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Biochemical Studies on Repair Cartilage Resurfacing Experimental Defects in the Rabbit Knee*†

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ABSTRACT: **Wounds penetrating articular cartilage to bone heal with cartilage described variably as either fibrous or hyaline. In the present study, such repair cartilage was induced in the rabbit for biochemical comparison with normal articular cartilage. The main collagen in the repair tissue after three weeks was type I. By six to eight weeks, type II had become predominant and continued to be enriched up to one year; but type I still persisted as a significant constituent of the repair tissue even after a year, so the repair cartilage never fully resembled normal articular cartilage. From radiochemical analysis, type II was determined to be the major collagen synthesized by the repair tissue after three to four weeks. After six months, the repair cartilage contained more collagen and less hexosamine than control cartilage, suggesting that the fibrous texture that often developed was due to a loss of proteoglycans rather than to a change in the type of collagen.**

CLINICAL RELEVANCE: **Procedures capable of inducing the differentiation of authentic articular cartilage to resurface degenerated human joints would be invaluable. Surgical methods, such as drilling through to subchondral bone, are often attempted. It is not known, however, whether the cartilage that forms is true articular cartilage or, for example, fibrocartilage. The present experimental study in rabbits compared the properties of such repair cartilage with those of normal articular cartilage.**

It has been well documented that articular cartilage of mature animals has little capacity for repair after traumatic injury. Cuts or other mechanical damage restricted to the cartilage alone do not repair. However, it has been observed both experimentally and clinically that defects that penetrate the subchondral plate to bone do eventually resurface with a form of cartilage[1,2,9,10,12,14,16]. Such deep lesions initially fill with granulation tissue, the fibroblasts of which differentiate into chondrocytes by seven to ten days after injury and establish new hyaline cartilage by four to six weeks[11]. Morphological studies demonstrated that this cartilaginous tissue can retain its hyaline appearance for at least several months[2,14]. However, it has been reported that degenerative lesions and a more fibrous texture develop by one year after surgery[14], suggesting that the repair cartilage is less durable than normal articular cartilage. To assess the clinical potential for resurfacing of joints, it is important to know whether the repair cartilage really resembles articular cartilage biochemically. Does its composition eventually change to that of fibrocartilage, for example, with a change from type-II to type-I collagen?

Three genetically distinct types of fibrillar collagen are known with certainty. Type I is the major collagen of mature bone, tendon, skin, and other coarsely fibered tissues. Type II is the major collagen of hyaline cartilage and a few related tissues[3,13]. Type III is widely distributed in smaller amounts but is missing from adult bone and most cartilages. Typical fibrocartilage, such as the semilunar meniscus of the knee, contains mainly type-I collagen[5], and so differs from hyaline cartilage biochemically. Type-II collagen contains more hydroxylysine and much more glycosylated hydroxylysine than type-I collagen, and these extra residues possibly endow type-II fibrils with special physical properties.

The experimental model used in the present study for inducing repair cartilage in rabbits was based mainly on the model used by DePalma et al. in dogs. Histological results from about 200 rabbits, including autoradiography with ^{3}H-thymidine to determine the source of the chondrocytes, have been published elsewhere[11]. By not shaving the articular surface and by drilling large holes (three millimeters in diameter) it was possible to sample pure repair cartilage for biochemical analyses[11]. The experiments

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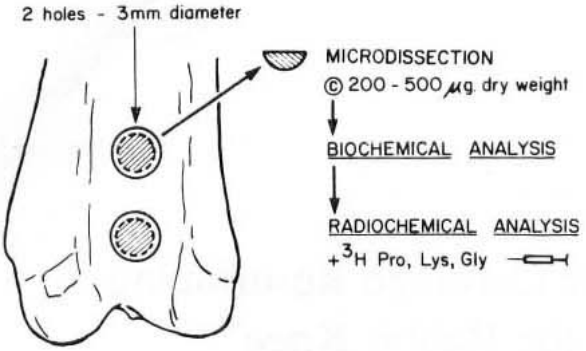


FIG. 1-A

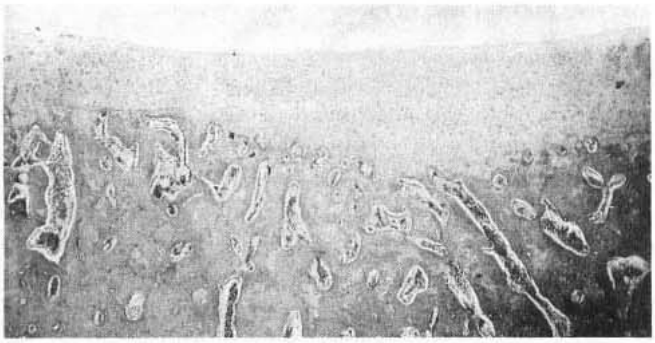


FIG. 1-B

Fig. 1-A: Diagram of the femur in the rabbit knee, showing the sites on the patellar groove where the two three-millimeter holes were drilled. Pure repair cartilage was sampled for biochemical analyses and, from joints that were labeled radioisotopically *in vivo*, for radiochemical analyses.

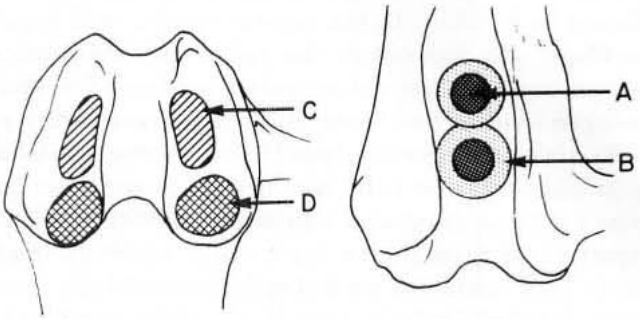
Fig. 1-B: Light microscopy of a repaired hole eight weeks after injury. Original articular cartilage can be seen at both edges of the photograph; new bone and cartilage fill the hole.

were designed to answer the following questions: Does the induced cartilaginous tissue contain amounts and types of macromolecules similar to those of normal articular cartilage? Do the cells in the tissue function as normal articular chondrocytes in their rate and quality of synthesis of collagen and proteoglycans? Do they continue to do so for a year or more after injury? Our aims were to determine how closely the repair cartilage resembles normal articular cartilage biochemically, and hence to evaluate its prospects of surviving as a replacement bearing surface.

