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CELL SURFACE COMPONENT(S) INVOLVED IN RAT HEPATOCYTE INTERCELLULAR ADHESION

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SUMMARY: Aggregation of rat hepatocytes was effectively inhibited by monovalent antibodies (Fab fragments) directed against hepatocyte plasma membranes, but monovalent antibodies against some distinct, known hepatocyte surface antigens had no effect. Surface antigens, which neutralized the Fab inhibiting effect on cell aggregation, could be solubilized from plasma membranes by limited proteolytic digestion. Thus, hepatocyte intercellular adhesion seems to involve specific cell surface components, which may be proteins or protein derivatives.

INTRODUCTION

Intercellular recognition and adhesion are of fundamental importance for the physiology of multicellular organisms, but our knowledge of the molecular mechanisms of these phenomena is still limited. Several models for how cells adhere to each other have been proposed (1), and essentially two fundamentally different groups of models can be distinguished. In the physical model cell adhesion is regarded as the result of a balance between non-specific repulsive and attractive physical forces acting on a large area of the cell surface (1). In the biochemical models cell adhesion is thought to be mediated by specific binding between cell surface-associated molecules in a receptor-ligand type of interaction (1). Recently, evidence supporting the biochemical model has been presented for cellular slime moulds (2) and for neural retina from chick embryos (3). In these studies an immunochemical method devised by Gerisch and his associates (4), employing monovalent antibodies directed against the cell surface was used. With a similar technique we demonstrate in the present communication that specific cell surface components are involved in the intercellular adhesion between mammalian cells also. These cells are rat hepatocytes, which we earlier have shown are well suited for the exploration of molecular mechanisms in mammalian cell adhesion phenomena (5-9).

Abbreviations: LEP, liver eigen protein; EDTA, ethylenediamine tetraacetic acid; DOC, sodium deoxycholate

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MATERIALS AND METHODS

Antisera and Fab fragments. Rabbit antisera against rat hepatocytes or rat liver plasma membranes were produced by subcutaneous injection of Freund's complete adjuvant either containing 10^7 cells, isolated by collagenase perfusion (5,7), or 1 mg (as protein) of plasma membranes, isolated according to Ray (10). Booster injections were given with the same amount of antigen in incomplete adjuvant every 3:rd - 4:th week. Antisera against LEP (= liver eigen protein, ref. 11), rat hepatocyte heparin receptor and bovine fibronectin were generous gifts of Drs. D.M. Neville, Jr., L. Kjellén and S. Johansson, respectively, which we gratefully acknowledge. The immunoglobulin fraction was isolated from antisera by precipitation with 50% saturated ammonium sulphate, and was digested with crystalline papain (P 3125, Sigma Chemical Co.; 1 mg enzyme per 50 mg protein in 0.03 M NaCl, 0.02 M tris-HCl, pH 7.4, 0.05 M cystein-HCl, 0.05 M EDTA) at 37° C for 15 hours. The digest was dialyzed against 0.15 M NaCl, 0.01 M phosphate, pH 7.4 and the Fab fraction was isolated by chromatography on Sephadex G-100.

Membrane solubilization. Detergent solubilization of plasma membranes (isolated according to Ray (10)) was performed with 0.12 M sodium deoxycholate (DOC) (Merck; 10 mg DOC per mg membrane protein) in 0.15 M NaCl, 0.01 M tris-HCl, pH 8.0. The solubilized membrane components were chromatographed on Sephadex G-100 equilibrated with 0.01% Tween 80 (Sigma Chemical Co.) to remove DOC. The DOC-free fractions were pooled and concentrated by pressure dialysis (Amicon PM 10 filter). Before testing in cell aggregation these components were dialyzed against a balanced salt solution (Buffer 3 in ref. 7) containing 0.01% Tween 80.

Limited proteolytic digestion of plasma membranes (isolated according to Ray (10)) was carried out with trypsin or papain. Trypsin digestion: plasma membranes in 0.15 M NaCl, 0.01 M phosphate, pH 7.4 at a concentration of 5 mg protein/ml were incubated with trypsin (DPCC-treated, T 1005, Sigma Chemical Co.; 0.04 mg enzyme per mg membrane protein) for 60 min at 37° C after which soybean trypsin inhibitor (T 9003, Sigma Chemical Co.; 2 mg per mg trypsin) was added. The membrane suspension was centrifuged and the supernatant was collected. Papain digestion: plasma membranes in 0.03 M NaCl, 0.02 M tris-HCl, pH 7.4, 0.05 M cystein-HCl, 0.05 M EDTA at a concentration of 5 mg protein/ml were incubated with crystalline papain (P 3125, Sigma Chemical Co.; 0.03 mg enzyme per mg membrane protein) for 60 min at 37° C. The membrane suspension was centrifuged and the supernatant was dialyzed against Buffer 3 at 4° C.

