Removal of proteoglycans from the surface of defects in articular cartilage transiently enhances coverage by repair cells

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Lesions within the articular cartilage layer of synovial joints do not heal spontaneously. Some repair cells may appear, but their failure to become established may be related to problems of adhesion to proteoglycan-rich surfaces. We therefore investigated whether controlled enzymatic degradation of surface proteoglycan molecules to a depth of about 1 μm, using chondroitinase ABC, would improve coverage by repair cells.

We created superficial lesions (1.0 × 0.2 × 5 mm) in the articular cartilage of mature rabbit knees and treated the surfaces with 1 U/ml of chondroitinase ABC for four minutes. The defects were studied by histomorphometry and electron microscopy at one, three and six months.

At one month, untreated lesions were covered to a mean extent of 28% by repair cells; this was enhanced to a mean of 53% after enzyme treatment. By three months, the mean coverage of both control and chondroitinase-ABC-treated defects had diminished dramatically to 0.2% and 13%, respectively, but at six months both untreated and treated lesions had a similar coverage of about 30%, not significantly different from that achieved in untreated knees at one month.

These findings suggest that, with time, chondrocytes near the surface of the defect may compensate for the loss of proteoglycans produced by enzyme treatment, thereby restoring the inhibitory properties of the matrix as regards cell adhesion. This supposition was confirmed by electron microscopy. Our results have important bearing on attempts made to induce healing responses by transplanting chondrogenic cells or by applying growth factors.

Materials and Methods

Preliminary experiments. To establish the conditions required for proteoglycan removal to a depth of about 1 μm from the cut surface within about four minutes, we prepared chondroitinase ABC (Sigma Chemical Company, St Louis, Missouri) at various concentrations in sodium-phosphate buffer and applied it to surface defects.
created in rabbit knees. Tissue specimens were fixed in the presence of ruthenium hexa-ammine trichloride (RHT; Johnson-Matthey and Brandenberger, Zürich, Switzerland) and embedded in epoxy resin. Ultrathin sections were prepared for electron microscopy. We assessed the extent of proteoglycan digestion by determining the loss of matrix granules.\textsuperscript{19,20} The desired effect was achieved by using chondroitinase ABC at a concentration of 1 U/ml.

**Operative procedure.** We used three groups of six skeletally mature (8 to 10 months) New Zealand White rabbits which were killed at one (group 1), three (group 2) and six (group 3) months after surgery. The rabbits were anaesthetised by intramuscular injection of Rompun/Ketalar, and both knees exposed by parapatellar medial approaches. Superficial cartilage lesions were then created on the lateral patellar groove and the medial femoral condyle.

The instruments used to create the superficial defects were made to our specifications. (Rolf Hänggi Engineering, Grenchen, Switzerland). Each defect was 1 mm wide with a depth of 0.2 mm. The length of each defect was controlled by the surgeon to be about 3 to 5 mm.

The defects in one knee of each rabbit were left untreated as controls. On the other side, chondroitinase ABC (1 U/ml) was applied topically for four minutes, before being removed with a sterile sponge. The defects on both sides were rinsed thoroughly with physiological saline, and the knees then closed in layers.

To rule out a specific effect of chondroitinase ABC, we treated similar defects in four additional rabbits with 2.5% trypsin (Sigma Chemical Company, St Louis, Missouri). In addition, to exclude the possibility of a systemically-transmitted effect of chondroitinase ABC from experimental-to-control knees, we studied another four control rabbits which had no enzyme treatment in either knee.

**Tissue processing.** The whole distal femur was carefully dissected free from soft tissues and fixed at ambient temperature\textsuperscript{2} hanging free in a glass container. We used a 2% (v/v) solution of glutaraldehyde (Grogg Chemie, Bern, Switzerland) containing 0.5% (w/v) RHT\textsuperscript{21} and 0.1 M sodium cacodylate (Grogg Chemie, Bern, Switzerland), which was adjusted to pH 7.4 with HCl/NaOH and to 330 mosmol with NaCl.

After fixation for one hour, the joint surfaces were sawn into parallel slices, 1 to 2 mm in thickness, perpendicular to the longitudinal axis of the defect. Chemical fixation was then continued for a further three to five hours, before rinsing tissue in isotonic buffer (0.1 M sodium cacodylate; pH 7.4). Tissue slices were postfixed for three to five hours in a 1% (w/v) OsO\textsubscript{4} solution (Oxken Limited, Mulberry, UK) containing 0.1 M sodium-cacodylate buffer (pH 7.4) and 0.5% RHT (w/v). After thorough rinsing in isotonic buffer, tissue was dehydrated in a graded series of increasing ethanol concentration, beginning at 70% (v/v), and embedded in Epon 812.

For light microscopy and morphometric analyses, semithin sections of 1 μm were cut on a Reichert OME3 ultramicrotome and stained with Toluidine Blue O. For electron microscopy, ultrathin sections were cut on a Reichert Ultracut E ultramicrotome, and stained with uranyl acetate and lead citrate.\textsuperscript{22}

**Sampling and morphometry.** On average, we obtained four to five sections of each defect. The position of the initial cut made at the proximal end was random,\textsuperscript{23} but subsequent cuts were taken at fixed intervals to permit estimation of the defect reference volume by the principle of Cavalieri.\textsuperscript{24,25} Morphometric estimations of the relative coverage of defect surfaces were made by light microscopy using 1 μm sections and a cycloid test system.\textsuperscript{26,27} The sectioning direction was varied systematically relative to the vertical plane (defined as perpendicular to the joint surface). Stained sections were photographed, and morphometric estimations made on glossy prints. Statistical analyses of differences between animal groups were based on Student’s t-test.

