On the ultrastructure of softened cartilage: a possible model for structural transformation

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(Accepted 8 January 1998)

ABSTRACT

The fibrillar architecture in the general matrix of softened cartilage has been compared with that of the normal matrix using both Nomarski light microscopy and transmission electron microscopy with combined stereoscopic reconstruction. A pseudorandom network developed from an overall radial arrangement of collagen fibrils is the most fundamental ultrastructural characteristic of the normal general matrix. This, in turn, provides an efficient entrapment system for the swelling proteoglycans. Conversely, the most distinctive feature of the softened matrix is the presence of parallel and relatively unentwined fibrils, strongly aligned in the radial direction. The presence of an optically resolvable fibrous texture in the softened cartilage matrix indicates the presence of discrete bundles of closely packed and aligned fibrils at the ultrastructural level of organisation. The general absence of such texture in the normal cartilage general matrix is consistent with the much greater degree of interconnectedness and related short-range obliquity in the fibrillar architecture, hence the importance of the term pseudorandom network. A mechanism of structural transformation is proposed based on the important property of lateral interconnectivity in the fibrils which involves both entwinement and nonentwinement based interactions. The previously reported difference in intrinsic mechanical strength between the normal and softened matrices is consistent with the transformation model proposed in this study.

Key words: Articular cartilage; collagen; ultrastructural transformation.

INTRODUCTION

The remarkable load-bearing properties of articular cartilage are a direct consequence of the biomechanical interplay between 2 primary structural entities possessing contrasting biophysical characteristics: the tension-resisting collagen fibrils and the heavily hydrated proteoglycan complexes, the latter being extremely weak in shear. When appropriately integrated, these 2 components yield a structural system capable of sustaining high levels of compressive loading. The 3-dimensional meshwork of fibrils in cartilage is crucial to its successful mechanical function. Without this meshwork, there would be no anchoring of the proteoglycan aggregates, thus leaving them to expand to their largest domain size with little ability to resist an applied load.

Ultrastructural studies employing thick/thin TEM sections in conjunction with stereoscopic imaging (Broom & Silyn-Roberts, 1989) have demonstrated that the 3-dimensional meshwork of collagen in the general matrix is developed primarily from an overall radial arrangement of fibrils which extend over large ultrastructural distances, and which repeatedly deflect laterally as short oblique segments. Neighbouring and near-neighbouring fibrils are seen to intertwine and associate at ‘nodal’ points in a transient manner to create an interconnected 3-dimensional architecture. It was proposed that these nodal sites represented regions where the functionally important characteristic of interfibrillar cohesion is established. Microrupture studies, involving the propagation of microscopic tears either along or across the primary radial direction, also support this structural model (Broom, 1984a; Broom & Silyn-Roberts 1990). In this scheme radial continuity along the length of the fibrils provides the matrix with resistance to the propagation of a rupture tangentially (i.e. in a direction parallel to...
the articular surface), since such propagation would require that the radial fibrils undergo tensile fracture. By contrast, resistance to the propagation of tears in the radial direction will be provided by the nodal linking regions (Broom, 1984a, b; Broom & Silyn-Roberts, 1990).

In terms of mechanical function, cartilage sustains mostly compressive loads, and only under extreme loading conditions will it be subjected to forces that might lead to its rupture or tearing. Compressive load-bearing, while exploiting the intrinsic strength or resistance to rupture of the matrix, also depends on another related property, namely its swelling stiffness. Essentially, the resistance to compressive deformation of a given region of the normally loaded joint surface is provided both by an intrinsic stiffness arising from the swelling of the hydrated proteoglycans against the constraining network of collagen fibrils, and by a complex process of consolidation involving the time-dependent exudation of fluid from a structure of very low hydraulic permeability (see review by Oloyede & Broom, 1996). The arrangement of the 3-dimensional fibrillar structure in the cartilage matrix will strongly influence its stiffness and therefore its ability to maintain a sufficient level of hydrated thickness under load. This hydrated thickness is functionally important. Previous studies in this laboratory have demonstrated experimentally its role of reducing the level of potentially damaging contact stresses that may be transmitted into the subchondral bone under both static and dynamic loading conditions (Broom & Oloyede, 1992).

