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Molecular approach to the understanding of osteoarthritis

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Connective tissue has generally been regarded as being rather inert with little direct influence on metabolism because its function is mainly a mechanical one. It has also been assumed that apart from the effects of aging, the connective tissues of different individuals are much the same. Both these assumptions are incorrect.

Firstly, it is becoming clear that connective tissue is by no means inert and although the cells within it are quite sparse they are active in synthesizing the extracellular constituents of the tissue and are strongly influenced by their immediate environment. Thus changes in connective tissue not only affect its mechanical properties but also the metabolic behaviour of the cells within it. For example, depletion of the matrix of chick embryonic cartilage in organ culture stimulated the cells to make good the loss within 4 days (Fitton Jackson, 1970) and when 80% of the chondroitin sulphate had been removed by digestion with hyaluronidase, the total synthesis of proteoglycan was five times above control levels (Hardingham *et al.*, 1972). It is also notable that conditions of culture influence the types of proteoglycan (Hascall *et al.*, 1976) and of collagen (Cheung *et al.*, 1976; Gay *et al.*, 1976) that are synthesized.

Secondly, the articular cartilage of different individuals aged between 26 and 60 years has been shown to vary considerably in the levels and topographical distribution of collagen, chondroitin sulphate, and keratan sulphate (Muir *et al.*, 1969; Kempson *et al.*, 1973). Such differences appear to be characteristic of the individual irrespective of age or sex and are probably genetically determined, and may be a factor predisposing some people to develop osteoarthritis. Thus, among nearly 20 femoral cartilages analysed, the collagen content of the superficial zone from the same area was more than 90% of the dry weight at one extreme and less than 45% at the other (Kempson *et al.*, 1973). Load-

bearing joints have to withstand very high localized repetitive compressive loads (Freeman and Kempson, 1973) which even during walking have been calculated to be several times body weight (Walker *et al.*, 1969). It is therefore possible that those cartilages with high levels of collagen in the superficial zone may withstand the stresses of impact loading better than those with lower levels.

Although rubbery in consistency, human articular cartilage consists of about 70% water (Muir *et al.*, 1969). When a load is applied, fluid pressure within the cartilage rises immediately but water is driven out only slowly because the proteoglycans, which are hydrophilic compounds, impede the flow of interstitial water as they are entrapped in the collagen network and remain within the cartilage (Freeman and Kempson, 1973). The resilience or compressive stiffness of cartilage is therefore directly correlated with the proteoglycan content (Kempson *et al.*, 1970).

Proteoglycans

The polyanions of connective tissue, termed glycosaminoglycans, possess sulphate and carboxyl groups. Most features of their chemical structures were established before 1950, mainly by the classical work of Karl Meyer. It was thought that being strongly acidic, glycosaminoglycans such as chondroitin sulphate interacted with basic groups of proteins but were not bound to protein by covalent bonds. In most investigations, however, to obtain good yields of glycosaminoglycans the tissue was digested with proteolytic enzymes. On the other hand, although poorer yields were obtained when cartilage was extracted without proteolysis, the products appeared to be of higher molecular weight and gave viscous solutions. While investigating such preparations of chondroitin sulphate from cartilage (Muir, 1958), I found that even after careful purifica-

tion of the chondroitin sulphate, there remained about 10% of protein which had a characteristic amino acid composition, in which serine, glycine, proline, and glutamic acid were predominant. Proteolytic enzymes, such as papain, rapidly destroyed the viscosity of solutions of such preparations of chondroitin sulphate and after exhaustive proteolysis all amino acids were greatly reduced with the exception of serine which was almost entirely retained. I therefore suggested that chondroitin sulphate was attached to serine residues of a specific protein by covalent bonds and not merely associated with protein by electrostatic interactions as had been proposed. This idea was consistent with

results of physicochemical studies of Mathews and Lozaityte (1958) and of Partridge *et al.* (1961), who suggested that 50–100 chondroitin sulphate chains were attached at one end to a protein core to form a bottle-brush shaped molecule (Fig. 1) (now termed proteoglycan). This structure is now generally accepted and has since been modified only in detail and has been shown to be essentially correct from electron micrographs of single proteoglycan molecules (Rosenberg *et al.*, 1970).

