INCREASED EXPRESSION OF THE Ed-B-CONTAINING FIBRONECTIN (AN EMBRYONIC ISOFORM OF FIBRONECTIN) IN HUMAN OSTEOARTHRITIC CARTILAGE

X. CHEVALIER, N. GROULT and W. HORNEBECK

Department of Rheumatology, Hopital Henri-Mondor and Laboratoire de Biochimie du Tissu Conjonctif, CNRS URA 1460, Creteil, France

SUMMARY

Fibronectin is non-collagenous protein which accumulates in osteoarthritic cartilage. The presence of fibronectin and its specific isoform containing the B sequence, Ed-B fibronectin (B.Fn), was studied in normal and osteoarthritic human cartilage using immunohistochemical and biochemical assays, with a specific monoclonal antibody. Results showed substantial amounts of B.Fn in osteoarthritic cartilage, especially in the superficial and middle layers. Western blot analysis confirmed the presence of B.Fn with a molecular mass of 220 and 55 kDa. In contrast, in normal cartilage, expression of B.Fn was extremely low. In conclusion, the expression of a specific isoform of fibronectin during the osteoarthritic process suggests that this isoform might have specific functions in extracellular matrix remodelling.

KEY WORDS: Fibronectin, Cartilage, Osteoarthritis, Ed-B fibronectin.

FIBRONECTIN is a cell adhesion protein that mediates a variety of cellular functions, including phagocytosis, cell migration, cell adhesion and cell differentiation [1, 2]. These biological activities result from its ability to interact with both cell surface and extracellular matrix (ECM) components, such as collagens, glycosaminoglycans, fibrin and heparin [1, 2]. Whereas fibronectin is encoded by a single gene on human chromosome 2 [3], biochemical analysis revealed a number of different protein isoforms that vary in molecular size [4]. Three alternative spliced regions have been found in rat and human mRNAs; this finding offers a partial explanation for the variety of fibronectin species observed [5, 6]. An alternatively spliced exon (referred to as V, variable region) is always expressed in liver, and thereby recovered in plasma [1, 2].

Two alternatively spliced exons (referred to as extra domain A and extra domain B) are type III elements. Exon Ed-A is present in cell-associated fibronectin, but is absent in plasma fibronectin produced by the liver [7]. The fibronectin containing the Ed-B sequence (B.Fn) is highly expressed in transformed human cells and during embryogenesis [8-10], but is only barely detectable in cultured medium from human fibroblasts [8-10]. This specific isoform might be involved in matrix remodelling [9, 10].

The ECM of cartilage is mainly composed of a network of types II and IX collagens and large chondroitin sulphate proteoglycans called aggrecans, both of which account for the tensile strength and loading resistance of the tissue [11]. Fibronectin has been found in elevated amounts in degenerated cartilage of different animal species [12, 13] and in human osteoarthritic joints [14, 15]. In normal joints, fibronectin is a minor component of normal cartilage ECM [15]. The structure of cartilage fibronectin may differ from fibronectin from other tissues since it can interact with specific components (collagen type II, aggrecans) of the cartilage ECM [16]. A previous investigation has shown that canine articular cartilage fibronectin differs from liver fibronectin in size, antigenic determinants and the number of type III homologue repeats [16]. Recently, it has been shown that cartilage fibronectin may include, or alternatively tightly bind to, a glycosaminoglycan chain [17].

The expression of B.Fn might account for the specific properties of fibronectins in ECM cartilage, especially in damaged cartilage. We therefore examined the distribution and presence of B.Fn in normal and osteoarthritic human cartilage, using immunohistochemical and biochemical studies.

PATIENTS AND METHODS

Patients

Cartilage tissues were obtained from the Department of Orthopaedic Surgery, Hopital Henri-Mondor, Creteil, at the time of joint replacement in patients with osteoarthritis (OA) of the hip or the knee (n = 10) (mean age 73 yr; six women, four men). All patients with hip or knee OA had typical radiological features of the disease. Normal cartilages were obtained from femoral head cartilages at the time of joint replacement for femoral neck fracture (n = 5). Each cartilage sample was immediately cut into small pieces and frozen in liquid nitrogen, cryoprotected with OCT (Miles, Naperville, IL) and stored at -80°C until used.

