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Interaction of intact type VI collagen with hyaluronan

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The capacity of non-pepsinyzed type VI collagen to bind to hyaluronan wa investigated. Type VI collagen was extracted from bovine meniscal cartilage with 6 M GuHCl and purified by extraction of PEG precipitates and dissociative Sephacryl S-500 HR chromatography. Type VI collagen, detected with a monoclonal antibody, bound in 0.5 M NaCl to hyaluronan-coated micro-wells, the degree of binding being higher at 37°C than 23°C and 4°C. Incubation of type VI collagen in competitive inhibition assays with testicular hyaluronidase digests of hyaluronan in liquid phase, reduced binding of the protein to hyaluronan-coated microwells to background levels. Thus, non-pepsinyzed type VI collagen binds to hyaluronan in vitro.

Collagen; Type VI collagen; Hyaluronan; Hyaluronic acid; Cartilage; Meniscus

1. INTRODUCTION

Type VI collagen is composed of three genetically distinct α chains that form a relatively short triple helix with large, globular domains at each extremity [1]. The non-helical portions of the molecule contain at least 15 repeat segments that bear homology with the collagen binding motif of von Willebrand factor, and a further segment that shares homology with the type III repeat of fibronectin [2–5]. Tetramers of the molecule appear to associate to form filaments [1] that make contact with, but seem independent of the main type I and III and type II collagen fibrils [6]. Consistent with these observations is the report that type VI collagen binds to fibrillar type I collagen [3]. These properties of type VI collagen imply a role for the protein as an intermolecular adhesion factor in the higher-ordered organization of macromolecules in the extracellular matrix of cartilage and other connective tissues.

In the course of immunolocalization studies of type VI collagen in articular cartilage, we noted a striking increase in staining of the protein after the tissue was digested with streptomyces hyaluronidase before reaction with an anti-type VI collagen monoclonal antibody. Similarly, Wu et al. [7] reported that digestion of bovine nucleus pulposus with streptomyces hyaluronidase, but not chondroitinase ABC, released substantial amounts of type VI collagen from the tissue. These observations suggest that hyaluronan may be one of the macromolecules to which type VI collagen binds in connective tissues.

In this study, we demonstrate that a highly purified, intact, non-pepsinyzed type VI collagen binds to hyaluronan in vitro.

Abbreviations: PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride; GuHCl, glanidine hydrochloride; BSA, bovine serum albumin.

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2. EXPERIMENTAL

2.1. *Materials*

Human umbilical cord hyaluronan, bovine testicular hyaluronidase (EC 3.2.1.35), PEG (average molecular weight 3350) and BSA (>98% pure) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Dextran (mol. wt. 70 000) was from Pharmacia Biotechnology Products, Piscataway, NJ USA. Pro-Bind assay plates (Falcon) were from Becton Dickinson Co, Lincoln Park, NJ. Anti-human type VI collagen monoclonal antibody (5D3, cell supernatant) was kindly supplied by Dr Eva Engvall, La Jolla CA. Goat anti-mouse IgG (H+L) alkaline phosphate conjugated, was from Bio-Rad Lab., Richmond, CA.

2.2. *Isolation of Type VI collagen*

Medial and lateral menisci from nine-month-old bovine knee joints were dissected free of adhering tissue, washed briefly with distilled water, diced with a scalpel blade and then ground to a granular consistency in liquid nitrogen. The ground menisci were extracted (1 g tissue: 10 ml solution) for 2 days at 4°C with 6 M GuHCl, 0.05 M sodium acetate pH 6.2, containing the following proteinase inhibitors: 0.01 M EDTA; 0.1 M ε-amino-*n*-caproic acid; 0.005 M benzamidine HCl; 0.001 M *N*-ethylmaleimide; and 0.001 M PMSF. The solutions were centrifuged (5000 rpm for 30 min) and the supernatant further clarified by filtration through a scintered glass funnel. The extract was diluted with 50 mM Tris-HCl, pH 7.2, containing the proteinase inhibitors, to a final concentration of 4 M GuHCl. This solution was mixed with an equal volume of 60% PEG (mol. wt. 3350) in 4 M GuHCl, 50 mM Tris-HCl, pH 7.2, gently by inversion and left overnight at 4°C in 12 ml capacity ultracentrifuge tubes. The fine precipitate that formed overnight was recovered by centrifugation at 100 000 × *g* for 30 min at 8°C in a Beckman SW 40 swing-out rotor and L8

