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Characterization of the repair tissue in articular cartilage defects using silver-enhanced colloidal gold immunostaining

N. REISSIS, S. DOWNES*, M. KAYSER, D. LEE, G. BENTLEY
The Institute of Orthopaedics and IRC in Biomedical Materials, University College London, Royal National Orthopaedic Hospital, Stanmore, Middlesex, HA7 4LP, UK

The newly developed silver-enhanced colloidal gold staining method was used in a rabbit model to characterize the repair tissue in large articular cartilage defects filled with a heterocyclic methacrylate polymer. By 6 weeks the resurfacing tissue consisted of highly organized hyaline-like articular cartilage, fully integrated with the adjacent normal cartilage. Immuno-histochemistry detected collagen type II, keratan sulphate, chondroitin 4-sulphate and chondroitin 6-sulphate in the matrix of the neocartilage. The level to which the polymer plug was recessed appeared to be critical to the overall quality of the repair tissue. Optimum results were obtained when the top surface of the biomaterial was at the level of the subchondral bone, below the level of the surrounding articular cartilage. Other technical aspects of implantation, that also affect the repair, are also discussed.

1. Introduction

The clinical need for more conservative treatment in reconstructive orthopaedic surgery has recently become demanding. Conventional joint replacements do not meet the needs of the younger and active patients because of the high rate of loosening of the implants and the need for revision surgery in these patients.

Biological resurfacing of articular cartilage defects is challenging, particularly in focal articular cartilage lesions with no ligamentous or bony damage. However, the quality of the repair is still unpredictable [1]. Attempts at cartilage repair using isolated chondrocytes [2, 3], organic support matrices [4–6] or synthetic polymers [7–9] have been reported in the literature. The repair tissue appears to be fibrocartilaginous in nature.

We have previously reported on a new heterocyclic methacrylate, based on poly-ethyl-methacrylate (PEMA) polymer powder and tetra-hydro-furfuryl-methacrylate (THFMA) monomer liquid, that appeared to enhance the biological resurfacing in large, full-thickness articular cartilage defects in a rabbit model [10]. The self-repair potential of the rabbits was also validated in the same study; the control empty holes repaired with significantly inferior tissue, both in structure and composition. The present experimental study also utilizes the PEMA/THFMA system and focuses on technical aspects of its implantation that appear to influence the quality of the repair in the animal model. In addition we have characterized the cartilage components of the repair tissue using the new, silver-enhanced colloidal gold technique [11]. The method is very sensitive and allows the use of routine histological counter-staining, such as haematoxylin and eosin, which helps in the study of tissue morphology.

2. Materials and methods

Thirty Sandy Lop rabbits were anaesthetized with 2% halothane and a 3:2 mixture of nitrous oxide and oxygen. Using a 4.5 mm drill bit, a 5 mm deep circular osteochondral defect was drilled by hand in the patella groove of the distal femur. Defects were thoroughly washed with normal saline until all osteochondral debris were removed.

Preparation of the biomaterial was performed by mixing 1 g PEMA with 0.6 ml THFMA (containing 2.5% V/V N,N-dimethyl-p-toluidine) for 2 min at room temperature. Using a sterile syringe, 0.1 ml of the dough was injected into the defect. Polymerization occurred *in situ*. By using the flat end of a 4.5 mm wide cylindrical metallic rod pushed into the defect, the surface level of the polymer plug was carefully controlled during polymerization to be either at the level of the surrounding articular cartilage, the thickness of which at this part of the joint is 1.4 mm (range 1.3–1.6 mm), or at the level of the underlying subchondral bone (2 mm or more below the surface of the adjacent articular cartilage). Bleeding did not interfere with polymer setting and plug fitting and it was minimal in most cases.

The rabbits were group-housed in floor pens [12] allowing complete freedom of movements pre and

* To whom correspondence should be addressed.

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postoperatively. This model is functional, the patella groove being an area of load-bearing with significant shear forces applied to the defect by the articulating patella. Rabbits were killed at 3 and 6 weeks post-implantation by intravenous injection of pentobarbitone sodium. The quality of the repair tissue was assessed by histology, histochemistry and immunohistochemistry.