Gel and ion exchange chromatography. Trypsin- or papain-solubilized membrane components, concentrated to 1 ml (Amicon, PM 10 filter) were applied to a 1x50 cm column of Sephadex G-200. The column was eluted with Buffer 3 at 4° C and fractions of 1 ml were collected. Pooled fractions were dialyzed against 0.1 M NaCl, 0.02 M tris-HCl, pH 7.4, concentrated to 1 ml and applied to a 1.5x20 cm column of DEAE-Sephadex A-50 equilibrated with the same buffer. The column was eluted at 4°C with a linear gradient made from 45 ml 0.1 M NaCl, 0.02 M tris-HCl, pH 7.4 and 45 ml 0.5 M NaCl, 0.02 M tris-HCl, pH 7.4, followed by 1 M NaCl, 0.02 M tris-HCl, pH 7.4. Fractions of 3 ml were collected and each fraction was dialyzed against Buffer 3.

Cell aggregation. Aggregation of rat hepatocytes, isolated by collagenase perfusion (5,7), was measured as the reduction in the number of single cells from a shaken suspension, essentially as described earlier (5). Suspensions of cells ($1x10^6$ cells in 0.5 ml of Buffer 3 supplemented with glucose (1 mg/ml) and DN:ase (DN-25, Sigma Chemical, Co.; 0.05 mg/ml)) were rotated at

80 rpm (New Brunswick G 24 environmental incubator shaker) in flat-bottomed plastic dishes (Linbro Sci. Co., Cat. No. FB 16-24-TC) at 37° C for various times. Aliquots of 0.1 ml were diluted with ice-cold Buffer 3 (40.0 g) and the number of single cells was determined with a Celloscope 302 (Lars Ljungberg & Co., Stockholm, Sweden) equipped with a capillary 190 µm in aperture diameter. Prior to incubation at 37° C, the cells were preincubated at 4 ° C for 30 min, with various Fab fragments which had or had not been incubated together with solubilized membrane components at 20° C for 30 min.

RESULTS AND DISCUSSION

Inhibition of cell aggregation by Fab fragments

Monovalent antibodies (Fab fragments) against rat hepatocytes or rat liver plasma membranes, effectively inhibited hepatocyte aggregation (Fig. 1), even at low concentrations (Fig. 2), whereas Fab fragments from non-immunized rabbits had no effect (Fig. 1). However, Fab fragments of antibodies (which were demonstrated to bind to the hepatocytes) directed against distinct hepatocyte surface antigens had no effect on cell aggregation (Fig. 1). These antigens, which include a liver specific plasma membrane protein (LEP, ref. 11), a partially purified rat hepatocyte heparin receptor (12), and fibronectin (see below), are apparently not involved in hepatocyte intercellular adhesion. Thus mere binding to the cell surface of any Fab molecules is not sufficient to prevent aggregation. Similarly, binding of other types of molecules to the hepatocyte surface did not affect aggregation, as demonstrated by the fact that addition of asialo-glycoproteins (galactoproteins), for which mammalian hepatocytes have receptors (13), was without effect. The inhibition of cell aggregation by Fab fragments directed against hepatocytes or plasma membranes was not due to a toxic effect on the cells, since hepatocytes both attached and spread on collagen and fibronectin in the presence of anti-plasma membrane Fab. This also argues against the possibility that inhibition of aggregation might be due to steric shielding of receptors by Fab molecules directed against a major cell surface component, which is unrelated to intercellular adhesion. It thus seems as if the inhibition of cell aggregation is due to specific blocking of surface molecules involved in intercellular adhesion.

Solubilized membrane antigens which neutralize the Fab-inhibition of cell aggregation

In order to follow the purification of the adhesive components of plasma membranes their activity was assayed by use of the anti-plasma membrane Fab

