FEBS Letters 363 (1995) 214–216 FEBS 15383

Thrombospondin 1 binds to the surface of bovine articular chondrocytes by a linear RGD-dependent mechanism

Raymond R. Miller, Cahir A. McDevitt*

Section of Musculoskeletal Biology (Wb3), Department of Biomedical Engineering, Cleveland Clinic Foundation Research Institute, 9500 Euclid Avenue, Cleveland, OH 44195-5254, USA

Received 13 February 1995

Abstract Thrombospondin 1 is present in articular cartilage and is synthesized by chondrocytes. Adult bovine articular chondrocytes in serum-free medium were evaluated in a solid-phase assay for their ability to attach to thrombospondin 1 isolated from human platelets. The chondrocytes attached to the thrombospondin 1 by a mechanism that was inhibited by a synthetic linear GRGDSP but not a GRGESP peptide. The cells, however, did not spread on thrombospondin 1, but did spread on fibronectin and Pep-Tite 2000, a synthetic RGD-containing peptide. Preincubation of thrombospondin 1 with EDTA irreversibly inhibited its capacity to attach to chondrocytes. We conclude that thrombospondin 1 binds to chondrocytes by its RGD sequence.

Key words: Thrombospondin; Chondrocyte; Cartilage; Cell attachment

1. Introduction

Chondrocytes in articular cartilage are surrounded by an abundant extracellular matrix consisting of collagens, proteoglycans and matrix proteins [1,2]. The cell surface and immediate pericellular environment of the condrocyte have critical roles in tethering the cells to their extracellular environment and in signal transduction processes. The pericellular matrix of mature articular cartilage, when viewed in the electron microscope, is clearly distinguishable from the dense fibrillar meshwork of the inter-territorial matrix [3]. Proteins that are present in cartilage and can attach to cells are good candidates for participation in these cell-pericellular matrix interactions. In a previous report we demonstrated that thrombospondin 1, a protein containing the amino acid sequence RGD, is present in articular cartilage and is synthesized by chondrocytes [4].

Thrombospondin 1 can now be categorized with type VI collagen and fibronectin as a member of the multidomain family of connecting, or adhesive proteins that bind to cells and other matrix molecules and play critical roles in organizing pericellular and extracellular matrices [5]. Thrombospondin 1 is a trimeric glycoprotein consisting of three identical monomer chains [6]. It was first identified as a component of platelet alpha granules [7]. Besides cartilage, thrombospondin 1 is present in meniscus, ligament, and intervertebral disc [8], as well as other connective tissues [9]. The RGD sequence in a variety of adhesive proteins often mediates their interaction with specific members of the integrin family of receptors [10]. Although thrombospondin 1 promotes cell attachment in both normal and transformed cells [11,12], some of these interactions are not inhibited by the RGD-containing peptide [13], suggesting that more than one mechanism exists in the protein for cell attachment to thrombospondin 1.

Thrombospondin 1 appears to play a role in wound healing and various pathological processes. In traumatized skeletal muscle, the temporal appearance of thrombospondin 1 is correlated with post-trauma generation [14]. Thrombospondin 1 has also been suggested to play a role in the early organization of the extracellular matrix of human skin [15] and corneal [16] wounds. In atherosclerotic lesions, thrombospondin 1 staining was especially prominent [9]. In a related study, interleukin-1, a cytokine believed to play a role in bone and cartilage destruction, specifically stimulates the expression of thrombospondin in rabbit articular chondrocytes while having no effect on fibronectin [17].

The capacity of chondrocytes to attach to thrombospondin 1 has not been investigated. We here report that chondrocytes isolated from adult bovine articular cartilage bind to, but do not spread, on thrombospondin 1 by an RGD-dependent mechanism, and that a divalent cation, presumably calcium, in the structure of thrombospondin 1 is essential for the interaction.

