COLD-INSOLUBLE GLOBULIN MEDIATES THE ADHESION OF RAT LIVER CELLS TO PLASTIC PETRI DISHES

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Received October 17, 1977

SUMMARY: Adhesion of rat hepatocytes to plastic culture dishes requires a factor present in normal plasma or serum which tentatively is identified as cold-insoluble globulin since *(i)* cold-insoluble globulin was the only native plasma protein tested showing cell-adhesion mediating activity, and *(ii)* plasma from which cold-insoluble globulin selectively had been removed lost its ability to induce cell attachment.

Under certain circumstances also asialoceruloplasmin became a potent cell adhesion mediating agent. However, cell attachment mediated by asialoceruloplasmin and cold-insoluble globulin, respectively, was demonstrated to involve separate mechanisms.

INTRODUCTION

The attachment of vertebrate cells to a substratum is a prerequisite for a number of normally occurring cell physiological phenomena, such as proliferation (so called anchorage dependence of growth) and motility. Fibroblastic cells adhere readily to a variety of different substrates (1-3), whereas rat hepatocytes were recently demonstrated to require a component present in calf serum to attach to plastic dishes (4). Preliminary experiments in an attempted purification of the serum adhesion factor suggested this to be a protein with properties similar to that of CIG[1)]. CIG is immunologically related to fibronectin, a cell surface-associated protein of cultivated fibroblasts and glia cells (for review see Refs 5 and 6), which is deleted on the malignant or transformed counterpart (7.8). CIG has previously been demonstrated to mediate the adhesion of BHK cells (9) to denaturated collagen. In this report evidence is presented suggesting that the serum dependent adhesion of rat liver parenchymal cells (hepatocytes) to culture dishes is mediated largeley by CIG.

1)Abbreviation: CIG, cold-insoluble globulin

ISSN 0006-291X

MATERIALS AND METHODS

Materials

Transferrin, albumin and gammaglobulin of human origin and fetuin prepared from fetal calf serum were kindly provided by Dr. T.C. Laurent, Department of Medical and Physiological Chemistry, University of Uppsala, and human antithrombin by Dr. I. Björk, Department of Medical and Physiological Chemistry, Swedish University of Agricultural Sciences.

Ceruloplasmin was purchased from AB Kabi, Stockholm. Sweden, and was further purified by chromatography on Sephadex G-200. Cold-insoluble globulin was isolated from human plasma according to the modified (10) procedure of Mosher (11).

Asialoceruloplasmin was obtained after digestion of ceruloplasmin with neuraminidase (12), followed by chromatography on Sephadex G-200.

Deacetylated asialo-oligosaccharides prepared from fetuin were a generous gift of Dr. B. Nilsson, Department of Clinical Chemistry, University of Lund, Lund, Sweden. Before use oligosaccharides were re-acetylated by treatment with acetic anhydride (13).

Human plasma was depleted of cold-insoluble globulin by passing the plasma through a column of gelatin-Sepharose as described (14). The resulting product contained no CIG detectable by radial immunodiffusion.

Plastic Petri dishes (Cat. No 1008) were obtained from Falcon Plastics, Los Angeles, California, U.S.A.

Methods

Protein was determined according to Lowry et. al. (15) using bovine serum albumin as a standard.

Attachment of cells to plastic dishes. Rat hepatocytes were isolated by perfusion with collagenase via the portal vein as previously described (16). The hepatocytes were incubated in a balanced salt solution (Buffer 3, Ref. 17) at 37°C in humidified air; 3×10^6 cells in 2 ml were incubated in 35 mm Petri dishes. After appropriate incubation times the buffer was removed and the dishes were washed with 1 ml of balanced salt solution. Cell attachment was followed by microscopy and quantitated from the activity of lactate dehydrogenase after lysis of the cells with Triton X-100 as described (4).

The effect of various components on cell attachment was investigated in one of two ways: *(i)* The component was added in soluble form directly to the incubation medium at the start of the incubation; *(ii)* The dishes were pre-coated by adding 10 µg of the component (or 5 µl of plasma) dissolved in 2 ml of water to each dish. The dishes were air-dried, washed extensively with water and air-dried again before being used in the attachment assay. All incubations were performed in duplicate.