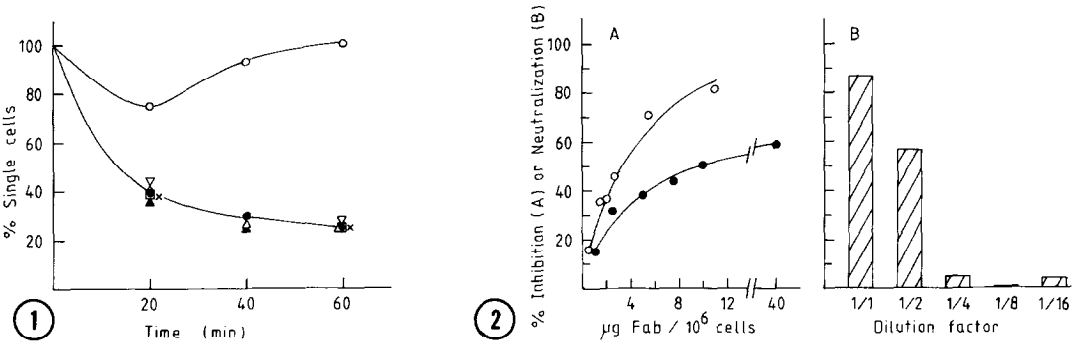

Fig. 1. Effects of various Fab fragments on rat hepatocyte aggregation. ●, preincubation in the absence of any added Fab fragments; O, preincubation with anti-plasma membrane Fab (1 mg/10^6 cells); □, preincubation with anti-plasma membrane Fab (1 mg/10^6 cells) absorbed with rat liver plasma membranes; Δ, preincubation with anti-LEP Fab (1.6 mg/10^6 cells; ∇, preincubation with antibovine fibronectin Fab (0.2 mg/10^6 cells); X, preincubation with anti-heparin receptor Fab (0.2 mg/10^6 cells); ▲, preincubation with normal rabbit IgG Fab (1.2 mg/10^6 cells).

Fig. 2. Fab-inhibition (A), and neutralization of Fab-inhibition (B) of hepatocyte aggregation. (A): Inhibition of aggregation resulting from preincubation with the indicated amounts of two different preparations (O,●) of anti-plasma membrane Fab. O, cell suspensions were rotated at 37° C for 30 min; ●, cell suspensions were rotated at 37° C for 40 min. Zero % inhibition is set to equal the number of single cells remaining in the absence of anti-plasma membrane Fab. One hundred % inhibition is set to equal the number of single cells present prior to the rotary incubation at 37° C. (B): Fab-neutralizing activity of plasma membranes, solubilized with papain. Two hundred µl of various dilutions of the soluble membrane components were incubated with 10 µg of anti-plasma membrane Fab fragments in a total volume of 400 µl for 30 min at 20° C. 1x10^6 cells in 100 µl of Buffer 3 were added and the cells were preincubated for 30 min at 4° C, after which rotary incubation was performed for 40 min at 37° C. Zero % neutralization of the Fab-inhibition of cell aggregation is set to equal the number of single cells remaining in the presence of 10 µg Fab fragments, without any added papainate. One hundred % neutralization is set to equal the number of single cells remaining in the absence of any Fab fragments.

fragments as outlined in Fig. 2. The cell aggregation inhibiting effect could be neutralized by preincubation of Fab with whole plasma membranes (Fig. 1). Plasma membranes solubilized with DOC also neutralized the Fab fragments. However, the latter results were difficult to interpret since the DOC-solubilized components themselves affected the hepatocyte aggregation in a non-reproducible manner. This effect of the DOC-solubilized components probably was due to the aggregation of the components which occurred when DOC was exchanged to the non-lytic detergent Tween 80, which was necessary before

