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Ultrastructural changes in articular cartilage after experimental section of the anterior cruciate ligament of the dog knee

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INTRODUCTION

Section of the anterior cruciate ligament of the knee (Pond & Nuki, 1973) produces joint laxity, leading eventually to degenerative changes in articular cartilage resembling those occurring naturally in dogs. Very early biochemical and morphological changes in the cartilage can be studied in such an experiment which help us to understand the reactions and adaptability of normal cartilage. They are also relevant to the development of joint abnormalities in man. For example, after cruciate section in the dog, biochemical changes occur in the proteoglycans (McDevitt & Muir, 1976) and the collagen (Eyre, McDevitt & Muir, 1975) similar to those in human degenerative joints (Mankin & Lippiello, 1970). In the dog, biochemical findings have been supplemented by a morphological study (McDevitt, Gilbertson & Muir, 1977) which has demonstrated early changes in the appearance, numbers and distribution of the cell population. Preliminary observations of ultrastructural changes in the cartilage have been reported (Muir, 1977) but these have been extended considerably in the present study.

MATERIAL AND METHODS

Cartilage was examined from twenty two female beagles, 3–5 years old, formerly used for breeding. Ligament section was performed in sixteen dogs. The anterior cruciate ligament was sectioned by the method of Pond & Nuki (1973), using a fine pointed scalpel blade (No. 11) through a stab incision on the lateral aspect of the right knee. The intact left knee was used as a control. Sham operations (stab incision without ligament section) were carried out on six dogs. The dogs were given systemic antibiotics (penicillin and streptomycin; Streptopen – Glaxovet Ltd) for three days after surgery. They were exercised for one hour per day from the second day after operation. Tissue from the dogs was sampled, after pentabarbitone euthanasia (Euthatal – May and Baker Ltd), at two days (2 animals), four days (2 animals), one week (5 animals), one month (2 animals), two–three months (3 animals) and 15–18 months (2 animals) after operation. Sham-operated material was examined at one week. The knee joints were opened from the lateral side, the operated joint being opened first. Blocks of the full thickness of area 'A' or central part of the surface of the medial tibial condyle (McDevitt *et al.* 1977) were placed in fixative and immediately sectioned normal to the articular surface into slices less than 1 mm thick. The

tissue was fixed in 6 % glutaraldehyde for 16 hours at 4 °C, washed in 10 % ethanol, post-fixed in 1 % osmium tetroxide, and embedded in Araldite. Sections 1 μm thick were cut normal to the articular surface and stained with toluidine blue–Azur A (Ito & Winchester, 1963) for preliminary light microscopy. Thin sections for electron microscopy were cut on a Reichert OMU 3 microtome, stained on uncoated copper grids with uranyl acetate and lead citrate, and examined at 60 kV in a Philips 301 electron microscope.

Frozen sections 10 μm thick of tissue from each joint were taken after fixation and coloured for lipid with Sudan black B.

Measurements

An attempt was made to assess changes in the superficial matrix of the cartilage semi-quantitatively by applying the point counting technique (Weibel, Kistler & Scherle, 1966). Electron micrographs ($\times$ 44000) of the superficial 5 μm of the cartilage were used. A 1·1 cm square lattice was laid over the photographs and points were counted where they lay over fibrils; the results were expressed as percentages of total points.

Diameters of the superficial fibrils were measured using the negatives of electron micrographs, magnified by $\times$ 22000. All fibrils lying within two 1·1 $\times$ 1·1 cm squares were measured in each photograph, using a travelling microscope with a magnification of $\times$ 20. Between 100 and 200 fibrils were measured in each specimen.

Measurements of the thickness of the nuclear fibrous lamina of the chondrocytes were also made on the electron micrographs.

The 1 μm thick Araldite sections stained with toluidine blue Azure A were used for cellularity and lipid estimations. With an eyepiece graticule in a Leitz Orthoplan binocular optical microscope at a magnification of $\times$ 500, cell nuclei were counted in rectangular areas of the section measuring 0·25 $\times$ 0·175 mm. Successive rectangles were located at increasing distances from the articular surface, commencing with the area adjoining the surface (Stockwell, 1971). Using a second 'stepped wedge' graticule, the numbers and sizes of intracellular lipid globules were estimated in the same rectangular areas, the globules presenting as pale yellow circular intracellular areas in the Araldite section. These procedures were carried out on at least two sections from each block (separated by approximately 50–100 μm in the thickness of the block) and on at least two blocks from each joint. Results were expressed as cells per mm^2 and as μm^2 lipid per cell.

RESULTS

Articular surface and superficial zone matrix

The surface contour of the articular cartilage of control joints showed irregular undulations, but these were not related to the sparse superficial cells which were rarely close to the surface in these specimens of dog tibial cartilage. Occasional small fissures in the surface were present, and at high magnification the surface was not completely smooth; electron-dense granular and amorphous material was inconspicuous in most control joint surfaces. The collagen fibrils lying within 5–10 μm of the surface (Fig. 1) were close-packed and mostly ran parallel to the surface.