Molecules of such structure would be of high molecular weight because each chondroitin sulphate chain has a molecular weight of about 20 000, and if 50–100 are attached to a protein backbone the resulting composite molecule or proteoglycan will have a molecular weight of 1–2 million (Luscombe and Phelps, 1967; Eyring and Yang, 1968; Rosenberg *et al.*, 1970). Cartilage proteoglycans are thus considerably larger even than fibrinogen or the tropocollagen molecule (collagen fibrils are composed of many molecules), and would therefore be entrapped in the collagen network. The mutual repulsion of multiple negative charges on the chondroitin sulphate chains ensures that proteoglycan molecules are fully extended in solution and occupy a large solvent volume. Being hydrophilic and immobilized because of their size, proteoglycans impede the flow of interstitial water when an external load is applied to cartilage. Thus the resilience of cartilage is explicable in terms of the molecular structure of proteoglycans.

The structure shown in Fig. 1 makes proteoglycans particularly vulnerable to proteolytic attack, because even when only a few peptide bonds are cleaved the whole molecule falls apart. This explains why the

Fig. 1 Diagram of cartilage proteoglycan molecule. HA = hyaluronic acid; CS = chondroitin sulphate; KS = keratan sulphate.

Fig. 2 Equilibrium density gradient centrifugation in caesium chloride. A Associative conditions Starting density usually 1.6 g/ml. Centrifuged at 100 000 g for 48 h at 20°C. B Dissociative conditions Lower fraction from associative gradient mixed with an equal volume of 7.5 M guanidinium chloride at pH 5.8. Starting density adjusted to 1.5 g/ml with caesium chloride. Centrifuged at 100 000 g for 48 h at 20°C.

release of proteolytic enzymes by inflammatory cells in the joint is particularly destructive and why after a single intravenous injection of papain rabbits' ears became floppy (Thomas, 1958). Proteoglycans in the ear cartilage became depleted after being attacked by papain because the resulting fragments were small enough to diffuse out of the cartilage supporting the ear, which as a result became flaccid because water was no longer held within it.

Aggregation of proteoglycans

A unique feature of proteoglycans is their ability to form multimolecular aggregates of very high molecular weight of the order of 50–100 million (reviewed by Muir and Hardingham, 1975). Such aggregates are phenotypic of cartilage, but until new dissociative procedures for the extraction of proteoglycans were introduced by Sajdera and Hascall (1969) this feature of cartilage proteoglycans was largely missed. With the use of dissociating solvents such as 4 M guanidine HCl or 2 M CaCl_2 , proteoglycans may be extracted in good yield without disruptive homogenization of the tissue which is otherwise necessary. Hascall and Sajdera (1969) were also the first to apply equilibrium density gradient centrifugation in caesium chloride to the purification of proteoglycans. Both innovations have enabled important advances to be made in the understanding of the structure of these complex molecules. These procedures are particularly suitable for handling macromolecules that are prone to irreversible changes that may be brought about by shearing forces, precipitation, or chromatographic adsorption.

When proteoglycans are extracted under dissociating conditions, such as with 4 M guanidine HCl, aggregated molecules are dissociated and on dialysis to low ionic strength they reassociate. The proteoglycans are then purified by density gradient centrifugation in caesium chloride when they separate at the bottom of the gradient from contaminating proteins (Fig. 2). In the analytical ultracentrifuge, such preparations contained a fast and a slower sedimenting component (Hascall and Sajdera, 1969). When subjected to a second density gradient centrifugation in caesium chloride under dissociative conditions in the presence of 4 M guanidine HCl aggregates become dissociated and the constituents of the aggregate separate at different buoyant densities, the proteoglycans going to the bottom of the gradient (Fig. 2). When examined in the analytical ultracentrifuge after this treatment followed by dialysis, the fast sedimenting component had disappeared and only the slower one remained (Hascall and Sajdera, 1969) indicating that aggrega-

Fig. 3 The proteoglycan-hyaluronic acid complex. The dimensions of the model were deduced from the stoichiometry of the interaction (Hardingham and Muir, 1972).

