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RESEARCH ARTICLE

Extracellular matrix protein fibronectin induces matrix metalloproteinases in human prostate adenocarcinoma cells PC-3

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Abstract

Studies on interaction of tumor cells with ECM components showed increased extracellular protease activity mediated by the family of matrix metalloproteinases (MMPs). Here we studied the effect of human prostate adenocarcinoma PC-3 cells–fibronectin (FN) interaction on MMPs and the underlying signaling pathways. Culturing of PC-3 cells on FN-coated surface upregulated MMP-9 and MMP-1. This response is abrogated by the blockade of α_s integrin. siRNA and inhibitor studies indicate possible involvement of phosphatidyl-inositol-3-kinase (PI-3K), focal adhesion kinase (FAK) and nuclear factor-kappaB (NF- κ B) in FN-induced upregulation of MMPs. FN treatment also enhanced phosphorylation of FAK, PI3K, protein kinase B (PKB or Akt), nuclear translocation of NF- κ B, surface expression of CD-44, and cell migration. Our findings indicate that, binding of PC-3 cells to FN, possibly via $\alpha_s\beta_1$ integrin, induces signaling involving FAK, PI-3K, Akt, NF- κ B followed by upregulation of MMP-9 and MMP-1. CD-44 may have role in modulating MMP-9 activity.

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Keywords: PC-3, signaling, ECM, fibronectin, integrin, MMP

INTRODUCTION

Tumor cells interact with extracellular matrix (ECM) by specific cell surface receptors and these interactions influence tumor development, cell survival, cell proliferation, and cell migration (Juliano, 2002). ECM is mainly filled with intricate network of macromolecules like glycoproteins, fibrous proteins, etc. Some important examples of ECM components are fibronectin (FN), laminin (LN), vitronectin, collagen-IV, etc. Among them FN is well characterized having binding sites for other matrix macromolecules, and for receptors on cell surfaces. The motifs of cell-ECM interaction are Arg-Gly-Asp (RGD) and Pro-His-Ser-Arg-Asn (PHSRN) in FN molecule (Akiyama et al., 1995). Among the cellsurface integrin receptors, $\alpha_{s}\beta_{1}$ is major FN receptor on most cells. Integrin-mediated responses to FN include adhesion, downstream signaling to cause cell migration, assembly of cytoskeleton, invasion, etc. (Akiyama et al., 1989; Sen et al., 2010).

Metastatic potential of cancer cells is determined by their ability to disrupt tissue architecture by ECM degradation and remodeling. Culturing cancer cells in presence of intact matrix proteins such as FN or LN

or matrix-derived peptides was found to increase invasive and metastatic potential (Terranova et al., 1984). Dysregulated proteolysis has been implicated in tumor invasion and metastasis in multiple model systems (Munshi & Stack, 2006). Metastatic cells produce several extracellular proteinases, including matrix metalloproteinases (MMPs) that process ECM components. Rapid expression and activation of MMP-2 and MMP-9 upon exposure of several human cancer cell lines to FN in serum-free medium was observed to be mediated by $\alpha_{\epsilon}\beta_{1}$ integrin and involve participation of signaling proteins like focal adhesion kinase (FAK), phosphatidylinositol-3-kinase (PI3K), extracellular-signal-regulated kinases (ERK), Nuclear Factor- kappa B (NF-KB), etc. (Das et al., 2008; Maity et al., 2010). In several types of cancer, high expression of MMP-1 has been correlated with the depth of tumor invasion, angiogenesis, lymphangiogenesis, poor prognosis, and the presence of local and distant metastases (Murray et al., 1998; Murray et al., 1996; Shiozawa et al., 2000). In human prostate cancer cell DU-145, interaction of $\alpha_{5}\beta_{1}$ integrin with the fibronectin PHSRN sequence was reported to induce activation of FAK, PI3K, and protein kinase C- δ (PKC δ) in the intracellular signaling pathway leading to MMP-1dependent invasion (Zeng et al., 2006).

Prostate adenocarcinoma is now the most common malignancy in men and the second leading cause of cancer-related deaths in the United States (Greenlee et al., 2001). In the European Union it is the second most common malignancy in men. Statistically significant increase

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in the incidence of prostate cancer has been registered in several cities in India (Yeole, 2008). The symptomatic phase of the disease is mainly due to occurrence of metastasis to the bone. PC-3 cell line, one of the classical cell lines of prostatic cancer, was originally derived from advanced androgen-independent bone metastasis. In this communication PC-3 cell line was used to study its response during interaction with ECM protein FN and decipher the intracellular signaling associated with the response.

