

Healing of osteochondral defects via endochondral ossification in an ovine model.

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Introduction

Synovial joint surface defects are important causes of Osteoarthritis (OA), the most prevalent disease of synovial joints, afflicting humans and animals and causing a huge healthcare, welfare and economic burden (Cross, et al. 2014). A considerable amount of research time and effort is directed at devising tissue engineering strategies to enhance joint surface defect healing, including using cell based therapies, tissue engineering solutions and small molecule treatment. However the development of optimal repair strategies is hampered by a lack of full understanding of the underlying mechanisms by which joint surface defects heal. Whilst a number of studies exist in rodents and small animals, relatively little is known of the healing mechanism of osteochondral defects in large animals and, by inference, in man.

Joint surface defects occur either in the cartilage ('chondral' defects) or develop in the cartilage and underlying bone ('osteochondral' defects). Osteochondral defects have healing capacity if they are small and are believed to heal by recruiting non-differentiated bone marrow-derived stromal/stem cells contained in the bone marrow into the damaged site. In contrast, chondral defects have a poor intrinsic healing capacity. Treatment strategies to heal chondral defects often utilise the bone marrow-derived stromal/stem cell population by accessing the bone marrow through microfracture techniques, which provide cost-effective and functional healing in many cases (de Mulder, et al. 2014, van Susante, et al. 2003, Miller, et al. 2015). The precise contribution of the stromal stem cell population to the healing of osteochondral defects, however, is not known, although they may have both a direct and/or indirect (modulatory) effect on the repair tissue.

The mechanism by which osteochondral defects heal has been described in a number of animals, with most work being performed in rabbits. Shapiro *et al* (1993) described the sequence of healing in 3mm diameter osteochondral defects of the femoral trochlea including an initial fibrin repair, mesenchymal cell recruitment, cartilage formation starting adjacent to the damaged cartilage and subsequent bone formation. Other work, in larger animals, has described cartilage formation adjacent to the residual damaged cartilage. It has been shown that a number of different parameters can affect both the efficiency of healing and possibly the mechanism of healing, including the position of the defect in the knee joint (with both anatomical considerations (Jung, et al. 2009, Orth, et al. 2013) and biomechanical differences (Duda, et al. 2005) being important), the size and depth of the defect (Nosewicz, et al. 2012) and the age of the animal (Bos, et al. 2006).

The sheep is a commonly used translational model for evaluating efficacy of potential treatments for osteochondral defects (Orth, Meyer, Goebel, Eldracher, Ong, Cucchiarini and Madry 2013, Getgood, et al. 2012, Novak, et al. 2016), however the mechanisms by which osteochondral defects heal in sheep are not described. The aim of this study was to describe the temporal sequence of healing of 7mm diameter osteochondral defects of the distal femur in the sheep in order to provide key information on those processes that are potential targets for therapy.

Materials and Methods

This study received approval from both the Animal Welfare and Ethical Review Board, Cambridge University and the UK Home Office (Project Licence number 70/8165) .

Animals: Forty skeletally mature female Welsh Mountain Sheep (mean age 3.2 \pm 0.8 years) were included in the study, representing the control (untreated empty defect) animals from two experiments conducted in our laboratory performed by the same surgical team. The experiments were conducted 12 months apart in three cohorts, with the surgical component of the work carried out in the same calendar month each year. The feed, husbandry and location of the sheep was the same in all animals. The animals were all obtained from the same supplier source.

Animal anaesthesia, preparation and surgical technique: General anaesthesia was induced with an injection of thiopentone (3mg/kg) and maintenance achieved via inhalational anaesthetic of a mixture of isoflurane, nitrous oxide and oxygen. Perioperative analgesia was provided by pre-operative intramuscular carprofen. Antibiotic prophylaxis was given via intramuscular procaine penicillin. The basic surgical procedure was identical for all subjects and performed under strict asepsis. Each stifle was physically examined for any abnormalities whilst anaesthetised. If any gross instability or pathology was found, the animal was excluded from participation within the study.

The animal was placed in a dorsal recumbency position and, following surgical preparation, the left stifle joint opened via a parapatellar approach. Following patella subluxation, a full thickness 7mm diameter x 6mm deep osteochondral defect was created

in the medial femoral condyle (MFC) using a hand drill. The joint was closed in a standard fashion. No splints, casts or immobilisation techniques were used in any animal. Postoperatively, animals were allowed to fully weight bear, but kept in small pens for 48 hours to reduce ambulation. All animals were housed indoors for the remaining study period in large pens or outdoor in fields, both of which permitted normal ambulation. Animals were killed with an overdose of injectable anaesthetic at 1, 2, 4, 8, 12, 18 and 26 weeks post surgery.

