Effect of Lectins and Chemical Modification on the Hemagglutinin Activity of Human Plasma Fibronectin

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Z. Naturforsch. **36 c,** 863–868 (1981); received March 30/June 5, 1981

Fibronectin, Hemagglutination, Lectins

Purified human plasma fibronectin has been shown to agglutinate protease-treated red cells [Vuento, Hoppe-Seyler's Z. Physiol. Chem. **360,** 1327–1333, (1979)]. The present report shows that the activity is inhibited by low concentrations of lectins and by macromolecular serum factors. Chemical modification of carboxyl groups of fibronectin strongly inhibited the activity, but modification of amino groups or guanidinium groups had little effect on the activity. The results suggest that fibronectin receptors on erythrocyte surface are carbohydrate-containing molecules. Humoral macromolecular factors may control the interaction of fibronectin with cell surfaces. Chemical modification studies indicate that the parts of the fibronectin molecule responsible for the hemagglutinin activity are different from those mediating the binding of fibronectin to collagen.

Introduction

Fibronectins are large molecular weight glycoproteins which mediate adhesion of cells to each other and to various substrata, especially to collagen (ref. [1, 2]). These proteins have been isolated from *in vitro*-cultured fibroblasts and from blood plasma. Fibronectins from both sources mediate cellular adhesion, although the plasma form of the protein may be slightly less active [3]. Both cellular and plasma fibronectins agglutinate sensitized erythrocytes [3–5]. This hemagglutination reaction provides a convenient model system for studies on the interaction of fibronectin with cell surfaces. The molecules on cell surface which interact with fibronectin have not been identified. In the present report we show that lectins and macromolecular serum factor(s) inhibit the hemagglutination. Data is also given on the effect of chemical modification on the hemagglutinating activity of fibronectin.

Materials and Methods

Purification of fibronectin

Fibronectin was purified from human blood plasma by affinity chromatography methods described earlier [6]. Fibronectin was labeled with ^{125}I by the Chloramine T-method [7] using modifications described earlier [8].

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0341-0382/81/0900-0863 $ 01.00/0

Chemical modification of fibronectin

Free amino groups of fibronectin were modified by succinylation [9]. The modification was carried out with 5 mg of succinic anhydride per 1 mg of fibronectin at pH 7.0–7.5 during an incubation time of 1 h. The reagents were removed by dialysis. Guanidinium groups of arginine residues were modified using 5 mM phenylglyoxal or 2,3-butanedione [10] in 50 mM borate, pH 7.5/0.9% NaCl. After an incubation time of 1 h, the reagents were removed by dialysis. Carboxyl groups were modified with 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide at pH 4.75 in the presence of 1 M glycine ethyl ester [11]. After an incubation time of 1 h, the reagents were removed as above.

Gel chromatography

Gel chromatography experiments were carried out on columns of Sepharose 4 B (Pharmacia, Uppsala, Sweden, 1.5 × 70 cm) or Sephacryl S 300 (Pharmacia, 0.9 × 96 cm), equilibrated with 50 mM Tris-HCl, pH 7.5/0.05% (w/v) sodium dodecylsulfate or with 10 mM Tris-HCl, pH 7.5/0.9% NaCl, respectively. The void volumes were determined with Blue dextran (Pharmacia).

Hemagglutination assay

The hemagglutination assay was carried out on plastic microtiter plates using trypsinized rabbit erythrocytes [4]. The erythrocytes were used as a 1%

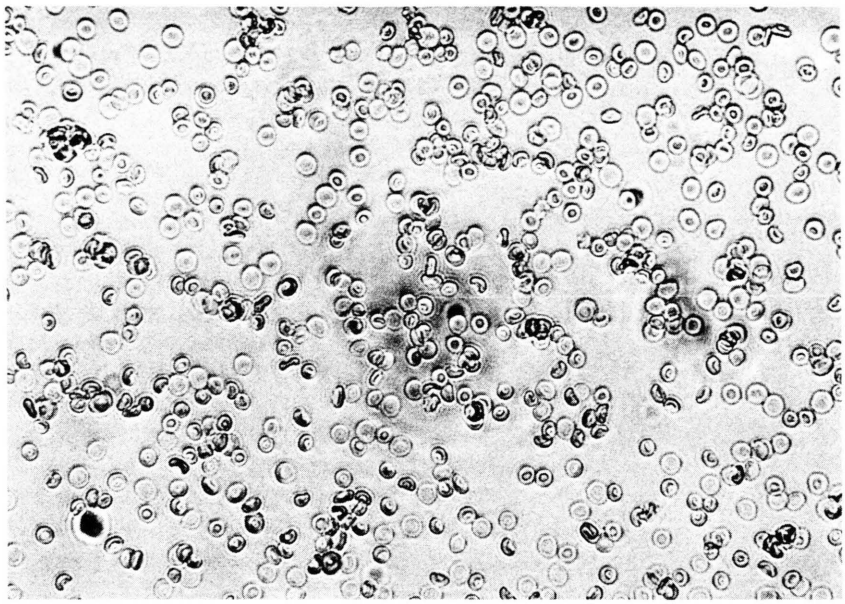

Fig. 1. Hemagglutination of trypsinized rabbit erythrocytes. A, erythrocytes in control buffer; B, erythrocytes in buffer containing 30 µg/ml of fibronectin.

