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The cartilage chondrolytic mechanism of fibronectin fragments involves MAP kinases: comparison of three fragments and native fibronectin

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Summary

Objective: To define the role of mitogen activated protein (MAP) kinases in fibronectin fragment (Fn-f) mediated matrix metalloproteinase (MMP) upregulation and damage to bovine cartilage and to compare activities of three Fn-fs with native fibronectin (Fn), which is inactive in terms of cartilage damage.

Methods: Bovine chondrocytes were cultured with three Fn-fs, an amino-terminal 29-kDa, a gelatin-binding 50-kDa and a central 140-kDa Fn-f or native Fn at concentrations from 0.01 to 1 μM, concentrations lower than those found in osteoarthritis synovial fluids. Lysates were probed for activation of MAP kinases, extracellular signal-regulated kinase 1/2 (ERK1/2), p38 and stress activated protein kinase/c-jun N-terminal kinase (SAPK/JNK). Confocal fluorescent microscopy was used to visualize movement of activated kinases. Kinase inhibitors were tested for their abilities to block Fn-f mediated protein upregulation of MMP-3 and MMP-13 and Fn-f induced depletion of cartilage proteoglycan (PG) from cultured explants.

Results: The 29-kDa, the most potent Fn-f in terms of cartilage damage, enhanced phosphorylation of ERK1/2, p38 and JNK1/2 within a 1-h incubation while the 50 and 140-kDa Fn-fs required up to 4 h for maximal activity and native Fn was only minimally active toward p38 and JNK, but did strongly activate ERK1/2. The activated kinases displayed a distribution toward the nuclear membrane and within the nucleus. MAP kinase inhibitors markedly decreased Fn-f mediated upregulation of MMP-3 or MMP-13 and Fn-f mediated cartilage PG depletion.

Conclusions: These results suggest that Fn-fs upregulate MMP-3 and MMP-13 in bovine chondrocytes through MAP kinases and that kinase inhibitors afford protection against this degenerative pathway.

Key words: Fibronectin, Fibronectin fragments, Chondrocytes, MAP kinases, Matrix metalloproteinases, Cartilage chondrolysis, Cartilage.

Abbreviations: Fn-f(s) fibronectin fragment(s), MMP matrix metalloproteinase, PG proteoglycan, Fn fibronectin, iNOS inducible NO synthetase, MAP mitogen activated protein, ERK extracellular signal-regulated kinase, JNK N-terminal kinase, NF-κB nuclear factor κB, DMEM Dulbecco's modified Eagle's medium, DAPI 4′-6-Diamidino-2-phenylindole, TRITC tetramethyl rhodamine iso-thiocyanate, SDS sodium dodecyl sulfate, DTT dithiothreitol, HRP horseradish peroxidase, SD standard deviation, DMB dimethylmethylene blue, ECM extracellular matrix, BLU Boehringer Light Unit (also integrated intensity), ERK1 44-kDa ERK, ERK2 42-kDa ERK, JNK-1 46-kDa JNK, JNK-2 54-kDa JNK, OA osteoarthritis.

Introduction

We have reported that fibronectin fragments (Fn-fs) have cartilage chondrolytic activities[1], elevate matrix metalloproteinase (MMP) expression[2–4], suppress proteoglycan (PG) synthesis in chondrocytes and cartilage[5–7] and enhance rates of PG loss from cartilage tissue in explant cultures[1–3,5–7]. These activities involve catabolic cytokines[8,9], interaction with the fibronectin (Fn) $\alpha_5\beta_1$ integrin receptor[10–12] and enhanced release of several MMPs[2–4,9]. Effects of Fn-fs on cytokines[13] and on MMPs and aggrecan degradation have also been confirmed in porcine chondrocyte models[14] as well as effects on MMPs and on protein kinase C (PKC) and proline-rich tyrosine kinase (PYK2) activation in human chondrocytes[15]. Our own studies of bovine chondrocytes have shown a role for MMP-3[2–4] as well as aggrecanases[16] which cleave within the aggrecan interglobular domain[17]. Several members of the A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family of proteins cleave aggrecan at this site[18–21], including ADAMTS-4 and ADAMTS-5[18,19] which are increased by inflammatory cytokines and which when overexpressed enhance cartilage matrix degradation[22–26]. Both forms are expressed in normal and osteoarthritis (OA) cartilage and synoviium[24] and their inhibition correlates with prevention of aggrecan degradation *in vitro*[24,27]. Thus, the ability of Fn-fs to induce aggrecanase like cleavage supports their potential relevance. Since the Fn-fs also upregulate inducible NO synthetase (iNOS)[28] as well as the Toll-like receptor 2[29], the Fn-fs have diversified effects in inflammatory pathways.

The potential consequences of the Fn-fs on cartilage tissue pathology *in vivo* are severe since PG depletion from mature cartilage explants caused by the Fn-fs *in vitro* appears to be irreversible[6,7]. The relevance of the Fn-f model is supported by our observations that injection of Fn-fs into normal rabbit knee joints causes a severe loss of articular cartilage PG[30,31]. Biochemical aspects of this model have been reviewed[32].

The physiologic relevance of Fn-fs to cartilage degeneration has been investigated in several ways. We have detected

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several 30–200-kDa Fn-fs in human OA cartilage using a monoclonal antibody specific for the N-terminus and demonstrated that IL-1 activates bovine cartilage to generate 30–200-kDa Fn-fs and that removal of Fn-fs from OA synovial fluids reduces the cartilage damaging activity of OA synovial fluids[33]. We also showed that fragmentation of Fn forms isolated from bovine synovial fluid, bovine cartilage or bovine plasma generates Fn-fs with similar cartilage damaging activities[33]. Others have recently shown that 30-kDa gelatin-binding Fn-fs isolated from OA cartilage enhance aggrecanase like cleavage of aggrecan in bovine cartilage[34]. Thus, it is highly likely that a broad range of cartilage damaging Fn-fs can be found in OA cartilage and synovial fluids.

Little is known of how the Fn-fs alter signal transduction pathways. Fn-fs might compete with native Fn and cause dissociation of the matrix from direct or indirect interaction with the Fn receptor. Detachment of Fn from matrix interaction has been shown in other cell types to alter signaling, including decreasing cellular proliferation[35], stimulating cyclin dependent kinases and DNA synthesis and activating mitogen activated protein (MAP) kinases[36]. Since detachment has been shown to enhance integrin internalization and degradation[37], Fn-fs might cause such detachment and alter or even abolish normal integrin signaling. In fact, it has been shown that Fn-fs can activate MAP kinases as correlated with NO production[38] and as correlated with MMP-13 production[39] as well as activate nuclear factor κB (NF-κB) as correlated with cytokine production[40] and MMP-13 upregulation[41]. However, there have not been any studies that correlate MAP kinase activation with Fn-f mediated cartilage damage.

We have compared MAP kinase activation properties of three different Fn-fs, with different MMP upregulation and cartilage damaging activities and differing abilities to bind Fn receptors and cartilage matrix, to determine how kinase activation correlates with cartilage damage. Comparisons have also been made to native Fn which has limited cartilage damaging activity and may actually serve as an inhibitor of the Fn-f pathway[32].

