Isolated epiphyseal chondrocyte allografts into joint surfaces

An experimental study in rabbits

GEORGE BENTLEY, AUDREY U. SMITH, AND RAMA MUKERJHEE

From the Nuffield Department of Orthopaedic Surgery, University of Oxford

SUMMARY Isolated epiphyseal chondrocytes from 5 week old female New Zealand white rabbits were transplanted as allografts into drill holes in the tibial articular surface of adult male New Zealand white rabbits. The grafts were examined after 8 weeks. Fresh chondrocytes which were partially separated from their matrix were more successful (47%) than completely separated cells (20%) and were significantly more successful ($P>0.05$) in skeletally mature (58%) as opposed to immature recipients (20%).

Storage of the cells at -79°C for 2 to 9 days or at -196°C for 36 to 58 weeks gave successful results of 23% and 33%, respectively. Control defects showed fibrocartilage filling the defect in 25%.

The factors affecting survival of chondrocyte allografts require further study before clinical application of the method.

Lexer (1908) and Judet (1908) were the first to attempt replacement of joint surfaces with osteochondral grafts of articular cartilage and a variable thickness of subchondral bone. Since that time sporadic attempts have been made to resurface joints in animals and man with osteochondral allografts and the results were reviewed by Aichroth *et al.* in 1971. The success rate of these experiments has not been sufficiently high to justify widespread clinical application of the method. The bony component is necessary to achieve fixation of the grafts to the host bone ends but failure has been due largely to difficulties related to this component. In general, autografts have survived but, despite some reported successes, allografts have undergone progressive breakdown due to immunological rejection of the bony component and subsequent mechanical failure of the cartilage surface.

In 1968, Chesterman and Smith attempted a new approach to the biological replacement of joint surfaces in animals. They isolated living chondrocytes from mature rabbit articular cartilage by removing the matrix with the enzymes papain, collagenase, and pronase. After slow centrifugation these cells were implanted into V-shaped defects cut in the articular surface of the humerus of adult

rabbits of the same breed. Six weeks later the defects were filled with fibrous tissue or fibrocartilage. The implanted chondrocytes had not formed adequate amounts of hyaline cartilage to repair the surface defects. There were 4 possible reasons for this failure.

(1) The isolated chondrocytes might not have remained in the defect in sufficient numbers to multiply and form matrix before the host repair and defence mechanisms destroyed them. (2) Isolated chondrocytes may be unable to survive in the environment of a moving joint containing synovial fluid. (3) The defects in the bone were cut with a dental drill which may have caused bone death and prejudiced survival of the cell grafts. (4) The transplanted cells were extracted from mature animals which have less capacity for cell multiplication than immature cells (Mankin, 1968).

In view of these considerations and the fact that cartilage has very low antigenicity compared with cancellous bone containing marrow cells, Bentley and Greer (1971) transplanted both isolated articular chondrocytes and epiphyseal chondrocytes taken from very young rabbits (6 weeks old) into defects drilled in the upper tibial articular surfaces of adult rabbits of the same breed. The articular chondrocytes survived in small numbers and were slowly rejected but the epiphyseal chondrocytes apparently

survived in most cases and laid down normal-staining hyaline matrix which filled the defects completely. This work raised interesting questions and possibilities and therefore an extended series of experiments was planned.

Materials and methods

Chondrocytes were isolated from the epiphyseal growth plates of 5 week old New Zealand white female rabbits weighing 500–1500 g (average 800 g). Over 1 million cells were extracted from the growth plates of 10 metatarsals. A total of 100 adult male New Zealand white rabbits, which were mature as judged by the absence of a growth plate on pre-operative radiographs, received grafts. Three of the animals died during the investigation, leaving a total of 97 for evaluation. Chondrocytes for grafting were prepared by 4 methods.

GRAFTING METHODS

1. 'Slow' isolation (partially separated cells)

Chondrocytes were isolated by a relatively slow technique, using low concentrations of enzymes (Lowe and Smith, 1975). The epiphyseal growth plates were removed from the 8 metatarsals of 5 week old New Zealand white rabbits and cut into small fragments 2 mm square. These fragments were agitated successively in flasks containing 4% papain solution (Sigma no. P-3375) dissolved in medium TC199 for 1 hour and 0.01% collagenase solution (Sigma no. C-0130) over a period of 12 hours in a shaking reaction incubator containing water at 37°C. When this process was completed the cells were partially separated from their matrix so that the medium contained isolated cells and clumps of 50–100 cells held together by gelatinous material derived from the original matrix (Fig. 1).

