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Sprifermin (rhFGF18) enables proliferation of chondrocytes producing a hyaline cartilage matrix

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SUMMARY

Objective: Fibroblast growth factor (FGF) 18 has been shown to increase cartilage volume when injected intra-articularly in animal models of osteoarthritis (OA) and in patients with knee OA (during clinical development of the recombinant human FGF18, sprifermin). However, the exact nature of this effect is still unknown. In this study, we aimed to investigate the effects of sprifermin at the cellular level.
Design: A combination of different chondrocyte culture systems was used and the effects of sprifermin on proliferation, the phenotype and matrix production were evaluated. The involvement of MAPKs in sprifermin signalling was also studied.
Results: In monolayer, we observed that sprifermin promoted a round cell morphology and stimulated both cellular proliferation and Sox9 expression while strongly decreasing type I collagen expression. In 3D culture, sprifermin increased the number of matrix-producing chondrocytes, improved the type II:I collagen ratio and enabled human OA chondrocytes to produce a hyaline extracellular matrix (ECM). Furthermore, we found that sprifermin displayed a 'hit and run' mode of action, with intermittent exposure required for the compound to fully exert its anabolic effect. Finally, sprifermin appeared to signal through activation of ERK.
Conclusions: Our results indicate that intermittent exposure to sprifermin leads to expansion of hyaline cartilage-producing chondrocytes. These *in vitro* findings are consistent with the increased cartilage volume observed in the knees of OA patients after intra-articular injection with sprifermin in clinical studies.

Introduction

Osteoarthritis (OA) is characterised by cartilage degradation mediated by increased catabolic activity of chondrocytes with a concomitant inappropriate repair response. The associated symptoms include pain and limited functionality of the affected joint, resulting in considerably reduced quality of life. Current pharmacological treatment options – which consist primarily of the use of acetaminophen, non-steroidal anti-inflammatory drugs, cyclooxygenase 2 inhibitors or intra-articular corticosteroid injection[1] – are limited, focussing on symptom alleviation but not hindering disease progression. Consequently, there is a strong need for disease-modifying osteoarthritis drugs (DMOADs) that deliver both structural improvement and symptom relief.

DMOADs can either prevent cartilage breakdown during the course of the joint disorder (anti-catabolic approach), promote tissue regeneration (anabolic approach), or both. The anabolic approach consists of reversing the cellular events that occur during OA, thereby promoting repair of injured cartilage. Anabolic compounds under investigation mainly include growth factors involved in cartilage development and homeostasis. Among these, insulin

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growth factor (IGF), transforming growth factor-β (TGFβ), bone morphogenic protein (BMP)-2, BMP7, fibroblast growth factor (FGF) 2 and FGF18 have been evaluated for cartilage engineering or regeneration. They all exhibit anabolic effects and have demonstrated efficacy in preclinical models of OA or cartilage repair[2–4]. However, because of deleterious side effects (such as osteophyte formation, cartilage mineralisation, joint fibrosis or synovial inflammation) or insufficient efficacy, only BMP7 and FGF18 (under the international non-proprietary name, sprifermin) have progressed to clinical trials[2].

FGF18 specifically activates FGFR3 in cartilage and chondrocytes[5,6] and is involved in chondrogenesis[6] and skeletal development[7], and has been shown to stimulate proliferation and extracellular matrix (ECM) production by healthy chondrocytes in monolayer[5]. Furthermore, intra-articular injections of FGF18 have been shown to stimulate cartilage repair in a rat meniscal tear model, inducing a dose-dependent increase in cartilage thickness and significant reduction of degeneration scores[8,9]. Finally, sprifermin injected intra-articularly into the knee of patients with OA has resulted in a dose-dependent reduction of cartilage loss[10].

Published information concerning the biological effects of FGF18 on chondrocytes is very limited[5] and its effects on chondrocyte phenotype or on OA chondrocytes (i.e., chondrocytes from OA patients) are largely unknown. The aim of the present study was to elucidate FGF18's action at the cellular level, to better understand what is observed in *in vivo* preclinical models and in humans[8–10]. Several experiments in monolayer and in 3D culture were conducted using the pharmacological compound sprifermin to: 1) investigate its impact on chondrocyte proliferation and phenotype, 2) evaluate the ability of sprifermin to promote production of a hyaline-like ECM in mature and OA chondrocytes, 3) determine if permanent exposure is necessary for optimal pharmacological activity, and 4) study the signalling pathways involved in FGF18 signalling.