Experimental procedures

ISOLATION OF Fn AND Fn-fs

Fn was isolated from human plasma and a well-characterized amino-terminal heparin-binding 29-kDa Fn-f, a gelatin-binding 50-kDa Fn-f and 70–140-kDa Fn-fs as well as smaller C-terminal Fn-fs isolated as described[1] as well as an MMP-3 digest, denoted MMP-3D[42]. We have identified the 29-kDa Fn-f in this mixture[27] and shown that this mixture has potent cartilage damaging activity[42,43].

EXPLANT CARTILAGE CULTURES AND CHONDROCYTES' CULTURES

Explant cartilage cultures and chondrocytes' cultures were prepared as described elsewhere[11,12]. At the end of the experiments, cultures were tested for cell viability using the LIVE/DEAD® Reduced Biohazard Viability/Cytotoxicity Kit #1 (L-7013) as directed by the manufacturer.

LABELING AND VISUALIZATION OF Fn-fs AND KINASES BY CONFOCAL FLUORESCENT MICROSCOPY

Protein was conjugated to rhodamine and added to chondrocytes plated in 10% serum/Dulbecco's modified Eagle's medium (DMEM) into eight well chamber slides with 0.15 million cells per well. Four hours after addition of rhodamine-labeled Fn-fs, cells were rinsed, fixed and visualized on a Zeiss 510 META confocal microscope equipped with a spectral META detector.

To visualize movement of kinases toward nuclear membrane, cells in chamber slides, after being blocked by 5% donkey serum diluted in 0.5% bovine serum albumin (BSA)–phosphate buffered saline (PBS), were probed with antibodies to kinases, followed by reaction with rhodamine [tetramethyl rhodamine iso-thiocyanate (TRITC)] conjugated AffiniPure donkey anti-rabbit IgG. Cell nuclei were stained with 300 nM 4′-6-diamidino-2-phenylindole (DAPI) dihydrochloride.

WESTERN BLOTTING AND CHEMILUMINESCENT DETECTION

For kinase detection, lysates were subjected to electrophoresis on 10% acrylamide gels, the gels blotted and blots were blocked for 1 h with 5% nonfat dry milk in 20 mM Tris buffer, pH 7.4, containing 140 mM NaCl [tris buffered saline (TBS)] and 0.1% TBS-Tween 20 (TBST). The blots were then incubated with the anti-total or anti-phosphospecific kinase antibodies in a 1:1000 dilution in 5% BSA in TBST (BSA/TBST) overnight and after washing, the blots were reacted with horseradish peroxidase (HRP) conjugated goat anti-rabbit immunoglobulin G (IgG) in a 1:2000 dilution in 5% BSA/TBST for 1 h, followed by addition of Supersignal West Dural Extended Duration substrate. Densities of bands were measured by a LumiImager® with Boehringer–Mannheim LumiAnalyst™ 3.0 software. For MMP detection, the blots were blocked with 3% BSA in TBS overnight and incubated with anti-MMP-3 or MMP-13 antibody in 1:5000 or 1:3000 dilution, respectively. The blots were then incubated with peroxidase conjugated goat anti-rabbit IgG from Sigma® 1:12,000 diluted in 1% BSA/TBST for 1 h. Subsequently blots were incubated with substrate and visualized as described above.

KINASE ACTIVATION STUDIES

For kinetic studies, cells were treated with 0.5 μM Fn or Fn-fs for various times and lysed in cold lysis buffer: 150 mM NaCl, 10 mM Tris, pH 7.5, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate, 1 mM ethylene glycol tetraacetic acid (EGTA), 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin with a 1:500 fold dilution of protease inhibitor cocktail II. Lysates were centrifuged at 2000 × *g* for 10 min at 4°C and supernatants recovered for analysis. For dose–response effects, cultures were adjusted to 10 nM to 1 μM Fn or Fn-fs and cell lysates were recovered at 1 or 4 h.

TESTS OF KINASE INHIBITORS ON MMP UPREGULATION BY THE 29-kDa Fn-f

The inhibitors tested were PD98059 for extracellular signal-regulated kinase 1/2 (ERK1/2); SB202190 and SB203580 for p38 and SP600125 for N-terminal kinase (JNK). Inhibitors were added over a 100 fold concentration range that included effective concentrations reported by others: 100 μM for PD98059[28,29], 20 μM for SB202190[29–31], 30 μM for SB203580 and 20 μM for SP600125[30,32]. Inhibitors were typically dissolved in dimethylsulfoxide (DMSO) to a final concentration in culture of 0.4% and inhibitors were added 2 h prior to addition of 0.5 μM 29-kDa Fn-f. After 24 h, media were collected, dialyzed against water, concentrated 10×, denatured with sample buffer and reduced with 0.05 M dithiothreitol (DTT) for electrophoresis and blotting.

TESTS OF EFFECTS OF MAP KINASE INHIBITORS ON Fn-f MEDIATED CARTILAGE DAMAGE, AS DEFINED BY DECREASES IN CARTILAGE PG CONTENT

Bovine cartilage explants in 10% serum/DMEM were preincubated with inhibitors for 24 h and then adjusted to 0.5 μM of the MMP-3 digest of Fn (MMP-3D). Media and reagents were renewed every other day. After 7 days in 10% serum/DMEM, cartilage was recovered, weighed, subjected to papain digestion and PG content was measured with dimethylmethylene blue (DMB) reagent as described[2]. The final mean and standard deviation (SD) values of PG content were compared using two-tailed unpaired Student's *t* tests.

MATERIALS

Total and phosphospecific ERK1/2 (Thr202/Tyr204), JNK (Thr183/Tyr185), p38 (Thr180/Tyr182) antibodies and HRP-conjugated goat anti-rabbit IgG were purchased from Cell Signaling Technology® (Danvers, MA). NHS-rhodamine [5-(and 6)-carboxylfluorescein, succinimidyl ester], the Super Signal Chemiluminescent Substrate kit for HRP and the Modified Lowry Protein Assay Kit were from Pierce Chemical Co. (Rockford, IL). Polyclonal antibodies to human MMP-3 and MMP-13 were from Chemicon International Inc. (Temecula, CA). DMEM and the secondary antibody used for MMP detection were purchased from Sigma–Aldrich (St. Louis, MO). The kinase inhibitors and protease inhibitor cocktail II were from CalBiochem (San Diego, CA). Rhodamine (TRITC)-conjugated AffiniPure donkey anti-rabbit IgG (H + L) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). DAPI dihydrochloride (FluoroPure™ grade) and the LIVE/DEAD® Reduced biohazard Viability/Cytotoxicity Kit #1 (L-7013) were purchased from Invitrogen™ (Carlsbad, CA).

