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Cellular and Metabolic Specificity in the Interaction of Adhesion Proteins With Collagen and With Cells

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Fibronectin mediates the adhesion of fibroblasts to collagen substrates, binding first to the collagen and then to the cells. We report here that the interaction of the cells with the fibronectin-collagen complex is blocked by specific gangliosides, GD_{1a} and GT_1, and that the sugar moieties of these gangliosides contain the inhibitory activity. The gangliosides act by binding to fibronectin, suggesting that they may be the cell surface receptor for fibronectin.

Evidence is presented that other adhesion proteins or mechanisms of attachment exist for chondrocytes, epidermal cells, and transformed tumorigenic cells, since adhesion of these cells is not stimulated by fibronectin. Chondrocytes adhere via a serum factor that is more temperature-sensitive and less basic than fibronectin. Unlike that of fibroblasts chondrocyte adhesion is stimulated by low levels of gangliosides. Epidermal cells adhere preferentially to type IV (basement membrane) collagen but at a much slower rate than fibroblasts or chondrocytes. This suggests that these epidermal cells synthesize their own specific adhesion factor. Metastatic cells cultured from the T241 fibrosarcoma adhere rapidly to type IV collagen in the absence of fibronectin and do not synthesize significant amounts of collagen or fibronectin. Their growth, in contrast to that of normal fibroblasts, is unaffected by a specific inhibitor of collagen synthesis. These data indicate the importance of specific collagens and adhesion proteins in the adhesion of certain cells and suggest that a reduction in the synthesis of collagen and of fibronectin is related to some of the abnormalities observed in transformed cells.

Key words: cell adhesion, adhesion proteins, fibronectin, chondronectin, collagen substrates, gangliosides, cell surface

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Fibroblasts use fibronectin, a large glycoprotein, to adhere to collagen substrates [1]. These cells synthesize their own fibronectin but under the usual culture conditions utilize the fibronectin present in serum to attach to collagen. The interaction follows a specific sequence of events starting with the binding of fibronectin to the collagen molecule [2]. The cells then bind to the fibronectin-collagen complex in a divalent cation-requiring, energy-dependent process [3]. The interaction of fibronectin with collagen is specific, and chromatography of fibronectin on collagen affinity columns is used to purify the protein in high yield from serum, tissue culture medium, or cell extracts [4, 5]. There appears to be a specific site within collagen to which fibronectin binds [6, 7]. Peptides from a similar locus on collagen types I, II, and III bind to fibronectin (Fig. 1). The binding site has been further located within a fragment of the α1(I) chain comprising residues 757–791. This sequence lacks carbohydrate and includes the bond cleaved by animal collagenase. The integrity of the collagenase-sensitive bond at residues 775–776 and of a chymotrypsin-sensitive bond at 779–780 are required for fibronectin binding [8]. The data suggest that a specific sequence of amino acids, perhaps containing a small number of residues, is the binding site for fibronectin on collagen. In addition, a specific binding site on fibronectin for collagen has been described [9, 10].

Here we report on the role of fibronectin and other serum proteins in the attachment of fibroblasts, chondrocytes, and epidermal cells to collagen. Further differences are noted in the attachment of cells from a metastatic fibrosarcoma. Finally, data are presented that are consistent with a role for certain complex gangliosides as the cell surface receptors for fibronectin.

MATERIALS AND METHODS

Materials

Collagen types I and IV were prepared from lathyritic rat skin [11] and a murine tumor [12], respectively, as previously described. Gangliosides were either obtained from Sigma Chemical Co. or Supelco, Inc., or were prepared as previously described [13].