2.1. Preparation for histology and histochemistry

The distal part of each femur, including the resurfaced defect, was fixed in 4% paraformaldehyde and 0.3% glutaraldehyde in 0.1 M sodium cacodylate for 2 days; decalcified with neutral EDTA and the end point of decalcification checked by radiography. After decalcification samples were washed in 0.1 M sodium cacodylate buffer for a further 24 h and dehydrated through a gradual series of ethanols and xylene for 24 h at room temperature. Impregnation into wax was performed over 24 h at 60 °C, before embedding into wax blocks. Ten sagittal sections, each 5 micrometres thick, including the whole of the repair tissue and the edges of the surrounding normal articular cartilage, were cut from each specimen. One was stained with haematoxylin/eosin, one was stained with safranin-0 and the rest were included in the immuno-histochemical study.

2.2. Preparation for immuno-histochemistry

The proprietary kit for the silver-enhanced colloidal gold staining was provided by Bioclin (Cardiff, Wales). Four selected monoclonal antibodies were applied individually: the anti-type II collagen antibody (CIICI, Development Studies Hybridoma Bank, Iowa, USA, dilution 1:1), the anti-keratan sulphate (5D4, ICN Biomedicals, Bucks, UK, dilution 1:500), the anti-chondroitin 4-sulphate antibody (2-B-6 ICN Biomedicals, Bucks, UK, dilution 1:100) and the anti-chondroitin 6-sulphate antibody (3-B-3, ICN Biomedicals, Bucks, UK, dilution 1:100).

Sections were dewaxed in xylene, rehydrated through graded series of ethanols and incubated with hyaluronidase (10 IU ml^{-1}, Sigma, Poole, UK) and chondroitinase ABC (0.25 IU ml^{-1}, Sigma, Poole, UK) for 2 h at 37 °C. Incubation with the primary antibody was made overnight at 4 °C in a dark, humidified atmosphere. The antibodies were diluted individually in phosphate-buffered saline with 0.6% bovine serum albumin (Sigma, Poole, UK). Sections were incubated with the colloidal gold conjugated anti-mouse Ig secondary antibody (dilution 1:50) for 2 h at room temperature in a dark, humidified atmosphere, followed by fixation in 1% glutaraldehyde. The colloidal gold was visualized by use of a physical silver development solution and the sections were counterstained with Mayer's haematoxylin.

3. Results

Two rabbits were excluded because they developed septic arthritis. No inflammatory or foreign body reaction was recorded in any of the synovium specimens studied.

At 3 weeks the repair tissue was predominantly fibrous. However, when the surface level of the polymer was recessed below the adjacent articular cartilage, into the subchondral bone, the reparative tissue contained many cells expressing chondrocyte-like phenotype (Fig. 1). A few of these were seen in lacunae, separated by an increased amount of pericellular matrix. Safranin-0 staining revealed proteoglycan synthesis forming a pericellular "halo" around these cells. Chondrocytic cells were larger and more numerous in the deeper layers of the repair tissue, forming, occasionally, small aggregations.

In contrast, when the top surface of the polymer was recessed higher up, at the level of the articular cartilage, the repair tissue was exclusively fibrous, with flattened, spindle-shaped cells lying parallel to the articular surface (Fig. 2). The structural characteristics of the resurfacing tissue in these specimens were poor, superficial laminations and fissures were present and the quality of bonding of the repair tissue to the adjacent normal cartilage was also poor.