Fig. 1A

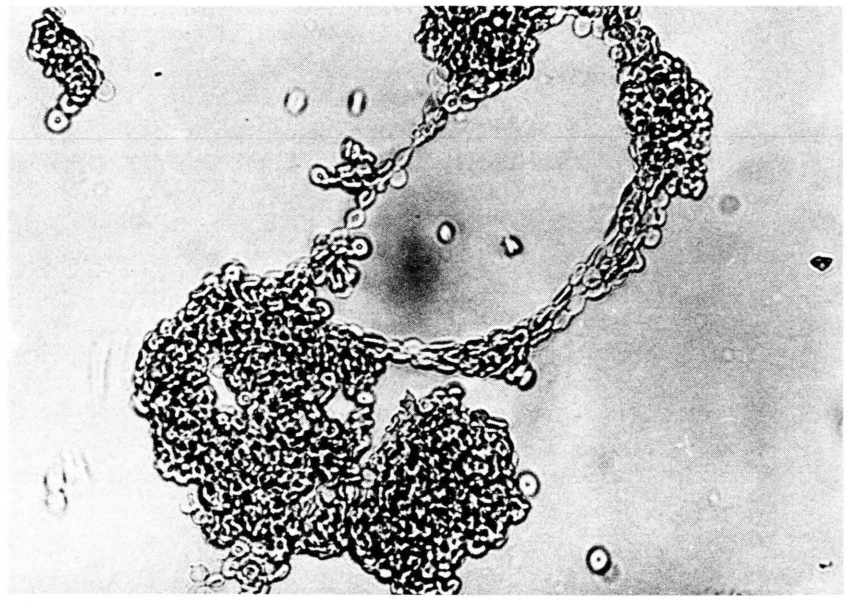

Fig. 1B

suspension in a total volume of 0.2 ml. Diluent buffer was 10 mM Tris-HCl, pH 7.5/0.9% NaCl/0.02% NaN_3 or 50 mM sodium borate, pH 7.5/0.9% NaCl/0.02% NaN_3. Bovine serum albumin was added to the buffers to a concentration of 0.1%. The plates were read after an incubation time of 2 h at room temperature. In accord with the earlier results [4] a strong hemagglutination was observed at fibronectin concentrations as low as 10–30 µg/ml (Fig. 1, Tables I–II). Erythrocyte ghosts were prepared by the method of Steck and Kant [12]. The ghosts were solubilized in 0.05% sodium dodecylsulfate (1 mg ghost protein per 1 ml of solution), and the solubilized membranes were tested for interaction with ^{125}I-labeled fibronectin by incubating aliquots of the labeled protein with the membrane preparation and by fractionation of the incubation mixture on Sepharose 4 B.

Lectins

Concanavalin A, Lotus tetragonolobus lectin, Kidney Bean phytohemagglutinin, Wheat Germ agglutinin and Soybean agglutinin were obtained from Sigma (St. Louis, Mo., USA).

Results

Inhibition of fibronectin-induced hemagglutination by lectins

Several plant lectins were tested for their ability to inhibit the fibronectin-induced hemagglutination of rabbit erythrocytes. The lectins themselves strongly agglutinated trypsinized rabbit red cells. The minimum lectin concentration giving a recognizeable agglutination was 15 ng/ml with Concanavalin A, 250 ng/ml with Kidney Bean phytohemagglutinin, 4 ng/ml with Wheat Germ agglutinin, and 0.2 ng/ml with Soybean agglutinin and Lotus tetragonolobus lectin. To test the effect of these lectins on the fibronectin-induced hemagglutination, the lectins were diluted fourfold under the minimum agglutinating concentration. Control experiments with such dilute lectin solutions showed no agglutination. The effect of low concentrations of lectins on the fibronectin-induced hemagglutination is shown in Table I. The reaction was not affected by Concanavalin A, by Kidney Bean phytohemagglutinin nor by Wheat Germ agglutinin. However, the hemagglutination was strongly inhibited by low concentrations of Soybean agglutinin and Lotus tetragonolobus lectin.