Materials and Methods

Surgical Procedure and Sampling of Tissue

Over a period of three years, about 300 New Zealand White rabbits were used in two age groups, six to seven months (3.2 to 3.6 kilograms) and ten to twelve months (4.1 to 4.5 kilograms). The latter were skeletally mature with closed epiphyses; the former were almost mature but their epiphyses were not all closed. Using surgical techniques, defects were cut through the surface of the patellar groove of the femur in both knee joints. Initially one hole — but in later experiments and for all biochemical analyses, two holes — were drilled using an orthopaedic hand drill (bit, 3.2 millimeters in diameter) through the subchondral plate[11]. Usually one knee was used for morphological studies and the other, for biochemical analyses. For biochemical analysis the repair tissue filling the holes was sampled at three, four, six, eight, twelve, twenty-four, and forty-eight weeks, taking special care to avoid contamination with the surrounding original cartilage or with underlying bone (Figs. 1-A and 1-B). The repair tissue was whiter in color than the surrounding cartilage, so the sampling of pure repair tissue was quite easy under the dissecting microscope. Samples of femoral cartilage from the same joint served as controls. From each joint, three separate sites were sampled: weight-bearing and non-weight-bearing areas of the femoral condyles and the patellar groove adjacent to the drilled holes (Fig. 2). Twenty-five animals in each age group were prepared for chemical analyses only. More than twenty-five animals in each age group were labeled radioisotopically to examine the amounts and types of matrix macromolecules synthesized by both control and repair cartilage. Tissue samples



A Repair Cartilage (3mm diameter, 0.5 mm deep)
B Surrounding Cartilage on Patellar Groove
C Non-weight Bearing Portion of Femoral Condyle
D Weight Bearing Portion of Femoral Condyle

FIG. 2

Diagram of the rabbit femoral condyles, showing the three sites where control cartilage was sampled. Comparable load-bearing and non-load-bearing surfaces of lateral and medial condyles were pooled.

were dried in a desiccator and, when necessary, weighed on a Cahn electrobalance.

Radioisotopic Labeling of Cartilage in Vivo

All radiochemicals (New England Nuclear, Boston, Massachusetts) were injected intra-articularly, dissolved in one milliliter of sterile 0.06-molar sodium phosphate buffer, pH 7.2. Twelve animals were injected with fifty microcuries of [L-2, 3-^{3}H(N)]-proline (twenty-eight curies per millimole) per knee joint, one from each of the two age groups at three, four, six, eight, twelve, and twenty-four weeks after surgery. Another twelve rabbits were injected with 250 microcuries of [L-4,5-^{3}H(N)]-lysine (sixty curies per millimole) at similar intervals. The rabbits were killed seventy-two hours later. The distal end of each femur was dissected and stored frozen at −20 degrees Celsius until required for analysis. Before we sliced the cartilage from the femur, all specimens were immersed in physiological saline at 4 degrees Celsius for two to three hours to swell

the cartilage. Another group of twelve animals (six young and six old) was injected intra-articularly in each knee with 100 microcuries of [6-^{3}H]-glucosamine in one milliliter of buffer and then killed after six hours. The femoral condyles of these rabbits were soaked in excess cold physiological saline containing the protease inhibitors ten-micromolar phenylmethanesulfonylfluoride, one-millimolar p-hydroxymercuribenzoate, and ten-millimolar EDTA, for eighteen hours at 4 degrees Celsius before the cartilage was sampled. A final group of six rabbits (three young and three old) was injected intra-articularly with 100 microcuries of [2-^{3}H]-glycine (fifteen curies per millimole) in each knee at four, twelve, and twenty-four weeks after surgery, and then killed five days later. Their cartilage was sampled directly, without washing.

Electron Microscopy

Control and repair tissue from animals five, six, and eight weeks after operation from both age groups was fixed for electron microscopy. We took repair tissue only from the very center of the holes and adjacent original tissue on the patellar groove was taken as a control.

Chemical and Radiochemical Analyses

Determination of ^{3}H-Proline and ^{3}H-Hydroxyproline

Specimens labeled with ^{3}H-proline (300 to 500 micrograms dry weight) were hydrolyzed in 6N hydrochloric acid (one milliliter) at 108 degrees Celsius for twenty-four hours under elemental nitrogen in partially evacuated, sealed glass tubes. The acid was removed on a Buchler Evapomix and the dried hydrolysate was dissolved in 0.2-molar sodium citrate buffer, pH 2.20. A portion was applied to a column (0.9 by ten centimeters) of amino-acid analyzer resin (Mark Instruments MR 205 resin) and eluted at fifty milliliters per hour with 0.2-molar sodium citrate, pH 3.25, at 40 degrees Celsius. About twenty fractions of 2.2 milliliters were collected; 2.0 milliliters of each was sampled to determine ^{3}H-activity by scintillation spectroscopy. Hydroxyproline and proline were completely resolved, each peak eluting in three to four fractions. Using another portion of the hydrolysate, a Beckman 121M amino-acid analyzer was used to measure chemically the hydroxyproline content of the tissue and the molar ratio of hydroxylysine per 100 residues of hydroxyproline.

Determination of Hydroxylysine and Hydroxylysine Glycosides

Dried and weighed cartilage slices (300 to 500 micrograms) from joints labeled *in vivo* with [^{3}H]-lysine were hydrolyzed in sealed polypropylene tubes with 0.2 milliliter of two-molar sodium hydroxide per milligram of sample at 108 degrees Celsius for twenty-four hours. Each hydrolysate was transferred to a small test tube and diluted with 1.4 milliliters of distilled water and 0.4 milliliter of one-molar citric acid to give a final concentration of 0.2-molar sodium citrate[5]. Any precipitate was removed by centrifugation and a portion of the solution was applied directly to a column (0.9 by sixty centimeters) of Mark Instruments 201 resin. Hydroxylysine and its glycosides were fractionated by chromatography, eluting at sixty milliliters per hour with 0.35-molar sodium citrate buffer, pH 5.28, at 50 degrees Celsius[5,7]. Fractions of 2.7 milliliters were collected and 2.0 milliliters was taken for determining ^{3}H-activity by scintillation spectroscopy. Hydroxylysine and its glycosides were measured chemically on the Beckman 121M amino-acid analyzer using similar elution conditions to those in the preparative procedure[8].

Digestion with Cyanogen Bromide

Weighed, freeze-dried samples of repair tissue and of control cartilage from the same joints were added to 70 per cent (volume per volume) formic acid (one milliliter per ten milligrams) and the mixture was flushed with elemental nitrogen. A weight of cyanogen bromide equal to the weight of the cartilage was added from a stock solution in 70 per cent formic acid (200 milligrams per milliliter), and the stoppered vial was shaken for sixteen hours at 25 degrees Celsius. The mixture was diluted tenfold with water, lyophilized, and dissolved in sample solution for slab-gel electrophoresis. Standards of pepsin-solubilized type-I and type-II collagen were prepared from rabbit cartilage and demineralized bone. These purified collagens and samples of complete bone matrix and articular cartilage were also cleaved with cyanogen bromide. Some were reacted for only four hours to check rates of peptide release.

