OsteoArthritis and Cartilage (2005) **13,** 537–544
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doi:10.1016/j.joca.2005.02.007

Osteoarthritis and Cartilage

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Promotion of the intrinsic damage–repair response in articular cartilage by fibroblastic growth factor-2[1]

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Summary

Objective: To identify the effect of fibroblastic growth factor-2 (FGF-2) on the intrinsic damage–repair response in articular cartilage *in vitro*.

Methods: Articular equine cartilage explants, without subchondral bone, had a single impact load of 500 g applied from a height of 2.5 cm. Explants were then cultured in 0, 12, 25, 50 or 100 ng/ml FGF-2 for up to 28 days. Unimpacted discs served as controls for each time-point. Histological and immunohistochemical techniques were used to quantify and characterise the response of putative chondrocyte progenitor cells (CPC) to damage and FGF-2 treatment.

Results: FGF-2 significantly accelerated the appearance and increased the numbers of *de novo* repair cells identified histologically at the cartilage surface. The response was affected by the dose of FGF-2. The repair cells were shown to be chondrocytes by their expression of collagen types II, IX/XI, but not of type I collagen. In addition, these cells, and those underlying the articular surface, were shown to be immunopositive for Notch-1 and PCNA, markers for proliferating cartilage progenitor cells.

Conclusions: The results of this study indicate that, following single impact load, CPC can be stimulated in mature articular cartilage *in vitro*. These CPC and the cells arising from them appear to represent the cartilage's response to damage. The timing of the appearance of CPC and their overall numbers can be significantly increased by FGF-2, providing further evidence for an important role for FGF-2 in modulating cartilage repair. These results indicate that further study into the mechanisms of repair in mature cartilage using this *in vitro* model are vital in understanding the repair capacity of mature cartilage.
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Key words: Cartilage, Repair, Progenitor cells, FGF-2.

Introduction

Defects in articular cartilage are classified as full or partial thickness, depending on whether the underlying subchondral bone is penetrated. Full thickness defects recruit bone mesenchymal stem cells to achieve fully functional healing[1], whilst partial thickness defects are considered unable to repair and form mature hyaline cartilage[2–5].

Despite a widely held previous belief that mature cartilage does not possess cartilage progenitor cells (CPC)[5], direct evidence for a CPC population has recently been reported[6]. These authors described the isolation of CPC from the surface zone of articular cartilage and their phenotypic plasticity in an embryonic chick plasticity tracking system. CPC have also been identified indirectly in normal[2] and diseased cartilage[7] and in isolated chondrocyte populations[5,8,9,10].

We have recently observed a novel intrinsic damage–repair response in mature articular cartilage explants following single impact load and subsequent culture[11,12]. This response was characterised by the appearance of *de novo* cells on the impacted surfaces of cartilage. These cells were chondrocytes, as demonstrated by their positive immunostaining for chondrocytic markers including collagen types II, IX/XI and fibronectin, indicating that they may be the products of CPC expansion and cell migration[12]. In order to further identify the mechanism leading to the appearance of these *de novo* cells it was considered important to determine the effect of possible modulators.

Although much research has focussed on the chondrogenic effects of growth factors, particularly transforming growth factor-beta (TGF-β), insulin-like growth factor-1 (IGF-1) and the bone morphogenic proteins (BMPs)[13,14], relatively few studies have recognised the importance of fibroblastic growth factor-2 (FGF-2). FGF-2 is regarded as a potent mitogen for chondrocytes *in vitro*[15] and a number of studies have demonstrated that FGF-2 may be involved in the cartilaginous repair response in full thickness articular cartilage defects[16–19]. FGF-2 is known to have a proven role in the control of bone-marrow derived stem cells, allowing their *in vitro* expansion and maintenance as chondro-osteo-progenitor cells[20], and Hiraki *et al.*[18] suggested that, in a full thickness defect repair model, FGF-2 may stimulate a selective expansion of CPC.

