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EXPERIMENTAL OSTEOARTHRITIC ARTICULAR CARTILAGE IS ENRICHED IN GUANIDINE-SOLUBLE TYPE VI COLLAGEN

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SUMMARY: Experimental osteoarthritis was surgically induced in the right knee joint of dogs; the left knee served as a control. Articular cartilage was extracted with 4 M guanidinium chloride, 0.05 M sodium acetate, pH 6.0, containing proteinase inhibitors and the proteins purified by associative CsCl density gradient centrifugation. Equal quantities of protein were electrophoresed in agarose-acrylamide gradient gels and the high molecular weight type VI collagen bands detected in immunoblots with a polyclonal antiserum. Type VI collagen bands between 185 and 220 kDa were evident in the pathological specimens of dogs sacrificed 3, 5, and 7 months after surgery and were either absent or only very weakly visible in the controls. These results demonstrate that experimental osteoarthritic cartilage is enriched in 4 M guanidine-soluble type VI collagen.

Normal articular cartilage is composed of proteoglycans and matrix glycoproteins enmeshed in a fibrillar matrix that is mainly type II collagen [1]. Osteoarthritis is characterized by a loss of articular cartilage from focal sites [2]. Animal models have demonstrated that early osteoarthritis is associated with both degenerative and reparative processes in cartilage [1]. The early degenerative processes in animal and human cartilage include a disorganized fibrillar meshwork as assessed by electron [3,4] and interference [5] microscopy, an increased water content [6], and a defective entrapment of proteoglycans [6,7]. The apparent reparative changes include an aggressive synthesis of matrix molecules [8] and an accumulation in the tissue of proteoglycans with larger chondroitin sulfate-rich domains [9].

Changes in the concentrations or stuctures of intermolecular adhesion molecules are possible events in both the degenerative and reparative processes. At least four such putative multi-domain, intermolecular, adhesion molecules have been identified in normal articular cartilage: fibronectin [10-12], thrombospondin [13], and collagens type IX [14] and type VI [15,16].

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The monomer form of type VI collagen is comprised of two globular domains joined by a relatively short collagenous triple helix [17,18]. This molecule is composed of one large chain of 180-260 kDa and two chains of 140 kDa (19). Type VI has been identified in many tissues [17], where it can assume a microfibrillar structure with a beaded periodicity of 100-110 nm [20]. While the function of type VI collagen is unknown, it has been implicated in interactions with cells [21] and proteoglycans [18,22]. In this report we demonstrate that early canine experimental osteoarthritic cartilage is enriched in guanidine-soluble type VI collagen.

MATERIALS AND METHODS

Source of reagents: Colored globular protein standards for electrophoresis and immunoblotting were obtained from Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, MD, USA. Sea Plaque agarose for electrophoresis was from FMC Bioproducts, Rockland, ME, USA. Nitrocellulose, gelatin (EIA grade), affinity purified goat anti-rabbit IgG (H+L), alkaline phosphatase conjugate, and reagents for color development were from Bio-Rad Laboratories, Rockville Center, NY, USA.

Animal surgery and extraction of proteins: Experimental osteoarthritis was induced in the right knee joints of skeletally mature dogs by section of the anterior cruciate ligament with the left joints serving as controls (6). Dogs were sacrificed at 3 (one dog), 5 (three dogs) and 7 (one dog) months after surgery. The guidelines of the Cleveland Clinic Foundation Animal Research Committee for the relief of pain and euthanasia of animals were stringently adhered to. Cartilage from the medial tibial plateau (fibrillated cartilage [6]) and femoral condyles (intact cartilage) was extracted for 24 hours with 10 times its weight of 4 M guanidinium chloride, 0.05 M sodium acetate, pH 6.0, and the following proteinase inhibitors: 0.1 M 6-aminocaproic acid; 0.5 M benzamidine HCl; 0.001 M phenylmethylsulfonyl fluoride; 0.01 M N-ethylmaleimide (modified from procedure in 23). The extracts were filtered through sintered glass discs in columns and dialyzed exhaustively at 4°C against 0.05 M sodium acetate, pH 6.0, containing the proteinase inhibitors.

Density Gradient Isolation of Matrix Proteins: CsCl was added to the extracts (starting density: 1.40 g/ml) and the material centrifuged at 100,000 g_{av} for 72 hours at 10°C. The tubes were then cut with a Beckman tube slicer into two fractions: the top fraction (A2 fraction; 3/5 of total volume) and the bottom 2/5 by volume (A1 fraction). The sides of the tubes of the A2 fraction were washed with 4 M guanidinium chloride to remove adhering insoluble material. The A2 fractions were dialyzed at 4°C for 24 hours against 0.05 M sodium acetate, pH 6.0, containing the proteinase inhibitors, then against water and lyophilized.