Histology: Specimens were decalcified in formic acid/sodium citrate over four weeks. Following complete decalcification, the specimens were dehydrated through a series of ethanol exchanges of increasing concentrations, cleared in xylene and then embedded in paraffin wax. Sections of 8 µm thickness were made through the central portion of the defect. Sections were stained with Toluidine Blue and/or Safranin O/Fast Green. A semi-quantitative histological analysis was performed as described by Manil-Varlet (Mainil-Varlet, et al. 2003), based on a semi-quantitative scoring system originally described by O'Driscoll (O'Driscoll, et al. 1988)(Table 1) . This scoring system has a maximum of 34 points and is suitable for demonstrating longitudinal healing of osteochondral defects in large animal species (Jung, Breusch, Daecke and Gotterbarm 2009). Scoring was carried out blindly by one observer (FH).

Immunohistochemistry: Immunohistochemistry was performed as described previously (Getgood, Henson, Skelton, Herrera, Brooks, Fortier and Rushton 2012) The following primary antibodies were used in this study; polyclonal rabbit anti type X collagen

(ab58632, Abcam, UK, 1 in 200 dilution) and monoclonal mouse anti-PCNA (DAKO, UK). Type X collagen immunostaining was detected with a FITC-conjugated secondary anti-rabbit secondary antibody (Sigma) and PCNA immunostaining was detected with a TRITC-conjugated secondary anti-mouse antibody (Sigma). Normal species-specific serum was used as a control for each antibody.

Results

Weeks 1-2: The osteochondral defect is infilled with fibrous tissue

At week one and two after injury the defect site had filled with blood clot and fibrous tissue. The fibrous tissue was organised with a visible surface layer. At the edge of the damaged articular cartilage there was a loss of normal architecture (Figure 1A). The normal chondrocyte arrangement was replaced with a zone of multicellular chondrocyte 'clusters' associated with a zone of proteoglycan loss as detected by a reduction in toluidine Blue staining at the site.

Weeks 4-8: Chondrocytes form adjacent to the edge of damaged cartilage and bone formation occurs through endochondral ossification.

Between weeks four and eight neo-cartilage was evident at the top edges of the defect immediately adjacent to the edge of the remaining cartilage and the cartilage clusters (Figures 1B and 2). The cells within the chondrocyte clusters were positively stained for PCNA immunoreactivity indicating active cycling. Close examination of the tissue suggested that these new chondrocytes derived primarily from differentiation of the

fibrous tissue clot adjacent to the damaged cartilage. However, there was some suggestion that chondrocytes at the edges of the damaged cartilage were contributing to repair as the orientation of the chondrocytes at the edges of the damaged cartilage was towards the neocartilage rather than the articular surface (Figure 2).

From week 4 onwards the area in which chondrocytes were seen was now extending towards the base of the defect following the edge of the bone. In defects of increasing age the chondrocytes reached deeper towards the base of the lesion until they joined with the chondrocytes arising from the opposite side of the lesion by approximately 8 weeks post-injury, effectively lining the exposed bone edges of the defect (Figure 3).

Chondrocytes adjacent to the exposed bone edge were observed to be larger and more rounded than the new chondrocytes that were arising towards the top of the defect with a morphology similar to hypertrophic chondrocytes. Positive type X collagen staining in these larger chondrocytes confirmed their identity as hypertrophic chondrocytes (Figure 3). No evidence of intramembranous ossification was detected in any of the sections studied, nor were any cartilage 'islands' observed within the fibrous tissue away from the damaged bone edges.

Week 8 to 12: Bone formation occurs through endochondral ossification from the base and edges of the lesion.

From week 8 onwards, the healing process continued via a process of endochondral ossification with a gradual increase in the amount of cartilage within the defect (Figure 4). The cartilage formed within the healing tissue did not show any recognisable

columnar structure at 8w but, by 12w, the chondrocytes were beginning to become more structured at the edge of the lesion close to the intact cartilage.

Week 18 New bone formation continues and cartilage is removed

At week 18 new bone had been formed at the edges and base of the defect: at this time point a characteristic 'v' shaped healing lesion was observed (Figure 4). There was evidence of tidemark reformation in the newly formed cartilage only at the edges of the defect and the chondrocytes within this newly formed cartilage had organised into an approximately columnar structure.

Week 26 Healing is nearly complete

Healing was very close to completed at 26 weeks (mean histological score 29.6/34). At 26 weeks the defect had been filled in with cartilage and bone. The cartilage/bone junction was positioned at the correct anatomical site within the cartilage/bone unit, however the tidemark was not completely reformed in any animal. Within the subchondral bone underneath the newly formed cartilage there were numerous cartilage remnants remaining which would require further resorption and remodelling in future. No evidence of osseous hypertrophy was found in any sample studied.