METHODS

Cell culture

PC-3 (human prostate cancer cell line) was obtained from National Centre for Cell Sciences, Pune. This cell line was grown and maintained in Nutrient mixture F12 Ham medium, containing 10% FBS (GIBCOTM-Invitrogen, Carlsbad, CA) in a 5% CO, incubator at 37°C.

Treatment of cells with FN

PC-3 cells were grown on FN(10 μ g/ml)-coated 35 mm petridishes (Corning, USA) in F12 SFCM or Serum-Free Culture Media (300000 cells/ml) for required time at 37°C, 5% CO₂.

Cell-adhesion assay

To assay the binding capacity of PC-3 cells to FN (440 kDa; Roche, Germany), microtitre plate wells (Corning, USA) were coated with the ligand (2.5 μ g/ml, 5 μ g/ml, 10 μ g/ml) in triplicate. Rest of the steps were performed as previously described (Das et al., 2008).

Gelatin zymography

The gelatinases were separated from the culture supernatants of control and experimental sets using gelatin sepharose 4B beads (GE Healthcare Biosciences AB, Uppsala, Sweden) with shaking for 2 h at 4°C. To assay the gelatinase activity, Gelatin Zymography was performed using a 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) co-polymerized with 0.1% gelatin (Sigma-Aldrich) as described in earlier report (Sen et al., 2009).

Study the effects of cell signaling inhibition on MMPs

PC-3 cells were untreated (con) or treated (exp) with anti- α_3 , anti- α_4 , and anti- α_5 integrin antibodies (1 µg/ml) or with chemical inhibitors of PI3K, ERK, p38 (LY294002, PD98059, SB203580, respectively, in the concentration of 50 µM each; Promega, Madison, USA) in SFCM (Das et al., 2008) for 1 h at 37°C, 5% CO₂. Bay 117085 (Alexis Biochemicals, Lausen, Switzerland), the NF-κB inhibitor, was treated in 5 μ M concentration (Dutta et al., 2010) for 24 h at 37°C, 5% CO₂. Then both the control and experimental sets were grown on FN-coated (10 μ g/ml) surface for 4 h in SFCM. The culture supernatant was subjected to gelatin zymography to visualize gelatinase activity and the cell extracts were subjected to western blot analysis to visualize MMP-1 expression.

Whole cell extraction

Control and treated cells were collected, washed with ice cold phosphate-buffered saline (PBS), and were extracted in lysis buffer [50 mM Tris pH 7.5, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% Deoxycholate, Protease inhibitor cocktail tablets (Roche, Germany), 1 mM sodium orthovanadate, and 1 mM Sodium fluoride] on ice and clarified by centrifugation. The protein concentrations were estimated by Lowry method and equal amounts of proteins were loaded for western blot assay.

Nuclear fraction isolation

PC-3 cells were grown in absence and presence of FN (10 μ g/ml) for 4 h. Cells were collected, washed twice with ice cold 1X PBS, and nuclear extract was prepared following previously described steps (Sen et al., 2010). Equal amounts of proteins from each fraction was loaded and analyzed by western blot assay.

Western blot analysis

Equal amounts (100 µg) of protein from whole cell or cytoplasmic or nuclear extracts from control and experimental sets were subjected to western blot analysis using primary antibodies [anti- α_5 , anti-FAK, anti-phospho-FAK (Tyr-397), anti-PI3K (p85), antiphospho-PI3K (Tyr-508), anti-NF-kB, anti-Akt, antiphospho-Akt (Thr-308), anti-phospho-Akt (Ser-473), anti-E-cadherin, anti-VEGF, anti-integrin-linked kinase (ILK), anti-phospho-ILK (Thr-173), and anti-MMP-1; 1 μ g/2 ml dilution for each] followed by the respective secondary antibodies $(1 \mu g/200 \text{ ml dilution})$ using methods discussed previously (Ganguly et al., 2012). Blots were reprobed with anti β -tubulin antibody as internal loading control. All of these antibodies were purchased from Santa Cruz Biotechnology, Inc., USA. Anti-TBP antibody, used for loading control for nuclear protein, was purchased from Abcam Inc, Cambridge, MA, USA. [ILK: Integrin-linked kinase, VEGF - Vascular endothelial growth factor, TBP: TATA box-binding protein]