2. Experimental

2.1. Materials

Gelatin-agarose and 3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyl tetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO). Heparin-Sepharose was purchased from Pharmacia (Uppsala, Sweden). Medium and cell culture reagents were obtained from Gibco (Grand Island, NY). Fetal bovine serum was purchased from Cell Culture Laboratories (Cleveland, OH). Pep-Tite 2000 was obtained from Telios Pharmaceuticals (San Diego, CA). Bio-Gel A 0.5 m was purchased from Bio-Rad (Richmond, CA). Outdated human platelets were obtained from the American Red Cross (Cleveland, OH). Monoclonal antibody against human platelet thrombospondin was kindly provided by Dr. Deane F. Mosher (University of Wisconsin, Madison, WI).

2.2. Preparation of thrombospondin 1

Thrombospondin 1 was purified from human platelets essentially as described by [4]. Outdated human platelets were centrifuged at $180 \times g_{av}$ for 15 min at room temperature to remove remaining blood cells. The supernatant was removed and 1/5 vol. of ACD (citric acid, 8 gm/l; sodium citrate, 22 gm/l; and glucose, 24.5 gm/l, pH 4.5) was added. The platelets were centrifuged at $1100 \times g_{av}$ for 10 min and resuspended in 0.15 M NaCl, 4.3 mM K_2HPO_4, 4.3 mM Na_2PO_4, 24 mM $NaHPO_4$, 5 mM glucose, pH 6.5. The platelets were washed twice by resuspending and centrifuging in the above buffer. The final pellet was resuspended in 0.15 M NaCl, 20 mM Tris-HCl, pH 7.5, 0.1 mM $CaCl_2$, 5 mM glucose. This suspension was warmed to 37°C, and thrombospondin 1 secretion induced by the addition of human thrombin (0.5 U/ml) for 2 min until large aggregates formed. After the addition of phenylmethylsulfonyl fluoride to inhibit the thrombin, the platelet aggregates were removed by centrifugation for 5 min at $200 \times g_{av}$ at 4°C. The superna-

*Corresponding author. Fax: (1) (216) 444 9198.

SSDI 0014-5793(95)00311-8

tant was then centrifuged at 20,000 × g_{av}. The final supernatant was removed and made 1 mM in $CaCl_2$ by adding an appropriate volume from a stock of 1 M $CaCl_2$ solution.

The solution was applied to a gelatin-agarose column (1 ml/10 U of platelets) equilibrated in affinity column buffer (20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1 mM $CaCl_2$) to remove fibronectin. The column was washed with 5 column vols. of buffer. The unbound material was applied directly to a heparin-Sepharose column (1 ml/2 U of platelets) equilibrated in the affinity column buffer. The material bound to the heparin-Sepharose was eluted with a stepwise gradient consisting of column buffer containing 0.25, 0.60, and 2.0 M NaCl. The majority of the thrombospondin 1 eluted in the 0.60 M fraction as assessed by an ELISA using a monoclonal antibody against human thrombospondin 1. The purified thrombospondin 1 migrated as a single band on SDS-PAGE at a molecular weight of 180 kDa and as a single band on a Western blot probed with the antibody [4]. This fraction was used for the attachment studies described below.

2.3. Chondrocyte culture

Chondrocytes were isolated essentially according to the method of Green [18]. Articular cartilage from an adult bovine ankle joint was dissected carefully to remove any adhering tissue. The dissected tissue was weighed and then incubated with testicular hyaluronidase (5 mg/g of tissue) in Hank's balanced salts solution (HBSS) for 15 min at 37°C. The tissue was washed with HBSS and subsequently digested with trypsin and collagenase (0.2% each in HBSS) for 1 h at 37°C followed by an overnight digestion with 0.2% collagenase. The cells were filtered through a 153 μm nylon sieve (Tetko, Elmsford, NY) and then washed twice by centrifugation at low speed (1000 × g_{av}) in HBBS. The final cell pellet was resuspended, counted with a hemocytometer and plated at a density of 2×10^6 cells per 100 mm dish in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin-B (0.25 μg/ml). Cultures were incubated at 37°C in an atmosphere of 10% CO_2 in a humidified incubator.

