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CELL ADHESION AND SPREADING FACTOR

CHEMICAL MODIFICATION STUDIES

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Summary

The purified fetal calf serum factor that promotes cell adhesion and spreading of baby hamster kidney cells on tissue culture substrata has been subjected to a variety of chemical modifications and then tested for activity. These studies have shown that modification of the carbohydrate portions of the factor by glycosidic enzymes or by periodate oxidation did not alter its ability to promote cell spreading. On the other hand, modification of some protein portions of the factor by proteolytic enzymes or by specific modification of —COOH groups, tyrosine residues, or tryptophan residues resulted in a marked inhibition of factor activity. Modification of protein —SH groups, $-NH_2$ groups, or methionine residues did not affect factor activity. Control experiments indicate that the various modifications were directed at the activity of the factor and not its adsorption onto the substrata.

Introduction

Cell adhesion and spreading factor is a ubiquitous serum component required for the adhesion and spreading of baby hamster kidney (BHK) cells and several other cell types onto tissue culture plastic [1,2]. The mechanism of action of factor depends upon its adsorption onto the substratum surface [1,2] and the adsorbed factor constitutes the sites to which cell adhesion occurs [2]. A variety of physical and chemical studies on the fetal calf factor have shown that it contains two active species, 12.5 and 9s; that it is a glycoprotein containing about 10% carbohydrate including ~3% sialic acid; and that it has a low isoelectric point, pI = 4.0 [2]. In human serum, the cell adhesion and spreading factor has been found to be very similar to cold insoluble globulin [3].

It is likely that cell interactions with the substratum involve short-range chemical interactions [4—6] and occur by specific receptor-ligand-like inter-

actions [6,7]. In order to gain some insight into the nature of the interaction between cells and adsorbed adhesion and spreading factor, a variety of experiments have been carried out in which the factor has been chemically modified and the functional activity of the modified factor determined. Since the factor must be adsorbed onto the substratum to be active, it was necessary to make sure that the modifications did not simply prevent its adsorption onto the substratum. Therefore, in all of the experiments, the factor was modified after its adsorption onto the substratum. It was also necessary to determine whether the modifications removed the factor, and this was accomplished in control experiments with radioactively labeled factor.

Materials and Methods

Baby hamster kidney cells which were adapted for growth in suspension culture were the gift of Dr. Adrian Chappel, Communicable Disease Center, Atlanta, Ga. Eagle's minimal essential medium (spinner modified) and fetal calf calf serum were obtained from GIBCo., Grand Island, N.Y. Tryptose phosphate broth was obtained from DIFCo Labs, Detroit, M.I. [^{3}H]Formaldehyde (specific activity 100 Ci/mol) was obtained from New England Nuclear, Boston, Mass. EDTA, sodium dodecyl sulfate (SDS) hydroxylamine-HCl, succinic anhydride, iodoacetic acid (free base), Triton X-100, trypsin inhibitor (type 1S, 1 mg inhibits 1.85 mg trypsin), trypsin (type XI, 7300 U/mg), pronase (type VI, 4 U/mg), papain (30 U/mg), neuraminidase (type VI, 6 U/mg), α-mannosidase (type III, 18 U/mg), *N*-acetylglucosaminidase (74 U/mg), β-galactosidase (grade IV, 700 U/mg), and hyaluronidase (type VI, 4900 U/mg) were obtained from Sigma Chemical Co., St. Louis, Mo. Collagenase (type III, 107 U/mg) was obtained from Worthington Biochem., Freehold, N.J. Lactoperoxidase (40 U/mg) was a product of PL Biochemicals, Milwaukee, Wisc. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl, glycine methyl ester HCl, and *N*-bromosuccinimide were obtained from Pierce Chemical Co., Rockford, Ill. Chloramine-T was obtained from Eastman Chemical Co., Rochester, N.Y. All other chemical reagents were obtained from Fisher Scientific, Houston, Texas.

Adhesion and spreading factor was prepared from fetal calf serum as described previously [2]. The final specific activity of the preparations used in all experiments was 150—200. Radioactively labeled factor was prepared by the reductive alkylation technique [8]. A summary of three different preparations is presented in Table I. The radiolabeled product was analyzed for spreading activity (see below), protein concentration [9], and radioactivity. Specific spreading activity (units/mg) and specific radioactivity (cpm/unit) were calculated.