Cartilage from the macroscopically intact joint can exhibit a wide range of mechanical properties. Palpable softening and roughening of the articular surface is observed in the tibial condyles of large mammals including humans over the age of about 16 y, especially in those regions not covered by the meniscus (Bullough et al. 1985). This softened tissue is almost invariably of greater thickness than the normal cartilage taken from contiguous or opposing joint sites. At a low level of microscopic resolution, the articular surface of this softened cartilage is frequently disrupted and may contain clefts that penetrate into the underlying intermediate and deeper zones. In our examination of numerous bovine knee joints over many years in this laboratory we have occasionally observed a similar softening and thickening of cartilage removed from the patella. At the light microscopic level of examination we have found close similarities between the appearance of the softened general matrix taken from both the central tibial regions, the medial and lateral facets of the patella, and from the lateral side of the patellar groove. Under the light microscope this softened cartilage exhibits, to varying degrees, a radially aligned, fibrous texture which is distinct from that of the more amorphous appearing normal matrix. The contrasting mechanical property differences between the soft tibial articular cartilage and the normal have been quantified using microcompressive experiments (Broom, 1982) and microrupture experiments (Broom, 1984a). Also we have carried out extensive light microscopy using Nomarski imaging on fresh, hydrated thin slices in order to compare the soft with the normal matrices in the bovine animal (Broom, 1982, 1986b). A gradation in both microcompressive stiffness and microrupture strength has also been demonstrated across the bovine patellar groove and correlated with changes in the collagen ultrastructure (Silyn-Roberts & Broom, 1988).

For a given level of interconnectivity in the collagenous network any decrease in the proteoglycan content should, in principle, result in a lower level of intrinsic swelling stiffness and therefore a more deformable matrix. Further, assuming a fixed level of proteoglycans, any loss of interconnectedness in the collagen network that might reduce the constraints on the swelling of the proteoglycan domains, would also lead to a lower matrix stiffness. Combined microscopic and microrupture experiments conducted on fully hydrated slices of cartilage have shown that in the softened matrix there is a greatly reduced resistance to rupture propagation in the radial direction relative to that of the normal matrix (Broom, 1984b). This would suggest a reduction in the density of fibril interconnections in this type of matrix.

This present paper therefore reports on the findings of a microscopic and ultramicroscopic investigation into the general matrix of softened articular cartilage. We attempt to elucidate more thoroughly the 3-dimensional arrangement of the fibrillar network, comparing it with that observed in the normal tissue, and offer a possible mechanism for structural transformation.

**MATERIALS AND METHODS**

Fresh samples of softened articular cartilage-on-bone were obtained from the tibial surfaces of 4 cattle aged ~ 2 y. The particular region of the tibial surface sampled was that corresponding to the central area of both the lateral and medial sides not normally covered by the meniscus. In contrast to the surrounding covered regions, tissue from this central zone appeared
Fig. 1. Hydrated, fixed, unstained normal (a) and softened (b) general matrix observed under Nomarski light microscopy. Arrows indicate radial direction. × 1130.

less smooth and manifested a palpable softening when gently probed. Normal control samples were obtained from the corresponding contact areas of the matching femoral condyles in each of the 4 joints. All of these femoral controls exhibited a smooth, glistening surface appearance indicating that, at least macroscopically, the articular surface was undisturbed. The retrieved samples were then stored frozen at approximately −20 °C. Prior to experimentation, the samples were thawed and equilibrated in 0.15 M saline at 4 °C.