Results

TESTING OF HIGHLY HOMOGENEOUS Fn-fs

The location within native Fn of the Fn-fs studied here is shown in Fig. 1(A) as well as potential interactions of Fn-fs with PG and collagen in the extracellular matrix (ECM). The

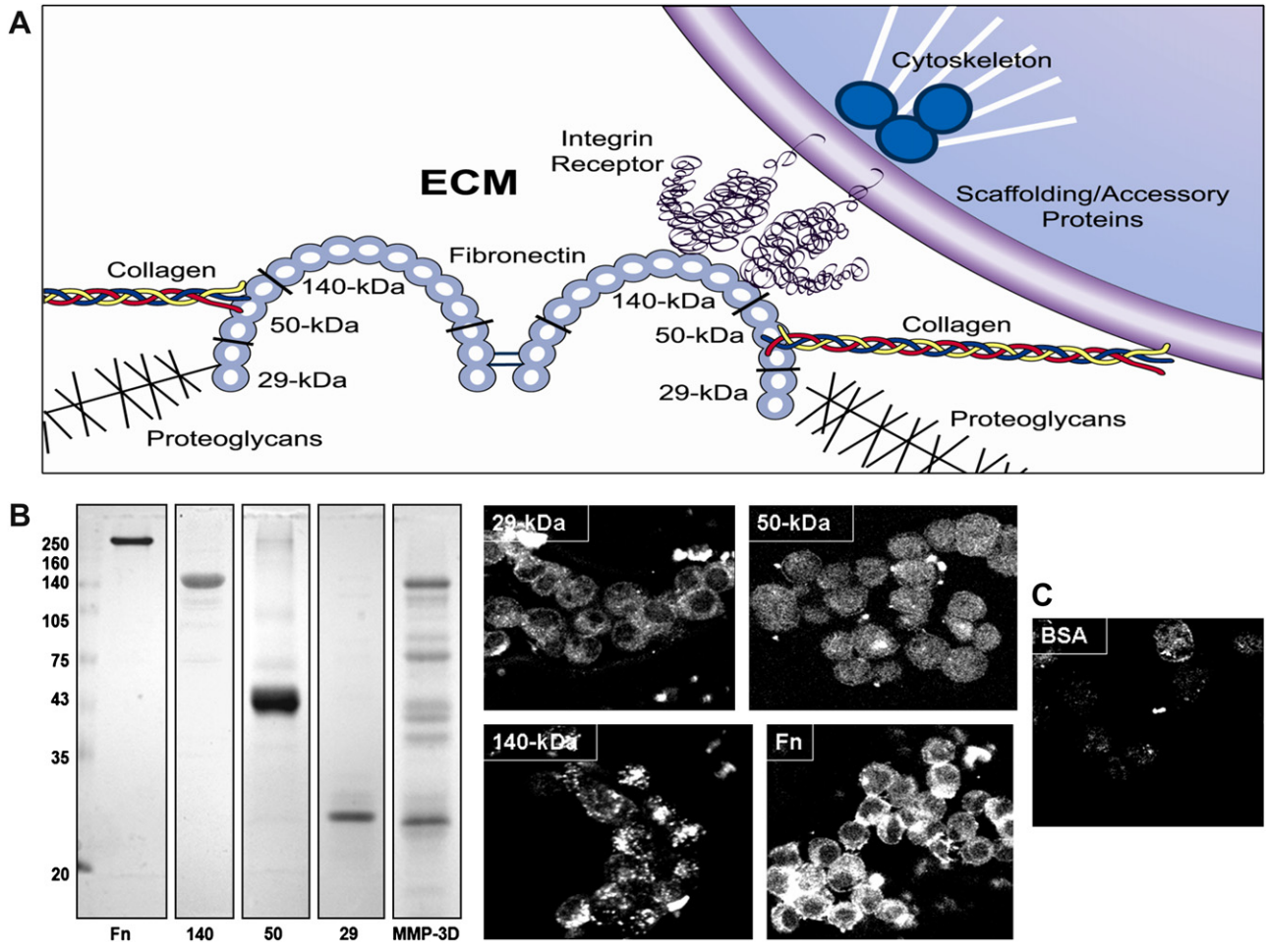

Fig. 1. Location of Fn-f domains, homogeneity of Fn-fs and demonstration of chondrocyte interaction. Panel A: the location of these Fn-fs in the intact polypeptide is shown in Fig. 1A as well as their potential interactions with PG and collagen in the ECM. Panel B: Fn-fs and a mixture of Fn-fs generated by MMP-3 digestion were generated from human plasma Fn. Fn and Fn-fs were subjected to SDS electrophoresis on two different 10% acrylamide gels with separator lanes and gels stained with Coomassie R-250. The empty lanes were cut and lanes merged to construct the montage. Lanes are the Fn solution used to generated Fn-fs (Fn lane), the 140-kDa Fn-f (140), the isolated 50-kDa Fn-f (50), the isolated 29-kDa Fn-f (29) and the MMP-3 digest (MMP-3D). Panel C: Fn-fs were rhodamine labeled and added to chondrocytes, followed by fluorescent confocal microscopy.

three Fn-fs differ in potencies in terms of causing cartilage damage and differ in interactions with the $\alpha_5\beta_1$ Fn receptor. Figure 1(B) shows the results of SDS gel electrophoresis on 10% acrylamide gels of reduced samples of proteins used for these studies. As shown, the sample of reduced Fn has subunits of approximately 250 kDa and is relatively devoid of Fn-fs while the 140-kDa Fn-f has some contaminating lower mass material and the 50-kDa Fn-f has additional higher mass material. The 29-kDa Fn-f contains some higher mass bands but is highly enriched. The MMP-3D mixture was generated by addition of MMP-3 to native Fn and has been partially characterized by us[42,43]. The MMP-3D lane shows an approximately 30-kDa Fn-f which is identical to the thrombin-generated 29-kDa Fn-f studied here as shown using mass spectrometry/sequence analysis[43].

Figure 1(C) demonstrates that rhodamine-labeled native Fn and Fn-fs have affinity to isolated chondrocytes and thus have the potential to affect cell signaling. Note that the Fn-fs appear to bind in different patterns and all bind to a greater extent than BSA used as a negative control. The 29-kDa Fn-f bound in more diffuse patterns, while the 140-kDa Fn-f bound in a more concentrated punctate pattern. Both show less diffuse cytosolic distribution than the 50-kDa Fn-f. Native Fn shows some diffuse binding but also bound mainly on the cell membrane. Thus, the three Fn-fs appear to differ in their interaction targets.

ALL THREE Fn-fs AND Fn KINETICALLY AND DOSE-DEPENDENTLY ENHANCED PHOSPHORYLATION OF ERK1/2 KINASES

As preliminary baseline data we found there was no enhanced phosphorylation of p38 or JNK with time in untreated chondrocytes, while basal phosphorylation of ERK1/2 activation appeared to decrease by 4 h (data not shown). In other experiments, 0.5 μM BSA as a negative protein control did not enhance activation (data not shown). Our preliminary data showed maximal effects of Fn-fs on MAP kinases between 0.3 and 1 μM so a midpoint concentration of 0.5 μM was used for the kinetic studies.

