Wellcome Film Project

The Biochemistry of Connective Tissue in Arthritic Disease

The Scientific Basis of Medicine

Presented by Dr Helen Muir, The Kennedy Institute of Rheumatology.

University of London Audio-Visual Centre, 1976.

Polarographic strain apparatus by courtesy of Mr M Tuke, Biomechanics Unit, Imperial College.
Produced by David R Clark.

Made for British Postgraduate Medical Federation.

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Duration: 00:43:55:06

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<**Opening credits**>

<**Dr Helen Muir to camera**>

The purpose of this talk is to try and explain what is currently known about the biochemistry of connective tissue, particularly cartilage, so that we may gain some insight into what may be happening in arthritic diseases. In these diseases, cartilage is one of the first tissues to be attacked.

Now, the definition of connective tissue is the supporting parts of the body and these include such tissues as bone, tendon, skin, cartilage, blood vessels. And they are characterised by containing rather few cells embedded in a matrix. Nevertheless, the quality of different types of connective tissue are obviously very different.

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<Muir over table showing different qualities of connective tissue, then shown seated by display board with tables. Camera moves between her and tables>

And these differences in quality are achieved, first of all, by changing the relative proportions of different constituents. The fibrous extracellular material is protein collagen and this is embedded in amorphous material which contains many proteins, the most important of which are a class of large glycoproteins called proteoglycans. Now, the relative proportion of collagen and proteoglycans changes in different tissues. In bone and tendon there is much more collagen than in other types of connective tissue. In cartilage, there is much more proteoglycan than in tendon and it is, in fact, the richest source of proteoglycans. Skin, on the other hand, is in between bone and cartilage. And, clearly, the properties of tissues are defined largely by the relative amount of collagen and other material that is present. One also has to consider the actual types of macromolecule which are present in connective tissue.

To begin with, I'll deal with collagen. There are, in fact, so far four types of collagen that have been identified. Type IV collagen is found only in basement membrane and I shan't be dealing with that. But the other three types, I, II, and III are found in most connective tissues as shown in this picture. The collagens differ from each other in amino acid sequence and the most common type of collagen is type I, which is found not only in bone and tendon but skin and blood vessels. And type III collagen is restricted to skin and blood vessels. Cartilage, however, has an exclusive type of collagen, type II, and this is not found in any other tissue.

<Muir over illustration of cross section of human finger joint>

Now, cartilage is clearly a very important tissue in joints and my talk is really going to try and deal with, first of all, its biomechanical properties and also its biochemistry to try and explain how it functions.

Now, this is a cross-section of a finger joint of a human hand. The two larger areas on either side are the bone, and this is covered by a small covering of cartilage, and

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the light area between the two sides is the joint space. Now, cartilage has a sort of stiff rubbery consistency and it is designed by nature, first of all, to provide a smooth gliding surface so that the bones can slip over each other. But it also has a very important function in distributing stresses over a larger area of the bone when force is applied, in other words, when the bone is bearing a load.

<**Muir over animated plastic models of the human femoral head**>

This effect of cartilage on bone is shown very nicely in a plastic model of the human femoral head. This is photo-elastic material and it is illuminated with polarised light. Lines of stress are shown by interference fringes. Now if the bone is covered with cartilage, the stresses are distributed over the whole of the femoral head, whereas when there's no cartilage, the stresses are concentrated. When this model is applied, a load is applied and it presses against the acetabulum at the top; you can see the lines of force developing – that is loaded, unloaded, loaded, unloaded. The cartilage in this case is a piece of rubber which has the same thickness as cartilage and the same consistency.

Now, the other model, there is no rubber at the top and it's being pressed on by the acetabulum, just in that small area where lines of stress are developing at the top. So that the small contact area is having to carry the entire load on that hip. The result of this that, in life, such concentrated stresses produce micro fractures in the subchondral bone and these eventually lead to changes in the shape of the bone underneath. Just to come back to the models here, on the right, which is without the cartilage in place – that's unloaded, loaded, unloaded and then loaded. If you compare the left and the right-hand side you can see very clearly that without cartilage present the stresses are concentrated.