In order to prevent distortion of the fresh, hydrated cartilage following release from its subchondral bone, the cartilage-on-bone samples were fixed with 2.5% glutaraldehyde in 0.1 M Sorensen’s phosphate buffer (pH 7.2) for 3 h. Following this fixation the cartilage was removed as a full thickness layer from its underlying bone. Surface-to-deep or radial slices incorporating the full depth of cartilage and approximately 0.1–0.15 mm in thickness were prepared using a special cutting procedure described earlier by Broom (1984a), and then examined optically with Nomarski imaging in their fully hydrated state. Additional samples of cartilage-on-bone, adjacent to where the above slices were taken, were postfixed in 1% osmium tetroxide for 2 h and then stained en bloc with 2% aqueous uranyl acetate overnight at 60 °C. After dehydration with ethanol the samples were processed through a standard propylene oxide/Epon sequence for final embedding, and then cured for 36 h at 60 °C. The embedded blocks were trimmed so as to sample only the deep matrix centred in the lower third of the full cartilage thickness. We have defined this region as the ‘general matrix’ in the present study. Thick/thin sections nominally 0.2 to 0.5 µm in thickness and with the section plane incorporating the radial direction were then prepared using conventional microtome techniques. These sections were then stained in aqueous 2% uranyl acetate and 4% lead citrate, and examined by transmission electron microscopy at 120 kV. Stereo pairs at 0° and 10° tilt were obtained from representative regions. These were especially useful in tracing the degree of fibril continuity over substantial ultrastructural distances in the thick/thin sections. Some thick Epon-embedded slices (nominally 2.5 µm) were stained with toluidine blue and examined using Nomarski light microscopy. Also, additional slices for Nomarski observation were taken from portions of cartilage that were fully hydrated but unfixed in order to examine the influence of fixation
on the optical appearance of the general matrix structure.

From each of the 8 normal and 8 softened cartilage samples 3 separate blocks were examined by TEM in order to obtain representative ultrastructural views.

RESULTS

Light microscopy

Nomarski imaging provides a rapid and convenient means of assessing the fully hydrated cartilage matrix up to medium levels of structural resolution. The normal general matrix was characterised by an almost nondirectional ground-glass or amorphous texture (Fig. 1a) whereas the softened general matrix exhibited, in many regions, a fine parallel fibrous texture (Fig. 1b) which often contained a superimposed crimp or waveform as was noted in earlier studies by Broom (1982). These same contrasting textures were resolved with even greater clarity in the fully processed tissue in the thick Epon sections when viewed under oil immersion (Fig. 2a, b). This consistent difference in the matrix textures observed in the fixed/hydrated, fixed/Epon-embedded samples and in the unfixed hydrated slices clearly demonstrates that fixation and embedding has little discernable influence on the appearance of the textures observed in either the normal or softened general matrices.

Transmission electron microscopy

General comparison of the normal and soft matrices

Figure 3 shows representative lower magnification ultrastructural views of the normal and soft general matrices for direct comparison. These were obtained from sections of similar thickness (nominally 0.2 μm) and illustrate how pronounced is the long range continuity of the fibrils in the soft compared with that in the normal general matrix when viewed in these comparably thin Epon sections.

Collagen architecture of the normal matrix and the importance of section thickness

Capturing the 3-dimensional fibrillar architecture of cartilage is difficult in thin sections. Figure 4a is a higher magnification view of Figure 3a. Figure 4b is a view of approximately the same region but from an Epon section of nominal thickness 0.5 μm. The 0.2 μm

Fig. 2. Appearance of general matrix in normal (a) and softened (b) cartilage viewed in thick Epon sections under Nomarski light microscopy. Arrows indicate radial direction. × 1865.
Fig. 3. Lower magnification ultrastructural views of normal general matrix (a) and softened general matrix (b) of cartilage showing the dramatic differences in structural arrangement when viewed in similar thin section planes. Site X shows a more confused pseudorandom configuration of fibrils and these will not be resolved optically (see text). Arrows indicate radial direction. Bar, 1 µm.

section (Fig. 4a) gives little indication of large scale fibril continuity in the radial direction. When viewed stereoscopically, most fibril elements were seen to pass obliquely through the thickness of the section with only a few exhibiting any degree of continuity. However, with the section thickness increased to 0.5 µm (Fig. 4b), a distinct radial continuity begins to emerge in the structure, thus providing a distinct impression of the pseudorandom nature of the fibrillar architecture in the normal matrix. It is therefore clear from these micrographs that overall radial fibril continuity in the normal matrix can only be captured when viewed in sections of sufficient thickness, and incurring, as a consequence, a substantial loss of image quality.

