Fibronectin Increases Matrix Metalloproteinase 9 Expression through Activation of c-Fos via Extracellular-regulated Kinase and Phosphatidylinositol 3-Kinase Pathways in Human Lung Carcinoma Cells

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Enhanced expression of matrix metalloproteinase-9 (MMP-9) is associated with human lung tumor invasion and/or metastasis. We have demonstrated that fibronectin (FN), a matrix glycoprotein, stimulates human non-small cell lung carcinoma (NSCLC) cell proliferation. The current study examines the effect of FN on MMP-9 expression in NSCLC cells. We show that FN increases MMP-9 protein, mRNA expression, and gelatinolytic activity in NSCLC cells. The integrin α5β1 mediated the effects of FN because α5 small interfering RNA blocked FN-stimulated MMP-9 protein expression, and abrogated FN-induced phosphorylation of ERK and phosphatidylinositol 3-kinase (PI3K) signals. The inhibitors of ERK, PD98095, and of PI3K, wortmannin, blocked MMP-9 expression and increased the expression of the AP-1 subunit c-Fos protein, whereas AP-1 inhibitor, nordihydroguaiaretic acid, and a c-Fos small interfering RNA eliminated the effect of FN on MMP-9 expression. This study indicates that binding to the integrin α5β1 receptor, stimulates the expression of MMP-9 through increased AP-1/DNA binding and c-Fos protein expression via ERK and PI3K signaling pathways. The data unveils a novel mechanism by which FN could promote NSCLC cell invasion and metastasis.

Matrix metalloproteinases (MMPs)2 are a family of zinc enzymes responsible for degradation of extracellular matrix components including basement membrane collagen, interstitial collagens, fibronectin, and various proteoglycans during normal remodeling processes (1, 2). The potent proteolytic activity of MMPs is mainly regulated by the combination of tissue inhibitors of matrix metalloproteinases (TIMPs) and by appropriate expression of their inhibitors. The process of tissue destructive remodeling has been implicated in the pathogenesis of tissue destructive disorders including lung disorders and cancers. MMP-9 is expressed in response to chronic lung diseases (2, 4). In addition, MMPs have been implicated in the pathogenesis of chronic obstructive pulmonary disease, like bronchial asthma, chronic obstructive pulmonary disease, and interstitial lung disease (2, 3). MMPs are also involved in cancer and various inflammatory conditions (2). MMP-9 expression is increased in malignant cancers when compared with benign or non-invasive ones, and there is compelling in vitro and in vivo evidence for a role of MMP-9 in tumor progression and angiogenesis (6, 7). Its dramatic overexpression in cancer and various inflammatory conditions suggest that this protease is a potential target for the development of novel therapeutic interventions (8).

Because of its perceived importance, our research focuses on the factors that increase MMP-9 expression in the lung. Our search led us to FN, a matrix glycoprotein highly expressed in tobacco-related lung disease that has been shown to stimulate lung carcinoma cell growth through several mechanisms (9–11). Cell adhesion to FN results in MMP secretion in normal and tumoral cells (12–14). These studies suggest that tumor cell interactions with FN might lead to MMP-9 expression thereby promoting cancer cell migration, invasion, and related processes. However, the mechanisms by which FN stim-

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2 The abbreviations used are: MMP, matrix metalloproteinase; ERK, extracellular-regulated kinase; FN, fibronectin; NDGA, nordihydroguaiaretic acid; CRE, cyclic AMP response element; JNK, c-Jun NH2-terminal kinase; mTOR, mammalian target of rapamycin; RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; siRNA, small interfering RNA.
ulates MMP-9 expression in tumor cells have not been explored in detail. Here, we report that FN, by binding to its α5β1 integrin receptor, stimulates MMP-9 expression through activation of extracellular-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) dual signaling pathways followed by induction of extracellular-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) dual signaling pathways followed by induc-

**MATERIALS AND METHODS**

* Cultures and Chemicals—The human non-small cell lung carcinoma cell lines H1838 and H2106 were obtained from American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, HEPES buffer, 50 IU/mL penicillin/streptomycin, and 1 μg/mL of amphotericin (complete medium) (15). Afterward, cells were harvested and replated in serum-free medium on FN-coated culture plates for experiments described later. [methyl-3H]Thymidine and poly(dI-dC) were purchased from Amersham Biosciences. [γ-32P]ATP was purchased from PerkinElmer Life Sciences, Inc. Collagen type 1 and polyclonal antibodies against the integrin α5 (H-104) and MMP-9 (H129) were purchased from Santa Cruz Biotechnology, Inc. Polyclonal antibodies specific for Akt, ERK1, ERK2, and their phosphorylated forms (p-Akt Thr308, p-ERK1/2, Thr202/Tyr204) and rapamycin were purchased from Cell Signaling Inc. (Beverly, MA). The AP-1 inhibitor, nordihydroguaiaretic acid (NDGA), the PI3K inhibitor wortmannin (10), JNK inhibitor SP600125, were obtained from Calbiochem. Gelatin zymography was performed by using a 9% SDS-PAGE gel saturated with 1 mg/mL gelatin (Sigma, 300 bloom) as previously described (16). Samples with equal protein concentration (10 μg/mL) were loaded onto the gel and electrophoresed at a constant 150 V for 1.5 h. The gels were stained or replaced by rabbit IgG. Blots were incubated with polyclonal antibodies raised against the protein kinase A inhibitor H89, and the c-Jun NH2-terminal kinase (JNK) inhibitor SP600125, followed by an overnight incubation at room temperature in 2.5% Triton X-100, and electrophoresed at a constant 150 V for 1.5 h. The gels were dried onto cellophane and scanned under a densitometer for determination of gelatinolytic activity.

