Delivering rhFGF-18 via a bilayer collagen membrane to enhance microfracture treatment of chondral defects in a large animal model.

Dr Daniel Howard, PhD, Orthopaedics Research Unit, University of Cambridge, Box 180, Addenbrooke's Hospital, Hill's Road, Cambridge, UK

Dr John Wardale, PhD, Orthopaedics Research Unit, University of Cambridge, Box 180, Addenbrooke's Hospital, Hill's Road, Cambridge, UK

Hans Guehring, MD, Merck, Darmstadt, Germany

Dr Frances Henson PhD, Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge, UK.

Telephone 0044 1223 337647, Fax 0044 1223 337610 fmdh1@cam.ac.uk

Running Title: rhFGF-18 delivered on a membrane potentiates microfracture healing

Abstract

Purpose: Augmented microfracture techniques use growth factors, cells and/or scaffolds to enhance the healing of microfracture treated cartilage defects. This study investigates the effect of delivering recombinant human fibroblastic growth factor 18 (rhFHF18, Sprifermin) via a collagen membrane on the healing of a chondral defect treated with microfracture in an ovine model. ***Methods:*** 8mm diameter chondral defects were created in the medial femoral condyle of 40 sheep (n=5/treatment group). Defects were treated with microfracture alone, microfracture + intra-articular rhFGF18 or microfracture + rhFGF-18 delivered on a membrane. Outcome measures included mechanical testing, weight bearing, International Cartilage Repair Society repair score, modified O'Driscoll score, qualitative histology and immunohistochemistry for types I and II collagen. ***Results:*** In animals treated with 32μg rhFGF-18 + membrane and intra-articularly there was a statistically significant improvement in weight bearing at 2 and 4 weeks post surgery and in the modified O'Driscoll score compared to controls. In addition repair tissue stained was more strongly stained for type II collagen than for type I collagen. ***Conclusion:*** rhFGF-18 delivered via a collagen membrane at the point of surgery potentiates the healing of a microfracture treated cartilage defect.

Key words: FGF18, chondral repair, cartilage, microfracture, growth factor

Introduction

Microfracture, first described by Steadman *et al* [1,2], permits bone marrow derived mesenchymal stem and progenitor cells into a chondral defect site [3] by making small holes through the subchondral bone plate to access the underlying subchondral bone marrow [4].

The progenitor cells have a multipotent differentiation capacity that includes the ability to form cells of the chondrocyte lineage; this differentiation capacity produces a cartilaginous repair tissue at the site of the defect. Of the many different surgical procedures which are in routine use worldwide in order to promote articular cartilage healing, microfracture is commonly performed [5] and often advocated as a first line of treatment for cartilage defect repair [6].

In the joint, bones are surfaced with hyaline cartilage. Whilst a number of treatment methods stimulate cartilage repair at the site of defects, the type of the repair tissue is crucial for restoration of normal joint function, with improved patient outcome directly correlated with repair tissue quality [7,8]. In microfracture healed defects, the initial tissue formed is granulation tissue which becomes replaced with fibrous repair tissue [4], biochemically and mechanically inferior to hyaline cartilage. Continuous loading of the fibrocartilagenous repair leads to degeneration of the repair tissue [9], with deteriorating results following microfracture at 24 month second-look arthroscopy and biopsy [8]. Thus, one goal of improving the efficacy of microfracture is to modify the repair tissue produced. A number of different strategies have been reported including the use of growth factors in combination with microfracture [10] in animal models – one example of an 'augmented microfracture' strategy. Growth factors used have included the bone morphogenic proteins (BMPs)[11,12], transforming growth factors (TGF-βs) [13] and platelet rich plasma (PRP) [14], with and without biomaterials [15]. Recently, our group reported significantly improved healing of a microfracture treated large animal chondral defect when intra-articular rhFGF-18 (Sprifermin) was administered post-surgery [16].

FGF-18 has been reported to be an anabolic growth factor [17,18], promoting chondrogenesis, osteogenesis and bone and cartilage repair [19-22]. Intra-articular rhFGF-18 has been shown to increase in *de novo* cartilage formation and reduce osteoarthritis (OA) in

rat surgical models of OA[23,24]. These results, in combination with our published data[16], indicate that intra-articular rhFGF18 has the potential to enhance hyaline cartilage repair in microfracture treatment of cartilage defects. However, whilst intra-articular injections are an efficacious treatment method, they are invasive, transiently painful and require repeated clinic visits for administration, leading to reduced patient compliance. Indeed, there is an increasing trend, within the clinic, towards development of 'one-step articular cartilage repair' treatments in order to simplify cartilage defect therapy [25]. The development of a single step system for the administration of FGF-18 to defects treated by microfracture would therefore represent a significant improvement over the intra-articular administration of rhFGF-18.