Semi-Quantitative analysis

A scoring system suitable for scoring the healing of experimental large animal osteochondral defects was used. This scoring system demonstrated that an increase in score correlated with the age of the lesion in these medial femoral condyle lesions (Figure

5). At 1, 2 and 4 weeks of healing the mean healing score (out of a possible total of 34) was 7.6 +/- 0.94, 7.3 +/-1.3 and 8.6 +/- 1.7 respectively, with the majority of the scoring coming from the integration of the infill material with the intact cartilage edges and the underlying bone. At 8w of healing the mean score had risen to a mean of 14.6 +/-1.7, increasing through a mean of 16.3 +/-2.5 at 12w, a mean of 25+/-0 at 18w and ending at 26w with a mean of 29.6 +/-0.63. Between 4w and 8w there was increased scoring for tissue infill and surface integrity and then increasing scoring for morphology, structure and matrix staining in the later time points. The tidemark was not full restored at 26w in any animal.

Discussion

Healing of joint surface defects remains a clinical challenge with significant research endeavour directed at optimising treatment strategies. Understanding the mechanisms behind normal healing is vital to inform therapy selection and experimental design. In this study we examined the temporal sequence of healing within an osteochondral defect experimentally created in the medial femoral condyle in the sheep and demonstrated that healing of the defect occurs via endochondral ossification. The defect size in this study was 7mm diameter, which was nearly fully healed within 26 weeks at this anatomical site (cartilage formed in the appropriate place but with some remodelling of subchondral bone and tidemark formation still occurring). This data indicates that a 7mm osteochondral defect in the medial femoral condyle is able to achieve spontaneous repair and is thus

considered as below a 'critical size' i.e. a defect of a size that cannot spontaneously heal (Rudert 2002).

Whether or not an experimental defect is small enough to achieve spontaneous repair is extremely important in the evaluation of therapeutic interventions.

Various factors play a role in determining whether a defect can spontaneously repair, including the anatomical site of the lesion in the joint (medial femoral condyle lesions are reported to heal more poorly than comparable lesions in the trochlea in the sheep (Orth, Meyer, Goebel, Eldracher, Ong, Cucchiarini and Madry 2013) but better in the mini-pig (Jung, Breusch, Daecke and Gotterbarm 2009). In the goat, it has been reported that a 6mm diameter defect in the medial femoral condyle does not undergo spontaneous repair (Jackson, et al. 2001), however, in other studies in sheep an 8.3mm diameter osteochondral defect in the medial femoral condyle did not heal after 6m (Nosewicz, Reilingh, van Dijk, Duda and Schell 2012), suggesting that a defect of approximately 8mm is the size below which spontaneous healing occurs in the medial femoral condyle of adult sheep at 6m.

These results show that ovine osteochondral healing occurs through a defined sequence of events. Initially the lesion is filled with a haematoma that then rapidly remodels to form a fibrous clot. The first sign of organ-specific tissue is the formation of neocartilage adjacent to the damaged edges of the cartilage. At this stage in the healing process, there was a very similar appearance to the healing defects in different animals at the same time post surgery. This neo-cartilage is then observed, over time, to stream down the edges of the defect supported by the

underlying bone, coalescing at the base of the lesion. New bone is produced circumferentially on the edges and base of the lesion via the process of endochondral ossification, as evidenced by the characteristic hypertrophic morphology of the chondrocytes and the expression of type X collagen. In this sheep osteochondral repair process, no evidence of MSC cell condensation was detected in the centre of the lesion as had been reported in the rabbit (Shapiro, et al. 1993). As healing progresses, particularly at weeks 8 and 12, there was a wider difference between individual animals with, at 12w the ICRS scoring varying between 13 and 19, primarily due to differences in the maturation state of the new cartilage formed, however, by 18w and then at 26w there was a marked reduction in variability, with all animals having a similar appearance. These results indicate that, at 26w post surgery animals have a similar histological appearance in 7mm defects in the medial femoral condyle.

The origin of the neocartilage and the cartilage that then covers the exposed bone surface is not known. The neocartilage originates at the junction between the fibrous clot and the damaged cartilage and could arise either from a) the damaged cartilage edge (via activation of cartilage progenitor cells (Nelson, et al. 2014)) or by b) recruitment and differentiation of MSC (released from the bone marrow). PCNA labelling indicated that the cells within the fibrous clot and the chondrocyte clusters in the damaged cartilage edge are actively cycling. Shapiro et al (Shapiro, Koide and Glimcher 1993) showed, using longitudinal tritiated thymidine labelling, that there was significant labelling of undifferentiated cells within the fibrous clot, as well as in

the damaged cartilage, and concluded that the undifferentiated cells primarily contributed to the regeneration of the defect. However, some histological sections in this study do appear to show a possible contribution of damaged cartilage to new cartilage production and further studies are required to investigate the precise contribution of the chondrocytes and undifferentiated cells in large animal osteochondral healing.