The repeating fibril segment obliquity, with elements essentially out of phase with each other and thus creating a pseudorandom network is, we believe,
one of the most fundamental ultrastructural characteristics of the normal general matrix and is consistent with previous findings in our laboratory (Broom & Silyn-Roberts, 1989). As noted in the introduction a key requirement for mechanical cohesion of this 3-dimensional architecture would be the repeating presence of some kind of ‘nodal’ point where many fibrils associate briefly, in effect, creating a substantial component of lateral linkage in the network.

Further insight into the overall ultrastructural arrangement in the normal matrix can be obtained by examining regions of the general matrix in the vicinity of a cut boundary. Figure 5a shows the exposed cut boundary of the cartilage in the deep matrix and perpendicular to the articular surface. Figure 5b shows the exposed cut boundary at approximately the same depth but now parallel to the articular surface. Both of these cuts were made originally on the fresh,
fully hydrated bulk cartilage and would therefore have exposed the fibrils in the immediate vicinity of the cut to a loss of constraint arising from any localised removal of ground substance. What is significant is that the exposed fibrils at the cut edge in Figure 5b (see region A) have reverted to a near radial, more linear arrangement (thus suggesting their overall ‘native’ orientation in the matrix), as distinct from those fibrils in more constrained, oblique configurations in the matrix proper (see region B). By contrast, in the perpendicular cut edge (see Fig. 5a) any fibrils that are released lie mostly in near-radial orientations rather than projecting out normal to the boundary as is observed in Figure 5b. We consider this to be strong evidence in support of the fibrillar architectural model for the general matrix of cartilage proposed earlier (Broom 1984a, 1986b), i.e. a largely radial arrangement of collagen fibrils suitably constrained over short range distances to create a pseudorandom network.
**The softened matrix**

The most distinctive feature of the softened matrix was the presence of extensive, parallel and relatively unentwined fibril segments, strongly aligned in the radial direction. These fibrils frequently formed discrete aggregates or bundles separated by regions of low electron density. These bundles either exhibited a pronounced waviness or crimp (Fig. 3b), or were nearly straight (Fig. 6). This aligned structure persisted over considerable ultrastructural distances, and is suggestive of some form of regular secondary interaction between the closely arrayed elements along their lengths. With respect to the straight arrays, provided the fibril direction was contained within the plane of the Epon section, the actual section thickness did not interfere with structural visualisation, and the large-scale radial continuity of the fibrils within the bundles was indeed dramatic.

However, if the alignment direction departed significantly from the plane of the section an illusion of breaks in the fibril bundle continuity appeared in the structure (Fig. 7a). We have illustrated this 3-dimensional visualisation effect schematically in
Fig. 7. (a) TEM of softened general matrix. Discontinuities in the large fibrillar bundles are a consequence of their alignment direction departing from the section plane. Arrow indicates radial direction. Bar, 1.5 µm. (b) Schematic illustration showing the influence on the projected TEM image when the fibril bundle direction passes obliquely through the section plane rather than parallel to it.
Figure 7b in order to emphasise just how important it is to consider the orientation of such aligned structures when viewing in thin section. The fibril bundles comprising the more crimped morphology exhibited a greater degree of disruption in the radial direction although overall continuity was generally evident as is shown in Figure 3b.

In plan view the straight and wavy bundles, where they were clearly separated, typically had a projected bundle diameter in the approximate range 0.1–0.5 µm. It should be noted that this dimension is of the same order of magnitude as the radially aligned fibrous structure observed at the light microscope level in the softened matrix (see Figs 1b, 2b). The cramped fibrillar morphology observed ultrastructurally is also consistent with the wavy fibrous structure seen in some areas of the softened general matrix at the light microscope level (Fig. 2b).