**RT-Polymerase Chain Reaction**—Total RNA was prepared from human lung carcinoma cells treated with FN or collagen type 1 (20 μg/mL each) using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. To amplify the 590-bp MMP-9 and 200-bp GAPDH cDNA fragments, the sequences of PCR primers (Sigma Genosys, Woodlands, Texas) were as follows: for MMP-9 promoter, 5’-CAGCTGCCACCCCCCT-CAGACC, and antisense, 5’-GCACATTGCGGCCGATA-AGG; and for GAPDH sense, 5’-CCATGGAAGAGGCTGGG, and antisense, 5’-CAGAGTGACATGATGCC, according to published data (15, 17). The RT-PCR was carried out as previously described (15). The samples were first denatured at 95 °C for 30 s, followed by 32 PCR cycles, each with temperature variations as follows: 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The last cycle was followed by an additional extension incubation of 7 min at 72 °C. Analysis of amplicons was accomplished on 1% agarose gel containing 0.2 μg/μL ethidium bromide and visualized under a UV transilluminator.

**Real-time RT-PCR**—This procedure was described previously (15). Briefly, total RNA was prepared from H1838 cells with TRIzol reagent according to the manufacturer’s instructions. Final results were expressed as n-fold differences in MMP-9 gene expression relative to the GAPDH gene. All PCR using the LightCycler-FastStart DNA Master SYBR Green I kit were performed in the Cepheid Smart Cycler real-time PCR cycler (Sunnyvale, CA) (15). Experiments were performed in triplicate for each data point.

**Western Blot Analysis**—The procedure was performed as previously described (18). Protein concentrations were determined by the Bio-Rad DC protein assay. Equal amounts of protein from whole cell extracts were solubilized in 2× SDS sample buffer (imidazole, pH 6.8, 4% SDS, 20% glycerol, 5–10% β-mercaptoethanol, 0.04% bromphenol blue), and separated on SDS-8% polyacrylamide gels. The separated proteins were blotted to nitrocellulose using a Bio-Rad TransBlot semidry transfer apparatus for 1 h at 25 volts, blocked with 5% nonfat dried milk in TBS and 0.1% Tween 20). Afterward, the blots were washed three times with TBS and incubated with a secondary antibody raised against rabbit IgG conjugated to horseradish peroxidase (1:2000, Cell Signaling) for 1 h at room temperature. The blots were washed, transferred to pre-made ECL solution (Amersham Biosciences) for 1 min and exposed to x-ray film. Protein bands were quantified by densitometric scanning using a Bio-Rad GS-800 calibrated densitometer. In controls, the antibodies were omitted or replaced by rabbit IgG.

**Plasmids**—The 0.67-kb fragment of the MMP-9 promoter construct, together with two deletion (−599 and −90) and three mutation constructs (AP-1 mutant: TGAGTCA to TTTGTCA; NF-κB mutant GGAATTCCCCC to TAAATTCCCCC; Sp-1 mutant CCGCCC to CCAACC) ligated to the firefly luciferase reporter gene has been reported previously (19). −599 and −90 fragments were inserted to the XbaI site of pGL3-SXI. Synthetic Renilla Luciferase Reporter Vector (pGL3-SV40) was obtained from Promega.

**Transient Transfection Assays**—Human NSCLC cells were seeded at a density of 1 × 105 cells/well in 6-well dishes and grown to 60% confluence. For each well, the plasmid DNA (1 to 2 μg) containing wild-type, or deleted or mutated MMP-9 promoter constructs, and 0.2 μg of the internal control plasmid phRL-SV40 (Renilla luciferase gene) were cotransfected into the cells using FuGENE 6 lipofection reagent as described in
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Figure 1. The effect of FN on MMP-9 expression in human lung carcinoma cells. A, dose-dependent effect of FN on MMP-9 protein and mRNA expression. B, time-dependent effect of FN on MMP-9 protein and mRNA expression. C, dose-dependent effect of FN on MMP-9 mRNA expression. D, effect of FN on MMP-9 mRNA expression as determined by real-time RT-PCR. Each bar graph represents the mean ± S.D. of three separate experiments. * indicates a significant difference from control (p < 0.05).

