IDENTIFICATION OF CARBOHYDRATES AND FUNCTIONAL GROUPS INVOLVED IN THE ADHESION OF NEOPLASTIC CELLS

Proto PIPPIA, Giorgio IVALDI and Augusto COGOLI+

Istituto di Fisiologia Generale, Università di Sassari, Via Muroni 25, 07100 Sassari, Italy and +*Laboratorium für Biochemie, ETH – Zentrum, Universitätstrasse 16, 8006 Zurich, Switzerland*

Received 9 April 1980
Revised version received 3 June 1980

1. Introduction

Some experimental data supports the hypothesis that the molecular components which regulate cell–cell recognition and related phenomena, as cell adhesion and control of mitosis, are localized on the cell surface [1,2]. In addition, common chemical structures could regulate cell–cell recognition, cell growth and development.

The purpose of our study was to establish a correlation between molecular structures on the cell surface and the phenomena mentioned above. Here we report the effect of chemical and enzymatic modification of components of the cell membrane on the cell adhesion.

As a 'test system' we used a strain of neoplastic cells [3] with high monolayer density, which permits a more accurate measurement of cell adhesion than with a strain with lower density as, e.g., fibroblasts.

The involvement of carbohydrates in cell adhesion was studied by degradation with specific enzymes and by oxidation with sodium periodate. The role of amino, carboxy and sulphydro groups was investigated with group-specific reagents.

2. Materials and methods

2.1. *Neoplastic cells*

The cell strain (SGS-2) used in this investigation was derived from a solid sarcome (Sarcome Galliera) of the rat [3].

2.2. *Adhesion assay*

The method in [4] was modified according to the properties of SGS-2 cells:

(i) The cell layers were prepared by inoculation of 9×10^4 cells/cm^2 as 30 h before the assay on Linbro 12-well tissue culture wells (Flow Labs.);

(ii) Single labeled cells (SLC) were prepared by exposing confluent monolayers to 2 μCi/ml [^{3}H]leucine (Amersham, spec. act. 52 Ci/mmol) for 24 h at 37°C, in medium A [5]. Thereafter a single cell suspension was obtained by treatment of monolayer with 0.25% trypsin (Gibco) in solution A for 7 min at 37°C. Under these conditions we obtained a label of 0.1/0.2 cpm/cell;

(iii) The adhesion was determined by the addition of 1.5 ml of SLC suspension (10^5–1.5×10^5 cells/ml) to each well containing a confluent monolayer followed by incubation at 37°C with shaking at 60 strokes/min. At given times non-adhering cells were removed by cautious aspiration through polytene capillaries of 0.4 mm diam. The monolayers were washed twice with medium B [5] without amino acids (0.5 ml/cm^2 of monolayer). The monolayers were lysed with 1.5 ml 2 M NH_4OH (3 washes of 0.5 ml each). The combined washes were mixed with 11 ml Instagel (Packard) for radioactivity determination.

2.3. *Enzymatic modification of the cell membrane*

SLC (at 2–4 $\times$ 10^6 cells/ml) were exposed to the enzyme chosen for the experiment at 37°C for 30 min in PBS (Ca^{2+}–MG^{2+}-free phosphate-buffered saline, pH 7.4). Thereafter cells were washed twice with PBS and resuspended in medium B for the adhesion assay. The enzymes used in this study were

neuraminidase from *Vibrio cholerae* (EC 3.2.1.18) (Behring Institut), galactose oxidase from *Polyporus circinatus* (EC 1.1.3.9) (type 1 from Sigma), L-fucosidase from calf kidney (Boerhinger), (EC 3.2.1.51).