Electrophoresis in Sodium Dodecylsulphate-Polyacrylamide

Electrophoresis of collagen cyanogen bromide-cleaved peptides was carried out in slabs of 10 per cent polyacrylamide gel (1.5 millimeters thick), using a continuous Tris-borate buffer system: 0.05-molar Tris, 0.03-molar boric acid, and 0.1 per cent sodium dodecylsulphate, pH 8.5[8,15]. Fifty to 100 micrograms of the cyanogen bromide-cleaved peptides were dissolved at two milligrams per milliliter in a solution of 0.05-molar Tris, 0.03-molar boric acid, 0.1 per cent sodium dodecylsulphate, and two-molar urea, pH 8.5, and denatured for five minutes in a water bath at 100 degrees Celsius. Each well was loaded with thirty to fifty microliters of sample solution. A current of fifteen milliamperes per gel was applied for about twenty minutes until the tracking medium passed through the stacking gel to the top of the separating gel. The current was then increased to thirty milliamperes per gel for two hours. The gels were stained for sixteen hours in 25 per cent isopropanol and 10 per cent acetic acid (volume per volume) containing 444 milligrams per liter of Coomassie Brilliant Blue 250, and then for six hours in 10 per cent isopropanol and 10 per cent acetic acid (volume per volume) containing thirty-five milligrams per liter of the stain. The gels were then de-stained for eighteen hours in a diffusion de-stainer and strips were scanned at 550 nanometers in a Gilford spectrophotometer.

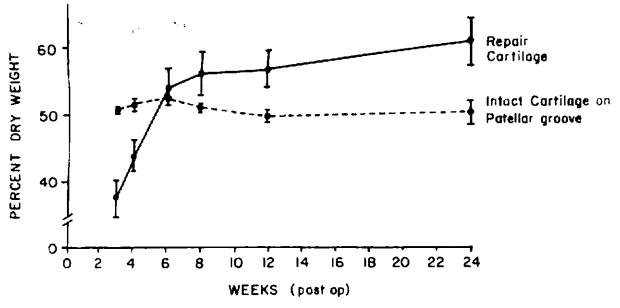


FIG. 3

Graph showing the increasing content of collagen in the rabbit repair tissue with time after injury compared with the normal range determined for samples of control articular cartilage taken from the surrounding patellar groove. Each point is the average ± standard deviation for six to eight independent samples.

Using a phosphate buffer, individual cylindrical gels were also run for some radiochemical measurements on sliced gels. Gels run on cyanogen bromide digests of samples labeled *in vivo* for five days with 100 microcuries of ^{3}H-glycine plus fifty microcuries of ^{3}H-proline were frozen after electrophoresis, cut into 1.3-millimeter slices, and hydrolyzed in sealed test tubes with 0.4-molar sodium hydroxide for sixteen hours at 55 degrees Celsius. Each hydrolysate was acidified with 0.06-molar hydrochloric acid and mixed with ten milliliters of scintillation fluid (Packard Instagel), and then its ^{3}H-activity was measured.

Hexosamine Analysis

Cartilage samples labeled *in vivo* with [6-^{3}H]-glucosamine were hydrolyzed with 1.0 milliliter of 6N hydrochloric acid at 100 degrees Celsius for three hours under nitrogen in partially evacuated, sealed glass tubes[17]. The ^{3}H-glucosamine and ^{3}H-galactosamine were completely resolved on a fifty by 0.9-centimeter column of amino-acid analyzer resin (Mark Instruments 201 resin), using conditions similar to those used to measure the hydroxylysine glycosides. Fractions of 2.7 milliliters were collected and ^{3}H-activity was determined on two-milliliter portions taken across the region of effluent in which the hexosamines were found to elute. The chemical content of hexosamine in the tissue was measured on another portion of the hydrolysate using a program on the Beckman 121M amino-acid analyzer with ten nanomoles per milliliter of δ-amino valeric acid included in the sample buffer as an internal standard[5].

Results

Tissue with the texture of cartilage could be sampled from the holes by three weeks after the operative procedure (two to three millimeters wide and 0.5 millimeter deep). The average dry weight for more than 100 samples of repair cartilage from individual holes was 400 (± 170) micrograms. Of 128 joints finally selected for biochemical studies, four (3 per cent) were infected after surgery, and these were excluded. After sampling the tissue for biochemical analysis, histological sections from randomly chosen joints were prepared for light microscopy to be certain that only repair tissue had been removed. No significant differences were found between the six to seven-month-old and the ten to twelve-month-old groups of rabbits in the morphology of repair or in any of the biochemical properties of the repair cartilage.

Collagen and Hexosamine Content of the Repair Cartilage

The changing contents of collagen and hexosamine in the repair cartilages with time after injury are plotted in Figures 3 and 4. Collagen was derived from the hydroxyproline content using a factor of 7.6 that we calculated for type-II collagen from the amino acid and hexose composition of the pure α1 (II) chain. Hexosamine was taken as the sum of glucosamine and galactosamine. At each stage of repair, the collagen content varied little among specimens from different animals. The average content of collagen in the repair cartilage rose steeply between three and six weeks to a plateau slightly higher than that of normal cartilage from the patellar groove. After six weeks the collagen content still increased but only gradually, probably reflecting a loss of proteoglycans. Thus, by twenty-four weeks the repair cartilage generally was richer in collagen than was the control cartilage from the patellar groove or the femoral condyles. Not surprisingly, therefore, there was a trend for the hexosamine content to fall between four and twenty-four weeks (Fig. 4). At twenty-four weeks the repair cartilage contained significantly less hexosamine than the control cartilage. However, among different specimens of repair cartilage taken at each stage, the hexosamine contents varied more than among the controls. The analyses established that the major load-bearing posterior surfaces of the femoral condyles were richer in hexosamine than their anterior surfaces (Fig. 4).

The molar ratio of galactosamine to glucosamine was higher in the repair cartilage than in samples of control articular cartilage taken from the same joints (Table I). This

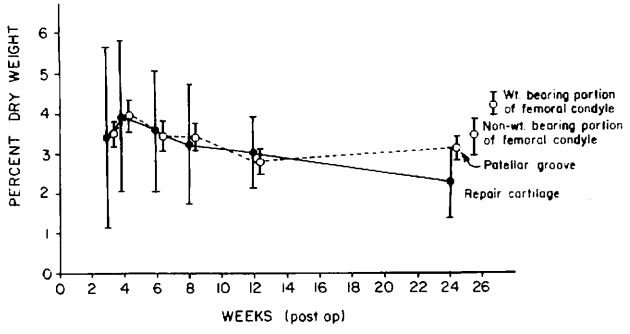


FIG. 4

Variations with time after injury in the hexosamine content of repair cartilage compared with control cartilage from the knee. Each point for the repair cartilage is the average ± standard deviation for four to six independent samples. The hexosamine contents of weight-bearing and non-weight-bearing control cartilage are plotted as averages for all animals with no relation to time. Each is the average ± standard deviation for eleven to thirteen independent samples from different animals. The measurements on control cartilage from the patellar groove are averages of two independent samples at each time-point plotted ± the standard deviation calculated for control samples as a whole. The lower hexosamine content of the repair cartilage at twenty-four weeks compared with control cartilage on the patellar groove or femoral condyles was statistically significant ($p < 0.05$.)

