Focal Adhesion Kinase and Mitogen-activated Protein Kinases Are Involved in Chondrocyte Activation by the 29-kDa Amino-terminal Fibronectin Fragment*

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The 29-kDa amino-terminal fibronectin fragment (FN-f) has a potent chondrolytic effect and is thought to be involved in cartilage degradation in arthritis. However, little is known about signal transduction pathways that are activated by FN-f. Here we demonstrated that FN-f induced nitric oxide (NO) production from human articular chondrocytes. Expression of inducible nitric-oxide synthase (iNOS) mRNA and NO production were observed at 6 and 48 h after FN-f treatment, respectively. Interleukin-1β (IL-1β) mRNA up-regulation was stimulated by FN-f in human chondrocytes. To address the possibility that FN-f-induced NO release is mediated by IL-1β production, the effect of IL-1 receptor antagonist (IL-1ra) was determined. IL-1ra partially inhibited FN-f-induced NO release although it almost completely inhibited IL-1β-induced NO release. Tyrosine phosphorylation of focal adhesion kinase was induced transiently by FN-f treatment. Blocking antibodies to α5 or β1 integrin and Arg-Gly-Asp-containing peptides did not inhibit FN-f-induced NO production. PP2, a Src family kinase inhibitor, or cytochalasin D, which selectively disrupts the network of actin filaments, inhibited both FAK phosphorylation and NO production induced by FN-f, but the phosphatidylinositol 3-kinase inhibitor, or cytochalasin D, which selectively disrupts the network of actin filaments, inhibited both FAK phosphorylation and NO production induced by FN-f, but the phosphatidylinositol 3-kinase inhibitor, or cytochalasin D, which selectively disrupts the network of actin filaments, inhibited both FAK phosphorylation and NO production induced by FN-f, but the phosphatidylinositol 3-kinase inhibitor, or cytochalasin D, which selectively disrupts the network of actin filaments, inhibited both FAK phosphorylation and NO production induced by FN-f, but the phosphatidylinositol 3-kinase inhibitor, or cytochalasin D, which selectively disrupts the network of actin filaments, inhibited both FAK phosphorylation and NO production induced by FN-f, but the phosphatidylinositol 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network of actin filaments, inhibited both FAK phosphorylation and NO production induced by FN-f, but the phosphatidylinosito

Experimental Procedures

Materials—Tissue culture reagents were purchased from Invitrogen (Grand Island, NY). Fetal calf serum was purchased from Omega Scientific (Tarzana, CA). FN-f, Arg-Gly-Asp-Ser (RGDS) were purchased from Sigma. Rabbit anti-p125FAK polyclonal antibody (C-20), horseradish peroxidase-conjugated goat anti-rabbit IgG and anti-mouse IgG have been reported to have the most potent chondrolytic activity among specific proteolytic fragments of fibronectin (1). It elevates matrix metalloproteinase, production (2), enhances rates of proteoglycan loss, and suppresses proteoglycan synthesis in cartilage explant culture (1). FN-f also increases the levels of tumor necrosis factor-α, interleukin-1β (IL-1β), and IL-1α in cultured human articular cartilage (3). The pathophysiological significance of these FN-f activities is supported by in vivo studies where injection of FN-f into rabbit knee joints caused depletion of cartilage proteoglycan (4).

Fibronectin is found in high concentrations in both synovial fluid and plasma of osteoarthritis (OA) and rheumatoid arthritis patients (5, 6). An increase in fibronectin concentrations in synovial fluid has also been observed in a canine model of OA (7). FN-f is produced by protease cleavage of native fibronectin (8) and has been reported to be increased in OA synovial fluid and plasma (5). Collectively, these results support the notion that FN-f is an inducer of cartilage destruction in OA and rheumatoid arthritis.

Activation of mitogen-activated protein kinases (MAPK) has been implicated in proinflammatory cytokine signaling in chondrocytes (9). These cytokines activate all three MAP kinase subgroups, extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 MAPK in human articular chondrocytes (10). However, the cellular receptors and signaling mechanisms that mediated the effects of FN-f have not been fully understood.

Cellular adhesion to fibronectin is mediated primarily by the αIβ1 integrin that interacts with Arg-Gly-Asp (RGD) sequence within fibronectin’s tenth type III module (11, 12). However, this motif is not present in the 29-kDa FN-f, which has the first five type I repeats, termed matrix assembly sites, that are required for fibronectin binding to cell surface receptors (13, 14). It has previously been reported that the 29- or 70-kDa amino-terminal fibronectin fragment can bind to the cell surface with the same affinity as intact fibronectin (15, 16). It thus remains unclear whether FN-f binds to the fibronectin receptor and can activate integrin-related signals.