In operated joints, constant differences were observed. The articular surface was roughened, with a 'fluffy' appearance (Fig. 2). Large amounts of electron-dense amorphous material had usually infiltrated the cartilage surface (Fig. 3). The super-

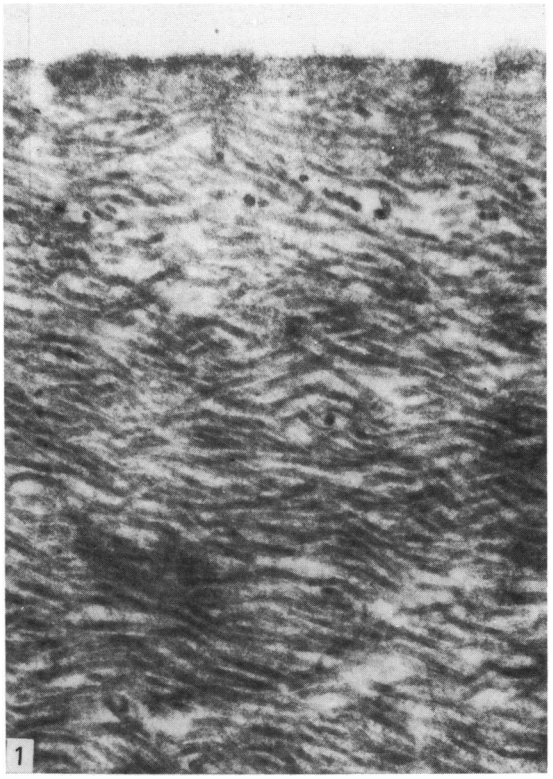

Fig. 1. Articular surface, control joint, one week. ×44000.

Fig. 2. Articular surface, operated joint, one week. Collagen fibrils are more widely spaced and the articular surface is less distinct than in controls. ×44000.

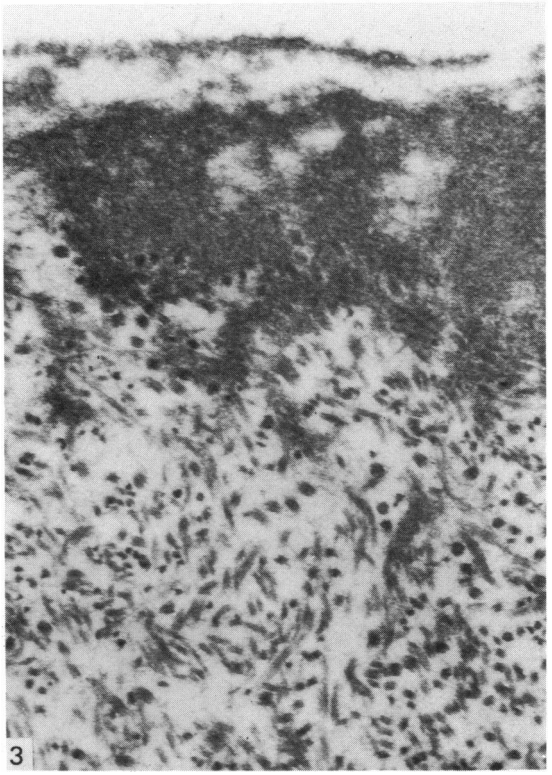

Fig. 3. Articular surface, operated joint, two months. Collagen fibrils are still widely spaced, and considerable amounts of electron-dense amorphous and filamentous material have infiltrated the superficial cartilage. × 44000.

Table 1. *Collagen fibre content in superficial matrix of operated and control tibial articular cartilage*

		Percentage of points overlying collagen fibres	
		Operated joint	Intact joint
All specimens (excluding sham-operated)	(n = 16)	64·2 ± 4·5 (t = 4·84, P < 0·001)	75·4 ± 8·1
One week (after cruciate ligament section)	(n = 5)	62·8 ± 3·9* (t = 6·91, P < 0·001)	79·1 ± 3·5
One week (after sham operation)	(n = 6)	80·6 ± 3·1* * Difference: t = 8·46, P < 0·001	78·4 ± 3·2

ficial collagen fibrils were abnormally widely separated although with no marked loss of orientation at first. These changes occurred as early as four days and persisted certainly until three months after operation.

The results of point counting confirmed the wider spacing of the fibrils (Table 1). In the operated group, the mean number of points lying over fibrils was reduced by 15 % compared with controls; conversely, the number of points overlying interfibrillar matrix had increased by more than 40 %. At one week, there was a significant difference between the results of cruciate ligament section and of sham operation, though not between sham-operated and the contralateral intact joints of the same

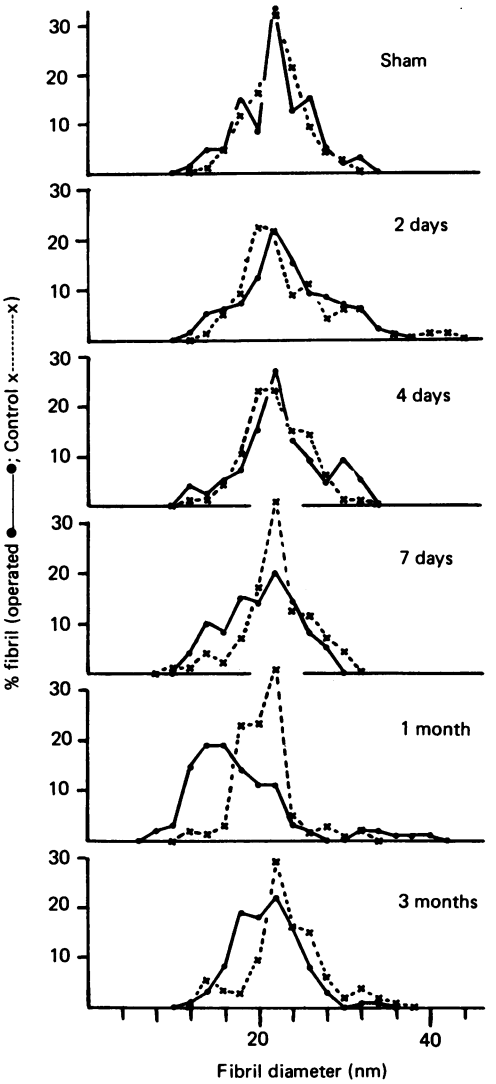

Fig. 4. Collagen fibrils in superficial articular cartilage of representative specimens. Diameter of the fibril population is narrower in operated than in control joints, from seven days after cruciate section.

animals. Diameters of superficial collagen fibrils (Fig. 4) ranged from 6–44 nm, with a peak at 22 nm. There were no changes in sham operations or until one week after cruciate ligament section. Thereafter, a shift towards a narrower fibril diameter occurred in operated joints and was most pronounced at one month.