2. 'Rapid' isolation (completely separated cells)

The alternative method involved the use of higher concentrations of collagenase. The 2 mm square fragments of epiphyseal cartilage were incubated with trypsin 0.25% (Sigma no. T-8253) followed by 0.025% collagenase over a total period of 2 hours (Bentley and Greer, 1971) during which time the cartilage and medium were agitated by shaking the flask mechanically in a shaking reaction incubator. By this technique the chondrocytes were completely isolated from their matrix (Fig. 2).

3. Stored, frozen chondrocytes

Cells which had been partially isolated by the slow technique were suspended in medium TC199 containing 10% DMSO (di-methyl sulphoxide), sealed in ampoules and cooled slowly to -79°C in

alcohol saturated with solid CO₂ and stored at -79°C for 2 to 9 days. A small group of 6 animals received cells which had been suspended in 10% DMSO and cooled slowly to -79°C and stored in liquid nitrogen at -196°C for 36 weeks in 2 cases of 58 weeks in 4 cases. Viability tests showed that 80% to 90% of the cells were alive after thawing and before transplantation.

The cells which had been completely isolated by the rapid technique using high concentrations of enzymes did not survive prolonged storage at -196°C after cooling and thawing by the same technique.

4. Dead chondrocytes

Chondrocytes prepared by both 'slow' and 'rapid' techniques were killed by rapid freezing and slow thawing. They were transplanted as controls for living cells.

GRAFTING PROCEDURE

After isolation of the cells and preparation a viability count was used using the eosin or trypan blue exclusion technique (Hoskins *et al.*, 1956). The cells were then slowly centrifuged at 500 rpm immediately before transplantation thus forming a clump in the bottom of the centrifuge tube. The recipient animal was anaesthetised with ether by inhalation. The lateral tibial articular surface of the right knee was exposed surgically and a drill hole was made 3 mm wide and 1–2 mm deep in the surface using a hand drill in the hope of avoiding excess heat and unnecessary death of bone. The hole was irrigated with sterile isotonic saline to remove any bone fragments and, after swabbing dry, the isolated cells were pipetted into the defect.

As a control the same procedure was performed on the left knee but no cells were injected. Joints were closed immediately with catgut and silk sutures and the animals allowed to move freely about their cages.

Previous studies (Bentley and Greer, 1971; Bentley, 1972) demonstrated that after 2 and 4 weeks from grafting the grafted defects showed abundant highly cellular cartilage which stained heavily with metachromatic stains and was heavily labelled with ³⁵SO₄ on autoradiographs compared with the control defects which contained fibrous tissue and took up neither metachromatic stain nor ³⁵SO₄. By 8 weeks from grafting there had been complete filling of the defects with chondrocytes which were becoming organised into zones similar to those in the surrounding host cartilage (Figs 3 and 4).

Therefore in this study 8 weeks after grafting the animals were killed by dislocation of the neck. The tibial condyles were removed and photographed. Representative specimens were incubated for 1

Fig. 1 (top) Photomicrograph of epiphyseal chondrocytes partially separated from their matrix suspended in medium TC 199 seen in clumps of 50–100 cells held together by gelatinous material derived from the original matrix. Stained with euchrysin and viewed by fluorescent light, $\times 160$.

Fig. 2 (bottom) Photomicrograph of epiphyseal chondrocytes completely separated from their matrix suspended in medium TC 199. Stained with euchrysin and viewed by fluorescent light, $\times 400$.

Fig. 3 *Allograft of epiphyseal chondrocytes into upper tibial articular surface of rabbit knee 8 weeks after implantation. Filling of the defect by chondrocytes which are filling the defect, producing matrix, and becoming incorporated is seen. Toluidine blue $\times 30$.*

Fig. 4 *A control drill hole shows a fibrous tissue plug in the defect which does not stain metachromatically with toluidine blue. Toluidine blue, $\times 30$.*

hours at 37°C in Eagle's basal medium to which had been added 100 $\mu\text{Ci/ml}$ $^{35}\text{SO}_4$ with 100 units/ml penicillin and 1 $\mu\text{g/ml}$ streptomycin. All specimens were washed, fixed in formalin, decalcified in EDTA (ethylene-diamine tetra-acetic acid) and embedded in paraffin. The sections were cut 6 μm thick. The slides for autoradiography were prepared by the dipping technique (Joftes 1959) using Kodak NTB₃ emulsion. They were stored in light-proof boxes in the dark at 4°C for 3 weeks. After they had been developed the autoradiographs were stained with haematoxylin and eosin. Corresponding sections were stained with haematoxylin and eosin and alcoholic toluidine blue to demonstrate the distribution of glycosaminoglycans in the articular cartilage and the grafts.