Figure 2. Western blot analysis of MMP-9 expression in H1838 cells exposed to FN. A, gelatin zymograph analysis of MMP-9 activity. B, Western blot analysis of MMP-9 protein expression. Each blot represents the mean ± S.D. of three separate experiments. * indicates a significant difference from control (p < 0.05).

Figure 3. Electrophoretic Mobility Shift Assay (EMSA) of CRE-decoy oligonucleotide binding to nuclear extracts from H1838 cells exposed to FN. A, gelatin zymograph analysis of MMP-9 activity. B, Western blot analysis of MMP-9 protein expression. Each blot represents the mean ± S.D. of three separate experiments. * indicates a significant difference from control (p < 0.05).

Figure 4. Dose-dependent effect of FN on MMP-9 mRNA expression. A, Gelatin zymograph analysis of MMP-9 activity. B, Western blot analysis of MMP-9 protein expression. Each blot represents the mean ± S.D. of three separate experiments. * indicates a significant difference from control (p < 0.05).

Reagents and Materials
- Firefly luciferase activity was measured using the Dual Luciferase Reporter Kit according to the manufacturer's instructions. The NF-κB, Sp1, and AP-1 oligonucleotides were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase as recommended by the manufacturer (Promega). Fifty microliters of nuclear proteins from control or treated cells were mixed with 50 µl of a solution containing 20 pmol of unlabeled consensus oligonucleotide and 10,000 cpm of [32P]ATP and incubated for 20 min at room temperature. After incubation, the samples were electrophoresed on a 4% native polyacrylamide gel containing 8% Tris glycine buffer. The gel was then dried and subjected to autoradiography.

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Introduction
Fibronectin (FN) is a multifunctional extracellular matrix protein that plays a critical role in cell adhesion, migration, and proliferation. Recent studies have suggested that FN also regulates the expression of matrix metalloproteinases (MMPs), which are involved in the degradation of extracellular matrix proteins.

Materials and Methods
- Human lung carcinoma cells (H1838) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were seeded at 0.25–1 × 10^5 cells/well in 6-well plates containing 0.5 mg/ml of FN or collagen type 1 (20 µg/ml each)-coated culture plates and grown to 70% confluence.

Results
- The effect of FN on MMP-9 expression was investigated using real-time RT-PCR. Total RNA was isolated from H1838 cells exposed to increased concentrations of FN (20 µg/ml) for 24 h. The MMP-9 mRNA levels were determined using quantitative RT-PCR with GAPDH as an internal control.

Discussion
- Our results suggest that FN induces the expression of MMP-9 in human lung carcinoma cells. The mechanism of this induction is not fully understood, but it is likely to involve the activation of signal transduction pathways that regulate MMP-9 expression.

Conclusion
- The induction of MMP-9 by FN in human lung carcinoma cells provides new insights into the role of FN in cancer progression.

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manufacturer’s instructions. Briefly, Lipofectamine 2000 reagent was incubated with serum-free medium for 10 min. Subsequently, a mixture of respective siRNA was added. After incubation for 15 min at room temperature, the mixture was diluted with culture medium and added to each well. The final concentration of siRNA in each well was 100 nM. After culturing for 30 h, cells were washed, resuspended in new culture media, and were exposed to the culture plates coated with FN for an additional 24 h. Afterward, cells were harvested by Western blot, transfection luciferase activity assays, and [H³]thymidine incorporation assays.

Statistical Analysis—All experiments were repeated a minimum of three times. All data collected from electrophoresis gel mobility shift assays, luciferase activity assays, real-time RT-PCR, and Western blot were expressed as mean ± S.D. The data presented in some figures are from a representative experiment, which was qualitatively similar in the replicate experiments. Statistical significance was determined with Student’s t test (two-tailed) comparison between two groups of data sets. Asterisks shown in the figures indicate significant differences of experimental groups in comparison with the corresponding control condition (p < 0.05, see figure legends).

RESULTS

FN Stimulates MMP-9 Gelatinolytic Activity and Gene Expression in Human NSCLC Cells—We began by exploring the effects of FN on MMP-9 gelatinolytic activity and protein expression in an analogous manner with molecular and cell biological tools used to characterize the role of FN in other cell systems including human lung carcinoma cells (7, 15, 22). FN had no effect on FN-induced MMP-9 (Fig. 2B). FN was also shown to affect several kinases including ERK and PI3K/Akt in H1838 cells (7, 15, 25). We found that FN on MMP-9 mRNA levels was confirmed by real-time RT-PCR (Fig. 3A). Similar results were obtained with H2106 cells (not shown), indicating that collagen type 1 had no effect. The FN-induced MMP-9 protein expression was also stimulated MMP-9 protein levels were evaluated. As shown in Fig. 3, we found that H1838 cells transfected with the wild-type MMP-9 promoter construct had no effect on induction of MMP-9 protein expression in α5 silenced cells, whereas control siRNA had no effect on FN-induced MMP-9 (Fig. 2A). FN has been shown to affect several kinases including ERK and PI3K/Akt in cell systems including human lung cancer cells (7, 15, 22). Here, we show that FN-induced phosphorylation of ERK1/2 (Fig. 2B) and Akt (Fig. 2C) were abrogated in α5 silenced NSCLC cells. This, together with the finding above, suggested that the α5 integrin signal was critical in mediating the effect of FN.