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<**Muir over: slide showing diagrammatic representation of cartilage; animated graphic representation of proteoglycan molecules; electron micrographs of cartilage from human knee**>

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Now, cartilage can be regarded as a collagen net filled with proteoglycan. Now this is clearly just diagrammatic, but here we have the collagen fibres as those rods and the blobs in between are proteoglycan molecules. And they have the property of being deformable, they can change in shape, but they are too big to actually move through the collagen network and this is a very important function that they have in cartilage.

The graphic representation of this is shown here and we have just a net without any force applied. Now, when this is loaded, the net depresses in the middle and some of the water that was held in the interstices of the net is expelled temporarily. When the force is released then the water will go back again. Whether this is true or not has been rather nicely illustrated in a scanning electron micrograph of cartilage taken from the mid zone of a human knee. This is from a paper by McCall. Now in the mid zone the collagen fibres of the cartilage are randomly oriented. At the surface they are parallel to the articular surface. But deep down they are random.

Now this shows the unloaded cartilage. Under load the fibres become oriented sideways. The load is from the top downwards. And you can see, going back to the first picture, that's the unloaded specimen, and that is loaded. This is scanning electron micrographs. That's loaded and unloaded. So the conclusion that one gets a network of collagen fibres being changed in orientation under load appears to be a true description of what is occurring.

Now, under load one should, theoretically, get some expression of water from the tissue.

<**Muir over graphs showing details of water expression from cartilage**>

And this graph is taken from a paper by Maroudas in which the amount of water expressed from cartilage under a load is shown by the graph that increases, labelled expression, and then when the load is removed, the water immediately re-imbibed by the tissue. And these two curves are inversely related to each other.

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Well, the problem arises is what happens: the water comes out but no proteoglycan is expressed at the same time because it's entrapped in the collagen network. Now, cartilage contains 70% water so this is a very unusual tissue, it is certainly not a sponge because very little water is squeezed out under load, but it is an extremely resilient tissue and it is thought that this is achieved by the proteoglycans which are immobilised in the collagen network.

Now, this graph shows, this is derived from a paper by Maroudas, and the bottom of the graph shows one to four, which are sections of cartilage cut from the articular surface at the left. And each line represents, first of all, the triangles of proteoglycan, the squares are collagen and hydraulic permeability is shown in circles. If the supposition is correct that proteoglycans arrest or impede the flow of interstitial water, then hydraulic permeability, which is a measure of water flow, should be inversely related to proteoglycan as, indeed, is shown in this graph. It does not appear to have very much correlation with collagen content.

<**Muir to camera, then over graphs showing correlations between collagen content and cartilage stiffness**>

So one can conclude, therefore, that one important function of cartilage is to distribute the load over the surface of the subchondral bone and that its resilience depends on the restriction of movement of water and that this is achieved by entrapping the proteoglycans in the collagen network. Now, if this conclusion is correct, one should be able to show that the stiffness of cartilage is directly related to the proteoglycans that it contains.

And in this graph, which is taken from a paper by Kempson and myself, we analysed human femoral cartilage for proteoglycan content and measured its compressive stiffness – in other words its resilience to a compressive load. And there is, as you can see, a good correlation with the proteoglycan content; in fact, the partial relation coefficient was as high as 0.85.

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A similar graph was also drawn of the collagen content of the cartilage and compared with compressive stiffness and there was clearly no correlation with collagen content. So that collagen, in fact, does not correlate with compressive stiffness. It is, however, important for tensile stiffness. Now, this graph shows the tensile stiffness and collagen content and there is, again, a good correlation with tensile stiffness and collagen content.

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The circles show normal cartilage. The dotted line is the average of the same correlation of areas of cartilage taken from joints that had osteoarthrosis, but from areas that were apparently normal; in other words, not where the lesion was, but surrounding tissue. And although the collagen is at least as much as that in normal cartilage, it is very much weaker in tension. And the reason for this is not fully understood.

<**Muir to camera, then over slides showing structure of cartilage proteoglycans molecules**>

So that one can therefore conclude that proteoglycans have a very important role to play in cartilage and so does collagen and that these two constituents have really opposite roles. One is to control tensile stiffness which is collagen, and the other compressive stiffness.

So much for the biomechanical function of cartilage. I now want to turn to the proteoglycans and their molecular structure.