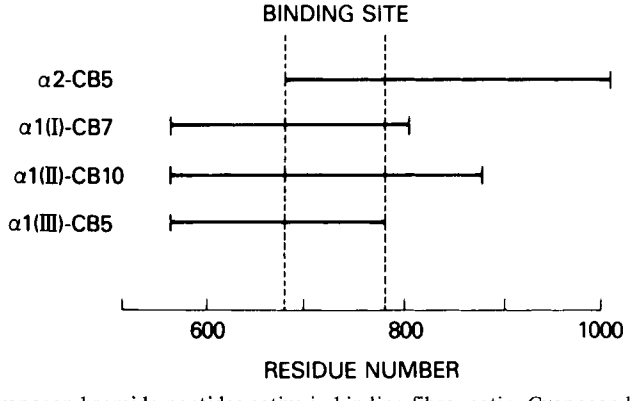

Fig. 1. Collagen cyanogen bromide peptides active in binding fibronectin. Cyanogen bromide peptides were prepared and isolated as already described and assayed for binding to fibronectin by either a radioimmunoassay or bioassay for activity in cell adhesion. Data on α2, α1 (I)-CB7, and α1 (II)-CB 10 from Dessau et al [7]; Data on α1 (I)-CB7 and α1 (II)-CB10 from Kleinman et al [6, 8]. Data on α1 (III)-CB5 from Kleinman and McGoodwin, unpublished observations.

Radioactive gangliosides were prepared as already described [14, 15]. Ceramides were obtained from Supelco, Inc., and oligosaccharides were obtained by ozonolysis of the ganglioside [16]. Cis-hydroxyproline was obtained from Sigma Chemical Co. Fibronectin was prepared by passing bovine serum (Colorado Serum Co.) over a denatured type I collagen Sepharose affinity column [4, 5] and eluting the bound fibronectin with 1 M KBr in 0.05 Tris containing 0.025 M 6-aminohexanoic acid, pH 5.3. The material that did not bind to the affinity column was considered to be fibronectin-free serum.

Cells

Chinese hamster ovary (CHO) cells were obtained from American Type Culture Collection. Chick sternal chondrocytes were obtained from day 14 embryonic chick sterna by dissociation with collagenase [17]. Epidermal cells were obtained from adult guinea pig epidermis by dissociation with trypsin [18]. Pulmonary metastatic tumor cells were selected in vivo from a pulmonary metastasis of the T241 fibrosarcoma [19].

Assay for Cell Adhesion

Collagen-coated bacteriologic plates [8] (35 mm) were preincubated for 1 h at 37° in 95% air, 5% CO_2 with Eagle's minimal essential medium (MEM) containing either serum, fibronectin, or fibronectin-free serum and 200 μg/ml of bovine serum albumin. The cells (usually 10^5) were then added for an additional incubation. The nonadherent cells were removed by gentle washing with 0.02 M phosphate-buffered saline (PBS). The attached cells were released with a solution of trypsin containing 0.1% ethylenediamine tetraacetic acid (EDTA) in PBS and were counted electronically.

Immunoprecipitation

Murine serum fibronectin (1 μg) was suspended in 1 ml MEM supplemented with albumin (20 μg) and was incubated with radioactive gangliosides for 60 min at 37° in 5% CO_2 (final pH 7.4). In some cases, the fibronectin was preincubated with denatured lathyritic rat skin collagen (2 μg) for 30 min at 37°. Rabbit antiserum (10 μl) against purified murine fibronectin was then added at a dilution of 1:5, a concentration previously shown capable of binding at least 5 μg of fibronectin. Samples were incubated for 2 h at 4°C. Samples were finally incubated with 100 μl of goat anti-rabbit immunoglobulin (1:4 dilution) (Miles Laboratories lot No. G105) at 4° overnight. No carrier was required and the blank was < 50 cpm. All incubations were performed in siliconized glass tubes. Samples were then spun over a sucrose cushion, dissolved in 0.5 ml of 0.5 N acetic acid, and counted.

