Isolation of a Serum Component that Stimulates the Spreading of Cells in Culture

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A foetal-calf-serum component has been isolated that stimulates cells in culture to adopt typical fibroblast morphology. This 'spreading' factor is a glycoprotein with a mol.wt. of approx. 62 000 and contains 12% carbohydrate; it has an isoelectric point of 4.8. The method described allows purification to homogeneity as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and analytical isoelectric focusing. Under cell-culture conditions the factor is active at 18 µg/ml.

Cells in culture require the presence of animal serum in order to proliferate. The serum stimulates cells to adopt a characteristic morphology; this process is often referred to as 'cell spreading' and seems to be promoted by specific serum components (Fisher *et al.*, 1958; Lieberman & Ove, 1958; Puck *et al.*, 1968; Grinnell *et al.*, 1977).

Previous reports implicated fetuin, an α -glycoprotein from foetal-calf serum (Pederson, 1944) as an effector of cell spreading (Fisher *et al.*, 1958). Lieberman & Ove (1959) found that DEAE-cellulose removed most of the activity from fetuin, and subsequently Grinnell (1976) suggested that a contaminating protein was the spreading factor. On the other hand, Puck *et al.* (1968) found that highly purified fetuin did promote cell spreading. Studies are complicated by the considerable microheterogeneity displayed by fetuin (Page, 1977).

Grinnell *et al.* (1977) have partially purified from foetal-calf serum a factor that stimulates cell spreading; it is a mixture of two glycoproteins, the larger of which is a component of mol.wt. 215 000 and has a sedimentation coefficient of 12.5S. The smaller component has a sedimentation coefficient of 9S, and SDS/polyacrylamide-gel electrophoresis shows it to be composed of three peptides of mol.wts. 94 000, 80 000 and 71 000.

An analogous preparation can be isolated from human serum, and this is found to be immunologically cross-reactive with cold-insoluble globulin (Grinnell & Hays, 1978). This plasma component is known to play a role in the adhesion of fibroblasts to collagen (Klebe, 1974) and of hepatocytes to tissue-culture plastic (Hook *et al.*, 1978).

Abbreviation used: SDS, sodium dodecyl sulphate.

Recent evidence suggests that there are a number of components in serum able to promote cell spreading (Knox *et al.*, 1979). In the present paper we describe the purification and properties of one such component from foetal-calf serum.

Materials and Methods

Materials

Foetal-calf serum and Ham's F10 medium were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K., as were BHK 21/C13 cells. Acrylamide and *NN'*-methylenebisacrylamide were purchased from Eastman-Kodak, Hemel Hempstead, Herts., U.K., and sodium dodecyl sulphate and *NNN'*-tetramethylethylenediamine from Bio-Rad, Watford, Herts., U.K. All other chemicals were of the highest grade available. Ampholine polyacrylamide (PAG) plates were purchased from LKB, Selsdon, Croydon, Surrey, U.K. Petri dishes were from Sterilin, Teddington, Middx., U.K. Phosphate-buffered saline was composed of KCl (0.2 g/litre, KH_2PO_4 , (0.2 g/litre), NaCl (8.0 g/litre), Na_2HPO_4 (1.15 g/litre), pH 7.4. Sephacryl S-200 was obtained from Pharmacia, London W.5, U.K. Anti-fibronectin was a gift from Dr. R. Hynes, Massachusetts Institute of Technology, Boston, U.S.A.

Cells

BHK 21/C13 cells were routinely cultured in Ham's F10 medium supplemented with 7.5% (v/v) foetal-calf serum. Cells were grown as monolayers on 90 mm Petri dishes at 37°C in a humidified atmosphere of air/CO₂ (19:1). Cultures were

regularly tested for mycoplasma by the method of Russell *et al.* (1975). Cells were subcultured by using 0.05% (w/v) trypsin and 0.5 mM-EDTA in phosphate-buffered saline.

