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Fibronectin fragments mediate matrix metalloproteinase upregulation and cartilage damage through proline rich tyrosine kinase 2, c-src, NF-κB and protein kinase Cδ

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Summary

Objective: Since fibronectin fragments (Fn-fs) enhance cartilage damage through integrins, the objective was to investigate the role of integrin linked kinases, focal adhesion kinase (FAK) and a soluble form of FAK, proline rich tyrosine kinase 2 (Pyk2) and cellular src kinase (c-src) and the transcription factor, nuclear factor κB (NF-κB) in cartilage damage.

Methods: Bovine chondrocytes were cultured with various concentrations of three different Fn-fs, an amino-terminal 29 kDa, a gelatin binding 50 kDa and a central 140-kDa Fn-fs, each with progressively weaker cartilage damaging activity, or with native fibronectin (Fn), and lysates probed for activation of the selected kinases. Confocal microscopy was used to visualize intracellular location of activated kinases and NF-κB. Various kinase inhibitors were tested for their effects on Fn-f mediated upregulation of matrix metalloproteinase (MMP)-3 and -13 and cartilage proteoglycan (PG) depletion.

Results: The Fn-fs kinetically enhanced phosphorylation of FAK but did not show a clear dose–response effect. The 29-kDa and 50-kDa Fn-fs enhanced phosphorylation of Pyk2, c-src and NF-κB to a much greater extent than the 140-kDa Fn-f and native Fn and did so as a function of dose. The 29-kDa Fn-f enhanced the phosphorylation of nuclear Pyk2 as compared with no treatment or native Fn. Inhibitors of Pyk2, c-src, NF-κB and protein kinase Cδ (PKCδ) decreased MMP upregulation and decreased Fn-f mediated damage to cartilage.

Conclusions: These studies enhance our knowledge of crucial factors in Fn-f mediated signaling in MMP upregulation and cartilage damage and because of the potential physiologic relevance of Fn-fs, provide a better knowledge of cartilage degeneration in general.

Key words: Cartilage, Chondrocytes, Kinases, Fibronectin, Fibronectin fragments, Matrix metalloproteinases, Cartilage damage, Intracellular signaling.

Abbreviations: Fn-f fibronectin fragment, Fn fibronectin, MMPs matrix metalloproteinases, FAK focal adhesion kinase, Pyk2 proline rich tyrosine kinase 2, NF-κB nuclear factor κB, PG proteoglycan, PI3K phosphoinositide 3-kinase, PKCδ protein kinase Cδ isoform, iNOS inducible nitric oxide synthase, MAP mitogen activated protein, MMP-13 matrix metalloproteinase-13, collagenase 3, ECL enhanced chemiluminescent, HRP horse radish peroxidase, TRITC tetramethyl rhodamine iso-thiocyanate, IgG immunoglobulin G, DAPI 4′-6-Diamidino-2-phenylindole, MMP-3D MMP-3 digest of Fn, EGTA ethylene glycol tetraacetic acid, PMSF phenylmethyl sulfonyl fluoride, DTT dithiothreitol, TBS 20 mM Tris buffer, pH 7.4, containing 140 mM NaCl, BSA bovine serum albumin, PBS phosphate buffered saline, TBST Tris buffered saline-Tween 20, SD standard deviation, DMSO dimethylsulfoxide, Grb2 growth factor receptor-bound protein 2, BAPTA/AM 1,2-bis(*o*-aminophenoxy)-ethane-*N,N,N′,N′*-tetraaceticacid tetra(acetoxymethyl) ester.

Introduction

Proteolytic cleavage of fibronectin (Fn) which occurs in cartilage degeneration creates fibronectin fragments (Fn-fs) that have cartilage chondrolytic activities[1] through their abilities to elevate matrix metalloproteinase (MMP) expression[2–4], suppress proteoglycan (PG) synthesis in cartilage[5–7], enhance rates of PG loss from cartilage tissue in explant cultures[1–3,5–7] and upregulate catabolic cytokines[8,9]. Fn-fs likely interfere with native Fn signaling through the classical $\alpha_5\beta_1$ integrin receptor, since analog peptides of integrin-binding sequences of native Fn block activities of the Fn-fs[10], Fn-fs can be chemically crosslinked to the α_5 subunit[11] and reduction of $\alpha_5\beta_1$ levels by antisense oligonucleotides reduces Fn-f activities[12]. The Fn-fs enhance expression of a number of MMPs[2–4] and enhance aggrecanase like cleavage[13]. Fn-fs also upregulate inducible nitric oxide synthase (iNOS)[14] and nitric oxide (NO) levels[15] as well as the toll-like receptor 2[16]. The combined effects of these actions on catabolic pathways are severe. Fn-fs at or below concentrations found in osteoarthritis (OA) synovial fluid, 0.1–1 μM, when added to cultures of cartilage explants, cause degradation and irreversible release of up to half of the matrix PG within days[6,7]. The relevance of these effects of the Fn-fs is supported by observations that injection of Fn-fs into normal rabbit knee joints causes a severe loss of articular cartilage PG[17,18]. Major aspects of this model have been reviewed[19].