TABLE I

COMPARISON OF THE MOLAR RATIOS OF GALACTOSAMINE TO GLUCOSAMINE IN REPAIR AND CONTROL ARTICULAR CARTILAGE FROM RABBIT KNEE JOINTS

Time after Operation	Young Rabbits (6-7 Months Old)		Adult Rabbits (10-12 Months Old)	
	Repair	Control*	Repair	Control*
3-4 weeks	3.3 ± 0.6(4)	3.1 ± 0.6(4)	4.6 ± 0.8(3)	1.8 ± 0.2(4)
6-8 weeks	3.3 ± 0.3(4)	2.5 ± 0.2(4)	3.9 ± 0.8(6)	2.0(1)
12 weeks	3.3 ± 0.3(3)	1.8 ± 0.05(3)	2.8 ± 0.6(2)	1.8 ± 0.3(3)
24 weeks	1.3 ± 0.3(3)	1.9 ± 0.1(3)	3.0 ± 0.3(2)	1.9 ± 0.2(3)

* Averages ± standard error of the mean of analyses on independent samples of original cartilage from patellar groove or femoral condyles (no significant differences between weight-bearing and non-weight-bearing surfaces). Numbers in parentheses indicate numbers of cartilage samples analyzed.

was especially evident in the older rabbits, for which the average increase is highly significant statistically. Note also the age-related fall in ratio for the normal cartilage of the younger rabbits but not for the adult group (Table I).

Incorporation of ^{3}H-Proline into Collagen

The rate of deposition of collagen by the repair cartilage was compared with that of control articular cartilage from the same joint by measuring their incorporation of ^{3}H-proline into ^{3}H-hydroxyproline. Non-weight-bearing anterior surfaces of the femoral condyles were analyzed as controls. The results are shown in Figure 5. Four weeks after operation, and three days after labeling the joint, about 43 per cent of the total ^{3}H-activity in the repair cartilage was present as ^{3}H-hydroxyproline. Assuming that collagen contains 120 residues of proline for every 100 residues of hydroxyproline, this means that about 95 per cent of the incorporated ^{3}H-proline was in collagen. In control cartilage from the same joint at four weeks, only 25 to 30 per cent of the incorporated ^{3}H-proline was in collagen. Both young and old rabbits showed similar differences in incorporation between control and repair cartilage. By twenty-four weeks, the fraction of ^{3}H-proline incorporated into collagen by the repair cartilage had fallen to control levels. However, for four to six weeks after operation even the control articular surfaces showed stimulated labeling of collagen compared with rabbits of similar age that were not operated on (Fig. 5), probably reflecting a stimulated synthesis of matrix macromolecules in response to the transient synovitis.

Hydroxylysine and Hydroxylysine Glycosides

Table II shows the molar ratio of hydroxylysine to hydroxyproline in the repair cartilage at various times after injury. At all stages, the ratio was lower than that of normal rabbit articular cartilage. This is explained in part by the presence of type-I collagen in the repair tissue.

Figure 6 shows the steady increase in glycosylation of hydroxylysine in repair cartilage collagen with time after injury, reaching a maximum by about twelve weeks. This plateau maximum is slightly lower than that of control normal articular cartilage. Assuming that 17 per cent of hydroxylysines in type-I collagen and 63 per cent in type-II collagen are glycosylated (determined for collagen of rabbit bone and rabbit articular cartilage), the repair cartilage includes about 10 per cent of its total collagen as type I at twelve weeks. However, type-I collagen of repair

TABLE II

HYDROXYLYSINE CONTENT OF COLLAGEN IN THE REPAIR ARTICULAR CARTILAGE OF RABBITS AT VARIOUS TIMES AFTER INJURY*

Time after Injury	Hydroxylysine per 100 Residues of Hydroxyproline (Molar Ratio)
3 weeks	15.3 ± 0.6(4)
4 weeks	15.0 ± 0.7(4)
6 weeks	15.9 ± 0.5(2)
8 weeks	14.3 ± 0.3(2)
12 weeks	15.2 ± 1.5(3)
24 weeks	16.2 ± 0.7(7)
Normal cartilage (patellar groove)	19.8 ± 0.1(12)

* Average values ± standard error of the mean of independent specimens from both age groups at each stage. Numbers in parentheses indicate numbers of cartilage samples analyzed.

tissue is probably more heavily glycosylated than type-I collagen of bone, so this estimate may be low, as suggested by the analyses of cyanogen bromide-cleaved peptides described later. The molar ratio of glucosylgalactosylhydroxylysine to galactosylhydroxylysine decreased rapidly between three and twelve weeks, as shown in Fig-

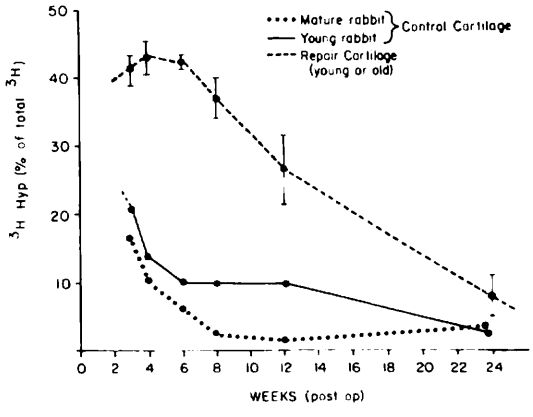


FIG. 5

Graphs showing changes in collagen synthesis in the repair and control cartilages with time after injury. Collagen synthesis is assessed by the incorporation of ^{3}H-proline into ^{3}H-hydroxyproline. Animals were killed seventy-two hours after injection of the ^{3}H-proline intra-articularly. Control cartilage was taken from the non-weight-bearing surface of the femoral condyles (area C in Fig. 2). The results for repair cartilage are averages of four to seven independent specimens from both age-groups plotted ± standard deviation. Single measurements are plotted for the samples of control cartilage. *Mature* refers to the animals that were ten to twelve months old at operation and *young*, to the six to seven-month-old animals.

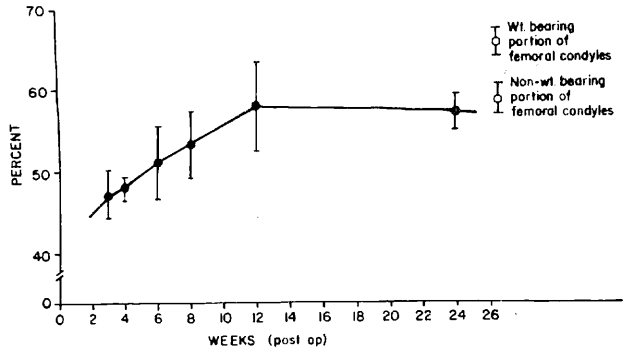


FIG. 6

Graph showing the increasing glycosylation of hydroxylysine in the total collagen of the repair cartilage with time after operation. The ordinate refers to the percentage of hydroxylysine residues that are glycosylated. Plotted values on the curve are averages ± standard deviation for four independent specimens. The ranges shown for the control articular surfaces are averages ± standard deviation determined on five to seven specimens from different animals.

ure 7, but even at twenty-four weeks the ratio was higher than in control cartilage.

It should also be noted in Figures 6 and 7 that the collagens of the weight-bearing and non-weight-bearing articular surfaces differed in degree of glycosylation, both in total content and in ratio of disaccharide to monosaccharide derivatives.