BHK-21-13s cells were grown in suspension culture. The medium was Eagle's minimal essential medium (spinner modified) with double the concentration of amino acids (except 1X glutamine) and vitamins and supplemented with Hepes buffer (20 mM), 0.1 g/l ferric nitrate, 2.0 g/l dextrose, 10% tryptose phosphate broth, and 10% fetal calf serum. The final sodium bicarbonate concentration in the medium was 0.5 g/l.

Cell spreading was measured as described previously [1,2]. Cells from sus-

TABLE I

RADIOACTIVE LABELING OF ADHESION AND SPREADING FACTOR

[^{3}H]Formaldehyde was added as indicated to a 1.0 ml solution of adhesion and spreading factor (5.4 mg, spec. act. = 186) in 0.1 M sodium phosphate, pH = 9.0. After a 30-s incubation at 4°, the reaction was completed by the addition of sodium borohydride (twice 50 µl of a 13.6 mg/ml solution). The solution was then brought to pH 6.8 by the addition of HCl and dialyzed exhaustively against 1.0 M sodium phosphate, pH 6.8. Other details are described in the text and Materials and Methods.

Expt.	[^{3}H] Formaldehyde		Activity of radiolabeled factor	
	µmol added	Ci/mol	Spreading (units/mg)	cpm/unit
1	16.5	12	147	3594
2	2.00	100	147	5396
3	2.00	100	122	2888

pension culture were collected by centrifugation at 500 × *g* for 2 min (Sorvall HL-4 rotor) and washed and resuspended in adhesion medium (150 mM NaCl; 3 mM KCl; 1 mM $CaCl_2 \cdot 2\ H_2O$; 0.5 mM $MgCl_2 \cdot 6\ H_2O$; 6 mM Na_2HPO_4; final pH = 7.3). Incubations of $0.5—1.0 \cdot 10^6$ cells in 1.0 ml of adhesion medium were carried out in Falcon No. 3001 (9.6 cm^2) tissue culture dishes for 45 min at 37°C. At the end of the incubations, the extent of cell spreading was determined with a Zeiss Invertoscope D inverted microscope equipped with phase contrast objectives by visually observing 100—200 attached cells and estimating the percentage of spread cells: 90—100% (4+), 65—85% (3+), 40—60% (2+), 5—35% (1+). With experience it has become possible to read the spreading assay with considerable reproducibility and little variation among three different observers. One unit of spreading activity has been defined as the minimum amount of factor required for the cells to obtain 4+ spreading [1,2].

In the experiments designed to test the effects of various modifications on adsorbed adhesion and spreading factor, the tissue culture dishes were pretreated with 1.0 ml adhesion medium containing 2 units factor (spec. act. ~150—200) for 5 min at 22°C. These 'activated' substrata were then rinsed with deionized water and, subsequently, the various modifying reagents were added to the dishes as indicated. The incubations were stopped by rinsing the dishes and then these 'modified activated' substrata were tested for cell spreading by adding cells in 1 ml adhesion medium as described above.

In experiments with radiolabeled adhesion and spreading factor, the extent of binding of the factor to the substrata was calculated by comparing the cpm in the supernatants before and after 5 min incubation of radioactive factor with the culture dishes. The release of adsorbed factor from the substratum during the modification reactions was measured by determining the cpm released into the incubation medium during the modification procedures. All radioactive determinations were carried out using 1.0 ml aqueous samples and 10 ml Aquasol (New England Nuclear) and counted in a Nuclear Chicago Mark II scintillation spectrophotometer.

Results

Effect of non-specific chemical treatments on adhesion and spreading factor activity

Substrata were pretreated with radiolabeled factor and the extent of factor binding was determined. Under these conditions (Table II) about 1000 cpm became adsorbed to the substratum. Subsequently, these substrata were treated with a variety of chemical reagents shown in Table II. The substrata were then tested for their ability to promote cell spreading and the supernatants from the treatments were tested to determine the extent of factor release from the substratum. The results are presented in Table II and indicate that the adsorbed factor was remarkably stable and at least partially resistant to most of the diverse treatments. Even treatment with 1% SDS or 1.0 M NaOH did not completely inhibit the activity of the factor. Moreover, the factor was very resistant to being removed from the substratum.

Effect of proteolytic and glycosidic enzyme treatments on adhesion and spreading for activity

The activity of the adsorbed factor was markedly inhibited by treatment with the proteolytic enzymes trypsin, pronase, and papain. On the other hand, collagenase was without effect. The results are presented in Table III. The inhibition could not be accounted for by removal of the factor from the substratum. The concentration dependence of trypsin and pronase inhibition was tested, and the results are presented in Fig. 1. Pronase was much more effective than trypsin and inhibited factor activity at a concentration as low as 0.01 mg/ml.