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FIG. 1.1.—Presence of fibronectin containing the Ed-B sequence (B.Fn) and of total fibronectin in normal cartilage. For details of the immunohistochemical technique, see Patients and methods. (A) Control section (×100) (see Patients and methods). (B) Presence of ‘total’ fibronectin (including all isoforms) (×100). (C) Presence of B.Fn localized in only a few chondrocytes in the superficial layer (dark arrow) (×100). Presence of fibronectin containing the Ed-B sequence (B.Fn) and of total fibronectin in normal cartilage stimulated for 24 h with 10 ng/ml of rIL-1β. (D) Control section (×100). (E) Presence of ‘total’ fibronectin (including all isoforms) under IL-1β stimulation (×100). Note the presence of patches of staining in pericellular areas from the different layers. (F) Presence of B.Fn in normal cartilage under IL-1β stimulation. B.Fn was localized in some chondrocytes from the superficial layer (×100).

**TABLE 1**

Intensity score for fibronectin containing the Ed-B sequence (B.Fn) among the different cartilage samples: 10 osteoarthritic cartilage samples and five normal cartilage samples. Number of cartilage samples referred to the intensity of staining, scored in four grades: 0, no staining; +, mild staining; ++, intense staining mostly around the cells; +++, intense patches of staining.

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<td>Normal samples</td>
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**Monoclonal antibodies**

We used monoclonal antibodies (MAb) from hybridoma conditioned medium (L. Zardi, Institute for Cancer Research, Genoa, Italy) to detect fibronectin. Characterization of the MAbs has been previously reported and there is no cross-reactivity with other glycoproteins such as tenasin [18]. The MAb BC-1 recognizes B.Fn, IST-9 recognizes A.Fn and IST-4 recognizes all different fibronectin isoforms (i.e. ‘total’ fibronectin).
Immunohistochemistry

Immunohistochemical localization of fibronectins in human cartilage was performed on 6 μm frozen sections. Slides were previously coated with L-polylysine and fixed in acetone at +4°C for 10 min. Sections were pre-treated with bovine testicular hyaluronidase (Serva, Heidelberg, Germany) (0.5 mg/ml: 1500 U/ml) at 37°C for 60 min in Tris-NaCl (pH 5.8) [19]. This step is required to allow the detection of fibronectin [12]. The sections were first incubated for 1 h with 1:10 anti-Ed-B fibronectin (BC-1) or with 1:100 MAb directed against all fibronectin isoforms (IST-4). After washing in TBS buffer [0.05 M Tris–HCl, 0.15 M NaCl (pH 7.4)], the slides were further incubated with biotinylated goat anti-mouse immunoglobulins (diluted 1/1000) for 30 min. Sections were then washed and incubated with a 1:50 dilution of a soluble complex of alkaline phosphatase-conjugated streptavidin (Dako kit) for 30 min at room temperature [20]. Immunoreactivity was revealed by the addition of a chromogenic substrate consisting of naphthol, fast-red solution and levamisole that gives a red to brown staining. The sections were counterstained with haematoxylin, dehydrated and mounted with glass coverslips. To determine the specificity of the staining, controls were established as follows: (1) omission of the primary MAb; (2) using the primary MAb alone; (3) using...
non-specific mouse IgG. Furthermore, another MAb from the same hybridoma conditioned medium directed against the Ed-A-containing fibronectin showed a lack of any staining in normal cartilage, thereby serving as a real negative control (Fig. 1.3).

The intensity of staining for B Fn was scored in four grades: 0, no staining; +, mild staining; ++, staining mostly around the cells; ++++, intense patches of staining.

On the same samples, toluidine blue dye staining was performed for proteoglycan.

Cartilage explants from two normal and from two osteoarthritic specimens were incubated in serum-free Dulbecco's modified Eagle's medium (DMEM). In some normal samples, recombinant human IL-1β (10 ng/ml) (Tebu Co., Paris) was added to the medium for 24 h. Cartilage explants were then collected and dipped in liquid nitrogen. Cryostat sections (6 μm) from these explants were examined for the presence of
fibronectins, as previously described. Proteoglycan staining was performed using toluidine blue dye.

