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ATTACHMENT OF RAT HEPATOCYTES TO COLLAGEN AND FIBRONECTIN; A STUDY USING ANTIBODIES DIRECTED AGAINST CELL SURFACE COMPONENTS

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SUMMARY

Attachment of rat hepatocytes to collagen but not to fibronectin substrata was efficiently inhibited by antibodies against the hepatocyte surface. Further analyses of this inhibition suggested that hepatocyte attachment to collagen involves cell surface antigens which are not identical to membrane bound fibronectin or collagen.

Rabbit antibodies against rat fibronectin inhibited hepatocyte attachment to rat fibronectin but not to collagen or rabbit fibronectin. After plasmin digestion of fibronectin, peptides were isolated that lacked affinity for collagen but could serve as a substratum for hepatocyte attachment. These results suggested that attachment to fibronectin does not involve membrane bound fibronectin or collagen.

INTRODUCTION

In the vertebrate organism most cells are associated with supportive structures composed of glycosaminoglycans, collagen, fibronectin and other glycoproteins. Studies in vitro have shown that attachment of vertebrate cells to a solid substratum is essential for normal cell physiology (1) and that both collagen and fibronectin can serve as substrata for cell adhesion (2,3). However, the interrelation between collagen and fibronectin in these reactions are poorly understood and seem to vary for different cell types (4-6). Furthermore, no cell surface molecules mediating physiological cell attachment have yet been identified.

In analyses of various cell adhesion phenomena, including intercellular adhesion of rat hepatocytes, antibodies against cell surface components have been used (7-10). This approach has now been employed in a study of the substratum attachment of rat hepatocytes and in the present communication we report how antibodies against plasma membranes and against rat fibronectin affect the attachment of hepatocytes to collagen and fibronectin, respectively.

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MATERIALS AND METHODS

Collagen, fibronectin and fibronectin fragments. Neutral salt soluble collagen, shown to be free of fibronectin (4), was prepared from rat skin (11). Fibronectin was isolated from bovine, rat, rabbit or human plasma by affinity chromatography on gelatin-Sepharose (12) followed by chromatography on DEAE-cellulose.

Fibronectin (50 mg; 2 mg/ml in phosphate buffered saline (PBS)) was incubated for 20 hours at 37°C with 2 ml of plasmin-Sepharose (50 clotting units of plasminogen was coupled to 1 g of CNBr-activated Sepharose and was activated by incubation with 10^4 plug units of streptokinase at 35°C for 60 min). The gel was sedimented and the supernatant, containing the fibronectin fragments, was chromatographed on a column of Sepharose 6B eluted with PBS.

Antibodies. Rabbits were immunized with rat liver plasma membranes (fraction M-L, Ref. 13) or fibronectin (300 µg) isolated from rat plasma as described (10). Antibodies raised against a partly purified heparan sulphate receptor (HSR) from rat liver plasma membranes (14; Å. Oldberg, L. Kjellén and M. Höök, unpublished) were kindly donated by Drs. L. Kjellén and Å. Oldberg of this institute. The IgG fractions of the antisera were isolated by affinity chromatography on Protein A Sepharose (15). Monovalent antibodies (Fab-fragments) were prepared by papain digestion of the IgG fraction essentially as described (16). Fc-fragments and intact IgG molecules not cleaved by the enzyme were removed by adsorption on Protein A-Sepharose.

The anti-fibronectin antibodies (covalently linked to Sepharose), bound a plasma protein that on PAGE had the apparent molecular weight expected for fibronectin. The antibodies obtained from rabbits immunized with plasma membranes or with the heparan sulfate receptor, respectively, reacted with the surface of isolated, intact hepatocytes as demonstrated by binding of ^{125}I-labeled Protein A to viable cells, which had been preincubated with the antibodies.