Immunocytochemical analysis

PC-3 cells were grown on coverslips in absence and presence of 10 μ g/ml FN for required time periods. After PBS wash, cells were fixed with 3.5% formalde-hyde and treated with 0.5% Triton X-100 (Amresco,

USA). Non-specific sites were blocked using 1% BSA solution. Cells were then incubated with anti-FAK and anti-CD-44 (Santa Cruz Biotechnology Inc., USA) primary antibodies (1 µg/ml), washed thrice with PBS and incubated with respective secondary antibodies (1 µg/ml dilution) in humid chamber. In the case of FAK, FITC labeled secondary antibody (Santa Cruz Biotechnology Inc., USA) was used. Cells were washed six times with PBS and coverslips were mounted on glass slides and observed under fluorescence microscope. In the case of CD-44, biotin coupled secondary antibody (Santa Cruz Biotechnology Inc., USA) was used. After PBS wash and 30 min incubation in an avidin-biotinylated peroxidase complex reagent (Vector Laboratories, Burlingame, CA), expressions were visualized with diaminobenzidin tetrahydrochloride (Pierce Biotechnology, USA) treatment. Cells were washed with PBS, coverslips were mounted on glass slides, viewed under bright field microscope.

Wound-healing assay

PC-3 cells (300000 cells/ml) were cultured in a monolayer in absence and presence of FN (10 μ g/ml, 4 h). The monolayer was then scratched with a sterile pipette tip, followed by washing with SFCM to remove cellular debris. The cells were maintained in fresh SFCM and cell migration was observed under microscope and photographed at successive time intervals (0, 15 and 24 h).

Transwell assay

24-well transwell plate (Corning, USA) with 12 inserts were taken and the lower chamber of each well was poured with 0.6 ml (as suggested by manufacturer) fresh F12 medium containing 5% FBS. Control and FN-treated (10 μ g/ml, 4 h) PC-3 cells (100000 cells/insert) were seeded in triplicate on membrane in the upper chamber of the insert and kept for 24 and 48 h 37°C, 5% CO₂. Further steps were performed as previously described (Sen et al., 2010).

Semi quantitative reverse transcriptase-polymerase chain reaction

RNA was extracted from 1×10^6 PC-3 cells grown in absence and in presence of FN (10 µg/ml) for 4 h. Cells were washed in PBS and total RNA was extracted (RNAqeous, Ambion, USA). Two-step reverse transcriptase-polymerase chain reaction (RT-PCR) (Retroscript, Ambion, USA) was done with equal amounts of total RNA using specific primers (Operon, Germany) for human MMP-9 and TIMP-1 genes. Glyceraldehyde phosphate dehydrogenase (GAPDH) primers (Operon, Germany) were used as control to normalize for mRNA integrity and equal loading. Equal amounts of each PCR products were run on a 2% agarose gel and bands were visualized under UV. The primer sequences and PCR cycles/conditions for each primer are given below. The PCR cycles in all cases were started with Taq activation at 94°C for 5 min and ended with final extension of 72°C for 7 min. The primer sequences are as follows:

hMMP-9: 5'-CGCTACCACCTCGAACTTTG-3' (forward); 5'GCCATTCACGTCGTCCTTAT-3'-(reverse); hTIMP-1: 5'-CACCCACAGACGGCCTTCTGC-3'-(forward); 5'-AGTGTAGGTCTTGGTGAAGCC-3'-(reverse); GAPDH:5'-CGGAGTCAACGGATTTGGTCGTAT-3' (forward); and 5'-AGCCTTCTCCATGGTGGTGAAGAC-3' (reverse).

Conditions used for PCR consisted of 40 cycles for MMP-9, and TIMP-1 at 94°C for 30 s, 58°C for 30 s, and 72°C for 90 s in thermal cycler (Ganguly et al., 2012).

