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Cell Biology

Identification of an adhesion factor for chondrocytes

(cell attachment/cartilage/chondronectin/cell matrix/collagen)

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ABSTRACT **The attachment of chondrocytes to collagen substrates is stimulated by serum but not by fibronectin. The active material in serum was partially purified and was shown to be a protein by its sensitivity to trypsin and heat and its chromatographic properties. This factor, which we have named *chondronectin*, is distinct from fibronectin and does not stimulate fibroblast attachment. Because material with similar attachment-enhancing activity is produced by chondrocytes and is extractable from cartilage, chondronectin may be a chondrocyte-specific attachment protein.**

Collagen substrates promote the attachment (1), growth (2), and differentiation (3, 4) of cells in culture and presumably in vivo. It is now known that fibroblasts and some other cell types do not bind directly to collagen (1, 5). Rather, fibronectin, a large glycoprotein produced by fibroblasts (6) and many other cell types (7–12), binds these cells to collagen (13). Fibronectin is also present in serum (12) at a high concentration (≈200–300 μg/ml) and attaches freshly trypsinized cells to their substrates. Fibronectin-free serum as well as pure fibronectin can be prepared by affinity chromatography on immobilized collagen (14, 15) and used to test the requirements of various cells for attachment to collagen.

Fibronectin, however, does not mediate attachment of all cell types to collagen. Epidermal cells, for example, adhere preferentially to type IV collagen by a process that is not stimulated by fibronectin (16). Many hours are required for epidermal cells to attach, suggesting that they synthesize their own attachment factor, although this has not yet been shown.

Although fibronectin is produced by limb mesenchymal cells (7, 17), it is not produced by differentiated chondrocytes in cartilage (7, 17, 18). Because chondrocytes exist as single cells separated by a matrix of type II collagen and other macromolecules, we have investigated the binding of chondrocytes to collagen and have found that fibronectin is not required for chondrocyte attachment. Rather, chondrocytes use a different protein for attachment that is present in both serum and extracts of cartilage.

EXPERIMENTAL PROCEDURES

Preparation of Cells for Attachment Assays. Chondrocytes were obtained from sternal cartilage of 13- to 16-day embryonic chickens. Sterna were digested with 0.4% collagenase (CLS II, Worthington) in Hanks' balanced salt solution at 37°C for 3 hr. The suspension of chondrocytes was washed with Ham's F12 culture medium (F12) in the absence of serum and filtered through several layers of 20-μm mesh Nitex nylon monofilament (Tetke, Elmsford, NY) to remove large clumps of cells and debris.

Chinese hamster ovary (CHO) cells were maintained in a 5% CO_2/95% air mixture at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1 mM L-proline, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were detached from tissue culture flasks with 0.1% trypsin/0.1% EDTA in phosphate-buffered saline, washed, and filtered as described for chondrocytes.

Attachment Assay. Collagen-coated dishes were prepared by allowing 10 μg of collagen in solution to air dry at room temperature on bacteriologic plastic petri dishes (35 mm, Falcon). Dishes prepared in this manner were preincubated for 60 min with 1 ml of F12 culture medium containing the material to be tested for attachment activity. Cells (1.5×10^5 in 0.1 ml of F12) were then added and incubated for an additional 90 min. Unattached cells were removed by rinsing the dishes with phosphate-buffered saline. Attached cells were detached with 0.1% trypsin/0.1% EDTA. The number of attached cells was determined by using an electronic cell counter (Coulter). Duplicate or triplicate assays were run for each determination. Except when noted, type II collagen, isolated from a rat chondrosarcoma (19) was used as a substrate. Type I collagen was isolated from the skin of lathrytic rats (20), type III from fetal calf skin (21), and type IV from a murine sarcoma (22).

Chromatographic Procedures. *Affinity chromatography.* Fibronectin was removed from serum by adsorption to a collagen-Sepharose 4B column (14, 15). In these experiments, the adsorbant was first equilibrated with F12 medium and then incubated with fetal calf serum (GIBCO) for 1 hr at room temperature. Unbound material was washed from the column with F12 medium, and bound fibronectin was eluted with 1 M KBr in 0.05 M Tris·HCl, pH 5.3, containing 0.025 M 6-aminohexanoic acid. The isolated fibronectin fraction was dialyzed against fresh F12 before use.

DEAE-cellulose column chromatography. Chondrocyte attachment activity was precipitated from serum with ammonium sulfate (30% of saturation). The precipitated material was redissolved in 6 M urea, dialyzed against 0.05 M Tris·HCl, pH 7.4, and chromatographed at 4°C on a DEAE-cellulose column equilibrated with the same buffer. Bound material was eluted from the column with a linear gradient of 0 to 1 M NaCl. Fractions (5 ml) were collected and aliquots of each fraction were dialyzed against F12 and assayed for attachment-enhancing activity for chondrocytes and for CHO cells.