Cell-spreading activity

To assess the spreading activity in serum or purified fraction, cells were subcultured into serum-less Ham's F10 medium at a density of 5×10^4 cells/ml. Cell suspension (1.8 ml) was added to 0.2 ml of material and the resulting suspension poured into 30 mm Petri dishes. After 1 h at 37°C, the Petri dishes were viewed with a Leitz Diavert inverted microscope. Assessment of cell spreading was carried out by independent observers; five separate fields were examined and 100 cells per field were counted. The criteria for cell spreading are that a cell should be flattened and asymmetric and both nucleus and nucleoli should be clearly visible.

Isolation of spreading factor

A 50 ml portion of foetal-calf serum was adjusted to 37.5%-satd. $(\text{NH}_4)_2\text{SO}_4$ by the addition of a saturated solution at pH 7.0. After equilibration at room temperature for 20 min, the precipitate was removed by centrifugation at 10000 *g* for 20 min. The supernatant was dialysed exhaustively against water, followed by dialysis for 36 h at 4°C against 5 litres of 0.05 M-sodium acetate/acetic acid buffer, pH 5.0. The contents of the dialysis sac were removed and after centrifugation the supernatant was diluted 1:3 with water and left at room temperature for 2 h. During this time a precipitate formed, and this was removed by centrifugation at 10000 *g* for 20 min. After washing the precipitate with ice-cold dialysis buffer it was dissolved in 2.5 ml of phosphate-buffered saline, pH 8.0, and dialysed against 0.05 M-sodium acetate buffer, pH 5.0, for 5 h at 4°C. A precipitate formed, and this was removed by centrifugation. The supernatant contained the isolated spreading factor.

Electrophoresis

Gel electrophoresis was carried out as described by Ornstein (1964) and Davis (1964). Polyacrylamide (7%, w/v) slab gels were used with a current of 22 mA/gel.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out essentially as described by Laemmli (1970) in vertical slab gels. Stacking gels contained 5% (w/v) acrylamide and 0.15% bisacrylamide; running gels contained 7.5% acrylamide and 0.2% bisacrylamide. Gels were run at 22 mA with a tracking dye of 0.01% Bromophenol Blue. Sample buffer consisted of 1% SDS, 10% (v/v) glycerol and 2 mM-mercaptoethanol in 0.1 M-Tris/HCl, pH 6.8. After heating to 100°C for 2 min, samples were cooled before being electrophoresed.

Standards of bovine serum albumin, creatine kinase and β -galactosidase were run in parallel with samples.

Samples (15–25 μ g) of protein were applied and gels were fixed in trichloroacetic acid (10%, w/v)/sulphosalicylic acid (3.5%, w/v) in methanol/water (3:7, v/v) for 1 h, stained for 16 h with 0.2% (w/v) Coomassie Brilliant Blue R250 in ethanol/acetic acid/water (55:16:134, by vol.) and destained in ethanol/acetic acid/water (25:8:67, by vol.).

Isoelectric focusing

Analytical isoelectric focusing was carried out by using commercial polyacrylamide plates and a LKB Multiphor unit. pH ranges of 3.5–9.5 and 4–6.5 were used. Gels were run according to makers' instructions.

Carbohydrate analysis

Total sugar analysis was carried out by the orcinol/ H_2SO_4 method as described by Marshall & Neuberger (1972).

N-Terminal amino acid

The N-terminal amino acid was determined by the method of Sanger (1945).

Amino acid analysis

Amino acid analysis was carried out with a Locarte analyser after hydrolysis of samples for 24 h at 110°C.

Determination of protein concentration

Protein determination was carried out by the methods of Gornall *et al.* (1949) or Lowry *et al.* (1951).

Molecular-sieve chromatography

Molecular-sieve chromatography of purified spreading factor was carried out as described by Knox *et al.* (1979). A sample (1 mg) of purified cell spreading factor was loaded on to a column (30 cm $\times$ 2 cm) of Sephacryl S-200 and was eluted with phosphate-buffered saline, pH 8.1. Fractions of 1 ml were collected.

