Ultrastructural localisation of fibronectin in human osteoarthritic articular cartilage

J A REES, S Y ALI, AND R A BROWN

From the Experimental Pathology Unit, Institute of Orthopaedics (University of London), Royal National Orthopaedic Hospital, Stanmore, Middlesex

SUMMARY A protein A-gold immunolocalisation technique has been used on sections of femoral head articular cartilage to localise fibronectin. Chondroitinase treatment enhanced gold staining, particularly when tissue samples were digested before fixation. The greatest accumulations of fibronectin were seen in the surface zone of osteoarthritic cartilage. Disease free cartilage contained very little fibronectin in this region. Cells which appeared to produce fibronectin were rare in normal specimens but common in the superficial region of osteoarthritic cartilage. These chondrocytes appeared to release fibronectin as part of an amorphous material which accumulated in the pericellular region. This is the first ultrastructural demonstration of fibronectin synthesis by articular cartilage chondrocytes.

Key word: chondrocytes

A number of groups have demonstrated the presence of fibronectin in normal cartilage tissues.^{1–5} Some studies identified fibronectin using immunofluorescence, in a pericellular halo around the chondrocytes in normal hyaline cartilage,¹ whereas others found no staining in the pericellular region but rather in the interterritorial matrix.² The location of fibronectin in cartilage is important as this may provide clues to its source and its role in the matrix. This is highlighted by reports of increased levels of fibronectin in cartilage from osteoarthritic human⁶ and canine joints.^{4,7}

The aims of this ultrastructural study were to identify where in the cartilage matrix fibronectin is located, where it accumulates in chondrocytes, and how these features differ in normal and osteoarthritic cartilage. The findings reported here support the contention that fibronectin is synthesised locally by chondrocytes.

Materials and methods

Osteoarthritic articular cartilage, type IV—residual cartilage as described by Ali and Bayliss⁸—was taken from the femoral heads peripheral to the area of exposed bone⁹ immediately after total hip

replacement. Normal femoral head cartilage (type I or II) was obtained after amputation or surgery for subcapital fracture of the femoral neck. Thin slices of tissue were fixed in 2.5% glutaraldehyde in 0.085 M sodium cacodylate buffer, pH 7.2, for one hour at room temperature. Secondary fixation was for one hour in 1% osmium tetroxide in the same buffer. Specimens were dehydrated, embedded in Araldite and sections placed on Formvar coated grids and examined unstained or stained with uranyl acetate and lead citrate using a Phillips 300 electron microscope.

IMMUNOELECTRON MICROSCOPY

Specimens were taken as described above. Pre-fixation digestion was with chondroitinase ABC (Sigma, Dorset) at 2 units/ml in 50 mM TRIS-HCl buffer, pH 8, for 45 min at 37°C. Both predigested and non-digested specimens were fixed in 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 for one hour at room temperature and washed in buffer for 15 minutes. Excess glutaraldehyde was neutralised by exposing the tissue to 0.5 M ammonium chloride for two hours, and specimens were partially dehydrated at 4°C in graded concentrations of methanol. Specimens were transferred to 70% methanol at –30°C for one hour, then into a 1:1 mixture of Lowicryl K4M resin:70% methanol overnight and finally, into four changes of pure resin for 48 hours. The resin was polymerised at

Accepted for publication 1 May 1987.

Correspondence to Dr R A Brown, Experimental Pathology Unit, Institute of Orthopaedics (University of London), Royal National Orthopaedic Hospital, Stanmore, Middlesex HA7 4LP.