2.4. Coating of microwell plates

Thrombospondin 1 and bovine serum albumin (BSA) were diluted to a final concentration of 20 μg/ml in HBSS. Pep-Tite (a synthetic RGD containing peptide) was diluted to a final concentration of 5 μg/ml in HBSS. Protein or peptide solutions (100 μl) were adsorbed onto Falcon microwell dishes overnight at 4°C. The next day the wells were washed three times with 100 μl of HBSS to remove any unadsorbed protein.

2.5. Attachment assay

Articular cartilage chondrocyte attachment was measured as previously described [19]. Briefly, chondrocytes were added to coated microwells at a density of 50,000 cells per microwell. After a 2 h attachment period, the wells were washed three times with HBSS to remove any unattached cells. MTT in serum-free DMEM without the Phenol red indicator (100 μl) was added to each well and incubated for 3 h at 37°C. The dye-containing medium was then aspirated and 100 μl of propanol was added to each well. The plates were vigorously shaken to solubilize the bound formazan, and the optical density of each well was measured in a Bio-Rad EIA reader at a wavelength of 550 nm.

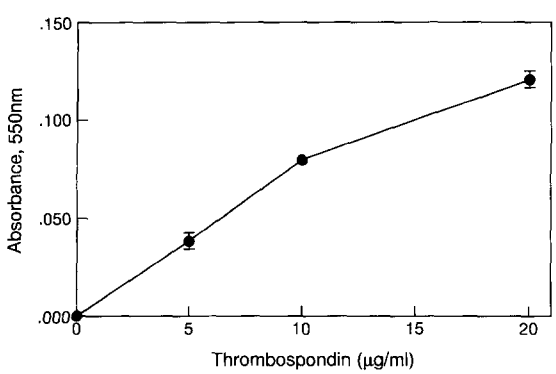

Fig. 1. Concentration dependence of chondrocyte attachment to thrombospondin 1. Microwells were coated with 0–20 μg/ml of thrombospondin 1 and attachment was assayed as described in section 2.

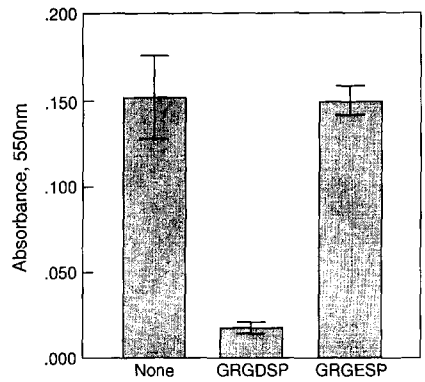

Fig. 2. Effect of synthetic peptides on the attachment of chondrocytes to thrombospondin 1. Microwells were coated with 20 μg/ml of thrombospondin 1 in the presence or absence of peptides (1 mM) and attachment was assayed as described in section 2.

3. Results

3.1. Cell attachment to thrombospondin 1

Chondrocytes isolated from bovine articular cartilage attached to thrombospondin 1-coated microwells (Fig. 1). The attachment of the chondrocytes to thrombospondin 1 was concentration dependent and saturable. As a negative control, BSA did not promote attachment at coating concentrations up to 1 mg/ml (data not shown). While chondrocytes attached to microwells coated with thrombospondin 1, almost all of the cells failed to spread in a 2 h incubation (data not shown). In contrast, more than 85% of the chondrocytes attached to wells coated with fibronectin or Pep-Tite 2000 spread in 2 h. In other studies, the vast majority of chondrocytes were attached and spread after 2 h on wells coated with type VI collagen or fibronectin [20].

3.2. Effect of synthetic peptides on chondrocyte attachment

The role of the cell binding RGD sequence of thrombospondin 1 in chondrocyte attachment was examined by adding the synthetic peptides GRGDSP and GRGESP in the attachment assay as potential competitive inhibitors. As seen in Fig. 2, at a concentration of 1 mM, the GRGDSP peptide completely inhibited chondrocyte attachment. In contrast, the GRGESP peptide had no effect when added at the same concentration.