Table I. Effect of lectins on the fibronectin-induced hemagglutination of trypsinized rabbit erythrocytes. The hemagglutinating activity of fibronectin was titrated in the absence or presence of low concentrations of lectins. The concentrations of lectins in ng/ml are given in the parentheses. At these concentrations, the lectins alone did not agglutinate trypsinized rabbit erythrocytes.

Lectin added [ng/ml]	Minimum concentration of fibronectin giving a recognizeable hemagglutination [μg/ml]
None	10
Concanavalin A (4)	10
Kidney Bean phytohemagglutinin (60)	10
Wheat Germ agglutinin (1)	10
Soybean agglutinin (0.05)	100
Lotus tetragonolobus lectin (0.05)	200

Inhibition of the hemagglutination by serum factors

The fibronectin-induced hemagglutination was inhibited by normal rabbit serum. Under the conditions of the assay, the minimum concentration of rabbit serum to give a clear inhibition was 0.6 μl (mean from 5 sera from different animals, range 0.12–1.2 μl). Sera from other species were also tested and found inhibitory. Thus 0.6 μl of human serum, 0.6 μl of bovine serum, 1.2 μl of horse serum, 2.5 μl of goat serum, 1.2 μl of mouse serum and 1.2 μl of chicken serum gave a recognizeable inhibition in the assay.

The inhibitory activity of rabbit serum was heat-labile, being completely destroyed by incubation of the serum at 65 °C for 30 min. Gel chromatography of rabbit serum on a column of Sephacryl S 300 is shown in Fig. 2. The inhibitory activity eluted in a macromolecular fraction between the void volume and the elution volume of the main protein peak (Fig. 2).

Demonstration of complex-formation between fibronectin and erythrocyte membrane components

A complex formation between ^{125}I-labeled fibronectin and components of solubilized rabbit red cell membranes could be demonstrated by gel chromatography on Sepharose 4 B column (Fig. 3). Labeled control fibronectin eluted from the column as a single peak. The preparation contained a small

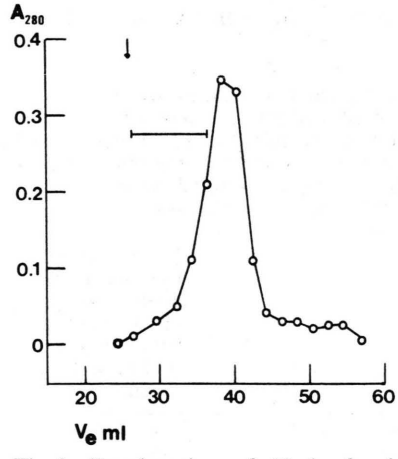

Fig. 2. Fractionation of 50 μl of rabbit serum by gel chromatography on a column of Sephacryl S 300. The void volume of the column is indicated by an arrow. The bar shows the elution position of the hemagglutination-inhibiting material.

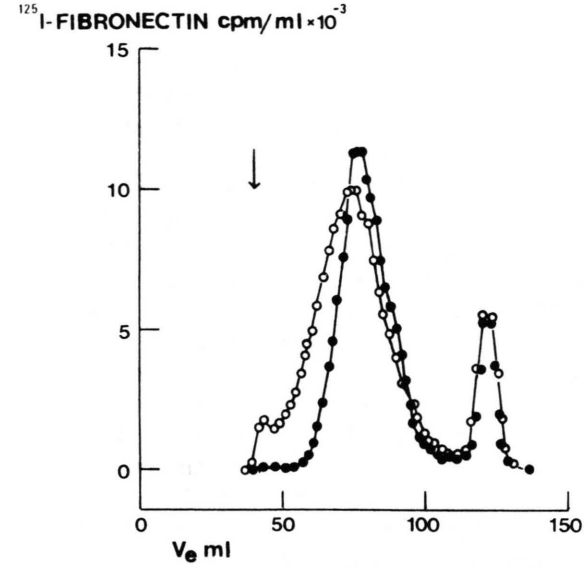

Fig. 3. Demonstration of complex formation between ^{125}I-labeled fibronectin and components of rabbit erythrocyte membranes. Rabbit erythrocyte ghosts were solubilized with 0.05% (w/v) sodium dodecylsulfate, incubated with labeled fibronectin and fractionated on a column of Sepharose 4 B in the presence of 0.05% sodium dodecylsulfate (○). Samples of labeled control fibronectin (●) were run under identical conditions. The void volume of the column is indicated by an arrow.

amount of free iodide which served as a marker for the total volume of the column. Fibronectin preincubated with solubilized erythrocyte membranes showed a different elution profile from that of control fibronectin. A portion of the radioactive protein now eluted in the void volume of the column, and much of the radioactive protein eluted between the void volume and the elution volume of control fibronectin (Fig. 3). This indicated a formation of high molecular weight complexes.

