Immunohistochemical demonstration of fibronectin in the most superficial layer of normal rabbit articular cartilage

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Abstract
Objective—To locate fibronectin ultrastructurally in the most superficial layer of normal articular cartilage of rabbits, in order to clarify its role in joint physiology.
Methods—Articular cartilage was obtained from the femoral condyle of seven normal adult rabbits and prepared by a method that included tannic acid fixation. Polyclonal antibodies against rabbit fibronectin were used in an immunohistochemical electron microscopic study, without any enzymatic digestion but with a pre-embedding method for the transmission electron microscopy.
Results—The cartilage surface was successfully preserved by tannic acid fixation. The most superficial layer in electron photomicrographs was approximately 200–300 nm thick, cell free, and appeared to have two parallel components: the more superficial lamina and the deeper lamina. Gold labelled fibronectin lined this layer in immunohistochemical electron photomicrographs.
Conclusions—Fibronectin covering the surface of the articular cartilage may have a role in joint lubrication and protection of the cartilage by binding with the collagenous matrix and hyaluronic acid in synovial fluid. Chondroitin sulphates may act as a charge barrier in close relationship with the collagen fibrils in the deeper lamina. Significant alteration in these functions may be one of the first causal steps leading to destruction of the articular cartilage.


Fibronectin is a normal product of mature chondrocytes and is also reported to be present in normal synovial fluids in low concentrations. Once inflammation occurs in the joint, production of fibronectin increases in the synovial cells and chondrocytes; synovial fluids from osteoarthritic or rheumatoid arthritic knees develop markedly increased concentrations. The presence of fibronectin in normal and osteoarthritic articular cartilage has also been demonstrated immunohistochemically and biochemically, but its function is not fully understood. At the ultrastructural level, there is only one report, which used a post-embedding method and demonstrated fibronectin in normal articular cartilage from two human patients. Our aim was to demonstrate the ultrastructural distribution of fibronectin in the most superficial layer in normal rabbit articular cartilage, in order to increase understanding of the role of fibronectin in the joint and the processes leading to osteoarthritis.

Materials and methods
Seven adult rabbits (all male, aged six months, weight approximately 3 kg each) that had been bred and raised by professional rabbit breeders were killed under general anaesthesia with intravenously administered sodium pentobarbitone two or three days after arrival at our laboratory. We removed the hind legs and resected thin sections of articular cartilage from the femoral condyle. The slices were immediately washed in buffered saline for 30 minutes to remove synovial fluids. Each rabbit contributed five specimens, three to the experimental group, and one each to control groups A and B. The specimens were fixed in 0.1 mol/l cacodylate buffered 2% glutaraldehyde containing 0.5% tannic acid.

Sections from all rabbits were treated with 10% rabbit serum for 10 minutes to accomplish non-specific binding and subjected to an immunogold staining technique. All specimens were incubated en bloc, the experimental group in phosphate buffered saline (PBS) containing goat antirabbit fibronectin antibodies (10–40 μg/ml) (Organon Teknika Co Ltd), control group A in PBS only, and control group B in 10% normal goat serum, for one hour at room temperature. All specimens were washed three times with PBS for 10 minutes, incubated with PBS containing antigoat IgG conjugated to 10 nm gold particles diluted 10× with 1% bovine serum albumin containing PBS (British Biocell International Co Ltd) for one hour at room temperature, and washed with PBS again. All blocks were post-fixed with 0.1 mol/l cacodylate buffered 2% glutaraldehyde, embedded in hydrophilic resin (LR-White), ultrathin sectioned, contrasted with aqueous uranyl acetate and lead citrate, and examined using a transmission electron microscope (Hitachi, type 7100).

Results
The most superficial layer was approximately 200–300 nm thick, cell free, and appeared to have two components (fig 1). The thickness of
Figure 1  Transmission electron microscopic observation of the most superficial layer of the articular cartilage, apparently having two parallel components: a more superficial lamina, approximately 50 nm thick, and a deeper lamina, approximately 200 nm thick. Horizontal bar represents 1 µm.

Figure 2  Immunohistochemical electron microscopic localisation of fibronectin on the more superficial lamina of pre-embedded and immunogold stained articular cartilage. A: Control specimen. B, C: 10 nm gold particles arranged along the serrated structure of the 'lamina splendens'. Note that the apparent differences in density of fibronectin (10 nm gold particles) correspond with different angles of cut. Horizontal bar represents 0.5 µm.
this layer varied in each section according to the angle of the cut relative to the articular surface when ultrathin sections were made. The more superficial lamina, approximately 50 nm thick, showed an electron dense and serrated structure devoid of collagen fibres. In the immunohistochemical electron micrographs, this was lined by a 10 nm layer of gold labelled fibronectin (fig 2). The concentration of PBS containing goat antirabbit fibronectin antibodies found to be most effective for transmission electron microscopy was 40 μg/ml. Non-specific binding to aldehyde sites of glutaraldehyde was negligible. The deeper layer, approximately 200 nm thick, was composed mainly of fine collagen fibrils oriented parallel to the articular surface.