−30°C for 24–48 hours with an ultraviolet light source. The blocks were stored desiccated, in sunlight, before sectioning. Gold/silver sections (70–100 nm thick) were mounted on Formvar coated copper grids and stained by the protein A-gold method as described by Mapp and Revell.¹⁰ Grids were treated for five minutes with 1% bovine serum albumin (Armour) in phosphate buffered saline—10 mM sodium phosphate, 0.15 M NaCl, pH 7.5 (BSA/PBS). Undigested specimens were digested with chondroitinase at this stage as described above, but for 30 minutes. Grids were then rinsed in BSA/PBS and treated with rabbit antihuman plasma fibronectin (Behring AG, Marburg, FDR), diluted 1:50 in BSA/PBS for one hour at room temperature, and washed repeatedly in BSA/PBS. Sites of antibody binding were localised by treatment for one hour at room temperature with protein A-gold using either 5 or 20 nm diameter particles (Janssen, Beerse, Belgium and E-Y Laboratories, San Mateo, USA). Grids were washed thoroughly in BSA/PBS and in distilled water and examined unstained or counterstained with aqueous uranyl acetate and lead citrate. Control sections were prepared (a) by omitting the primary antifibronectin antibody treatment, (b) by incubating sections with non-immune rabbit serum in place of antifibronectin, and (c) by pretreating the rabbit antifibronectin with excess, purified human fibronectin.

Results

Specimens of femoral head articular cartilage were examined from the periphery of eroded areas⁹ of four osteoarthritic (two men aged 78 and 60 years,

two women aged 61 and 55 years) and two normal joints (one man aged 22 years, one woman aged 83 years). Type IV⁸ full depth and slope zone cartilage showed many of the typical degenerative changes associated with osteoarthritis, such as surface fibrillation, loss of matrix staining for proteoglycans, cell clusters, and a somewhat disorientated collagen network in the superficial zone.¹¹

CONNECTIVE TISSUE LOCALISATION

In normal articular cartilage the matrix staining for fibronectin was negligible. A deposit of electron dense material, probably a layer of adsorbed protein, was seen in a normal (man aged 22 years, amputation) cartilage specimen at the surface (Fig. 1). This material stained strongly with gold particles, indicating a high and localised concentration of fibronectin. It was notable, however, that there was no evidence of penetration of fibronectin from this surface film into the underlying cartilage matrix. The absence of staining in the superficial zone is in contrast with staining in osteoarthritic specimens. The collagen matrix appeared normal with a regular arrangement of fibrils of constant diameter, which were orientated tangentially to the articular surface (Fig. 1). This contrasted with the osteoarthritic specimens, where orientation was more variable and a range of fibril diameters was present (Fig. 2).

The heaviest accumulations of fibronectin were seen in superficial zone cartilage matrix from all of the osteoarthritic specimens examined (Fig. 2a). This accumulation appeared as a band of intense gold staining reaching 20 to 60 µm below the articular surface. No specific accumulations were

Fig. 1 Normal surface articular cartilage matrix stained for fibronectin. Electron micrograph of the surface of normal (man aged 22 years, amputation specimen) femoral head cartilage showing the absence of gold particles, hence fibronectin, in the matrix. Note that the thin band of non-collagenous material overlying the cartilage (arrow) is stained strongly with 20 nm gold particles (see inset). Sections were treated with chondroitinase ABC and counterstained with uranyl acetate and lead citrate.

Fig. 2 Osteoarthritic surface cartilage matrix stained for fibronectin. Electron micrograph of the surface region of osteoarthritic (61 year old woman) femoral head cartilage. The cartilage matrix stained strongly with gold particles and had been incubated with (a) rabbit antihuman fibronectin and (b) non-immune rabbit serum before treatment with 20 nm protein A-gold particles. Sections were treated with chondroitinase ABC and counterstained with uranyl acetate and lead citrate.

seen, for example, adjacent to surface fibrillations or clefts. Light counterstaining of this area with uranyl acetate and lead citrate indicated that the main matrix component in this area was fibrillar collagen. Although there was a tendency for gold particles to lie on collagen fibrils, no pattern of association was visible, either with fibrils of a particular size or with the collagen striations. Some gold staining was also seen in deeper areas of matrix in some osteoarthritic specimens. In all the above cases control sections showed only small amounts of non-specific gold binding (Fig. 2b).