The purpose of this study was to investigate whether delivering rhFGF-18 via a bilayer collagen membrane at the point of surgery to a microfracture treated chondral defect would demonstrate improved articular cartilage repair compared to microfracture alone or rhFGF-18 administered intra-articularly in an ovine chondral defect model.

Methods

This study received approval from both local research ethics committee and the Home Office.

Animals: A total of forty skeletally mature female Welsh Mountain Sheep (mean age 3.9 years) were included in the study. Each sheep weighed between 40 and 42kg at the start of the experiment with no significant differences in weight between groups. Each experimental group contained five sheep. This number was derived from a Power calculation using the results from previous similar experiments[16].

Experimental design: For all animals, full thickness chondral defects of 8mm diameter were created in the medial femoral condyle (MFC) of the right stifle joint. A microfracture awl was then used to create seven evenly spaced microfracture holes (1.5mm diameter, 3mm deep) in each defect. Eight experimental groups were created (Table 1).

Surgical technique:

The basic surgical procedure was as described previously [16]. An 8mm diameter chondral defect was created 10 mm distal to the condyle groove junction and aligned with the medial crest of the trochlear groove.

rhFGF-18 administration: rhFGF-18 was applied either at point of surgery delivered adsorbed to a membrane or as intra-articular injections. Previous experiments in our group had demonstrated a statistically significant effect of 30μg rhFGF18 administered intra-articularly [16]. **Membrane delivered rhFGF-18:** rhFGF-18 was applied to an 8mm diameter bilayer collagen membrane (Chondrogide, Geistlich) at concentrations between 0.064μg and 32μg (Table 1). The membrane/growth factor construct was applied to the chondral defect and glued in place using Tisseel tissue glue at the periphery of the membrane (Baxter). **Intra-articular rhFGF-18** 30μg rhFGF18 was injected into the medial femoro-tibial joint once a week for 3 weeks at 4, 5 and 6 weeks post-operatively and 16, 17 and 18 weeks post-operatively.

Force plate analysis of weight bearing A force plate (Accusway, AMTI, USA) was used to quantify the weight bearing of the operated limb. Weight bearing was measured at a walking gait prior to surgery, 2 weeks, 4 weeks, 2 months, 3 months, 4 months and 5 months after surgery. At each time point each animal had 10 recordings acquired and a mean weight bearing value calculated. Each measurement was converted into N/kg force and calculated as

a percentage of weight bearing pre-surgery for each individual animal. Weight bearing data was grouped into treatment groups for final analysis.

Necropsy: Animals were humanely sacrificed at 13 or 26 weeks postoperatively using a lethal dose of sodium pentobarbital.

Gross Morphology: The joints were photographed and the surface of the osteochondral defect sites blindly scored using the International Cartilage Repair Society score (Table 2).

Mechanical testing: After the gross morphological observations were made, each implant site underwent non-destructive mechanical testing to determine changes to the cartilage surface surrounding the implant or empty defect. Hardness measurements were taken in duplicate from the centre of the chondral defect, and at a distance of 1 mm inside the original edge of the created chondral defect at the 12, 3, 6, and 9 o'clock positions, and 1mm from the edge in the perilesional cartilage, using a handheld digital durometer (Shore S1, M scale, Instron Ltd, UK). A number between 0-100 would be given with an inbuilt calibrated error of +/-5. These measurements were then repeated in the contralateral limb in the same anatomic sites giving a surrogate measure of hardness of the reparative tissue by expressing the result as a percentage relative to the control cartilage in the contralateral limb, and the perilesional cartilage of the ipsilateral limb.

Histology: Following mechanical testing the specimens were decalcified in formic acid/sodium citrate over four weeks, prior to routine paraffin processing. Sections of 10 μm thickness were made through the central portion of the defect. Sections were stained with Toluidine Blue and Safranin O/Fast Green. The histology sections were blindly scored by one investigator, using a modified O'Driscoll score (Table 3).

