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The Roles of Matrix Molecules in Mediating Chondrocyte Aggregation, Attachment, and Spreading

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Abstract The most abundant macromolecules in cartilage are hyaluronan, collagen, aggrecan, and link protein, which are believed to play roles in maintaining a unique three-dimensional network for a functional joint. This study was designed to investigate the roles of the major extracellular molecules in mediating chondrocyte-matrix interactions. We employed specific approaches to remove components individually or in combination: hyaluronan was digested with hyaluronidase; type II collagen was digested with collagenase; aggrecan expression was inhibited with antisense and β-xyloside approaches; and link protein expression was inhibited with antisense oligonucleotides. Digestion of hyaluronan induced chondrocyte attachment to tissue culture plates, collagen-coated plates, and fibroblast-like chondrocyte cultures, and induced chondrocyte aggregation. Treated chondrocytes exhibited a fibroblast-like morphology, and the effects of hyaluronidase were dose-dependent. Conversely, the effect of collagenase on chondrocyte adhesion and aggregation was far less pronounced. Treatment with Arg-Gly-Asp peptide inhibited chondrocyte-collagen interaction. Chondrocyte attachment was enhanced by antisense oligonucleotides complementary to aggrecan and link protein and by β-xyloside treatment. Nevertheless, hyaluronan seems to predominate over the other molecules in mediating chondrocyte-matrix interactions. J. Cell. Biochem. 79:322–333, 2000.

Key words: chondrocyte; matrix; cartilage; dedifferentiation; interaction

INTRODUCTION

Cartilage is composed of chondrocytes and extracellular matrix (ECM). The ECM molecules are secreted by the cells and in return the matrix protects the cells from damage. In addition, the matrix helps maintain the cell's shape and phenotype and is also a barrier to materials that reach the cells [Buckwalter and Mankin, 1998]. Collagens, noncollageous glycoproteins, and proteoglycans are assembled in a highly organized fashion in the matrix [Wight et al., 1991]. Chondrocytes typically express large quantities of hyaluronan, aggrecan, and type II collagen [Morgelin et al., 1988; Cancedda et al., 1992]. Hyaluronan, originally thought to be a filler in the ECM, had been revealed over the years to be associated with aggregation, proliferation, inflammation, locomotion, and migration activities [Laurent, 1970; Toole, 1984; Miyake et al., 1990; Catterall et al., 1995]. As part of the matrix, hyaluronan interacts with other matrix molecules. It has been reported that hyaluronan interacts with link protein and aggrecan, via tandem repeats [Goetinck et al., 1987; Grover et al., 1994; Watanabe et al., 1997]. This interaction forms a stable ternary complex that is crucial in maintaining cartilage stability [Buckwalter et al., 1984]. The importance of aggrecan has been illustrated in two animal models, nanomelia [Stirpe et al., 1987; Li et al., 1993; Vertel et al., 1993] and mouse cartilage deficiency [Watanabe et al., 1994]. These lethal diseases demonstrate that aggrecan is crucial for cartilage development. Type II collagen is predominately expressed in cartilage. The importance of this molecule lies in its tensile properties, which allow formation of a fibrillar

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meshwork. This fibril meshwork also binds and traps proteoglycans and noncollagenous glycoproteins [Aumailley and Gayraud, 1998; Buckwalter and Mankin, 1998]. Furthermore, collagen also binds integrin [Dürr et al., 1993; Enomoto et al., 1993].

This study was designed to examine the effects of matrix molecules in mediating chondrocyte activities. Because of the intimate relationship between chondrocytes and ECM, changes in the matrix will affect cell properties. In this study matrix molecules were degraded or their synthesis was inhibited and the resulting effects on cell-matrix interactions were monitored. We demonstrated that removal of hyaluronan or type II collagen, or reduction in biosynthesis of aggrecan or link protein, resulted in cell-matrix destabilization in the form of adherent or aggregating cells. Of the matrix molecules studied, hyaluronan's effects were predominant.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), trypsin/EDTA (Cat. No. 15305-014), and oligopeptides (e.g. RGD) were purchased from GIBCO Laboratory (Grand Island, NY). Hyaluronan from human umbilical cord (Cat. No. H1751), hyaluronidase Type 1-S from bovine testes (Cat. No. H3506), hyaluronidase from *Streptomyces hyalurolyticus* (Cat. No. H1136), collagenase (which degrades different types of collagens, Cat. No. C6885), Type II collagen from chicken sterna (Cat. No. C9301), anti-integrin β1 antibody, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, reagents for color development (3-amino-9-ethylcarazole, AEC and 3,3'-diaminobenzidine, DAB), and all chemicals were from Sigma (St. Louis, MO). Hyaluronidase (*Streptomyces hyalurolyticus*) was also purchased from Calbiochem-Novabiochem Corp. (Cat. No. 389561). Antitype II collagen antibody (Clone II6B3) and anti-N-CAM antibody were from Developmental Studies Hybridoma Bank (Iowa University). Antisense and sense oligonucleotides were synthesized by ACGT Corp. (Toronto, Canada). Tissue culture plates (100 mM, 6-well, and 24-well) were from Nalge Nunc International (Naperville, IL). Nylon cell strainers were from Becton Dickinson.

Chicken sterna from 18–19 day embryos (Hamburger and Hamilton strains from Brampton Chick Hatchery Co. Ltd., Brampton, Canada) were removed and rinsed with PBS. Each sternum was cut into pieces and incubated in dissociation medium (0.3% collagenase in HBSS) at 37°C for 30 min. The dissociation medium was changed and the incubation continued for a further 1.5 h. An equal volume of medium (DMEM containing 10% FBS) was added to stop the enzymatic reaction. Cells were collected by passing through cell strainers (40 μm) and centrifuged at 1,100 rpm for 10 min. The newly-isolated chondrocytes were grown as suspension cultures in bacterial petri dishes.

Chondrocyte Aggregation Assay

24-well tissue culture plates were coated with 1.5% agarose gel, on which suspension chondrocytes were plated at a density of 10^5 cells/ml. Hyaluronidase was added to each well at 0–50 units/ml and maintained at 37°C for 24 h in a tissue culture incubator. Each treatment was performed in triplicate. Chondrocyte aggregation was monitored using an inverted light microscope and then photographed. Cells aggregated as conglomerates and were removed by passage through 40 μm cell strainers. The individual chondrocytes were suspended in culture medium and cell number, determined using a cytometer, was subtracted from the control number.