Methods

Sprifermin (recombinant human FGF18) production

Sprifermin, a recombinant human FGF18, was expressed in *Escherichia coli* and purified as previously described[5]. Sprifermin is a truncated, 170 amino acid form of FGF18 (molecular weight = 19.83 kDa), from which the signal sequence, and the 11 C-terminal amino acids, have been removed. Consequently, sprifermin starts with a methionine followed by amino acid 28 (Glu) and ends with amino acid 196 (Lys) of the wild-type human FGF18. The stock solution was in 4.7 mg/mL in 7 mM Na_2HPO_4, 1 mM KH_2PO_4, 2.7 mM KCL, pH 7.3 and was diluted directly in culture medium at wished working concentrations.

Porcine chondrocyte culture

Porcine chondrocytes were isolated from the femoral heads of pigs, approximately 1 year of age, obtained from a local slaughterhouse.

For the monolayer, cells were first cultured 1 week in monolayer in HAM's F12 supplemented with 10% foetal calf serum (FCS) (Promocell GmbH), 1% Penicillin/Streptomycin and 50 μg/mL ascorbate-2-phosphate (Sigma–Aldrich). Cells were then passaged and further cultured in the same medium with increasing sprifermin concentrations from 0.1 to 10,000 ng/mL. After 5 days, cells were stained for actin (see Supplementary Methods) and after 7 days cells were counted with a Vi-CELL™ Analyzer counter (Beckman Coulter Inc.) and glycosaminoglycan (GAG, see Supplementary Methods) concentration was measured in the medium. Gene expression analysis was performed by real-time polymerase chain reaction (PCR).

For the 3D culture, chondrocytes were cultured in suspension in DMEMHG, 10% serum (FCS from Promocell GmbH or foetal bovine serum from Merck Millipore), 50 μg/mL ascorbate-2-phosphate and 0.4 mM Proline. Cells were cultured for 1 week without treatment and then with 10 or 100 ng/mL sprifermin for 4 weeks according to the scheme Fig. 2(A). As a control, 3D constructs were also left untreated. At the end of the culture period, the 3D constructs were either used for biochemical analysis (deoxyribonucleic acid [DNA], GAG and hydroxyproline [HPro] content), gene expression (real-time PCR), or histological analysis. Prior to biochemical analysis the constructs were digested overnight at 60°C with Papain 0.125 mg/mL (Merck KGaA, Cat. No 1.07144.0025) in 0.1 M Na_2HPO_4, 0.01 M EDTA and 5 mM L-Cysteine.

Human chondrocyte 3D culture

For the culture of human cells, human material from patients who underwent total knee replacement was provided by the Universitätsmedizin Mannheim or the Orthopaedic University Hospital Friedrichsheim in Frankfurt, and obtained with full, ethical, written consent (for Frankfurt ethical approval No. 433/11; for Mannheim ethical approval No. 2013-576N-MA). For the 3D culture, the level of cartilage deterioration (chondromalacia) of the tibial plateau, the condyles and the patellofemoral groove was mapped according to the gross appearance of the cartilage, with Grade I describing the least and IV the most damaged tissue[11]. Cells from three different patients and different grades were isolated separately to obtain three different cell suspensions: two with chondrocytes isolated from Grade II cartilage (from patients 1 and 2), assigned 'Grade II-1' and 'Grade II-2', and one with chondrocytes isolated from Grade III–IV chondrocytes (pooled from patients 2 and 3), assigned 'Grade III–IV'.

Cells were first cultured for 4–5 days in monolayer, harvested and further cultured in 3D as previously described for the porcine chondrocytes or used for the mitogen-activated protein kinase (MAPK) array assay.

Analysis of signalling pathways

Human OA chondrocytes were cultured at 1.5×10^6 cell/T25 flask for 24 h to allow adherence to the flask, before the addition of medium containing 0.5% FCS with or without rhFGF18 100 ng/mL for 15 min. Afterwards, the cells were lysed and the analysis of phosphorylated kinases with the MAPK array was performed according to the recommendation of the manufacturer, 160 μg protein was loaded for each sample. Image acquisition was performed with a Chemidoc XRS+ (Biorad).

For the testing of the different MAPK inhibitors, porcine cells were cultured in monolayer as mentioned above with sprifermin 100 ng/mL in the presence of various inhibitors (see Supplementary Table I). After 5 days, cells were stained for actin (see Supplementary Methods) and after 7 days type I collagen expression was evaluated by real-time-PCR. All inhibition assays were performed at 3% O_2, a condition that was found to better preserve the cell phenotype in monolayer.

Real-time PCR

The cells were homogenised in RLT buffer (from the RNeasy Mini Kit, Qiagen, 3D construct were additionally treated with proteinase