FAK inhibition by siRNA treatment

PC-3 cells were seeded on 35 mm dishes and grown to 60% confluence. For the transfection process, FAK siRNA and negative control siRNA were transfected using Lipofectamine[™] 2000 following the manufacturer's protocol. The transfection agent (Lipofectamine 2000; Invitrogen, Life Technologies, USA) was mixed with serum-free culture medium. Subsequently, respective siRNA [FAK siRNA (h), sense- GCAUGUGGCCUGCUAUGG; antisense- CCAUAGCAGGCCACAUGC; Santa Cruz Biotechnology] mixed with serum-free culture medium (SFCM) was added to the Lipofectamine medium and incubated at room temperature for additional 45 min. The mixtures were then diluted in serum-free culture medium and added to each dish so that the final concentration of the siRNA in each plate was 60 nM. The mixture was overlaid on the cells in full media without serum and without antibiotic and incubated at 37° C in presence of 5% CO₂ for overnight. Then the transfection mixture was replaced with fresh media supplemented with 10% FBS and antibiotic and the transfected cells were allowed to grow for 24 h. Cells were exposed to FN in SFCM for 4 h. The SFCM was subjected to gelatin zymography and cells were extracted for western blot.

RESULTS

FN induces gelatinolytic activity and mRNA expression of MMP-9 with possible involvement of α_{s} integrin in PC-3 cells

Cell-adhesion assay showed that PC-3 cells bind to FN and the percentage of adhesion increases with the increase in concentration of FN (Figure 1A). PC-3 cells grown on FN-coated surface showed increase in MMP-9 gelatinolytic activity in the culture supernatant with appreciable upregulation at 10 μ g/ml for 4 h. Treatment of PC-3 cells with coated LN, another ECM component,



Figure 1. Effect of FN on MMP-9, TIMP-1, and α_5 integrin in PC-3 cells: A. Cell-adhesion assay was done with different concentrations (2.5, 5, and 10 µg/ml) of FN and percentages of adhesion were plotted. B. PC-3 cells were grown in SFCM in absence (Lane 1) and in presence of 10 µg/ml of FN for 2 h (Lane 2), for 4 h (Lane 3), and 10 µg/ml of LN for 4 h (Lane 4). Culture supernatant of HT-1080 cells grown in SFCM for 24 h was used as MMP-9 (92 kDa)/MMP-2 (72 kDa) marker (Lane -M). The culture supernatants in all the cases were subjected to gelatin zymography. C. PC-3 cells were grown in serum-free culture medium (SFCM) in absence (0) and in presence of 10 µg/ml FN for 4 h. 2 steps RT-PCR was done with equal amounts of total RNA, using MMP-9 and TIMP-1 specific primers for PCR. GAPDH primers were used to confirm equal loading. D. PC-3 cells were treated without (Lane 1) or with 1 µg/ml anti- α_3 (Lane 2), anti- α_4 (Lane 3), and anti- α_5 (Lane 4) integrin antibodies for 1 h and then all the sets were grown in presence of 10 µg/ml FN for 4 h. Gelatin zymography of the culture supernatants were performed. E. Following FN treatment (10 µg/ml for 4 h), PC-3 cells were collected, extracted, and equal amounts of protein (100 µg) was subjected to western blot analysis with anti- α_5 antibody (1 µg/2 ml dilution) and respective HRP-coupled secondary antibody (1 µg/200 ml dilution) for ECL method. β -tubulin was used as internal control.

at 10 µg/ml concentration for 4 h does not show detectable gelatinolytic activity in gelatin zymography. Thus it shows specificity of FN to upregulate secreted MMP-9 activity in PC-3 cells (Figure 1B). RT-PCR analysis of FN-treated PC-3 cells (10 µg/ml for 4 h) indicates increase in expression of MMP-9 mRNA and decrease in TIMP-1 mRNA (Figure 1C). α_3 , α_4 , α_5 integrins were masked using their respective antibodies (1 µg/ml, for 1 h at 37°C in CO₂ incubator) before culturing the PC-3 cells on FN-coated surface (10 µg/ml, 4 h). Gelatin zymography of culture supernatant showed appreciable downregulation of FN-induced MMP-9 activity in α_5 integrin-blocked cells (Figure 1D). FN at 10 µg/ml for 4 h was also shown to enhance α_5 protein expression in whole cell extracts by western blot assay (Figure 1E).