Hydroxylysine Glycoside Content of Newly Synthesized Collagen

By labeling the joint *in vivo* with ^{3}H-lysine, the degree of hydroxylysine glycosylation in the newly synthesized collagen could be measured. The three radioactive hydroxylysine compounds were well resolved on the 0.9 by sixty-centimeter column (Fig. 8), and their relative activities could be readily measured up to twelve weeks after injury. After twelve weeks, because of the low uptake of ^{3}H-lysine by the cartilage and the small fraction of activity in collagen, the analysis became too inaccurate to be useful. Table III gives the radiochemical analyses of hydroxylysine glycosylation in samples taken three to twelve

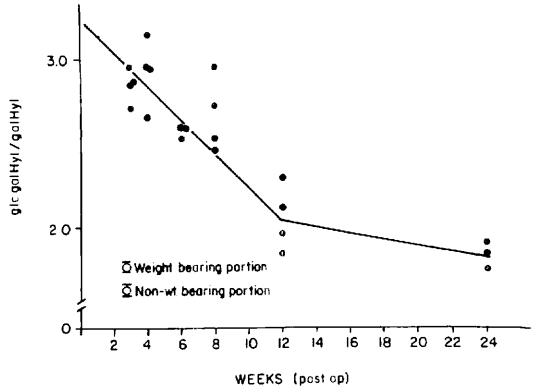


FIG. 7

Graph showing the changes with time after operation in the molar ratio of glucosylgalactosylhydroxylysine to galactosylhydroxylysine in the repair articular cartilage. Measurements on individual specimens are plotted to indicate the scatter. The averages ± standard deviation for five to seven independent samples of the control articular surfaces are also shown.

weeks after injury. The highest glycosylation was reached at six to eight weeks, when most of the cells in the repair cartilage appeared to be producing type-II collagen although type-I collagen was still present chemically.

Analysis of Type-I and II Collagen by Electrophoresis of Cyanogen Bromide-Cleaved Peptides on Sodium Dodecylsulphate-Polyacrylamide Gels

Total Collagen

The molecular types of collagen present in the repair cartilage were assessed semiquantitatively by electrophoresis of their cyanogen bromide-cleaved peptides on

TABLE III

PERCENTAGE GLYCOSYLATION OF HYDROXYLYSINE OF THE NEWLY SYNTHESIZED FRACTION COMPARED WITH THE TOTAL COLLAGEN OF THE RABBIT REPAIR CARTILAGE AT VARIOUS TIMES*

Time after Injury	Newly Synthesized† (Radiochemical)	Total‡ (Chemical)
3 weeks	40 ± 3(6)	47 ± 1.5(4)
4 weeks	44 ± 2(4)	47 ± 0.7(4)
6 weeks	50 ± 5(3)	51 ± 2.6(3)
8 weeks	57 ± 5(5)	53 ± 2.0(4)
12 weeks, 24 weeks	47 ± 3(9)	57 ± 1.5(4)
Control§	52 ± 2(5)	62 ± 0.6(12)

* Averages ± standard error of the mean for specimens of control and repair cartilage from two rabbits (one in each age group) at each stage after operation. No difference between rabbits of different ages was detected, so the results were averaged.

† Measured on ^{3}H-lysine-labeled cartilage by expressing the ^{3}H-activity recovered in ^{3}H-glucosylgalactosylhydroxylysine + ^{3}H-galactosylhydroxylysine as a percentage of the total ^{3}H-hydroxylysine.

‡ By chemical analysis of glucosylgalactosylhydroxylysine, galactosylhydroxylysine, and hydroxylysine on the amino-acid analyzer.

§ Cartilage from the patellar groove surrounding the hole.

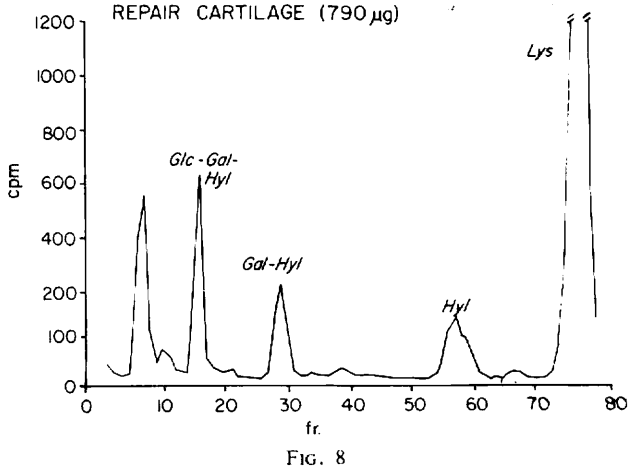


FIG. 8

Typical elution profile determined on an amino-acid analyzer of radioactivity in a base hydrolysate of repair cartilage labeled *in vivo* with ^{3}H-lysine.

sodium dodecylsulphate-slab gels, comparing the profiles with those of standard type-I and type-II collagen. A photograph of a typical stained gel is shown in Figure 9. To quantify the relative amounts of type-I and II collagen in the regenerated cartilage, several of their characteristic cyanogen bromide-cleaved peptides were assayed in the

TABLE IV

RELATIVE AMOUNT OF TYPE-I AND II COLLAGEN IN THE RABBIT REPAIR CARTILAGE AT VARIOUS TIMES AFTER INJURY

Time after Injury	Type II as a Percentage of Type-I and II Collagens*
3 weeks	30
4 weeks	39
6 weeks	53
8 weeks	59
12 weeks	57
24 weeks	76
48 weeks	81

* Calculated from densitograms of stained cyanogen bromide-cleaved peptides on sodium dodecylsulphate-polyacrylamide electrophoresis (Fig. 9).

gels densitometrically. The peptides α1(II)CB10 and α2CB3,5 were relied on mainly[6,7]. Although α2CB3,5 may be a partial cleavage product, its high and reproducible yield could be exploited as a useful measure of type-I collagen[7]. Scans of standard preparations of type-I and II collagen and of digests of normal articular cartilage and bone were run as controls to calibrate the relative intensities of their bands (Table IV).

The results confirm that a gradual increase in type-II collagen is responsible for the significant rise in content of hydroxylysine glycosides with time. At all stages the repair cartilage contained both type-I and type-II collagen, but the proportion of type-II collagen increased markedly with time, especially in the three to eight-week period. It is important to note, however, that even at the latest stage type-I collagen was still a significant component of the repair tissue, unlike normal rabbit articular cartilage in which no type-I collagen could be detected (at most, less than 1 per cent of the collagen).