Chondrocytes were also treated with combinations of hyaluronidase and collagenase. In 1.5% agarose-coated 24-well plates, various concentrations of collagenase (0–0.5 mg/ml) were added to untreated cells or cells treated with 50 units per ml of hyaluronidase (at 37 °C for four h). In another set, various concentrations of hyaluronidase (0–50 units/ml) were added to untreated cells or cells treated with 0.05 mg/ml of collagenase. The degree of aggregation was determined as above.

Chondrocyte Adhesion Assay

Suspension chondrocytes were seeded to 24-well tissue culture plates at a density of 10^4 cells/ml, to which hyaluronidase was added to final concentrations of 0–50 units/ml. Each treatment was performed in triplicate. The cultures were maintained at 37°C for 24 h. Attachment of chondrocytes to the tissue culture

plates was monitored using an inverted light microscope. Unattached chondrocytes were removed by aspiration, and the cultures were washed with PBS to remove weakly adherent chondrocytes. Spreading of the adherent chondrocytes was photographed. The cells were then harvested by incubation with 100–500 μl EDTA (20 mM) and cell number was either determined by using a cytometer or by counting cells in randomly selected fields.

The effect of hyaluronidase on chondrocyte adhesion was also tested using 5 units/ml on 24-well plates with a density of 2.5×10^5 cells/ml. The cultures were maintained in an incubator for various time periods, and cell adhesion was assayed as above. Suspension chondrocytes were also incubated with 0.2 mg/ml collagenase for various time periods to test its effect on chondrocyte adhesion.

Chondrocytes were also treated with combinations of hyaluronidase and collagenase. Various concentrations of collagenase (0–0.2 mg/ml) were added to untreated cells or cells (5×10^4 cells/ml) treated with 50 units per ml of hyaluronidase. In another experiment, various concentrations of hyaluronidase (1–50 units/ml) were added to untreated cells or cells treated with 0.025 mg/ml of collagenase. The number of adherent chondrocytes was counted as above.

Suspension chondrocytes (5×10^4 cells/ml) were cultured on tissue culture plates (100 mM) and bacterial petri dishes in the presence of hyaluronidase (20 units/ml) at 37°C overnight in an incubator. Cells in suspension were removed and the plates were washed with PBS once to remove unattached cells. The number of adherent cells remaining on the plates was counted.

Hyaluronan or type II collagen was coated on 24-well tissue culture plates at a concentration of 1 mg/ml at 4°C overnight. The plates were rinsed with standard medium (DMEM plus 5% FBS). Suspension chondrocytes at a density of 10^5 cells/ml were treated with hyaluronidase (20 units/ml) and then incubated in collagen-coated plates at 37°C for one day, or treated with collagenase (0.1 mg/ml) and then incubated in hyaluronan-coated plates at 37°C for four days. The number of adherent cells per randomly selected field was counted.

Chondrocyte-Chondrocyte Interaction Assay

Suspension chondrocytes (at a density of 10^5 cells/ml) were incubated with hyaluronidase (200 units/ml), collagenase (1 mg/ml), or culture medium at 37°C for 30 min, followed by incubation with fibroblast-like chondrocyte monolayers for one h. After incubation, unbound chondrocytes were removed, and the cultures were washed with PBS. The interaction of suspension chondrocytes with fibroblast-like chondrocytes was examined and photographed. The number of suspension chondrocytes attached was determined and analyzed by *t*-test.

Hyaluronidase-treated chondrocytes were incubated with fibroblast-like chondrocyte monolayer cultures in the presence of antibody against integrin, N-CAM, or normal serum. After incubation, unbound chondrocytes were removed and washed with PBS as above. The number of suspension chondrocytes attached to the monolayer chondrocytes was determined. Statistic analysis was performed by *t*-test.

Chondrocytes (2×10^5 cells in 100 μl) were incubated with or without 10 μl RGD peptide (1 mg/ml) for one h. They were then diluted to 1 ml with medium, treated with or without hyaluronidase (20 units/ml), introduced into 24-well plates (coated with type II collagen, 1 mg/ml, overnight at 4°C), and maintained for three h. The cultures were washed briefly with PBS and adherent cells were counted in random fields. In another experiment, 2×10^5 chondrocytes (per ml) treated with or without hyaluronidase (20 units/ml at 37°C for one h) were introduced into 24-well tissue culture plates coated with or without RGD peptide (1 mg/ml) and maintained for three h. The cultures were washed briefly with PBS and adherent cells were counted in random fields and analyzed by *t*-test.

Treatment With Antisense and β-xyloside

The antisense technique has been successfully used in our studies [Zhang et al., 1998; Zhang et al., 1999]. Sense (gctctgctgacccccg, gaagtgatgtatgggat, and gtcgaccagactgtcag) and antisense (gccgatccactgata, atctggagcactggt, and agacgtggcagcagtgtc) oligonucleotides complementary to aggrecan were synthesized based on the sequence reported [Li et al., 1993]. Sense (tcaacagctggctcaggaacccacgaaatccagg and agaccagtaacagctgcagcccc) and antisense (atactctccgtagctctcactgtg and ggggctgcagctgttactgggtct) oligonucleotides were synthesized based on link protein sequence reported [Deak et al., 1986]. Chondrocytes (10^3 cells/well) were treated with oligonucleotides at a final concen-

tration of 4.2 μM. Cell attachment was examined four days later.

p-Nitrophenyl β-D-xylopyranoside was used as a false acceptor for chondroitin sulfate [Robinson and Gospodarowicz, 1984; Potter-Perigo et al., 1992]. Suspension chondrocytes at a density of 10^4 cells/ml were seeded to 24-well tissue culture plates. Cells were treated with xyloside at concentrations of 0, 0.5, 1.0, and 2.0 mM for four days. DMSO, a xyloside solvent, was added to cultures at the same volume as a negative control.

Analysis with Flow Cytometry

Analysis of interactions of extracellular molecules with chondrocyte cell surfaces was performed using a technique previously described by us [Cao and Yang, 1999]. These matrix molecules interact with either cell surface receptors or cell surface binding molecules and can be immunostained by antibodies specific against the molecules. Briefly, cells were incubated with hyaluronidase. The primary antibodies (antilink protein or antitype II collagen) were introduced and incubated for one hour at 4°C. Cells were briefly washed with PBS, and the FITC-conjugated goat antimouse IgG was added and incubated in the dark for one hour at 4°C. Untreated cells (without hyaluronidase treatment) and cells lacking primary antibody treatment were used as controls. After washing, the cells were analyzed with flow cytometry.