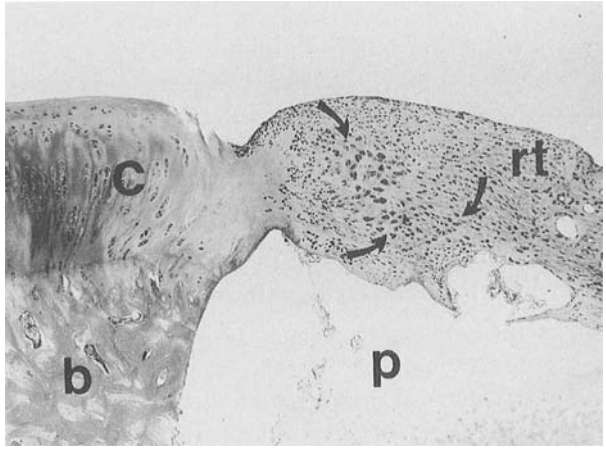

Figure 1 At 3 weeks the repair tissue is fibrocartilaginous, with numerous chondrocyte aggregations in the middle and deep layers (p = polymer, b = bone, c = normal articular cartilage, rt = repair tissue, the arrows show the chondrocyte aggregations) [Haematoxylin-Eosin staining, ×13.2].

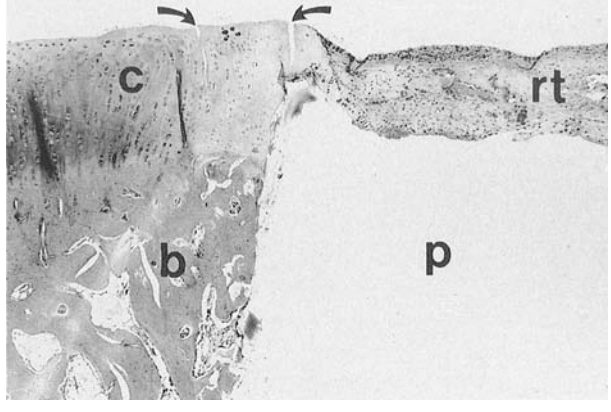

Figure 2 When the surface level of the polymer is placed too high, the repair tissue at 3 weeks is exclusively fibroblastic and structurally inferior. Deep fissures can be seen in both the repair tissue and the adjacent articular cartilage (p = polymer, b = bone, c = normal articular cartilage, rt = repair tissue, the arrows show the fissures). [Haematoxylin-Eosin staining, ×13.2]

The silver-enhanced colloidal gold staining technique detected no cartilage components in the repair tissue of any of the 3-week specimens when the polymer surface was recessed at the level of the surrounding articular cartilage. By contrast, when the polymer was fitted well into the subchondral bone, keratan sulphate, chondroitin 4-sulphate and chondroitin 6-sulphate were detected in certain areas within the reparative tissue.

At 6 weeks all defects were completely resurfaced. The quality of the repair tissue, however, depended strongly upon the level of the polymer. When it was set below the level of the surrounding articular cartilage, a full-thickness repair of highly organized hyaline -like articular cartilage was formed (Fig. 3). Chondrocytes were evenly distributed throughout the matrix, forming occasionally, particularly in the deeper layers, vertical columns. The structural integrity of the neocartilage was normal, no superficial laminations nor fissures were noted and its surface was smooth and intact. This newly formed cartilage was fully integrated with the surrounding normal cartilage. In a few specimens the sections showed empty lacunae, more numerous in the deep zones of the repair tissue (Fig. 3), which were stained pink using Schiff's staining, suggesting they were lipid droplets. Safranin-0 staining (Fig. 4) showed proteoglycans, evenly distributed in the matrix and around chondrocytes and immuno-histochemistry detected collagen type II, keratan sulphate, chondroitin 4-sulphate and chondroitin 6-sulphate in the matrix, particularly in the middle and deep layers (Fig. 5).

In contrast, when the biomaterial was placed at the level of the articular cartilage, the repair tissue appeared predominantly fibroblastic, resembling the type of repair noted in the 3-week specimens (Fig. 6). The structure was inferior, deep fissures and tissue disruption were seen and the bonding of the reparative tissue to the surrounding cartilage was incomplete. The silver-enhanced colloidal gold staining showed the presence of proteoglycans around the few rounded chondrocytic cells found in the deep layer of the repair tissue, but not throughout the matrix. Collagen type II was not detected in any specimen.