RESULTS

Characteristics of Chondrocyte Attachment. The role of serum and of fibronectin in the attachment of chondrocytes to collagen was investigated. These were compared to CHO cells, which are known to require fibronectin for attachment to collagen (ref. 1 and Fig. 1). About 20% of the chondrocytes and 10% of the CHO cells attached over a 3-hr period to the collagen substrate in the absence of serum. Serum stimulated the adhesion of both cell types, although a greater serum concentration was required for maximal attachment of chondrocytes than of CHO cells. In addition, the CHO cells attached more rapidly

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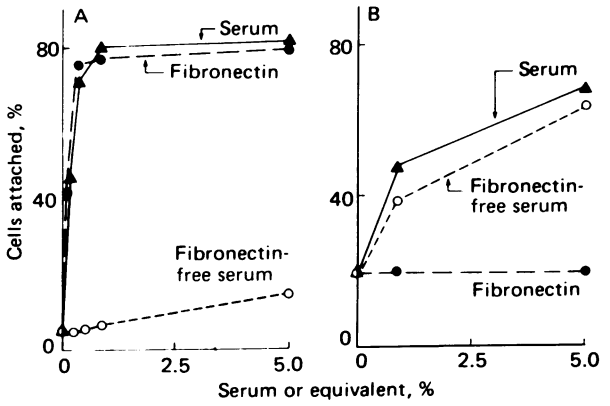

FIG. 1. Effect of serum and fibronectin on CHO cell (*A*) and chondrocyte (*B*) attachment. Assays were performed in the presence of increasing concentrations of fetal calf serum or its equivalent concentration of fibronectin-free serum or fibronectin. Data represent the means of duplicate measurements, which did not differ by more than 10%.

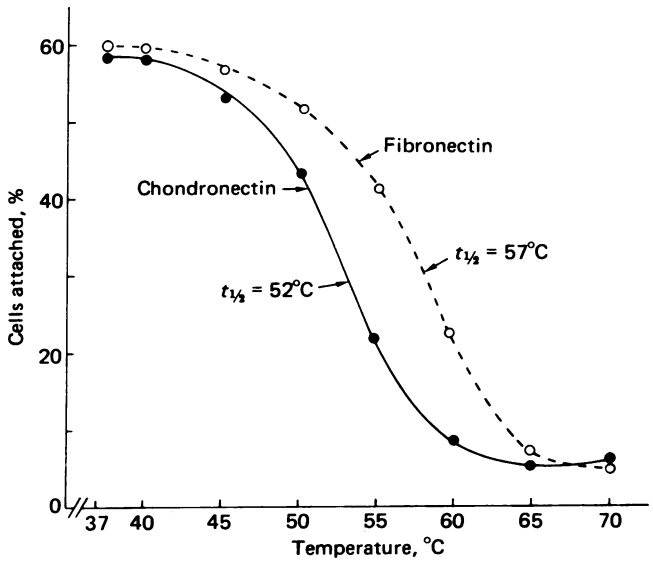

FIG. 2. Heat stability of attachment factors. Ten percent fetal calf serum in F12 medium was incubated at various temperatures for 20 min after equilibration. Attachment assays were carried out at 37°C. The temperatures at which the chondrocyte attachment factor and fibronectin lost 50% of their attachment-enhancing activity are indicated. Data represent the means of duplicate measurements, which differed by 10% or less.

than the chondrocytes. In 10% fetal calf serum, half of the CHO cells attached within 15 minutes, whereas the chondrocytes required three times as long to reach a similar level (data not shown). Attachment of both cell types to collagen substrates was temperature dependent, and less than 5% of the cells attached when assays were carried out at 4°C. Similar effects of temperature have been reported for fibroblast attachment (23).

As expected, serum from which fibronectin had been removed did not stimulate CHO cell attachment (Fig. 1). However, the fibronectin-free serum supported near maximal attachment of chondrocytes. Purified fibronectin did not stimulate the attachment of chondrocytes but did stimulate the attachment of CHO cells. These results indicate that serum contains a factor that enhances chondrocyte attachment and that this factor is distinct from fibronectin.

Characteristics of the Chondrocyte Attachment Factor. Preliminary studies showed that both the chondrocyte attachment factor and fibronectin were precipitated by 30% saturated $(NH_4)_2SO_4$ and also that both were retained by an Amicon XM-300 filter. These studies suggest that the chondrocyte factor is a large macromolecule(s). Incubation of F12 medium containing 10% serum with trypsin (1 mg/ml) for 30 min at 37°C followed by the addition of an excess of soybean trypsin inhibitor destroyed chondrocyte attachment activity. These results indicate that the chondrocyte factor is a protein.