The present study analyzes intracellular signaling pathways that are activated in human articular chondrocytes by FN-f. We address the following questions: 1) if integrin-related signaling is activated; 2) if MAP kinase subgroups are activated; and 3) which signaling pathways are responsible for FN-f-induced nitric oxide (NO) production.
were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phospho-ERK, anti-phospho-p38 MAP kinase polyclonal antibody, and anti-phospho-SAPK/JNK polyclonal antibody were purchased from New England Biolabs (Beverly, MA). Mouse anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). Inhibitors anti-human β1 integrin monoclonal antibody (mAb13) was kindly provided by Dr. K. M. Yamada (National Institutes of Health, Bethesda, MD). Mouse anti-α5, β1 integrin monoclonal antibody (JB BS5) was purchased from Chemicon International (Temecula, CA). PD98059, SB203580, PP2, wortmannin, cycloheximide, and fibronectin were purchased from Calbiochem (San Diego, CA). Recombinant human IL-1ra was purchased from R&D Systems (Minneapolis, MN). All other reagents were chemical grade and purchased from Sigma.

Chondrocyte Isolation and Culture—Human cartilage was obtained at autopsy from donors without known history of joint disease. For all experiments reported here, cartilage from the femoral condyles and tibial plateaus of the knee joints was used. Chondrocytes were isolated by collagenase digestion of cartilage and cultured at high density in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a 95% air, 5% CO2 incubator at 37 °C (17). Cells were used for experiments in first passage and seeded at 30,000 cells/well in 96-well plates for analysis of NO production or at 1,000,000 cells per a 100-mm Petri dish for Western blotting. After cells became confluent, they were cultured in serum-free medium for 24 h prior to stimulation with FN-f.

Preparation of Cell lysates—Cells were lysed in lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.5, 0.1% SDS, 1% Triton X-100, 1 mM sodium deoxycholate, 1 mM EDTA, 50 mM NaF, 1 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 30 min at 4 °C and insoluble material was removed by centrifugation at 14,000 rpm for 10 min at 4 °C. The supernatants were transferred into fresh tubes and the protein concentration was determined by Bradford assay. Immunoprecipitation of pp125Fak—Equal amounts of protein were used for immunoprecipitation. Following preclearing with rabbit IgG-agarose beads, whole cell lysates were incubated with 2 μg of anti-pp125Fak antibody for 1 h at 4 °C and then with protein A-Sepharose beads for 1 h at 4 °C. The beads were sedimented by centrifugation at 8,000 rpm for 2 min and washed 3 times with ice-cold lysis buffer. Sodium dodecyl sulfate sample buffer containing β-mercaptoethanol was added and the beads were then boiled for 4 min to dissociate the proteins.

Western Blotting—Immunoprecipitated proteins or whole cell lysates were separated by 8 or 10% SDS-PAGE, respectively. After electrophoresis, proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membranes were first blocked in 5% powdered milk/Tris-buffered saline with Tween (TBS-T) (12.5 mM Tris/HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) for 1 h. The membranes were then rinsed once with TBS-T and incubated in 2.5% bovine serum albumin, 2.5% powdered milk/TBS-T, or 5% bovine serum albumin for 1 h at 4 °C. The membranes were then rinsed three times with TBS-T and then further incubated with horseradish peroxidase-conjugated secondary antibody in 5% powdered milk/TBS-T for 1 h. Afterward the membranes were washed three times with TBS-T and developed according to the supplier’s protocol using Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and exposed to x-ray film.

Reverse Transcriptase-PCR—Total RNA was isolated from 2 × 106 chondrocytes using the STAT-60 reagent (Tel-Test, Friendswood, TX) and reverse transcribed using the Superscript Preamplification System (Invitrogen, Gaithersburg, MD) with random hexamers. One μl of the reverse transcription reactions was subjected to PCR analysis with primers specific for IL-1α (387-bp product); sense, GAGCTCGCCAGTGGAAATGATGGC; antisense, CAAGCTTTTTGCTGTGACTCCCG (36 cycles); inducible nitric-oxide synthase (iNOS: 36 cycles): sense, CTTTACTGATGTTGAGTCC; antisense, GAAGGCTGTAGTCTGCG (32 cycles); iNOS cDNA was amplified using Taq polymerase. PCR was performed as follows: 94 °C for 30 s denaturation, 60 °C for 30 s annealing, 72 °C for 30 s extension. The PCR products were separated on 1.5% agarose gels containing ethidium bromide.