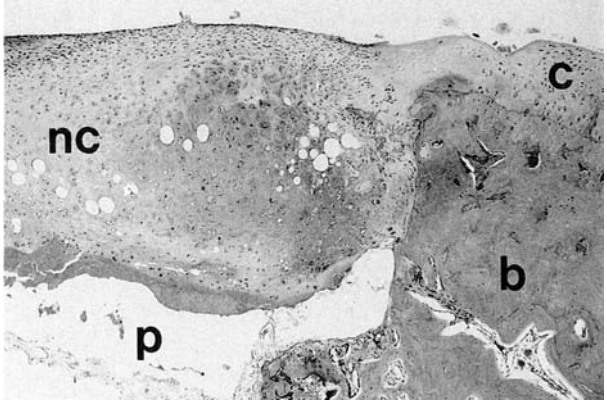

Figure 3 At 6 weeks the repair tissue consists of highly organized, hyaline-like articular cartilage, fully integrated with the surrounding articular cartilage (p = polymer, b = bone, c = normal articular cartilage, nc = neocartilage, the empty lacunae are lipid droplets). [Haematoxylin-Eosin staining, ×13.2]

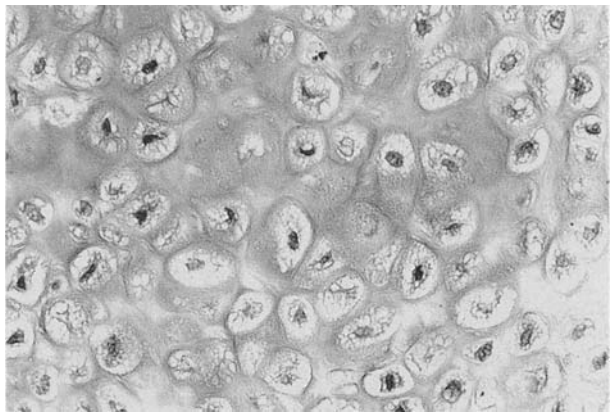

Figure 4 Detection of proteoglycans throughout the matrix and around the chondrocytes in the neocartilage seen in Fig. 3. [Safranin-0 staining, ×132]

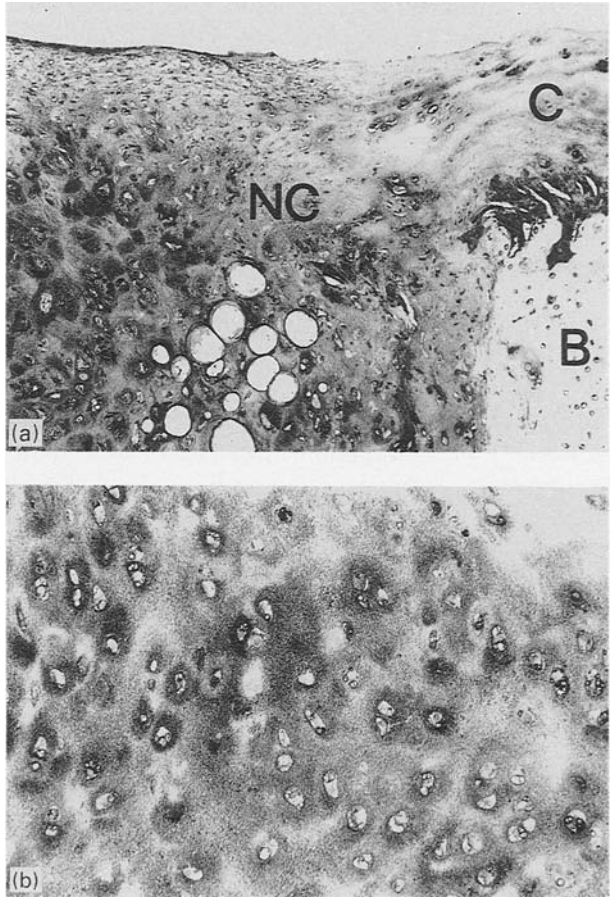

Figure 5 (a) Immunolocalization of collagen type II in the neocartilage seen in Fig. 3 (b = bone, c = normal articular cartilage, nc = neocartilage, the empty lacunae are lipid droplets. [Silver-enhanced Colloidal Gold staining, ×33] (b) High magnification in the middle of the neocartilage seen in Fig. 5a. Collagen type II can be seen around the chondrocytes and throughout the matrix of the neocartilage. [Silver-enhanced colloidal gold staining, ×66]