Cell attachment assay. The assay employed has been described in detail elsewhere (4). Briefly, rat hepatocytes (1 x 10^6 cells) isolated by collagenase perfusion were seeded in 2 ml of a calcium and magnesium containing medium on plastic Petri dishes (Falcon Plastics, Cat No 1008) coated with 150 µg of heat-denatured collagen or with 20 µg of intact fibronectin or fibronectin peptides. The cells were incubated for 1 hour at 37°C in humidified air after which the medium was removed and the dishes were washed once. The number of cells attached to the substratum was determined from the activity of lactate dehydrogenase in a Triton X-100 lysate of attached cells.

The effects of antibodies on cell attachment were tested by preincubating cells (1 x 10^6 cells/ml) for 30 min at 4°C in antibody-containing medium and then seeding 1 ml of the incubation mixture on petri dishes coated with the appropriate protein and continuing the incubation as described above.

RESULTS

Rabbit antibodies, both monovalent Fab fragments and intact IgG molecules, directed against rat liver plasma membranes inhibited the adhesion of rat hepatocytes to collagen (Fig. 1). At the concentration of antibodies required to completely inhibit the adhesion of cells to collagen, the adhesion to fibronectin was unaffected (Fig. 1). The antibodies exerted their effect by binding to cell surface components, since cells, which were preincubated with antibodies and then washed,

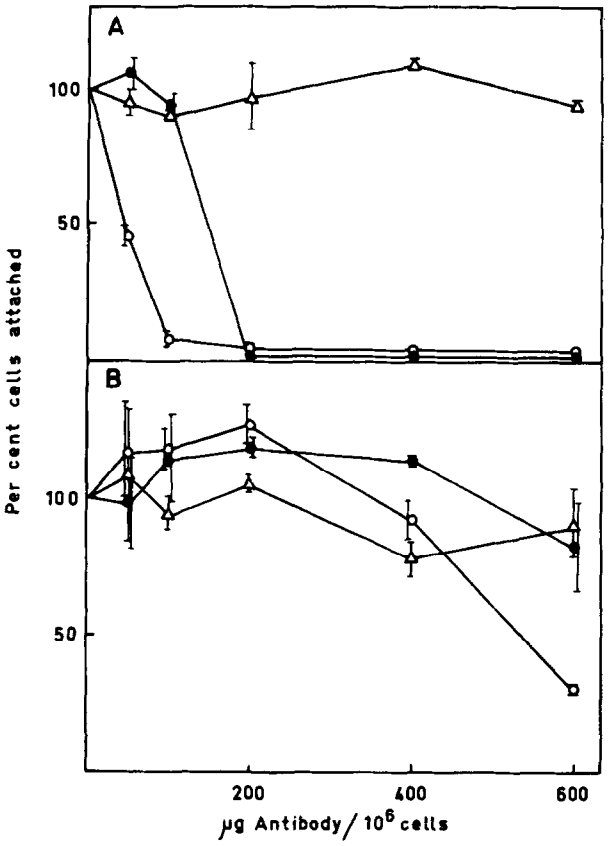

Fig. 1. Effects of anti-plasma membrane antibodies on attachment of hepatocytes to denatured collagen (A) or to rat fibronectin (B).
Hepatocytes (1 x 10^6 cells/ml of Buffer 3) were preincubated for 30 min at 4°C with the indicated amounts of antibodies. 1 x 10^6 cells (still in the presence of antibodies) were added to each dish and incubated at 37°C for 20 min. The medium was aspirated off and the dishes were washed with 1 ml of Buffer 3. Attached cells were determined as described in the Methods section. Attachment is given as the percentage of cells attached, with 100% set to equal the number of cells attached in the absence of antibodies. The range obtained from incubation of duplicate dishes is shown. o, incubation with anti-plasma membrane IgG; •, incubation with anti-plasma membrane Fab; Δ, incubation with antibodies (IgG) against the partially purified heparin receptor (HSR) from hepatocyte plasma membranes. IgG isolated from non-immune rabbits had no effect on cell attachment.