Extraction of fibronectins from cartilage

Cartilage samples were minced into small pieces under sterile conditions in phosphate-buffered saline (pH 7.4) containing penicillin (20 U/ml) and streptomycin (10 µg/ml), and 3 µm cryostat sections of tissue were homogenized. Samples were weighted to obtain an average of 150-200 mg of wet tissue per sample. Cartilage samples were extracted first with 0.05 M phosphate buffer (pH 7.4) containing 2 M urea and 1 mg/ml heparin [1], in the presence of protease inhibitors (phenylmethylsulphonyl fluoride, sodium iodoacetate, N-ethylmaleimide and EDTA) at a 2 mM final concentration. After centrifugation, the tissue residue was again extracted for 24 h with 5 M guanidinium chloride (pH 7.2), containing protease inhibitors. Urea and guanidinium salts were further removed by gel filtration through a Sephadex G-25 column or by exhaustive dialysis against Tris-buffer containing protease inhibitors (at the same concentrations as described above). Extracts were subjected to electrophoresis and fibronectin was revealed by Western blotting.

Western blotting

Aliquots of dissociative cartilage extracts were separated by 6% SDS-PAGE gel electrophoresis under reducing conditions, and proteins were electrotransferred onto a nitrocellulose sheet. The same conditions as previously described were used to reveal fibronectin antigens. The anti-B Fn MAb was used at 1/20 dilution. Controls without primary MAb were all negative.

RESULTS

Immunohistochemical studies

Normal cartilages (Fig. 1.1). In normal cartilage samples, B Fn was only detected in the superficial layer of two samples where a few chondrocytes appeared immunoreactive (Fig. 1.1C; Table I). B Fn was always absent in the middle and deep layers (Fig. 1.1C). In contrast, unspliced fibronectin (designated as total fibronectin) was present with a more diffuse distribution (Fig. 1.1B). Staining for total fibronectin was substantial on the cartilage surface.

Normal cartilage explants stimulated with IL-1β (Fig. 1.1). To determine whether the lack of detection of B Fn in normal cartilage could be due to masking by proteoglycan molecules, normal cartilage explants were stimulated with 10 ng/ml of IL-1β. This treatment induced an increase in the content of 'total' fibronectin which accumulated around the chondrocytes in all layers (Fig. 1.1E). This distribution did not strictly correlate with proteoglycan depletion, which predominated in the superficial areas (Fig. 1.2). Under IL-1 treatment, a few chondrocytes (pericellular areas) of the superficial layer appeared positive for B Fn (Fig. 1.1F).

Osteoarthritic cartilages. B Fn was present in the matrix of altered cartilage. In the majority of the OA samples (n = 7), B Fn was present around the cartilage surface lesions and in pericellular areas of chondrocytes of different layers (Fig. 2; Table I). Its distribution in the matrix distant from the chondrocyte was lighter, if
FIG. 4.—Immunoreactive chondrocytes for fibronectin containing the Ed-B sequence (B Fn) in human osteoarthritic cartilage. For details of the immunohistochemical technique, see Patients and methods. (A) B Fn is localized within the clone of chondrocytes (×400). (B) Control case with MAb alone: lack of staining (×400) (omitting the primary monoclonal antibody). (C) B Fn seems to be localized within the chondrocyte (×1000).
any. In some OA cartilage samples \((n = 3)\), staining for B.Fn was more intense and patchy (Fig. 3C). It was also present in superficial and middle layers of the cartilage. In some areas, B.Fn was found to accumulate near the fibrillated clefts. Proteoglycan staining showed a loss of proteoglycan which predominated in superficial and middle layers in all OA samples. The difference in proteoglycan staining among OA cartilage samples could not allow definitive conclusions to be drawn to explain the differences observed in staining for B.Fn. Using microscopic magnification, B.Fn staining seems to be localized in the chondrocyte (Fig. 4).

In comparison, 'total' fibronectin distribution showed a diffuse pattern, widely distributed throughout the matrix of the different cartilage layers (Figs 2 and 3).

**Osteoarthritic cartilage explants stimulated with IL-1β (Fig. 2).** In osteoarthritic cartilage samples, IL-1β induced an increase in pericellular staining for total fibronectin and to a lesser extent for B.Fn, when compared with unstimulated cartilage explants. This staining predominated in the zone underneath the cartilage surface, but distant from it (superficial–middle layers).