Electrophoresis: Samples for electrophoresis were dissolved at a concentration of 5 mg dry weight/ml in a sample buffer comprised of 2% SDS, 0.05 M Tris HCl, pH 8.5, and 15% glycerol. Exactly 50 μg of protein, as assessed by a micro-well modification [24] of the bicinchoninic acid procedure, was loaded onto each lane of the gel. SDS electrophoresis (starting conditions: 100 V; 25 mA per gel) of nonreduced and dithiothreitol-reduced samples was conducted for 17 hours in a slab gel (0.78 mm thickness) composed of a polyacrylamide gradient (20-3%) in one direction and an agarose gradient (0-0.4%) in the other direction. Gels were electroblotted (starting conditions: 70 V; 21 mA) for 10 hours at 4°C onto nitrocellulose sheets (25). The type VI collagen bands were detected with anti-(type VI collagen) antiserum and goat anti-rabbit alkaline phosphatase-conjugated secondary antibody.

Preparation of type VI collagen antigen: Type VI antigen was purified from pepsin digests of bovine uterus [26] by precipitation with 2 M NaCl, pH 2.5, and chromatography on a Sepharose CL-4B column in 4 M guanidinium chloride as described [27]. Electrophoresis under reducing conditions confirmed the presence of bands at 50 kDa, 45 kDa and 35 kDa characteristic of the α1(VI)p, α2(VI)p, and α3(VI)p pepsinized chains respectively [28,29]. An additional band at 60 kDa identified by peptide mapping as a less extensively pepsinized α3(VI) chain with a longer noncollagenous domain was also present.

Preparation of anti-(type VI collagen) antiserum: An antiserum against the purified pepsinized form of type VI collagen was raised in rabbits as previously described [27]. Immunoblots confirmed that the antiserum reacted preferentially with the 60 kDa component, indicating that the majority of the epitopes were in the noncollagenous domain of the α3(VI) pepsin-derived chain (Ayad, unpublished). The antiserum showed a positive reaction against type VI collagen up to a dilution of 1:50,000. No cross-reactivity was detected with collagen types I, II, III, IV, V, IX, and XI, or fibronectin at the working dilution of 1:1000, as assayed by direct ELISA [30].

RESULTS

Dialysis of the guanidinium extracts yielded insoluble material that was considerably more abundant in the pathological specimens. Soluble and insoluble material was processed together in all subsequent procedures. Again, after associative density gradient centrifugation, an insoluble gel formed near the top of the gradient with the pathological specimens. This gel was essentially absent in the control samples.

Figure 1 shows an immunoblot of protein A2 fractions that were isolated from the site on the tibial cartilage that regularly becomes mildly damaged in this experimental model [6]. The anti-(type VI collagen) antiserum recognized a variety of bands in the proteins from the pathological site (Fig. 1, lane 1; OA). A cluster of four bands at 220 kDa, 200 kDa, 190 kDa, and 185 kDa, characteristic of the high molecular weight chains of type VI collagen, was evident. The most prominent band was the 185 kDa species. Lower molecular weight bands at 170 kDa, 140 kDa (possibly representing the lower molecular weight chain of type VI collagen), a prominent band at 125 kDa and trace constituents at 120 kDa, 95 kDa, 75 kDa, and 66 kDa were also present. In striking contrast, the anti-(type VI collagen) antiserum recognized only the diffuse 125 kDa band in the protein fraction from the contralateral control joint (Fig. 1, lane 2; CON). When the pathological specimen (OA) was incubated with nonimmune rabbit serum at the 1:1000 dilution used in lanes 1 and 2, no bands were detected (Fig. 1, lane 3). The anti-(type VI collagen) antiserum failed to recognize the bands when electrophoresis was conducted under nonreduced conditions (data not shown).

We wished to establish whether the guanidine-soluble type VI collagen was enriched in experimental osteoarthritic cartilage at different stages of the experimental disease. Figure 2 shows immunoblots of proteins isolated from the apparently intact femoral cartilage of operated (OA) and control (C) joints