Staining of Chondrocyte Cultures

Culture medium was removed by absorption (using strips of filter paper) and then air-dried. In this way, chondrocytes and the matrix network surrounding the chondrocytes were lowered to the surface of the plates with minimal disruption. Chondrocytes plus matrix were fixed with 95% ethanol (prechilled at –20°C) for 10 min followed by 4% paraformaldehyde for 10 min. The fixed cells were stained with 1:1000 diluted mouse antilink protein monoclonal antibody 4B6 [Binette et al., 1994] or 1:100 diluted antitype II collagen followed by HRP-conjugated goat antimouse IgG antibody. AEC or DAB was used for color development according to the manufacturer's instructions (Sigma).

To examine the distribution of aggrecan in the network, glycosaminoglycan chains were stained with alcian blue. Chondrocytes and matrix were fixed as above. The fixed cultures were washed with 3% glacial acetic acid and then stained with 0.5% alcian blue in 3% glacial acetic acid for one hour. The stained cultures were destained with 3% glacial acetic acid until the background was clear, followed by a brief rinse with PBS. The stained cultures were examined and photographed.

RESULTS

Effect of Hyaluronidase on Chondrocyte Aggregation and Spreading

It has been difficult to study chondrocyte behaviors in culture since the cells tend to attach to tissue culture plates after cell isolation. We have previously developed a technique to culture chondrocytes in suspension. With this technique, it is possible to study how the behavior of chondrocytes is affected by the matrix molecules. Chondrocytes cultured in this way produce high levels of matrix molecules in which chondrocytes are embedded inside the matrix; therefore this system mimics chondrocytes embedded in cartilage.

Suspension chondrocytes were treated with hyaluronidase (HAse) and incubated in agarose-coated 24-well tissue culture plates. While chondrocytes in the control cultures still grew as suspension cultures (Fig. 1A), chondrocytes treated with HAse (5 units/ml) began to aggregate after one day of incubation (Fig. 1B). The size and tightness of these aggregates grew with increasing concentrations of hyaluronidase (Fig. 1 C-F). The treatment had a dose-dependent effect on chondrocyte aggregation, reaching a plateau at 20 units per ml (Fig. 1M).

The suspension chondrocytes were also treated with hyaluronidase at the same concentrations in tissue culture plates (24-well). After one day of incubation, chondrocytes started to attach to the plates. As the concentrations of hyaluronidase increased, not only did more chondrocytes become attached, but the cells also began to elongate (Fig. 1G-L). The effect was dose-dependent, reaching a plateau at 20 units per ml (Fig. 1N).

Suspension chondrocytes were treated with hyaluronidase, and the binding of aggrecan, link protein, and type II collagen to the chondrocyte surface was investigated. Hyaluronidase treatment diminished the intensity of alcian blue staining (Fig. 2A-F), indicating a reduction in proteoglycan (aggrecan) binding. Cultures were also probed with antilink protein

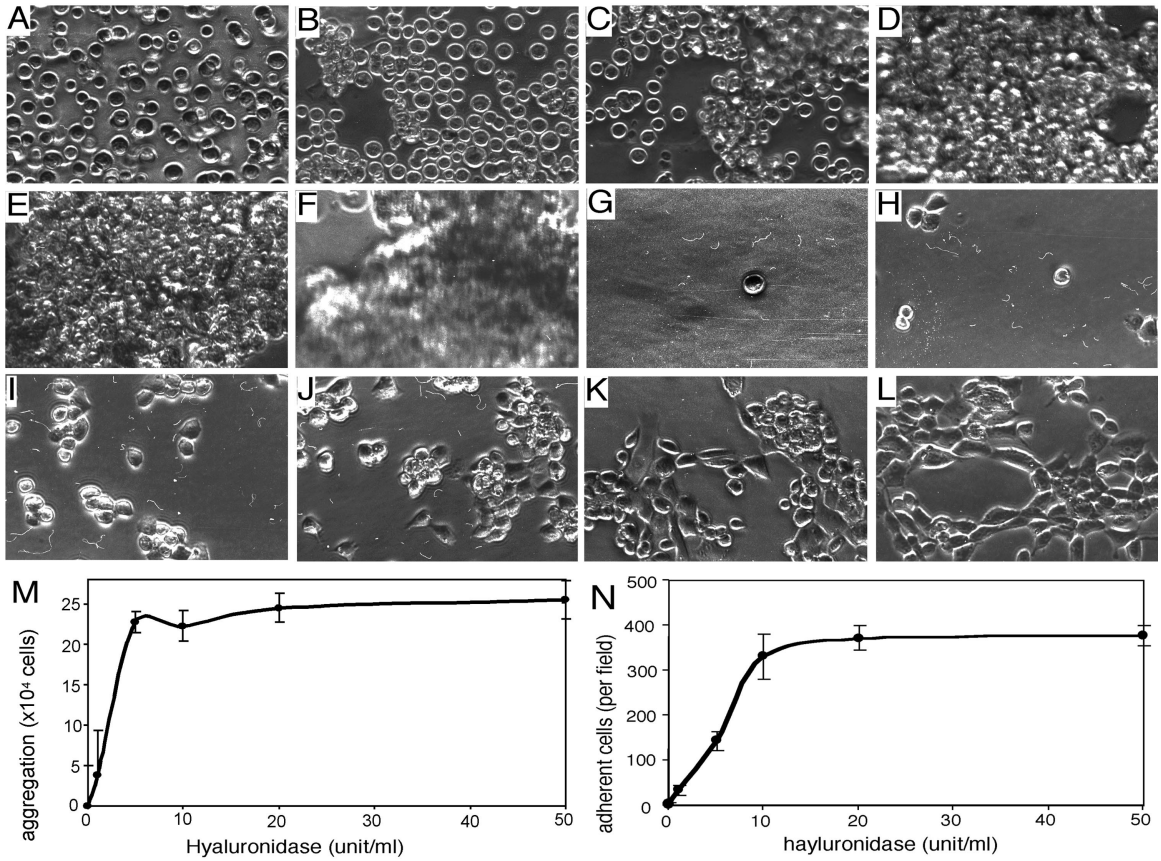

Fig. 1. Hyaluronidase affects chondrocyte aggregation and adhesion. Suspension chondrocytes, maintained in agarose-coated 24-well tissue culture plates at a density of 10^5 cells/ml, were treated at 37°C for 24 h with hyaluronidase at the following concentrations (units/ml): 0 (**A**), 1 (**B**), 5 (**C**), 10 (**D**), 20 (**E**), and 50 (**F**). Hyaluronidase treatment had a dose-dependent effect on aggregation, reaching a plateau at 20 units/ml. In attachment assays, suspension chondrocytes cultured on tissue culture plates were treated with hyaluronidase (units/ml): 0 (**G**), 1 (**H**), 5 (**I**), 10 (**J**), 20 (**K**), and 50 (**L**). Hyaluronidase treatment triggered chondrocyte attachment and spreading in a dose-dependent manner, reaching a plateau at 20 units/ml. Aggregating cells (**M**) and adherent cells (**N**) were counted. Hyaluronidase at a concentration of 5 units/ml was sufficient to trigger chondrocyte aggregation or attachment.