The adsorbed factor was also treated with a variety of glycosidic enzymes including neuraminidase (1 unit/ml), α-mannosidase (3.4 units/ml), *N*-acetylglucosamidase (3.2 units/ml), β-galactosidase (15 units/ml) and hyaluronidase (600 units/ml). All treatments were for 10 min at 37°C. None of these enzyme treatment inhibited spreading activity of the factor.

TABLE II

EFFECT OF VARIOUS CHEMICAL TREATMENTS ON ABSORBED ADHESION AND SPREADING FACTOR

Substrata were pretreated with adhesion and spreading factor and subsequently with 1.0 ml of the reagents indicated for 5 min at 22°C. The substrata were then rinsed with deionized water and spreading activity was determined. Other details are described in the text and Materials and Methods.

Additions (concentration)	Spreading *	Bound radioactivity released ± S.D. * (%)
EDTA (1.0 mM)	4+	0.18 ± 0.31
KI (1.0 M)	3+	2.20 ± 0.79
NaOH (0.1 M)	2.3+	9.70 ± 4.30
NaOH (1.0 M)	1.2+	20.00 ± 11.00
HCl (0.1 M)	2+	0.58 ± 0.35
HCl (1.0 M)	3+	0.12 ± 0.09
Triton X-100 (1%)	4+	1.40 ± 1.50
SDS (1%)	1.3+	25.00 ± 5.20

* Average of three determinations.

TABLE III

EFFECT OF VARIOUS PROTEOLYTIC TREATMENTS ON ADSORBED ADHESION AND SPREADING FACTOR

Substrata were pretreated with adhesion and spreading factor and subsequently with 1.0 ml of the reagents indicated for 10 min at room temperature. The substrata were then rinsed with 1.0 ml of a soybean trypsin inhibitor solution (2.0 mg/ml) and then with deionized water. Spreading activity was then determined. Other details are as described in the text and Materials and Methods.

Additions (concentration)	Spreading *	Bound radioactivity released ± S.D. * (%)
H_2O	4+	0.30 ± 0.15
Trypsin inhibitor (2 mg/ml in H_2O)	4+	0.52 ± 0.05
Trypsin (1 mg/ml in H_2O)	0.6+	9.50 ± 6.00
Pronase (1 mg/ml in H_2O)	0	22.00 ± 8.50
Papain (1 mg/ml in H_2O)	0.7+	1.90 ± 3.60
Collagenase (1 mg/ml in Dulbecco's phosphate-buffered saline)	3+	n.d. **

* Average of four determinations.
** n.d. = not determined.

Effect of specific chemical modification reactions on adhesion and spreading factor activity

Modification of free carboxyl groups. The adsorbed factor was treated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl, a soluble carbodiimide, in the presence of glycine methyl ester as described in Table IV. This treatment, which is known to block free —COOH groups [10], resulted in an inhibition of cell spreading. Glycine methyl ester was not required and the soluble carbodiimide was inhibitory by itself at a concentration as low as 0.0025 M. Control experiments with radiolabeled factor demonstrated that the carbodiimide treatment (0.025 M) resulted in removal of only 3.7% of the adsorbed factor from the substratum.

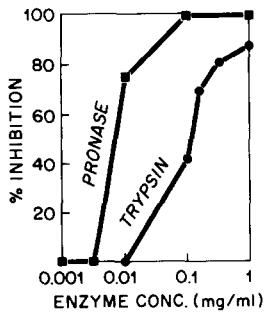

Fig. 1. Proteolytic inactivation of adsorbed adhesion and spreading factor. Substrata were pretreated with 1.0 ml adhesion medium containing 2 units of factor for 5 min at 22°C. The substrata were then rinsed with deionized water and treated with 1.0 ml solutions of pronase or trypsin at the concentrations indicated. After 10 min at 22°C, the substrata were rinsed with deionized water and spreading activity was measured. Spreading was estimated on a 0—4+ scale and the percent inhibition of spreading calculated. Other details are described in the text and in Materials and Methods.