RESULTS

Use of a Proline Analog to Distinguish Collagen-Dependent Cell Attachment

Studies by Kao and Prockop [20] have shown that cis-4-hydroxyproline prevents the growth of fibroblasts in culture. This compound is incorporated into peptide chains in place of proline and prevents the normal formation of trans-4-hydroxyproline in collagen. As a result, the collagen molecules do not form a triple helix and are not deposited into fibers [21]. Due to the known action of cis-hydroxyproline, the effect of this compound on the growth of fibroblasts is most likely due to its inhibition of the deposition of a collagen matrix. This proline analog can therefore be used to test whether collagen is

necessary for the growth of cells in culture. In contrast to the previous observations on a tissue culture substrate where cell growth is inhibited in the presence of this analog, we found normal growth of fibroblasts on a collagen substrate in the presence of cis-4-hydroxyproline [22]. Thus, a collagen substrate is necessary for the growth of normal cells. However, the growth of cells from a metastatic tumor on tissue culture dishes was not altered by cis-4-hydroxyproline. These cells make little or no collagen or fibronectin (Table I) and attach preferentially to basement membrane collagen (Fig. 3). A low production of collagen is frequently observed with transformed cells [23], as is reduced production or surface retention [24] of fibronectin. The altered production of matrix components may be associated with cells that grow and spread abnormally.

Attachment Characteristics of Fibroblasts, Chondrocytes, Epidermal Cells, and Metastatic Tumorigenic Cells

As stated earlier, the attachment of trypsinized fibroblasts to collagen substrates is stimulated by serum and fibronectin (Fig. 2). The adherence of chondrocytes, epidermal cells and metastatic tumorigenic cells to collagen is not fibronectin-dependent (Figs. 2 and 3). Chondrocytes adhere well to type I collagen via a factor (presumably a protein) in the serum other than fibronectin (Fig. 2). This factor, which we have named chondronectin, is readily distinguished from fibronectin, since it is more temperature-sensitive than fibronectin ($t_{1/2} = 52°$ vs $t_{1/2} = 57°$ for 30 min), is separable from fibronectin by DEAE-cellulose column chromatography, and is produced by chondrocytes [25] in culture.

Epidermal cells adhere preferentially to type IV collagen, but their attachment is not stimulated by serum, fibronectin, or fibronectin-free serum (Fig. 3). These cells attach slowly to type IV collagen in comparison to fibroblasts or chondrocytes when incubated with serum [18]. Epidermal cells probably make their own specific attachment protein as indicated by their preferential binding to type IV collagen substrates and the extended time required for their attachment.

Cells from a fibrosarcoma (PMT-T241) were found to adhere preferentially to type IV collagen without the requirement for serum or fibronectin. Attachment under these conditions was rapid, suggesting that there was a preformed receptor on the cells. However, serum and fibronectin did stimulate the adhesion of these cells to type I collagen (Fig. 3). These observations indicate that these cells have the cell surface receptor for fibronectin but, in contrast to fibroblasts, do not synthesize significant amounts of fibronectin or of collagen (Table I).

TABLE I. Collagen and Fibronectin Synthesis and Cis-Hydroxyproline Sensitivity of Normal and Metastatic Cells

Cells	% Cells remaining after 5-day exposure to 25 μg/ml cis-hydroxyproline	% Collagenase-sensitive[a] ^{3}H-proline-labeled macromolecules	Fibronectin synthesis[b] (ng/cell/day)
Adult connective tissue cells	10	5.8	600
Pulmonary metastatic tumor cells	100[c]	None detected[d]	None detected

[a]Collagen determined by method of Peterkofsky and Diegelmann [32].
[b]Fibronectin determined by ELISA assay [33].
[c]Value indicates no difference in cell number between treated and untreated cultures.
[d]Chemical analyses could detect very low levels of hydroxyproline.

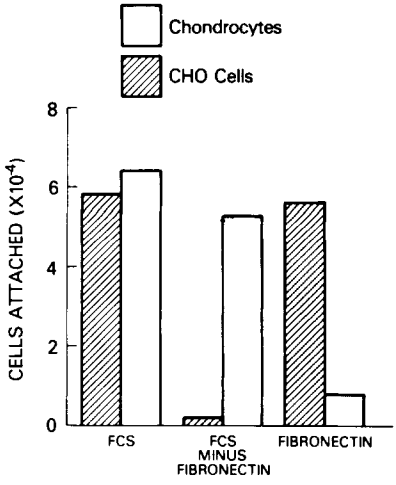

Fig. 2. Attachment properties of fibroblasts and chondrocytes on collagen type I in the presence of fetal calf serum (FCS), fibronectin, or serum minus fibronectin. Attachment assays were carried out as described in the Materials and Methods. Each point represents the mean of duplicate measurements, which did not differ by more than 10%.