In an attempt to demonstrate the donor origin of the cartilage cells in the grafted defects samples of isolated cells were labelled by suspension in Eagle's basal medium containing tritiated thymidine for 48 hours (Fig. 5). The labelled cells were transplanted into 6 animals which were sacrificed at 14 days, and autoradiographs prepared. No label could be seen in the defects. This was thought to be due to the rapid division of the cells in the defect which diluted the T³H label. In addition attempts were made to recognise Barr bodies of female cells transplanted

to male recipients as described by Peer (1958). Although Barr bodies were identified in the donor cells at $\times 1000$ magnification the difference between the numbers in donor and recipient cartilage was not clear enough to be unequivocal. Other authors have found difficulty in employing this technique.

GRADING OF RESULTS

The grafted areas were placed in two grades according to the extent of filling of the defects: (1) *successful*—complete or near complete filling of defect by cartilage (Fig. 6); (2) *unsuccessful*—deep cartilage only or fibrous tissue (Fig. 7).

Some control defects which had been drilled and received no grafts showed fibrocartilage in the defect in a proportion of cases. This was less cellular than the grafts, sometimes showed fragments of bone from the drilling of the defect and was clearly distinguishable from the grafts (Fig. 8). To achieve some comparability with the grafts, those control defects which showed complete filling by fibrocartilage were labelled 'successful' and those which contained deep fibrocartilage or fibrous tissue were labelled 'unsuccessful'. It was presumed however that the cartilage tissue in the control defects had arisen by metaplasia of cells from the subchondral bone marrow.

Fig. 5 Epiphyseal chondrocytes in cell culture after incubation with tritiated thymidine for 48 hours. Labelling of a high proportion of cells has occurred. $\times 400$.

Fig. 6 A successful allograft of partially separated epiphyseal chondrocytes showing high cellularity, heavy meta-chromatic staining of the matrix, and incorporation into the drill hole in the upper tibial articular surface. Toluidine blue, $\times 30$.

Fig. 7 An unsuccessful allograft of partially-separated epiphyseal chondrocytes. Fibrous tissue only occupies the defect. Toluidine blue, $\times 30$.

Fig. 8 A control defect which shows fibrocartilage filling but labelled 'successful'. The cartilage is less cellular than the successful grafts, stains less heavily with toluidine blue, and contains some bone fragments. Toluidine blue, $\times 30$.

Results (Table 1)

FRESH CHONDROCYTES PARTIALLY SEPARATED FROM THE MATRIX

A total of 34 joints received allografts of isolated chondrocytes partially separated from their matrix. Of these, 16 (47%) showed successful filling with hyaline cartilage whilst 18 (53%) showed only deep cartilage or fibrous tissue.

Table 1 *Allografts of isolated epiphyseal chondrocytes into the knee of mature rabbits*

Cell preparation	Total	Successful*	Unsuccessful†
<i>Fully isolated</i>			
(1) Partial separation	34	16 (47%)	18 (53%)
(2) Complete separation	10	2 (20%)	8 (80%)
(3) Frozen at -79°C for 2-9 days	32	8 (23%)	24 (77%)
(4) Frozen at -196°C for 36-58 weeks	6	2 (33%)	4 (67%)
(5) Dead cells	15	6 (40%)	9 (60%)
(6) Control defects	97	24 (25%)	73 (75%)
$P < 0.2$			

*Successful—complete or near-complete filling with cartilage.

†Unsuccessful—half-filling with cartilage or fibrous.

FRESH CHONDROCYTES COMPLETELY SEPARATED FROM THE MATRIX

Ten joints received cells which were completely separated from their matrix. Only 2 (20%) were successfully filled with hyaline cartilage whilst 8 (80%) were unsuccessful.

PARTIALLY SEPARATED CHONDROCYTES STORED AT -79°C FOR 2 TO 9 DAYS

Thirty-two allografts of cells stored at -79°C for 2 to 9 days and showing 80-90% viability on trypan blue staining were implanted. Eight (23%) were successfully filled with hyaline cartilage and 24 (77%) were unsuccessful.