The presence of an optically resolvable fibrous texture in the softened cartilage matrix, whether straight or crimped, therefore indicates the presence of discrete bundles of closely packed and aligned fibrils at the ultrastructural level of organisation. Conversely, in the normal cartilage general matrix the general absence of such texture is consistent with a much greater degree of interconnectedness and related short-range obliquity in the fibrillar architecture as is illustrated in Figure 4b. This would also correlate with its higher mechanical stiffness and intrinsic strength as has been previously noted by Broom (1982, 1984a).

It was also observed that under the optical microscope there were many regions in the softened general matrix where the fibrous texture was rather difficult to resolve. We suggest 2 possible explanations for this. One is that they contain the strongly-aligned arrays of fibrils that have not formed discrete, optically resolvable bundles well separated by collagen-free regions. In this situation the regular, parallel spacing of the fibrils would not present a discrete, aggregated structure of sufficient size for resolution with the light microscope. Alternatively, the fibrils in such regions are arranged in a more confused pseudorandom configuration as for example at site X in Figure 3b, and therefore lacking optical resolution.

Superimposed on the strongly aligned and parallel structure dominating the softened matrix was a much reduced degree of fibril interaction compared with that observed in the normal matrix. These interactions occurred at several levels. Firstly, whole bundles of parallel fibrils were seen to fan out into more diffuse configurations (see region A in Fig. 8). Secondly, some parallel arrays appeared to cross over and
interact with adjacent arrays also lying in the same direction (e.g. fibril marked B in Fig. 8). A third distinctive morphology involved both crossover and random tangling of individual fibrils (see Figs 8, 9). By tracing their paths stereoscopically some of the fibrillar elements in these tangled regions were observed to have their origin in the parallel bundles. On exiting and tangling laterally with neighbouring elements, they were seen to re-enter another or their original bundle (see Fig. 9). This may constitute one of the mechanisms for establishing a degree of transverse cohesion in the softened matrix. An important feature of these tangled regions was their extensive lateral development (Fig. 6), in effect producing a type of repeating secondary fibrillar structure extending approximately at right angles to the primary radial array.

**DISCUSSION**

An important question to be addressed is how this distinctly aligned structure might have arisen in the softened general matrix. Is it a structure that has been laid down developmentally in a form that has persisted through to the mature matrix, or is it a result of some kind of transformation of the normal matrix? In consideration of this important question, it should be noted that this softened tissue is consistently found on both tibial plateaux of the normal bovine joint. It is also found consistently in articular cartilage from the tibial plateau of humans at least over the age of 16 y, and in normal dogs aged at least 1 y (Bullough et al. 1985). Previous studies in our laboratory have also shown that there are similar, although less dramatic, differences in the collagen arrangement in the general matrix in traversing from the medial to the lateral sides of the normal bovine patellar groove (Silyn-Roberts & Broom, 1988). We therefore argue that based on the broad amount of evidence available, our softened tissue would appear to be a ‘common’ variant of the range of cartilage matrices found in the normal mammalian joint system, reflecting more the influence that the partial covering of the meniscus has on the pattern of mechanical loading of the tibial plateau, as has also been suggested by Bullough et al. (1985), rather than the effects of pathological change.

Earlier studies by one of the present authors have demonstrated that regions of strongly aligned fibrils could be induced in the normal general matrix either by repeated compressive impacting of cartilage-on-bone samples (Broom, 1986a), or by exposing thin slices of cartilage to limited digestion with collagenase (Broom, 1988). That such a major structural transformation can be induced artificially in the normal general matrix, either by direct mechanical trauma or by enzymatic means, suggests that it might also be able to occur in vivo. If such a transformation has in fact taken place then this would require that much of the interconnectedness between the fibrils in the normal matrix be destroyed so that major realignment into closely associated parallel arrays and bundles can then occur. Fibrils that interconnect laterally by physical tangling and entwinement would obviously resist such rearrangement up to the point of their fracture.
Fig. 10. A possible mechanism for ultrastructural transformation of the normal fibrillar architecture into the softened configuration. Large scale development of parallel fibrillar bundles with inter-bundle crossovers and tangling in the softened architecture is derived from 2 different kinds of fibril interconnectivity in the normal matrix architecture. The non-tangling interactions are indicated by dots.