Fissuring of the surface was common from about two months and by fifteen to eighteen months after operation the cartilage was markedly fibrillated (Fig. 5) with clefts running into the superficial one third of the cartilage thickness. The surface of the fibrillated cartilage was locally smooth between the clefts and the underlying collagen mesh closer packed than hitherto, with entangled fibrils lacking the normal tangential orientation.

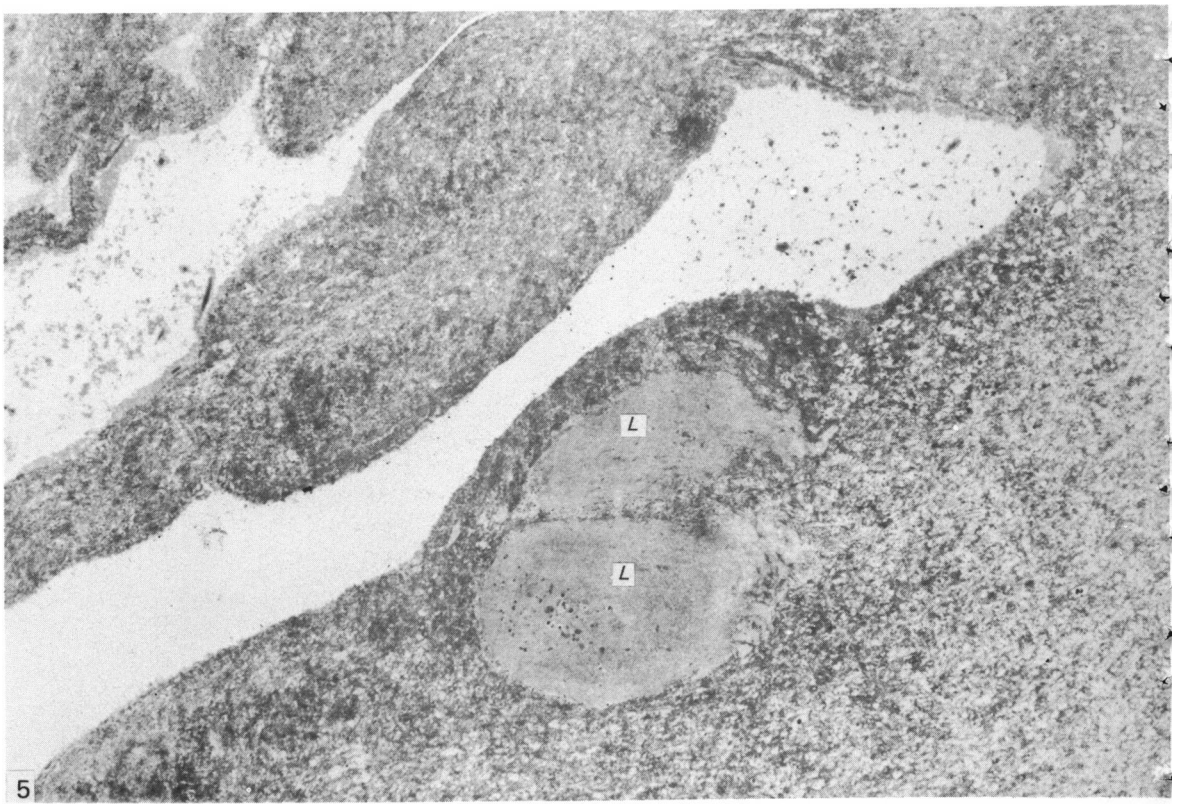

Fig. 5. Articular surface, operated joint, 18 months. The surface is irregular and fissured. Two areas ('empty' lacunae, *L*) previously occupied by living cells lie just beneath the surface. ×4000.

Superficial zone cells

In control tissue, most cells within about 0·2 mm of the surface occurred singly and were spheroidal rather than elongated or flattened. The cell lacuna contained finely-textured matrix without banded collagen fibrils; small membrane-bound dense bodies and granules lay scattered in the fibrillar matrix near the lacunar rim, but large masses of this material were rarely seen. The cells (Fig. 6) contained slightly crenated oval nuclei, glycogen, and small amounts of granular endoplasmic reticulum, Golgi membranes and mitochondria. In addition, 10 nm diameter microfilaments, myelin figures and lipid droplets were occasionally present.