Involvement of FAK and PI3K in FN-induced MMP-9 activity

To identify the cell signaling components involved in FN-induced MMP-9 upregulation, specific chemical inhibitors were used to treat the cells prior to culturing with FN. FN-induced MMP-9 gelatinolytic activity was considerably decreased with treatment of the cells with PI-3K inhibitor (LY 294002), whereas no appreciable effect was observed upon treatment with ERK inhibitor (PD 98059) and p38 inhibitor (SB 203580) (Figure 2A).

Western blot analysis reveals that, FN treatment appreciably induces phosphorylation of FAK at Tyr-397 and phosphorylation of PI3K at its p85 subunit within 4 h (Figure 2B). FN treatment on PC-3 cells for various time periods induces gradual migration of FAK to the focal complex (white arrow) and nucleus (yellow arrow) as visualized by immunocytochemistry (Figure 2C). When FAK was silenced by siRNA treatment, endogenous FAK expression was reduced in FN-treated cells. FN-mediated upregulation of MMP-9 was decreased in these cells, as visualized in zymography of the culture supernatant. FAK silencing was also found to reduce PI3K phosphorylation at p85 in FN-treated cells indicating position of FAK upstream of PI3K in this signaling pathway (Figure 2D).

Effect of FN on Akt, NF-KB in PC-3 cells

Akt expression and its phosphorylation status at Thr-308 position remains almost same, but appreciably enhanced at Ser-473 position in FN-treated PC-3 cells than the untreated cells as found in western blot analysis (Figure 3A). Western blot of nuclear fraction isolated from FN-treated PC-3 cells shows presence of elevated level of NF- κ B than the untreated cells (Figure 3B). FNinduced MMP-9 gelatinolytic activity was considerably decreased in cells treated with NF- κ B inhibitor (BAY-11-7085) before growing on FN (Figure 3C).



Figure 2. Effect of FN on FAK and PI3K, and their involvement in FN-induced MMP-9 activity: A. PC-3 cells, untreated (Lane 1) or treated with PI-3K inhibitor LY 294002 (Lane 2); ERK inhibitor PD 98059 (Lane 3) and p38 inhibitor SB 203580 (Lane 4), were grown in presence of FN and gelatin zymography of the culture supernatants were performed. B. PC-3 cells were grown in SFCM in absence (0) and in presence of 10 μ g/ml FN for 4 h. Equal amounts of proteins (100 μ g) of cell extracts were subjected to western blot analysis with anti-FAK, anti-p-FAK, anti-p-FAK, anti-p-PI3K antibodies. β -tubulin was used as internal control. C. PC-3 cells were grown on coverslips in absence (Control) and presence of FN (10 μ g/ml) for 1, 2, 4 h. Immunocytochemistry was preformed with anti-FAK (1 μ g/ml dilution) primary and respective FITC-labeled secondary antibody (1 μ g/ml dilution). White arrows and yellow arrows show localization of FAK at focal points and nucleus, respectively. D. PC-3 cells were transfected with control and FAK siRNA (60 nM each) followed by FN treatment and then culture supernatant was subjected to zymography (d) and cell extracts were subjected to western blot analysis of FAK (a), p-PI3K (b). β -tubulin (c) was used as internal control for western blot. Lane 1 is control lane without FN or siRNA treatment, Lane 2 denotes treatment of FN (10 μ g/ml for 4 h) without siRNA treatment, Lane 3 is with FN and FAK siRNA treatment, Lane 4 is with FN and control siRNA treatment.

Effect of FN on migration, invasion, and CD-44 surface expression of PC-3 cells

Photographs taken at 0, 15, and 24 h during woundhealing assay of control (-FN) and FN-treated PC-3 cells indicate that cells in presence of FN are able to migrate and heal experimentally scratched wounds more efficiently than the cells in absence of FN can do (Figure 4A). When control and FN-treated PC-3 cells were seeded on the membrane of transwell inserts, the FN-treated cells were observed to migrate more efficiently through the membrane after 24 and 48 h (Figure 4B). In FN-treated PC-3 cells, CD-44 molecules were observed to be localized throughout the surface making the cell periphery darker and more prominent than the untreated control cells (Figure 4C).