PARTIALLY SEPARATED CHONDROCYTES STORED AT -196°C FOR 38 TO 56 WEEKS

Six joints received cells stored at -196°C for long periods of 36 to 58 weeks. Two (33%) were successfully filled with hyaline cartilage and 4 (67%) were unsuccessful.

DEAD CHONDROCYTES

Fifteen allografts of dead chondrocytes were transplanted. Six (40%) showed filling of the defects by

fibrocartilage corresponding to successful grafts whilst 9 (60%) contained fibrous tissue.

CONTROL DEFECTS

The 97 drill holes which received no cells showed fibrocartilage corresponding to successful grafts in 24 (25%). Deep fibrocartilage or fibrous tissue was present in 73% (75%).

On analysis the differences between groups 1-6 were not significant ($P < 0.2$).

FACTORS INFLUENCING RESULTS

Maturity of recipients (Tables 2 and 3)

Although the recipient animals were 2 or more years old, 10 were skeletally immature histologically as judged by the presence of an epiphyseal growth plate in the upper end of the tibia or the absence of a 'tide mark' between zones 3 and 4 of the articular cartilage. The animals concerned all had received allografts of partially separated chondrocytes but only 2 (20%) were successfully filled with cartilage compared with 14 out of 24 (58%) in mature recipients, a significant difference ($P < 0.05$). Interestingly, out of the 10 controls in immature animals, 6 (60%) showed large amounts of fibrocartilage in the defects compared with 7 out of 24 (29%) in mature animals. However, this difference is not significant ($P < 0.1$).

Table 2 *Allografts of partially-separated epiphyseal chondrocytes—mature and immature recipients*

	Total	Successful*	Unsuccessful*
Mature recipients	24	14 (58%)	10 (42%)
Immature recipients	10	2 (20%)	8 (80%)

$P < 0.05$

*See Table 1 for definitions.

Table 3 *Control defects in mature and immature recipients*

	Total	Successful*	Unsuccessful*
Mature recipients	24	7 (29%)	17 (71%)
Immature recipients	10	6 (60%)	4 (40%)

$P < 0.1$

*See Table 1 for definitions.

Table 4 *Allografts of isolated chondrocytes—drill hole depth and results*

Drill hole depth	Cell preparation	Total	Successful*	Unsuccessful*
Normal	Fresh, partially separated	15	8 (53%)	7 (47%)
Deep	Fresh, partially separated	14	7 (50%)	7 (50%)
Shallow	Fresh, partially separated	6	1 (17%)	5 (83%)

$P < 0.3$

*See Table 1 for definitions.

These findings suggested a vigorous repair response by metaplasia from the subcondral marrow cells in the immature animals and also suggested that for partially separated cells an immature recipient tolerated the allografts less well than a mature one.

Depth of drill hole (Table 4)

Previous experiments (Bentley, 1972) showed that for graft success the defect must penetrate the articular cartilage into the subcondral bone. Thus a normal drill hole entered the subcondral bone for 2 mm. A deep drill hole, which entered the bone marrow cavity of the tibia, was thought to be prejudicial to allograft success because bleeding occurred from the hole which tended to wash the cells out into the joint cavity and to expose them to plasma enzymes such as plasmin which would damage them (Lack and Rogers, 1958). A very shallow drill hole which just entered the subcondral bone might lead to the washing out of the cells from the defect by synovial fluid.

The only group which was large enough for comparison of this factor was that in which the recipients received allografts of partially separated fresh chondrocytes. Out of 14 with a deep drill hole, 7 (50%) showed successful filling compared with 8 out of 15 (53%) with a normal depth and 1 out of 6 (17%) where the hole was judged to be very shallow. The differences are not significant ($P < 0.3$) but the findings suggested that a very shallow drill hole was prejudicial to survival and establishment of the graft.

Host lymphocyte accumulation around the allografts

Previous work demonstrated intense lymphocyte accumulation around rejected grafts of articular cartilage (Bentley and Greer, 1971). Therefore an attempt was made to correlate the numbers of lymphocytes seen in the bone marrow around the grafts with the results. No difference could be detected between successful grafts, unsuccessful grafts and control defects. The 8-week interval between grafting and sacrifice may have caused this result since a lymphocyte response in the subcondral bone, if it had occurred, would probably have taken place within the early weeks after grafting.