In the first few months after operation, little change occurred in cell contour or in the lacunar matrix. In the cells (Fig. 7) pairs of granular endoplasmic reticulum membranes became more numerous though they were no more dilated than in controls, and Golgi membranes and secretion vacuoles were usually more profuse; in any one specimen, however, only one of these organelles (granular endoplasmic reticulum or Golgi) tended to be augmented. Intracellular lipid globules were more common and larger in operated than in control cartilage, an observation confirmed by staining frozen sections for lipid with Sudan black B. Where a large amount of glycogen was present in control chondrocytes, it seemed to be reduced, or broken up into smaller areas, in cells of operated cartilage. These changes were detectable from four days and maximal at one to three months after operation, but were not observed

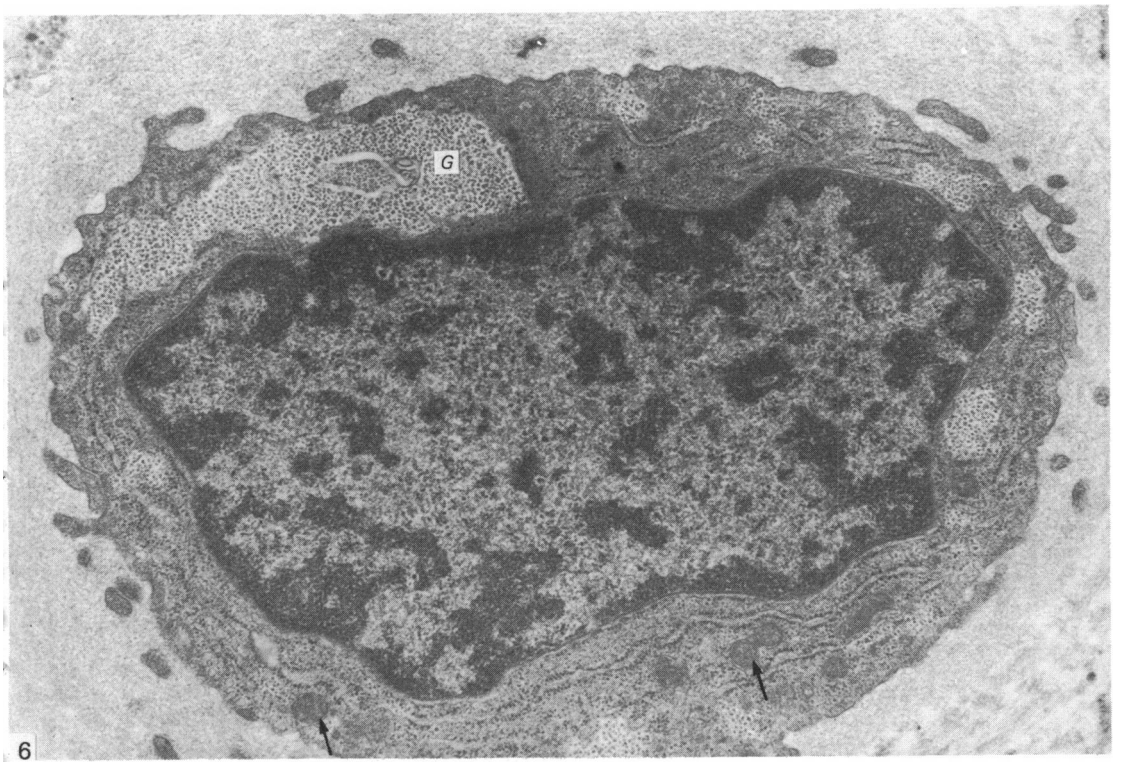

Fig. 6. Superficial zone chondrocyte, control joint, one week. Abundant glycogen (*G*), a few rough endoplasmic reticulum paired membranes and mitochondria (arrows) are present. In most sections of these cells, a small Golgi area is present but is not seen here. × 19000.

in sham-operated joints. Although mitochondria were sometimes less electron-dense, and myelin figures were more common, the overall picture in the first few months after operation was of active healthy cells. No mitoses were observed, although an occasional binucleate cell was found.

At fifteen to eighteen months after operation, the increase in granular endoplasmic reticulum and Golgi membranes persisted, but there was little difference in fat content between operated and control cells. The lacunae were enlarged, sometimes showing a concentric 'lamination' (Fig. 8), with larger numbers of matrix granules, 70 nm diameter, than in controls. Close to the fibrillated articular surface, 'empty' lacunae containing only a little cell debris were common, sometimes causing a hump in the surface contour (Fig. 5).

Middle zone cells

With increasing distance (0·2–0·6 mm) from the articular surface, control chondrocytes acquired a more irregular shape with deeply crenated nuclei. The nuclear fibrous lamina, the dense lamina on the internal aspect of the nuclear envelope (Fig. 7), was marginally thicker in superficial zone (29·8 ± 7·7 nm) than in middle zone (26·6 ± 5·0 nm, $t = 2{\cdot}24$, $P < 0{\cdot}05$) chondrocytes ($n = 50$) of intact joints. The Golgi membranes and secretion vacuoles were often more prominent than in superficial cells but the granular endoplasmic reticulum was more sparse. Glycogen and filaments were more abundant (Fig. 9), though variable in quantity. Lipid droplets