In addition to an involvement in the modulation of connective tissue progenitor cell behaviour, recent *in vitro*

[1]Supported by the BBSRC and The PetPlan Charitable Trust, UK.
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Received 27 August 2004; revision accepted 6 February 2005.

studies have identified a role for FGF-2 in immediate signalling following loading damage[21,22], making it a good candidate for involvement in repair of mechanically induced damage. The aim of this study was to investigate the effects of FGF-2 on *de novo* cell numbers in a single impact load model of intrinsic damage−repair response in articular cartilage.

Materials and methods

COLLECTION OF SAMPLES

Cartilage was obtained from horses ($n = 8$, age range 18 months−16 years) that were humanely destroyed for reasons other than orthopaedic disease. Discs of articular cartilage (7 mm diameter) were aseptically harvested using a cork borer from the trochlear ridges of the distal tibia and from the lateral and medial trochlear ridges of the distal femur. The depth of the discs was approximately 1 mm, and included all zones of the cartilage from the articular surface to the deep zone down to, but not including, subchondral bone. All explants were immediately placed into sterile phosphate buffered saline (PBS) containing antibiotics and antimycotics (200 IU/ml penicillin, 2.5 μg/ml fungizone, 100 μg/ml streptomycin), then washed 3 times in sterile PBS.

SINGLE IMPACT LOADING

Cartilage was impacted using a drop tower device as described by Bowe *et al.*[23]. Samples were impacted with 0.175 J at 0.7 m/s (500 g dropped from 2.5 cm[24]), corresponding to a nominal stress of approximately 8 MPa[25]. Unimpacted discs served as controls.

CULTURE OF CARTILAGE DISCS

After impact, explants were cultured for 1, 3, 5, 7, 10, 20 and 28 days in Dulbecco's Modified Eagles Medium (DMEM) containing 10% foetal calf serum and antibiotics and antimycotics as detailed above. Explants were divided into four groups: untreated, impacted and non-impacted controls and impacted and non-impacted FGF-2 supplemented groups to which recombinant human FGF-2 (Sigma, UK) was added to a final concentration of 12, 25, 50 or 100 ng/ml. The FGF-2 treatment was continuous, medium with freshly added FGF-2 being changed every 7 days, to avoid unnecessary disturbance of the cartilage explants. Following culture, cartilage discs were either placed into 4% formal saline solution and processed for routine histological examination or embedded in Tissue Tek OCT and snap-frozen in liquid nitrogen. Paraffin and cryostat sections (7 μm) were used in histological and immunohistochemical studies.

HISTOLOGICAL ANALYSIS

Sections were stained with Haematoxylin & Eosin (H&E) or Toluidine blue. Routine light microscopy was used to demonstrate cartilage architecture, the extent of cartilage damage, changes in chondrocyte morphology and to estimate proteoglycan (PG) content within the extra-cellular matrix (ECM).

DE NOVO CELL QUANTIFICATION

The appearance of *de novo* cells was quantified by manually counting the number of *de novo* cells per ×20 field at three separate, randomly selected places on the articular surface per section at each experimental time-point in each animal. Values were expressed as mean numbers (±standard deviation) of *de novo* cells observed at the articular surface. A paired Student's *t* test was used to establish statistical significance between groups.

IMMUNOHISTOCHEMICAL ANALYSIS

Immunohistochemical analysis was performed as described previously[11,26]. The following primary antibodies were used; polyclonal rabbit anti-rat collagen IX/XI (Calbiochem), polyclonal rabbit anti-porcine type II collagen, polyclonal mouse anti-bovine collagen I (Sigma−Aldrich) and monoclonal mouse anti-proliferating nuclear antigen (PCNA) (Sigma−Aldrich) and goat anti-human Notch-1 (Santa Cruz). Horseradish peroxidase-conjugated secondary anti-rabbit, mouse and goat 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.

Results

CARTILAGE DAMAGE FOLLOWING IMPACT LOAD

Following dissection, single impact load and subsequent culture, cartilage showed characteristic damage at the articular surfaces. The damage pattern immediately post-impact was found to be variable across the surface of the impacted cartilage and included mild, intermittent surface roughening with little loss of metachromatic PG staining [Fig. 1(A)], isolated 45° fissures with slight PG loss [Fig. 1(B)] and multiple 90° mini-fissures [Fig. 1(C)]. Between the damage sites the cartilage appeared histologically normal. All control, unimpacted cartilage samples presented a smooth, intact articular surface along the entire length of the explant, with uniform metachromatic staining and no loss of PG [Fig. 1(D)]. This typical pattern seen immediately after impact did not change during culture and was similar in cartilage from different sites and from different aged animals, within the range used in this study.