We then tested whether regulation of MMP-9 protein and activity by FN was mediated by ERK and PI3K. We found MMP-9-related gelatinolytic activity present in conditioned medium with maximal stimulation at 20 ng/ml in 24 h as shown. Note that collagen type 1 had no effect. This, together with the finding above, suggested that the α5 integrin signal was critical in mediating the effect of FN.

The effects of FN on MMP-9 gelatinolytic activity and gene expression are shown in Fig. 2A and 2B. FN increases MMP-9 gelatinolytic activity and gene expression in a dose-dependent manner as assessed by RT-PCR, and Western blot analysis with antibodies against MMP-9 and actin proteins.

FN Increases MMP-9 Promoter Activity—We next examined whether the effects of FN on MMP-9 expression occur at the transcriptional level. The MMP-9 promoter constructs used contain multiple transcription factor binding sites including NF-κB, Sp1, and AP-1 (Fig. 4, A and B). These sites have been previously identified as a role for the mTOR signal cascade in mediating the effect of FN on NSCLC cell growth (9). However, we found that rapamycin, an inhibitor of mTOR, had no effect on basal MMP-9 expression (Fig. 3A) and mRNA expression (Fig. 3D). In contrast, the inhibitor of Rho kinase, Y27632, and that of protein kinase A, H89, did not affect FN-induced MMP-9 protein and mRNA expression (Fig. 3, D–F). We previously identified a role for the mTOR signal cascade in mediating the effect of FN on NSCLC cell growth (9). However, we found that rapamycin, an inhibitor of mTOR, had no effect on induction of MMP-9 expression in α5 silenced cells, whereas control siRNA had no effect on FN-induced MMP-9 (Fig. 2A). FN has been shown to affect several kinases including ERK and PI3K/Akt in cell systems including human lung carcinoma cells (7, 15, 22). Here, we show that FN-induced phosphorylation of ERK1/2 (Fig. 2B) and Akt (Fig. 2C) were abrogated in α5 silenced NSCLC cells. This, together with the finding above, suggested that the α5 integrin signal was critical in mediating the effect of FN.

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was still observed with one MMP-9 deletion construct (−90/ +34 bp). However, there was a decreased response to FN with the smallest MMP-9 deletion construct (−73/+34 bp) (Fig. 4C). By using site-directed mutated MMP-9 promoter constructs connected to the luciferase reporter gene in which the NF-κB, Sp1, and AP-1 sites were separately mutated, we found that FN had no effect on MMP-9 promoter activity when the AP-1 site was mutated, whereas FN-induced activity was detected when the other two were tested (Fig. 4D). This suggests the involvement of AP-1 in mediating the stimulatory effect of FN on MMP-9 expression. Similar results were obtained with H2106 cells (not shown).

AP-1, but Not NF-κB or Sp1 Sites in the MMP-9 Promoter, Is Involved in FN-induced MMP-9 Gene Expression—To further explore FN regulation of MMP-9 promoter activity, electrophoretic mobility shift assays were performed to identify the transcription factors regulated by FN. As shown in Fig. 5, H1838 cells exposed to FN (20 μg/ml) for 24 h showed a significant increase in AP-1 DNA binding (A), whereas very little effect on Sp1 (B) and NF-κB (C) nuclear protein binding activities was noted when compared with solvent controls. In contrast, collagen type 1 (20 μg/ml) had no effect. The specific bands for AP-1, Sp-1, or NF-κB were attenuated by a 100-fold molar excess of unlabeled consensus oligonucleotides, but were not inhibited by the mutated unlabeled oligonucleotide (−34 bp) (Fig. 5). Synthetic double-stranded phosphorothioate oligonucleotides containing mutated AP-1 (Mut AP-1, Mut Sp1), NF-κB (Mut NF-κB) sites were end labeled with [γ-32P]ATP and used as another control to specificity. The addition of anti-c-Fos supershift band, whereas an anti-c-Jun antibody had a lesser effect (Fig. 5D). The natural product, NDGA, has been shown to supershift band, whereas an anti-c-Jun antibody had a lesser effect (Fig. 5D). The natural product, NDGA, has been shown to inhibit binding of the transcription factors to DNA and promote growth inhibition and alter gene expression. The CRE oligonucleotides have been shown to inhibit CRE- and AP-1-directed gene transcription and promote growth inhibition in vitro and in vivo in a broad spectrum of cancer cells (25). Using CRE-decoy oligonucleotides transfected into cells, we found that the CRE palindromic oligonucleotides eliminated the FN-induced AP-1 DNA binding (Fig. 6C) and MMP-9 promoter activity (Fig. 6D), whereas the control CRE oligonucleotides had no effects. This suggests that the CRE-decoy competed with AP-1 and interfered with FN-induced AP-1/DNA binding and MMP-9 promoter activity. Similar results were obtained with H2106 cells (not shown).