Immunodiffusion

Immunodiffusion was carried out as described by Hudson & Hay (1976).

Results

Stability of spreading activity in foetal-calf serum

The effect of pH and dilution on the spreading activity of foetal calf serum was examined. Table 1 shows the activity that remains when the serum is returned to its initial pH and concentration. At a

Table 1. *Effect of dilution and pH on spreading activity of foetal-calf serum*

Portions (10 ml) of foetal-calf serum were diluted with water and the pH altered with 1 M-NaOH or 1 M-HCl. After 16 h, at 4°C, samples were centrifuged, re-adjusted to pH 7.0 and concentrated to 10 ml with Amicon Centriflo concentration cones. Spreading assays were carried out at a serum concentration of 5% (v/v) in Ham's F10 medium. Spreading activity: +++, 90% spreading; ++, 60–90%; +, 20–60%; –, <20%.

pH	Dilution	...	Spreading activity				
			0	1:5	1:10	1:30	1:50
2			+++	+++	+++	+++	+
3			+++	+++	+++	+++	–
4			+++	+	++	+++	–
5			+++	++	+	+++	–
6			+++	+++	+++	+++	+
7			+++	+++	+++	+++	+
8			+++	+++	+++	++	–

Table 2. *Conditions for the formation of acid precipitate with spreading activity*

Portions (10 ml) of foetal-calf serum were diluted with water and the pH adjusted with 1.0 M-HCl. After 25 min at room temperature, the precipitate was removed by centrifugation at 10 000 g for 20 min. Precipitates were redissolved in 5 ml of phosphate-buffered saline, pH 8.0, and spreading activity was determined at a serum concentration of 10% (v/v) in Ham's F10 medium. Spreading activity: +++, 90% cell spreading; ++, 60–90%; +, 20–60%; –, <20%.

pH	Dilution	...	Spreading activity					
			1:1	1:2	1:3	1:4	1:5	1:10
3.0			–	–	–	–	–	–
3.5			–	–	+	+	–	–
4.0			+	+	+++	+++	+++	++
4.5			+	+	+++	+++	+++	+
5.0			–	–	++	+++	++	–
5.5			–	–	+	+	+	–
6.0			–	–	–	–	–	–

dilution of 1:50 there is an almost complete loss of activity at a range of pH values. The loss of activity is probably a result of low salt concentration, since dilution to 1:50 with phosphate-buffered saline does not cause loss of spreading activity.

When serum was diluted to 1:5 or 1:10 at pH 4.0 or 5.0, there was a smaller, but significant, loss of spreading activity. Under these conditions of dilution and pH, significant precipitates were formed; these were not soluble in phosphate-buffered saline at pH 7.0, but completely dissolved in phosphate-buffered saline at pH 8.0. These solutions were found to contain potent spreading activity that would stimulate the spreading of >95% of cells at 100 µg/ml. (No precipitation occurs at dilutions of 1:50, and so this is not an explanation for the loss of activity at these dilutions.)

A finer range of dilution and pH was used to determine the optimum conditions for acid precipitation of spreading activity. Table 2 shows that the optimal range of pH is 4.0–5.0 and that of dilution is 1:3–1:5. Again, salt concentration is critical, and no precipitate is produced in the range

pH 4.0–5.0 if the serum is diluted with phosphate-buffered saline, 1% KCl, 1% NaCl or 1% (NH₄)₂SO₄.

Use of dialysis to precipitate spreading activity

Dialysis can be used to change both salt concentration and pH and thus precipitates spreading activity from serum. Table 3 shows dialysis buffers that will result in a precipitate with spreading activity; again a pH of less than 5.5 is necessary.

When examined by SDS/polyacrylamide-gel electrophoresis, the precipitate that results from dialysis of foetal-calf serum against 0.05 M-sodium acetate buffer, pH 5.0, was heterogeneous (see Fig. 1). In order to achieve a more homogeneous preparation, a solution of the first precipitate was subjected to a second dialysis. A small precipitate formed, but the spreading activity was found in the supernatant rather than in the precipitate. SDS/polyacrylamide-gel electrophoresis of the supernatant revealed one major and one minor band (see Fig. 1).