or antitype II collagen antibody. The level of staining was greatest with untreated chondrocytes. In the treated cells, link protein (Fig. 2G-I) and type II collagen (Fig. 2J-L) staining decreased as the concentrations of hyaluronidase increased. To confirm these results, suspension chondrocytes treated with or without hyaluronidase were probed with antilink protein or antitype II collagen antibodies and analyzed by flow cytometry. The levels of link protein binding (Fig. 2M) and type II collagen binding (Fig. 2N) to chondrocytes were reduced as the concentrations of hyaluronidase increased.

Effect of Matrix Molecules on Chondrocyte Attachment and Aggregation

In cartilage, aggrecan and link protein are the major proteoglycan and glycoprotein macromolecules, respectively, and so we also examined the effect of these molecules on chondrocyte attachment. Inhibition of aggrecan expression was performed by treatment with antisense oligonucleotides complementary to aggrecan. Chondrocyte adhesion increased by antisense treatment (Fig. 3A). Antisense oligonucleotides complementary to link protein also enhanced chondrocyte attachment, but to a lesser extent (Fig. 3B). In an alternate experiment, xylosides were introduced to prevent glycosaminoglycan (GAG) chain attachment to the core protein of aggrecan. Xyloside treatment stimulated chondrocyte attachment (Fig. 3C).

The effects of collagen on chondrocyte aggregation and attachment were tested using collagenase, either alone or combined with hyaluronidase. In attachment assays, increased

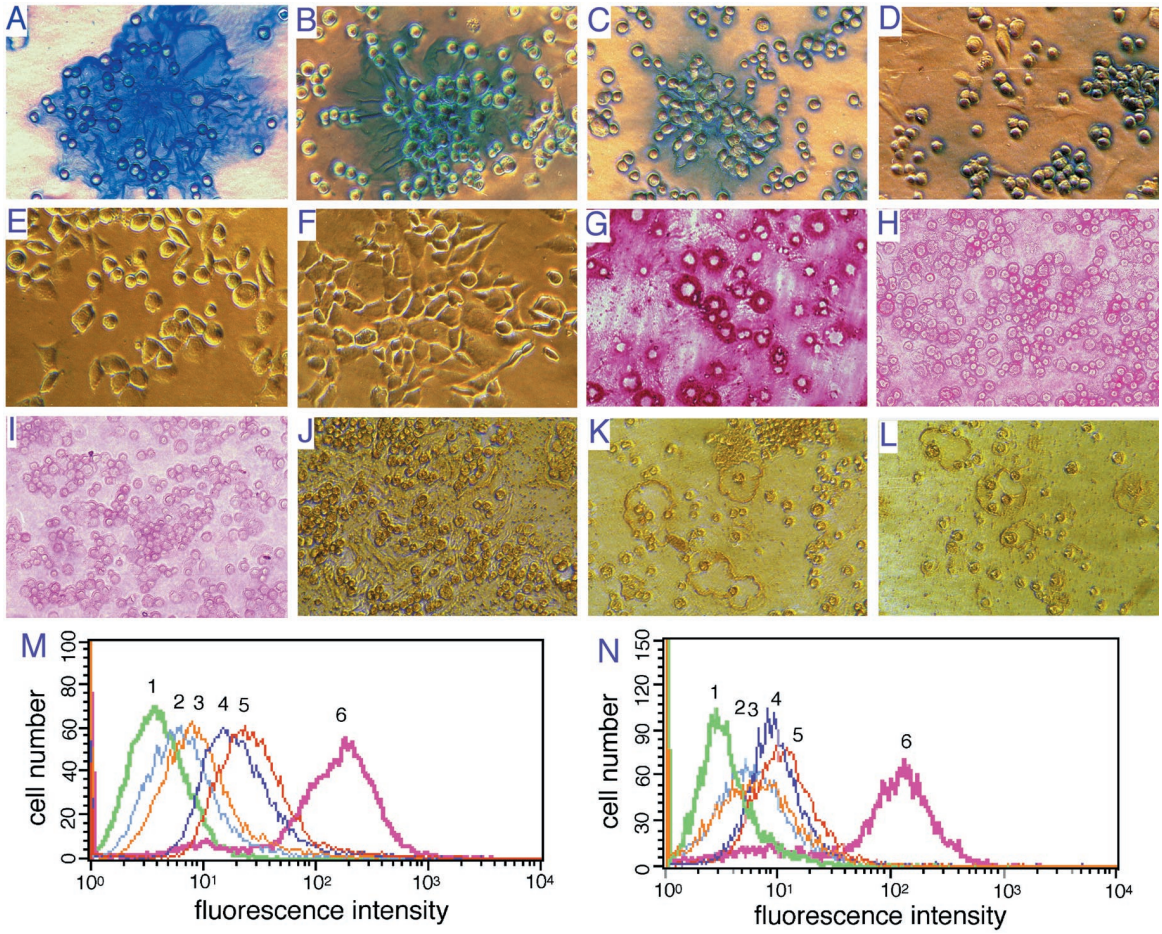

Fig. 2. Hyaluronidase affects binding of aggrecan, type II collagen, and link protein to chondrocyte cell surfaces. Suspension chondrocytes were incubated with hyaluronidase (units/ml): 0 (**A**), 1 (**B**), 5 (**C**), 10 (**D**), 20 (**E**), and 50 (**F**) and fixed after one day with 95% ethanol and stained with alcian blue. Levels of staining decreased as the hyaluronidase concentrations increased. Chondrocytes treated with hyaluronidase at 0 (**G** and **J**), 5 (**H** and **K**), and 10 (**I** and **L**) units/ml were also stained with monoclonal antibody against link protein (**G-I**) and type II collagen (**J-L**). Hyaluronidase treatment reduced link protein and type II collagen staining. Chondrocytes treated with hyaluronidase (0–50 units/ml) were probed with monoclonal antibody against link protein (**M**) and type II collagen (**N**) and analyzed by flow cytometry. The levels of staining decreased as the concentrations of hyaluronidase increased. Peak 1, negative control (no primary antibody); 2, cells treated with 50 units hyaluronidase; 3, 10 units; 4, 5 units; 5, 1 unit and 6, 0 unit.