Conversely, fibrils that interconnect via some non-tangling mechanism could, potentially, ‘break rank’ without fracturing in order reform as parallel arrays.

What evidence do we have for either of these mechanisms? Firstly, in terms of measured rupture strength of the general matrix, a major role for the physical entwinement mechanism seems less favoured. As noted earlier, microrupture studies have demonstrated a characteristic ease of propagation radially through the general matrix as opposed to the very considerable difficulty of achieving transverse rupture (Broom, 1984a). High levels of entwinement would mean that radial propagation could proceed only if the multitude of fibrils involved in such tangles were fractured and this would increase considerably the rupture propagation stresses, perhaps making radial propagation as difficult as in the transverse direction. Secondly, the evidence obtained from both our present ultrastructural studies and those conducted earlier on the normal matrix (Broom & Marra, 1986; Broom & Silyn-Roberts, 1989), whilst demonstrating the presence of some tangling and entwinement, does not suggest a particularly high density of such interactions. The ultrastructural picture we have been able to construct so far is more consistent with an interconnecting mechanism involving repeated fibril associations that are largely nontangling, and therefore potentially disconnectable (and possibly reconnectable). If this is so then the bulk of the fibrillar architecture in the general matrix is potentially transformable except for a residue that will remain largely unchanged, because it involves physical entwinement.

A possible model for structural transformation

We therefore propose that the component of laterally spreading tangled fibrils superimposed on the strongly aligned radial arrays in the softened matrix (Figs 6, 9) represents those fibrils, present in the original matrix prior to its transformation, that are physically entwined. This concept is illustrated in Figure 10.

What then might constitute a nonentwinement based mechanism for achieving high levels of transverse cohesion between the radial fibrils in the general matrix of normal cartilage? Although we cannot rule out the possibility that the collagen in the soft matrix may not all be of the main type II, the interactions associated with this main collagen of articular cartilage would still play a key role in any structural transformation.

Various models of mediated interaction between the primary type II collagen fibrils have been proposed. Interest has particularly focused on the role of the minor collagen types IX and XI. Molecules of type IX collagen have been shown to ‘decorate’ the surface of thin fibrils of type II collagen in cartilage (Müller-Glauser et al. 1986; Vaughan et al. 1988) and the formation of covalent cross-links between type II and type IX collagen has also been demonstrated (Eyre et al. 1987; Van der Rest & Mayne, 1988; Wu & Eyre, 1989). Type IX collagen molecules are also covalently cross-linked to other type IX molecules (Wu et al. 1992). Type XI collagen is a fibril-forming molecule of the same class as types I, II, III, and V (Miller & Gay, 1987). It has been shown that the type XI fibrils can be heteropolymeric structures embodying both type II and IX collagens (Smith et al. 1985, 1987; Mendler et al. 1989; Eikenberry et al. 1992; Petit et al. 1993). Studies have also shown that the type XI collagen molecules are cross-linked primarily to each other (Wu & Eyre, 1995).

As a result of this very close association of the collagen types II, IX and XI, and it has been proposed that the loss of integrity of the fibrillar framework in the cartilage matrix may be brought about by the selective proteolysis (possibly via stromelysin-induced cleavage) of both the type IX and XI collagens (see Wu & Eyre, 1995). This would leave the bulk of the type II fibrillar content in place but lead to a dramatic loss of interconnectivity in the 3-dimensional network of type II fibrils comprising the normal matrix. These fibrils would then be relatively free to assume a more
aligned configuration in response to the swelling tendency of the matrix as a whole (see schematic in Fig. 10). Such a scheme is consistent with the ultrastructural evidence reported in the present study.

ACKNOWLEDGEMENT

This work was supported by a project grant from the NZ Medical Research Council.

REFERENCES