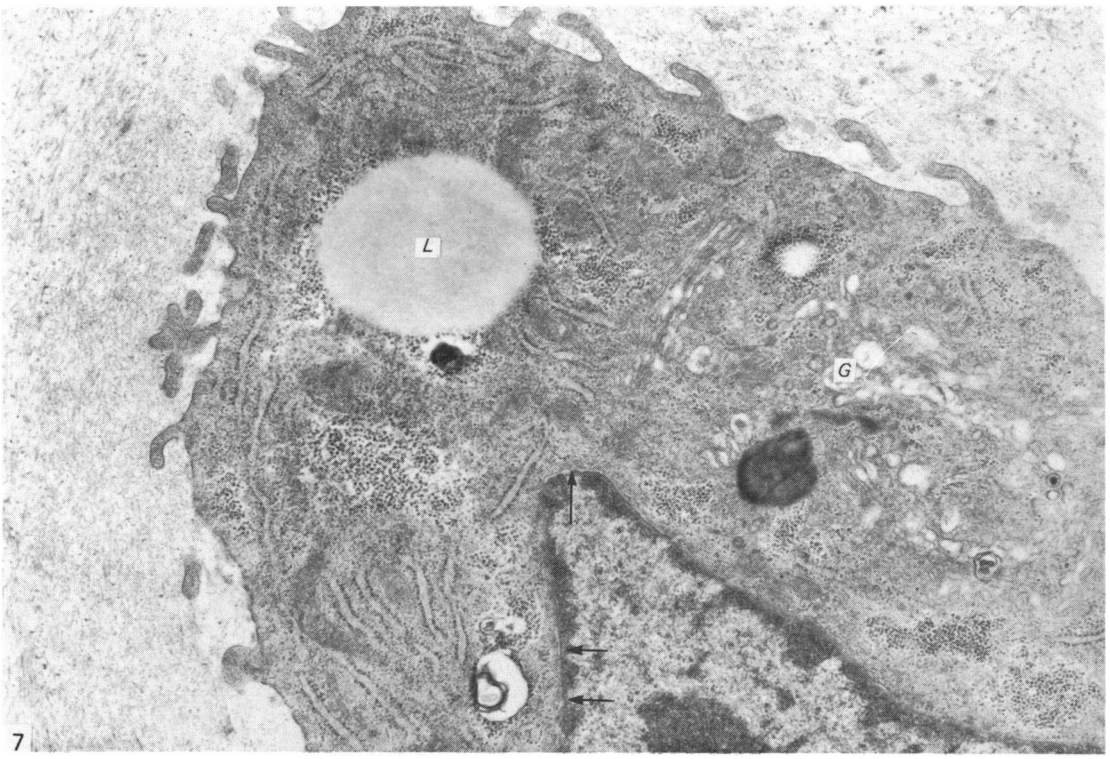

Fig. 7. Superficial zone chondrocyte, operated joint, one week. Glycogen areas are smaller, and the granular endoplasmic reticulum and the Golgi apparatus (*G*) are more prominent than in control cells. Lipid droplets (*L*) are common in cells from operated joints. Arrows indicate nuclear fibrous lamina. × 19000.

were occasionally present in middle zone cells. Cell lacunae were wider and collections of electron-dense membrane-bound and granular particles at the lacunar rim were more common.

Post-operative structural changes were first detected in middle zone chondrocytes at one week and most pronounced at one to two months, and they continued to be seen up to eighteen months after cruciate ligament section. The granular endoplasmic reticulum was much more abundant, with widely dilated cisternae (Fig. 10). Golgi membranes were more extensive and secretion vacuoles more numerous. Glycogen areas were reduced but little or no change in fat content occurred. Mitochondria seemed to be less electron-dense but otherwise normal; myelin figures and lysosomes were a little more numerous. Outside the cell, matrix granules and concentric lamination of the lacunar matrix were much more common. There was little evidence of cell degeneration and only an occasional binucleate cell was observed. The nuclear fibrous lamina was slightly thicker in cells of operated (28·9 ± 3·3 nm) than of control joints (25·3 ± 5·1 nm, $t = 2{\cdot}39$, $P < 0{\cdot}025$).

The middle zone cells of sham-operated joints were similar to those of controls.

Cellularity and lipid measurements

Taking the data from all 16 dogs with real-operated joints, the cell density of the superficial zone was 60 % higher than that of the middle zone in their control joints

