancer cells in G2-M stage (48). Taken together, these observations suggest that DPPIV blocks the signaling pathway coordinated by bFGF and ERK1/2 in prostate cancer cells.

Tumor cell migration and invasion depends largely on an enzymatic cascade of proteolysis that involves uPA and its receptor (uPAR). This cascade is an essential step for malignant cell transport. Growth factors including bFGF and activated ERK1/2 have been shown to promote uPA production in various tumoral and normal cell types (32–35). Elevated levels of uPA are found in patients with advanced stages of prostate cancer and it is a strong indicator of poor prognosis (34–36). It is interesting to note that uPA activates plasminogen to generate plasmin that degrades extracellular matrix components including fibronectin, a required event for malignant cell metastasis. Growth factors, polypeptide growth factors that are associated with activation of MAP kinase signaling pathways play a critical role in survival and migration of tumor cells (28, 29). Indeed, bFGF-deficient endothelial cells lack ERK1/2 activation and loose their migratory potential (28). Activation of ERK1/2 is shown to be associated with progression of prostate cancer to the lethal androgen-independent state (30, 31). However, the causal role of these downstream kinases in androgen-independent prostate cancer is not completely understood. Our data show that DPPIV expression leads to decreased levels of phosphorylated ERK1/2 resulting in inhibition of migratory and invasive potential of prostate cancer cells (DU-145). This effect was abrogated upon DPPIV silencing by siRNA treatment of the same cells demonstrating the specific effects of DPPIV on bFGF mediated ERK1/2 activation. Interestingly, silencing of DPPIV in LNCaP cells also resulted in increased bFGF expression and activation of ERK1/2. This indicates that regardless of androgen receptor expression, DPPIV regulates bFGF and ERK1/2 signaling in prostate cancer cells. This observation is of importance because most clinical samples of hormone refractory disease still express androgen receptor and continue to survive and proliferate probably through activating alternative survival and proliferative signaling pathways (1–5). There is evidence to show that, among other factors, polypeptide growth factors that are associated with activation of MAP kinase signaling pathways play a critical role in this phenomenon (4). Our present studies link DPPIV with blocking of bFGF-mediated signaling pathway implicated in prostate cancer progression.

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DPPIV may render tumor cells less adhesive thereby promoting detachment of cells from primary tumors initiating metastasis. However, in vivo studies will further strengthen the role of DPPIV in blocking metastatic potential of prostate cancer cells.

Given the ability of DPPIV to regulate the cell proliferation, differentiation, apoptosis, and cell motility through its regulation of bFGF provides a new and potentially significant approach for better understanding prostate cancer progression and development of treatment strategies aimed at blocking mitogenic and angiogenic signaling.

Acknowledgments


Grant support: Department of Microbiology and Molecular Genetics, Lake Champlain Cancer Research Organization, Vermont Cancer Center at the University of Vermont.

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We thank Emma Hewes and Scott Tighe at shared Core Facility of Vermont Cancer Center for technical help in construction of siRNA plasmids and flow cytometric analysis and Drs. David Nanus, Cedric Wesley, Alan Houghton, Nick Heintz, Mark Plante, Brooke Mossman, and Susan Wallace for helpful discussions.

References


Dipeptidyl Peptidase Inhibits Malignant Phenotype of Prostate Cancer Cells by Blocking Basic Fibroblast Growth Factor Signaling Pathway

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