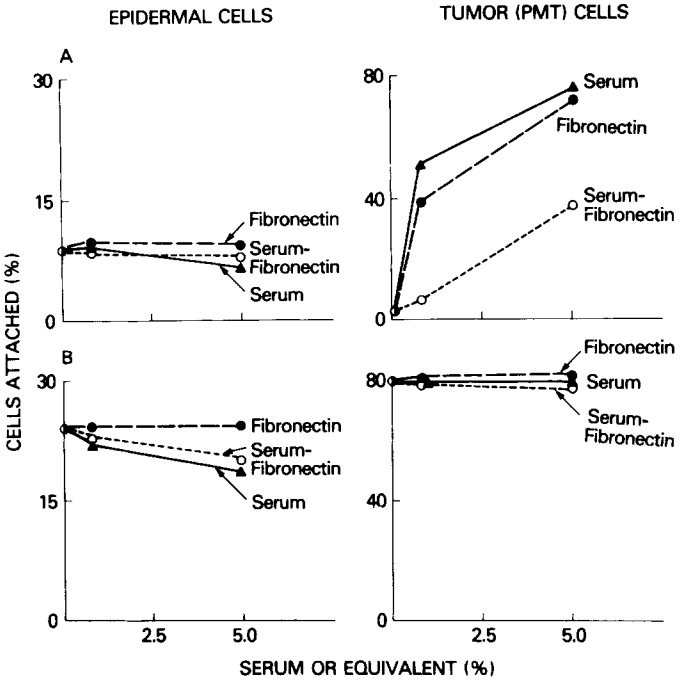

Fig. 3. Attachment properties of epidermal cells and metastatic tumor cells on collagen type I (A) and type IV (B) in the presence of increasing amounts of serum, fibronectin, or serum minus fibronectin. Attachment assays were carried out as described in Materials and Methods, except for the epidermal cells, which were allowed to attach for 18 h. Similar trends were observed with the epidermal cells after an incubation of 1.5 h, but the level of attachment was very low. The data on cell attachment are plotted as the amount of fibronectin present in that the percentage of serum indicated on the abscissa. Each point represents the mean of duplicate measurements, which did not differ by more than 10%.

Effect of Gangliosides on Cell Adhesion

The cell surface receptor(s) for fibronectin have not been identified. Because ganglioside synthesis [26] and fibronectin binding [27] are reduced in transformed cells, we have investigated the possible role of gangliosides in the interaction of fibronectin with the cell membrane. We studied the influence of a variety of gangliosides on CHO cell adhesion. As seen in Table II, GM_3, the simplest ganglioside tested, had no effect on cell adhesion and GM_2 and GM_1 were not as active in inhibiting cell adhesion as a mixture of gangliosides. GD_{1a} and GD_{1b} were more active, but GD_{1b}, which is an isomer of GD_{1a}, had less activity, suggesting the importance of the position of sialic acid. GT_1 was the most active ganglioside in inhibiting cell adhesion.

The activity of the gangliosides resided in the sugar portion since purified ceramides had no effect upon cell adhesion (Table II), while purified oligosaccharides from the ganglioside mixture retained some, but not all, of the activity. Since the position of sialic acid appeared important in the effect of gangliosides on cell adhesion, we next modified the sialic acid residues by mild periodate exposure. Oxidation of sialic acid residues from either mixed gangliosides or purified GD_{1a} reduced the activity significantly (Table II). Reduction of the oxidized products did not restore activity. The structure of the gangliosides as measured by thin-layer chromatography was not destroyed by the oxidation and reduction processes (not shown). In addition, free sugars, including sialic acid, glucose, galactose, N-acetyl glucosamine, and N-acetyl galactosamine, had no effect upon cell adhesion. These results suggest the specificity of the inhibition by gangliosides.