This is a model of the current view of the structure of cartilage proteoglycans. These are rather unusual molecules and those in cartilage are unique and distinct from those in other connective tissues. The molecule has a protein core down the middle and tied to this are chains of chondroitin sulphate and keratan sulphate. Now these are sulphated polysaccharides which belong to a class of compound known as glycosaminoglycans. The protein core has a molecular weight in the order of 200,000

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and each chondroitin sulphate chain has a molecular weight of about 20,000. The keratan sulphate chains are shorter. The distribution of chondroitin sulphate and keratan sulphate is not entirely random but there is, in addition to this area of the molecule, a globular end which interacts with hyaluronic acid and this I'm going to discuss in a moment. The point about this molecule is that it looks like a bottlebrush with the carbohydrate chain sticking out all around it and, therefore, it occupies a very large volume of solvent when it is in solution.

The overall molecular weight varies between one and five million. And all this data this model was based on, entirely chemical data. However, in 1970, Rosenberg published an electron micrograph of proteoglycan and, as you can see, this rather closely resembles the model that I showed before. This is a negatively stained picture and it only reveals the carbohydrate chains, not the protein part of the molecule. But clearly the overall shape and dimensions of this molecule is very similar to the diagrammatic representation shown here.

<**Muir over graph comparing size of proteoglycans with other proteins; to camera; over graph showing effect of proteolytic enzymes on proteoglycans**>

Now, the relative size of proteoglycans compared with other proteins is shown in this picture. They are very large molecules, for example, the largest constituent of plasma is fibrinogen and that looks quite small compared with proteoglycan. The collagen molecule shown here is clearly not the fibrous filament, but the single tropocollagen molecule which self-aggregates to form the filaments. So compared with the soluble tropocollagen, cartilage proteoglycans are even larger.

The integrity of proteoglycans is clearly very important in maintaining the normal properties of cartilage and a molecule shaped as that one I showed in that diagram is very vulnerable to proteolytic attack. One has a core protein down the middle and even one or two peptide bonds, if they were broken the whole of this molecule would fall apart and its properties would change grossly and it would then be able to diffuse through the collagen network. To try and show that this is, in fact, a true representation of what might happen in vivo, we made a solution of proteoglycan and

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presented it with proteolytic enzymes and the effect on the viscosity is shown on this graph here.

If one has a very asymmetric molecule it will obviously be viscous, it's large and it's asymmetric and it will not flow through a constriction with any ease. In other words, it will produce a solution of high viscosity. Now when papain was introduced, within five minutes the original viscosity, measured as flow time, was decreased by 80%. The top curve is heat-inactivated enzyme and clearly there was no effect. Now, papain, of course, is not a natural protease in animals but cathepsin D has been identified in cartilage, and this is taken from a paper by Dingle, and the relative viscosity is plotted against time. And again, here, the cathepsin D reduced the viscosity of a solution of proteoglycan, Now, when the molecule is broken up into smaller pieces, it will clearly lose quite a lot of its viscosity because the molecules are smaller and they can therefore pass through the constriction more readily. The small amount of viscosity left behind, just shown by the arrow, is that due to the fragments.

<**Muir to camera**>

In inflammation, the inflammatory cells release a whole host of proteolytic enzymes which can attack the proteoglycan and the fragments are leeched out into the circulation. The cartilage is then more permeable to enzymes and these can then get in and actually attack the collagen fibrous network with the result that the cartilage is totally destroyed in the end, and this happens in rheumatoid arthritis and also in the later stages of osteoarthrosis.

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I now want to turn to a very peculiar property of proteoglycans of cartilage. These are unique amongst this class of proteoglycans in that they can form very large molecular aggregates, multi-molecular aggregates with molecular weights up to 100 million or more. The phenomenon of aggregation was first identified by Hascall and Sajdera in 1969, and they showed that in 4 molar guanidine, proteoglycan aggregates could be dissociated and if the dissociation products were placed on a

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caesium chloride density gradient and run in the ultra centrifuge, the constituents of the aggregate separated from each other. The proteoglycans went down to the bottom of the density tube and at the top there was a protein component, named protein link, which they thought caused the aggregation. However, T E Hardingham, in my laboratory, showed that, in fact, aggregation depended on the interaction of proteoglycans with a very small amount of hyaluronic acid which is present in cartilage. It had not previously been found there and we spent considerable effort to identify it and it counts for less than 1% of the total uronic acid in the tissue.