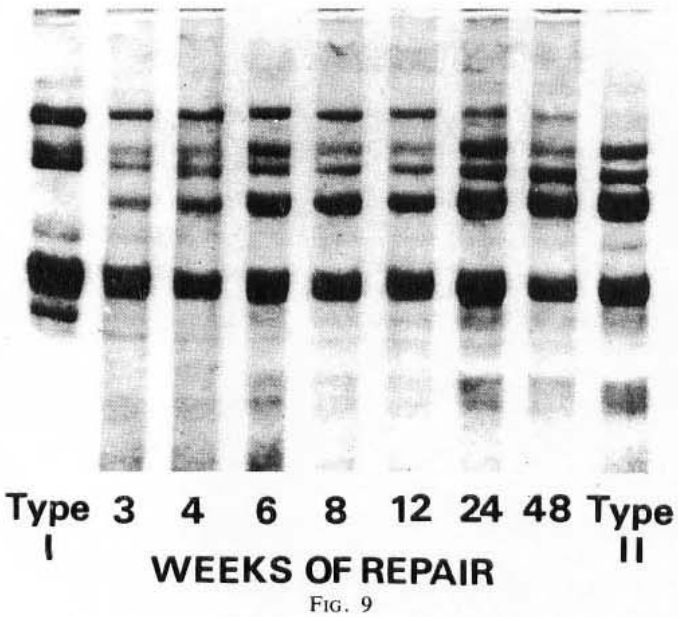


FIG. 9

Slab electrophoresis in sodium dodecylsulphate-polyacrylamide of cyanogen bromide digests of repair cartilage sampled at various intervals after injury. Standards of type-I and II rabbit collagens from decalcified bone and normal articular cartilage, respectively, were run for comparison. The major cyanogen bromide-cleaved peptides of types I and II have previously been identified in this type of separation[8]. The steadily increasing proportion of type-II collagen and decrease in type I is evident between three weeks and one year.

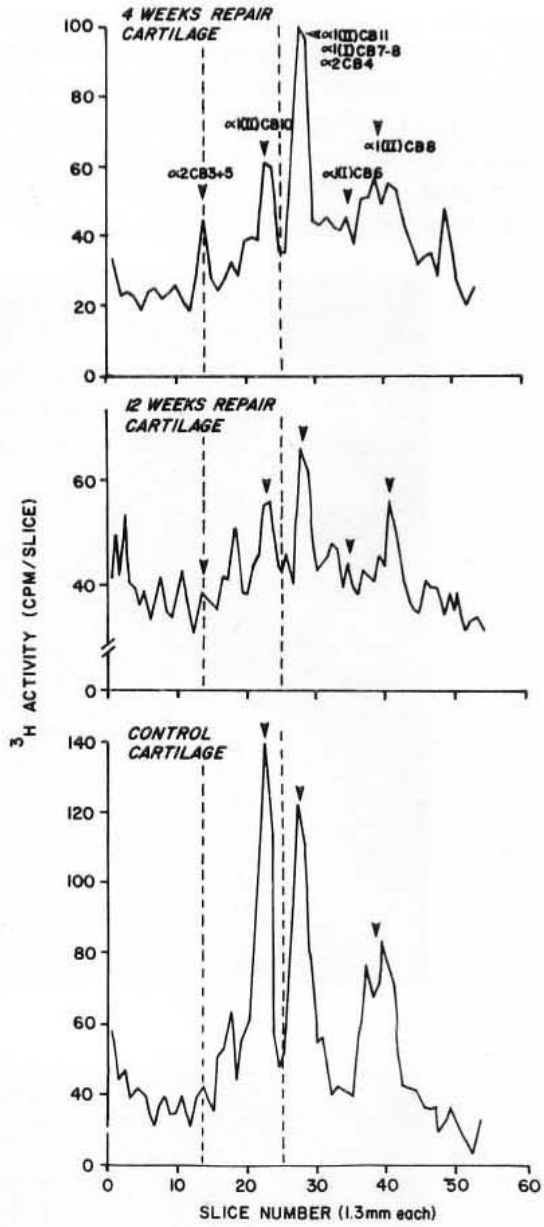


FIG. 10

Slab electrophoresis in sodium dodecylsulphate-polyacrylamide of cyanogen bromide digests of repair articular cartilage labeled *in vivo* with ^{3}H-proline plus ^{3}H-glycine. ^{3}H-activity was measured in serial slices of gel. An analysis of control articular cartilage from femoral condyles is compared with analyses of repair cartilage at four weeks and twelve weeks. The results confirm that mainly type-II collagen with some type-I collagen is being made at four weeks. Although much less label is incorporated into collagen by twelve weeks, type II is still the major product.

^{3}H-Labeled, Newly Synthesized Collagen

The cyanogen bromide-cleaved peptides of radioactive collagen labeled *in vivo* with ^{3}H-glycine and ^{3}H-proline were fractionated by electrophoresis on cylindrical gels of sodium dodecylsulphate-7 per cent polyacrylamide, and were detected by measuring ^{3}H-activity in serial slices.

Figure 10 compares a profile for control cartilage with profiles for repair cartilage at four weeks and twelve weeks

after injury. At four weeks, prominent peaks of radioactive α2CB3,5, αl(II)CB10, and the mixture of αl(II)CB11 and αl(I)CB8 were observed. At this stage the tissue was producing both type-I and type-II collagen at a ratio of about 40 per cent to 60 per cent. However, at twelve weeks the radioactive peak of α2CB3,5 was smaller and that of αl(II)CB10 was much larger, indicating an almost complete commitment to synthesizing type-II collagen in accord with the results with ^{3}H-lysine.

Incorporation of ^{3}H-Glucosamine

More than 95 per cent of the ^{3}H-label in the washed cartilage samples from animals labeled with ^{3}H-glucosamine was macromolecular. This was proved by