TABLE IV

MODIFICATION OF ADSORBED ADHESION AND SPREADING FACTOR BY A SOLUBLE CARBODIIMIDE

Substrata were pretreated with adhesion and spreading factor and subsequently with a 1.0 ml solution containing glycine methyl ester and 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide HCl at the concentrations indicated, final pH = 4.75, for 10 min at 22°C. The substrata were then rinsed with deionized water and spreading activity was determined. Other details are described in the text and Materials and Methods.

Expt.	Additions (concentration)	Activity
1	No additions	4+
	1.0 M ester + 0.25 M carbodiimide	0
	1.0 M ester	4+
	0.25 M carbodiimide	0
2	No additions	4+
	0.25 M carbodiimide	0
	0.025 M carbodiimide	0
	0.0025 M carbodiimide	1+
	0.00025 M carbodiimide	3+

Modification of tyrosine groups. Tyrosine residues were modified by treatment of the adsorbed factor by the chloramine-T technique [11] or the lactoperoxidase technique [12]. The results are presented in Tables V and VI, respectively. Both of these treatments were able to completely inhibit the activity of the factor. Moreover, the inhibitory treatments resulted in removal of less than 3% of the adsorbed factor from the substratum.

Modification of tryptophan groups. Tryptophan residues were modified by treatment of the adsorbed factor with *N*-bromosuccinimide [13]. The results

TABLE V

MODIFICATION OF ADSORBED ADHESION AND SPREADING FACTOR BY CHLORAMINE T AND NaI

Substrata were pretreated with adhesion and spreading factor and subsequently with 1.0 ml 0.1 M sodium phosphate, pH 7.1, containing the additions shown for 5 min at 22°C. Subsequently the substrata were rinsed with 1.0 ml of sodium metabisulfite (0.48 mg/ml) and then with deionized water. The spreading activity was then determined. Other details are described in the text and Materials and Methods.

Additions (concentration)	Spreading
No additions	4+
Chloramine T (0.4 mg/ml)	4+
Chloramine T (0.4 mg/ml):	
+ NaI (1 mM)	0
+ NaI (0.1 mM)	1+
+ NaI (0.01 mM)	2+
+ NaI (0.001 mM)	3+
Chloramine T (0.04 mg/ml):	
+ NaI (1 mM)	1+
+ NaI (0.1 mM)	1+
+ NaI (0.01 mM)	3+
+ NaI (0.001 mM)	4+

TABLE VI

MODIFICATION OF ADSORBED ADHESION AND SPREADING FACTOR BY LACTOPEROXIDASE AND NaI

Substrata were pretreated with adhesion and spreading factor and subsequently with 1.0 ml 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.0, containing the additions indicated. The reactions were carried out for a total of 10 min at 22°C. H_2O_2 was added in two separate equal aliquots at the beginning of the incubations and after 5 min. The substrata were then rinsed with deionized water and spreading activity was determined. Other details are described in the text and Materials and Methods.

Additions (concentration)	Spreading
Control	4+
Lactoperidase (0.1 mg/ml)	4+
Lactoperidase (0.1 mg/ml) + NaI (1 mM)	4+
H_2O_2 (0.1 mM)	4+
Lactoperidase (0.1 mg/ml) + NaI (1 mM) + H_2O_2 (0.1 mM)	1+
Lactoperidase (0.1 mg/ml) + NaI (0.1 mM) + H_2O_2 (0.1 mM)	0
Lactoperidase (0.01 mg/ml) + NaI (1 mM) + H_2O_2 (0.1 mM)	0
Lactoperidase (0.01 mg/ml) + NaI (0.1 M) + H_2O_2 (0.1 mM)	1+

are presented in Table VII. This treatment completely inhibited the activity of the factor. Moreover, the inhibitory conditions resulted in removal of only 3% of the adsorbed factor from the substratum.

Modification of free amino acid groups, free sulfhydryl groups, methionine residues, and carbohydrate groups. Other experiments were carried out with succinic anhydride (1 mg/ml in saturated sodium acetate; 10 min at 22°C) and acetic anhydride (1—10 mM in 50% saturated sodium acetate; 10 min at 22°C) to modify free amino groups [14,15], iodoacetic acid (1—20 mM; pH 5, 7, and 9; 10 min at 22°C) to modify free sulfhydryl groups [16], H_2O_2 (1%; pH 2.5 and 7; 10 min at 22°C) to oxidize methionine residues [17], and sodium periodate (0.2 M in sodium acetate; pH 4.5 and 7.0; 30 min at 22°C) to modify carbohydrate groups. None of these treatments resulted in inhibition of the activity of the factor.