<**Muir over electron micrograph of proteoglycan aggregates, then over graphs showing relative viscosity of proteoglycan aggregates plotted against hyaluronic acid concentration**>

Now, before hyaluronic acid was identified in cartilage, an electron micrograph had been published by Rosenberg's group of proteoglycan aggregates. This is shown here. Now, this electron micrograph is at a magnification 5 times less than the electron micrograph of the single proteoglycan molecule which I showed previously. Here, the arms represent single proteoglycan molecules and not the carbohydrate side chains. The overall dimensions are shown here and the molecular size is, clearly, very large indeed. We proposed that those proteoglycans sticking out each side were held together by interacting with a single chain of hyaluronic acid represented there by the white line down the middle. This is a drawing superimposed on the electron micrograph of the aggregate.

Now, if one has an interaction of this sort, and your chain in the molecular size from a few million to many millions, the effect on the physical properties will be very great and we were able to show that hyaluronic acid and proteoglycans interacted in the way we thought using viscometric methods. By increasing the relative amount of hyaluronic acid and proteoglycan, one could get a measure of the maximum change in the relative viscosity as the hyaluronic acid proportion increased.

This graph shows that at 1%, that is the hyaluronic acid concentration in the mixture at the bottom, at 1% there is almost maximum effect on the viscosity so that this

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gives you a measure of the stoichiometry of this kind of interaction. We have also shown that in interacting with hyaluronic acid, one can show a change in molecular size by gel chromatograph, gel permeation chromatography or gel chromatography.

This graph shows the elusion of single proteoglycan molecules from Sepharose 2B column. The void volume of the column is shown at V0 and most of the proteoglycan penetrates into the gel and is eluted as a Gaussian distribution. Proteoglycans are not, by any means, homogeneous in molecular size and that Gaussian distribution represents the range of molecular sizes which this particular preparation contained. Now, when it has interacted with hyaluronic acid, the proteoglycan is shifted now into the void volume and most of it has interacted. The equation at the top of that represents what we think is happening. Many proteoglycan molecules in the left interact with a single molecule of hyaluronic acid to make a very large composite molecule.

This can be shown, also, by comparing the effect on gel chromatography of increasing proportions of hyaluronic acid. If one measures proteoglycan and hyaluronic acid in a weight ratio, again the maximum effect on the elusion profile is obtained when the proportion of proteoglycan is 100 to 1, that is, only 1% of hyaluronic acid is there. Now, this would fit the proposal that we made that many […]

<Muir over table summarising the way in which proteoglycans interact with a single chain>

[…] proteoglycans interact with a single chain, as summarised here. There are many, we can't say how many because this would depend on the length of the hyaluronic acid chain, and hyaluronic acid is not at all uniform in length in different tissues. The binding is not an entanglement phenomenon because it occurs at very low concentrations of hyaluronic acid, nor is it strictly electrostatic and it is extremely specific to hyaluronic acid, no other comparably similar polysaccharide, isomeric polysaccharide, will take the place of hyaluronic acid. And it appears that there is only a single site on each proteoglycan molecule for interaction with hyaluronate. In

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other words, they cannot cross link and form a gel by interacting with more than one molecule of hyaluronate.

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<**Muir over diagrams showing stoichiometry of proteoglycan hyaluronic acid reaction** >

Using both viscometric and gel chromatographic methods, we were able to produce the stoichiometry of proteoglycan hyaluronic acid interaction and proposed a model for the complex with overall dimensions shown in this picture here. The length is about 1200 nm and the diameter about 300 to 400 nm so the overall size of this complex is very large. The space between single proteoglycans is between 20 and 50 nm. Such a large molecule clearly would be entrapped in the collagen network, even more than the single proteoglycan. We don't really know at this time, however, exactly what the function of aggregation is. The link protein which separates the top of the gradient interacts both with proteoglycan and hyaluronic acid. Now this diagram shows the complex by itself, it is superimposed on the other, and the binding region of each proteoglycan is shown by that hook. Now we know from viscometric and gel chromatic methods that this equation, proteoglycan plus hyaluronate to the complex on the right, the equilibrium is very far on the right-hand side. Nevertheless, it is an equilibrium and if one removes proteoglycans one at a time then it will dissociate towards the left. The function of the link protein, which interacts both with the hyaluronic acid and the proteoglycan, is to lock the complex and prevent the equilibrium from going backwards.

This is shown diagrammatically here. The triangles are the link protein, which is a relatively small globular protein. The proposal that it stabilises the complex was shown in several experiments by Hardingham and I have illustrated it in one example here.