concentrations of collagenase promoted chondrocyte attachment, suggesting that collagen is required to maintain chondrocytes in suspension cultures (Fig. 4A). In the presence of hyaluronidase, increased concentrations of collagenase reduced chondrocyte attachment, suggesting further that collagen played a role in mediating chondrocyte attachment (Fig. 4A). In aggregation assays, collagenase alone had little effect on chondrocyte aggregation. In the presence of hyaluronidase (50 units/ml), however, low concentrations of collagenase seemed to induce chondrocyte aggregation, but this effect decreased as the concentrations of collagenase increased (Fig. 4B).

In the opposite scenario, collagenase concentration was kept constant while hyaluronidase concentration varied. In attachment assays, addition of hyaluronidase did not change the adhesion trend. In the presence of a low concentration of collagenase (0.05 mg/ml), hyaluronidase-induced chondrocyte attachment was enhanced (Fig. 4C). As expected, hyaluronidase induced chondrocyte aggregation with or without collagenase, suggesting a predominant effect of hyaluronidase on chondrocyte aggregation (Fig. 4D). It should be noted that at the low concentrations of hyaluronidase (1–5 units/ml), a low concentration of collagenase (0.05 mg/ml) enhanced the aggregation, suggesting that colla-

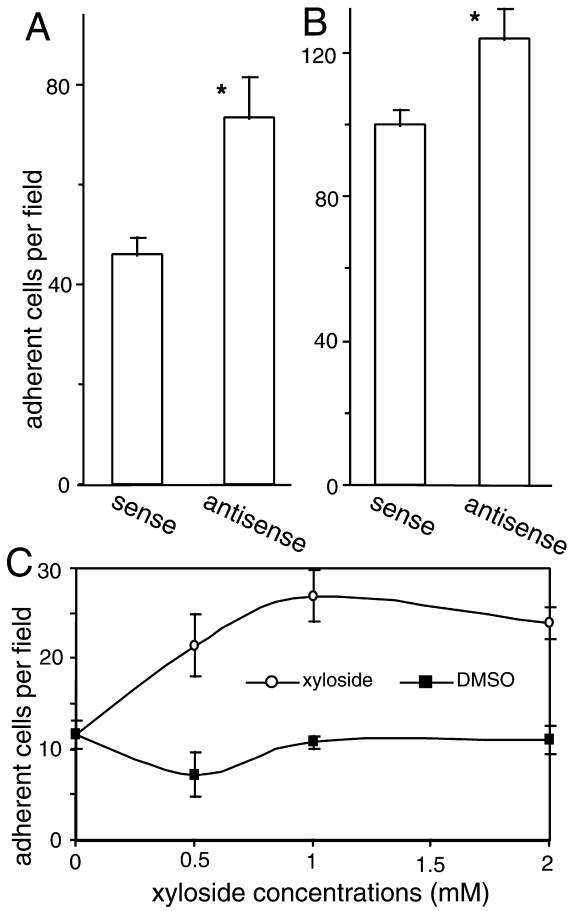

Fig. 3. Effect of aggrecan on chondrocyte adhesion. Sense and antisense oligonucleotides complementary to aggrecan (**A**) and link protein (**B**) were added to the culture. After four days, the number of adherent chondrocytes was greater in antisense treatment. Incubation with β-xyloside, which inhibits elongation of GAG chains, increased chondrocyte attachment (**C**).

gen also played a role in mediating chondrocyte aggregation (Fig. 4D).

The effects of hyaluronan and collagen were then compared by treating suspension chondrocytes with high concentrations of hyaluronidase (50 unit/ml) and collagenase (0.2 mg/ml). Dramatic increases in cell attachment were observed over the entire time period of hyaluronidase treatment. However, in the case of collagenase treatment alone, minimal changes were observed (Fig. 4E), again suggesting that hyaluronidase had a greater effect on chondrocyte adhesion than collagenase.

Hyaluronan Reduced Chondrocyte Attachment to Various Substrates

To test chondrocyte attachment under different conditions, various substrata were used. Collagen-coated wells were employed to test the effect of hyaluronidase digestion on chondrocyte attachment. The results indicated that the number of adherent chondrocytes was significantly greater with hyaluronidase treatment as compared to the untreated chondrocytes (Fig. 5A). In addition, chondrocytes treated with collagenase and incubated in hyaluronan-coated wells also showed increased attachment versus untreated cells (Fig. 5B). In another test, bacterial petri dishes versus tissue culture plates were compared. Suspension chondrocytes were seeded to bacterial petri dishes and tissue culture plates were treated with hyaluronidase to test cell attachment. The number of adherent chondrocytes was slightly higher when incubated in tissue culture plates (Fig. 5C). However, the number of chondrocytes attached to bacterial plates was still significantly greater than that observed in untreated cells.

The effects of hyaluronan and collagen were also tested using chondrocyte-chondrocyte interaction assays. Suspension chondrocytes treated with hyaluronidase or collagenase were introduced into fibroblast-like chondrocyte cultures. The effect of hyaluronidase on suspension chondrocyte attachment versus collagenase was several fold greater (Fig. 6A). To investigate candidate molecules mediating this cell-cell interaction, antibody against β1-integrin or N-CAM was included with hyaluronidase-treated suspension chondrocytes before addition to fibroblasts-like chondrocyte cultures. After treatment was performed, as described above, it was observed that anti-integrin antibody inhibited cell-cell interaction (Fig. 6B). The proportion of adherent cells observed in control cultures (Fig. 6C) and cultures treated with hyaluronidase plus anti-integrin (Fig. 6E) was similar. Cell adhesion increased when the suspension culture was treated only with hyaluronidase (Fig. 6D).