Table II. Effect of chemical modification on the hemagglutinating activity of fibronectin. The modification reactions were carried out as described in the Materials and Methods. Hemagglutination assay was performed in Tris-buffered saline* or in borate-buffered saline**, both containing 1 mg/ml of bovine serum albumin.

Preparation	Lowest concentration (μg/ml) giving a recognizeable agglutination of trypsinized rabbit erythrocytes
Control fibronectin*	15
Control fibronectin**	30
Succinylated fibronectin*	25
Phenylglyoxal-treated fibronectin*	45
Carbodiimide-treated fibronectin*	125
2,3-Butanedione-treated fibronectin**	70

The effect of chemical modification on the hemagglutination activity of fibronectin

The results from experiments employing chemical modification of fibronectin are shown in Table II. Succinylation of amino groups had only a slight effect. Treatment of fibronectin with the arginyl reagents 2,3-butanedione and phenylglyoxal reduced the activity to some extent. The activity was strongly reduced by a modification of carboxyl groups (Table II).

Discussion

We have shown that the hemagglutinating activity of fibronectin can be inhibited by low concentrations of specific lectins (Table I). Since *Lotus tetragonolobus* lectin and Soybean agglutinin are known to bind to carbohydrate structures containing L-fucose [13] or N-acetylgalactosamine [14], respectively, our results suggest that carbohydrate groups may play a role in the fibronectin-induced hemagglutination. Because fibronectin contains no fucose nor galactosamine [15], it is unlikely that the observed effect of lectins could be due to a binding of the lectins to fibronectin. It appears that the lectins bind to some carbohydrate-containing structures on the erythrocyte membrane, and prevent the interaction of these molecules with fibronectin, thus inhibiting the hemagglutination. It has been recently reported that ricin similarly inhibits fibronectin-mediated adhesion of fibroblasts [16]. Thus it seems that carbohydrate-containing structures are generally important in fibronectin-cell surface interactions.

The molecules on red cell membrane which bind fibronectin remain to be identified. In the present experiments an interaction of membrane components solubilized in sodium dodecylsulfate was demonstrated (Fig. 3). From Fig. 3, a formation of large-molecular weight complexes between fibronectin and the receptor(s) is evident. This suggests that the receptors are relatively large molecules.

Sera from various species inhibited the hemagglutination. The inhibitory activity of rabbit sera was heat-labile, and resided in a macromolecular

fraction, as shown by gel chromatography experiments (Fig. 2). The elution position of the factor suggests a molecular weight larger than that of albumin. However, the exact molecular weight of the factor remains to be determined.

These results raise the possibility that the interaction of fibronectin with cell surfaces could be regulated by macromolecular humoral factors. Further work to characterize the inhibitory factors of serum is in progress.

Chemical modification experiments were carried out in order to identify chemical groups of fibronectin essential for the hemagglutinating activity. The results indicate (Table II) that succinylation of free amino groups had no significant effect on the hemagglutinating activity, suggesting that amino groups are not essential for the activity. Treatment of fibronectin with the specific arginyl reagents 2,3-butanedione and phenylglyoxal caused a clear but not complete reduction in activity. Modification of carboxyl groups by carbodiimide caused an effective inactivation. This could mean that carboxyl groups of fibronectin are directly involved in the hemagglutination, or that they are important for keeping up a native conformation essential for the activity. We have shown earlier that the hemagglutinating activity of fibronectin is heat-labile, suggesting that a native conformation is important for the activity [17]. Modification of carboxyl groups of fibronectin has been shown to lead to conformational changes [18].

It has been shown by other workers that a chemical modification of sulfhydryl and amino groups of cell adhesion and spreading factor (a protein closely related to fibronectin) had no effect on its cellular adhesion-mediating activity, while a modification of carboxyl groups resulted in a loss of activity [19]. Thus the stability of the hemagglutinating activity of fibronectin is very similar to that of cellular adhesion-mediating activity. This further supports the validity of the hemagglutination system as a model for fibronectin-cell surface interaction.

In another communication [18] we have shown that succinylation performed under conditions identical with those used in this study completely inhibited the binding of fibronectin to collagen. Similarly, modification of arginyl groups inactivated the collagen-binding activity. Thus it seems that the parts of fibronectin molecules that interact with cell surfaces are different from those mediating the binding to collagen. This conclusion is also supported by the finding that the two activities have different thermal stability [17]. The cellular adhesion-mediating and collagen-binding activities have also been identified in different peptides after a limited proteolytic digestion of fibronectin [20]. The existence of different binding sites on fibronectin molecule for cell surface and for collagen probably is essential for the adhesion-mediating function of this protein.

Acknowledgement

We thank Ms. Airi Visuri for secretarial assistance during the preparation of the manuscript.

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