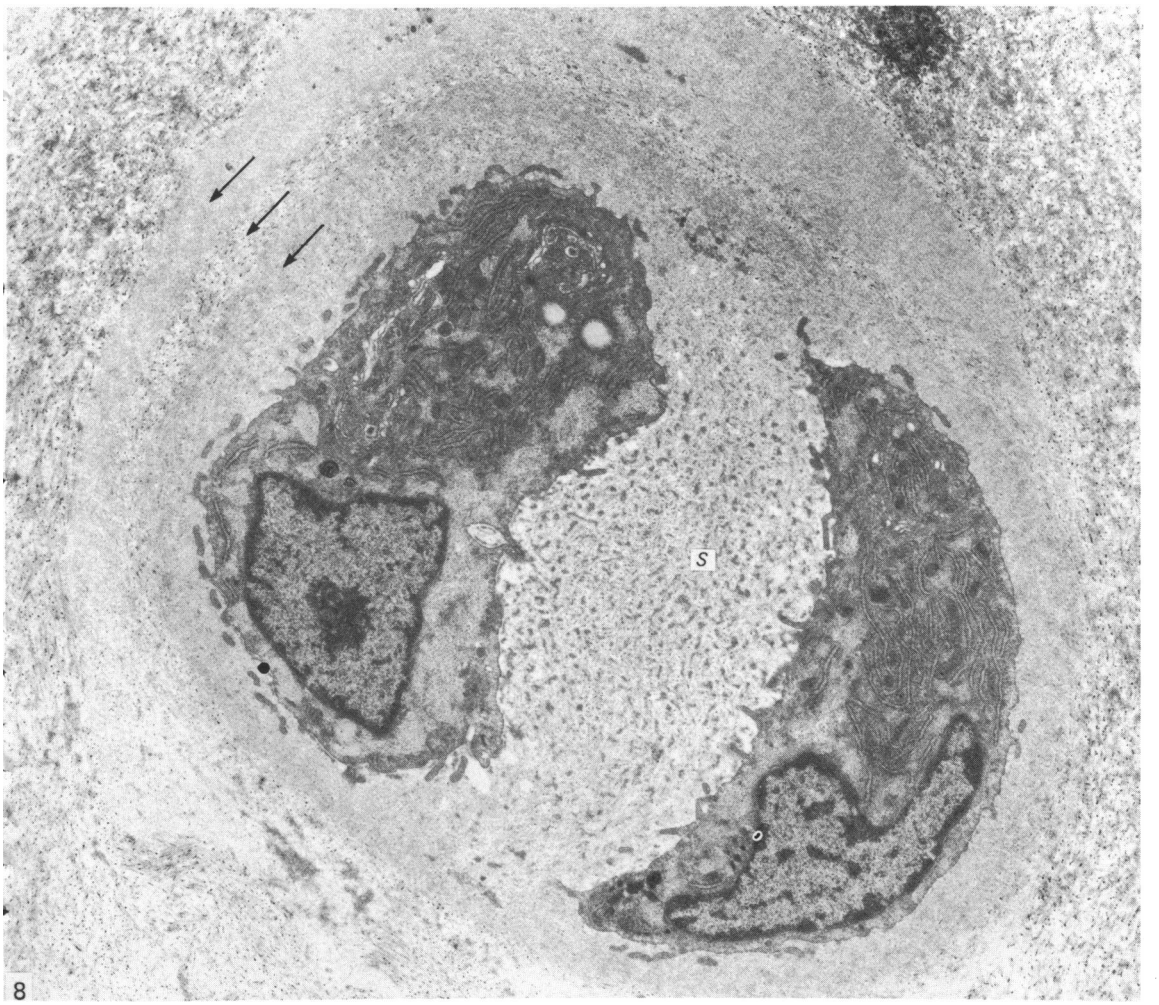

Fig. 8. Superficial chondrocytes, operated joint, 18 months. The cells contain profuse granular endoplasmic reticulum and Golgi membranes. Abundant secretion material (*S*) and matrix granules lie between and around the cells, where the matrix shows a laminated texture (arrows). ×9000.

but 82% higher in their operated joints, although there was no significant change in the middle zone (Table 2). However, if analysis were restricted to data obtained at one week after operation, there were no statistically significant differences between real- and sham-operated joints (Table 3A) or between sham-operated joints and their controls (Table 3B).

Intracellular lipid content was higher in superficial than in middle zone chondrocytes and although there were no differences between control and sham-operated joints (Table 3B), the mean value for operated joint cartilage was twice that of controls (Tables 2, 3A). No significant change occurred in the middle zone.

In control joint sections, differences were found between locally smooth and locally minimally fibrillated areas of the surface. In the superficial zone, intracellular lipid content was higher beneath fibrillated areas, compared with the tissue beneath smooth surfaces (Table 4). Again, no differences were observed in the middle zone.

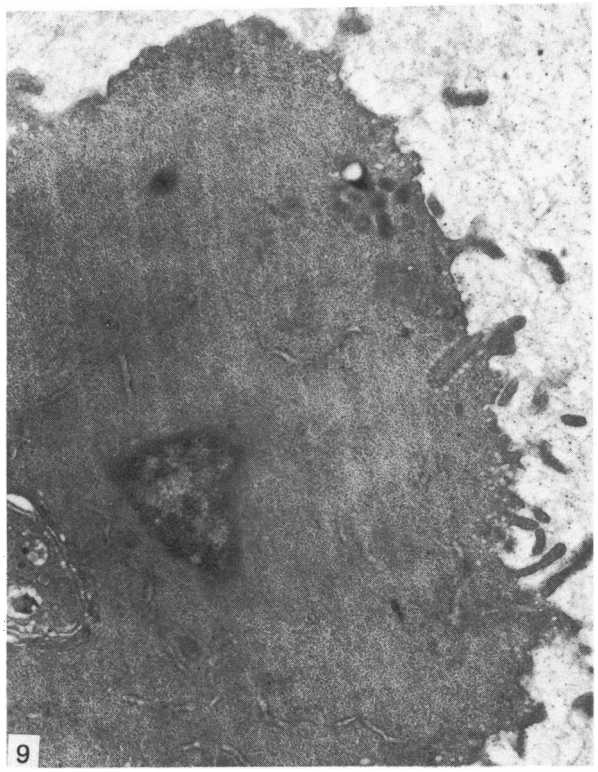

Fig. 9. Middle zone chondrocyte, control joint, one week. This cell is packed with small glycogen granules and few cytoplasmic organelles are present. ×12000.

Table 2. *Intracellular lipid and cellularity of operated and control tibial articular cartilage*

	Lipid/cell (μm^2)		Cells/mm^2	
	Operated ($n = 16$)	Control ($n = 16$)	Operated ($n = 16$)	Control ($n = 16$)
Superficial zone (0–0·175 mm)	0·89±0·65	0·39±0·31 ($t = 2·78$, $P < 0·01$)	642±144	540±112 ($t = 2·25$, $P < 0·05$)
Middle zone (0·175–0·53 mm)	0·26±0·20	0·20±0·09	352±62	336±62

DISCUSSION

In previous experiments, area A of the medial tibial condyle exhibited the earliest and most pronounced changes after cruciate ligament section. In control joints, this region appears to differ from typical normal articular cartilage elsewhere (Stockwell & Meachim, 1979). The articular surface may show minor fissuring, the superficial chondrocytes are spherical rather than flattened and discoidal, and the cell density of the superficial zone is lower than is commonly found. Possibly the greater vulnerability of area A to experimental cruciate ligament section is due to these pre-existing unusual features and/or there may be a greater susceptibility to mechanical stress inherent in the position of area A.