NO Assay—The concentration of nitrates, the stable end products of NO breakdown, in conditioned media from chondrocytes was determined by the Griess reaction using NaNO2 as standard.

Statistical Analysis—Data are expressed as mean ± S.E. as indicated from at least three independent experiments. Statistical analysis was performed by Student’s t-test.

RESULTS

FN-f Induces iNOS Expression and NO Production—NO has been suggested to mediate some of the effects of IL-1 in cartilage degradation (18). As FN-f induces IL-1-like changes in cartilage, we determined whether FN-f can induce NO production. As shown in Fig. 1, iNOS mRNA expression was induced by FN-f stimulation in human articular chondrocytes. FN-f also increased NO production in a dose-dependent manner (Fig. 1). The induction of NO by FN-f was observed in 16 of 29 normal human chondrocyte preparations and was statistically significant (p < 0.01). The levels of NO induced by optimal concentrations of FN-f were approximately half of that induced by IL-1β. FN-f-induced NO production was completely inhibited by pretreatment with cycloheximide (50 μg/ml). Thus, FN-f stimulated NO production by inducing iNOS mRNA expression and de novo synthesis of iNOS protein.

Interactions between FN-f and IL-1β—It has been reported that chondrocytes can produce IL-1β and FN-f and both cytokines can stimulate cartilage degradation. First, we examined whether IL-1β, FN-f-induced NO production was mediated by IL-1β, the effect of IL-1 receptor antagonist (IL-1ra) was determined. IL-1ra partially (80%) reduced FN-f-induced NO production while the same concentrations of IL-1ra inhibited the high levels of NO production by IL-1β stimulation by 80% (Table 1). These findings indicate that FN-f can induce IL-1β but this
cytokine has only a minor role in the FN-f activation of chondrocytes as measured by NO release. We also analyzed the chondrocyte response to combinations of IL-1β and FN-f to determine whether these stimuli can synergize. The results showed additive effects but did not reveal any indication of synergy (Fig. 3).

**FN-f Activates Integrin-related Signals**—The α5β1 integrin is the major fibronectin receptor in chondrocytes (19, 20). Therefore, we first determined if FN-f activates FAK since FAK is tyrosine phosphorylated in response to integrin engagement (21). Tyrosine phosphorylation of FAK was detected 2.5–5 min after FN-f stimulation. This phosphorylation was transient and after 15 min of incubation with FN-f, FAK phosphorylation was reduced to basal levels (Fig. 4).

We next determined if integrin blocking antibodies or synthetic peptides containing the Arg-Gly-Asp cell binding region of fibronectin, affect FN-f induced NO production. However, neither α5 (mAb JBS5) nor β1 (mAb13) integrin blocking antibodies nor the RGD-containing synthetic peptide, RGDS, inhibited FN-f induced NO production (Fig. 5). This suggests that the α5β1 integrin does not mediate the effects of FN-f. Consistent with this is the observation that native fibronectin did not antagonize NO production induced by FN-f stimulation (Fig. 5).

Furthermore, native fibronectin did not induce NO production by itself (data not shown). Thus, FN-f induces FAK phosphorylation, but the α5β1 fibronectin receptor does not mediate this effect. We next determined if FAK activation is involved in FN-f-induced NO production in human chondrocytes. It has been reported that Src family tyrosine kinases or cytoskeletal organization were necessary for FAK phosphorylation (22). PP2 a selective Src family tyrosine-protein kinase inhibitor dose-dependently inhibited NO production and it completely inhibited NO production at 10 μM. Cytochalasin D, which disrupts actin polymerization, also partially (by ~50%) inhibited NO production (Fig. 5).

The phosphatidylinositol 3-kinase (PI-3K) is one of the FAK-related signaling pathways (23). However, the PI-3K inhibitor wortmannin did not inhibit NO production by FN-f. Taken together, these results suggest that FAK activation is necessary for FN-f-induced NO production in human chondrocytes, but this does not involve PI-3K.