**Discussion**

The surface layer of articular cartilage presents very different appearances under phase contrast microscopy, scanning electron microscopy, and transmission electron microscopy.

The most superficial layer of the articular cartilage was first examined with phase contrast microscopy and termed the 'lamina splendens' by MacConaill in 1951. Later (1979), Aspden and Hukins suggested that MacConaill's lamina splendens may be merely an artefact of that microscope technique. Furthermore, they reported diffraction effects manifesting as the appearance of fringes when the microscope was defocused. We agree with these conclusions concerning their observations at that level of magnification. The 'lamina splendens' described is many times thicker than the most superficial layer that is actually distinct from the underlying structure when observed via electron microscopy.

More recently (1990), Clarke observed the articular surface from the joint cavity by scanning electron microscopy. He found the so called lamina splendens to be composed of closely packed mesh-like collagen fibrils of smaller diameter than those forming the underlying collagen fibrils. The more superficial lamina in our study using transmission electron microscopy appeared to be a matrix of presumably non-collagenous material. Collagen fibrils were not distinct. We suspect that the very fine serrations at the surface are composed of some kind of proteoglycan or glycoprotein and supported by underlying fibrils. Such a deposit at the surface would be very thin (50–100 nm) and may not have appeared as a distinct layer of material under scanning electron microscopy even with good preservation.

Fibronectin was the first high molecular weight glycoprotein to be isolated that has binding sites for collagen fibres or glycosaminoglycans, especially hyaluronic acid and heparin. The presence of fibronectin in normal and osteoarthritic articular cartilage has also been demonstrated immunohistochemically and biochemically. Clemmensen et al. used a light microscope and indirect immunoperoxidase technique to observe the fibronectin covering the rabbit articular cartilage and synovial membrane, and Wurster and Lust and demonstrated the presence of fibronectin in the articular surface of osteoarthritic and disease free rabbit articular cartilage. Furthermore, they reported that fibronectin was localised to the thin band of non-collagenous material overlying the cartilage. It is important to maintain the exact morphological structure of the most superficial layer in order to examine its precise nature immunohistochemically. Great care must be taken not to damage this delicate layer in resecting samples, fixing, and embedding. The surface of the cartilage may not be touched before embedding. In our study, tannic acid, which is generally known to congeal albumin or starch, effectively maintained the structure of the articular surface. We avoided the use of enzymic digestion, which is likely to destroy proteins, and our technique of fixation with tannic acid, staining, and embedding successfully preserved the fibronectin binding sites for the antifibronectin antigen. Gold labelled fibronectin was observed lining the serrations of the most superficial layer of this normal articular cartilage. Fibronectin covering the superficial lamina may have a role in joint lubrication and protect the cartilage by binding with hyaluronic acid in the synovial fluid, as described by Clemmensen et al.

Part of the deeper lamina is reported to be composed of chondroitin sulphates. These may act as a charge barrier in close relationship with the size barrier formed by the collagen fibrils. Alteration in these functions of the more superficial lamina following injury or aging could change the nutritional balance of the cartilage and increase the friction ratio of the cartilage surface. Kobayashi et al. (15th Biotechnology Symposium, Tokyo, 1995) proposed that a marked increase in the friction ratio may be the key change that disrupts the normal dynamics of the joint. These alterations could lead to changes in mechanical stresses, which may in turn influence the chondrocytes to begin excess production of fibronectin as an attempted repair. This would partly explain the markedly increased concentrations of fibronectin seen in synovial fluids from osteoarthritic or rheumatoid arthritic knees. Fibrinogen fragments are also known to induce production of several cytokines, such as interleukin-1, interleukin-6, and tumour necrosis factor α, which can depress proteoglycan synthesis or cause secondary synovitis. It seems reasonable to conclude that loss of or damage to the superficial lamina may contribute to the deterioration of articular cartilage.

We thank N Kishimoto in the Central Research Laboratory of Okayama University Medical School for technical assistance with the electron microscopy.

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Ann Rheum Dis 1995 54: 995-998
doi: 10.1136/ard.54.12.995

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