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FGF-2 is bound to perlecan in the pericellular matrix of articular cartilage, where it acts as a chondrocyte mechanotransducer[1]

T. L. Vincent M.D., Ph.D.†*, C. J. McLean B.Sc.†, L. E. Full B.Sc.†, D. Peston B.Sc.‡ and J. Saklatvala Ph.D.†

† *The Kennedy Institute of Rheumatology, Faculty of Medicine, Imperial College School of Science, Technology, and Medicine, London, UK*

‡ *Department of Histopathology, Charing Cross Hospital, London, UK*

Summary

Objective: We have shown previously that cutting or loading articular cartilage resulted in a fibroblast growth factor-2 (FGF-2) dependent activation of the extracellularly regulated kinase (ERK), and induction of a number of chondrocyte regulatory proteins including tissue inhibitor of metalloproteinase-1 and matrix metalloproteinases 1 and 3. An extracellular matrix-bound pool of FGF-2 was apparent, which could be liberated from the tissue by heparitinase (Vincent *et al*., Proc Natl Acad Sci U S A 2002;99(12):8259–64, Vincent *et al*., Arthritis Rheum 2004 Feb;50(2):526–33). Our objectives were to determine where FGF-2 was stored in articular cartilage, to which proteoglycan it was bound, and to elucidate its role in chondrocyte mechanotransduction.

Methods: Immunohistochemistry and confocal microscopy were used to localise FGF-2 in the tissue. *In vitro* binding studies were performed using IASYS surface plasmon resonance. To study the role of pericellular FGF-2 in mechanotransduction cartilage explants or articular chondrocytes encapsulated in alginate were loaded using an in house loading rig. The loading response was assessed by the activation of ERK, in the presence or absence of a specific FGFR inhibitor.

Results: Here we have identified perlecan as the heparan sulphate proteoglycan that sequesters FGF-2 in articular cartilage. Perlecan and FGF-2 co-localised within the type VI collagen-rich pericellular matrix of porcine and human articular cartilage. Chondrocytes encapsulated in alginate were able to accumulate pericellular perlecan and FGF-2 in culture, and deliver an FGF-dependent activation of ERK when loaded.

Conclusion: Loading-induced ERK activation was dependent upon the presence and concentration of pericellular FGF-2, suggesting a functional role for this matrix-bound growth factor in chondrocyte mechanotransduction.
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Key words: Basic fibroblast growth factor, Perlecan, Pericellular matrix, Mechanotransduction.

Abbreviations: FGF-2 basic fibroblast growth factor, HS heparan sulphate, CS chondroitin sulphate, PG proteoglycan, GAG glycosaminoglycan, pERK phosphorylated extracellularly regulated kinase.

Introduction

The ability of the cells of articular cartilage to sense and respond to mechanical force is undisputed. Not only is it well accepted that excessive mechanical stress increases the risk of developing osteoarthritis, but intermittent loads associated with modest weight bearing activity appear to be important for anabolic tissue responses. This is illustrated by the observation that 50% of knee cartilage volume is lost in patients within 6 months of sustaining a spinal cord injury[1], and a recent imaging study in which patients, randomised to moderate exercise, demonstrated increased glycosmaninoglycan (GAG) content in their knee cartilage using delayed gadolinium-enhanced magnetic resonance imaging[2]. These clinical responses are consistent with data showing regulation *in vitro* of matrix proteins such as aggrecan and type II collagen, as well as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), in response to loading cartilage explants and chondrocytes[3–7]. It should be stressed that regulation is complex, and is affected by the frequency, magnitude and duration of the applied load[8–10].

Surprisingly little is known about how mechanical signals are transduced through cartilage to change chondrocyte gene expression. Integrins are favoured mechanical transducers in cartilage: through their extracellular domains they form multiple connections with the extracellular matrix (reviewed in Ref.[11]), and intracellularly, they are thought to recruit signalling molecules (they have no intrinsic signalling activity themselves) to activate signalling pathways[12–14]. Indeed, hyperpolarisation of human articular chondroctyes in response to stretch, and a transforming growth factor (TGF) beta-dependent incorporation of $^{35}SO_4$ in compressed monolayer chondrocytes were both inhibited by alpha 5 beta 1 integrin blockade[15–17].

Intracellular signalling events in response to loading chondrocytes also include activation of ion- and stretch-

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*Address correspondence and reprint requests to: Tonia Vincent, M.D., Ph.D., The Kennedy Institute of Rheumatology, Faculty of Medicine, Imperial College School of Science, Technology, and Medicine, 1, Aspenlea Road, London W6 8LH, UK. Tel: 44-020-8383-4475; Fax: 44-020-8383-4499; E-mail: t.vincent@imperial.ac.uk

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activated ion channels: intracellular calcium flux was detected in chondrocytes in agarose in response to cyclical load[18], and blocking stretch-activated channels, and calcium flux with gadolinium or nifedipine, respectively, inhibited the biological responses to stretch of primary chondrocytes seeded in a collagen matrix[19]. Voltage-activated calcium channels and epithelial sodium channels have been shown to co-localise with beta 1 integrin in 'mechanoreceptor complexes' in loaded limb bud chondrocytes[20].

It is also possible that tissue growth factors act as mechanotransducers. Previously, we found that there was an fibroblast growth factor (FGF)-dependent activation of the extracellularly regulated kinase (ERK) upon cutting or loading articular cartilage[7,21]. FGF-2 was present in the extracellular matrix of articular cartilage and was released following treatment with the heparin degrading enzyme heparitinase, suggesting that it was bound to a heparan sulphate proteoglycan (HSPG). Perlecan, originally described as the proteoglycan of basement membranes, is highly expressed in cartilage[22–24] and is therefore a good candidate for sequestering FGF-2.