were unable to attach to the collagen substratum whereas preincubation of the substrata did not reduce the number of cells attaching. Rabbit immunoglobulin from un-immunized animals or antibodies directed against the partially purified HSR did not affect attachment to either substratum (Fig. 1). These results suggest that the inhibitory effect of anti-plasma membrane antibodies on the cell-collagen adhesion was not caused by a decreased viability of the cells or by a complete inability of hepatocytes to adhere to a substratum. Furthermore, any

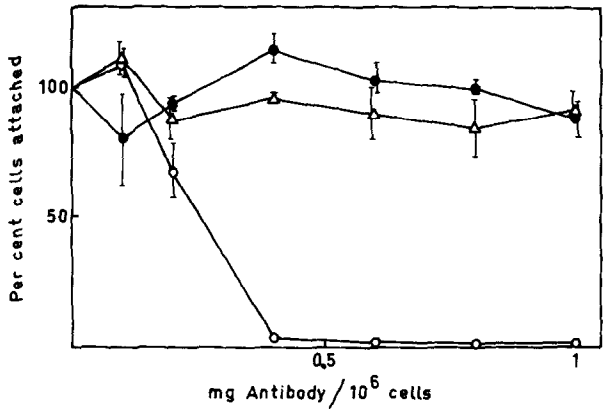

Fig. 2. Effects of anti-rat fibronectin antibodies (IgG) on attachment of hepatocytes to denatured collagen (●), rat fibronectin (o) or rabbit fibronectin (Δ).
For details, see the legend to Fig. 1.

antibody binding to the hepatocyte did not inhibit the adhesion of cells to collagen. The observed effect of the anti-plasma membrane antibodies might be due to a blockage of such structures present on the cell surface that are involved in the attachment of cells to collagen and points to the presence of hepatocyte receptors for collagen. Absorption of the anti-plasma membrane antibodies with immobilized rat fibronectin or collagen did not decrease the inhibition of cell attachment to collagen, suggesting that neither membrane associated fibronectin nor membrane associated collagen mediate the adhesion of cells to collagen.

Rabbit antibodies directed against rat fibronectin inhibited the attachment of hepatocytes to rat fibronectin- but not to collagen-coated dishes (Fig. 2). The effect of these antibodies on the adhesion of cells to fibronectin was presumably caused by blockage of substratum molecules rather than by cell surface components, since the adhesion to rabbit fibronectin (which did not react with the antibodies) was unaffected by the presence of the antibodies (Fig. 2). This result might indicate that cell surface bound fibronectin does not serve as the receptor in the adhesion of cells to a fibronectin substratum.

A direct demonstration that an appropriate antibody is able to interfere with the binding between collagen and fibronectin is presented in Fig. 3. Thus, anti-rat fibronectin antibodies but not anti-plasma membrane antibodies effectively inhibited binding of [^{125}I] rat fibronectin to immobilized collagen. These results provide further support to the conclusion that hepatocyte attachment to collagen does not involve a collagen-fibronectin interaction.

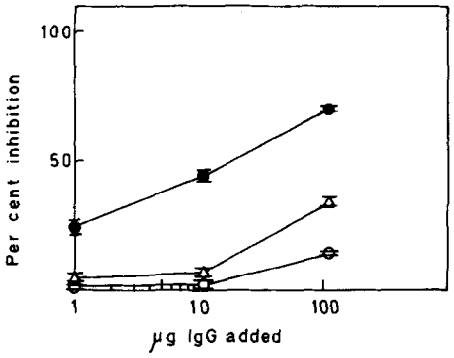

Fig. 3 *Effects of antibodies on collagen-fibronectin binding.* Collagen covalently linked to Sephadex G-25 was incubated with ^{125}I-labeled human plasma fibronectin in the presence or absence of various antibodies. The incubation was performed in PBS containing bovine serum albumin (2 mg/ml) and NaN_3 (0.2 mg/ml) for 140 min at 22°C. The beads were washed three times in the incubation solution and the activity of ^{125}I was determined in a Beckman gamma spectrometer. To 1 ml of a collagen-Sephadex suspension (corresponding to approximately 0.75 µg of collagen) 100 µl of ^{125}I fibronectin (10^5 cpm; approximately 20 ng of fibronectin) and 200 µl of the respective IgG containing solution were added. Zero % inhibition was set to equal the binding in the absence of any antibodies and 100% inhibition was set to equal no binding. o, anti-plasma membrane IgG; •, anti-rat fibronectin IgG; Δ, non-immune IgG.