Effect of FN on E-cadherin, VEGF, ILK

PC-3 cells were grown without (0) or with 10 μ g/ml FN for 4 h and cell lysates were subjected to western blot assay, which shows downregulation of E-cadherin



Figure 3. Effect of FN on Akt, NF-κB: A. PC-3 cells were grown in SFCM in absence (0) and in presence of 10 µg/ml FN for 4 h. Equal amounts of protein (100 µg) of cell extracts were subjected to western blot analysis with anti-Akt, anti-p-Akt (Thr-308), anti-p-Akt (Ser-473) antibodies. β-tubulin was used as internal control. B. Nuclear and cytoplasmic extracts of control (0) and FN-treated (10 µg/ml) cells were subjected to western blot analysis with anti-NF-κB antibody. TBP was used as internal control for nuclear proteins. C. PC-3 cells, untreated (Control) or treated with NF-κB inhibitor BAY-11-7085 (5 µM for 24 h), were allowed to grow on FN (10 µg/ml for 4 h in SFCM). The culture supernatants were collected and gelatin zymography was performed.

Figure 4. Effect of FN on PC-3 cell migration, invasion and cell surface expression of CD-44: A. Wound-healing assay of PC-3 cells grown without (-FN) or with FN (+ FN). B. Transwell assay. C. Immunocytochemistry was preformed with anti-CD-44 (1 μ g/ml dilution) primary and respective biotin-labeled secondary antibody (1 μ g/ml dilution).

expression, upregulation of expression of VEGF and phosphorylation of ILK in FN-treated cells in comparison with the untreated cells (Figure 5).

Effect of FN on MMP-1 expression and associated cell signaling proteins

In western blot assay of cell lysates of PC-3 cells, both the pro (52 kDa) and active form (43 kDa) of MMP-1 expressions were found to be upregulated in FN-treated (10 µg/ml for 4 h) cells in comparison with control cells, grown without FN (Figure 6A). Appreciable downregulation of FN-induced MMP-1 expression was observed in PC-3 cells, where α_5 integrin was masked (Figure 6B). FN-induced MMP-1 expression was considerably decreased with treatment of the cells with PI-3K inhibitor (LY 294002) and NF- κ B inhibitor (BAY-11-7085), whereas little or no appreciable effect was observed upon treatment with ERK inhibitor (PD 98059) (Figure 6C).

DISCUSSION

Many physiological processes viz wound healing, tissue remodeling, angiogenesis, embryo development, etc. are mediated by regulated degradation of ECM proteins by MMPs (Yan & Boyd, 2007). A loss of activity control of MMPs, which are precisely regulated at the level of transcription, activation of the precursor zymogens, interaction with specific ECM components, and inhibition by endogenous inhibitors, may result in a range of diseases including cancer (Visse & Nagase, 2003). Our interest is to study the effect of tumor cell–ECM interaction and its downstream signaling that control the expression and activity of MMPs in various cancer cell lines (Das et al., 2008; Sen et al., 2010; Maity et al., 2011a,b). In the pres-

0

E-cadherin

VEGF

ILK

p-ILK

β-tubulin

FN 4 hrs

10

µg/ml

120 kC

- 42 kD

59 kD

← 59 kD

← 55 kD







Figure 6. Culture of PC-3 cells on FN-coated surface modulate MMP-1: A. PC-3 cells (300 000 cells/ml) were grown in SFCM in absence (0) and in presence of 10 µg/ml FN for 4 h. Equal amounts of protein (100 µg) of cell extracts were subjected to western blot analysis with anti-MMP-1 antibody. B. PC-3 cells, untreated or treated with anti- α_5 integrin antibody (1 µg/ml for 1 h), were allowed to grow in presence of FN (10 µg/ml for 4 h). Cell extracts (100 µg) were subjected to western blot analysis with anti-MMP-1 antibody. C. PC-3 cells, untreated (Lane 1) or treated with ERK inhibitor PD98059 (Lane 2), PI-3K inhibitor LY294002 (Lane 3) (50 µM each in SFCM for 1 h) and NF-κB inhibitor (Lane 4) BAY-11-7085 (5 µM for 24 h), were allowed to grow in presence of FN (10 µg/ml for 4 h). Cell extracts (100 µg) were subjected to western blot analysis with anti-MMP-1 antibody. C. PC-3 cells, untreated (Lane 1) or treated with ERK inhibitor PD98059 (Lane 2), PI-3K inhibitor LY294002 (Lane 3) (50 µM each in SFCM for 1 h) and NF-κB inhibitor (Lane 4) BAY-11-7085 (5 µM for 24 h), were allowed to grow in presence of FN (10 µg/ml for 4 h). Cell extracts (100 µg) were subjected to western blot analysis with anti-MMP-1 antibody. β-tubulin was used as internal control.

ent study highly metastatic prostate adenocarcinoma cell line PC-3 is found to bind ECM protein FN in dosedependent manner. As a result of this adhesion-mediated signaling expression and activity of MMP-9 and MMP-1 are enhanced appreciably.