The heat stabilities of the proteins responsible for CHO cell and chondrocyte attachment activities were compared. In these studies, medium containing 10% serum was incubated for 20 min at various temperatures and then tested for cell attachment activity. A significant difference in the temperature sensitivity of the serum for the attachment of the two cell types was observed (Fig. 2). In a typical experiment, half of the chondrocyte attachment activity was lost at 50–52°C, whereas 80% of the fibroblast attachment activity was retained. After treatment at 57–60°C the serum was essentially inactive in supporting chondrocyte attachment but retained 50% of the original fibroblast attachment activity.

Serum was fractionated on DEAE-cellulose as an initial step in purification of chondronectin. Because the protein and activity profiles of serum were very complex, the ion-exchange step was preceded by ammonium sulfate fractionation. Both chondrocyte and CHO cell attachment activities were precipitated by 30% $(NH_4)_2SO_4$ saturation. Although this material eluted from the DEAE-cellulose column essentially as one major peak (Fig. 3), the peak was a mixture of proteins that varied from the leading edge to the trailing edge of the peak (data not shown). This heterogeneity was confirmed by the separation of chondrocyte attachment activity from that of CHO cells that require fibronectin. The chondrocyte factor eluted earlier and as a single peak of activity.

Interaction of the Serum Factor with Collagen and Chondrocytes. In the presence of serum, chondrocytes attached best to type II (60% attachment) collagen substrates, although they also attached to types I (47%), III (45%), and IV (30%) collagens. CHO cells bound equally well to all four collagens (5). It has previously been shown that prior incubation of the collagen substrate with serum at either 4°C or 37°C allows fibronectin to bind to collagen and supports the subsequent attachment of cells to washed plates at 37°C (ref. 1 and this study, data not shown). In contrast to CHO cells, rinsed dishes supported chondrocyte attachment only if the dishes had been incubated for long periods of time (Table 1). This indicates that the binding of the chondrocyte factor to collagen occurs more slowly than the collagen–fibronectin interaction. Furthermore, no chondrocyte attachment was observed when the dishes were incubated at 4°C for up to 24 hr, rinsed, and subsequently assayed for attachment at 37°C. Under the same conditions, fibronectin binds to collagen. It was observed that chondrocyte attachment was prompted by preincubating the cells with serum (Table 1). However, because concurrent incubation of the cells with cycloheximide largely reduced attachment, it seems likely that the chondrocytes were producing an attachment factor during the preincubation period.

Extraction of Chondrocyte Attachment Activity from Cartilage. Embryonic chicken sternal cartilage and a rat chondrosarcoma were extracted with F12 medium overnight in the cold. Aliquots were then assayed for attachment activity by using both CHO cells and chondrocytes (Table 2). The extract of the chondrosarcoma stimulated the attachment of both cell types. On the other hand, the extract of embryonic sternal cartilage enhanced only chondrocyte attachment. Similar activity was found in the medium used to culture chondrocytes for two days (not shown), further indicating that this attachment factor is produced by chondrocytes.

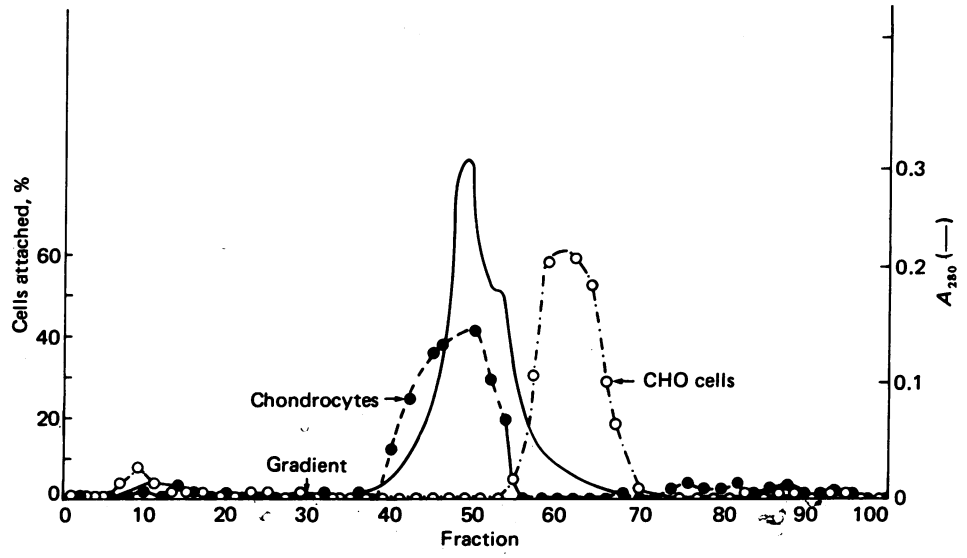

FIG. 3. Fractionation of the 30% saturated $(NH_4)_2SO_4$ insoluble fraction of fetal calf serum by DEAE-cellulose ion-exchange column chromatography using a 0.0–1.0 M NaCl gradient in 0.05 M Tris·HCl, pH 7.4, in a total volume of 400 ml. Five-milliliter fractions were collected. Superimposed on the protein elution profile is the pattern of attachment activity for CHO cells and for chondrocytes found in 1.0-ml aliquots of each fraction.