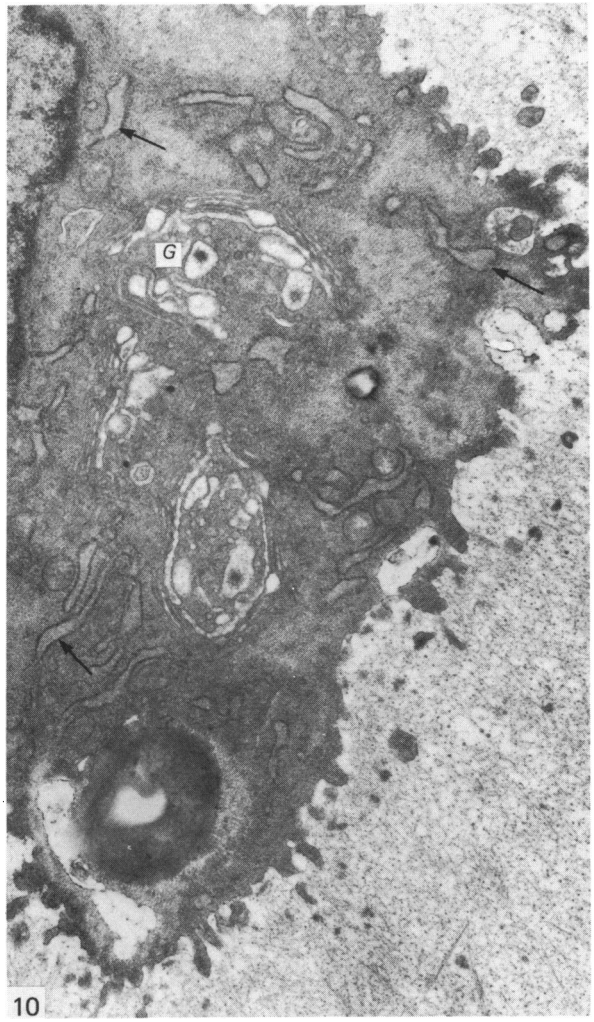

Fig. 10. Middle zone chondrocyte, operated joint, one month. Granular endoplasmic reticulum membranes with dilated cisternae (arrows), and the prominent Golgi complex (*G*) suggest enhanced synthetic activity in this cell. × 14000.

Abnormal distension of the collagen mesh seen after cruciate ligament section is presumably associated with increased hydration of the cartilage. The swelling pressure of proteoglycans is resisted by the collagen meshwork, and any loosening allows more water into the matrix. This is the explanation suggested for the increased water content of superficially fibrillated human articular cartilage (Maroudas, 1976). It would also explain the increased water content of dog tibial cartilage at three weeks and eight days after operation, determined, respectively, by analysis (Muir, 1977) and interference microscopy (Chayen, 1981, personal communication).

Ultrastructural studies of human cartilage show collagen fibrils to be unusually widely spaced beneath articular surfaces that are no longer intact when viewed in the electron microscope (Meachim & Roy, 1969). In the present experiment, splitting of the articular surface appears a few weeks after changes in the matrix.

Appearing almost simultaneously with increased interfibrillar spacing, the occur-

Table 3. *Intracellular lipid and cellularity of tibial articular cartilage one week after operation*

((*A*) Comparison of effects of cruciate ligament section and sham operation.)

	Lipid/cell (μm^3)		Cells/mm^3	
	Cruciate ligament section ($n = 5$)	Sham operations ($n = 6$)	Cruciate ligament section ($n = 5$)	Sham operations ($n = 6$)
Superficial zone (0–0·175 mm)	1·14±0·62	0·50±0·18 ($t = 2·46, P < 0·05$)	692±146	629±105
Middle zone (0·175–0·53 mm)	0·27±0·15	0·40±0·20	371±57	284±39

((*B*) Comparison of sham operations with their controls.)

	Lipid/cell (μm^3)		Cells/mm^3	
	Operated ($n = 6$)	Control ($n = 6$)	Operated ($n = 6$)	Control ($n = 6$)
Superficial zone (0–0·175 mm)	0·50±0·18	0·42±0·33	629±105	547±121
Middle zone (0·175–0·53 mm)	0·40±0·20	0·40±0·12	284±39	327±54

Table 4. *Intracellular lipid and cellularity underlying locally smooth and locally fibrillated surfaces of control tibial articular cartilage*

	Lipid/cell (μm^3)		Cells/mm^3	
Location in cartilage (distance from articular surface)	Smooth ($n = 8$)	Fibrillated ($n = 8$)	Smooth ($n = 8$)	Fibrillated ($n = 8$)
Superficial zone (0–0·175 mm)	0·37±0·17	0·67±0·27 ($t = 2·69, P < 0·02$)	553±66	606±27
Middle zone (0·175–0·53 mm)	0·29±0·15	0·36±0·27	338±38	306±54

rence of narrower collagen fibrils could be due either to splitting of existing fibrils, or to renewed fibrogenesis, because collagen synthesis is enhanced after cruciate ligament section (Eyre, McDevitt, Billingham & Muir, 1980). Similar causes have been postulated in early fibrillation of cartilage of the human knee where the superficial zone contains abnormally fine fibrils (Weiss & Mirow, 1972).