HISTOLOGICAL APPEARANCE OF ENDOGENOUS REPAIR RESPONSE IN CARTILAGE

Cartilage samples that had been impacted and then cultured showed evidence of a repair response after 10 days in culture. At this time small numbers of *de novo* cells were observed at the articular surface. No such cells were seen at 10 days in unimpacted controls. With increasing time in culture (approximately 20 days) cell numbers increased and cells were found accumulated particularly at sites of fissure formation [Fig. 2(A)]. By 28 days in culture the *de novo* cells appeared to be forming a continuous layer along the articular surface between the damage fissures [Fig. 2(B)].

Towards the end of the culture period some *de novo* cells were also observed along the cut edges of both impacted and unimpacted cartilage, indicating a response to damage caused by dissection.

No differences in damage response were apparent in this study between cartilage from different aged animals or from different joints.

The *de novo* cells were shown by immunohistochemistry to be producing PGs, collagens types II and IX/XI but not

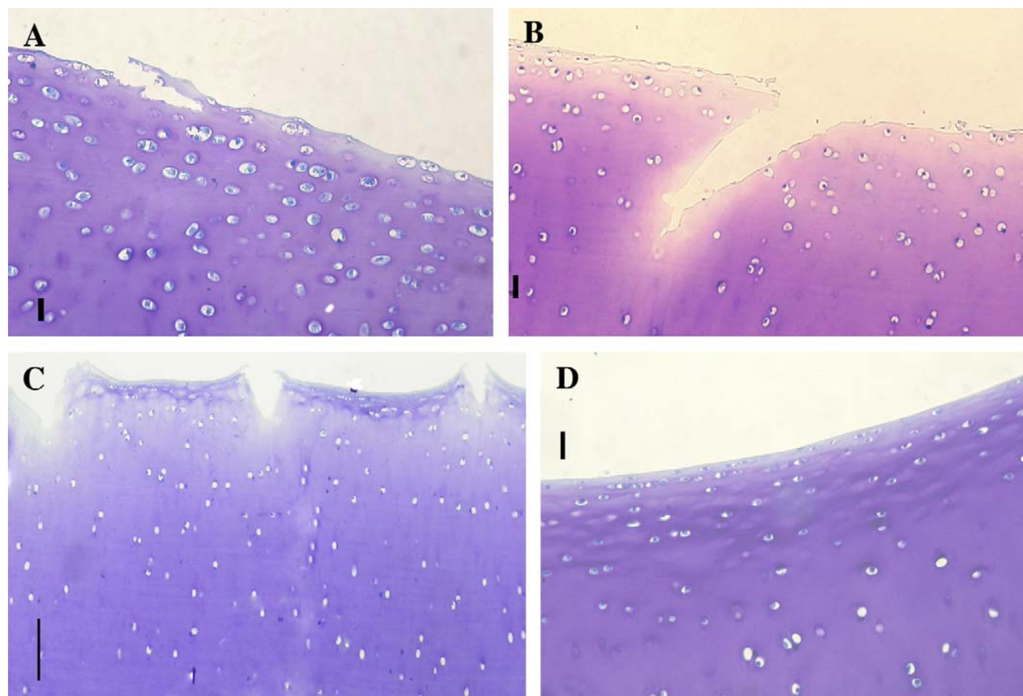

Fig. 1. Effect of a single impact load (500 g from 2.5 cm) on articular cartilage. (A) Impact causes surface roughening. Scale bar = 10 μm. (B) Impact causes formation of deep 45° fissures. Scale bar = 10 μm. (C) Articular cartilage after 7 days in culture. Impact causes multiple fissure formation. Scale bar = 10 μm. (D) Control section with no impact load applied. Scale bar = 10 μm. All sections stained with Toluidine blue.

type I[11,12], and were therefore assumed to be chondrocytes.