As shown in Fig. 2(A), all three Fn-fs and Fn at 0.5 μM enhanced phosphorylation of 44-kDa ERK (ERK1) and 42-kDa ERK (ERK2). The effects were maximal within 15 min and elevated for at least 4 h with effects of the 140-kDa Fn-f and Fn decreasing by 12 h. Figure 2(B) shows that all three Fn-fs had a concentration dependency with

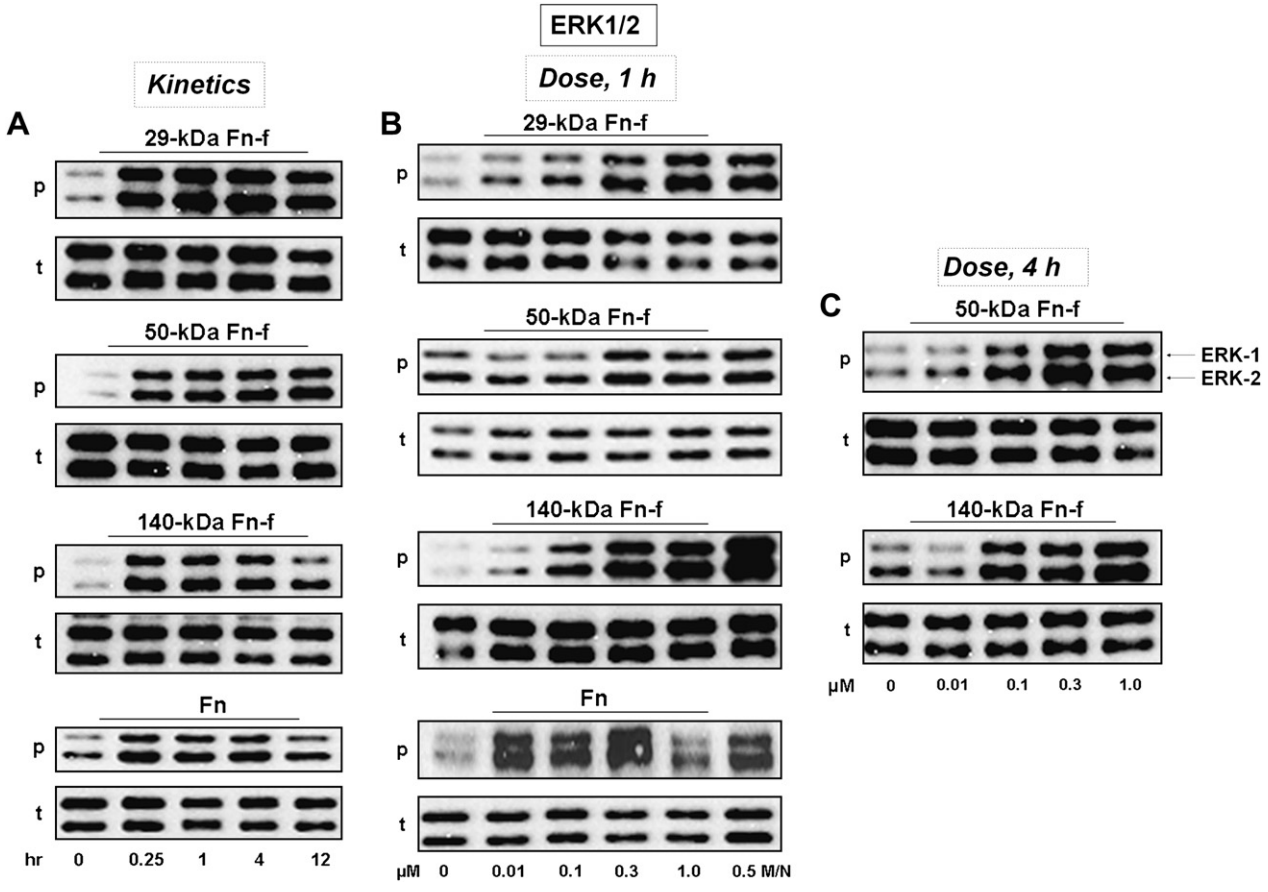

Fig. 2. Effect of Fn-fs and Fn on kinetics of activation of ERK1/2 and dose dependency. After 22 h of serum starvation, (A) cells were treated with 0.5 μM of each Fn-f or Fn and lysed at 0, 0.25, 1.0, 4.0 and 12.0 h; or (B) cells were treated with the Fn-fs or Fn at concentrations ranging from 0.01 to 1.0 μM and cell lysates were recovered at 1 h; or (C) cells were treated with the same concentration range of the 50-kDa or 140-kDa Fn-f, but cell lysates were recovered at 4 h. Proteins were first reduced with 0.5 M DTT and then resolved on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) and the blots probed with anti-total (t-) or phospho (p) ERK1/2 antibody. HRP linked goat anti-rabbit antibody and chemiluminescent substrate was used to visualize ERK1/2 bands on a nitrocellulose membrane. In (B), in the 29-kDa strip, the M/N lane corresponds to lysates from MMP-3 digest treated cells such that the two activities could be compared and in the 50-kDa, 140-kDa and Fn strips, the M/N corresponds to 0.5 μM 29-kDa Fn-f treated lysate so the activities of the other proteins could be compared to the most potent Fn-f, the 29-kDa Fn-f.

maximal effects between 0.1 and 1 μM, although the increases in 50-kDa Fn-f samples were less than for the other Fn-fs. The far right lane of the gel of the 29-kDa Fn-f samples corresponds to a 1-h 0.5 μM MMP-3D treatment (0.5 M/N) and shows activity similar to the 0.3–1 μM 29-kDa Fn-f. The far right lanes in gels of the 50-kDa, 140-kDa Fn-f and Fn samples (0.5 M/N) correspond to a 1-h 0.5 μM 29-kDa Fn-f incubation as a positive control. The effects of Fn were evident at 0.01 μM, while 1 μM appeared to decrease phosphorylation. The effects of 0.5 μM 29-kDa Fn-f at 1 h were quantified as increases in intensity for the ERK2 bands as compared with controls. With three experiments the effects of ERK2 were 9.8 ± 4 (S.E.M.) fold. As shown in Fig. 2(C), the dose dependent effects of the 50 and 140-kDa Fn-fs still occurred at 4 h. BSA negative controls (not shown) had no detectable effects. Similar data were obtained with three different chondrocyte preparations.

THE THREE Fn-fs HAD DIFFERENTIAL EFFECTS ON 46-kDa JNK (JNK-1) AND 54-kDa JNK (JNK-2)

As shown in Fig. 3(A), all three Fn-fs at 0.5 μM enhanced phosphorylation of 46-kDa JNK-1 by 15 min, while native Fn had far weaker effects. This Fn-f burst subsequently decreased to baseline by 12 h. The 29-kDa Fn-f also increased activation of JNK-2 (p54) at 1 h while the 50-kDa or 140-kDa Fn-fs only had weak effects. Probing for β-actin as a secondary loading control confirmed even loading of protein for not only the JNK blots but other kinase blots as well.