Immunohistochemistry: Immunohistochemistry was performed as described previously [26]. The following primary antibodies were used in this study; monoclonal mouse anti human

type I collagen (MP Biomedicals, US, 1 in 200 dilution) and monoclonal mouse anti human type II collagen (MP Biomedicals, US, 1 in 100 dilution). Horseradish peroxidase-conjugated secondary anti-rabbit and mouse immunoglobulins were used as appropriate, and the colour reaction developed with 0.1% 3′, 3-diaminobenzidine tetrachloride (DAB)/0.01% hydrogen peroxide. Normal species-specific serum was used as a control in all experiments.

Analysis of rhFGF-18 concentrations in serum and synovial fluid Blood samples and synovial fluid from the operated joint were obtained from animals in which 32µg rh FGF-18 was administered on the membrane to the chondral defect treated by microfracture (Group H). Samples were obtained at weekly intervals week 1 – 12.

Synovial fluid samples were analysed using a qualified three step immunoassay sandwich method performed on a Gyrolab platform. Samples were treated with 20 µg/mL Hyaluronidase, incubated for 30 minutes at 22±2°C in shaking and centrifuged prior to dilution with assay buffer and analysis. A biotinylated mouse monoclonal antibody against rhFGF-18 (clone F44A2, 0.1 mg/mL, Merck Serono) was used as capture reagent, and an Alexa Fluor-647 labelled monoclonal antibody against rhFGF-18 (clone F5A2, 20 nM, Merck Serono) was used as a detection reagent. The specifically-bound analyte was quantified by laser-induced fluorescence detection.

Statistical analysis: Statistical significance between groups and within groups for each end point was determined using a one-way analysis of variance (ANOVA) and Bonferroni's post hoc test. Where data sets within groups were not found to be normally distributed, a non-parametric Kruskal-Wallis test was instead used, with a post hoc Dunns multiple comparisons test. GraphPad Prism 5 statistical software package (Graphpad Software Inc, La Jolla, CA) was used for data analysis.

Results

Surgery: The surgical procedures and recovery from surgery was uneventful.

rhFGF-18 concentrations: rhFGF-18 was detected in the synovial fluid of all 5 animals at week 1 post surgery (mean 3466.44 pg/ml +/- 1735.94 pg/ml). No rhFGF-18 could be detected in the synovial fluid after week 1 and no rhFGF-18 was detected in the serum at any time point.

Force plate analysis: Using a force plate, the peak vertical force of the operated leg was measured pre and post surgery. In all operated animals there was a reduction in weight bearing at 2 weeks post surgery (Fig 1a and b) and then a recovery in weight bearing with time.

There was a significant difference between weight bearing in animals that received 0μg rhFGF-18 and animals that received 6.4μg rhFGF-18 delivered on the membrane at 2 weeks post-operatively and a significant difference between weight bearing in animals that received 0μg rhFGF-18 and animals that received 32μg rhFGF-18 delivered on the membrane at 2 and 4 weeks post-operatively i.e. animals that received rhFGF-18 had increased weight bearing following surgery. No difference was observed between other experimental groups.

Gross morphology: No adverse effects, for example, osteophyte formation or joint degeneration was found in any of the animals. The quality of repair at the site of the defect was assessed using the macroscopic ICRS scoring scale. No significant difference was found between treatment groups (Fig. 2).

Mechanical testing: At 6m there was no significant difference between the treatment groups, either between the contralateral limb or the perilesional cartilage in the operated limb (Fig. 3).

Quantitative Histology

Modified O'Driscoll total histology scores: All samples were scored using the modified O'Driscoll score (Fig. 4). No differences were detected between the two control groups (i/a vehicle injections and membrane applied with no rhFGF-18 added). The administration of two cycles of i/a rhFGF-18 significantly improved the modified O'Driscoll score. In addition, there was a statistically significant increase in modified O'Driscoll score when either 6.4µg and 32µg rhFGF-18 were loaded onto the Chondrogide membrane when compared to controls. There was no difference between the intra-articular injected rhFGF-18 and 32µg rhFGF-18 loaded onto the membrane at the point of surgery

Histological evaluation and immunohistochemistry In the control sections and those animals receiving 0.064 and 0.64µg rhFGF-18 there was little evidence of cartilage repair (Fig. 5A), as indicated by the modified O'Driscoll score. Most of these samples showed no repair, with denuded subchondral bone still present even at 6 months over much of the damaged zone. In contrast, in the membrane + 32µg rhFGF-18 and 30µg rhFGF-18 administered intra-articularly there was evidence of repair tissue with characteristic features of hyaline cartilage extending over a wider area of the defect with evidence of zonal organisation of the chondrocytes (Figs 5B and C).

