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Definition of a Critical Size Osteochondral Knee Defect and its Negative Effect on the Surrounding Articular Cartilage in the Rat

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SUMMARY

Background: Joint trauma is predisposing to the incidence of osteoarthritis (OA) of the knee. There is a limited knowledge on the impact of posttraumatic osteochondral defects on the whole joint. This study was designed to define a critical size osteochondral defect in the knee of rats and to investigate a possible association between osteochondral defects and degeneration of the surrounding joint surface.
Methods: Cylindrical osteochondral defects of different sizes were created in the knee joint of rats. The natural course of these lesions was studied by macroscopic observation, histology, and immunohistochemistry. Gene expression of the articular cartilage surrounding the defects *in vivo* and of articular chondrocytes cultured *in vitro* in IL1β and fibroblast growth factor 2 (FGF2) supplemented media was evaluated by quantitative polymerase chain reaction (qPCR).
Results: In defects of 0.9 mm diameter, spontaneous joint surface healing was observed but also upward advancing of the subchondral bone plate at 16 weeks. Larger 1.4 mm diameter defects were critical size, not resulting in successful healing at any time point. Importantly, the articular cartilage surrounding the defects expressed FGF2 and IL1β, but not ACAN and Col2. Chondrocytes cultured in IL1β and FGF2 supplemented media lost the natural fibroblast growth factor receptors – FGFr1/FGFr3 balance and showed decreased viability.
Conclusions: A critical size osteochondral defect was defined as 1.4 mm in diameter in rat. Subchondral bone plate advancement occured rapidly. The articular cartilage surrounding osteochondral defects showed catabolic activity with expression of IL1β, FGF2 and a disturbed FGFr1/FGFr3 balance, potentially initiating a process of early osteoarthritic disease.

Introduction

Knee arthroscopies performed in over 900,000 patients in the USA revealed osteochondral injuries in up to 60% of patients. From the patient's perspective of quality of life, osteochondral defects were estimated to be as painful and debilitating as late stage osteoarthritis (OA)[1–3]. Since articular cartilage has little capacity to repair itself, several treatments have been attempted to restore joint surface defects, such as microfracture, the osteochondral autograft transplant system (OATS), and autologous chondrocyte implantation (ACI)[4,5]. The results of OATS showed a high rate of return to sport on the short term, while significantly more failures occurred with OATS than with ACI at 10 years[6]. ACI gives very reasonable outcomes in prospective randomized studies[7–9], however some challenges remain including fibrocartilage formation with little hyaline cartilage restoration leading to joint deterioration over time and the high cost of this treatment[10,11]. Despite new strategies for osteochondral injuries repair have increasingly been attempted, treating these defects is still an unsolved challenge[11].

Several animal models have been investigated to be able to address the challenges for osteochondral repair[12,13]. An osteochondral defect model in rats seems very attractive in providing proof-of-concept data. First, the rat model displays an economic advantage as rats are relatively cheap and easy to care for. Second, the rat model is perceived as more clinically relevant than the

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mouse model, since the articular cartilage in rats displays typically also a zonal structure that resembles the one in human joints, less so for the mouse model[14]. In addition, immune-deficient rats are now also commercially available, providing an opportunity to study the regenerative potential of human cells in this model. Overall, the rat model seems appropriate for initial *in vivo* testing, however there is a need to better understand the biology of osteochondral defects. The spontaneous repair of osteochondral defects of different sizes has not been studied in a systematic approach[15]. Since the joint surface has some regeneration ability, a critical size osteochondral defect model for the rat should be defined. Although subchondral bone plate advancement towards the joint surface is a major and growing concern in osteochondral repair, this has not been studied in chronological order in rats[16,17]. Currently, no strategy seems to avoid subchondral bone plate advancement and fibrocartilage formation that leads to treatment failure.

As the incidence of OA has been associated with joint trauma, osteochondral defects may not remain restricted to a local wound, and they could also trigger a whole joint disease. In fact, there is some evidence that the presence of asymptomatic osteochondral defects or full thickness chondral defect leads to articular cartilage loss and to the development of early OA[18–20]. In this case, the anabolic activity of chondrocytes seems to be reduced in both normal and OA-articular cartilage[21], which can be explained, at least partially, by cartilage's limited capacity to recover from damage and exposure to inflammation[22]. Moreover, fibroblast growth factors (FGF) and fibroblast growth factor receptors (FGFr) also show regulatory effects on both catabolic and anabolic processes of the joint[23]. Therefore, therapies targeting inflammatory cytokines and FGFs are assumed to be of great relevance in the treatment of joint diseases and in the prevention of trauma related OA. However, our knowledge about the mechanisms triggering degeneration of the cartilage surrounding osteochondral defects is still incomplete and further understanding is required[11].