To evaluate the possibility that adhesion of hepatocytes to fibronectin is mediated by cell bound collagen, attempts were made to separate the fibronectin peptides containing the cell and the collagen binding sites, respectively. Fibronectin isolated from bovine plasma was incubated with immobilized plasmin and the degradation products were applied to a column of gelatin-Sepharose. Fibronectin peptides not binding to the gelatin-Sepharose and those eluted from the column with 4 M urea were compared in the cell attachment assay by using dishes coated with varying amounts of the two peptides (Fig. 4). The peptides not binding to gelatin-Sepharose had essentially the same attachment-mediating activity as had intact fibronectin or the peptides binding to gelatin-Sepharose. This result demonstrates that the sites in the fibronectin molecule binding to collagen and cells, respectively, are not identical.

To further characterize the fibronectin degradation products the plasmin-digested fibronectin was subjected to chromatography on Sepharose 6B. The fibronectin peptides were eluted in two peaks, A (K_{av} 0.29-0.48) and B (K_{av} 0.71-0.83) that were pooled separately. The ability of these peptides to bind to collagen was investigated by affinity chromatography on gelatin-Sepharose (12). Ninetyfive per cent of the material in fraction A but only 15% of that

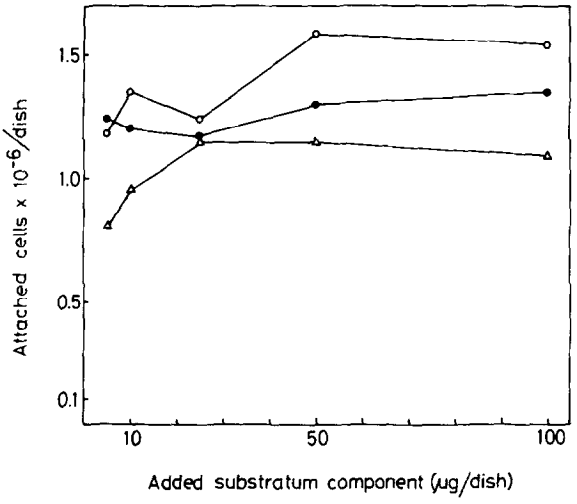

Fig 4 Attachment of rat hepatocytes to intact fibronectin and fibronectin peptides.

Bovine plasma fibronectin was digested with immobilized plasmin and applied to a column of gelatin-Sepharose. The column was extensively washed with phosphate buffered saline (PBS) and eluted with 4 M urea in PBS. In a control experiment the capacity of the gelatin-Sepharose was investigated by applying undigested fibronectin to an identical column. All of the applied fibronectin bound to the column and could be eluted with 4 M urea. Plastic culture dishes were coated with various concentrations of intact fibronectin (●), peptides not adsorbed to the gelatin-Sepharose (Δ) and peptides eluted from the column with 4 M urea (o), respectively. Hepatocytes (2 x 10^6 cells in 2 ml of Buffer 3) were seeded on the dishes which were incubated as described in the Method section.

in fraction B had affinity for gelatin-Sepharose. The B-peptides which lacked affinity for gelatin- Sepharose as well as the unfractionated A-peptides mediated cell attachment. The B-peptide mixture not binding to gelatin-Sepharose contained three dominating peptides with apparent molecular weights of 19,000, 25,000 and 29,000 on SDS-PAGE (Fig. 5).