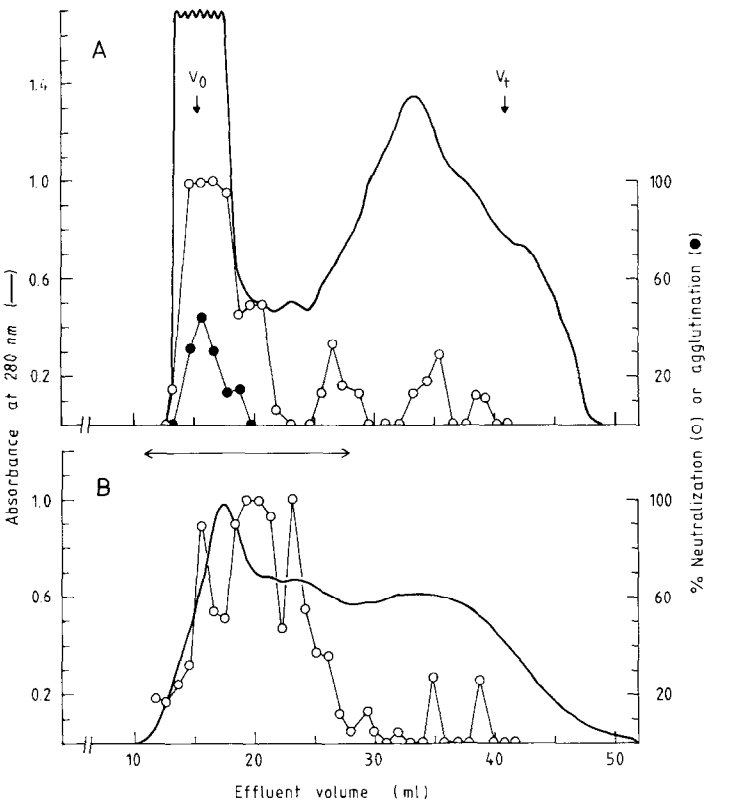

Fig. 3. Fab neutralizing and agglutinating activities of plasma membrane components solubilized by trypsin (A) or papain (B); fractionation by gel chromatography. Plasma membranes were digested with (A) trypsin (155 mg membrane protein) or (B) papain (180 mg membrane protein) and the soluble components were chromatographed on Sephadex G-200. Fifty µl of each fraction were tested for Fab neutralizing activity (○) as described in Fig. 2B. The agglutinating activity (●) of the soluble components was measured (without Fab fragments) by addition of 50 µl of the respective fractions to the aggregation assay. The agglutinating activity is given as the increase, in per cent, of the disappearance of single cells, over that obtained in the absence of any fractions. The papainate had no agglutinating activity of its own. The horisontal arrow indicates the fractions which were pooled and subjected to ion exchange chromatography as described in Fig. 4.

adding the solubilized components to viable hepatocytes. In order to circumvent these difficulties plasma membranes were treated by limited proteolytic digestion with trypsin or papain.

Both enzymes released Fab neutralizing acitivity into solution (Fig. 2), leaving no activity in the membrane residues. In addition the trypsinate itself had hepatocyte-agglutinating activity (Fig. 3), suggesting that

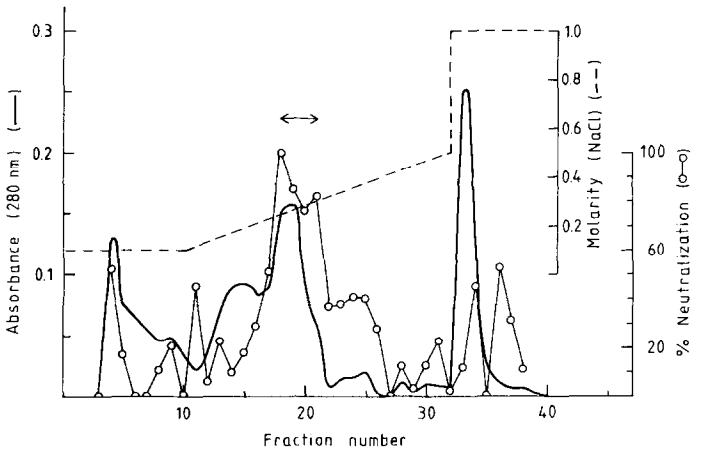

Fig. 4. Ion exchange chromatography of plasma membrane components solubilized by papain. The pooled fractions from the G-200 chromatography (Fig. 3B) were chromatographed on DEAE-Sephadex. Two hundred µl of each dialyzed fraction were tested for Fab neutralizing activity (O). The fractions indicated by the horisontal arrow were pooled, dialyzed against distilled water, lyophilized and analyzed by SDS polyacrylamide gel electrophoresis according to Neville (15).

multivalent components were released. The trypsin-released material with Fab neutralizing activity was of high molecular weight and heterogenous in size as demonstrated by chromatography on Sephadex G-200 (Fig. 3). The active components released by papain were somewhat smaller than those released by trypsin and showed no agglutinating activity of their own (Fig. 3). After Sephadex chromatography of the papainate the active material was pooled and subjected to ion exchange chromatography on DEAE-Sephadex, which resulted in the elution of the Fab neutralizing activity as one major and a few minor peaks (Fig. 4). SDS disc gel electrophoresis of the pooled major activity peak, under non-reducing conditions, revealed several protein bands. However, under reducing conditions only one major band, with a higher electrophoretic mobility than the majority of the non-reduced components, was observed.

Conclusion

Rat hepatocyte intercellular adhesion seems to be mediated by specific cell surface components, thus demonstrating that the biochemical model of cell-cell adhesion is applicable also to mammalian cells. Since both trypsin and papain released soluble fragments of these components from isolated plasma membranes, they are probably proteins or protein derivatives.

Fibronectin has been ascribed a role in both cell-cell and cell-substratum adhesion (see ref. 14 for a review). Previous studies in our laboratory have

demonstrated that rat hepatocytes very efficiently adhere to fibronectin-coated substrata (8) but that fibronectin does not mediate hepatocyte attachment to collagen (9). The rat hepatocytes might contain small amounts of surface-associated fibronectin (9), but further experiments are required to definitely show that fibronectin is a true cell surface constituent of rat hepatocytes. However, even if fibronectin proves to be a surface component, the present results indicate that it is not required for the formation of adhesive bonds between rat hepatocytes. Furthermore, the adhesive components are apparently not identical with LEP, the galactoprotein receptor or the heparin receptor, which are identified hepatocyte surface constituents.

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