<**Muir over graphs showing changes in viscosity of proteoglycan hyaluronic acid complex**>

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This graph shows a change in relative viscosity when solutions of the proteoglycan hyaluronic acid complex, the aggregate and the proteoglycan monomer were heated from 20 to 60 degrees. The top curve, shown with squares, shows the change in viscosity when the proteoglycan hyaluronic complex was warmed. And you can see there is a gradual reduction in relative viscosity up to 60 degrees, implying that the equilibrium between proteoglycan complex and the dissociation products is being shifted towards dissociation. The aggregate, however, which is shown by the triangles, next graph down, remains unchanged, the relative viscosity doesn't change until a critical viscosity is reached, about 55 degrees, when there is a sharp fall in viscosity. We interpret this as being due to a stabilisation of the proteoglycan hyaluronic acid complex by the link protein which binds both to the hyaluronate and the proteoglycan. The proteoglycan by itself does not change, the viscosity remains virtually unchanged on heating. So that the function of the link protein is to stabilise the hyaluronate proteoglycan complex. Now the function of the aggregate, which is the thing that occurs naturally, can only be surmised. One result will clearly be to immobilise proteoglycans in the collagen network, even more effectively than if they were not, if the proteoglycans were free. Another function, which can be shown in vitro, the proteoglycan aggregate is more resistant both to proteolytic enzymes and to enzymes that attack the carbohydrate moiety of this complex. So it has another function, perhaps, in vivo that it makes this all-important compound, the proteoglycan, less vulnerable to attack by degradative enzymes.

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<**Muir to camera**>

I now want to turn to another biochemical aspect of cartilage and that is the synthesis of the constituents of the matrix. Cartilage cells are capable of synthesising all the constituents of the matrix as far as we know; both the hyaluronic acid, the proteoglycan and the collagen. Now in adult tissues, the relative rates of turnover of these constituents is rather low. But in inflammatory conditions they are likely to be increased considerably. One of the ways of studying biosynthesis is to use

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radioactive precursors and, as far as proteoglycans are concerned, one clearly very useful and relatively cheap precursor is radioactively labelled sulphate. Using radioactive sulphate you can measure the rate of synthesis of proteoglycan. Now, autoradiography is a technique for showing the exact location of newly synthesised molecules.

<**Muir over autoradiograph of molecules**>

The silver grains which fall in the photographic film are exactly over the radioactive atoms. The experiment that we carried out consisted of taking living cartilage slices and incubating them with radioactive sulphate for 10 minutes. Some of the tissue was fixed immediately and other portions were placed in non-radioactive medium for varying periods of time. Now this picture shows the slice that was incubated for 10 minutes and then immediately fixed and you can see that the silver grains are located over the cells. When the cartilage was taken and placed in non-radioactive medium for 10 minutes the silver grains have now moved out from within the cells to the periphery nearby. Half an hour later the silver grains have moved even further from the cells and are located quite a long way from them. 3 hours later, which is shown next, the grains are really quite far away from the cartilage cells so that one can suppose, then, that cartilage cells synthesise proteoglycans which are then secreted into the medium and these newly formed molecules gradually move out from the cartilage cells right into the deeper parts of the matrix.

<**Muir to camera**>

I now want to consider osteoarthrosis, which is a non-inflammatory disease of wide-spread occurrence in man and many other species. One or several joints can be affected and the first changes that seem to happen is that the cartilage becomes softer and eventually it is attacked and destroyed and the subchondral bone changes its shape. This disease seems to happen to a greater or lesser extent in many people as they get older.

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It was, for a long time, thought to be a degenerative disease. But we have come to the conclusion, in studying the early changes in the biochemistry, that this may not be the case. The problem with osteoarthrosis is, you don't know that anyone has the disease until it is so advanced that it appears in radiographs. So the problem really is to try and devise an experimental model which can be examined at the very early stages before lesions have become so advanced.

In collaboration with a veterinary surgeon from Glasgow University, Elizabeth Gilbertson, my colleague Dr McDevitt and I have been examining the very early stages of an experimental osteoarthrosis in which one ligament of the knee joint was cut. The hyper-laxity of the joint which results from this leads on to osteoarthrosis. Now this occurrence can also happen naturally both in man and other animals, and when this happens, for example, to footballers or skiers, it can lead to osteoarthrosis.