c-Fos, but Not c-Jun, Mediates the Effects of FN on MMP-9 Expression—In Fig. 5D, we showed that FN stimulates c-Fos protein. Next, we found that FN increased nuclear c-Fos protein expression in a time-dependent manner (Fig. 7, A and B). The inhibitor of ERK1/2 blocked FN-induced c-Fos mRNA (Fig. 7D). To determine the effects of FN on c-Jun, we found that NDGA increased c-Jun expression (Fig. 7D). To determine the effects of FN on the CRE DNA binding, we investigated the promoter activity, c-Fos siRNA eliminated the CRE binding (Fig. 7E), but not the CRE binding (Fig. 7F). NDGA had no effect on FN-induced MMP-9 promoter activity (Fig. 7F).

Figure 3. Involvement of PI3K, ERK, and mTOR in FN-induced MMP-9 activity. Effect of inhibitors of ERK1/2 and PI3K on FN-induced MMP-9 activity. Total RNA was isolated from H1838 cells incubated for up to 2 h in the presence or absence of PD98059 (25 μM) and used as another control to confirm the binding to DNA. GAPDH served as internal control for normalization purposes. *, denotes significant differences from control (p < 0.05). Con, indicates untreated control cells. D, effect of protein kinase A and Rho kinase inhibitors on FN-induced MMP-9 gelatinolytic activity and protein. Culture medium and cellular protein were isolated from H1838 cells incubated for up to 2 h in the presence or absence of PD98059 (25 μM) before exposure of the cells to FN-coated culture plates for an additional 24 h, then subjected to gelatin zymograph for gelatinolytic activity and Western blot analysis for MMP-9 protein. F, effect of PD98059 or wortmannin (100 nM) on FN-induced MMP-9 promoter activity. Total RNA was isolated from H1838 cells cultured for up to 2 h in the presence of PD98059 (25 μM) or wortmannin (100 nM) before exposure of the cells to FN-coated culture plates for an additional 24 h, followed by real-time PCR analysis. GAPDH served as internal control for normalization purposes. **, indicates significance of combination treatment with FN alone (p < 0.05). Cor, indicates untreated control cells. D, effect of protein kinase A and Rho kinase inhibitors on FN-induced MMP-9 gelatinolytic activity and protein. Culture medium and cellular protein were isolated from H1838 cells incubated for up to 24 h in the presence or absence of PD98059 (25 μM) or wortmannin (100 nM) before exposure of the cells to FN-coated culture plates for an additional 24 h, then subjected to gelatin zymograph for gelatinolytic activity and Western blot analysis for MMP-9 protein. H, effect of mTOR inhibitor on FN-induced MMP-9 mRNA levels. Total RNA was isolated from H1838 cells cultured for up to 24 h in the presence or absence of rapamycin (10 nM), before exposure of the cells to FN-coated culture plates for an additional 24 h, and then subjected to gelatin zymograph for gelatinolytic activity and Western blot analysis for MMP-9 protein. J, effect of mTOR inhibitor on FN-induced MMP-9 mRNA levels. Total RNA was isolated from H1838 cells incubated for up to 24 h in the presence or absence of rapamycin (10 nM) before exposure of the cells to FN-coated culture plates for an additional 24 h, followed by real-time PCR analysis. GAPDH expression was evaluated as internal control for normalization purposes. *, denotes significant differences from control (p < 0.05). J, effect of inhibitors of JNK on FN-induced MMP-9 protein. Cellular protein was isolated from H1838 cells incubated for up to 2 h in the presence or absence of SP600125 (10 μM) before exposure of the cells to FN-coated culture plates for an additional 24 h, then subjected to Western blot analysis for MMP-9 protein. Actin served as internal control for normalization purposes. K, effect of JNK inhibitor on FN-induced MMP-9 mRNA levels. Total RNA was isolated from H1838 cells cultured for up to 24 h in the presence or absence of SP600125 (10 μM) before exposure of the cells to FN-coated culture plates for an additional 24 h, and then subjected to RT-PCR analysis. GAPDH expression was evaluated as internal control. Cor, indicates untreated control cells.
MMP-9 expression in cancer cells is an independent prognostic factor in operable NSCLC, so MMP-9 may be considered as a target for adjuvant anticancer therapy in operable NSCLC using selective MMP inhibitors with high specificity for MMP-9. MMP-9 inhibitors and an adenosine-mediated transfer of antisense MMP-9 have been shown to inhibit invasion, angiogenesis, growth, and metastasis in NSCLC cells (29, 30). In human lung cancer cells, high expression of MMP-9 is associated with growth, metastasis, and progression, and blockade of MMP-9 has been shown to control lymph node metastasis and prolong the life span of lung cancer patients (29–32). At present, the mechanisms responsible for the regulation of MMP-9 in tumors remain incompletely elucidated.