EFFECT OF FGF-2 ON THE ENDOGENOUS REPAIR RESPONSE

FGF-2 at the lower concentrations of 12 and 25 ng/ml had little effect on the number of *de novo* cells, but present at 50 and 100 ng/ml, it was noted that considerably increased numbers of *de novo* cells were present on the impacted cartilage and that there was evidence of clonal proliferation in the cartilage underlying the articular surface (considered as multiple nuclei within a single lacuna and evidence of mitotic bodies [Fig. 3(A)]). In addition, a number of cells within the ECM were noted to have an altered phenotype, having an elongated spindle shape instead of the usual rounded chondrocyte shape. These spindle-shaped cells were often orientated towards the articular surface and, in some instances, appeared to represent cells migrating out onto the surface [Fig. 3(B)].

At these higher concentrations of FGF-2 some depletion of PG from the extracellular matrix was apparent in some samples, as assessed by loss of staining with Toluidine blue [Fig. 3(A,B)]. This was not a consistent finding, however. More evident was the reproducible strong staining of the *de novo* cells, indicating continuing production of PG.

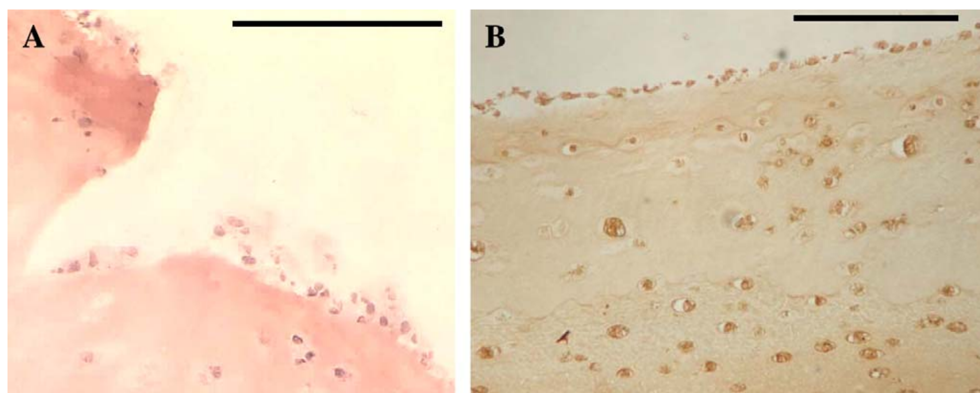

Fig. 2. Articular cartilage following impact, showing response to damage. (A) After 21 days in culture post-impact (0 ng/ml FGF-2) showing *de novo* cells at the site of fissure damage. Stained with H&E. Scale bar = 160 μm. (B) After 28 days in culture post-impact (0 ng/ml FGF-2) showing *de novo* cells covering the articular surface. Immunohistochemical staining with antiserum to collagen type IX/XI. Scale bar = 100 μm.