INTRACELLULAR AND PERICELLULAR LOCALISATION

Intracellular and pericellular staining for fibronectin

were most pronounced in the superficial zone of osteoarthritic specimens, though local accumulations were seen in and around a few cells of disease free cartilage. Clusters of three or four biosynthetically active cells producing fibronectin were seen in the superficial zone and occasionally in the mid or deep zones of osteoarthritic cartilage. Figs 3 and 4 illustrate chondrocytes producing an amorphous material which contained fibronectin, as shown by the localisation of gold particles. Similar material was also seen intracellularly in secretory vacuoles. Although this fibronectin did not appear to be the main constituent of the secreted material, its localisation within the amorphous material and secretory vacuoles was striking. The same amorphous material, seen in control sections treated with

Fig. 3 Secretion of fibronectin containing material: chondroitinase digestion of the section. (a) Low power appearance of a cluster of chondrocytes in the superficial zone of human osteoarthritic articular cartilage. ger=granular endoplasmic reticulum. Note amorphous material (arrows) in the lacuna (pericellular space), see below and Fig. 4. (b) Detail of the surface from an active secretory chondrocyte releasing an amorphous material (arrowed) which stained for fibronectin with 5 nm gold particles. p=cell processes. (c) Non-immune serum treated control section showing the same amorphous material which did not bind gold. Specimens were digested on the grid with chondroitinase ABC, treated with 5 nm gold, and counterstained with uranyl acetate/lead citrate.

non-immune serum, did not bind gold particles (Fig. 3c). The amorphous areas of secretory product were intact further from the cell (Figs 4a and 4c), though there was no evidence of an enclosing membrane. Even where this amorphous material was some distance from the chondrocyte, fibronectin was clearly localised as a component (Fig. 4c). A few cells surrounded by fibronectin containing material were seen in a specimen from a 22 year old normal man.

Figs 3 and 4 illustrate the effects on the section of digesting the tissue with chondroitinase either before fixation and embedding or after processing. Although localisation was similar by both methods,

prefixation digestion appeared to give a generally higher level of staining. Staining for fibronectin was very weak in the absence of any digestion stage.

Fibronectin was localised specifically in the endoplasmic reticulum, Golgi region, dilated cisternae of the rough endoplasmic reticulum, and in membrane bound and apparently secretory vesicles (Fig. 5) of active chondrocytes in osteoarthritic, surface zone cartilage. Similar membrane bound secretory vesicles have been described in chondrocytes by other workers.¹¹ Treatment of serial sections of fibronectin positive cells with non-immune serum, in place of antifibronectin, failed to produce staining above a low background level (Fig. 5c).

Fig. 4 Secretion and release of fibronectin containing material: chondroitinase digestion on block. (a) Electron micrograph showing cell surface (lower right hand) and pericellular area (lacuna) of an active chondrocyte. Amorphous material was visible both surrounded by cell processes and further from the cell (78 year old man, osteoarthritis). (b) Detail from (a) (lower box) of cell processes surrounding amorphous material which stained for fibronectin with 5 nm gold (arrowed). Note the absence of 5 nm gold in areas away from the amorphous material. (c) Detail of pericellular amorphous material at a distance from the cell surface seen in (a) (upper box) showing intense localisation of fibronectin (see also Fig. 3a). Once again, gold bound only to this amorphous material. Specimens were digested with chondroitinase ABC before fixation/embedding sections were stained using 5 nm protein A-gold and counterstained with uranyl acetate/lead citrate.

Discussion

Fibronectin was present in much larger amounts in cartilage from osteoarthritic, rather than from normal joints. Cells producing fibronectin were seen only rarely in normal specimens. The greatest accumulations were seen in the superficial zone of osteoarthritic cartilage and this may, in part, have been synthesised locally by chondrocytes. Certainly, considerable numbers of these cells, in osteoarthritic cartilage, synthesised and released fibronectin as part of a characteristic, amorphous material which accumulated in the pericellular region. A proportion of the fibronectin at the surface may also originate from synovial fluid, which is rich in this protein.¹² The proportional contribution by synovial fluid is unknown, however. Penetration of fibronectin into the surface of normal articular cartilage was minimal in both normal specimens. This, and the observation

of a fine layer containing fibronectin on the surface of normal cartilage, are consistent with a previous report that labelled fibronectin did not penetrate the surface of normal cartilage explants.¹³

The nature of other components present in the amorphous secretory material is unknown, although the improvement in antigen location after chondroitinase digestion implies that some proteoglycan is present. The nature of this material is currently under investigation. Similar amorphous material has been described by Geerts *et al* at or near the surface of liver cells.¹⁴ In this case both fibronectin and heparan sulphate were present.