In this study, we show that the matrix component FN increased the expression and gelatinolytic activity of MMP-9 in NSCLC cells. FN is a heterodimeric extracellular matrix glycoprotein implicated in a number of physiological events during embryogenesis, angiogenesis, thrombosis, inflammation, and tumor invasion (33, 34). The adhesion of lung carcinoma cells to FN enhances cell survival and confers resistance to apoptosis induced by chemotherapeutic agents (35). FN has been shown to affect invasion and gene expression in several cancers (36, 37). For example, monocyte chemotactic protein-1 (MCP-1) mediated by binding to its receptor CCR2 on the cancer cell surface enhances migration of carcinoma cells and blood monocytes (38). FN has been shown to activate several kinase signals in different cell systems (7, 15, 22, 39, 40). We previously demonstrated that this molecule increased NSCLC cell growth by activating the ERK and PI3K signals (9, 15). Both signal pathways are catalyzing the effects of FN on MMP-9 expression have been shown to be important in several other studies suggesting that activation of these dual signaling pathways is required for the FN-dependent activation of MMP-9 secretion and gene expression (14, 37, 41). Knockdown of Akt using siRNA also abrogated FN-induced MMP-9 expression (not shown).

The expression of MMP-9 is regulated by growth factors, hormones, cytokines, and cellular transformation. MMP-9 is highly regulated at three different levels: transcriptional regulation, activation of latent MMP-9, and inhibition of MMP-9 activity. Compared with that of control subjects, MMP-9 levels are significantly higher in the plasma of NSCLC patients (26). The enhanced expression of MMP-9 is associated with human lung cancer invasion and/or metastasis (26, 27). MMP-9 null mice showed an 81% reduction in Lewis lung carcinoma tumor as compared with wild-type controls (28). Homogeneous induced MMP-9 expression (Fig. 7G). Similar results were obtained with H2106 cells (not shown).

**DISCUSSION**

The expression of MMP-9 is regulated by growth factors, hormones, cytokines, and cellular transformation. MMP-9 is highly regulated at three different levels: transcriptional regulation, activation of latent MMP-9, and inhibition of MMP-9 activity. Compared with that of control subjects, MMP-9 levels are significantly higher in the plasma of NSCLC patients (26). The enhanced expression of MMP-9 is associated with human lung cancer invasion and/or metastasis (26, 27). MMP-9 null mice showed an 81% reduction in Lewis lung carcinoma tumor as compared with wild-type controls (28). Homogeneous
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![Figure 5. EMSA to determine DNA binding activities to FN.](image)

**A**. Effect of FN on DNA binding of AP-1 site. Oligonucleotides containing the AP-1 site were end labeled with [γ-32P]ATP and incubated with nuclear extracts (5 μg) from H1838 cells treated with FN- or collagen type 1 (20 μg/ml each)-coated culture plates for an additional 24 h. C. Effect of FN on DNA binding of Sp1 site. Oligonucleotides containing the Sp1 site were end labeled with [γ-32P]ATP and incubated with nuclear extracts (5 μg) from H1838 cells treated with FN- or collagen type 1 (20 μg/ml each)-coated culture plates for an additional 24 h. D. Effect of FN on DNA binding of NF-κB site. Oligonucleotides containing the NF-κB site were end labeled with [γ-32P]ATP and incubated with nuclear extracts (5 μg) from H1838 cells treated with FN- or collagen type 1 (20 μg/ml each)-coated culture plates for an additional 24 h.

**B**. The induction of MMP-9 by FN is mediated through its integrin receptor α5β1. We have found that blockade of this receptor eliminated the stimulatory effect of FN on MMP-9 expression. In contrast, the effects of FN on MMP-9 were not affected by blockade of Rho kinase, mTOR, and JNK signals, although these signals are involved in controlling cell migration, invasion, and gene expression, and in promoting NSCLC cell growth by FN (11, 43, 44, 45).