ultracentrifuge. The pellet was dispersed in 12 ml of cold water, gently rotated at 4°C for 1 h and then recentrifuged as before. This procedure was repeated once. The pellet was then dispersed in 12 ml of 0.5 M NaCl, 1.0 mM *n*-ethylmaleimide, 50 mM Tris-HCl, pH 6.2 for 4 h at 4°C. The pellet was again recovered by centrifugation and the procedure repeated. The pellet was then washed twice with distilled water as before to remove any remaining salt. The pellet was then dispersed and gently shaken for 4 h in 0.1% octyl β-glucoside, 1.0 mM *n*-ethylmaleimide, 50 mM Tris-HCl, pH 6.2, recentrifuged and washed three times with distilled water. Each precipitate was dissolved overnight in 3.0 ml of 4 M GuHCl, 0.05 M Tris-HCl, pH 6.2 containing the proteinase inhibitors. Insoluble material was removed by centrifugation and the supernatant containing [^{3}H]H_2O was chromatographed on a column (1.0 × 185 cm) Sephacryl S-500 HR equilibrated in and eluted with 4 M GuHCl, 0.05 M Tris HCl, pH 7.3 at a flow rate of 8 ml/h. Fractions (2 ml) were collected and monitored for type VI collagen by ELISA and for radioactivity to determine the K_v of the column. The purity of the type VI collagen was evaluated by electrophoresis in polyacrylamide (3–20%)-agarose (0.4–0%) gradient gels, with and without reduction with DTT, and stained with Coomassie blue. Gels were transblotted onto Immobilon-P (Millipore Corp., Bedford, MA) and either stained briefly with Coomassie blue or reacted with the anti-human type VI collagen monoclonal antibody and the secondary antibody.

For interaction studies, a solution of pure type VI in 4 M GuHCl, 0.05 M Tris-HCl, pH 7.3 at the concentration of 1.97 mg/ml, as assessed by bicinchoninic acid assay with BSA as standard [8], was used as a stock solution. This solution was diluted 30-fold with the assay buffer (see below) to yield a final type VI collagen concentration of 65.6 μg/ml and a GuHCl concentration of 0.13 M.

2.3. *Hyaluronan*

Human umbilical cord hyaluronan was further purified by ethanol precipitation [9]. The final product was homogeneous in polyacrylamide-agarose gel electrophoresis [10] stained with Stains All [11].

Oligosaccharides were generated by dissolving 25 mg of purified hyaluronan in 2 ml of 0.1 M sodium acetate, 0.15 M NaCl pH 5.0 overnight and digesting with 1000 U of bovine testicular hyaluronidase for 3 h at 37°C [12]. The digestion was terminated by boiling, and the solution was then centrifuged and the supernatant brought to pH 7.0 with 10% NaOH.

2.4. *ELISA for type VI collagen-hyaluronan interaction*

Polystyrene 96-well plates were coated overnight at 4°C with 100 μl of intact hyaluronan in solution (10 μg/100 μl of 20 mM Na_2CO_3, 0.02% NaN_3, pH 9.6). The wells were then washed three times with 0.5 M NaCl, Tris-HCl pH 7.5 (hereafter referred to as TBS). A systematic study of different proteins at different concentrations established that the optimum blocking conditions involved freshly prepared 0.75% bovine serum albumin (BSA) in TBS for 1 h at room temperature. Type VI collagen was diluted from the stock solution in 4 M GuHCl into TBS and incubated with the blocked plates for 2 h, initially at 4°C, 23°C, or 37°C, and in subsequent experiments at 37°C only (see section 3). Anti-human type VI collagen monoclonal antibody was diluted 1:5 with TTBS–1% BSA and incubated on the wells with gentle rocking for 1 h at room temperature. The wells were washed three times, 10 min per wash, with TBS containing 0.5% Tween-20 (TTBS). The wells were incubated with the secondary antibody, diluted 1:1000 with TTBS–1% BSA, for 1 h at room temperature. The wells were washed three times with TTBS. The substrate, *p*-nitrophenylphosphate, was added and the plates read in a Bio-Rad ELISA Plate Reader at 405 nm. All assays were performed in quadruplicate and the standard deviation calculated.

All assays contained the following negative controls: (a) the addition of type VI collagen was omitted to a set of hyaluronan-coated wells to establish non-specific binding of antibodies to the hyaluronan-coated wells. This binding was established as negligible in all assays. (b) Wells without hyaluronan were coated with BSA in the blocking step to establish the degree of binding to the BSA blocker. The optimum blocking conditions yielded a degree of binding of type VI collagen to the BSA that was markedly below that to hyaluronan (see section 3).