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There are only a few studies on Fn-f signal transduction pathways that relate to catabolic pathways in chondrocytes. It has been shown that focal adhesion kinase (FAK) and mitogen activated protein (MAP) kinases mediate NO production by a 29-kDa amino-terminal Fn-f added to human knee cartilage chondrocytes[15]. Further, MAP kinase and protein kinase C (PKC) dependent activation of proline rich tyrosine kinase 2 (Pyk2) were implicated in matrix metalloproteinase-13 (MMP-13) upregulation by a 110-kDa Fn-f added to human chondrocytes[20,21]. In our ongoing work, we have confirmed in detail the kinetics and dose dependencies of MAP kinase activation in Fn-f mediated MMP-3 and -13 upregulation and cartilage damage in a bovine chondrocyte and explant culture model and shown that three different Fn-fs, each with differing ability to enhance cartilage matrix damage, showed differential effects on activation of MAP kinases[22]. In this work we have extended our analysis to other kinases, including FAK, Pyk2, protein kinase Cδ (PKCδ), cellular src kinase (c-src) and nuclear factor κB (NF-κB), to determine whether all three Fn-fs utilize the same players both in enhancing MMPs and in causing cartilage damage.

Experimental procedures

MATERIALS

The LIVE/DEAD® Reduced biohazard Viablity/Cytotoxicity Kit #1 (L-7013) was purchased from Invitrogen™ (Eugene, OR; Carlsbad, CA). Antibodies to β-actin, total and phospho-FAK (Tyr576), total and phospho-Pyk2 (Tyr402), total and phospho-src family (Tyr416), total and phospho p65 NF-κB (Ser536) antibodies and horse radish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) were purchased from Cell Signaling Technology® (Danvers, MA). The secondary antibody for MMP detection, HRP-linked goat anti-rabbit IgG, was from Sigma Chem. Co. (St. Louis, MO). Polyclonal antibodies to human MMP-3 and -13 were from Chemicon International Inc. (Temecula, CA). All kinase inhibitors and the protease Inhibitor cocktail II reagent were from CalBiochem (San Diego, CA).

ISOLATION OF Fn-fs

A well-characterized amino-terminal heparin-binding 29-kDa Fn-f and a gelatin-binding 50-kDa Fn-f were isolated by sequential cathepsin D and thrombin digests of human plasma Fn as described[1]. A mixture of non-gelatin binding C-terminal Fn-containing the cell-binding 70 to 140-kDa Fn-fs as well as smaller C-terminal Fn-fs, referred to as the 140-kDa Fn-f were also studied. The 29-kDa Fn-f has been identified in extracts from OA cartilage[23] and in MMP-3 generated digests of Fn[24].

KINASE ACTIVATION STUDIES

Chondrocyte cultures were established and cells treated with 0.5 μM Fn or Fn-fs for various times and lysed at 0, 15 min, 1 h, 4 h and 12 h in cold lysis buffer as described[22]. To study dose–response effects, cultures were adjusted to 10 nM to 1 μM Fn or Fn-f and cell lysates recovered at 1 h. At the end of experiments and after up to 24 h, wells treated in parallel were tested for cell viability using a viability/cytotoxicity kit from Invitrogen (Carlsbad, CA). Several fields of over 3000 cells per field were visualized using an Olympus D-71 fluorescent microscope. Western blotting and chemiluminescent detection for kinases and MMPs were as described[22]. Probing for total kinase served as a loading control as well as probing, in some cases, for β-actin.

CONFOCAL MICROSCOPY PROBING FOR KINASES

To visualize intracellular location of kinases, cells were first fixed and after being blocked by 5% donkey serum diluted in 0.5% bovine serum albumin (BSA) in phosphate buffered saline (PBS), probed with antibodies to phosphorylated kinases using dilutions of 1:75, followed by reaction with tetramethyl rhodamine iso-thiocyanate (TRITC) conjugated AffiniPure donkey anti-rabbit IgG (H + L) using dilutions of 1:200. After washing, cells were incubated in 300 nM 4′-6-diamidino-2-phenylindole (DAPI) solution for 3–5 min at room temperature and rinsed with PBS 2–3 times before mounting with VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA).

TESTS OF KINASE INHIBITORS ON MMP UPREGULATION BY THE 29-KDa Fn-f AND ON Fn-f MEDIATED PG DEPLETION

To test effects of inhibitors on MMPs, monolayer cultures were incubated with inhibitors over a 100-fold concentration range including concentrations reported by others to be maximally effective[20–22]. Inhibitors were typically dissolved in dimethylsulfoxide (DMSO) such that the final concentration in culture media would be 0.4% and to control for effects of DMSO, a DMSO alone control was run. At the end of experiments, wells treated in parallel were tested for cell viability. To test effects on PG depletion, cartilage slices from bovine metacarpophalangeal joints were cultured as described in 10% serum/DMEM[5–8] and were preincubated with kinase inhibitors for 24 h, then adjusted to 1 μM 29-kDa Fn-f and PG content assayed at 7 d as described[2]. The final mean and standard deviation (SD) values were calculated and control vs experimental data were compared using two-tailed unpaired Student's *t* tests. A P value <0.05 was considered significant.

Results

Fn-fs HAD ONLY WEAK EFFECTS ON ACTIVATION OF FAK

Initial control experiments showed that untreated cells or cultures adjusted to 0.5 μM BSA as a protein control did not show significant activation (data not shown). The experiments were performed with three different chondrocyte preparations with similar results and representative blots are shown. The different primary cultures led to some variability, especially in untreated controls and exposure times of blots were varied to allow for visualization of more features which also allowed for apparent variability of controls. Nonetheless, each chondrocyte preparation gave internally consistent data. Figure 1(A) shows that there were only weak kinetic effects by Fn-fs and Fn on activation of FAK. Note that the loading control strips for total kinase, labeled t, show even loading. The p strips correspond to phosphorylation strips. We next examined the dose–response relationship at a 1 h time point, since other preliminary studies had shown a maximal effect of the 29-kDa Fn-f on p38 MAP kinase at that time point. Figure 1(B) shows that the three Fn-fs and Fn, from 0.01 to 1 μM, did not enhance phosphorylation at 1 h.