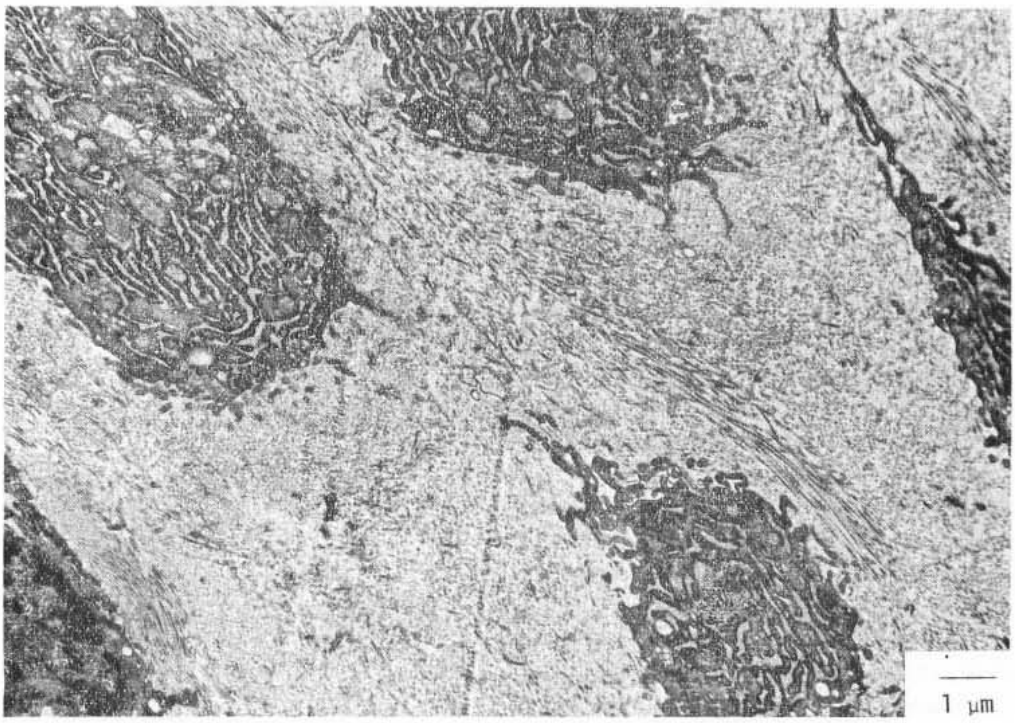


FIG. 11-A

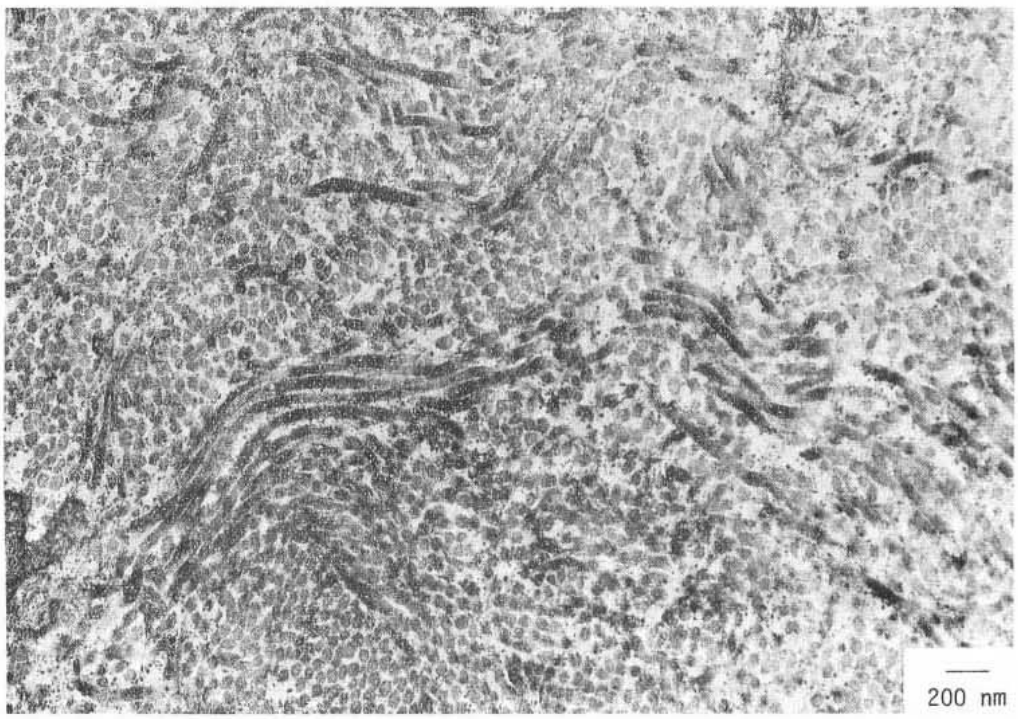


FIG. 11-B

Figs. 11-A, 11-B, and 11-C: Electron microscopy of the collagen architecture of repair and control cartilage six weeks after injury. The control cartilage was taken from the articular surface of the patellar groove surrounding the hole (uranyl acetate and lead citrate).
Figs. 11-A and 11-B: Repair cartilage.

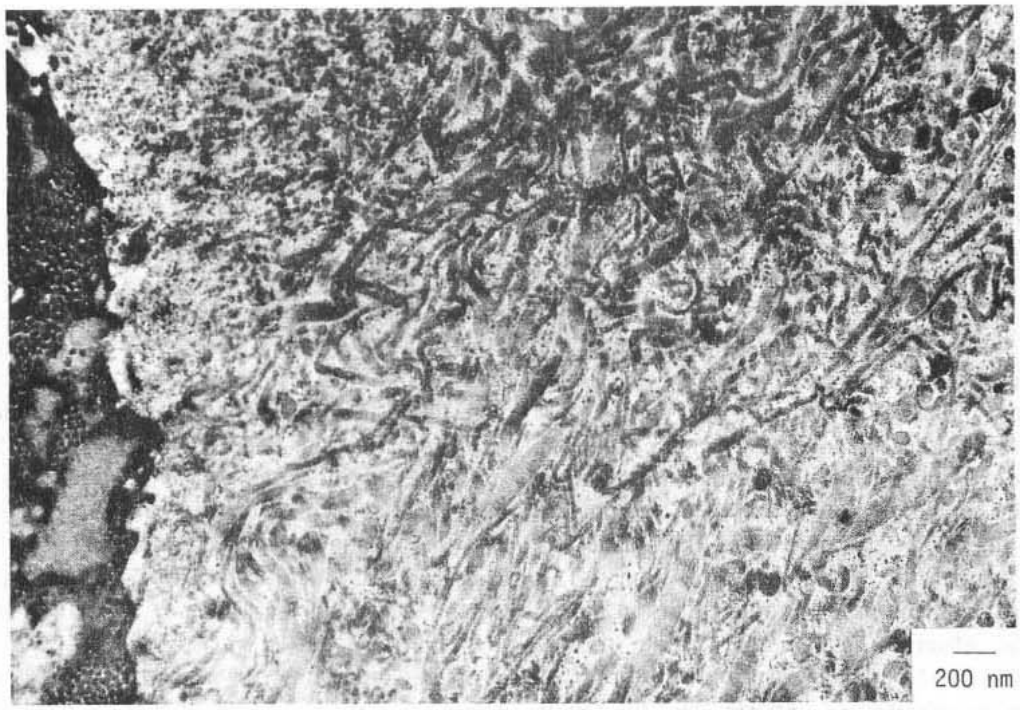


FIG. 11-C
Control cartilage.

eluting papain digests of the tissue from a column of Bio-Gel P-2 in dilute acetic acid, and measuring the activity in the void (macromolecular) and the included (free ^{3}H-glucosamine) peaks.

The ratios of ^{3}H-galactosamine activity to ^{3}H-glucosamine activity at various times during repair are listed in Table V. Averages include tissue from both young and old rabbits. There appeared to be a trend to a lower ratio with time after operation, although individual samples varied greatly. A statistical comparison of the three to four-week groups with the twelve to twenty-four-week groups showed that the fall in ratio was significant ($p < 0.05$). The sum of ^{3}H-activities in galactosamine plus glucosamine per unit of dry weight of cartilage showed no significant change with time after operation. However, an important observation is that the uptake was always much higher in the repair cartilage than in the adjacent, control cartilage on the patellar groove. The load-bearing sites on the femoral condyles always incorporated ^{3}H-glucosamine at about the same rate as the repair cartilage, however. This result did not appear to be a function of where the isotope was injected into the joint cavity, but seemed to reflect inherent differences between the various joint surfaces.