The yield of factor varies with batches of foetal-calf serum and could sometimes be increased by

Table 3. Use of dialysis to precipitate spreading activity
Portions (20ml) of foetal-calf serum dialysed for 16h at 4°C against different buffers. Precipitates were removed by centrifugation at 10000g for 20min and redissolved in 5ml of phosphate-buffered saline, pH 8.0. Spreading activity was determined at a serum concentration of 10% (v/v). Spreading activity: +++, 90% cell spreading; ++, 60–90%; +, 20–60%; –, <20%.

Buffer	Spreading activity of redissolved precipitates
0.05 M-Sodium acetate, pH 6.0	+
0.05 M-Sodium acetate, pH 5.0	+++
0.05 M-Sodium acetate, pH 4.0	+++
0.1 M-Sodium acetate, pH 5.0	+
0.2 M-Sodium acetate, pH 5.0	—
0.3 M-Sodium acetate, pH 5.0	—
0.1 M-Sodium phosphate, pH 6.8	—
0.05 M-Sodium phosphate, pH 6.8	—
0.025 M-Sodium acetate, pH 5.0	—

Fig. 1. SDS/polyacrylamide-gel electrophoresis of protein samples

(a) Acid precipitate produced by a single dialysis of foetal-calf serum against 0.05 M-sodium acetate buffer, pH 5.0; (b) supernatant from second acid dialysis of initial acid precipitate; (c) purified spreading factor; (d) albumin; (e) fetuin.

diluting (2-fold with water) the foetal-calf serum after the first acid dialysis.

Isolation of a homogeneous glycoprotein from foetal-calf serum that stimulates spreading

(NH₄)₂SO₄ precipitation has previously been used in the isolation of spreading factors from foetal-calf serum (Fisher *et al.*, 1958; Puck *et al.*, 1968; Grinnell *et al.*, 1977). A combination of (NH₄)₂SO₄ precipitation and the double dialysis described above results in the isolation of an apparently homogeneous glycoprotein that has spreading activity. Table 4 gives the spreading activities found at each step in the purification; no step is completely efficient. Fig. 1 is a representation of an SDS/polyacrylamide gel and shows the purified component running as a single band.

Table 4. Purification of spreading activity from foetal calf serum

	Concentration needed to stimulate cell spreading (μg/ml)
Foetal-calf serum	200
Supernatant from 37.5%-satd.-(NH ₄) ₂ SO ₄ precipitation	150
Precipitate from first acid dialysis	25
Supernatant from second acid dialysis	18

Fig. 2. Gel electrophoresis of protein samples
(a) Foetal-calf serum; (b) albumin; (c) fetuin; (d) purified spreading factor.

Table 5. Amino acid composition of purified spreading factor

Amino acid	Content (μg/100 μg)	Amino acid	Content (μg/100 μg)
Ala	5.3	Lys	7.4
Arg	5.6	Met	0.8
Asp	9.0	Orn	1.1
Cys	3.1	Phe	5.1
Glu	14.7	Pro	4.4
Gly	5.6	Ser	6.1
His	5.9	Thr	4.7
Ile	3.3	Tyr	3.6
Leu	8.4	Val	5.9

Properties of the spreading factor

On SDS/polyacrylamide-gel electrophoresis, the spreading factor runs close to the leading edge of albumin and has an apparent mol.wt. of 62 000 (Fig. 1). On gel electrophoresis without detergent, the glycoprotein also runs close to albumin (Fig. 2). It also gives a single band on analytical isoelectric focusing (Plate 1); the pI value is 4.8.

On molecular-sieve chromatography, with Sephacryl S-200, purified spreading factor gave a single protein peak corresponding exactly to the recovered cell spreading activity. The apparent molecular weight was in the range of 70 000–75 000.