Fig. 4 Diagram of proteoglycan aggregation.

tion was not a simple self-association but involved other components that had been removed in the second dissociative density gradient. It was at first thought that the protein that separated at the top of the dissociative gradient, termed 'protein link', was responsible for aggregation (Hascall and Sajdera, 1969). In 1972, Hardingham and Muir found that dissociated proteoglycans interacted with hyaluronic acid in a unique manner, in which many proteoglycan

molecules became bound to a single chain of hyaluronic acid. Subsequently, when proteoglycan aggregates were dissociated, hyaluronic acid was identified in the middle of the second dissociative density gradient (Hardingham and Muir 1974a). Hyaluronic acid had not before been found in cartilage and it accounted for less than 1% of the total glycosaminoglycan. That aggregation depends on a highly specific interaction of proteoglycans with hyaluronate is now recognized, but as far as is known it is a property unique to cartilage proteoglycans. The interaction has been examined in detail by Hardingham and Muir (1972) by viscometry and gel chromatography, since it leads to a large increase in molecular size. From such studies and using published molecular weights for proteoglycan and hyaluronic acid, a model for the complex was deduced (Hardingham and Muir, 1974b) (Fig. 3) whose dimensions agreed quite well with those calculated from electron micrographs of aggregates (Rosenberg *et al.*, 1970, 1975).

The third component of the aggregate, the 'protein link' which separates at the top of the dissociative gradient, appears to stabilize the proteoglycan hyaluronate complex and prevent its dissociation (Hascall and Heinegard, 1974b; Hardingham and Muir, 1975). The hyaluronate binding region of the proteoglycan which has a molecular weight of about 90 000 (Heinegard and Hascall, 1974) possesses a tertiary structure that depends on 5–7 intramolecular disulphide bridges (Hardingham *et al.*, 1976). The inter-relationship of the three components of the aggregate is shown in Fig. 4. The function of aggregation is not known, however. It presumably helps to immobilize proteoglycans in the collagen network and it may protect them to some extent from proteolytic breakdown (Hascall and Heinegard, 1974a; Heinegard and Hascall, 1974). Not all the proteoglycan in cartilage is aggregated, however, or capable of interacting with hyaluronate, and the proportion varies in different types of cartilage and during development.

Hyaluronic acid itself, when free and not combined with proteoglycan, has an inhibitory effect on proteoglycan synthesis by cultures of chondrocytes from adult (Wiebkin *et al.*, 1975) and embryonic cartilage (Toole, 1973; Solursh *et al.*, 1974). Since it had no effect on cultures of fibroblasts (Wiebkin *et al.*, 1975) or chondroblasts (Kosher *et al.*, 1973) its effect appears to be specific to differentiated chondrocytes.

Osteoarthritis

Osteoarthritis is generally a slowly progressive destructive disease of joints, of varying severity that

is widespread in man and other species. As the disease is asymptomatic in its initial phases, it is detectable only at later stages when radiological changes have developed. The biochemical and cellular changes that have been recorded are those of the later clinically recognizable stages of the disease. Because the time of onset is not known, the initial events are virtually impossible to investigate except in experimentally induced models of osteoarthritis.

In our work, carried out by my colleague C. A. McDevitt in collaboration with Dr. E. Gilbertson of the Department of Veterinary Surgery, University of Glasgow, osteoarthritis was produced surgically by cutting the anterior cruciate ligament of the knee of the dog (Pond and Nuki, 1973). This produces instability in the joint that leads to biochemical (McDevitt and Muir, 1976) and histological changes in the articular cartilage and to the development of osteophytes at the joint margins (McDevitt *et al.*, 1977). These changes are indistinguishable from those of natural osteoarthritis in the dog, and moreover, whenever this ligament is ruptured accidentally osteoarthritis usually results (Tirgari and Vaughan, 1975). This experimental model of osteoarthritis has several advantages, the time of onset is known, the joint is not exposed as only a stab incision is made

Fig. 5 A diagram of the tibial surface of the right knee of a dog. The cartilage of the areas within the dotted lines (A and B on the medial condyle, C on the lateral condyle) was taken for analysis. Cartilage from corresponding areas of the left (control) knee was analysed.