Electron Microscopy

Figures 11-A, 11-B, and 11-C compare typical electron micrographs of the collagen architecture of repair and control cartilage. The examples shown came from the ten to twelve-month-old age group, but repair tissue from the younger group looked similar. Tissue from animals at five, six, and eight weeks after operation was examined. All specimens showed active chondrocytes with a fine, uniform mesh of collagen fibrils. The uniformity in diameter of the collagen fibrils in the repair cartilage was most striking compared with the fibrils of the surrounding cartilage in the patellar groove. The fibrillar pattern of the control cartilage was typical of adult articular cartilage, with a wide range of diameters, random orientation, and great variation in intensity of staining of the characteristic colla-

TABLE V

INCORPORATION OF ^{3}H-GLUCOSAMINE INTO MACROMOLECULES BY THE REPAIR AND CONTROL RABBIT ARTICULAR CARTILAGE *IN VIVO**

	Total ^{3}H-Activity in Galactosamine + Glucosamine (*cpm/mg dry tissue*)	^{3}H-Galactosamine/ ^{3}H-Glucosamine (*cpm/cpm*)
3 weeks	5040 ± 2120	3.1 ± 0.5(3)
4 weeks	5100 ± 1140	2.8 ± 0.4(4)
6 weeks	3690 ± 455	2.5 ± 0.5(4)
8 weeks	3160 ± 750	2.6 ± 0.3(2)
12 weeks	2610 ± 110	1.9 ± 0.6(2)
24 weeks	3800 ± 370	1.9 ± 0.4(4)
Normal cartilage B†	660 ± 120	2.0 ± 0.2(5)
Normal cartilage D‡	2690 ± 318	2.1 ± 0.2(6)

* Animals were injected intra-articularly six hours before sampling. Values shown are averages ± standard error of the mean for individual samples from different animals in both age groups.

† Average ± standard error of the mean of specimens from the patellar groove of three animals from the six to seven-month-old age group and two from the ten to twelve-month-old group (see sampling site, Fig. 2).

‡ Average ± standard error of the mean of specimens from the posterior major load-bearing third of the femoral condyles of three animals from the younger age group and three from the older age group (see sampling site, Fig. 2).

gen banding. The abundance of rough endoplasmic reticulum in the repair chondrocytes attests to how actively they were synthesizing protein.

Discussion

The present surgical approach differs from many earlier ones in that the original articular surface was not shaved off before drilling, and wider holes were drilled so that pure repair cartilage could be sampled for biochemical analyses.

The results show that by eight weeks after injury the cartilage resurfacing the holes is predominantly hyaline by morphological and biochemical criteria, yet type-I collagen, characteristic of fibrocartilage and fibrous tissue in general, persists even up to one year after injury. However, based on the content of type-II collagen, the quality would appear to improve — become more like normal articular cartilage — with increasing time after injury. The presence of both type-I and type-II collagen fits in nicely with histological observations on the repair tissue. Meachim and Roberts, from their histological studies of similar lesions in the rabbit knee, reported that the repair tissue showed a so-called streaky, fibrocartilaginous texture, with chondroid regions and more fibrous regions closely intermingled. They concluded that although this could reflect divergent pathways of tissue differentiation, it seemed more likely that the heterogeneity reflected progressive stages in the development of a true cartilaginous texture. The trend shown in the present study for a progressive increase in type-II collagen for up to forty-eight weeks after injury supports this concept. Presumably, with time the initial fibrous matrix that is rich in type-I collagen is gradually resorbed and is replaced by a matrix in which the collagen is type II.

The histological studies forming the foundation for the present project[11] confirmed our observations. On average, the texture of the repair cartilage became more hyaline or chondroid with time after injury up to twenty-four weeks. Not all animals or joints behaved uniformly, however. For instance, about one in ten holes retained a deep plug of cartilage that in parts was often overtly fibrocartilaginous and in which bone failed to grow up and fill the hole to near its original level as it usually did. No satisfactory explanation could be found for these occasional failures. In many joints, after about twenty-four weeks there was a tendency for the repaired surface to split and fibrillate, rather like the changes seen in osteoarthrosis. This behavior again was not a uniform response. Several forty-eight-week specimens looked just as healthy when stained with safranin O as others did at eight weeks. The present biochemical findings indicate that these late morphological changes to a more fibrous texture may result from a loss of proteoglycans rather than from an overt transition of the complete tissue to fibrocartilage. The persistence of significant amounts of type-I collagen in the repair cartilage even after one year suggests that authentic articular cartilage never really develops, and this may be the reason why it fails mechanically. The presence of type-I collagen is not necessarily undesirable, however, since adult avian articular cartilage contains predominantly type-I collagen[8].

Despite the significant rise in type-II collagen with time after operation, the hydroxylysine content of the total collagen in the repair cartilage stayed roughly constant (Table II). This can be explained if the type-I collagen of the granulation tissue initially deposited was almost as rich in hydroxylysine as the type-II collagen that was laid down later, which in turn would have to contain less hydroxylysine than type-II collagen of normal articular cartilage. A tissue-dependent, variable hydroxylation of type-I and II collagen has been well documented. Thus, although it is generally accepted that type-II collagen is richer than type-I collagen in hydroxylysine, it is known, for example, that in adult humans type-I collagen of fibrocartilage and type-II collagen of articular cartilage contain similar amounts of hydroxylysine, at about twelve residues per α chain each[7].

The uniformity in diameter of the collagen fibrils around the repair chondrocytes at six weeks after operation may reflect differences in the chemistry of the type-II collagen, or in the quality of associated interfibrillar macromolecules, compared with normal cartilage. The ultrastructure of repair cartilage a year after injury was not examined. It would be useful to know whether the same changes that occur in normal articular cartilage with age are evident in maturing repair tissue: does the average diameter and the range of diameters of collagen fibrils increase, and does the fibrillar arrangement become more random? If so, this model might be useful for studying the course of these physical changes — for example, whether they take place in the original type-II collagen framework, or require turnover and synthesis of new collagenous matrix.

It is notable that the load-bearing and non-load-bearing surfaces of the normal femoral condyles differed significantly in the degree of glycosylation of *their collagens* (Figs. 6 and 7). Normal rabbits, not operated on, showed similar differences (unpublished observations). This may reflect variations in the post-translational control of collagen synthesis due to differences in mechanical load, and lends support to the previously observed difference in glycosylation of newly synthesized type-II collagen in osteoarthrotic cartilage[4]. The difference is not due to variable amounts of type-I collagen in the normal articular cartilage. By analysis of cyanogen bromide-cleaved peptides, we could not detect type-I collagen in amounts that would account for the changes in glycosylation between load-bearing and non-load-bearing surfaces.

How chondrogenesis is regulated in this model is not clear. Mechanical factors are probably important. Thus, Salter et al. found that continuous passive motion of rabbit knees after subchondral holes were drilled hastened the repair with hyaline tissue. Once the cartilage has developed, continuous mechanical loading may help to maintain its

differentiated state.

It should also be considered that although chondrocytes in the adjacent, original cartilage play no direct part in the repair, materials diffusing from it into the repair tissue may be regulatory. It is not clear, for instance, why the new bone stops growing upward roughly adjacent to the base of the surrounding articular cartilage.

The factors promoting chondrogenesis in the adult and maintaining the cartilage phenotype could be profitably studied with this model.

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