We next determined the mechanism by which the gangliosides were blocking cell adhesion. The ability of gangliosides to inhibit cell adhesion by blocking fibronectin binding to collagen or by blocking cell binding to the fibronectin-collagen complex was measured. Collagen-coated dishes were preincubated with gangliosides, rinsed to remove the unbound material, and then were used for the usual adhesion assays. Results from these experiments

TABLE II. Effect of Gangliosides and Their Components on CHO Cell Adhesion

Test compound	Amount required for 50% inhibition of cell adhesion (nmoles/100 μl)
Ganglioside mixture[a]	40
GM_3[b]	No activity
GM_2	89
GM_1	61
GD_{1b}	38
GD_{1a}	32
GT_1	<<< 26[c]
Periodate-treated ganglioside mixture	169
Periodate-treated and reduced ganglioside mixture	135
Periodate-treated and reduced GD_{1a}[d]	159
Oligosaccharides from ganglioside mixture	120
Ceramides[d]	>>> 350[e]

[a]Obtained from Sigma Chemical Co.
[b]Highly purified gangliosides.
[c]That level blocked cell adhesion 100%.
[d]Obtained from Supelco, Inc.
[e]That level had no effect upon cell adhesion.

demonstrated no inhibition of cell adhesion by gangliosides (Table IIIA). However, when the fibronectin-collagen complex was preincubated with gangliosides and then washed, cell adhesion was inhibited (Table IIIB). This suggests that gangliosides bind to the fibronectin-collagen complex. A direct demonstration of ganglioside binding to fibronectin was seen in preliminary studies in which radioactive gangliosides were mixed with fibronectin and antibodies against fibronectin were used to precipitate fibronectin and attached ganglioside. In these studies, we found that GD_{1a} bound better to fibronectin than did GM_1 (Table IV) in confirmation of the attachment data. The presence of collagen is not required for ganglioside binding. These studies indicate that gangliosides inhibit fibronectin-mediated cell adhesion by binding through their sugar residues to fibronectin.

The effect of gangliosides on chondrocyte cell adhesion was tested to determine how a cell whose adhesion is not mediated by fibronectin would respond to gangliosides. As seen in Figure 4, these cells responded differently. Chondrocyte adhesion was stimulated by low levels of gangliosides, and at higher concentrations cell adhesion returned to control levels. This confirms the cell specificity of the inhibition of gangliosides on fibronectin-mediated cell adhesiveness.

DISCUSSION

Many cells synthesize fibronectin in culture [27], and this protein is widely distributed in various anatomic sites [28]. However, cartilage [29] and various epithelial tissues do not appear to contain fibronectin. These observations correlate well with our survey of the attachment properties of various cells to collagen in vitro. Cells such as fibroblasts, hepatocytes, and periosteal cells produce fibronectin and use it to bind to collagen substrates [30]. Freshly isolated chondrocytes as well as cultured chondrocytes (unpublished observations) do not use fibronectin to attach to collagen [25]. Rather, they appear to use a different cell attachment factor, which we have named chondronectin. Chondrocytes, however, can synthesize fibronectin in culture [29], particularly when cultured with medium containing exogenous fibronectin. Fibronectin alters the morphology and biosynthetic activities of chondrocytes and appears to be a major factor accounting for their loss of chondrocyte

TABLE III. Mechanism of Ganglioside Inhibition of Fibronectin-Mediated Cell Adhesion

	A. Effect on fibronectin binding to collagen (cells × 10^4 attached: after fibronectin addition: % inhibition)[a]	B. Effect of cell binding to fibronectin-collagen complex (cells × 10^4 attached: % inhibition)[b]
No additions	18 (0)	24 (0)
Plus gangliosides	7 (61)	9 (62)
Plus gangliosides, then wash	18 (0)	10 (58)

Values represent mean of triplicate measurements that did not differ by more than 10%. In these experiments a quantity of ganglioside (0.58 μmole/ml) was used that is known to inhibit cell attachment by 60% in the presence of 0.2% serum or the equivalent amount of fibronectin.

[a]Collagen-coated dishes were incubated with a ganglioside mixture for one hour. Where indicated, the plates were rinsed several times, followed by the addition of serum or purified fibronectin and cell adhesion was measured as already described in Materials and Methods.