2.5. *Competitive inhibition ELISA assay*

Type VI collagen, diluted 1:15 from the 4 M GuHCl solution was mixed in glass vials with equal volumes of the following solutions: (a) the oligosaccharide digest; (b) 0.1 M sodium acetate, 0.15 M NaCl, pH 7.0, employed as a buffer in digestion (positive control); (c) dextran, 12.5 mg/ml of digestion buffer (negative control). The mixtures were incubated at 37°C for 1 h. Aliquots (100 μl) of the mixtures were added to the hyaluronan and BSA coated plates and evaluated for the degree of binding by the ELISA assay above.

3. RESULTS

The procedure described for the isolation of intact type VI collagen represents the results of a systematic study that included evaluation of different technologies and different precipitating agents (ammonium sulfate, cesium chloride, PEG of varying molecular weights and concentrations) for the purification of this relatively insoluble protein. As isolated, the type VI collagen migrated in electrophoretic gels (Fig. 1, lane 2) under reduced conditions as a 'ladder' of four bands (185–245 kDa) and an intense, slightly broadened band at 140 kDa, consistent with previous observations [7,13]. Importantly, no material was detected at the top of the gel,

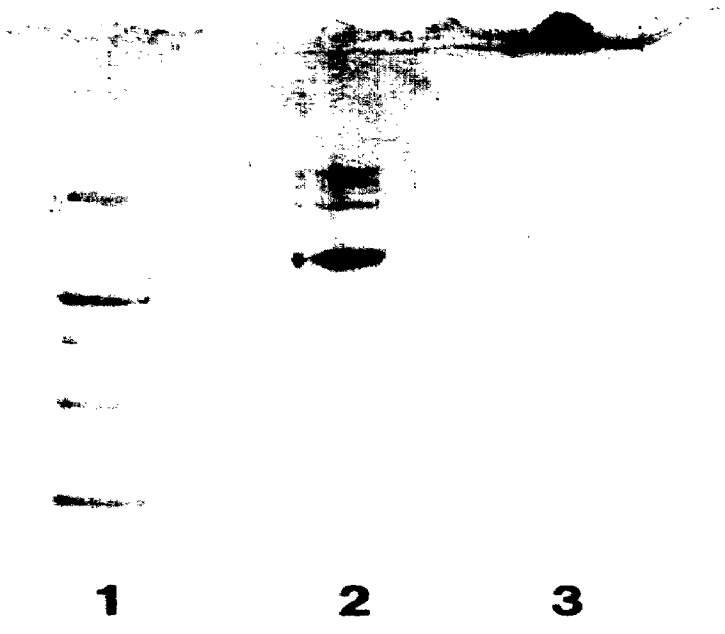

Fig. 1. Polyacrylamide (3–20%) - agarose (0.4–0%) gradient electrophoresis. Gels were stained with Coomassie blue. Lane 1, reduced standards, mol. wts 200 kDa, 116 kDa, 97 kDa, 66 kDa and 45 kDa. Lane 2, reduced type VI collagen showing ladder of bands around 200 kDa and intensely stained band at 140 kDa. Lane 3, type VI collagen under non-reduced conditions, with material exclusively located at top of gel.

Fig. 2. As in Fig. 1, with exception that material on gel was transblotted onto an Immobilon-P membrane. Lanes 1, 2 and 3 were stained with Coomassie blue. Lanes 4 and 5 were immunostained with anti-human type VI collagen antibody. Lane 1, type VI collagen under non-reduced conditions. lane 2, reduced standards, 200 kDa, 116 kDa, 97 kDa and 66 kDa. Lane 3 reduced type VI collagen. Lane 4, reduced type VI collagen. Lane 5, non-reduced type VI collagen. Some minor, degradation products of type VI collagen are evident in Lane 4.

an apparent contaminant in other studies, or within the gel under these conditions. By analogy with previous reports, the ladder around 200 kDa represents molecular variants of the α3(VI) chain and the 140 kDa band a mixture of the α1(VI) and α2(VI) chains [13]. Under non-reducing conditions (Fig. 1, lane 3) the material stained with Coomassie blue was exclusively at the top of gel, as anticipated for pure type VI collagen. Immunolocalization of western blots with the 5D3 monoclonal antibody that recognizes an epitope(s) on the α3(VI)

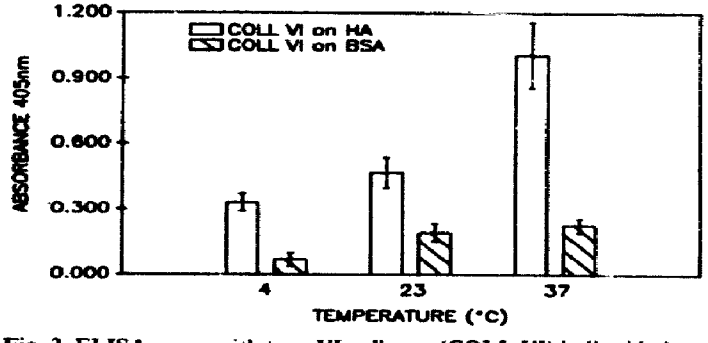

Fig. 3. ELISA assay with type VI collagen (COLL VI) in liquid phase and hyaluronan (HA) or BSA in solid phase. Interaction was performed at 4°C, 23°C and 37°C