The superficial cartilage matrix is composed largely of collagen, aligned tangentially to the surface. Although gold particles tended to lie on collagen fibrils in osteoarthritic specimens, there was no evidence of a regular or symmetrical pattern of distribution on collagen, as reported by Shiozawa

Fig. 5 Intracellular localisation of fibronectin with 20 nm gold. (a) Low power view of a cluster of active chondrocytes in the superficial zone of human osteoarthritic articular cartilage (61 year old woman). ger=granular endoplasmic reticulum. (b) High power detail of chondrocytes synthesising fibronectin. Note the localisation of fibronectin on the granular endoplasmic reticulum and in a dilated cisterna (arrow). Inset shows the concentration of gold particles on Golgi vacuoles. (c) Control treatment, with non-immune serum, of a similar region in the same cluster of cells. Sections were treated with chondroitinase ABC, stained with 20 nm protein A-gold, and counterstained with uranyl acetate and lead citrate.

et al in rheumatoid joints.¹⁵ Postembedding localisation used in this study is not ideal for staining of an antigen on collagen fibrils.

In any attempt to determine the relation between fibronectin accumulation and degenerative cartilage changes it is important to distinguish between penetration of exogenous protein and its synthesis by chondrocytes. Increased synthesis of fibronectin by superficial zone chondrocytes may represent part of a cellular response aimed at repair of the matrix, perhaps by stabilising local proteoglycan loss. This would be analogous to its production as part of the wound repair response in other tissues.¹⁶ Alternatively, cartilage fibronectin may be produced, in conjunction with cell surface proteoglycans, as part

of a pericellular matrix similar to that isolated from fibroblasts.^{17 18} This seems less likely in view of our observation that discrete areas of fibronectin-rich amorphous material were often some distance from the nearest cell. In addition, it has been suggested that the increased synthesis of fibronectin represents a subtle change in phenotype (perhaps with no direct function),⁷ reflecting the presence of de-differentiated chondrocytes.

Penetration and accumulation of exogenous fibronectin, in contrast, have a different significance. Molecules of the proportions of fibronectin have negligible access to the matrix of normal cartilage,¹⁹ as demonstrated in this and previous studies.^{9 13} Thus penetration of fibronectin for short

distances into canine osteoarthritic cartilage¹³ is likely to be a result of disruption of the collagen network. The consequences of this accumulation are unknown, though there is some evidence that chondrocyte behaviour may be altered.^{20 21}

This is the first description of intracellular fibronectin in osteoarthritic cartilage at the ultra-structural level and provides confirmation of previous radiotracer and biochemical evidence that fibronectin is synthesised *in vivo* by chondrocytes.^{4 7}

The differences in pericellular distribution between normal and osteoarthritic cartilage are consistent with the suggestion that there is a local signal for its synthesis as actively synthesising cells were seen in isolated groups,⁹ mainly in one region, the superficial zone. Furthermore, cells with and without fibronectin were frequently adjacent to one another. Initiation of this localised response may be signalled by changes in the structural matrix, for example through altered permeability or mechanical properties. The questions of how fibronectin production is stimulated and what its function or consequences are to the matrix are presently under investigation.

We are grateful to Miss Linda Adams for preparation of the manuscript. This work was supported by the Arthritis and Rheumatism Council.