RESULTS AND DISCUSSION

Plasma proteins mediating cell-substrate adhesion

Rat hepatocytes incubated in the balanced salt solution in Ham's F-10 medium require the assistance of component(s) present in normal plasma or serum to adhere to plastic dishes (both "bacterial" and "tissue culture" dishes (4)). To tentatively identify factors responsible for the adhesion mediating activity of plasma the potency of various plasma pro-

Table 1. Adhesion of rat hepatocytes to plastic culture dishes in the presence of plasma proteins

Protein	Number of cells attached x 10^{-4}		
	Protein conc. 25 µg/ml	Protein conc. 2.5 µg/ml	Protein films
None	11		40
Transferrin	2	17	4
Albumin	2	7	3
Fetuin	3	9	2
Antithrombin	2	5	2
Gamma globulin	3	13	38
Ceruloplasmin	6	9	4
Asialoceruloplasmin	11	51	240
CIG	170	130	240

Rat hepatocytes (3×10^6) were incubated for 60 min in 2 ml balanced salt solution in Petri dishes in the presence of soluble proteins at stated concentrations or alternatively in Petri dishes precoated with protein. (For further experimental details, see the Method section.)

teins as attachment mediators was investigated. Proteins were either added directly to the incubation mixture, or dishes precoated with protein films were used in the adhesion assay. In both cases CIG was the only native plasma protein tested which was capable of mediating cell-substrate adhesion (Table 1). However, cells readily attached to plastic dishes coated with asialoceruloplasmin. Ashwell, Morell and co-workers have demonstrated that plasma glycoproteins from which the terminal sialic acid residues have been removed thus exposing the penultimate galactose units, are rapidly cleared from the circulation. This uptake of the asialoglycoprotein is initiated by binding of oligosaccharide prosthetic groups, having galactose at their non-reducing ends, to a specific receptor present on the surface of hepatocytes (for a review see Ref. 18). It seems likely that this asialoglycoprotein receptor participates in the asialoceruloplasmin mediated cell-substrate adhesion. The possibility that the same receptor is involved in adhesion mediated by CIG had to be considered

Table 2. Effect of asialo-oligosaccharides on mediated cell attachment to plastic dishes

Attachment mediator	Oligosacch. conc. (µg/ml)	Number of cells attached x 10^{-4}	% cells attached[a)]
Asialoceruloplasmin	0	210	100
"	10	36	17
"	50	1	0.5
CIG	0	240	100
"	10	220	92
"	50	190	79
Plasma	0	220	100
"	10	190	86
"	50	180	82

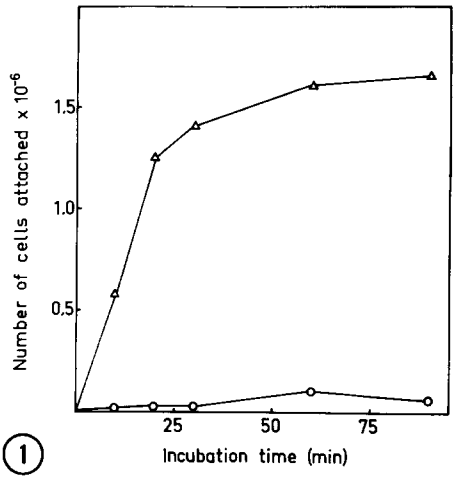
a) The number of cells attached in the absence of asialo-oligosaccharide was set to 100%

Cells (3×10^6) were incubated for 60 min in 2 ml balanced salt solution in plastic Petri dishes pre-coated with attachment mediator protein. (For further experimental details. see the Method section.)

since this glycoprotein may contain oligosaccharides where galactose is the terminal sugar unit (19). On addition of asialo-oligosaccharides, prepared from fetuin, to the medium the adhesion activity of asialoceruloplasmin was almost completely abolished whereas cell-substrate adhesion mediated by CIG or intact plasma was not drastically affected (Table 2). Thus the asialoglycoprotein receptor is presumably involved in the adhesion of hepatocytes to substratum mediated by asialoceruloplasmin but is probably not essential for adhesion mediated by CIG or intact plasma.

Preliminary characterization of CIG mediated adhesion of hepatocytes to substratum

Adhesion of rat hepatocytes to substratum mediated by CIG is shown to be a time-dependent process (Fig. 1). In the presence of 10 µg of CIG/ml the number of attached cells increased for at least 30 min but incubations performed for longer time than 60 min did not result in a significant further increase.