3.3. Effect of EDTA pretreatment of thrombospondin 1 on chondrocyte attachment

The structure of thrombospondin 1 is sensitive to the presence of calcium [21]. Removal of calcium from thrombospondin 1 causes an irreversible change in the molecule. The importance of this calcium sensitivity of thrombospondin 1 on the attachment of chondrocytes was examined by adsorbing thrombospondin 1 to microwells in the presence of 2.5 mM EDTA. The wells were washed to remove the EDTA and attachment then measured. As shown in Fig. 3, this pre-treatment of the thrombospondin 1 with EDTA completely abolished attachment. When calcium was added to the EDTA-treated

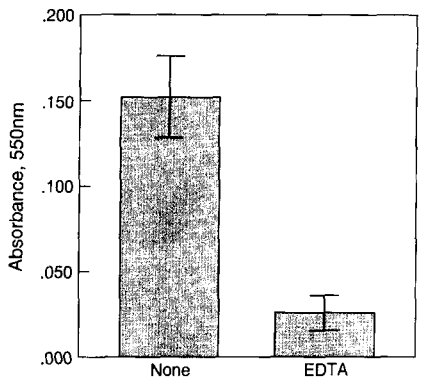

Fig. 3. Effect of pretreatment with EDTA on the attachment of chondrocytes to thrombospondin 1. Microwells were coated with 20 μg/ml of thrombospondin 1 either in the presence or absence of EDTA (2.5 mM) and attachment was assayed as described in section 2.

thrombospondin 1, the ability to attach chondrocytes was not restored (data not shown). This observation is probably not due to a reduced ability of calcium-depleted thrombospondin 1 to bind to plastic since Lawler et al. [21] observed equivalent binding of radiolabeled thrombospondin 1 to plastic in the presence or absence of calcium. The failure of the exogenous calcium to restore attachment activity to the EDTA-treated chondrocytes is also consistent with previous observations mentioned above. These observations clearly emphasize the importance of not exposing thrombospondin 1 to metal chelators during purification.

4. Discussion

We have demonstrated the ability of thrombospondin 1 isolated from human platelets to mediate the attachment of chondrocytes from adult bovine articular cartilage in a solid-phase assay. The attachment of chondrocytes to thrombospondin 1 was inhibited by the linear peptide GRGDSP, demonstrating the requirement for the RGD sequence of thrombospondin 1 in this attachment. In this respect the mechanism of attachment of thrombospondin 1 to chondrocytes was similar to that of fibronectin, but differed to that of type VI collagen, another RGD-containing protein [20]. Attachment also depended upon the presence of calcium, since removal of calcium from thrombospondin 1 by treatment with EDTA completely inhibited the subsequent attachment of chondrocytes. A recent study [22] reported that bovine cartilage oligomeric protein (COMP) attached to articular cartilage chondrocytes, but that thrombospondin 1 did not. However, it should be noted that the thrombospondin 1 in that study was exposed to EDTA during its isolation, a procedure that irreversibly abolishes its capacity to attach chondrocytes.

Thrombospondin 1 can now be added to a growing list of matrix molecules that can bind to chondrocytes. Other members of this group include type VI collagen and fibronectin [20] and a range of matrix proteins [23]. However, unlike most other members of this group, thrombospondin 1 does not appear to cause chondrocytes to spread, at least under the conditions employed in our study. This observation supports those of Murphy-Ullrich and Höök [24] in which they showed that thrombospondin 1 destabilizes cell adhesion by preventing focal adhesion formation and by loss of preformed focal adhesions. They suggested that thrombospondin 1 may function by priming cells for mitosis by inhibiting the formation of focal adhesion plaques. This is supported by findings that thrombospondin 1 is involved in the mechanisms of cellular proliferation [12,25]. The functions of thrombospondin 1 in normal and pathological articular and meniscal cartilage remain to be established.

Acknowledgements: Support from NIH Grant AR 39569 is acknowledged. We thank Dr. D.F. Mosher for a monoclonal antibody to human platelet thrombospondin.

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