Proteoglycan loss attributable to enzymatic degradation was assessed qualitatively on electron micrographs by estimating changes in the numerical density of matrix granules.\textsuperscript{19,20} Similar analyses were performed on all control material.

An articular cartilage defect at one month after local treatment with chondroitinase ABC. Most of its surface is covered by a layer of predominantly mesenchymal-like cells (arrowheads), with local variations in its thickness (S, superficial zone; R, radial zone; C, calcified cartilage; arrows, tidemark; bar = 100 μm).
Results

The superficial defects were U-shaped (Fig. 1), usually penetrating the lower radial zone. The topical application of chondroitinase ABC at a concentration of 1 U/ml for four minutes was confirmed to degrade proteoglycan molecules to a depth of about 1 μm, as shown by the loss of matrix granules (Fig. 2).

Isolated groups of cells were regularly seen along the surfaces of both enzyme-treated and untreated defects. Most of these showed the elongated form characteristic of fibroblasts or undifferentiated mesenchymal connective-tissue cells (Fig. 3), but there was a smaller population of cells with a more rounded profile and abundant cytoplasm with vacuoles (phagosomes). These resembled macrophages of monocytic lineage. This adhering population of cells will be referred to collectively as ‘repair cells’.

They were preferentially located either in the corners of the defects (Fig. 4), where they formed multilayered, wedge-shaped masses, or in micro-grooves produced by irregularities in the cutting process. They were also seen as single or double layers along plane surfaces, but to a less degree in untreated defects than in enzyme-treated ones. The coverage by repair cells was quantified morphometrically, and a summary of these findings is represented graphically in Figure 5. One month after surgery, the defects treated with chondroitinase ABC showed an approximately 25% greater coverage with repair cells than did untreated defects (p < 0.05).

At three months after surgery, the mean cell coverage in both control (0.2%) and enzyme-treated (13%) defects was dramatically reduced, the difference between these two being significant (p < 0.05). By six months, the situation had stabilised, and both control (31%) and enzyme-treated (33%) defects showed a similar degree of coverage. These proportions were not significantly different from those in untreated controls at one month (see Fig. 5).

The morphological characteristics of these cells did not change during the period of investigation: there were no signs of differentiation into chondrocytes. In addition to the superficial population of mesenchymal-like cells, clusters of chondrocytes were observed sporadically within the articular cartilage adjacent to the lesions; these sometimes protruded a short distance into the defect (see Fig. 4c).

The trend for increased coverage shown between the first and third months after treatment with chondroitinase ABC was mirrored in trypsin-treated defects, but these were not studied at six months. The type of repair cell involved was also similar (data not presented). The control animals with untreated defects in both knees showed the same cell coverage as that documented for unilateral control lesions, thereby excluding the possibility of systemic transmission of enzyme effects (data not presented).

Discussion

It is generally recognised that partial-thickness defects in articular cartilage do not heal. The only spontaneous repair reaction is the transient proliferation of chondrocytes near the defect surfaces. Similar cell clusters have been reported in the early stages of osteoarthritis and have been referred to as cell-clones or brut-capsules. Their size...
remains within constrained limits, and they never prolifere significantly into the void of the lesion, although some matrix has been reported to accumulate therein. Our previous observations led us to suspect that the lack of repair of cartilage tissue stems from the anti-cell-adhesion properties of the proteoglycans within its matrix, a feature for which these molecules are renowned. We therefore studied the effect of their removal from the surface of the defect, by degrading them with chondroitinase ABC. This treatment elicited an approximately 25% increase in coverage by repair cells at one month, but this never exceeded more than a few layers and was highly variable.

Fig. 3

Sections showing repair cells adhering to the surface of the defect at one month after treatment with chondroitinase ABC. Most of the adhering cells have a mesenchymal-like appearance (arrowheads) and build up uni- (A), bi- or occasionally trilayers (B). In some locations, however, particularly within microgrooves (C), multiple layers are also seen. Macrophages (M) are also encountered most commonly in these microgrooves (C, chondrocyte; CM, cartilage matrix; DS, defect surface; V, void of defect; bar = 20 μm (A, B and C)).
The lesion never became filled with repair tissue.

By the third month, cell coverage had decreased considerably, in controls by about 99% and in enzyme-treated defects by about 75%. At first, this finding seemed unaccountable, but more mature consideration suggested that native chondrocytes near the surface of the defect were probably stimulated anabolically by the trauma of surgery, causing an increase in proteoglycan synthesis. By six months, the situation had stabilised, with about 31% cell coverage in untreated defects and about 33% in enzyme-
vium, but the mechanism of migration remains to be clarified. Repair cells may well be passively transported within the cavity of the joint, but chemotactic attraction could also play a role, particularly after the enzymatic degradation; proteoglycan-degradation products are known to have a chemotactic effect on various cell types in vitro. Both mechanisms may be implicated; the high variance which we found suggests the influence of a number of, as yet, unknown factors.

Our finding that enzymatic removal of proteoglycans elicits only a transient increase in the local population of repair cells has important clinical implications. Attempts to induce a healing response in superficial defects of articular cartilage, for example by the local transplantation of chondrogenic cells or by the introduction of a suitable growth factor, are more likely to be effective when instigated within one month of treatment with chondroitinase ABC.

We wish to thank N. Pattiselanno for her technical assistance, and gratefully acknowledge help from the Surgical Research Unit (Department of Clinical Research and Clinic for Large Animals) of the University of Bern. We are also indebted to C. England for constructive criticism and editing of the manuscript. This work was supported by grants from the AO-IASIF-Foundation, Switzerland and Orthogene Inc, California, USA.

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References