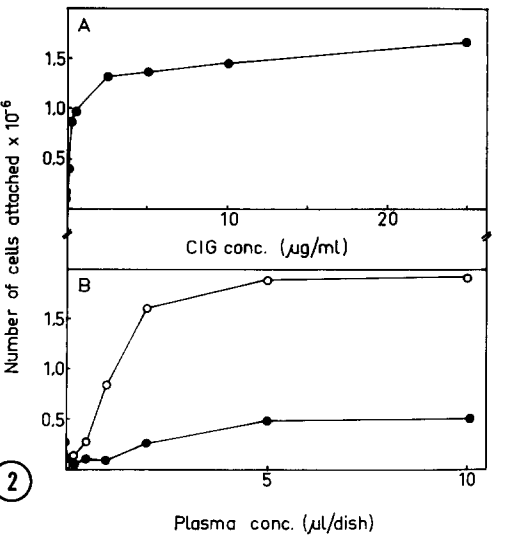


Fig. 1. Time course of CIG mediated cell attachment

Cells (3×10^6) in 2 ml of balanced salt solution were incubated in plastic Petri dishes in the presence (Δ—Δ) or absence (o—o) of CIG (10 μg/ml) for the indicated periods of time. The amounts of cells attached to the dishes were estimated as described in the Method section. Under these conditions 50-60% of the cells attached in the presence of CIG. However, 90-100% of the cells were able to attach when seeded at lower cell density ($<0.6 \times 10^6$/ml).

Fig. 2. Cell attachment as a function of CIG concentration

Cells (3×10^6) in 2 ml of balanced salt solution were incubated (A) in dishes after addition of CIG to yield the indicated concentrations or (B) in dishes precoated with the indicated amounts of normal (o—o) and CIG deficient (●—●) plasma. After 60 min incubation period the numbers of cells attached to the dishes were quantitated as described in the Method section.

The number of cells adhering to the culture dishes increased rapidly with the amount of CIG present in the medium up to a protein concentration of about 2.5 μg/ml (Fig. 2A); only a limited further increase of adhered cells was observed at high concentrations (25 μg/ml) of CIG.

The possibility that CIG is responsible for the cell-substrate mediating activity of plasma was investigated in experiments where cells were incubated in dishes coated with complete human plasma[2)] and the same plasma

[2)] Addition of plasma to the incubation medium caused an agglutination of cells, a phenomenon not observed in experiments using Petri dishes precoated with plasma. For this reason only the latter type of dishes were used when the effects of plasma were investigated.