Fig. 6 Percentage change ($OA/CON \times 100$) in water content of osteoarthrotic cartilage. Encircled figures represent grade of articular surface.

no foreign material is introduced into the joint and most important, the lesion appears in the same region of the tibial surface in every dog so that this area can be sampled before lesions have developed and the events leading up to the development of lesions followed. To circumvent variations between individual animals, the operation was performed on the right knee, the left serving as a control. Sham operations consisted of making a stab incision but leaving the ligament intact. Mature dogs of various breeds were used in which skeletal maturity and the absence of natural osteoarthritis was established by clinical and radiological examination (Gilbertson, 1975). The dogs were killed after various times from 1 to 48 weeks and the severity of lesions graded by Meachim's (1972) Indian ink staining method.

The tibial cartilage was divided into 3 areas (Fig. 5). Area A, which is not covered by the meniscus, was the site where lesions developed first.

The cartilage of the femur and patella were also sampled and here lesions tended to develop later. Deterioration of the cartilage surface and histological changes became progressively more severe with time and were first detectable in area A of the tibial surface as soon as one week after surgery (McDevitt *et al.*, 1977). There was a progressive deterioration of the cartilage surface which became frayed and then developed deep clefts, and cell density gradually increased. Cell lacunae with two or more nuclei appeared in all regions one week after surgery. These changes increased in extent and severity with time and were most pronounced in area A of the tibia. Sixteen weeks after surgery the cartilage matrix was highly cellular with clones of cells particularly abundant around severely fibrillated sites. Osteophytes were seen at the joint margins after 8 weeks (McDevitt *et al.*, 1977).

WATER

The earliest biochemical change was an increase in water content in area A of the tibia as well as in the femoral cartilage 3 weeks after the operation, although histological changes were minimal (Fig. 6). After 6 weeks the water content of areas B and C increased, and after 9 weeks particularly that of the femoral cartilage. However, with progression of the lesion in area A the water content in this area began to decrease. Since the proteoglycan content did not diminish until the tissue was severely fibrillated it suggests that either the collagen network had loosened or that the swelling pressure exerted by proteoglycans had increased. It is notable that in human osteoarthritis the cartilage is abnormally hydrated (Bollet and Nance, 1966; Mankin and Thrasher, 1975) and Maroudas (1976) has shown recently that human articular cartilage from areas surrounding fibrillated regions imbibed water and swelled when placed in normal saline, whereas normal cartilage did not. It therefore appears that increase in hydration of cartilage may be crucial in initiating the sequence of biochemical and histological changes which lead to osteoarthritis.

Despite the normal histological appearance of the tibial cartilage of area A 2 weeks after the operation, electron micrographs of the superficial region showed the collagen fibres to be randomly orientated and no longer approximately parallel to the surface. Compared with controls, the fibres were thicker and the spaces between them wider. These morphological changes are comparable with those observed in human osteoarthritis (Weiss, 1973). When proteoglycans were labelled *in vivo* with $^{35}\text{SO}_4^{2-}$, 24 hours and 48 hours before death, autoradiographs showed that the newly synthesized proteoglycan had migrated far away from the cells, whereas it was

still located near them in the control cartilage. Presumably this difference arose from the increased water content of the cartilage of the experimental joint since permeability of cartilage is related to water content (Maroudas, 1973). Increased water content may also partly explain why the total proteoglycan extracted from the cartilage of operated joints was greater than that from controls, a change that increased progressively with time (Fig. 6) and occurred throughout the cartilage of the joint even where lesions did not develop. In control cartilage only about half the total proteoglycan was extractable with 2 M CaCl_2 but in the operated joint the amount increased to 80–90% (Fig. 7). Similarly, in natural canine osteoarthritis about 85% of the total proteoglycan was extractable from cartilage of

the knee (McDevitt and Muir, 1974). This suggests that the proteoglycans were less effectively immobilized in the collagen network and that perhaps their association with collagen had also diminished. In the sham operated joints, however, there was no increase in water content or in the proportion of extractable proteoglycan.