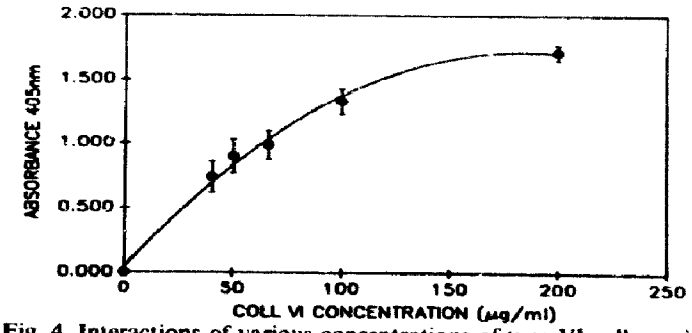

Fig. 4. Interactions of various concentrations of type VI collagen in liquid phase with hyaluronan-coated plates assessed by ELISA assay. The concentration of the stock type VI collagen solution was measured by the bicinchoninic acid assay.

chain, confirmed the ladder in reduced gels as type VI collagen. (Fig. 2, lane 4)

Negatively stained electron micrograph of the type VI collagen preparation revealed the characteristic, dumbbell shaped structure of the protein, with a length of about 110 nm as previously observed (data not shown).

Type VI collagen bound to hyaluronan-coated microwells (Fig. 3). The degree of binding was temperature-dependent and was greatest at 37°C and least at 4°C. The amount of type VI collagen that bound to BSA-coated microwells under the same conditions was minimal in comparison (Fig. 3). All subsequent type VI collagen–hyaluronan interactions, therefore, were performed at 37°C.

When 100 μl of a solution containing type VI collagen at concentrations from 40 μg/ml to 200 μg/ml was added to the hyaluronan-coated microwells, the amount of type VI collagen bound was a function of the concentration added (Fig. 4).

Fig. 5 shows the result of the competitive inhibition assay, with type VI incubated with HA oligosaccharides, dextran or buffer in the liquid phase before being added to the solid phase hyaluronan. The type VI collagen that was pre-incubated with buffer, bound to the hyaluronan on the microwells. In contrast pre-incuba-

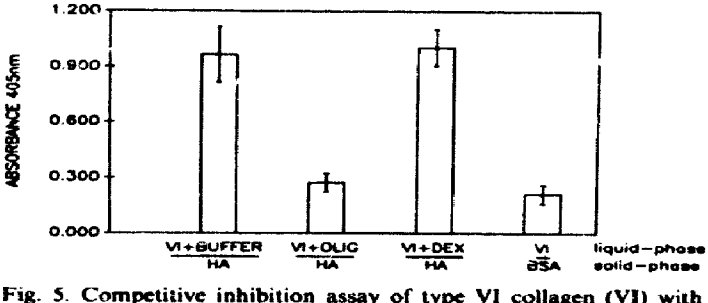

Fig. 5. Competitive inhibition assay of type VI collagen (VI) with buffer, hyaluronan oligosaccharides (OLIG) or dextran (DEX) in liquid phase. Mixtures were then incubated on plates coated with hyaluronan (HA) as solid phase. A BSA coated-plate served to establish non-specific binding to the blocker.

tion of type VI collagen with hyaluronan oligosaccharides markedly reduced the quantity of the protein that bound to the solid phase hyaluronan. (Fig. 5) The amount of type VI bound to the hyaluronan-coated wells under these conditions was indistinguishable from that bound to the BSA coated wells. Pre-incubation of type VI collagen with dextran at a concentration equivalent to that of the hyaluronan oligosaccharides, had no effect on the binding.

4. DISCUSSION

Our results confirm that purified, intact type VI collagen binds to hyaluronan. It is notable that the binding was evident in 0.5 M NaCl, conditions that would eliminate simple, electrostatic interactions. The inhibition of the binding by hyaluronan oligosaccharides in the liquid phase confirms the validity of the solid phase assays.

Type VI collagen must therefore be added to the group of proteins that bind to hyaluronan. These include proteoglycans and link proteins [14], a rat chondrosarcoma protein [15], hyaluronectin [16], fibrinogen [17], as well as cell surface and other proteins [18]. Our results support a role for type VI collagen in the organization of extracellular macromolecules into higher ordered structures in connective tissues.

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