The pathways involving the FN-induced upregulation of MMP-9 and MMP-1 in PC-3 cell line were not fully elucidated. To decipher it we initially studied α_{ϵ} integrin, which is normally expressed in these cells but upregulated further in presence of FN. This is consistent with our previous finding in a different cell line (Sen et al., 2010). Masking of integrin α_5 drastically reduce the MMP-9 expression. In earlier reports $\alpha_{5}\beta_{1}$ integrin, receptor for FN, was shown to play major role in FNmediated downstream signaling leading to upregulation of MMPs in different other cell lines (Das et al., 2008; Jia et al., 2004; Maity et al., 2010; Pal et al., 2012; Sen et al., 2010). Integrins activate various protein tyrosine kinases including FAK, which is an important component of focal adhesion. Localization of FAK at focal adhesion points in presence of FN was observed in our study. Upon activation, FAK is phosphorylated at Tyr-397 (Giancotti & Ruoslahti, 1999) and is evident in the current study in presence of FN. Activation of FAK is coupled to assembly of focal adhesions, which plays major role in cell attachment and migration (Gilmore & Romer, 1996; Mitra et al., 2005). In our study, FN-treated cells showed enhanced migration. FAK is also a potent regulator of MMP-9 expression and activity (Ganguly et al., 2012; Sein et al., 2000). Silencing the FAK by siRNA appreciably reduced gelatinolytic activity of FN-induced MMP-9. Thus FAK may play a very pivotal role in FN-induced upregulation of MMP-9 in PC-3 cells. Apart from these, FAK may have some other effects also in these cells. Some recent findings implicate involvement of FAK in regulating gene expression directly (Golubovskaya et al., 2009). Nuclear translocation of FAK (in addition to its localization to the focal complex), in presence of FN in our study indicatates possibility of its additional involvements in gene regulation, but further research is needed in this field.

Among the downstream cellular signaling targets of activated FAK, PI3K is found to be phosphorylated at its p85 regulatory subunit in response to FN treatment in our study. The major autophosphorylation site of FAK (Tyr-397) is one of the binding sites for the SH2 domains of p85 and is responsible for the in vivo association of FAK with PI3K (Chen et al., 1996). Inhibition of PI3K downregulates MMP-9 activity in FN-treated PC-3 cells. Silencing FAK also reduced PI3K phosphorylation and MMP-9 activity. These may indicate position of FAK upstream of PI3K in the FN-induced FAK/PI3K signaling leading to MMP-9 upregulation.

The serine threonine kinase Akt (Protein kinase B or PKB) is one of the downstream targets of PI3K (Corvera & Czech, 1998). Activation of Akt is thought to involve a conformational change and phosphorylation on two residues. The major phosphorylation site lies within the kinase domain activation loop (Thr 308). In addition, a second phosphorylation site in the C-terminus (Ser 473) is required for full or maximal activity. We observed that FN-upregulated phosphorylation of Akt (Ser 473) appreciably. This phosphorylation event may be done by ILK directly or indirectly by favoring PKB/Akt autophosphorylation (Persad et al., 2001). ILK is a Ser/Thr kinase that can interact with the cytoplasmic domains of β_1 integrins