IHC for collagen types I and II was performed on all of the samples. In the control samples interpretation of the results was hampered because little repair tissue was present, so that there was minimal tissue present to be stained with either antibody. In the presence of both membrane + 32µg rhFGF-18 and 30µg rhFGF-18, the repair tissue was strongly stained for type II collagen with minimal type I collagen staining, indicating a mature hyaline-like cartilage repair tissue had been produced (Figs. 6a-d).

Discussion

This study demonstrates that a combination of microfracture and 32µg rhFGF-18 applied via a collagen membrane at the point of surgery in a 'one step cartilage repair' - results in significantly improved cartilage repair tissue compared to microfracture, in an ovine chondral defect model. The results seen were comparable to the administration of two cycles of intra-articular 30µg rhFGF-18 in this study and those previously reported by our group [16].

In this study significant improvements were detected in weight bearing in the 2 and 4 week post-operative period and the modified O'Driscoll histology score. In addition, the tissue produced in the presence of rhFGF18 showed a repair tissue phenotype with features typical of hyaline cartilage, namely strong type II collagen immunoreactivity and little or no type I collagen immunoreactivity. In addition, no adverse events were found either with administration of rhFGF-18 on the membrane or with the intra-articular administration of the growth factor, indicating that this treatment does not raise any safety concerns in the joint environment.

Retention of intra-articular medication within the joint is a separate safety concern. Intra-articular medication enters the circulation via both vascular and lymphatic routes and can have potentially significant effects [27,28]. In this study no rhFGF-18 was detected in the systemic circulation in a 12 week experimental period, indicating that the rhFGF-18 was retained within the joint. In contrast, rhFGF-18 was detected within the synovial fluid of the treated joint at 1 week post-surgery. This finding compares favourably with studies of other intra-articular treatment modalities including hyaluronan [29], autologous conditioned serum [30] and interleukin-1 receptor antagonist [31], all of whom are detectable in the joint for less time than detected in this study. This indicates that the collagen membrane vehicle is likely

to have retained the rhFGF-18 locally, providing sustained release of the drug, as has been reported with other growth factors applied to collagen membranes [32,33].

The concept of an‘augmented microfracture’ procedure as a one step cartilage repair is an active area of current research. Recently, enhancement of microfracture techniques by application of stem cells [25], collagen membranes [34], ECM biomembranes [35] and chitosan-based BST-CarGel [36] have all shown superior healing compared to microfracture alone. That the presence of a scaffold or membrane alone leads to increased healing has led to the suggestion that these additions are stabilising or protecting the blood clots formed by the microfracture procedure, supporting the healing of the damaged tissue [37]. In this study, in contrast, we found no difference in any healing outcomes between groups that had microfracture alone and microfracture plus membrane, indicating that, in this model, application of the membrane did not provide any protective effect to the repairing tissue. Significant increases in healing were only detected in the presence of 6.4 and 32µg rhFGF-18.

In this study, three components of healing were examined. In addition to the standard gross findings (ICRS score) and histological analysis (modified O’Driscoll score, immunohistology), we used two functional measures of joint healing, weight bearing and durometer measurements. Durometer measurements indicate the stiffness of the healed cartilage relative to the undamaged cartilage. In this study we did not find a statistically significant difference between treatment groups, similar to that observed by our group in a previous, similar study [16]. These results, taken together, indicate that durometer measurements in this model may be of little functional value perhaps due to the influence of the underlying bone.

In contrast, we have demonstrated that animals that received 32µg rhFGF-18, applied on a membrane at the point of surgery, had significantly increased weight bearing on the operated leg at weeks 2 and 4 post surgery compared to controls and had returned to pre-operative levels of weight bearing by week 8 post surgery. The timing of this increased weight bearing is likely to be too early to be attributed to enhanced healing of the defects and may, perhaps, indicate that rhFGF-18 might have analgesic actions post surgery. However, it must be noted that the sample size used in the study (n=5 per experimental group) was determined using a power calculation designed to allow differences in histological features, not joint loading. Further work is needed in this area to establish the validity of the observation and the mechanisms underlying it.