We hypothesised that in articular cartilage FGF-2 is stored in the extracellular matrix bound to perlecan, and that it is this pool of FGF that mediates FGF receptor activation upon mechanical stimulation. Here we show that perlecan is an abundant HSPG in human articular cartilage, and that perlecan and FGF-2 co-localise within the pericellular matrix of articular cartilage. Activation of ERK in loaded alginate encapsulated chondrocytes is dependent upon the presence and concentration of pericellular FGF-2.

Methods

TISSUE

Porcine articular cartilage was from the metacarpophalangeal joints, and knee joints of 3- to 6-month-old pigs obtained from a local abattoir 4–8 h post-slaughter. Normal human articular cartilage was from the knee joint of individuals (ages 55 and 60 years) undergoing surgery at the RNOH, Stanmore, for removal of bone tumours.

MATERIALS

Antibodies to perlecan (rat monoclonal, MAB1948) were obtained from Chemicon International (Temecla, CA). FGF-2 antibodies (mouse monoclonal, 05-118), and phosphotyrosine clone 4G10 were from Upstate Cell signalling solutions, Milton Keynes. Collagen type VI anti-serum (rabbit) was kindly provided by Thomas Aigner, Erlangen, Germany. Monoclonal antibodies to beta 1 integrin were purchased from Biohit, Helsinki, Finland. Basic FGF was obtained from Peprotec, London, insulin growth factor (IGF)-1 was from R and D Systems, MN. Heparitinase I, Chondroitinase ABC and the heparan sulphate (HS) stub antibody (3G10) (Seikagaku, Japan) were purchased through Calbiochem, Nottingham, UK. For heparitinase, enzyme was reconstituted upon arrival, aliquotted, then re-lyophilised and stored at −70°C until required. SB402451 (also known as PD173074)[41] was kindly provided by Stephen Skaper, Glaxo Wellcome. All other reagents including hyaluronidase and Insulin Transferrin Selenium (ITS) + 1 liquid media were purchased from Sigma, UK.

CONFOCAL MICROSCOPY

Six-micrometre frozen sections were cut onto Surgipath snowcoat Xtra slides, air dried for 1 h and then left for a further hour at 60°C to ensure tissue adherence. Slides were fixed in acetone for 10 min. Sections were pre-treated with hyaluronidase for 1 h (0.01% hyaluronidase/tris-buffered saline at 37°C) with or without heparitinase (10 mu/ml), blocked with 5% bovine serum albumin/1% goat serum/phosphate-buffered saline (PBS) for 1 h at 37°C, then incubated for a further 1 h at 37°C with antibodies to perlecan (1:1000), FGF-2 (1:1000), type VI collagen (1:5000), beta 1 integrin (1:1000), heparan sulphate stub (3G10) (1:1000) either alone or in combination. Sections were washed with PBS three times, then incubated for 1 h with secondary fluorescein iso-thiocyanate (FITC) labelled antibodies (Alexa 488-conjugated anti-mouse IgG and Alexa 568-conjugated anti-rat IgG). Sections were further washed and mounted. Fluorescence signals were detected by Ultraview confocal microscopy (Perkin–Elmer, 60× oil immersion lens).

IMMUNOHISTOCHEMISTRY

For perlecan/FGF-2 staining, 5-μm frozen sections were prepared as above. Sections were blocked with 10% normal serum and were incubated with primary antibodies to perlecan (1:1000) or FGF-2 (1:1000). Antigens were visualised using streptavidin conjugated peroxidase (ABC kit, Vector) according to the manufacturer's recommendations.

IMMUNOSTAINING OF ALGINATE ENCAPSULATED CHONDROCYTES

Ten alginate beads were dissolved in ethylenediaminetetraacetic acid (EDTA) (100 μl, 50 mM), washed in NaCl (150 mM) and then resuspended in NaCl (20 μl) and loaded onto Lab-Tek II chamber slides (Nalge Nunc Int, Denmark). The slide was air dried, fixed in methanol for 15 min and then stained as above.

PURIFICATION OF PERLECAN AND AGGRECAN

Perlecan and aggrecan were purified from batches of 10 g of articular knee cartilage. Tissue was dissected into a 50-ml falcon and washed three times with sterile PBS. A total of 30 ml of 6 M GnHCl was added to the tissue bringing the volume up to 45 ml, and final concentration to 4 M GnHCl. Tissue was left rotating at 4°C for 48 h. Insoluble material was removed by centrifugation. Purification was as described by Govindraj *et al.*[28], using dot blot to confirm position of proteins. From 10 g of starting material, assuming 99% purity, 0.429 μg of perlecan and 56.42 μg of aggrecan were obtained.

PLASMON SURFACE RESONANCE (IASYS)

This technique, similar to Biacore analysis, was used to detect binding of heparan sulphate proteoglycan to immobilised FGF-2. One microgram of basic FGF or IGF (control) was covalently coupled to carboxymethyl dextran sensor chips (Affinity Sensors, Cambridge, UK). Remaining activated sites on the sensor surface were blocked with 1 M Tris/HCl, pH 8.0. Bound levels of protein were calculated to be between 1 and 6 ng/mm^2. The cuvette was equilibrated with PBS, and binding characteristics were observed

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