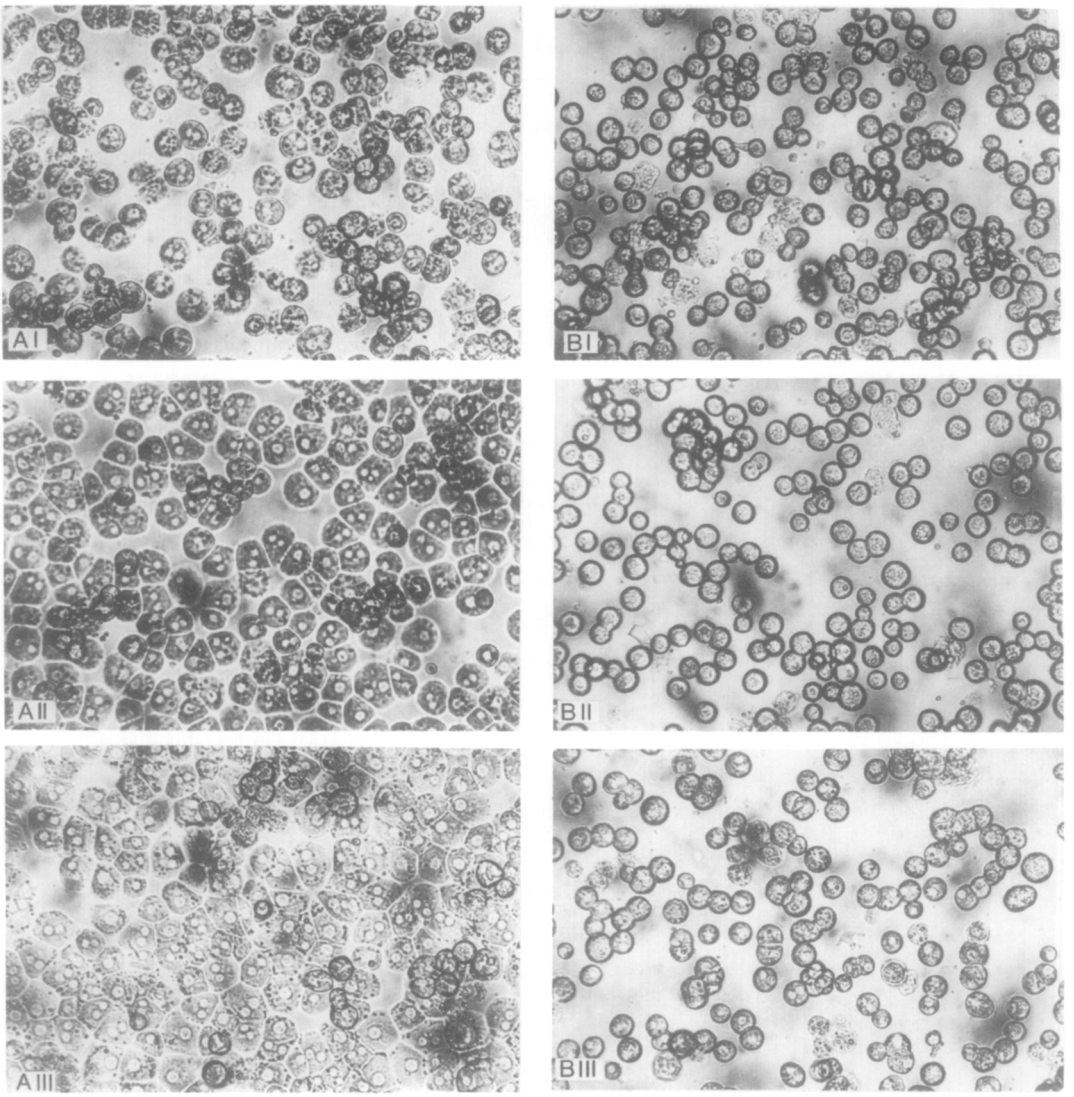


Fig. 3. Cell spreading of rat hepatocytes attached to protein films

Cells (3×10^6) in 2 ml of balanced salt solution were incubated at 37°C in dishes precoated with CIG (A) or asialoceruloplasmin (B). After 30 min the attached cells were washed with 3 x 1 ml of F-10 medium (27) supplemented with 100 U/ml of penicillin and 50 µg/ml of streptomycin and incubation was continued at 37°C in F-10 medium. The cells were photographed in a phase contrast microscope at 0 (I), 1 (II) and 7 (III) hours after the initial 30 min attachment period.

selectively depleted of CIG, respectively. The results show that cells adhered efficiently to films prepared from intact plasma but not to films prepared from CIG deficient plasma (Fig. 2B). Thus the cell substrate adhesion mediating activity of plasma appears to be at least partly exerted by CIG. Incubations in the presence of goat gammaglobulin raised against CIG did not affect the attachment of cells to CIG or plasma coated dishes.

Effect of CIG on cell-spreading

Under appropriate culture conditions rat hepatocytes change morphology from a rounded to a more polygonal shape. In view of the recent finding of a cell spreading factor present in plasma and which tentatively has been characterized as CIG (20; F. Grinell and D.G. Hays, personal communication) and of reported effects of urea solubilized cell surface fibronectin on the morphology of transformed fibroblasts (21) the shape of rat hepatocytes cultured on dishes coated with plasma proteins was examined. The results showed that cells cultured on purified CIG spread efficiently whereas cells cultured on asialoceruloplasmin retained their rounded shape (Fig. 3).

The physiological role of CIG in relation to hepatocytes is unclear. A previous report suggests that CIG mediates the attachment of fibroblasts to collagen (9) but the rat hepatocytes both attach to and spread on CIG-free collagen in the absence of CIG (4,22). Rat hepatocytes isolated with collagenase perfusion do not seem to contain fibronectin on their surface (22,23). However, fibronectin occurs associated with collagen structures (24) and in the liver both fibronectin (25) and collagen type III (26) are associated with the sinusoidal surfaces of the hepatocytes, where reticular fibres have been localized. Hence, both fibronectin and collagen may be involved in the anchorage of hepatocytes *in vivo*.

ACKNOWLEDGEMENTS

We wish to thank Drs. F. Grinell and D.G. Hays for sending us manuscripts in advance of their publication.

The skilful technical assistance of Miss I. Pettersson is gratefully acknowledged.

This investigation was supported by grants from the Swedish Medical Research Council (05197, 02309, 03X-4, 13X-05200), Gustaf V:s 80-årsfond, Svenska livförsäkringsbolagens nämnd för medicinsk forskning, and grant No. CA 17373 awarded by the National Cancer Institute, USPHS.

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