Changes in the collagen fibre meshwork could be due to mechanical or chemical factors. Sectioning the anterior cruciate ligament would change the mechanics of the joint, so causing stress fractures of the collagen. Alternatively, damage to soft tissues of the joint might release catabolic factors, demonstrable *in vitro* (Fell & Jubb, 1977), which appear to activate prostaglandin synthesis and enzyme cascade systems mediating partial degradation of collagen and/or its associated molecules (Meats, McGuire & Russell, 1980).

The ultrastructural changes seen in the middle zone cells and the increased numbers of matrix granules and the concentric lamination of the pericellular matrix, suggest that the synthesis and secretion of matrix macromolecules is increased. There

may be a new population of molecules synthesised, because those from operated joints have higher ratios of chondroitin sulphate:keratan sulphate than those from control joints (McDevitt & Muir, 1975, 1976). Hypertrophy of cytoplasmic organelles also occurs in human chondrocytes in early cartilage fibrillation (Weiss & Mirow, 1972).

What stimulates cellular activity is uncertain. Depletion of matrix proteoglycans stimulates synthesis of macromolecules (McElligott & Potter, 1960; Fitton Jackson, 1970), possibly mediated via the cell surface (Nevo & Dorfman 1972; Wiebkin & Muir, 1975). Loss of metachromasia in the superficial cartilage occurs after cruciate ligament section (Pond & Nuki, 1973; McDevitt, Muir & Pond, 1974), and could cause the cell reaction.

The increased cell density of the superficial zone noted previously (McDevitt *et al.* 1977) may be a transitory sequel to injury, because cell degeneration is a prominent feature in the later stages of the experiment.

Thickening of the nuclear fibrous lamina in middle zone cells may be associated with damage to the cartilage, as in torn menisci of human knees (Ghadially, Dick & Lalonde, 1980) and also in menisci of operated canine joints (Stockwell, unpublished observation). The function of the nuclear fibrous lamina is not known, however,

Although lipid globules are considered to be normal and constant inclusions in mature chondrocytes, the reasons why so much fat is stored in these cells have never been fully understood. In the present experiment, the early increase in ultracellular lipid of superficial cartilage of operated joints might be due either to a change in metabolism favouring lipid synthesis or to an increase in the supply of lipid precursors. Thus, it has been suggested that a significant part of the energy metabolism of normal dog cartilage is derived from the oxidation of fatty acids (Chayen, 1981, personal communication); any decrease in this activity, for example, in response to increased pentose shunt activity, should leave unused fatty acids to be stored as triglyceride. Although the amount of intracellular glycogen seems to be reduced after cruciate ligament section, no definite statement can be made until more is known about the intermediary metabolism of the chondrocyte.

Alternatively, an increase in the supply of fatty acids to the cell pool could come from various extracellular sources. Thus, lipid might be released into the synovial fluid either from damaged synovial membrane or from haemorrhage at operation, and be taken up by the chondrocytes. Indeed, experimental lipo-arthrosis causes loss of ground substance from the matrix of articular cartilage (Stockwell & Sprinz, 1981) and lipid accumulates in the superficial chondrocytes (Ghadially, Mehta & Kirkaldy-Willis, 1970) which take up fatty acids from the synovial fluid (Sprinz & Stockwell, 1976). Other factors may also be involved; increased hydration would imply greater cartilage permeability, and hence an increased flux of fatty acids to the chondrocytes from the synovial fluid, which normally contains a small amount of lipid (Bole, 1962). Cell degeneration could also provide a source of lipid within the matrix. However, cell death appears to be a late event, whereas increase in intracellular lipid occurs very early, and the amount of lipidic debris near the lacunar rim does not change significantly at early post-operative stages. Fatty acid involvement in collagen cross-linking has been suggested (Light & Bailey, 1980), and loosening of the bonds of the mesh might simultaneously release fatty acids. It seems unlikely that lipid is derived from autophagy of cytoplasmic lipoprotein membranes, because hypertrophy rather than autophagy of cytoplasmic organelles is most evident after cruciate ligament section.

Whatever the explanation, the changes seen after operation and also beneath areas of abnormal articular surface in the dog control joints suggest that the amount of intracellular lipid in superficial articular chondrocytes may be related to minor episodes of trauma or other types of lesion in the past history of the joint.

It should be noted that changes in articular cartilage following cruciate ligament section take place no earlier than the initiation of osteophyte formation at the articular margin (Gilbertson, 1975). Structural changes occur as early in chondrocytes themselves as in matrix and hence a purely mechanical cause of matrix changes and of the eventual degenerative fibrillation of articular cartilage may not be entirely valid. Sokoloff (1969) has pointed out that the joint is an internally interacting system which has to be regarded as a functional unit, and the degenerative process is unlikely to be focussed on one single factor.

SUMMARY

Ultrastructural changes in articular cartilage were studied in joint laxity induced by severing the anterior cruciate ligament of the right knee in sixteen mature dogs. The left knees provided controls; sham operations on six other dogs consisted of stab incision only, leaving the ligament intact. Cartilage from the medial tibial condyles was examined at intervals from two days to eighteen months later. In the superficial zone of the cartilage, collagen fibrils became abnormally widely spaced at four days, and narrower fibrils appeared from seven days after operation. Chondrocytes, particularly in the middle zone, became more active, with hypertrophy of cytoplasmic organelles detectable from four days. Superficial cells were initially healthy and became more numerous while their lipid content increased. The articular surface was fissured from two months and cell degeneration was rarely seen until several months after operation. These findings correlate with previous biochemical studies and are similar to early changes noted in degeneration of human articular cartilage.

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