[b]Collagen-coated dishes, which had been preincubated with purified fibronectin for 1 h, were incubated with a ganglioside mixture for an additional hour. The plates were then washed several times where indicated and cell adhension was measured as already described in Materials and Methods.

phenotype [31]. The behavior of other cells in culture may also be altered by the high levels of fibronectin present in serum. In addition to direct effects on the metabolism of cells, fibronectin may alter the population of cells retained in culture after passage of the cells.

Both quantitative and qualitative differences are noted in the attachment and binding of transformed cells to collagen. A line of highly metastatic mouse sarcoma cells (PMT)

TABLE IV. Measurement of Gangliosides Binding to Fibronectin by Immunoprecipitation of Labeled Gangliosides in the Presence of Fibronectin and Anti-fibronectin

	% Precipitated
A. ^{3}H-GM_1	
+ Fibronectin	16
+ Fibronectin + collagen	15
+ Collagen	3
B. ^{3}H-GD_{1a}	
+ Fibronectin	31
+ Fibronectin + collagen	30
+ Collagen	7

Immunoprecipitation of ^{3}H-ganglioside by anti-fibronectin: Each ganglioside was incubated with fibronectin, collagen, or both, followed by reaction with rabbit antifibronectin and precipitation by goat anti-rabbit IgG. Each value is the mean of two precipitations.

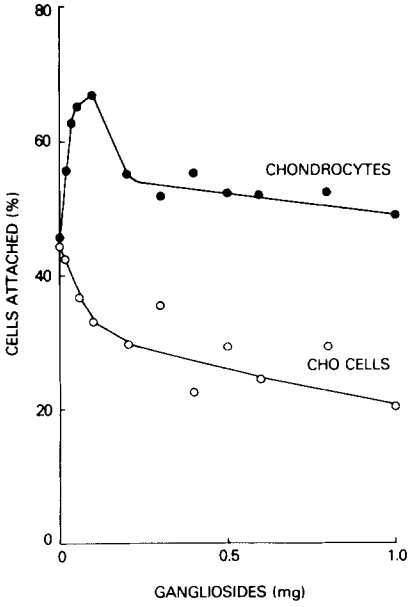

Fig. 4. Attachment of CHO cells and chondrocytes in the presence of increasing amounts of a ganglioside mixture. Collagen-coated dishes were incubated for 1 h with 1.5% fetal calf serum and the amount of ganglioside indicated. Then the cells were added and attachment was measured as described in Materials and Methods. The amount of serum employed is enough to support maximal attachment of CHO cells and 50% attachment of chondrocytes. The 50% attachment level of CHO cells is 0.2% serum. The gangliosides inhibit cell adhesion more at lower levels of serum. Each point represents the mean of duplicate measurements, which did not differ by more than 10%.

makes little or no fibronectin or collagen. In the presence of exogenous fibronectin, these cells can bind to a variety of collagen types. In the absence of fibronectin, PMT cells bind preferentially to type IV collagen. These differences may be related to the metastatic behavior of these cells. The lack of fibronectin and collagen may make these cells more peripatetic. The ability of these cells to bind to type IV collagen may allow circulating PMT cells to attach and penetrate endothelial basement membranes to initiate new growth. Other transformed but nonmetastatic cells produce less collagen and fibronectin than normal cells and do not show preferential binding to type IV collagen. Additionally, they retain less fibronectin on their surface [24]. These two changes appear to be related to the phenotypic changes associated with transformation.

As noted earlier, certain complex gangliosides have activities consistent with their role as cell surface binding sites for fibronectin. The studies reported here indicate that certain gangliosides, including GD_{1a} and GT_1, bind to fibronectin and block subsequent attachment of cells to the protein. The data also indicate that this activity resides in the oligosaccharide portion of the gangliosides and is dependent on intact sialic acid residues. Since the synthesis of these complex gangliosides is impaired in transformed cells [26], this could account for their reduced retention of fibronectin, and in turn could affect their malignant behavior.

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