and couple integrins and growth factor receptors to downstream signaling pathways promoting suppression of apoptosis, cell survival, cell migration, and invasion (Yoganathan et al., 2002). FN-induced upregulation of p-ILK, found in our study, may also contribute to activation of Akt in presence of FN in PC-3 cells in addition to FAK/PI3K. PI3K-Akt signals control several transcription factors, including NF-KB (Bader et al., 2005). In human cancer cells, NF-KB positively regulates cell survival, proliferation, chemoresistance, angiogenesis, cellular invasion, and oncogenesis (Karin et al., 2002; Nakanishi & Toi, 2005). NF- κ B is negatively regulated by I κ B, which binds NF- κ B in the cytoplasm. Positive regulation occurs through IKB kinase (IKK), which may be activated by active Akt. When IKK phosphorylates IkB, NF-kB is released and translocated to the nucleus to form a transcriptional activator of several regulatory proteins (Kane et al., 1999). The present work shows that, FN treatment induces nuclear translocation of NF-KB in PC-3 cells and NF-kB inhibition reduces FN-induced MMP-9 activity. In several cell lines MMP-9 upregulation was found to be dependent on transcriptional modulation of MMP-9 gene, requiring involvement of NF-kB (Tai et al., 2008). Taken together these informations indicate that, NF-KB may be an essential component in FN-mediated signaling in PC-3 cells and possibly involved in transcriptional modulation to upregulate MMP-9 level. Comparative RT-PCR assay indicates increase in MMP-9 gene expression and decrease in TIMP-1 gene expression, which is a negative regulator of MMP-9, in FN-treated PC-3 cells and cumulatively exhibits enhanced gelatinolytic activity of MMP-9.

Effect of upregulated MMP-9 expression is enhanced by its increased activation, which is seemed to be mediated by CD-44 because of its elevated cell-surface localization in FN-induced cells. CD-44 can function as docking molecule for MMP-9 activity and help in cell migration (Karadag et al., 2005; Samanna et al., 2007). Reduction of surface level of CD-44 reduces MMP-9 activity, whereas MMP-9 knocked down PC-3 cells show reduced CD-44 at cell surface. Hence CD-44 surface expression and MMP-9 activation on cell surface are interdependent (Desai et al., 2009). Treatments that enhance surface localization of CD-44, which is FN induction in our study, should favor enhanced MMP-9 activity. Such activity may become crucial for cell invasion and metastasis through degradation of the basement membrane required for tumor cell intravasation or extravasation. This fact is further supported by the transwell assay.

It has been reported that $\alpha_5\beta_1$ integrin-mediated signaling upregulate MMP-1 in human breast cancer (Jia et al., 2004). In PC-3 cells we have observed appreciable upregulation of MMP-1 expression and activity in FNinduced cells, but not in α_5 integrin masked cells. Interestingly, inhibition of PI3K and NF- κ B have downregulated FN-induced MMP-1 expression indicating similar pathway of MMP-9 regulation may be involved in modulation of MMP-1 activity. We extended our study to find the effect of FN-induced signaling on some of the key proteins which play important roles in angiogenesis and metastasis, thereby cancer progression namely VEGF, E-cadherin. VEGF is known to be a potent inducer of angiogenesis. The switch from vascular quiescence to angiogenesis involves MMP-9, which is upregulated in angiogenic islets and tumors, rendering VEGF more available to its receptors (Bergers et al., 2000). MMP-9 is capable to release biologically active VEGF from the ECM of cancer cells (Hawinkels et al., 2008). In our system we have showed upregulation of both MMP-9 and VEGF in response to FN, drawing a possible connection between FN-mediated signaling and induction of angiogenic switch. E-cadherin is a calcium-dependent cell-cell adhesion molecule which with its cytoplasmic tail forms a powerful invasion suppressor complex (Noë et al., 2001). In ovarian cancer, loss of E-cadherin followed by upregulation of α_{s} integrin was found to enhance metastasis (Sawada et al., 2008). Downregulation of E-cadherin and upregulation of α_{s} integrin in presence of FN in our study may increase invasive property of cancer cells.

CONCLUSION

Therefore it may be concluded that, FN induces signaling through $\alpha_{s}\beta_{1}$ integrin in PC-3 cells and possibly mediates signaling through FAK/PI3K/Akt/NF-KB pathway leading to upregulation of MMP-9 and MMP-1. Apart from the FAK/PI3K signaling, full activation of Akt may require involvement of ILK. FN treatment induces cell-surface localization of CD-44, which may enhance MMP-9 activity. Upregulation of intracellular VEGF levels and downregulation of E-cadherin level in presence of FN may favor angiogenesis and metastasis, respectively. This model study may be implicated in broader sense to understand the role of tumor microenvironment in progression of prostate cancer and to provide scopes for future clinical interventions by targeting these signaling pathways of FN-induced MMP upregulation and invasion of prostate cancer.

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