**Western blots**

Immunohistochemical identification of the protein in OA as B.Fn was confirmed by immunoblot analysis of cartilage extracts. Two bands were detectable using the anti-B.Fn MAAb: a band of 220 kDa \(M_r\), in keeping with the size of Fn monomer, and a band of 55 kDa, corresponding to a degraded form of Fn (Fig. 5). There was variation in the degree of B.Fn fragmentation between the OA samples.

In the normal cartilage extract sample, no band reacted with MAAb against B.Fn.

**DISCUSSION**

The main finding of this study is that B.Fn accumulates in OA cartilage. This indicates that in the altered cartilage a specific isoform of the fibronectin molecule is present that is usually found during embryonic development.

In normal cartilage samples, B.Fn was present in very small amounts, restricted to a few chondrocytes of the superficial layer. Treatment of cartilage explants with IL-1 (in order to reveal Fn epitopes by depleting the matrix of proteoglycans) induced the appearance of 'total' fibronectin and to a much lesser extent of B.Fn in the superficial layer. Part of the fibronectin staining may be related to PG depletion, including the deep layers where PG loss was not so marked. On the other hand, IL-1β may upregulate the expression of fibronectin, but it seems more unlikely that IL-1-treated human OA cartilage synthesizes enough fibronectin to be detectable by immuno-localization.

In OA cartilage, part of the fibronectin expressed the Ed-B sequence, which accumulates in pericellular areas. However, immunohistochemical techniques do not allow us to ascertain intracytoplasmic staining (meaning local synthesis) and electron microscopy would be necessary to confirm it. Diffusion of B.Fn from the SF may also contribute to its presence in the cartilage matrix. It has been shown that fibronectin isoforms (A.Fn and B.Fn) are expressed in the synovial membrane [21]. The presence of B.Fn was confirmed in OA cartilage extracts by biochemical assays, in keeping with a previous study [22].

The presence of B.Fn in OA cartilage reflects an alternative splicing of fibronectin mRNA. During chick embryo chondrogenesis, the fibronectin splicing pattern was found to vary from A + B + to B + A – fibronectins [23]. No mRNA, including the exon coding for the Ed-A sequence, could be detected after the maturation of cartilage [23]. In so-called normal human cartilages, which in fact correspond to senescent cartilages [24], only very small amounts of B.Fn could be detected. This indicates that in human cartilage, expression of B.Fn reflects pathological changes rather than age-related changes [25, 26].

Several mediators may participate in the accumulation of B.Fn in OA cartilage matrix. Splicing of fibronectin mRNA might be upregulated by IL-1 towards expression of exon IIIIB [27]. However, treatment of cartilage with IL-1β did not result in a significant increase in B.Fn. Transforming growth factor (TGF) β is a growth factor with antagonist effects compared to IL-1 action in cartilage [28–30]. It increases the synthesis of fibronectin in organotypic cultures of chondrocytes [29] and may therefore contribute to Fn accumulation in OA cartilage. Furthermore, TGFβ1 influences fibronectin gene...

**Fig. 5.**—Immunoblotting on cartilage extract samples from three specimens of human osteoarthritic cartilage samples. Presence of fibronectin containing the Ed-B sequence (B.Fn) which appeared as bands of 220 and 55 kDa \(M_r\). Note the inter-individual variations in intensity of the bands. Molecular weights are indicated on the left side of the figure. Lane 1, OA cartilage extract; lane 2, OA cartilage extract; lane 3, OA cartilage extract.
expression towards the expression of the two exons coding for the Ed-A and Ed-B sequences [31].

The functions of B.Fn in damaged cartilage are as yet unknown. Its increased expression in wound healing [32], in vascular lesions [33, 34], at several sites of inflammation [35], as well as in OA cartilage, suggests that B.Fn may play a role in matrix remodelling. Exon IIIIB (by inducing conformational changes) [36] might modify the binding properties of the molecule, thereby modulating the cell-matrix interactions.

In conclusion, these results show that a specific isoform of fibronectin, B.Fn, is increased in damaged cartilage and is only weakly detectable in normal cartilage. This indicates specific splicing of fibronectin mRNA in osteoarthritic disease.

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REFERENCES