Figure 3(B) shows dose–response data for a 1-h incubation and shows that the 29-kDa Fn-f was effective from 0.1 to 1.0 μM in mostly enhancing the JNK-1 isoform although weak effects on JNK-2 were detectable. The 50 kDa at 1 μM enhanced JNK-1 while the effects of the 140-kDa and Fn were far weaker. Neither the 50 or 140-kDa Fn-fs, nor Fn, had detectable effects on JNK-2 at 1 h. The far right lane in the gel for 29-kDa Fn-f treatment in panel B for a 1-h 0.5 μM MMP-3D incubation suggests similar activities for both treatments while the far right lanes in gels for 50 or 140-kDa Fn-f samples for a 1-h 0.5 μM 29-kDa Fn-f incubation show the much greater activity of the 29-kDa Fn-f. The effects of 1 μM 29-kDa Fn-f at 1 h were a 9.7 ± 2.6 fold increase for JNK-1 as compared with untreated control at 1 h. The effects of 0.5 μM 29-kDa Fn-f were compared with those of a 1-h incubation with human interleukin-1β (hIL-1β) from 0.02 to 5 ng/ml. Dose dependent effects of the cytokine were observed and effects at 5 ng/ml were similar in intensity and JNK isoform distribution to that of the 29-kDa Fn-f (data not shown). Panel C shows that at 4 h, 1 μM 50 and 140-kDa Fn-f increased phosphorylation of not only JNK-1, but also JNK-2.

THE THREE Fn-fs ENHANCED p38 MAP KINASE ACTIVATION

As shown in Fig. 4(A), the three Fn-fs enhanced p38 phosphorylation by 15 min which was prolonged through

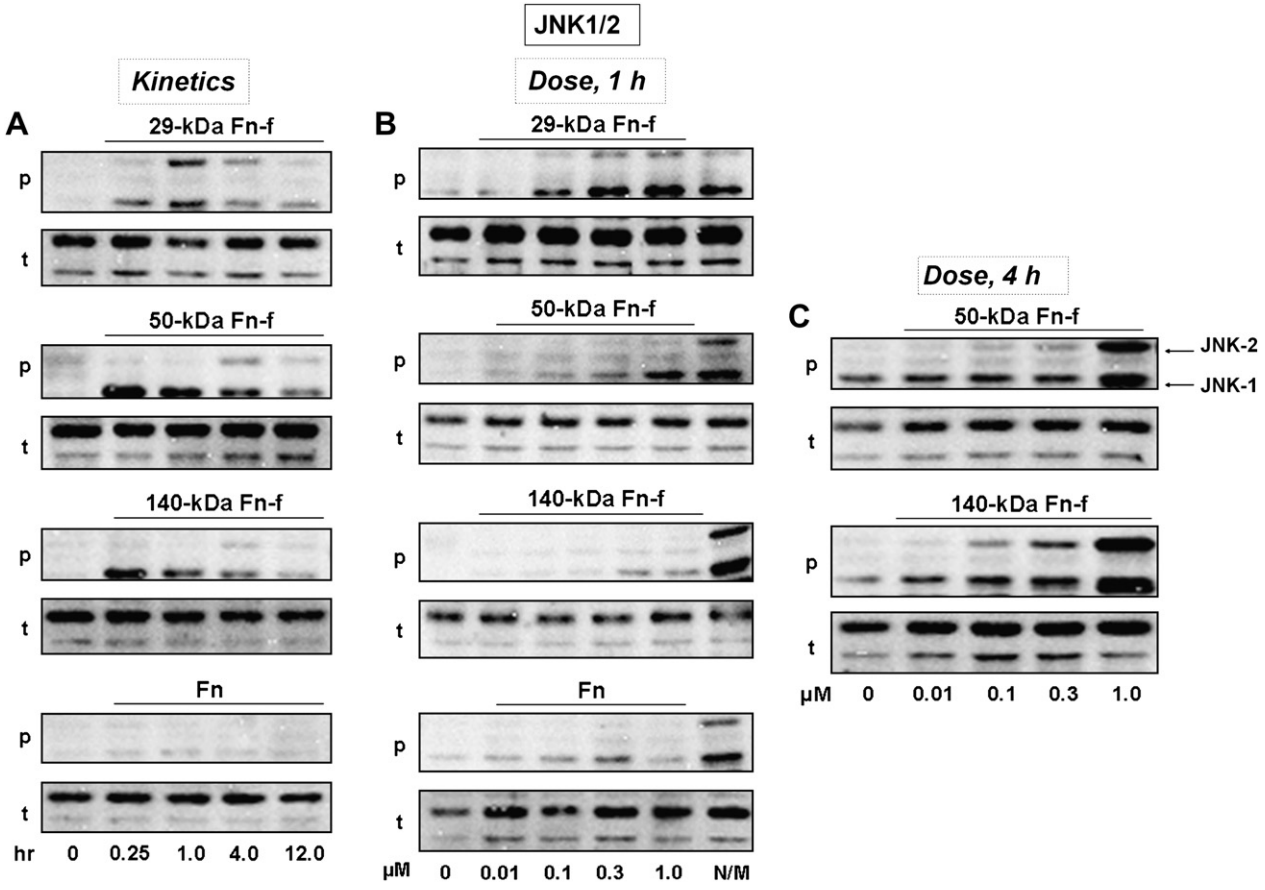

Fig. 3. Effect of Fn-fs and Fn on kinetics of activation of JNK1/2 and dose dependency – conditions were as in Fig. 2. Panel A represents kinetic data, panel B represents dose–response data at 1 h and panel C represents dose–response data at 4 h.