In this study, osteochondral defects of different sizes were used to evaluate the natural course of spontaneous osteochondral healing over time, not only within the defect area but also in the cartilage surrounding the defect. The aim of the present study was: (1) to define the critical size of an osteochondral defect in rats, (2) to define the course of subchondral bone plate advancement, (3) and to examine the association between osteochondral defects and degeneration of the surrounding cartilage.

Materials and methods

Animal experiments

All animal procedures were approved by the local ethical committee for Animal Research (KU Leuven). The animals were housed according to the guidelines of the Animalium Leuven (KU Leuven). Wild-type Lewis male rats (Charles River Laboratories, Den Bosch, Netherlands) at 9–11 weeks olds were used. Each rat received surgery on both knees. A small incision in the articular capsule was made along the patella and patellar tendon. Subsequently, the patella was dislocated to the lateral side according to previous reports[24,25]. Cylindrical osteochondral defects of different sizes (0.5 mm, 0.9 mm, 1.4 mm or 2.1 mm diameter × 1.0 mm depth) were created, as indicated in Fig. 1(A). After wound closure in two layers, the rats were allowed to walk freely in the cage before being sacrificed at 4, 8, and 16 weeks ($n = 4$ for histology and $n = 3$ for gene expression analyses). Eight age matched rats were sacrificed for non-operated knees.

Macroscopic evaluation

The distal part of the femur end was carefully collected at 4, 8 and 12 weeks after operation. The macroscopic appearance of defects was scored using the Goebel's semi-quantitative macroscopic scoring system[26].

Histological examination and immunohistochemistry

Safranin-o (SafO) staining and all immunohistochemistry were performed as described previously[27,28]. The specimens were visualized using a Leica DMR microscope (Leica, Wetzlar, Germany) using the same settings for all samples. Defects were evaluated using the Sellers's score[29,30]. The antibodies used are indicated in Supplementary Table 1.

Automated histomorphometrical measurements

The SafO positive area, subchondral bone area and subchondral bone plate distance were measured automatically from SafO stained slides using ImageJ. A color threshold for positive SafO staining was defined within a reference area of the native articular cartilage at an unaffected site. A region of interest (ROI) of a size comparable to the original defect was drawn. Subsequently, the SafO positive area within the ROI was measured in injured knees using ImageJ software ($n = 4$). In parallel, an intact control area was measured within the same size of ROI in native intact cartilage. The percentage of SafO positive area was calculated by the SafO positive area in the osteochondral defect divided by the intact control area.

A subchondral bone area was automatically chosen by the color threshold for a SafO negative region (excluding cysts) and measured in the ROI by ImageJ software. The subchondral bone area rate was calculated by the SafO negative area in the defect divided by the native subchondral bone area.

Distance from the surface to the closest point of subchondral bone in the center of the defect was measured. The subchondral bone plate distance was defined by the distance from surface to the closest point of subchondral bone minus 245 nm as the average native cartilage thickness which was measured.

Total ribonucleic acid (RNA) extraction and quantitative reverse transcription–polymerase chain reaction analysis

The regenerated tissue in the defect, the articular cartilage surrounding the defect, and the intact native cartilage (non-operated positive control) were harvested with a surgical knife under an inverted microscope (Supplementary Fig. 1). Isolation of total RNA, synthesizing complementary deoxyribonucleic acid (DNA), and running quantitative polymerase chain reaction (qPCR) were performed as described previously[31]. Each sample was tested in duplicate and compared with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression that allowed normalization of results. Relative differences in expression were calculated using the $2^{-\Delta\Delta CT}$ method. The sequences used to construct the primers are listed in Supplementary Table 2.

Isolation and culture of rat chondrocytes

Intact cartilage (non-operated positive control) from rat knee joints was dissected and digested with 0.2% type 2 collagenase (Thermo Fisher Scientific) in DMEM/F12 for 3 h at 37°C. Primary digested chondrocytes obtained from two knees were seeded in 19 cm^2 (5 wells in 12 well plates) at an initial cell density of approx. 500 cells/cm^2 and cultured in complete culture medium (DMEM/F12; 5% FBS; 1 μl/ml human transferrin; Sodium selenite; penicillin,