INVESTIGATION OF THE ROLE OF THE ALPHA5BETA1 INTEGRIN IN CHONDROCYTIC CHONDROLYSIS BY FIBRONECTIN FRAGMENTS

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Introduction

Fibronectin fragments (Fn-fs) act on cartilage tissue in vitro to cause responses intrinsic to degenerating cartilage tissue, cytokines and matrix metalloproteinases are elevated, proteoglycan (PG) synthesis is temporarily suppressed and the cartilage PG content is severely depressed. These activities are likely relevant to osteoarthritis (OA) since Fn-fs are found in OA synovial fluid and cartilage and Fn-fs injected into rabbit knee joints cause OA like damage. Thus, delineating the mechanism for the Fn-fs and identifying potential targets for intervention may be important in modulating their activity in OA.. We have focused initially on the potential involvement of fibronectin (Fn) receptors.

Materials and Methods

Bovine articular cartilage explant or high density chondrocyte cultures were used. The Fn-fs studied included an amino-terminal 29-kDa, gelatin-binding 50-kDa and a 100-140-kDa enriched cell-binding Fn-f. Fn-fs were rhodamine or biotin labeled using NHS rhodamine [5-(and 6)-carboxylfluorescein, succinimidyl ester] or sulfo-NHS-LC-Biotin(sulfosuccinimidyl-6-(biotinamido) hexa-oate). Crosslinking was performed with DTSSP (dithiobis(sulfosuccinimidyl propionate)). Photoaffinity labeling was performed with a reversible disulfide containing label: SAED, sulfosuccinimidyl 2-(7-azio-4-methylcoumarin-3-acetamido) ethyl 1,3'dithiopropionate.

Results

The Fn-fs and Fn bound to cells in monolayer culture, or in agarose cultures or to cells in cartilage tissue – In order to confirm that the Fn-fs interacted with chondrocytes, rhodamine labeled or biotinylated Fn-fs were added to various types of cultures. All three Fn-fs as well as native Fn surrounded cells in all three culture systems. Figure 1 shows the interaction of biotinylated 29-kDa Fn-f with chondrocytes in bovine cartilage. Cells were cleary outlined with staining on articualr surface. The interaction in agarose cultures was blocked by chondroitinase and keratanase digestion, or by reduction of disulfide containing Fn-fs.

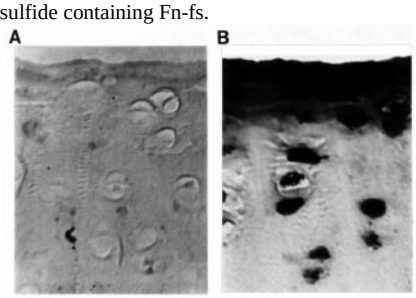

Figure 1. Bovine cartilage was cultured with biotinylated Fn-f in 10% serum/DMEM for 7 days, followed by cryosectioning and reaction with avidin-HRP and substrate. A (control with out Fn-f); B (Fn-f treated).

RGDS peptides mimicked the action of the Fn-fs, while mutants blocked Fn-f activity – In order to test whether the $alpha_5beta_1$ fibronectin (Fn) receptor, which binds the RGDS cell recognition sequence in Fn, mediated cartilage damage, RGDS and mutant peptides, RFDS and GRADSPK, were tested in depletion of carrtilage PG content. The RGDS peptide at 1 mM had activity similar to 100 nM Fn-fs.. However, mutants of this sequence, such as RFDS and GRADSPK, were inactive by themselves, but at 1-10 uM blocked Fn-f activity as shown in Figure 2.

An antibody to the $alpha_5beta_1$ Fn receptor enhanced MMP levels and suppressed PG synthesis – Antibodies to the Fn receptor at 10 ng/ml induced 43 and 98-kDa gelatinases and suppressed PG synthesis to 47 ((+/-) 7% of control values, similar to 100 nM Fn-fs.

The Fn-fs could be crosslinked to the $alpha_5$ subunit – To test whether Fn-fs bound near the receptor, chondrocytes in monolayer cultures were incubated with biotinylated Fn-fs and after 48 hours, DTSSP crosslinker was added. Cells were lysed with octylglucoside and the cell lysate applied to an anti-$alpha_5$ affinity column. The eluted material was probed on a Western blot with avidin-HRP. High mass complexes of 200 to 500-kDa were observed in Fn-f and Fn treated cultures but not in controls (Figure 3) and the $alpha_5$ subunit and Fn-fs were liberated upon reduction.. The reverse, addition of lysate to avidin-agarose and blotting the bound material with avidin-HRP, showed a weaker interaction. In other experiments, photo-affinity labeled Fn-fs were added to cultures and after UV irradiation, lysates analyzed for conversion of the $alpha_5$ subunit into complexes. This more specific analysis confirmed that all three Fn-fs and Fn bound near the $alpha_5$ subunit

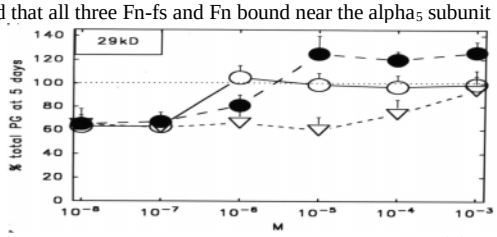

Figure 2. Bovine cartilage was incubated with 100 nM Fn-f for 5 days with increasing concentrations (X asix) of RFDS (open circle) or GRADSPK (filled circle) or GGR (triangle) and PG content of cartilage determined.

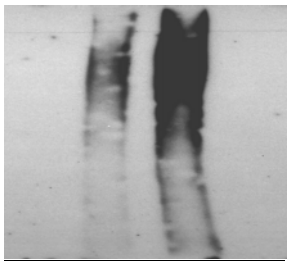

Figure 3. Lysates from crosslinked cultures were applied to anti-$alpha_5$-agarose, bound material blotted with avidin HRP to detect $alpha_5$ protein with attached biotinylated probe. Lane 1 shows no crosslinking with BSA; lane 2 shows crosslinking with 29-kDa Fn-f and lane 3 shows crosslinking with Fn; lane 4 shows control with no biotinylated protein..

Binding of Fn-fs to partially or fully purified Fn receptor – Since the previous data suggested the possibility of direct interaction of Fn-fs with the Fn receptor, cell lysates were applied to anti-$alpha_5$ resin to isolate receptor complexes. Biotinylated Fn-fs were then added to resin and the bound material blotted and probed with avidin-HRP to detect the presence of Fn-fs.. Interaction of all three Fn-fs and Fn was demonstrated. In other experiments, cell lysates were applied to Fn-f-Sepharoses to determine whether soluble receptor would bind each Fn-f. Bound material was eluted, run on an SDS gel and blotted with anti-$alpha_5$. Weak interaction could be demonstrated. Current work is directed toward testing of binding of Fn-fs to highly purified receptor.

Summary

Our preliminary data are consistent with the possibility that the Fn-fs bind to the $alpha_5beta_1$ Fn receptor and mediate their activities through this integrin. Current efforts are directed toward determining whether the Fn-fs bind other receptors and matrix molecules, and confirming the role of this receptor by testing whether anti-sense olignucleotides to the $alpha_5$ subunit block Fn-f activity. Whether the Fn-fs cause internalization of the Fn receptor, disorganization of microfilaments and disruption of Fn receptor clustering is also being tested. Our preliminary data do suggest the Fn receptor is a potential target for intervention of degradative Fn-f pathways in the cartilage disease process.

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