References

- 1 Glant T T, Hadhazy Cs, Mikecz K, Sipos A. Appearance and persistence of fibronectin in cartilage. *Histochemistry* 1985; **82**: 149–58.
- 2 Evans H B, Ayad S, Abedin M Z, *et al*. Localisation of collagen types and fibronectin in cartilage by immunofluorescence. *Ann Rheum Dis* 1983; **42**: 575–81.
- 3 Weiss R E, Reddi A H. Appearance of fibronectin during the differentiation of cartilage, bone and bone marrow. *J Cell Biol* 1981; **88**: 630–6.
- 4 Wurster N B, Lust G. Deposition of fibronectin in articular cartilage of canine osteoarthritic joints. *Am J Vet Res* 1985; **46**: 2542–5.
- 5 Wurster N B, Butler M, Harter M, *et al*. Presence of fibronectin in articular cartilage in two animal models of osteoarthritis. *J Rheumatol* 1986; **13**: 175–82.
- 6 Miller D R, Mankin H J, Shoji H, D'Ambrosia R L. Identification of fibronectin in preparations of osteoarthritic human cartilage. *Connect Tissue Res* 1984; **12**: 267–75.
- 7 Wurster N B, Lust G. Fibronectin in osteoarthritic cartilage—possible indication of phenotypic modulation of the chondrocyte. In: Verbruggen G, Veys E M, eds. *Degenerative joints*. Vol. 2. Amsterdam: Elsevier, 1985: 141–7.
- 8 Ali S Y, Bayliss M T. Enzymic changes in human osteoarthritic cartilage. In: Ali S Y, Elves M W, Leaback D H, eds. *Normal and osteoarthritic articular cartilage*. London: Institute of Orthopaedics, 1974: 189–205.
- 9 Jones K L, Brown M, Ali S Y, Brown R A. An immunohistochemical study of fibronectin in human osteoarthritic and disease free articular cartilage. *Ann Rheum Dis* 1987; **46**: 809–15.
- 10 Mapp P I, Revell P A. Fibronectin production by synovial intimal cells. *Rheumatol Int* 1985; **5**: 229–37.
- 11 Stockwell R A, Meachim G. The chondrocytes. In: Freeman M A R, ed. *Adult articular cartilage*. Tunbridge Wells: Pitman Medical, 1979: 69–144.
- 12 Scott D L, Farr M, Crockson A P, Walton K W. Synovial fluid and plasma fibronectin levels in rheumatoid arthritis. *Clin Sci* 1982; **62**: 71–6.
- 13 Wurster N B, Lust G. Incorporation of purified fibronectin into explants of articular cartilage from disease-free and osteoarthritic canine joints. *J Orthop Res* 1986; **4**: 409–19.
- 14 Geerts A, Geuze H J, Slot J W *et al*. Immunogold localisation of procollagen III, fibronectin and heparan sulphate proteoglycan on ultra thin frozen sections of normal rat liver. *Histochemistry* 1986; **84**: 355–62.
- 15 Shiozawa K, Shiozawa S, Shimizu S, Fujita T. Fibronectin on the surface of articular cartilage in rheumatoid arthritis. *Arthritis Rheum* 1984; **27**: 615–22.
- 16 Grinnell F. Fibronectin and wound healing. *J Cell Biochem* 1984; **26**: 107–16.
- 17 Yamagata H, Yamada K M, Yoneda M, Suzuki S, Kimata K. Chondroitin sulphate proteoglycan (PG-M-like proteoglycan) is involved in the binding of hyaluronic acid to cellular fibronectin. *J Biol Chem* 1986; **261**: 13526–35.
- 18 Hedman K, Kurkinen M, Alitalo K, Vaheri A, Johansson S, Hook M. Isolation of the pericellular matrix of human fibroblast cultures. *J Cell Biol* 1979; **81**: 83–91.
- 19 Maroudas A. Distribution and diffusion of solutes in articular cartilage. *Biophys J* 1970; **10**: 365–79.
- 20 West C M, Lanza R, Rozenbloom J, Lowe M, Holtzer H. Fibronectin alters the phenotypic properties of cultured chick embryo chondroblasts. *Cell* 1979; **17**: 491–501.
- 21 Gibson G J, Kielly C M, Garner C, Schor S L, Grant M E. Identification and partial characterisation of three low molecular weight collagenous polypeptides synthesised by chondrocytes cultured within collagen gels in the absence and in the presence of fibronectin. *Biochem J* 1983; **211**: 417–20.