PROTEOGLYCANS

The hexosamine in cartilage is largely attributable to glycosaminoglycans. Since chondroitin sulphate contains galactosamine and keratan sulphate glucosamine, molar ratios of these amino sugars provide an estimate of the relative proportions of chondroitin sulphate to keratan sulphate in the tissue. In all regions of cartilage of joints that had been operated on, irrespective of where lesions developed, there was a general increase in the molar ratio of galactosamine to glucosamine of the whole tissue which occurred soon after the operation (McDevitt and Muir, 1974, 1976; McDevitt *et al.*, 1977). In the extracted proteoglycans this change was even more pronounced. The extracted and purified proteoglycans from cartilage of control and operated joints were compared on dissociative density gradient centrifugation. They were distributed in the gradient in the same way and therefore had similar buoyant densities. About 75% of the proteoglycans separated at the bottom of the gradient in each case but those extracted from the cartilage of joints that had been operated on contained considerably more chondroitin sulphate relative to keratan sulphate than those from control cartilage. It therefore appeared that in the course of a few weeks a considerable proportion of the pre-existing proteoglycan in the cartilage had been replaced by proteoglycans that contained less keratan sulphate and in this respect resembled those of immature cartilage. In human osteoarthritis the proportion of keratan sulphate relative to chondroitin sulphate decreases in much the same way (Benmaman *et al.*, 1969; Mankin and Lippiello, 1971). The proteoglycans were more easily extracted and therefore they appeared to be more loosely associated with collagen compared with the proteoglycans of control cartilage (McDevitt and Muir, 1974, 1976). None of these changes occurred, however, in sham operated joints (McDevitt *et al.*, 1977).

In the analytical ultracentrifuge, the purified proteoglycans from the cartilage of operated joints consisted of a smaller proportion of aggregates and both aggregates and monomers were more polydisperse than corresponding molecular species from control cartilage. Preliminary results suggest that in human osteoarthritis of the knee aggregated proteoglycans were not detectable (McDevitt and

Fig. 7 Extraction of proteoglycans from tibial cartilage with 2 M CaCl_2 . The ordinate represents the proportion of the proteoglycans extracted expressed as a percentage of the total hexuronate. Encircled figures represent grade (Meachim, 1972) of articular surface.

Muir, unpublished results), in agreement with the results of Bayliss on osteoarthritis of human femoral heads (Bayliss, 1976).

Loss of aggregation could be due to three factors: reduction or absence of hyaluronate on which aggregation depends; the loss of the ability of proteoglycans to interact with hyaluronate; and the loss or inability of the 'protein link' component to stabilize the proteoglycan-hyaluronate complex. The reduction or absence of aggregates would not only affect the mechanical properties of cartilage but might also influence its metabolism. Hyaluronic acid has an inhibitory effect on the synthesis of proteoglycan by chondrocyte cultures when not combined with proteoglycan (Wiebkin *et al.*, 1975). If aggregation were reduced because of the inability of proteoglycans to interact with hyaluronic acid then uncombined hyaluronate in the tissue would increase.

COLLAGEN

Collagen accounts for the major proportion of the dry weight of cartilage and its contribution to the mechanical and physicochemical properties of the tissue have already been touched on. Cartilage contains type II collagen (Miller and Matukas, 1974) which is a type unique to hyaline and elastic cartilages (Eyre and Muir, 1975) and intervertebral discs (Eyre and Muir, 1974, 1976). It has been claimed that there is a switch in collagen synthesis from type II to type I in osteoarthritis (Nimni and Deshmukh, 1973). Since type II collagen is phenotypic of cartilage, this suggests a reversion to a less differentiated state. Collagen synthesis in experimental osteoarthritis was examined *in vivo* using as a precursor [^3H]-proline given by intra-articular injection 8 weeks after the operation (Eyre *et al.*, 1975). Collagen synthesis was determined by the formation of labelled hydroxyproline and the type of collagen synthesized by the isolation of specific peptides produced by cyanogen bromide cleavage of collagen (Eyre and Muir, 1974). In control cartilage after extraction of proteoglycans, collagen synthesis accounted for only a few per cent of total protein synthesis, whereas in the cartilage of operated joints 10 weeks afterwards, it accounted for more than half. All the newly formed collagen, however, was type II, and no type I collagen could be detected by chemical methods (Fig. 8). Nevertheless, with the use of fluorescent antibodies specific for each type of collagen, type I collagen has been detected in human articular cartilage in advanced stages of osteoarthritis as fluorescence within and as halos around the cells that had undergone cell division and formed clusters near deep clefts in the cartilage (Gay *et al.*, 1976). Fluorescence due to