2.4. *Chemical modification*

The oxidation with sodium periodate was done essentially according to the conditions in [6] for the stimulation of lymphocytes. SLC ($2–4 \times 10^6$/ml) in PBS were treated with 1 or 2 mM sodium periodate for 30 min at 0°C with occasional shaking. Thereafter the excess of oxidant was removed by washing twice with medium B. When the oxidation was followed by reduction with $NaBH_4$ the cells were washed and resuspended in PBS as above and exposed to 2 mM $NaBH_4$ (Fluka purum) for 30 min at 0°C. Excess of reducent was removed by washing twice with medium B. Reactions with iodacetamide (Merck), *N*-cyclohexil-*N'*-(2-(4-morpholinyl) ethyl) carbodiimid-methyl-*p*-toluol sulfonate (o CMC) (Sigma), dimethylsuberimidate 2 HCl (o DMS) (Sigma), dimethyladipimidate 2 MHCl (o DMA) (Sigma) were performed by incubating SLC resuspended in PBS ($2–4 \times 10^6$ cells/ml) with the given concentrations of the reagent at 0°C for 30 min. Excess of reagent was removed by 2 washes in medium B. In the experiment with CMC the activation of carboxyl groups was performed in the presence of 150% excess of NH_4OH with respect to CMC. The cell suspension was buffered at pH 7.3 with diluted HCl.

Either enzymatically or chemically modified cells were viable as controlled by Tripan blue exclusion. In addition they retained their capability of forming monolayers.

3. Results and discussion

The results of the experiments described here provide evidence that carbohydrates and other functional groups of membrane components are involved in the adhesion phenomena. In all experiments viability and cell growth were not affected by the treatment. This permits one to conclude that the results observed are not due to toxic effects of the enzymes or reagents employed.

3.1. *Carbohydrates*

The adhesion of SLC to monolayers is increased by 58% by treatment with neuraminidase (fig.1.1.B). On the other hand, simultaneous treatment with neuraminidase and galactose oxidase [8], which removes sialic acid residues localized at the end of the carbohydrate chain, exposing galactose residues to enzymatic oxidation, causes a loss of adhesion capacity, with respect to the cells depleted of sialic acid (fig.1.1.C). Although sodium periodate could potentially react with a number of vicinal alcoholic groups or vicinals amino alcohols present on carbohydrates or lipids, sialic acid is one of the components on the cell membrane which is most exposed to the oxidant. Periodate selectively splits the $C_6–C_7$ and $C_7–C_8$ bonds of sialic acid with formation of aldehyde groups on C_6 and C_7, respectively [7]. Treatment of SLC with sodium periodate results in a 68% decrease of their adhesion capacity to monolayers (fig.1.2.B).

However when the aldehide groups are reduced with $NaBH_4$ to alcoholic residues, a significant amount of adhesivity is recovered: the percent of adhering cells which was reduced to 32% after periodate treatment recovers to 50% when periodate-modified cells are exposed to $NaBH_4$ (fig.1.2.C).

The discussion of our results relies on the assumption that neuraminidase and galactose oxidase do not cross the cell membrane because of their molecular size, and that the same is true for IO_4^- due to their charge and to the temperature of incubation (0°C). We suggest that sialic acid, which is localized at the very end of the carbohydrate chain on the cell surface has a regulatory function on cellular adhesion, acting as 'lubricant' which modulates the interaction between ligands which are sterically masked as, e.g., galactose. This property could be due to the anionic character of sialic acid [9]. In fact the higher adhesivity of cells deprived of sialic acid is supported by experimental observations demonstrating its regulatory function in the interaction between some glycoproteins and the cell membrane [9,10]. The increase of adhesion following exposure of galactose residues indicates that they are directly involved in the process. This is confirmed by the loss of adhesion after enzymatic oxidation of galactose. Periodate profoundly modifies the chemical structure of sialic acid without splitting it off from the carbohydrate moiety of membrane glycoproteins in a different way from neuraminidase. This alteration of the structure is probably responsible for the lower adhesivity of periodate-modified cells (fig.1.2). This interpretation is supported by the recovery of adhesivity after