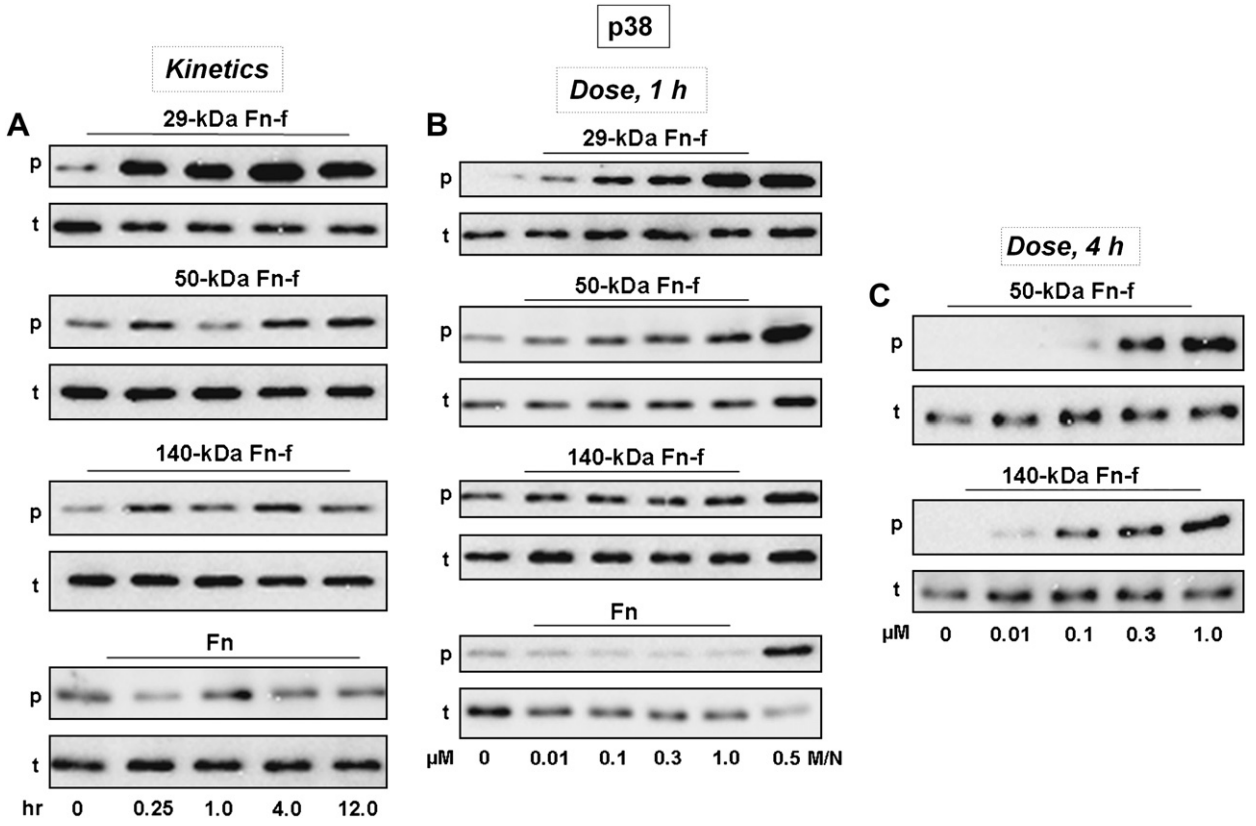

Fig. 4. Effect of Fn-fs and Fn on kinetics of activation of p38 and dose dependency — conditions as in Fig. 2.

12 h. For the 50-kDa and 140-kDa Fn-f treatments a reproducible decrease was observed at 1 h, as also observed for the JNK1/2 blots. Native Fn did not show enhanced p38 activation even with a 12-h incubation.

Figure 4(B) shows that with a 1-h incubation, the 29-kDa Fn-f was effective from 0.01 to 1 μM. The MMP-3D Fn-f mixture showed effects similar to those of the 29-kDa Fn-f. However, the 50 and 140-kDa Fn-fs had very weak effects as compared to the 29-kDa Fn-f treatment, shown in the far right lanes. Fn was ineffective over the same concentration range as compared with the 29-kDa Fn-f positive control in the far right lane. The fold increases as compared with controls for the 1 μM 29-kDa Fn-f at 1 h were 11.6 ± 3.3. The intensity of the signal for 0.5 μM 29-kDa Fn-f was similar to that of 5 ng/ml hIL-1β (data not shown). With a 4-h incubation [Fig. 4(C)], the 50 and 140-kDa Fn-fs did show dose dependent effects and were active at concentrations as low as 0.1 μM.

IN THE PRESENCE OF Fn-fs, PHOSPHORYLATED MAP KINASES HAD A NUCLEAR AND PLASMA MEMBRANE DISTRIBUTION

Cells were treated with the 29-kDa Fn-f for 4 h, fixed and then reacted with the nuclear staining dye DAPI as well as antibodies to phosphorylated kinases. Figure 5 shows in untreated cells a clear nuclear blue pattern with little staining with TRITC labeled anti-phospho-kinases. In contrast, treatment with the 29-kDa Fn-f for 4 h markedly enhanced colocalization of phospho-ERK, p38 or JNK with the nuclear membrane and within the nucleus. Note the red tint in the otherwise blue nucleus and the formation of red bodies within the nucleus. Some red staining of the cell membrane of Fn-f treated cells is also obvious with all three antibodies. The far two columns show some of the cells enlarged (enl) by a few fold and the images further illustrate the marked effects of the Fn-fs. Fn treated cells showed patterns more similar to untreated cells for p38, JNK1/2 and ERK1/2 (data not shown).

THREE Fn-fs UPREGULATED MMP-3 AND MMP-13 AT DIFFERENT RATES BUT Fn DID NOT ENHANCE MMP EXPRESSION AND MAP KINASE INHIBITORS DECREASED 29-kDa Fn-f MEDIATED UPREGULATION OF MMPs

To provide baseline data for testing effects of MAP kinase inhibitors, chondrocyte cultures were treated with Fn-fs or Fn and at various times media were probed for MMPs. Figure 6 (top) shows that neither MMP-3 nor MMP-13 was upregulated over a 48-h period in untreated controls as compared with a 24-h 0.5 μM 29-kDa Fn-f treatment (far right lane, *24). However, treatment with the 29-kDa Fn-f upregulated MMP-3 by 8 h and MMP-13 by 24 h. The major MMP-3 and MMP-13 bands corresponded to their zymogen forms at 59 and 60-kDa, respectively. The 50 and 140-kDa Fn-fs had similar effects and enhanced both MMPs by 24 h. Native Fn had no detectable effects on MMP-3 and MMP-13. The far right lane in the Fn gel corresponds to a 24-h incubation of 0.5 μM 29-kDa Fn-f as a positive control. Because of the significant effect by 24 h for all three Fn-fs, this time point was used for further studies.

Cultures were next adjusted to various concentrations of kinase inhibitors and expression of MMP-3 and MMP-13