DISCUSSION

We have shown that the attachment of chondrocytes to a collagen substrate is mediated by a factor in serum that we call *chondronectin* (in analogy to *fibronectin*, which mediates the attachment of fibroblasts to collagen). Because a higher serum concentration is necessary for chondrocyte than for fibroblast attachment, serum may contain less chondronectin than fibronectin. Although chondronectin has not yet been isolated, its retention by an Amicon XM-300 filter, exclusion from a Sephacryl S-200 column (not shown), and lability to trypsin and heat suggest that it is a large protein. Although the source of the chondronectin in serum is not known, a factor with similar attachment-enhancing activity is produced by chondrocytes and can be extracted from cartilage. The material from chondrocyte-conditioned medium and chondrosarcoma extracts is probably similar to the serum-derived factor because the attachment-enhancing activity from all three sources elutes from a DEAE-cellulose column at the same salt concentration and is excluded from a Sephacryl S-200 column (unpublished observations). These observations suggest that the material from these three sources is similar. Because chondronectin is found in cartilage, it is likely that chondrocytes use this protein to bind to their matrix. Preliminary data indicate that chondrocytes attach preferentially to type II collagen. These findings suggest that the interaction of chondrocytes with chondronectin and type II collagen is essential for the formation and maintenance of cartilage.

Previous studies demonstrated that fibronectin binds to a collagen substrate. Fibronectin-requiring cells can then bind to this complex (1). Similar data were obtained with chondronectin except that binding of chondronectin to type II collagen requires longer periods of incubation at physiological temperatures. Further, fibronectin is adsorbed from serum by brief exposure to immobilized type I collagen (14, 15) whereas

Table 1. Mechanism of chondronectin-mediated attachment

Series	Experimental conditions: Cells	Experimental conditions: Dishes	Chondrocytes attached, %: 0% serum	Chondrocytes attached, %: 10% serum
A	Freshly dissociated	Preincubated (1 hr, 37°C), not rinsed	10	62
B	Freshly dissociated	Preincubated (37°C) and rinsed		
		1 hr	12	15
		24 hr	10	50
C	Preincubated and rinsed 1 hr, 37°C	Not preincubated	30	45
	1 hr, 37°C, with cycloheximide (10 μg/ml)		15	25

All assays were performed on dishes coated with type II collagen; 1.5×10^5 cells were used per assay. Preincubation of dishes and cells was with F12 medium with or without serum as indicated in the column headed "Chondrocytes attached." Series A represents the routine experimental conditions as described in *Experimental Procedures*. In series B, dishes were preincubated at 37°C for the indicated times in either the presence or absence of serum. At the end of this period, the dishes were rinsed and the assays were performed in F12 medium minus serum by using freshly dissociated cells. The cells used in series C were preincubated in either the presence or the absence of serum for 1 hr at 37°C and then rinsed. These assays were performed in the absence of serum on dishes that had not been preincubated. In the cycloheximide experiments, the drug was present during dissociation of the sterna, preincubation of the cells, and assays of attachment. Each point represents the means obtained from the assay of triplicate samples, which differed by no more than 10% of the given value.

Table 2. Attachment-enhancing activity in extracts of embryonic chicken sternal cartilage and rat chondrosarcoma

Addition to F12 medium	Cells attached, %	
	CHO cell	Chondrocytes
None	3	10
1% fetal calf serum	81	37
Chondrosarcoma extract	60	33
Sternum extract	0	30

Fragments of embryonic chicken sternal cartilage (100 mg/ml) and rat chondrosarcoma (500 mg/ml) were extracted overnight at 4°C with F12 culture medium. Attachment assays were performed with aliquots of extract diluted to a final volume of 1 ml with F12 medium (chondrosarcoma extract: 20 μg of protein in 20 μl; sternal extract: 20 μg of protein in 100 μl).

including hyaluronic acid and heparan sulfate, alter the binding chondronectin is not. Recent studies suggest that other factors, of fibronectin to collagen (24). Possibly the interaction between collagen and chondronectin also involves other factors present in cartilage.

Cell-specific macromolecules, termed *cognins*, that are able to bring about homotypic aggregation of cells, have been described in embryonic tissues (25). Fibronectin and chondronectin may be examples of a family of proteins that mediate the association of specific cells with their matrices. Such specific interactions could explain the localization of the cells in tissues.

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