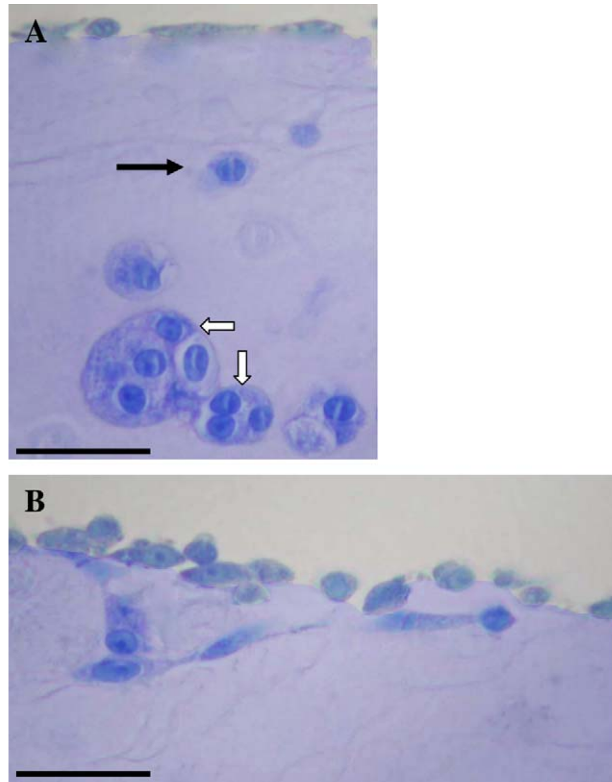

Fig. 3. Articular cartilage following impact damage and cultured for 7 days in the presence of 50 ng/ml FGF-2. (A) A single layer of *de novo* cells is present on the articular surface of the cartilage. In the cartilage beneath the articular surface, cells appear to be undergoing mitosis (arrow) and clonal expansion (white arrows). Scale bar = 45 μm. Stained with Toluidine blue. (B) A number of spindle-shaped cells are apparent immediately below the *de novo* cells. Scale bar = 45 μm. Stained with Toluidine blue.

TIME AND DOSE-DEPENDENCY STUDIES

Fig. 4(A) shows the change with time in the mean number of *de novo* cells present on the articular surface of impacted and non-impacted (control) cartilage in response to different concentrations of FGF-2 during *in vitro* culture. At day 1 in culture no *de novo* cells were present on impacted cartilage explants cultured either in the presence or absence of FGF-2. *De novo* cells were first detected, in small numbers, after 3 days in culture post-impact [Fig. 4(B)] in the presence of 50 and 100 ng/ml FGF-2. At lower concentrations of FGF-2 (12 and 25 ng/ml), and in controls (0 ng/ml FGF-2), no *de novo* cells were detected on cartilage until after day 10. By 20–28 days in culture with low concentrations of FGF-2 small numbers of *de novo* cells began to appear also at the cut edges of impacted and non-impacted cartilage, as described above. This delayed increase in cell numbers was insignificant compared to the dramatic increase caused by FGF-2 from day 7 onwards.

From 7 days in culture onwards a statistically significant ($P = 0.018$) increase in the numbers of *de novo* cells was detected on impacted cartilage explants cultured in the presence of 50 and 100 ng/ml FGF-2 [Fig. 4(C)], compared to lower doses of FGF-2 and to control explants (0 ng/ml FGF-2) [Fig. 4(A)]. The effective concentration of FGF-2 for promoting a highly significant difference in *de novo* cell numbers seemed to be 50 ng/ml. No discernable difference in *de novo* cell numbers was noted between 50 and 100 ng/ml FGF-2.

In unimpacted FGF-2 treated (50 ng/ml) controls *de novo* cells were present after day 7 but their numbers were significantly lower than the corresponding impacted samples [Fig. 4(A)].

At 28 days the numbers of *de novo* cells at these lower doses (mean 5.9 cells observed per ×20 field) were similar to the numbers detected in the presence of 50 and 100 ng/ml at day 7 (mean 7.3 cells observed per ×20 field), clearly demonstrating the acceleration in response due to FGF-2.

These FGF-2 time-course and dose–response experiments were performed with impacted cartilage samples from eight different aged horses. Unimpacted cartilage from four of these horses served as controls. Since no significant age differences were noted, data from the individual experiments were pooled to provide the plot in Fig. 4(A).

CHARACTERISATION OF *DE NOVO* CELLS

Immunohistochemical staining revealed that the *de novo* cells stained positively for collagen type II [Fig. 5(A)] and collagen types IX/XI [Fig. 5(B)]. Importantly no staining was detected for collagen type I, a marker of the fibroblastic phenotype. In addition, the *de novo* cells also stained strongly for the marker of cell proliferation, PCNA and for the CPC marker, Notch-1 [Fig. 5(C,D)]. The spindle-shaped cells in the cartilage underlying the articular surface were also stained strongly by anti-collagen type II, collagen types IX/XI [Fig. 5(A,B)], weakly by anti-Notch antiserum and not at all by anti-collagen type I antiserum.

Discussion

The data reported here indicate that FGF-2 significantly accelerates the appearance, and increases the numbers, of *de novo* cells identified histologically in this experimental model of endogenous cartilage repair. This response depends on the concentration of FGF-2, with little effect being seen at less than 50 ng/ml. Such dose-responsiveness of chondrocytes to FGF-2 has been previously reported in other experimental systems[27].