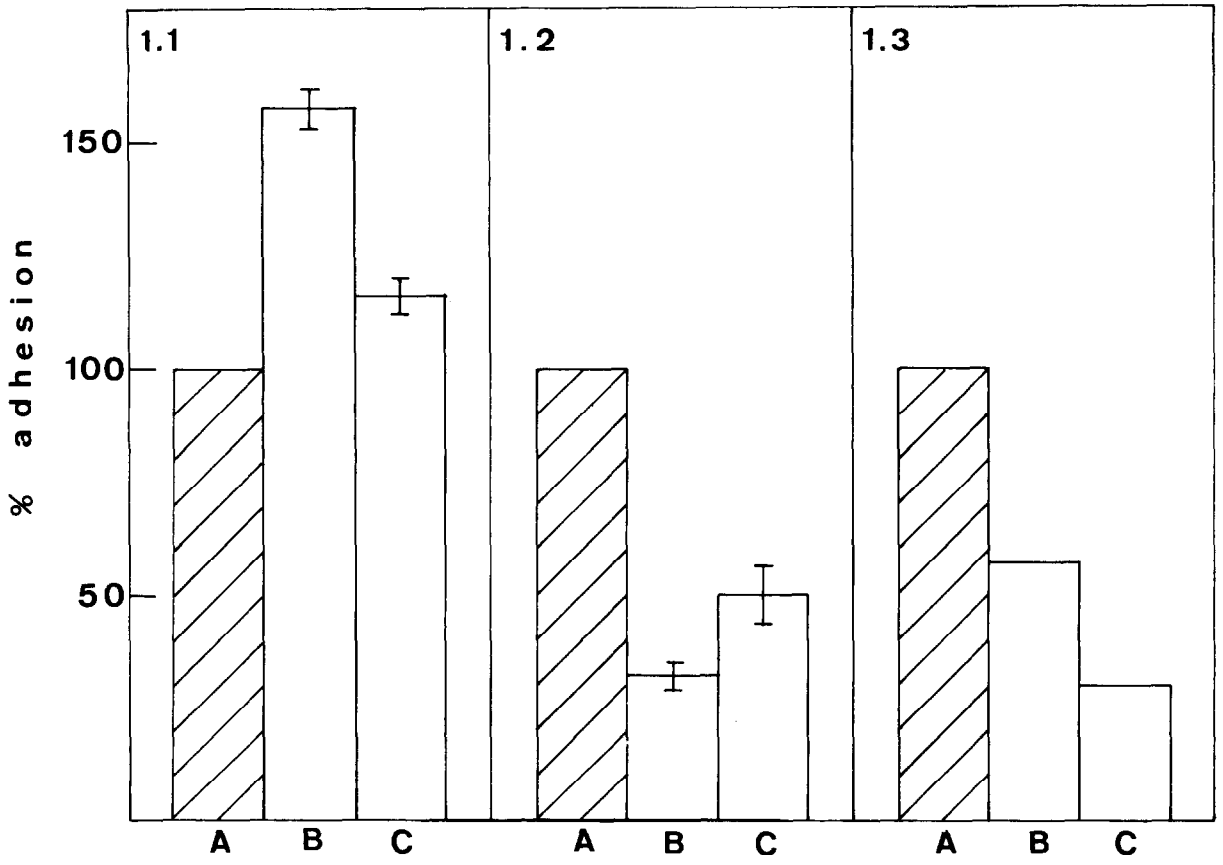

Fig.1. Effect of enzymatic and chemical reagents modifying the carbohydrate moiety of membrane glycoproteins on cell–cell adhesion: 1.1 (A) control, (B) neuraminidase (0.27 U/ml), (C) neuraminidase (0.27 U/ml) + galactose oxidase (2.8 U/ml); 1.2; (A) control, (B) sodium periodate 1 mM, (C) sodium periodate 1 mM + sodium borhydride 2 mM; 1.3, (A) control, (B) L-fucosidase (153 μg/ml), (C) L-fucosidase (180 μg/ml). The adhesion of the controls is taken as 100%. In 1.1 and 1.2 the standard deviation is calculated from 3 expt. In 1.3 the means of 2 expt are given: the two values for 1.3.B are 54% and 60%, and for 1.3.C are 28.8% and 31.2%. The data given here are taken from kinetics experiments in which the adhesion was measured every 10 min for 1 h and refer to an incubation time of 40 min. This time corresponds to the end of the linear phase [5] (see also fig.2).

introducing alcoholic groups on C_6 and C_7 of the modified sialic acid by reduction with sodium borhydride. A chemical environment close to the native one is probably restored.