To further confirm that the binding of integrin to collagen mediates chondrocyte-collagen interaction, chondrocytes, with or without hyaluronidase pretreatment, were cultured on collagen-coated plates, to which RGD peptide (ligand of integrin) was added. Addition of RGD peptide inhibited the attachment of hyaluronidase-treated chondrocytes to collagen-coated plates (Fig. 7A). In an alternative assay, RGD peptide was coated on tissue culture plates, to which chondrocytes, pretreated with or without hyaluronidase, were added. More

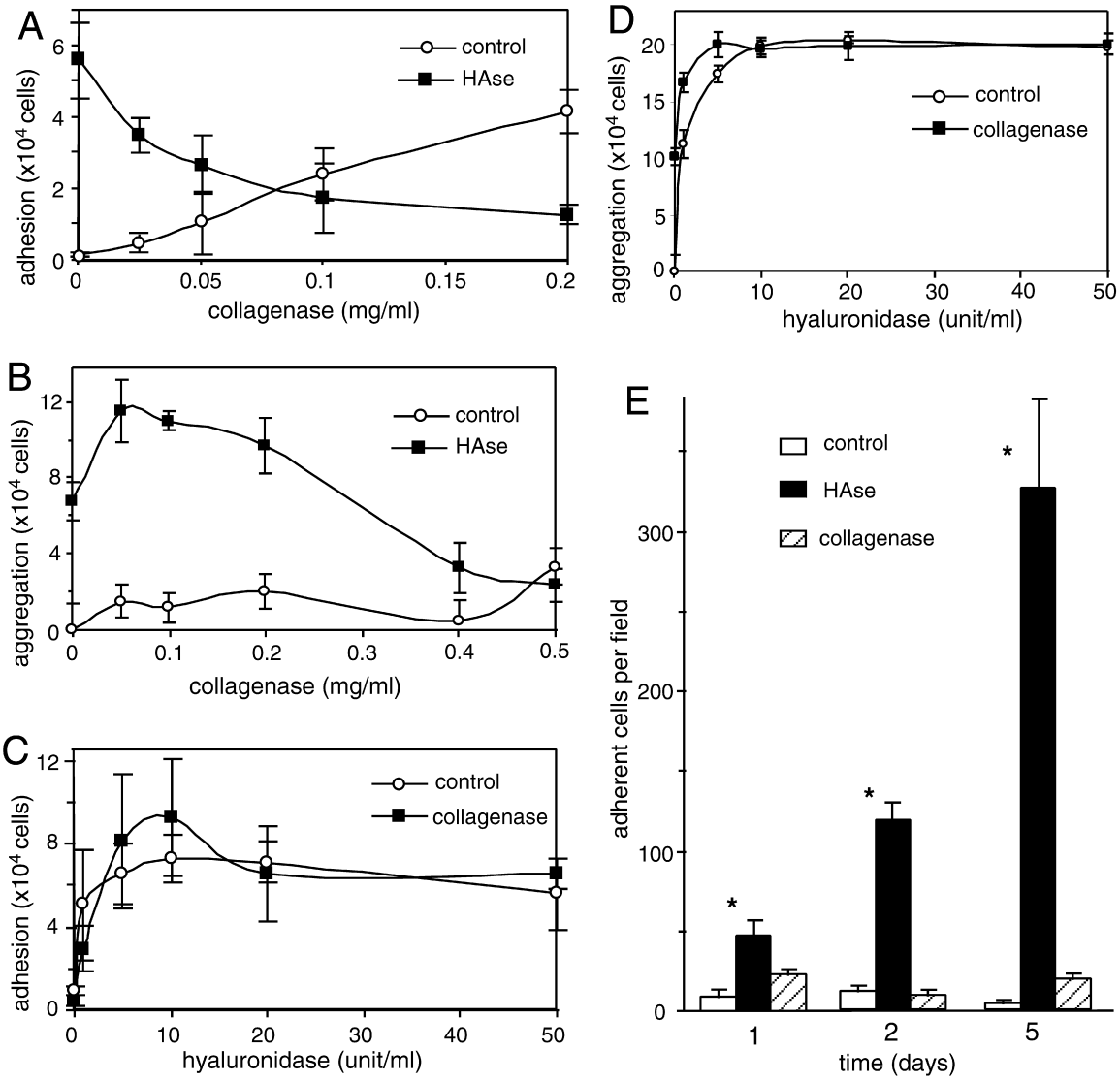

Fig. 4. Effect of simultaneous addition of hyaluronidase (HAse) and collagenase on chondrocyte aggregation and attachment. Suspension chondrocytes were treated with HAse and collagenase simultaneously for four h. **A** and **C**: untreated plates. **B** and **D**: agarose-coated plates. In the absence of HAse, increased concentration of collagenase enhanced chondrocyte attachment (**A**). Combined with 50 units (per ml) of HAse, increased concentrations of collagenase reduced cell attachment. In aggregation assay (**B**), nonaggregating cells were counted and subtracted from the control value. Treatment with collagenase alone had little effect on cell aggregation. However, in the presence of HAse, low concentrations of collagenase induced cell aggregation, while high concentrations inhibited cell aggregation. **C** and **D**: The effects of HAse on chondrocyte attachment and aggregation were tested. In cell attachment assays, increased concentrations of HAse stimulated chondrocyte attachment, and collagenase, at low concentrations (0.025 mg/ml), also promoted this process (**C**). In aggregation assays, increased concentrations of HAse stimulated chondrocyte aggregation. At low concentrations of HAse (1–5 units per ml), collagenase enhanced the aggregation (**D**). **E**: Cell attachment was determined at 1, 2, and 5 days. HAse had significantly greater effect on chondrocyte adhesion than did collagenase ($n = 3$; * $P < 0.05$).

chondrocytes bound to RGD peptide-coated plates than to uncoated plates (Fig. 7B).

DISCUSSION

Cartilage in the joints supports the weight of the whole body. The polymeric network of cartilage is an essential structural requirement for its load-bearing properties [Wight et al., 1991]. In this network, the most abundant macromolecules are type II collagen, proteoglycans, and hyaluronan, and the major proteoglycan is aggrecan. Cartilage, once injured by trauma or disease, is not repaired. After damage occurs, chondrocyte population increases