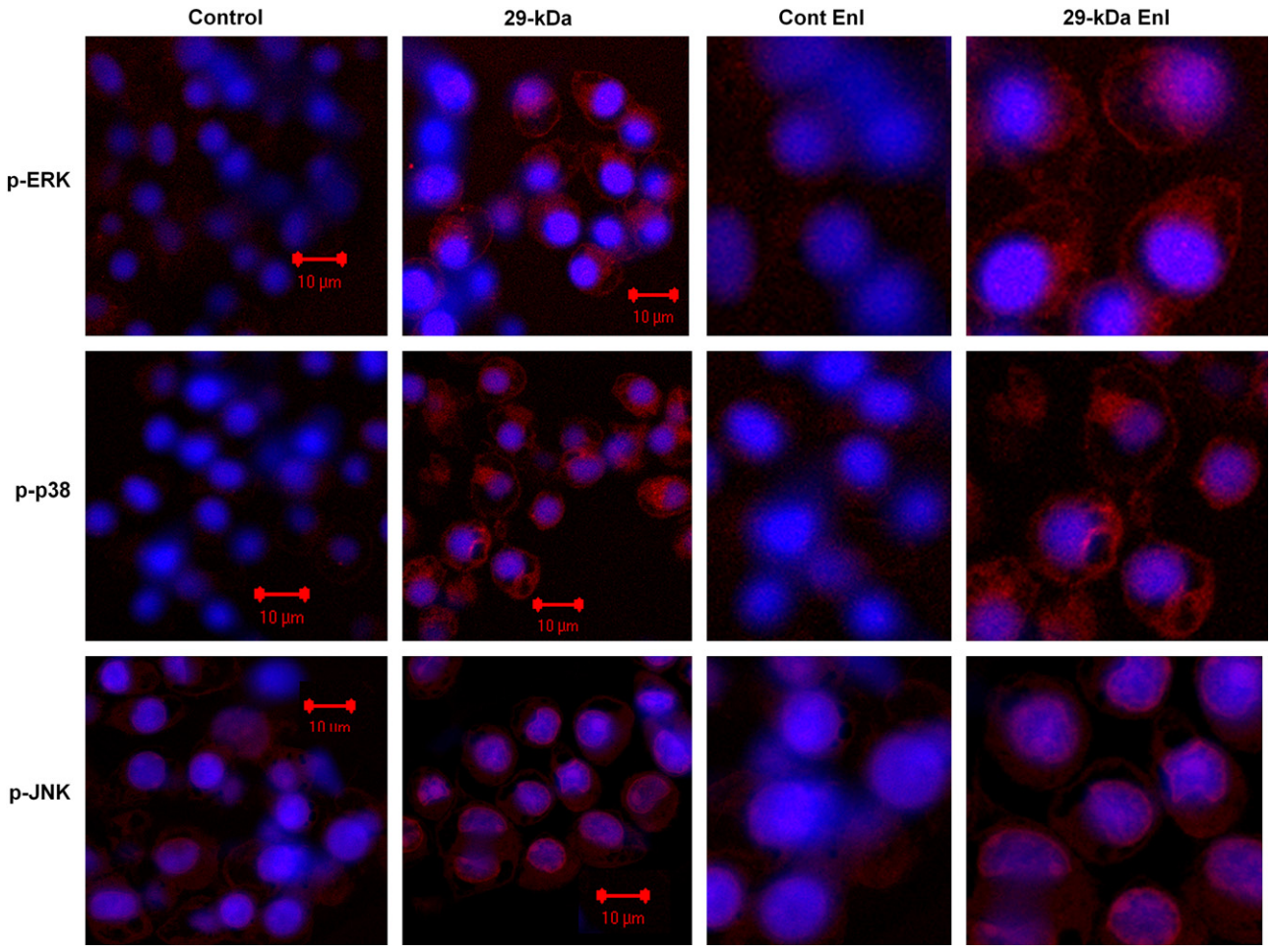

Fig. 5. Effect of Fn-f on intracellular distribution of phosphorylated MAP kinases. Cells were incubated in DMEM with 10% serum for 4–5 days in eight well chamber slides and then serum deprived for 22 h. The 29-kDa Fn-f was added and after 4 h cells were fixed and analyzed for distribution of p-ERK1/2, p-p38 and p-JNK by fluorescent confocal microscopy. Cells were counterstained with DAPI to visualize the nucleus. Control cells were untreated. Cont Enl and 29-kDa Enl were arbitrarily chosen fields that were enlarged to enhance visualization.

was probed 24 h after addition of the 29-kDa Fn-f. As shown in Fig. 7, the DMSO carrier had no effect on MMP expression and the inhibitors by themselves at the highest concentrations tested had no effect on MMP expression. However, the MAP kinase inhibitors decreased the effects of the 29-kDa Fn-f on both MMPs.

MAP KINASE INHIBITORS ALSO BLOCKED Fn-f MEDIATED CARTILAGE DAMAGE

To confirm an active role for the MAP kinases in Fn-f mediated damage to cartilage, explants were cultured in the presence of MAP kinase inhibitors for 24 h prior to the MMP-3D. As shown in Fig. 8, addition of Fn-f in 0.4% DMSO in media (D + f) or Fn-f in media (f) decreased PG content to a significant extent by 7 days as compared to cartilage only treated with 0.4% DMSO in media (D). The inhibitors by themselves in DMSO (PD, SP and SB) did not significantly decrease PG content. However, the ERK1/2 inhibitor (PD98059) in the presence of Fn-f increased PG content to above DMSO treated levels (D). The JNK1/2 inhibitor (SP600125) and the p38 inhibitor (SB202190) decreased the PG depletion activity of the Fn-f.

Discussion

Our objectives were to characterize the role of MAP kinases in Fn-f mediated MMP upregulation and cartilage by comparing three different Fn-fs with different cartilage damage potentials and with different affinities for cartilage tissues and for the isolated $\alpha_5\beta_1$ integrin. The 29-kDa Fn-f is the most potent damaging Fn-f, followed by the 50-kDa Fn-f and finally the 140-kDa Fn-f[1]. The 29-kDa Fn-f has affinity to heparin[1,44], the 50-kDa Fn-f can bind tightly to gelatin and collagen[1], and the 140-kDa Fn-f binds weakly to heparin[44] and to the protein core of heparin sulfate PG[45]. Since only the 140-kDa Fn-f has cell binding activities, yet all three alter intracellular signaling and upregulate MMPs, it is likely that Fn-f interaction with the matrix itself indirectly activates integrins.

The concentration range studied here, up to μM, was within the range we have reported for 30–200-kDa Fn-fs detected in OA synovial fluids[46] and for similar sized Fn-fs found in OA cartilage extracts as detected with an amino-terminal specific antibody[33]. The amino-terminal 29-kDa Fn-f studied here might be very relevant to the chondrolytic state in OA, since a 30-kDa amino-terminal Fn-f can be

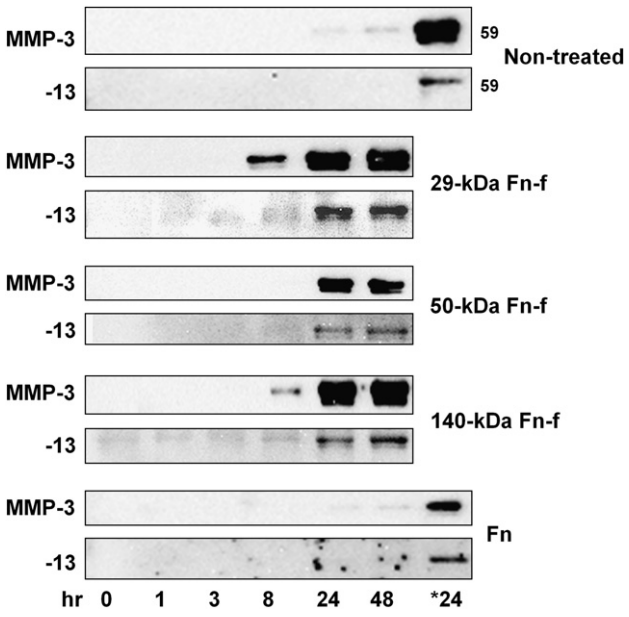

Fig. 6. Effect of Fn-fs and Fn on kinetics of upregulation of MMP-3 and MMP-13. Cells were incubated in DMEM with 10% serum for 4–5 days until cells reached 70–80% confluency and then serum deprived for 22 h. Media were changed to fresh DMEM 2 h prior to the addition of Fn-fs or Fn. Media were recovered at 0, 1, 3, 8, 24 and 48 h. After dialysis against water, media were concentrated to 10×, denatured with 2× sample buffer and then reduced with 0.05 M DTT. Equal amounts of sample were reduced with 0.5 M DTT and loaded onto a 10% SDS-PAGE gel. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and probed with anti-MMP-3 or MMP-13 antibody. Sample treated by the 29-kDa Fn-f for 24 h was loaded onto the last lane of Fn as a positive control. The nontreated controls' panels show the 59-kDa positions of MMP-3 and MMP-13.

detected in OA cartilage extracts by amino-terminal specific antibodies[33].

All three Fn-fs bound to chondrocytes consistent with their abilities to alter cellular signaling. The three Fn-fs and Fn enhanced activation of ERK1/2 within 15 min with sustained effects up to 12 h. Fn appeared to have similar effects but was active at concentrations lower than the Fn-fs. The observation that Fn-fs enhanced ERK activation is interesting since ERK is involved in growth factor pathways, although recent observations also suggest catabolic roles[47–51].