4. Discussion

This study utilized the new, room temperature polymerizing heterocyclic methacrylate PEMA/THFMA

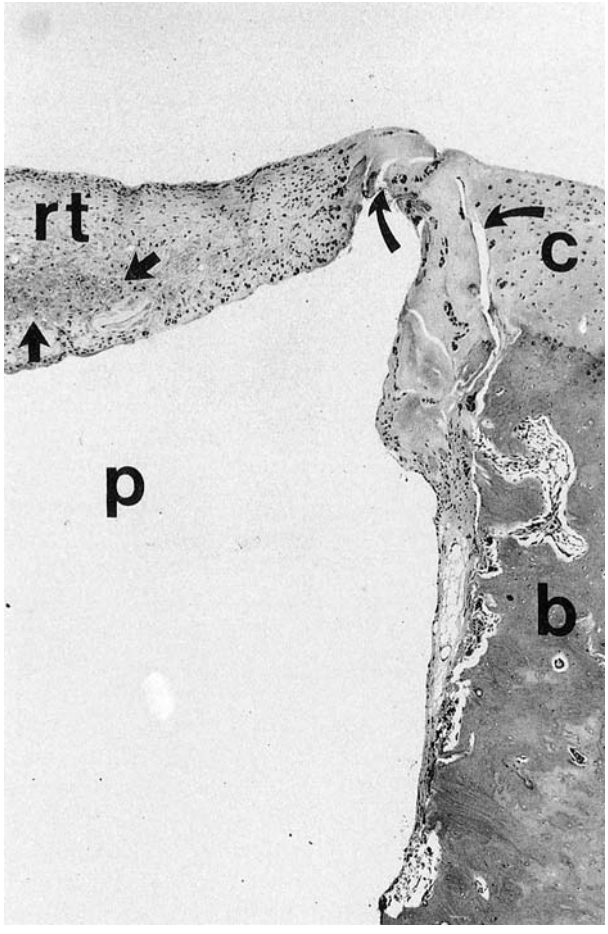

Figure 6 When the surface level of the biomaterial is placed too high, the repair tissue at 6 weeks is fibrocartilaginous, incompletely bonded to the adjacent articular cartilage (p = polymer, b = bone, c = normal articular cartilage, rt = repair tissue, the thin arrows show the incomplete bonding, the thick arrows show the chondrocyte aggregations). [Haematoxylin-Eosin staining, ×13.2]

to improve the biological repair in large, full-thickness articular cartilage defects. The material is biocompatible [13] and its implantation is technically simple. In the present study, when the surface level of the polymer was recessed below the level of the adjacent normal cartilage, into the subchondral bone, the defects were completely resurfaced and by 6 weeks the repair tissue consisted of hyaline-like articular cartilage, fully integrated with the surrounding articular cartilage. This early favourable result was not noted when the biomaterial was placed higher up, at the level of the articular cartilage.

The material may swell slightly during polymerization *in situ* due to its hydrophilicity [14] which, apparently, does not change the dimensions of the polymer plug. The top surface of the biomaterial must be even and smooth to allow resurfacing with full-thickness cartilage across the whole defect. This reparative tissue will allow more physiological, even distribution of the mechanical loads over the resurfaced area, thus improving the durability of the neocartilage [15].

When the surface of the polymer was recessed to the level of the surrounding articular cartilage, a zone of acellular tissue lying at the junction between the normal articular cartilage and the repair tissue was noted in a few specimens at both 3 and 6 weeks. This is a mechanically weak area, where overloading can easily be generated by the articulating patella and this may be responsible for the superficial laminations and deep fissures noted in these specimens.

It should be noted that the thickness of the articular cartilage can vary across the joint surface and, therefore, when the polymer is implanted into large defects, it is critical to adjust its surface level accordingly.

5. Conclusions

PEMA/THFMA is a heterocyclic methacrylate system that appears to be promising in the resurfacing of articular cartilage defects with new hyaline articular cartilage. Silver-enhanced colloidal gold is a sensitive immuno-staining method and can help in the study of the repair tissue.

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