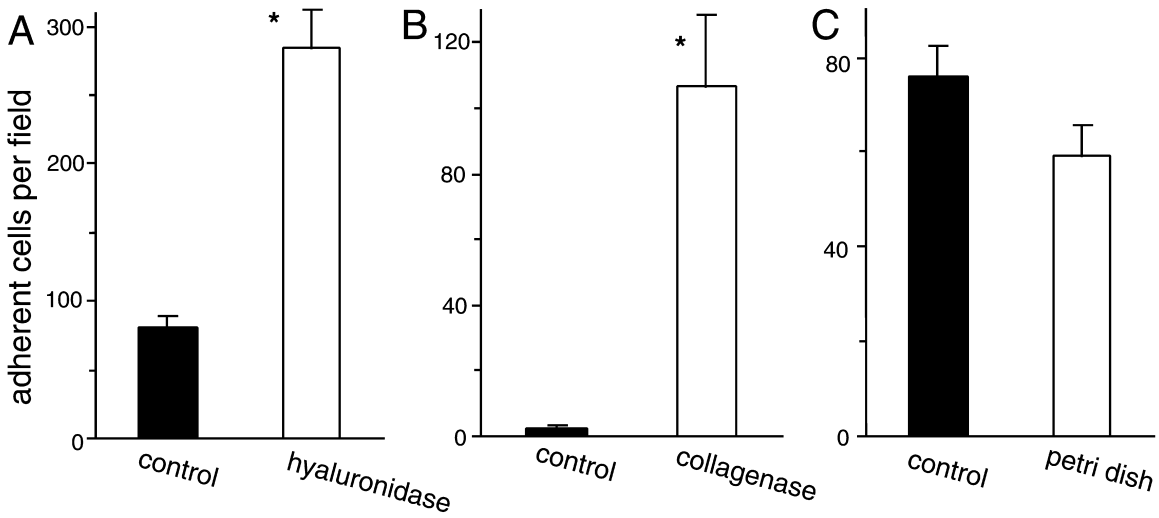

Fig. 5. Chondrocyte adhesion to collagen, hyaluronan (HA), and petri dishes. **A**: Suspension chondrocytes were incubated in collagen-coated plates in the presence of hyaluronidase (20 units/ml). Untreated chondrocytes were used as controls. Treatment with hyaluronidase significantly enhanced attachment. **B**: Chondrocytes were incubated in hyaluronan-coated plates at 37°C for four days in the presence of collagenase (0.1 mg/ml). Treatments with collagenase enhanced the attachment. **C**: Chondrocytes were seeded to bacterial petri dishes in the presence of hyaluronidase (20 units/ml) and incubated at 37°C overnight. Tissue culture plates were used as controls. Hyaluronidase treatment induced chondrocyte attachment to both types of plates. (n = 3; * $P < 0.05$)

through proliferation of chondrocytes and/or chondrogenesis of mesenchymal cells. However, these newly formed cells cannot migrate to the damaged area because they are buried within the extracellular matrix. By uncovering the mechanism of cell-matrix interaction, therefore, better therapeutic techniques may be developed.

One of the ECM's most important roles is to maintain tissue structure, and this is particularly relevant in cartilage where ECM molecules occupy the majority of cartilage volume, and chondrocytes are sparsely embedded within the matrix structure. Over-expression of some of these matrix molecules leads to cell-matrix destabilization [Yang et al., 1997; Cao et al., 1998], while removal of matrix results in apoptosis [Meredith et al., 1993; Frisch et al., 1994; McGill et al., 1997]. In this study, we used enzymatic techniques to degrade selected ECM components, and antisense to inhibit synthesis of matrix molecules. The resulting effects on cell-matrix interaction were monitored.

In physiological conditions, the matrix-chondrocyte interaction is very important. In cell culture, chondrocytes can be maintained as suspension culture only when there are sufficient amounts of matrix molecules to form three-dimensional conformations. This is supported by our finding that removal of matrix leads to apoptosis of chondrocytes [Cao and Yang, 1999; Cao et al., 1999]. The focus of this research is to understand the role of the matrix in maintaining cells in suspension. Previous studies from our laboratory demonstrated that aggrecan and link protein mediate cell adhesion, and that over-expression of these matrix molecules results in either chondrocyte aggregation or adhesion to plates. This suggests that any imbalance in matrix molecule expression will lead to perturbation of the three-dimensional structure and hence to cell-matrix destabilization. The current study confirms and extends these findings using inhibition instead of over-expression of matrix components.

In a functional cartilage, a ternary complex of aggrecan, hyaluronan, and link protein with low dissociation constant is thought to maintain the stable matrix structure [Hascall and Hascall, 1983]. Previous work indicated that exogenous expression of aggrecan or link proteins through transfection of cells reduces cell-substratum adhesion. However, introduction of both link protein and aggrecan did not yield significant change in chondrocyte adhesion [Yang et al, 1997]. In our present study, removal of aggrecan or link protein using anti-