Table 5 shows the amino acid composition of the spreading factor, and alanine was found to be the single *N*-terminal amino acid. Analysis of total sugar

EXPLANATION OF PLATE 1

Analytical isoelectric focusing

(a) Foetal-calf serum; (b) fetuin; (c) purified spreading factor.

EXPLANATION OF PLATE 2

Spreading of BHK cells

BHK cells were plated out in the presence of (a) no protein, (b) 7.5% (v/v) foetal-calf serum, and (c) 18 μ g of purified spreading factor/ml. Photomicrographs were taken at 1 h. Magnification $\times 200$.

Fig. 3. Dose-response curve for purified factor and foetal-calf serum

A series of cell-spreading assays were carried out as described in the Materials and Methods section, with foetal-calf serum (Δ) or purified cell-spreading factor ($\blacktriangle$). The results represent the means for five experiments using different preparations of factor. Error bars represent 1 S.D.

shows that the glycoprotein contains 12% (w/w) carbohydrate.

Biological activity of the spreading factor

The spreading factor stimulates spreading in a dose-dependent fashion. Thus at 18 $\mu\text{g/ml}$, more than 95% of BHK cells become completely spread and bipolar; at 10 $\mu\text{g/ml}$ the value is only 15%. A significant feature of the factor is that it stimulates complete spreading more rapidly than does control serum.

Plate 2 shows photomicrographs of cells that have spread in the presence of serum or in the presence of the isolated spreading factor at a concentration of 18 $\mu\text{g/ml}$.

The glycoprotein is also effective with other fibroblastic cell types and it will stimulate spreading of human skin fibroblasts and chick-embryo fibroblasts.

Fig. 3 shows a dose-response curve for foetal-calf serum and purified spreading factor.

In view of the cell-spreading activity that has been ascribed to fibronectin, an antiserum to the latter was used to determine whether the spreading factor isolated by us is related to fibronectin. In contrast with foetal-calf serum, the purified factor at a range of concentration up to 10 mg/ml failed to give a precipitin line after immunodiffusion.

Discussion

A simple method is reported for the isolation from foetal-calf serum of a glycoprotein that will promote the spreading of fibroblasts in culture. The glycoprotein has an apparent mol.wt. of 62 000 and contains 12% (w/w) carbohydrate.

The acid-precipitation procedure presumably relies on the fact that under conditions of low ionic strength, proteins will precipitate close to their isoelectric point. This technique may have other applications, especially where proteins have high or low isoelectric points.

The acid precipitate will only dissolve at pH 8.0, and it may be significant that Michl (1959) found that spreading activity can only be recovered from DEAE-cellulose if fractions are first brought to pH 7.8. In fact the separation of spreading activity by ion-exchange chromatography is likely to be extremely sensitive to elution conditions, since under conditions of low ionic strength the activity might be precipitated or denatured.

The spreading factor isolated by us is in no way similar to the 'adhesion and spreading factor' of Grinnell *et al.* (1977), although it may be significant that this partially purified preparation contains a polypeptide of mol.wt. 62 000. It is more likely that different workers have isolated separate spreading activities, since a recent report has shown that gel filtration can be used to distinguish between different spreading activities in serum (Knox *et al.*, 1979).

The relationship between the isolated spreading factor and fetuin is unclear. Fetuin gives a diffuse band on both disc-gel and SDS/polyacrylamide-gel electrophoresis and the heterogeneity is clear from isoelectric-focusing gels (Page, 1977). There may be a band within fetuin with the same pI as the purified factor (see Plate 2), and this might explain the spreading activity ascribed to fetuin. Clearly the term 'fetuin' is used to describe a group of glycoproteins rather than a homogeneous protein, and this might explain the lack of agreement as to its ability to promote cell spreading.

A purified spreading factor should make it possible to investigate the biochemical mechanisms underlying the process of serum-stimulated cell spreading.

Since the original submission of the manuscript, we have found that, for preparation to homogeneity, double dialysis after 37.5–45% satd. $(\text{NH}_4)_2\text{SO}_4$ fractionation produces an improved yield of spreading factor.

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