Discussion

It is established that chondrocytes have antigens but that cartilage matrix is almost non-antigenic (Elves, 1974). The success of the preliminary experiments with isolated epiphyseal chondrocytes (Bentley and Greer, 1971) was thought to be due to the fact that the cells placed in the defects multiplied rapidly and laid down matrix; this protected the chondrocytes from the allograft reaction which takes 7–10 days to develop (Heyner, 1969).

FRESH EPIPHYSEAL CHONDROCYTE ALLOGRAFTS

The most successful allografts in this experiment were of freshly-prepared partially isolated chondrocytes (47%). Total separation of the cells produced less successful results (20%) despite a 90% viability count with the eosin or trypan blue exclusion test before transplantation. This viability technique does not distinguish precisely between living and dying cells and it must be concluded that rapid, total removal of matrix from chondrocytes is often harmful to cell membranes. Moreover, variability in the potency and toxicity of collagenase has been reported by several workers (Lowe and Smith, 1975).

Partially separated chondrocytes were more successful in mature recipients than immature (see Table 2). The better filling of the control defects by fibrous tissue in the immature animals demonstrated a more vigorous intrinsic repair response in the immature, as recorded by other workers (Calandruccio and Gilmer, 1962; de Palma *et al.*, 1966; Bentley, 1972).

EFFECT OF STORAGE AT LOW TEMPERATURES ON CHONDROCYTES

The poor performance of chondrocytes stored at low temperatures as allografts is of interest since viability counts in those preparations stored for up to 1 year at -196°C in 10% DMSO showed a survival rate of 80–90%. This may indicate damage to the membranes of internal organelles of the cells produced by the cooling and thawing process and to inadequacy of the viability tests used. Recent work on the interaction of rates of cooling and re-warming and concentration of protective agents on lymphocytes and other cells, and even whole embryos, might have a bearing on the problem of freezing and thawing chondrocytes. Alternatively, other protective agents including glycerol and sugars or mixtures of protective agents in low concentrations which have been used successfully for banking spermatozoa might improve the viability of frozen chondrocytes (Smith, 1970).

The relative failure of cells stored by cooling to

-79°C or -196°C to form satisfactory grafts indicates that more sophisticated techniques such as electron microscopy should be used to assess the damage to chondrocytes after freezing, banking and thawing by different methods. Other tests of viability including tissue culture and the capacity of the thawed cells to lay down matrix would also be useful. The problem of storage of chondrocytes for long periods might be overcome more easily in the meantime by culturing the cells *in vitro*.

Another interesting finding was the presence of fibrocartilage in 6 out of 15 (40%) defects which had received allografts of dead chondrocytes. This might be interpreted as an inductive phenomenon. Alternatively, it could be postulated that in all cases the dead cells were rapidly absorbed by the synovial membrane and did not influence the filling of the defect. The cartilage present at necropsy 8 weeks later would then be due to intrinsic healing as seen in some of the control knees.

This study shows that reconstitution by hyaline cartilage of drilled defects, 3 mm in diameter, in the articular surface of the tibia in adult rabbits occurred in 47% of cases following allografts of partially separated epiphyseal chondrocytes taken from young animals of the same species. The success rate was significantly better when the recipients were skeletally mature. Repair of the control defects with fibrocartilage occurred in 25% of cases and this was greater in immature animals. Implantation of dead cells produced repair of the surface by fibrocartilage in 40% of cases possibly by an inductive phenomenon.

The donor origin of the hyaline cartilage in the grafted defects was not unequivocally proven but the high cellular density of the grafts was clearly distinguishable from the more fibrous and less cellular fibrocartilage which took up metachromatic stain and $^{35}\text{SO}_4$ less markedly.

In future, tissue typing of donors and recipients of chondrocytes could be carried out and should increase survival rates of allografts. The clinical potential of chondrocyte allografts might still be considerable in treatment of early cartilage defects which are seen frequently in the course of surgical exploration of joints.

There is yet potential for the establishment of a bank of chondrocytes stored at -79°C or -196°C which could be used for clinical purposes but the factors which affect survival of cells after storage at such temperatures require further study.

We wish to thank Professor R. B. Duthie for encouragement and advice throughout this study. Able technical assistance was given throughout by Mrs C. Lowe and Mrs B. Brewster. George Bentley

was generously supported by a grant from the Arthritis and Rheumatism Council, No. 0/74/77. The Medical Research Council supported Dr Smith at the Royal National Orthopaedic Hospital where the low temperature work was carried out.

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