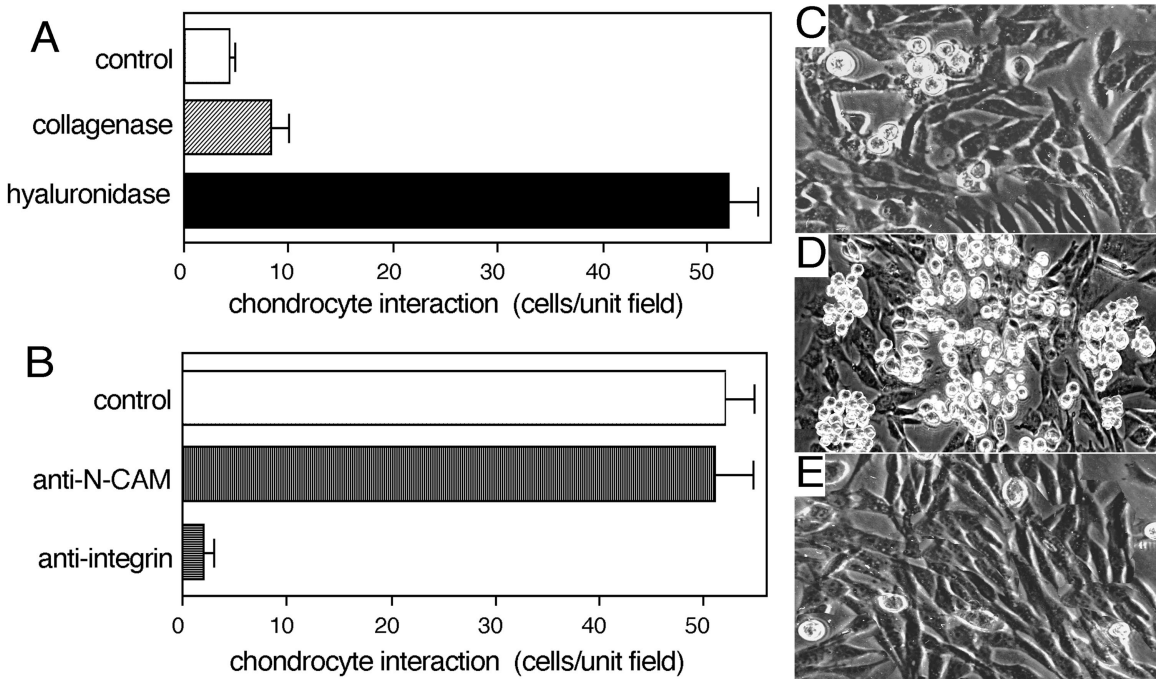

Fig. 6. Effect of hyaluronidase and anti-β 1 integrin antibody on chondrocyte interactions. **A**: Suspension chondrocytes were incubated with hyaluronidase (200 units/ml), collagenase (1 mg/ml) or culture medium at 37°C for 30 min. The treated chondrocytes were added to subconfluent fibroblast-like chondrocytes and maintained at 37 °C for one h. Treatment with hyaluronidase significantly enhanced the interaction as compared with the untreated control. Treatment with collagenase had a lesser effect. **B**: Antibody against integrin or N-CAM was added to hyaluronidase-treated suspension chondrocytes when these cells were mixed with the fibroblasts-like chondrocytes. Anti-integrin antibody inhibited the interaction as compared with a control treated with regular serum or anti-N-CAM antibody. Typical results of the interaction between the suspension chondrocytes and the fibroblast-like chondrocytes in the presence of normal serum (**C**), hyaluronidase (**D**), and anti-integrin antibody + hyaluronidase (**E**) are shown (n = 4).

sense oligonucleotides had a similar outcome. Furthermore, this effect is greatly magnified by the third molecule of the ternary complex: hyaluronan degradation produced a high degree of chondrocyte attachment. Given that one hyaluronan molecule has the capacity to bind several molecules of link protein and aggrecan, this dramatic effect is easily understood. It should be noted that we compared the effects of hyaluronidase that was taken from different sources (i.e. bovine testes and *Streptomyces hyalurolyticus*), and no major difference could be detected (data not shown). This suggests that the observed results of hyaluronidase in cell aggregation and adhesion occurred because of its effect on hyaluronan rather than on chondroitin sulfate proteoglycans. Due to the large quantities of hyaluronidase needed, we had to use bovine hyaluronidase in our experiments for cost-effective reasons.

Besides receptors for hyaluronan, receptors for collagen such as integrins are also present

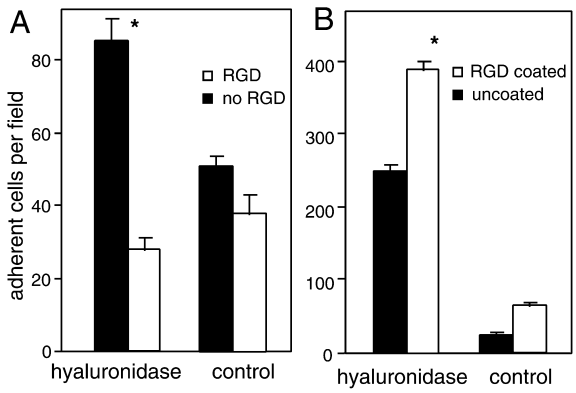

Fig. 7. RGD peptide inhibits chondrocyte-collagen interaction. **A:** Chondrocytes were pretreated with or without RGD peptide (0.1 μg/ml) and/or hyaluronidase (50 units/ml) then added to collagen-coated plates and incubated at 37°C for one h. Addition of RGD peptide abolished the effect of hyaluronidase. **B**: Chondrocytes treated with or without hyaluronidase were added to RGD peptide-coated plates (1 mg/ml). More chondrocytes bound to the coated plates under both hyaluronidase-treated and untreated conditions than to the non-coated plates (n =3; * $P < 0.05$).

on the cell surface. Integrins, heterodimers of α and β subunits, mediate cell activities such as migration and differentiation by outside-in signaling. In suspension cultures maintained on agarose-coated plates, treatment with hyaluronidase detached cells from the matrix, resulting in cell aggregation since the chondrocytes cannot attach to agarose. This cell-cell interaction is mediated by integrins. Integrins are known to bind the ECM molecules such as type I and type II collagens, and fibronectin [Dürr et al., 1993; Loeser, 1993; Enomoto et al., 1993]. We demonstrate here that blockage of integrin with RGD peptides or β1 integrin antibody prevents hypertrophic chondrocytes from interacting with collagen-coated plates or fibroblast-like chondrocytes, respectively. Therefore, this implies that type II collagen and β1 integrins mediate the chondrocyte-chondrocyte interaction we observed.

Aside from its role in chondrocyte adhesion, hyaluronan influences other matrix molecules. A reduction of the pericellular matrix volume occurs when digested with hyaluronidase. We demonstrated that the interactions of aggrecan, type II collagen, and link protein with cell surface decreased when hyaluronan is removed. Since aggrecan and link protein bind to hyaluronan, removal or degradation of hyaluronan causes them to dissociate from the cell proximity. Matrix metalloproteases (MMP) have also been identified as being responsible for matrix degradation and are controlled by a specific tissue inhibitor of metalloprotease (TIMP). Hyaluronan is known to stimulate TIMP-1 production, and high molecular weight hyaluronan is more efficient at eliciting TIMP-1 production than that of lower molecular weights [Yasui et al., 1992; Kikuchi et al., 1996]. Following hyaluronidase treatment, the low molecular weight hyaluronan fails to generate sufficient TIMP-1 to prevent MMP activity. Collagenase also belongs to the MMP family, so it is likely that type II collagen will be diminished as well. Furthermore, studies have shown that expression of type II collagen is also dependent on chondrocyte phenotype [Cancedda et al., 1992]. In cells with elongated and flattened morphologies, type II collagen synthesis is arrested. Cleavage of hyaluronan with hyaluronidase resulted in chondrocyte attachment to the tissue culture plates, and elongated morphologies ensued. These observations correspond to the results from our study.

The extracellular matrix is important in mediating chondrocytes in a three-dimensional network. Removal of just one type of matrix molecule (i.e. hyaluronan) can cause a cascade effect, preventing other matrix molecules from interacting with cells and severing cell-matrix interaction. This ultimately leads to the destruction of the three-dimensional network. Our studies also indicated that hyaluronan played a predominant role in maintaining chondrocytes in suspension culture. Degradation of hyaluronan has a far greater effect on inducing chondrocyte aggregation, attachment, and spreading than any other matrix molecule tested in this study.

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