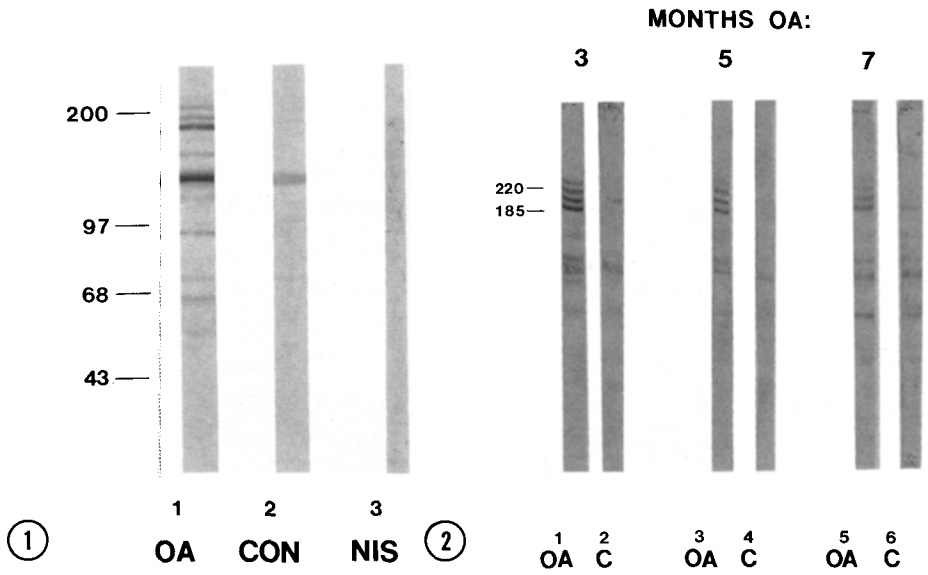

Fig. 1 Immunoblot with rabbit antiserum (1:1000 dilution) against type VI collagen. The proteins were isolated as A2 fractions from the fibrillated site (medial tibial plateau) of experimental osteoarthritic cartilage (OA) and the corresponding site on the control joint (CON). Equal quantities of protein from the pathological (lane 1) and control specimens (lane 2) were reduced and electrophoresed in SDS double-gradient polyacrylamide- (20-3%) agarose (0-0.4%) gels. Lane 3 contained the reduced pathological proteins that were incubated with nonimmune rabbit serum (1:1000 dilution). OA: experimental osteoarthritis. CON: control. NIS: nonimmune rabbit serum.

Fig. 2 Immunoblot with rabbit antiserum (1:1000 dilution) of proteins run under reduced conditions in double-gradient polyacrylamide-agarose electrophoretic gels as described in Figure 1. Specimens were A2 fractions from the apparently intact femoral condylar cartilage of experimental osteoarthritic (OA) and control (C) joints of dogs sacrificed 3 (lanes 1 and 2), 5 (lanes 3 and 4), and 7 (lanes 5 and 6) months after surgery. The three to four bands between 220 kDa and 185 kDa characteristic of the α 3 chain of type VI collagen are identified.

of dogs sacrificed 3 (lanes 1 and 2), 5 (lanes 3 and 4), and 7 (lanes 5 and 6) months after surgery. The type VI antiserum recognized the four characteristic type VI collagen bands between 220 kDa and 185 kDa. In contrast to the bands isolated from the fibrillated tibial site (Fig. 1, lane 1), the 185 kDa and the 190 kDa bands of the intact femoral site were of equal intensity (Fig. 2, lanes 1, 5 and 7). The bands at 220-185 kDa were either absent or markedly reduced in concentration in the control specimens. Several lower molecular weight bands that were evident in Figure 1 (lane 1) were also present in the pathological and, to a lesser extent, the control specimens.

Electrophoresis and immunoblots (not shown) confirmed that the type VI collagen was preferentially located in the insoluble material after dialysis of the 4 M guanidinium extract and in the insoluble gel near the top of the

density gradients. This observation is consistent with our conclusion that the experimental osteoarthritic cartilage was enriched in type VI collagen.

DISCUSSION

The high molecular weight bands between 220 kDa and 185 kDa identified in the osteoarthritic cartilage proteins by the anti-(type VI collagen) antiserum in our study were similar to those observed in cell cultures or tissue extracts of human placenta [31], fetal bovine ligaments [32,33] and immature bovine nuclei pulposi [22]. Current evidence suggests that the cluster of four chains represent modified forms of a single peptide chain that is distinct from the 140 kDa component widely reported for type VI collagen [22,33].

The spectrum of bands recognized by the anti-(type VI collagen) antiserum in our study between 170 kDa and 66 kDa, other than the 140 kDa species, presumably represents degradation products of the larger type VI peptides. Control experiments confirmed that these lower molecular weight peptides are not artifacts of the handling conditions for electrophoresis (McDevitt and Pahl, unpublished). As proteinase inhibitors and low temperature conditions were employed throughout the isolation of our A2 fractions, we tentatively assume that degradation products of larger type VI chains were probably generated in-vivo and may be significant in the role this molecule plays in osteoarthritis.

Our observation that type VI collagen is enriched in the early experimental osteoarthritic cartilage of the animal model we employed is apparently relevant to the pathogenesis of the human disease. Garrone and Tollier observed typical type VI collagen beaded fibrils in electron micrographs of human osteoarthritic cartilage [34].

The function of type VI collagen and its enrichment in early experimental osteoarthritic cartilage remain unknown. The two large globular domains of the molecule and its capacity to form fibrils, apparently noncross-linked [22], suggest it may be an important intermolecular or cell matrix adhesion molecule in cartilage.

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