The function of FN is largely mediated through its integrin receptor α5β1. We have found that blockade of this receptor eliminated the stimulatory effect of FN on MMP-9 and on phosphorylation of ERK and PI3K/Akt signals suggesting α5β1 is critical in mediating the effect of FN on these processes. Blockade of this integrin receptor has been shown to affect FN-mediated cell adhesion, survival, proliferation, and gene expression (9, 46–48). Monocyte adhesion was abrogated in cells treated with specific neutralizing anti-α5β1 integrin monoclonal antibody (46). Antibodies against α5β1 have been shown to block the effect of FN on phosphorylation of p70 S6K, a downstream signal of mTOR (9). Antisense oligonucleotides to the integrin receptor subunit α5 decreased FN fragment-mediated cartilage chondrolysis (49). Chondroscarcoma-derived chondrocytic cell adhesion to FN was abolished by a blocking antibody against α5β1 integrin, but not by one against -αβ3 integrin, suggesting the specificity of this integrin in mediating many FN functions (50).

The MMP-9 promoter contains multiple transcription factor binding sites including NF-κB, Sp1, and AP-1. These sites have been shown to be differentially responsive to stimuli (23, 51). MMP-9 has been demonstrated to be regulated by gene transcription in various cell types (23, 52). To further FN-mediated induction of MMP-9, we investigated whether FN-mediated expression of this gene, we performed transfection experiments utilizing human MMP-9 promoter constructs connected to a reporter gene. We found that FN, not collagen type 1, increased MMP-9 promoter activity. Furthermore, we provide evidence in support of AP-1 controlling MMP-9 expression. Data obtained from different experimental models in vitro and in vivo indicate that the AP-1 protein functions as an important regulator of cell proliferation, differentiation, apoptosis, and transformation (53). AP-1 activity is regulated at the level of gene transcription in various stimuli (23, 51). MMP-9 has been shown to be differentially responsive to stimuli (23, 51). MMP-9 has been demonstrated to be regulated by gene transcription in various cell types (23, 52). To further FN-mediated induction of MMP-9, we investigated whether FN-mediated expression of this gene, we performed transfection experiments utilizing human MMP-9 promoter constructs connected to a reporter gene. We found that FN, not collagen type 1, increased MMP-9 promoter activity. Furthermore, we provide evidence in support of AP-1 controlling MMP-9 expression. Data obtained from different experimental models in vitro and in vivo indicate that the AP-1 protein functions as an important regulator of cell proliferation, differentiation, apoptosis, and transformation (53). AP-1 activity is regulated in a given cell by a broad range of physiological and pathological stimuli including cytokines, growth factors, stress signals, and infections, as well as oncogenic stimuli (54). Mutation of the AP-1 site in the MMP-9 promoter region severely diminishes the stimulatory effect of FN on MMP-9 expression; this indicates that increased AP-1 DNA binding activity is necessary for up-regulation of MMP-9 by FN. The role of the AP-1 site in regulation of MMP-9 expression has been reported by others in other systems (19, 55). Serum amyloid A-activating factor-1, a novel transcription factor, and the AP-1 family of proteins cooperatively regulate cytotoxic-mediated induction of MMP-9 in the resident cells of the joint capsule, whereas the mutation of these two elements results in severe reduction in cytokine responsiveness of the MMP-9 promoter activity (23). The ginseng saponin metabolite suppresses phorbol ester-induced matrix MMP-9 expression through inhibi-
The role of transcription factor AP-1 in FN induction of MMP-9

A, effect of AP-1 inhibitor on FN-induced MMP-9 protein expression. Cellular protein was isolated from H1838 cells treated with a chemical AP-1 inhibitor, NDGA (0.5 μM), for 24 h before exposure of the cells to FN (20 μg/ml)-coated culture plates for an additional 24 h. Afterward, Western blot analysis was performed using polyclonal antibodies against c-Fos and c-Jun. Effects of the AP-1 inhibitor on FN-induced c-Fos and c-Jun protein expression. Cellular protein was isolated from H1838 cells transfected with control c-Fos siRNA and mutant AP-1 (20 μg/ml)-coated culture plates for an additional 24 h. Cellular protein was isolated from H1838 cells treated with PD98095 (25 μM) and wortmannin (0.1 μM) for 2 h before exposure of the cells to FN (20 μg/ml)-coated culture plates for an additional 24 h. Afterward, Western blot analysis was performed using a polyclonal antibody against c-Fos.

The role of c-Fos in FN induction of MMP-9.