The *de novo* cells described here and by Bowe and coworkers[11,12], must, by experimental design, be of cartilage origin, as no synovial tissue or underlying bone was included in the harvested explants. These cells, and the underlying spindle-shaped cells are Notch-1 and PCNA immunopositive. Cells immunopositive for PCNA are considered to be actively proliferating, whilst Notch-1 is reported to be a marker for chondroprogenitor cells[6,10]. The results reported here thus provide further evidence for the presence of CPC in articular cartilage and indicate that CPC may be identifiable, under certain conditions, in mature articular cartilage, in addition to neonatal cartilage as previously described[6]. In this experimental system the cartilage has been stimulated by both mechanical damage and FGF-2. Our results, and those reported by Bowe and coworkers[12], show that mechanical damage induces the appearance of *de novo* cells, whilst FGF-2 clearly accelerates and potentiates the response.

Histologically, these results provide evidence that FGF-2 is stimulating *de novo* cell production. The *de novo* cells appear to be derived from division of cells in the area of cartilage immediately underlying the articular surface. In this region there is evidence of mitosis and numbers of cells are seen undergoing clonal expansion. We would speculate

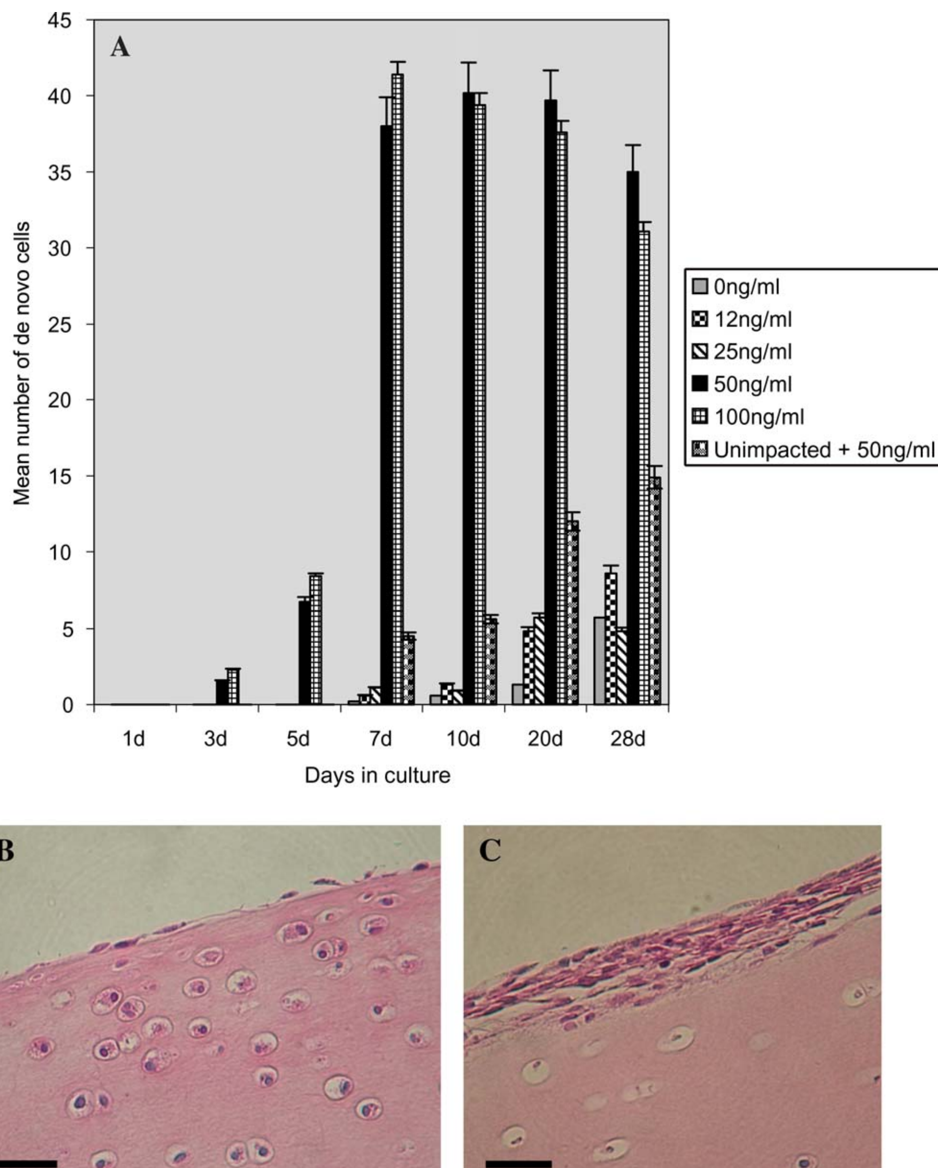

Fig. 4. Time-course and dose–response of FGF-2 in impacted and unimpacted articular cartilage. (A) Mean number of *de novo* cells (±standard deviations) detected at the articular surface of cartilage explants in the presence of various concentrations of FGF-2. Each experimental point represents summed data from eight horses (impacted cartilage) and from four horses (unimpacted cartilage). (B) A small number of *de novo* cells on the surface of the articular cartilage after 3 days in culture post-impact in the presence of 50 ng/ml FGF-2. Scale bar = 20 μm. (C) Increased numbers of *de novo* cells on the surface of the articular cartilage after 7 days in culture post-impact in the presence of 50 ng/ml FGF-2. Scale bar = 20 μm.