The three Fn-fs activated JNK-1 within 15 min to varying degrees and with subsequent declining effects. In contrast, the Fn-fs had more prolonged effects on p38. The 29-kDa Fn-f appeared to have the greatest effects on both kinases and at lower concentrations. The 29-kDa Fn-f had maximal effects within 1 h, while the 50 and 140-kDa Fn-f required higher concentrations and up to 4 h for maximal effects, especially for JNK isoforms. Native Fn was minimally active toward p38 and JNK consistent with the observation that Fn does not upregulate MMPs nor enhance cartilage damage[1]. The activation of the MAP kinases by Fn-fs was confirmed by observation of phosphorylated kinases on the nuclear membrane a known property of activated MAP kinases[54–56].

The observation that JNK-2 was enhanced by the 29-kDa Fn-f within 1 h but only weakly by the other two Fn-fs at 4 h was especially interesting since JNK isoforms have been reported to vary in terms of their interaction with activating transcription factor 2 (ATF2), Elk-1 and Jun transcription factors[52] and JNK plays a key role in MMP expression and joint destruction in inflammatory arthritis[53].

The longer time required for maximal effects of the 50 and 140-kDa Fn-fs and the enhancement of p38 activity up to 12 h bring into question the role of secondary pathways that might explain some of these observations. For example, cytokine pathways might contribute to Fn-f mediated MAP kinase activation. We have shown that Fn-fs enhance levels of tissue necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) protein

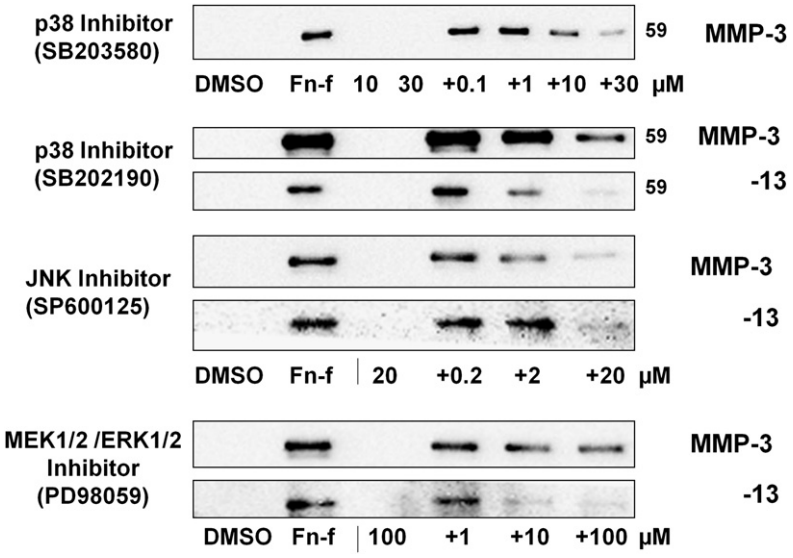

Fig. 7. Effect of MAP kinase inhibitors on Fn-f mediated MMP-3 and MMP-13 upregulation. Cells were cultured and treated as in Fig. 6, except some cultures were preincubated with a series of concentration of inhibitors, SB203580, SB202190, SP600125 or PD98059 for 2 h and then treated with or without 0.5 μM 29-kDa Fn-f for 24 h. Lanes shown are for DMSO alone (0.4% DMSO), addition of Fn-f with DMSO (Fn-f) or addition of inhibitor in DMSO for 2 h, followed by addition of Fn-f and analysis after 24 h (lanes with + sign). Then media were analyzed as in Fig. 6. The top strips show the positions of 59-kDa MMP-3 and MMP-13.

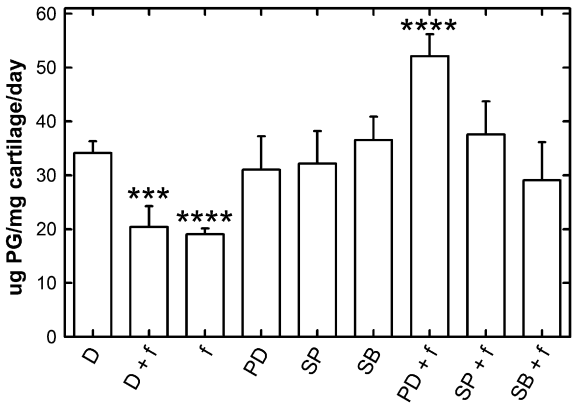

Fig. 8. Effect of MAP kinase inhibitors on Fn-f mediated cartilage matrix PG depletion in 10% serum 7 day cultures. Bovine cartilage in 10% serum/DMEM and 0.4% DMSO was cultured with inhibitors to MAP kinases for 24 h before Fn-f treatment and after 7 days, cartilage was recovered, weighed, subjected to papain digestion and PG content measured with DMB reagent. D represents cartilage treated with 0.4% DMSO, D + f is cartilage treated with DMSO and Fn-f, f is Fn-f alone, PD is PD98059 for inhibition of MAP kinase kinase 1/2 (MEK 1/2), SP is SP600125 for inhibition of JNK and SB202190 (SB) is for inhibition of p38. Bars are SD values. $^{**}P < 0.01$ and $^{***}P < 0.005$ as compared to control untreated.

and that they play active roles in Fn-f mediated cartilage damage[8,9]. Early kinase activation might lead to cytokine upregulation and downstream participation of a cytokine/MAP kinase driven catabolic pathway.

MMP-3 was a focus because we have shown it to be a major player in Fn-f mediated cartilage damage[3] and MMP-13 was of particular interest since it mimics, in a transgenic mouse model, joint changes characteristic of OA[57,58]. All three Fn-fs upregulated both MMPs by 8 h. As with differences in kinetics of activation of MAP kinases, the strongest cartilage damaging Fn-f, the 29-kDa Fn-f, upregulated MMPs faster than the other two Fn-fs. Tests of MAP kinase inhibitors showed an active role for the MAP kinases and that MMP-3 and MMP-13 production could be decreased in a parallel fashion. While our data suggest parallel effects, a single MAP kinase inhibitor does not always affect these two MMPs in parallel[47,50,59,60].

A recent report has shown that the p38 inhibitor, SB202190, above 5 μM also inhibits JNK[61]. Thus, our inhibition of p38 at 2 μM might have been partly due to a small effect on JNK, however SB203580, another p38 kinase inhibitor, also inhibited Fn-f activity and this inhibitor has been reported by the manufacturer to be specific at 100 μM, 10 times the concentration studied here. Further, the JNK inhibitor we used, SP600125, only has nonspecific effects toward p38 at 25 μM[62] and our studies were at 2 μM. However, it is still possible that these kinase inhibitors did inhibit the alternate kinase to a small extent. These inhibitors also decreased cartilage damage by Fn-fs, a significant observation, since there are few studies showing efficacy of kinase inhibitors in decreasing cartilage damage although there is much interest in targeting OA with kinase inhibitors[63].

Conflict of interest

The authors have no financial and personal relationships with entities that could have influenced this work.

Acknowledgments

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