Treatment of SLC with L-fucosidase decreases their adhesivity by 43% or 70% depending on the enzyme concentration (fig.1.3). Since L-fucose, like sialic acid, is localized at the end of carbohydrate chains, it could be involved in cell–cell adhesion. However its function must be somehow different from that of sialic acid.

3.2. *Amino groups*

To block amino groups we used the bisimido esters of adipic (DMA) and suberic acid (DMS). The inhibition of adhesion is proportional to the concentration of the reagent; 10 mM DMA and DMS inhibit the adhesion by 49% and 56%, respectively. The inhibitory effect could be due either to the transformation of amino into amide groups or to the possible cross-linking of membrane proteins. In the first instance elimination of positive charges, in the second reduced motility of membrane components could account for the loss of adhesivity.

3.3. *Carboxyl groups*

Were transformed into amides by activation with water soluble carbodiimide (CMC) in the presence of

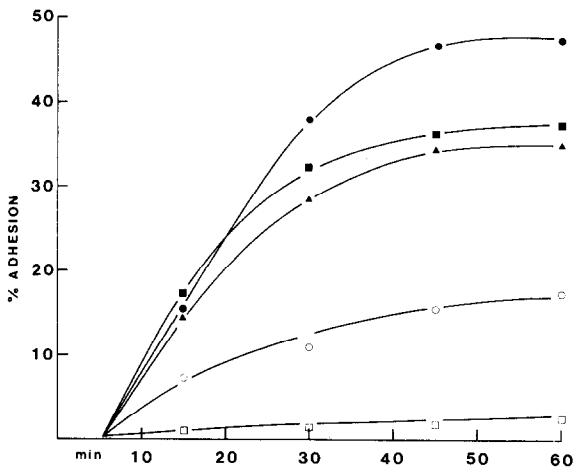

Fig.2. Time-course of the inhibition of cell–cell adhesion by iodoacetamide: (●) control; (■) 1 mM; (▲) 2 mM; (○) 5 mM; (□) 10 mM. Adhesion is expressed as % of single labeled cells adhering to the monolayer. Incubation was at 37°C.

NH_4OH. Again, inhibition of adhesion was observed. It ranged from 20–39% for 1–20 mM CMC. The relatively low inhibition could be attributed to minor alterations of the membrane surface rather than to the blocking of specific groups involved in adhesion.

3.4. *Sulphydro groups*

Under the conditions employed in this work iodacetamide will react predominantly with -SH groups [11]. A remarkable inhibition of adhesion is observed at 1–10 mM (fig.2). This result is in agreement with [12] which demonstrate that the blocking of -SH groups prevents the aggregation of hepatic and kidney cells of chicken embryos. The effect is interpreted in [12] as a lack of alignment between sterically complementary molecules.

4. Conclusions

This investigation clearly shows that some of the functional groups studied are involved in cell–cell adhesion. We provide evidence that sialic acid plays a regulatory role, whereas galactose residue may have a ligand function. Moreover, the strong inhibition observed after blocking -SH groups without affecting cell viability and growth, suggest that cystein residues are involved in the adhesive process. A further step in this line of experiments will consist of the identification of the membrane components involved in the process. In this context, one of the most important molecules is fibronectine [13] which carries functional groups susceptible to the chemical and enzymatic modifications described here. The relevance of fibronectine is supported by its sensitivity to neoplastic transformation, and consequently by its involvement in the loss of contact inhibition observed in tumor cells. Finally, it is our intention to perform an analogous investigation also on the corresponding non-transformed cells. This should permit a better understanding of the adhesion phenomena and of contact inhibition.

Acknowledgements

The skilful technical assistence of Mrs Mirjam Valluchi is gratefully acknowledged. This work was supported by the Swiss National Science Foundation, Berne (grant no. 3.109.077) and by the Italian CNR, Rome (grant no. 2.260.662).

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