that these cells then migrate out onto the surface of the cartilage, in response to the combined effect of damage and FGF-2 treatment, presumably to initiate repair. Evidence of such a repair response was provided by the location of the *de novo* cells that appeared to be coating the damaged articular surface and lining the fissures in an apparent attempt to repair the damage. It is interesting that, in addition to increasing cell numbers, FGF-2 has been implicated in the potentiation of migration of chondrocytes in monolayer culture *in vitro*, enhancing the velocity of cell migration[28].

These induced *de novo* cells are phenotypically chondrocytes, as demonstrated by their positive staining for the chondrocyte markers, PG, and collagen types II[29], IX/XI[30]. In another study (unpublished observations) we have also shown that the chondrocyte-specific cartilage matrix components fibronectin[31] and Cartilage oligomeric matrix protein (COMP)[30] are also expressed by these cells. No staining for type I collagen was detected. This is significant as collagen type I is a marker of a fibroblastic phenotype, rather than a chondrocytic phenotype, thus indicating that the spindle-shaped cells (which are likely to represent cells undergoing migration through the ECM) had a chondrocyte phenotype. Additionally, it can be concluded that in this study, incubation with FGF-2 at concentrations up to 100 ng/ml for up to 28 days did not induce a change from a chondrocytic phenotype to a fibroblastic phenotype. This result is in agreement with other workers who previously