DISCUSSION

In the present paper we report that antibodies raised against isolated rat liver plasma membranes inhibited the attachment of cells to collagen at concentrations where the adhesion of cells to fibronectin was unaffected. Furthermore, antibodies raised against the partly purified heparan sulfate receptor (HSR) did not inhibit the attachment of cells to collagen although the antibodies reacted with several cell surface components as demonstrated by immuno-precipitation (Å. Oldberg, M. Höök; to be published). In view of these results it seems reasonable to conclude that the inhibitory effect of the anti-plasma membrane antibodies on cell collagen adhesion is caused by

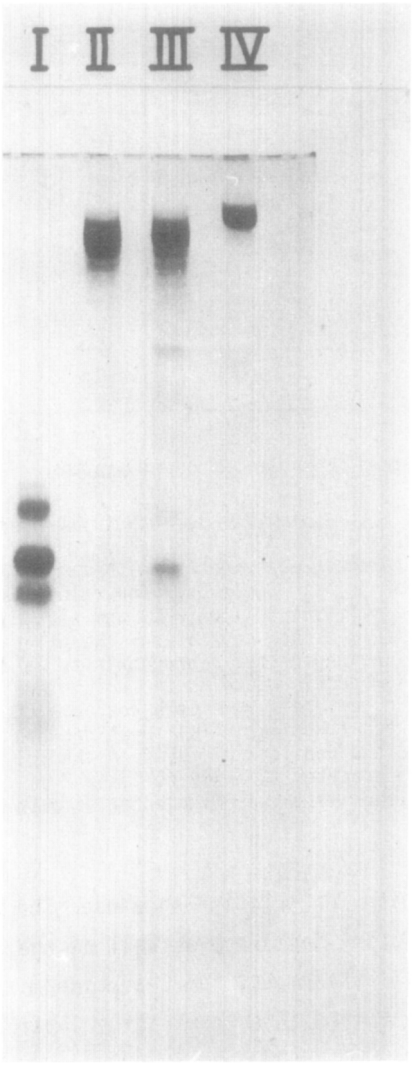

Fig 5 Gel electrophoresis of intact and plasmin-digested fibronectin

Track I; B-peptides which did not bind to gelatin-Sepharose
Track II; A-peptides which bound to gelatin-Sepharose
Track III; Plasmin-digested fibronectin
Track IV; Intact fibronectin.

Before analysis the samples were treated with 5% SDS and 10% β-merkaptoethanol for 3 min in a boiling water bath. The polypeptides were separated on 5% polyacrylamide slab gels using the buffer system described by Laemmli (19) and the gels were stained with Coomassie Brilliant Blue.

a blockage of cell surface components engaged in the attachment of cells to collagen and the presence of collagen receptors at the hepatocyte surface is suggested.

Antibodies against rat fibronectin did not affect the adhesion of cells to collagen and absorption of the anti-plasma membrane antibodies with immobilized fibronectin did not abolish the inhibition of cell collagen attachment. On the other hand, anti-rat fibronectin antibodies but not anti-plasma membrane antibodies interfered with the collagen-fibronectin interaction in a model system (Fig. 3). These results suggest that cell-surface associated fibronectin which has been suggested to mediate the adhesion of platelets to collagen (17), does not constitute the collagen receptor on hepatocytes, although it might be argued that the inhibitory antibodies could bind to antigenic determinants in cell associated fibronectin which are not exposed in the plasma form of fibronectin.

A collagen-fibronectin interaction in the hepatocyte attachment to fibronectin also appears unlikely since it was possible to isolate fibronectin peptides which lacked affinity for collagen but could serve as substrate for cell attachment. Similar results have also been reported by others (18).

The different effects of the anti-plasma membrane antibodies on the attachment of hepatocytes to collagen and fibronectin, respectively, is compatible with the idea that separate surface components are involved in the attachment of cells to dishes coated with the two proteins. However, the identification of the adhesion molecules involved in attachment of cells to collagen and fibronectin, respectively, must await their isolation. Such work is now in progress in our laboratory. Thus, the inhibitory effect of the anti-plasma membrane antibodies on attachment of cells to collagen is used to follow the purification of the collagen receptor(s) and preliminary results show that hepatocyte membrane components, which can be solubilized by detergents and purified by affinity chromatography on immobilized collagen, are able to neutralize the inhibitory activity of the antibodies (to be published).

Acknowledgements

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