Fig. 8 Chromatography on CM-cellulose of CNBr-peptides of collagen from dog articular cartilage labelled *in vivo* with ^3H proline.

antibodies to type II collagen was seen, however, throughout the cartilage matrix, and hence this evidence does not indicate that in osteoarthritis type II collagen is replaced by type I collagen as a structural component of the matrix. The reduced tensile strength of cartilage in osteoarthritis in visibly normal areas contiguous to lesions (Kempson *et al.*, 1973) must therefore be due to some other factor.

Osteoarthritis is more prevalent with advancing age. Age itself may be one factor in its genesis since a reasonable correlation with age and fatigue failure to cyclical loading of articular cartilage has been shown (Weightman, 1976). An important factor may, however, be the change in collagen-proteoglycan interactions reflected in the increase in extractable proteoglycans in osteoarthritis. This may reduce the tensile strength of collagen, which in turn could hasten fatigue failure.

General conclusions

The experimentally induced osteoarthritis that we have studied reproduces the changes in the joint and the biochemical changes in the cartilage that are seen in naturally occurring canine osteoarthritis as summarized in the Table. These changes are similar to those observed in human osteoarthritis (Mankin, 1976). The experimental model enables the early events to be studied so that a chronological sequence may be established. Changes occur first in the areas where lesions will later develop, they then

Table *Similarity of natural and experimental canine osteoarthritis*

Cartilage	(1) Location of lesions (2) Macroscopical appearance of lesions (3) Histology (4) Progression in severity (5) Biochemical changes in cartilage
	Cartilage softens and holds more water Proteoglycans more extractable synthesis increases composition changes aggregation reduced
Bone	Osteophytes
Meniscus	Rupture of medial meniscus
Synovial membrane	Discoloration and vascular proliferation

spread to areas immediately surrounding lesions and finally the entire cartilage of the joint is involved even where lesions do not develop.

In the present experimental model of osteoarthrosis there appear to be three phases. *Phase 1* (1–3 weeks after surgery). Minimal histological changes take place and only area A of the tibial cartilage and a focal area of the femoral cartilage are affected. The changes include increased water content and extractability of proteoglycans and increased molar ratio of galactosamine: glucosamine of the tissue.

Phase 2 (3–12 weeks after surgery). Erosion of the cartilage of area A develops and is accompanied by some loss of water and proteoglycans from this region. Elsewhere the changes that are localized in the first phase spread to all the remaining cartilage of the joint.

Phase 3 (12–48 weeks after surgery). Severe erosion of focal sites now develops and in these sites water content decreases and there is a marked loss of proteoglycan. Throughout the remaining cartilage the changes seen in phase 2 becomes more marked, particularly the increase in extractability of proteoglycans. Only in the third phase would the disease be recognized by its pathology or radiology as osteoarthritis.

Osteoarthritis is a particularly difficult disease to study experimentally, but as it occurs in many species, unlike rheumatoid arthritis, it is possible to study the genuine disease in animals. An opportunity thus exists to search for drugs that may delay its progress or even prevent the disease, although it is extremely unlikely that at advanced stages it is reversible to any significant extent. Because it is such a widespread disease, the benefit to the community would be enormous if its progress could be arrested or even delayed when clinical signs are first noted. The drugs that are at present prescribed for osteoarthritis have been developed for the treatment of entirely different diseases. Whether they are of real benefit to patients

is questionable and some may even be harmful particularly if given for long periods of time. To develop drugs specifically for osteoarthritis therefore presents a challenge to the pharmaceutical industry, but the thinking that has gone into the development of antirheumatic drugs should be discarded.

In its initial stages osteoarthritis appears to involve profound changes in the metabolism of cartilage cells set in train by increased hydration of the tissue; only at advanced stages does it become degenerative. At what stage the metabolic changes are reversible is a question of crucial importance in understanding the disease process and in attempting to develop effective therapeutic measures.

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