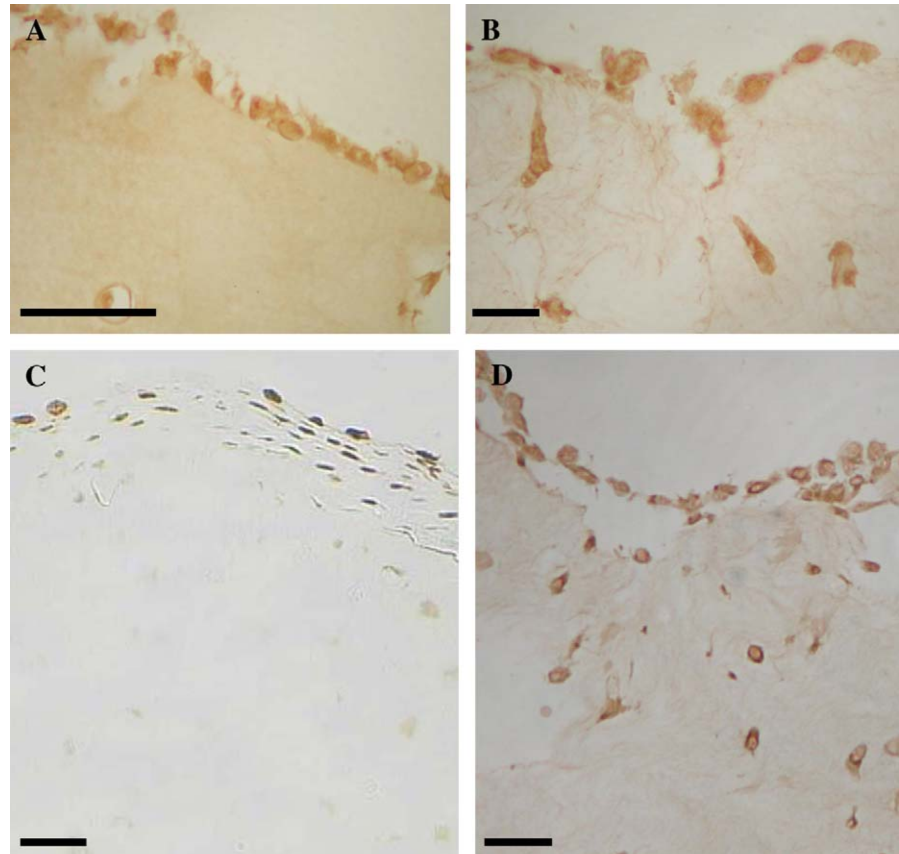

Fig. 5. Characterisation of *de novo* cells on the surface of the articular cartilage after 7 days in culture post-impact in the presence of 50 ng/ml FGF-2. (A) Immunolocalisation with anti-type II collagen antiserum. The cartilage extracellular matrix and the *de novo* cells are positively stained. Scale bar = 45 μm. (B) Immunolocalisation with anti-type IX/XI collagen antiserum. The cartilage extracellular matrix and the *de novo* cells are positively stained, as are the spindle-shaped cells underneath the articular surface. Scale bar = 35 μm. (C) Immunolocalisation was performed with anti-PCNA antiserum. Positive staining is present within the *de novo* cells and within cells underlying the articular surface. Scale bar = 150 μm. (D) Immunolocalisation was performed with anti-Notch-1 antiserum. Positive staining is present within the dividing cells within the cartilage and, to a lesser extent, in the *de novo* cells. Scale bar = 100 μm.

reported that FGF-2 had a stabilising effect on the differentiated chondrocyte phenotype in an agarose repair model[19]. Mandl *et al.*[32] also demonstrated that FGF-2-stimulated-chondrocytes had a better re-differentiation capacity and produced less type I collagen than cells treated with other growth factors. We suggest that these chondrocytes, which re-located to sites of impact damage, are capable of synthesising cartilage matrix components and thus have the potential for cartilage repair.

Connective tissue stem cells are known to be a target for FGF-2; in full thickness defects *in vivo*, FGF-2 stimulates prechondrogenic mesenchymal cells, giving rise to selective clonal expansion[18]. It has been reported that FGF-2 is the most effective growth factor for promoting growth of bone-marrow stromal cells *in vitro*[20] and that FGF-2 plays a crucial role in the self-renewal of these MSC[33]. The demonstration of a restricted zone of proliferating Notch-1 positive cells immediately beneath the articular surface of damaged, FGF-2 treated cartilage confirms both the presence and location of CPC. Although we might speculate that these are the cells that migrate out to sites of damage, the mechanism of action of FGF-2 on CPC and cell migration is as yet unknown. FGF-2 stimulates a wide response from target cells, including, in cartilage, the activation of the extra-cellular signal-regulated kinase 1/2 and p38 MAPK pathways[34]. In this experimental model FGF-2 may be acting, in part, via SOX-9, a transcription factor essential for chondrocyte differentiation and cartilage formation[27,35]. FGF-2 is a particularly potent agonist in this process[36], increasing SOX-9 expression through all four FGF-receptors via an MAPK regulatory pathway[37].

The results of this study, together with those previously reported by our group (Bowe and coworkers[12]) indicate that, following a single impact load, CPC can be stimulated in mature articular cartilage *in vitro*. These CPC and the cells arising from them represent the cartilage's repair response to damage. The timing of the appearance of CPC and their overall numbers can be significantly increased by FGF-2, providing further evidence for an important role for FGF-2 in modulating cartilage repair. These results indicate that further study into the mechanisms of repair in mature cartilage using this *in vitro* model may be vital in understanding the repair capacity of mature cartilage.

Acknowledgements

We would like to acknowledge the financial support of The PetPlan Charitable Trust and the BBSRC. We would also like to thank Ms J. Caddick and Ms L. Lavers for their technical assistance.

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