**FN-f Activates MAPK Pathways**—MAP kinase pathways are important in cell signaling in response to cytokine stimulation and integrin activation (24, 25). We thus determined if FN-f activates MAPK pathways by detecting the phosphorylated forms of ERK, JNK, and p38, using phospho-specific antibodies (Fig. 6). Increased phosphorylation of ERK and JNK was observed from 5 to 60 min after FN-f stimulation. Phosphorylation of p38 was also observed at 5 min after FN-f stimulation but it was reduced to basal levels at 30 min. Little or no activation of ERK, JNK, and p38 MAPK was detected in un-stimulated chondrocytes. Thus, FN-f is capable of activating all three MAPK pathways in human articular chondrocytes.

To further investigate whether ERK, JNK, and p38 MAPK are involved in FN-f-induced NO production in human chondrocytes, we used selective protein kinase inhibitors: PD98059 a specific MEK1/2 inhibitor that blocks the ERK signaling cascade, and SB203580 which selectively inhibits p38 MAPK at low concentration, but which inhibits both p38 MAPK and JNK at high concentrations (26). As shown in Fig. 7, FN-f-induced NO production was inhibited by 50% at 10 μM PD98059 and by 80% at 10 μM SB203580. These findings suggest that the MAPK, particularly ERK and JNK, mediate FN-f induced chondrocyte activation.

**DISCUSSION**

FN-f has potent chondrolytic activity and is present at increased levels in synovial fluid of OA and rheumatoid arthritis patients (1, 5, 6). FN-f may thus play an important role in cartilage destruction in arthritis. In this study, we examined signaling events that are activated by FN-f. We showed that...

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**TABLE I**

| Nitrite % | n  |
|----------|----|
| Control  | 2.25 ± 0.32 | 5  |
| IL-1β    | 32.67 ± 6.25 | 5  |
| +IL1ra   | 7.52 ± 4.49  | 23 |
| Control  | 2.43 ± 0.34 | 4  |
| FN-f     | 6.64 ± 1.03  | 100|
| +IL1ra   | 4.62 ± 1.02  | 70 |
Results represent mean values ± S.E. from three to nine different experiments.

Our results on intracellular signaling events showed that FN-f transiently induced tyrosine phosphorylation of FAK. We then analyzed signaling pathways that are involved with FN-f-induced NO production and determined the effect of PP2, which selectively inhibits Src family tyrosine-protein kinases (33), or cytochalasin D, an agent which disrupts actin polymerization (34). FAK may function to direct phosphorylation of cellular substrates by recruitment of Src kinases (21, 35). Cytochalasin D has been shown to prevent adhesion-dependent tyrosine phosphorylation (36, 37). PP2 and cytochalasin D inhibited FN-f-induced NO production in human chondrocytes. We next determined if the PI-3K pathway, which is a major substrate of FAK (22), is involved in FN-f-induced NO production. However, the PI-3K inhibitor wortmannin did not inhibit NO production. These data suggest that activation of FAK is required for FN-f-induced NO production but this does not involve PI-3K.

We next demonstrated that FN-f activated the three MAPK pathways, ERK, JNK, and p38. Inhibition of FN-f-induced NO production was only observed with high concentrations of PD98059 (10 \( \mu \)M) or SB203580 (10 \( \mu \)M). Particularly, SB203580 inhibits only p38 MAPK at low concentration (≤1 \( \mu \)M) but inhibits both p38 MAPK and JNK at high concentrations (10–25 \( \mu \)M) (25). Moreover, the amount of p38 phosphorylation by FN-f was relatively smaller than that of JNK. It has been recently reported that the specific Src family kinase inhibitor, PP2, inhibited JNK activation but had no effect on ERK and p38 MAPK activation (38). Our data also demonstrates that PP2 strongly inhibited FN-f-induced NO production. Therefore, inhibition of NO production by high concentrations of SB203580 appears to be mediated by JNK inhibition. Similarly, only high concentrations of PD98059 could partially inhibit FN-f-induced NO production. Almeida et al. (39) recently reported that FAK can activate both JNK and ERK in synovial fibroblasts. Wang and Brecher (40) suggested that ERK did not regulate iNOS mRNA expression but possibly influenced post-transcriptional events. Therefore, it is possible that ERK might promote FN-f-induced NO production, through the stabilization of iNOS mRNA.

In conclusion, this study demonstrates that FN-f induces NO production by up-regulating iNOS mRNA in human articular chondrocytes. Although FN-f increases IL-1\( \beta \) mRNA levels,
IL-1β plays only a minor role in FN-f-induced NO production. FN-f can activate FAK. However, this does not seem to be mediated by the α5β1 integrin, a major fibronectin receptor. Activation of MAP kinases, particularly ERK and JNK, are central signaling events in the chondrocyte response to FN-f.

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