TABLE VII

MODIFICAITION OF ADSORBED ADHESION AND SPREADING FACTOR BY *N*-BROMOSUCCINIMIDE

Substrata were pretreated with adhesion and spreading factor and subsequently with 1.0 ml of the reagents indicated for 5 min at 22°C. The substrata were then rinsed with deionized water and spreading activity was determined. Other details are described in the text and Materials and Methods.

Additions (concentrations)	Spreading
Sodium acetate (0.1 M, pH 4.45)	4+
Sodium acetate (0.1 M, pH 4.45):	
+ *N*-bromosuccinimide (1.0 mM)	0
+ *N*-bromosuccinimide (0.1 mM)	0
+ *N*-bromosuccinimide (0.01 mM)	4+

Discussion

The findings presented in this paper are an important step towards determining the chemical nature of the interaction of cells with adhesion and spreading adsorbed on the substratum. The results indicate that treatment of the adsorbed factor with proteolytic enzymes or with reagents that alter carboxyl groups, tyrosine residues or tryptophan residues cause loss of biological activity. On the other hand, treatments of the adsorbed factor expected to modify amino groups, sulfhydryl groups, methionine residues or carbohydrate groups did not change biological activity of the factor. However, the latter results can only be considered tentative since the adsorbed factor was not re-isolated and shown to be modified in the predicted manner. Based upon the results, we suggest that the spreading activity of cell adhesion and spreading factor depends upon specific protein portions of the adsorbed factor.

The sensitivity of free carboxyl groups was peculiar in that carbodiimide alone inhibited activity in the absence of any added amino compound. It is anticipated that the carbodiimide catalyzes cross-linking between adjacent —COOH and $-NH_2$ groups. Since the $-NH_2$ group containing compound did not have to be added, it seems likely that an intramolecular cross-linking occurred between and essential —COOH group and a nearby $-NH_2$ group. It is unlikely that the $-NH_2$ group itself was important in the activity because of the lack of inhibition by succinic anhydride or acetic anhydride. Another possibility was that a direct adduct formed between the carbodiimide and tyrosine —OH groups. This reaction can occur, but is reversible by the addition of hydroxylamine [18]. However, the inhibition caused by the carbodiimide could not be reversed by a 60-min treatment with 0.5 M hydroxylamine.

Finally, in all of the modification experiments, the factor was adsorbed to the substratum prior to modification; therefore, alteration in activity was not an indirect effect on the ability of the factor to adsorb to the substratum. Moreover, control experiments utilizing radioactively labeled factor revealed that the modifications did not result in removal of the factor from the substratum that could account for the inhibition of activity. Indeed, even treatment of the adsorbed factor with 1.0 M NaOH or 1% SDS only removed a small portion of the adsorbed material.

References

1 Grinnell, F. (1976) Exp. Cell Res. 102, 51—62
2 Grinnell, F., Minter, D. and Hays, D. (1977) Exp. Cell Res. 110, 175—190
3 Grinnell, F. and Hays, D.G. (1978) Exp. Cell Res. 115, 221—229
4 Grinnell, F., Milam, M. and Srere, P.A. (1971) Arch. Biochem. Biophys. 153, 193—198
5 Maroudas, N.G. (1975) J. Theor. Biol. 49, 417—424
6 Bell, G.I. (1978) Science 200, 618—627
7 Grinnell, F. (1978) Int. Rev. Cytol. 53, 65—144
8 Rice, R.H. and Means, G.E. (1971) J. Biol. Chem. 246, 831—832
9 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R. (1951) J. Biol. Chem. 193, 265—275
10 Hoare, D.G. and Koshland, Jr., D.E. (1967) J. Biol. Chem. 242, 2447—2453
11 McConahey, P.J. and Dixon, F.J. (1966) Int. Arch. Allergy 29, 185—189
12 Phillips, D.R. and Morrison, M. (1971) Biochemistry 10, 1766—1771
13 Spanoe, T.F. and Witkop, B. (1967) Methods Enzymol. XI, 506—522
14 Riordan, J.F. and Vallee, B.L. (1967) Methods Enzymol. XI, 565—570
15 Klapper, M.H. and Klotz, I.M. (1972) Methods Enzymol. XXV, 531—536
16 Gurd, F.R.N. (1967) Methods Enzymol. XI, 532—541
17 Neumann, N.P. (1972) Methods Enzymol. XXV, 393—400
18 Carraway, K.L. and Koshland, Jr., D.E. (1972) Methods Enzymol. XXV, 616—623