<**Muir over histological sections of control and of natural osteoarthrosis, then table detailing the development of osteoarthrosis**>

The experiments were conducted in such a way that we used a single individual to act as a control. The advantage of this experimental model is that the lesion appears in exactly the same place in each joint and we can therefore examine the cartilage surface both where the lesion is going to happen, after it has happened and the areas which are surrounding the lesion.

We have here histological sections of control and natural osteoarthrosis. You can see in the controls side that the surface of the cartilage is smooth and there are rather few cells and it looks very homogeneous. In osteoarthrosis, the cartilage surface – the two cartilages are facing towards each other, of course, in this picture – the cartilage surface is damaged, is breaking away; the staining is very uneven – this was ruthenium red staining. And there was a very great number of cells, far more than in the control. In the experimental osteoarthrosis, the histological appearance of the cartilage is rather similar. This is 16 weeks after the lesion was induced and there are many more cells than in the control, the surface is breaking away and, as in natural osteoarthrosis, the staining at the surface is less than deeper down. As I said

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before, this particular model of osteoarthrosis has the advantage that the lesion appears in the same part of the joint surface in each animal.

Area A is where the lesion develops first, and we have sampled the three areas A, B and C. Area A is not covered by the meniscus, this is a tibial condyle. The grade of osteoarthrosis is measured by an Indian ink staining procedure, developed by Meecham. Now, one of the very earliest changes which we have observed in the whole of the joint surface, irrespective of whether a lesion was going to develop or not, was an increase in water content. Now this is very important if you consider that cartilage contains about 70% of water in normal situations and if it gets wetter, it clearly is going to get softer.

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<**Muir over graphs detailing the development of osteoarthrosis**>

The grade of osteoarthrosis is shown by the numbers below and the areas A, B and C are shown above and below. We also sampled, in various specimens, both the femur and the patella. Now, you can see that area A develops a lesion, grade 2, at the time of 6 weeks when areas B and C have relatively minor changes. This graph, these histograms (the osteoarthritic specimens are in the solid columns and the diagonally striped columns are the controls), we have measured the amount of proteoglycans that can be extracted from the tissue under standard conditions using salts of high ionic strength. Normal cartilage, the proteoglycans tend to be rather difficult to extract and even under very severe conditions of extraction, using guanidine as a solvent, only about half of the total proteoglycan can be extracted. And in osteoarthrosis, one of the first changes we observe is that the proteoglycans become more readily extractible and this is, no doubt, related to the increase in water content. Now this change is really quite considerable, even at 6 weeks after the ligament was severed. There has been an increase from about 50% to 70% of the total proteoglycan that can be extracted from area A. This change is progressive with time and at 9 weeks there is an even greater amount of the total proteoglycan that can be extracted.

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Now, area A now has developed a lesion of grade 3. Area C is beginning to develop a lesion of grade 2. And there are accompanying changes in the femur and patella where there are still no obvious lesions. At 22 weeks, this change has persisted and by now the surface of the cartilage in area A has broken down and is looking rather like the histological specimen that I showed.

<**Muir to camera, then over table showing main features of osteoarthrosis**>

The other change that we have seen is that the proteoglycan synthesis is increased and that the new proteoglycans differ in chemical compositions from those that pre-existed. In addition to increased synthesis of proteoglycan, collagen synthesis is also increased. In controlled cartilage, of the total protein synthesised, only about 20% is collagen synthesis; whereas in osteoarthritis, the protein synthesis has been switched towards collagen synthesis so that between 80 and 88% of the total protein is now being diverted to collagen synthesis. In addition, there is a reduction in the number and proportion of aggregates that we have been able to obtain in the proteoglycans extracted from osteoarthritic cartilage.

Now, these changes have been seen in all areas of the joint cartilage, even where lesions have not yet developed. And it would therefore suggest that osteoarthrosis is not a degenerative disease; cartilage softens and holds more water; the proteoglycans become more extractable and their synthesis increases, they change in chemical composition and the degree of aggregation is reduced. At the same time, collagen synthesis is increased.

All these changes have occurred before lesions which a pathologist would recognise as osteoarthrosis. Although such lesions develop later on, the biochemical changes precede these lesions.

And, therefore, I would like to conclude that in the early stages osteoarthrosis is not a degenerative disease but a profound change in the metabolism of articular cartilage.

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