FIGURE 6. The role of transcription factor AP-1 in FN induction of MMP-9. A, effect of AP-1 inhibitor on FN-induced MMP-9 protein expression. Cellular protein was isolated from H1838 cells treated with a chemical AP-1 inhibitor, NDGA (0.5 μM), for 24 h before exposure of the cells to FN (20 μg/ml)-coated culture plates for an additional 24 h. Afterward, Western blot analysis was performed using polyclonal antibodies against c-Fos and c-Jun. Effect of blocking c-Fos signal on FN-induced MMP-9 protein expression. Cellular protein was isolated from H1838 cells transfected with control or c-Fos siRNA (100 nM each) for 30 h before exposure of the cells to FN (20 μg/ml)-coated culture plates for an additional 24 h, and then subjected to Western blot analysis for c-Fos protein. E, effect of blocking c-Jun signal on FN-induced MMP-9 protein expression. Cellular protein was isolated from H1838 cells transfected with control or c-Jun siRNA (100 nM each) for 30 h before exposure of the cells to FN (20 μg/ml)-coated culture plates for an additional 24 h, and then subjected to Western blot analysis for c-Jun and MMP-9 proteins. Actin served as internal control for normalization purposes. Con, indicates untreated control cells.
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Fibronectin
α5β1
Mak-1/Erk
PI3-K/Akt
NSCLC
AP-1 (c-Fos)
CRE-decoy
c-Fos siRNA
Nucleus
MMP-9 gene
Invasion Proliferation Metastasis

**FIGURE 8.** Schematic representation of signal pathways triggered in NSCLC in response to FN. By binding to its α5β1 integrin receptor, FN stimulates the expression of MMP-9 through increasing AP-1/DNA binding activity and c-Fos protein expression through activation of ERK and PI3K/Akt signaling pathways. This induces MMP-9 gene transcription and protein expression that may promote invasion, proliferation, and metastasis. The CRE-decoy abrogated the FN-induced AP-1/DNA binding and FN-enhanced MMP-9 promoter activity. Blockade of c-Fos expression alleviated the stimulatory effects of FN on MMP-9 promoter activity and protein expression.

Decoy oligonucleotides may provide a powerful new means of combating cancers, and other pathological conditions by regulating the expression of cAMP-responsive genes (25). Phenylephrine stimulated DNA binding activity of AP-1 and increased protein synthesis in cardiomyocytes of adult rats (58). Increased AP-1 binding activity by CRE-decoy abolished both of these growth responses (59). CRE-decoy oligonucleotide treatment of ovarian cancer cell growth and control CRE oligonucleotide ablated the stimulatory effect of FN (58). CRE-decoy abrogated the FN-induced AP-1/DNA binding and FN-enhanced MMP-9 promoter activity. These observations suggest that the CRE-decoy competed with AP-1 binding activity by CRE-decoy oligonucleotides blocked the FN-induced AP-1/DNA binding and FN-enhanced MMP-9 promoter activity. Blockade of c-Fos siRNA alleviated the effects of FN on MMP-9 promoter activity. In summary, our study indicates that FN, by binding to the integrin α5β1 receptor, stimulates the expression of MMP-9 through increasing AP-1/DNA binding activity and c-Fos protein expression that may promote invasion, proliferation, and metastasis. Inhibition of c-Fos expression alleviated the stimulatory effects of FN on MMP-9 promoter activity and protein expression.

We also found that the AP-1 subunits, in addition to their pro-apoptotic function, are also critically involved in survival signaling. c-Fos expression negatively correlates with increased neuronal cell death in the hippocampus during kainic acid-induced seizure, indicating an anti-apoptotic role for the protein in this scenario (60). Osteoblast binding to extracellular matrix proteins such as FN through integrins induces c-Fos and c-Jun expression via protein kinase C and phosphotyrosine kinase signaling pathways (61). FN is required for vascular endothelial growth factor-induced c-Fos induction, mitogenic response, and cell migration in T47D breast cancer cells (62). We found that the inhibitors of ERK, PD98095, of PI3K, wortmannin, and of AP-1, NDGA, blocked FN-induced c-Fos protein expression. Cellular interactions with the extracellular matrix play an important role in activating ERK and c-Fos-dependent processes. There is a positive correlation between survival and adhesion to FN, as well as activation of PI3K and ERK, and induced expression of c-Fos in bone marrow-derived mast cells, and wortmannin blocked these effects (63). Increased c-Fos expression was largely affected by inhibition of ERK and/or PI3K signals in other studies as well (64, 65). We also found that c-Fos siRNA alleviated the effects of FN on enhancing MMP-9 promoter activity. The c-Fos antisense oligonucleotides blocked thrombin-induced expression of MMP-9 mRNA as well as AP-1 binding activities in cultured human mesangial cells (59). Together, these studies show that c-Fos is a critical transactivator for MMP-9 gene expression. Note that the MMP-9 basal activity was still observed in c-Fos-silenced cells, suggesting that transcription factors other than AP-1 (e.g. Sp1 and NF-κB) might play a role in maintaining the MMP-9 basal promoter activity (19, 20). FN had little effect on c-Jun expression and silencing c-Jun by siRNA did not block the FN-induced MMP-9 protein expression. These results indicated that c-Jun played no significant role in mediating the effect of FN on MMP-9 expression.

In summary, our study indicates that FN, by binding to the integrin α5β1 receptor, stimulates the expression of MMP-9 through increasing AP-1/DNA binding activity and c-Fos protein expression. Inhibition of c-Fos expression alleviated the effects of FN on MMP-9 promoter activity and protein expression.
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