Whatever proves to be the source of the neocartilage, this study demonstrates the importance of the damaged tissue in directing healing. Neocartilage is initially formed adjacent to the damaged cartilage, indicating that, if not directly contributing to the neocartilage production, the damaged cartilage is providing an appropriate structural support for neocartilage production and/or secreting local trophic factors to induce differentiation of undifferentiated cells into cartilage. It is known that adult articular cartilage is a rich source of morphogenic signals upon injury (Dell'Accio, et al. 2006) (Watt, et al. 2013, Burleigh, et al. 2012) and cartilage damage induces activation of factors including bone morphogenic proteins (BMP) and Wnt signalling proteins within damaged cartilage ((Dell'Accio, et al. 2006, Dell'Accio, et al. 2008), (Dell'Accio, De Bari, El Tail, Barone, Mitsiadis, O'Dowd and Pitzalis 2006), and released into the local environment, for example fibroblastic growth factors (FGF) (Chong, et al. 2013).

The importance of the damaged tissue in directing repair is also apparent in the progression of cartilage formation as it lines the edge of the damaged bone prior to infilling the defect. This observation that endochondral ossification occurs upon the damaged bone is unlike the situation during fracture repair, where the cartilage callus is laid down through endochondral ossification on the intact periosteal bone surface rather than on the fractured bone ends (Ford, et al. 2004), demonstrating that osteochondral repair, in these animals at this anatomical site, occurs in a dissimilar repair environment to that which occurs in fracture repair.

As previously discussed regarding the role of damaged cartilage in neocartilage formation, whether the bone is acting as a physical support and/or as a producer of trophic mediators for chondrogenesis is not known. Differentiation of MSC into chondrocytes is enhanced by increased load (Angele, et al. 2003, Terraciano, et al. 2007): we could hypothesise that the junction between fibrous tissue and bone experiences increased load relative to elsewhere in the fibrous tissue within the defect and that the MSC in this area are being influenced by this load differential. The repair tissue/bone interface also represents an area of altered vascularity and oxygen tension within the damaged tissue and it has been demonstrated, by many authors that these variables, among many others, can affect cartilage repair processes (Babarina, et al. 2011) (Shang, et al. 2014, Gaut and Sugaya 2015). Clearly, further studies are required to fully characterise the role of bone in osteochondral repair.

There have been few studies that describe longitudinal observations of healing in large animal models and, to the authors' knowledge, there have been no previous studies of longitudinal healing in the medial femoral condyle of the sheep. Jackson *et al* (Jackson, Lalor, Aberman and Simon 2001) demonstrated, over 52 weeks in a goat model, that a 6mm diameter osteochondral defect fails to heal and that progressive, resorption of the defect and subsequent collapse of the defect occur. As observed in this study, clustering of chondrocytes at the edges of the damaged cartilage was noted but endochondral ossification (as determined by toluidine blue staining) was only detected at the base of the lesion. Gotterbarn *et al* (Gotterbarn, et al. 2008) reported that during healing of the medial aspect of the lateral trochlea ridge in mini-pigs endochondral ossification and cartilage streaming was observed at the upper edges of the lesion only.

The results of this study show that endochondral ossification is the mechanism by which osteochondral defects heal in the sheep. The quantitative analysis of the healing shows that there is little variability in the initial stages of healing between animals, but that variability is more marked between 8 and 12w of repair. However, by the end point of this study, 26w the variability was reduced and the appearance of the healing defects similar. In sheep, unlike in laboratory animals such as mice, there are far more genetic differences between individuals. In this study we have sought to eliminate individual differences as much as is practically possible, using female sheep of a similar age of the same breed from the same supplier, however,

these studies were conducted over a 3 year period (drawn from the control animals of different studies) and the results may be confounded by individual variations.

This finding provides information that may assist the design of tissue engineering strategies to heal osteochondral defects. However, this finding also poses a potential dilemma for strategies that have been used to reduce the deleterious osseous overgrowth that can occur during joint surface defect repair (Blanke, et al. 2009, Klinger, et al. 2011). On the basis of the findings reported here, future regenerative therapies should be directed at maintaining chondrocyte phenotype in the chondral layer of the defect once the subchondral bone plate is restored and strategies to inhibit endochondral ossification should be used cautiously.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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