Improving the quantity and quality of microfracture repair tissue is a clear clinical need [8]. In this, and a previous study [16], we have observed that rhFGF18 significantly improves the quality of healing post defect creation, whether applied at the point of surgery or delivered via intra-articular injection. However, in this study the macroscopic ICRS healing score was not significantly different between rhFGF-18 i/a and controls, as we have demonstrated previously. Whilst the mean ICRS score was higher in animals that had received rhFGF-18 i/a compared to controls, there was a wide variance in the data (all animals were included). This is likely due to biological variance between animals and reflects the lower number of animals used in this study (5 compared to 16 per group in the previous study [16],), as noted for the weight bearing data. Previous data from *in vivo* damage/repair models [22] and FGF-18 over-expression models [38,39], support the observation that rhFGF-18 drives the formation of increased and higher quality cartilage *in vivo.* FGF18 is has ‘anabolic’ effects in cartilage [40] and work in our group has shown that rhFGF-18 alters ECM metabolism and also reduces apoptosis in response to damage [41]. FGF-18 has also been shown to have a

potential chondroprotective role, possibly via regulation of Tissue Inhibitor of Metalloproteinases -1 (TIMP-1) [24].

In conclusion, the administration of rhFGF18 on a collagen membrane significantly enhances the healing of a microfracture treated cartilage defect. This augmented microfracture technique should be considered as a potential novel therapy for articular cartilage repair. Within a clinical setting administration of rhFGF-18 via a membrane to a microfracture treated lesion would allow a 'point of service' application of a novel biological factor that has demonstrable capacity to enhance cartilage healing.

Acknowledgements: The authors would like to acknowledge research grant funding support from Merck and would like to state that Dr Guehring is an employee of Merck.

Table Legends

Table 1: Eight treatment groups were used, with all animals undergoing the microfracture procedure (n=40 total). Groups C was the control i.e. microfracture only, Groups A and B had microfracture plus intra-articular injections, Groups D to H had microfracture plus membrane +/- recombinant human fibroblastic growth factor (rhFGF18). Duration of experiment for Groups A to G 6m, Group H, 3m

Table 2: ICRS macroscopic scoring system

Table 3: Modified O'Driscoll scoring system

299 **Figure Legends**

300 **Fig. 1** Weight bearing in the operated limb as measured using an Accugait force plate. The
301 results presented are the mean +/- SD of the values for 5 animals per group pre surgery and
302 2,4,8,12,16 and 20 weeks post surgery. **Fig 1A.** Weight bearing in animals that had a
303 microfracture treated chondral defect combined with rhFGF-18 delivered via a collagen
304 membrane at the point of surgery. There is a significant difference in the weight bearing in
305 animals that received 32μg rhFGF-18 compared to lower concentrations of rhFGF-18 and the
306 control (0μg rhFGF-18) at weeks 2 and 4 post surgery. **Fig 1B.** Weight bearing in animals
307 that received 0 or 30μg rhFGF18 injected into the medial femoro-tibial joint once a week for
308 3 weeks at 4, 5 and 6 weeks post-operatively and 16, 17 and 18 weeks post-operatively.
309 There is no significant difference between the two groups. * = significant difference at this
310 time point.

311

312 **Fig. 2** The effect of rhFGF18 on the total modified ICRS macroscopic score. There is no
313 statistically significant difference between groups.

314

315 **Fig. 3** The effect of rhFGF19 on the stiffness of the repaired cartilage as a percentage of the
316 contalateral limb. There is no difference between the groups.

317

318 **Fig. 4** The effect of rhFGF18 on the Modified O'Driscoll score. There was a statistically
319 significant increase in modified O'Driscoll score in the animals treated with intra-articular
320 30μg rhFGF18 (*) and those treated with 6.4μg and 32μg rhFGF-18 (*) applied on a collagen
321 membrane at the point of surgery compared to controls and lower doses of rhFGF-18.

322

Fig. 5. Safranin O stained sections. A Control – membrane + 0μg rhFGF18. No hyaline cartilage is present at the lesion site. B Membrane + 32μg rhGFG-18 applied via a membrane showing good hyaline cartilage production at the lesion site. C Intra-articular 30μg rhFGF-18 showing good hyaline cartilage production similar to that seen in B.

Figure 6. Immunohistochemistry. Collagen was visualised using a DAB (brown) stain. **Figs. 6a and b** 2 cycles rhFGF18 at 6 months. Immunohistochemistry of type I and type II collagen **Figs. 6c and d** 32μg rhGFG applied on bilayer membrane at 6 months. Immunohistochemistry of type I and type II collagen. In both treatments the repair cartilage is strongly positive for type II collagen and weakly positive for type I collagen indicating that the cartilage is similar to hyaline cartilage.

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