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FIBRONECTIN-INDEPENDENT MYOBLAST FUSION IN SUSPENSION CULTURES

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SUMMARY : Myoblasts cultivated in suspension in serum-free medium were used to examine whether fibronectin influences myoblast fusion. No effect on cell fusion was observed when the medium was supplemented with antibodies against fibronectin (at a concentration effective in inhibiting the myoblast attachment to gelatinized dishes mediated by 1 % horse serum). Purified horse serum fibronectin (70 µg/ml) also had no effect. The assay did, however, detect both inhibition of fusion in low-calcium medium and stimulation of fusion with added embryo extract, horse serum, and fibronectin-depleted horse serum. Thus, although fibronectin may influence cell motility or other processes necessary for fusion in monolayer cultures, it does not affect the fusion process itself.

Studies of myogenic cells grown in serum-containing medium as monolayer cultures have been interpreted as suggesting that fibronectin might influence the rate or extent of myoblast fusion (1-4). Fibronectin is a component of vertebrate sera and is also synthesized by a variety of cells, including fibroblasts; fibronectin released by cells is found as a soluble component of the culture medium, on cell surfaces, and in extracellular matrices (5,6). Using myoblasts from serum-free suspension cultures, we demonstrated that fibronectin is the component of serum responsible for mediating attachment of myoblasts to gelatinized dishes (7,8); purified fibronectin promotes myoblast attachment, while antibodies against fibronectin block serum-mediated attachment. There is evidence that fibronectin affects cell motility (9,10). Fibronectin may therefore affect not myoblast fusion per se, but some process or processes, related to cell-substratum attachment or cell migration, essential for fusion in monolayer cultures. Recently, several groups of workers (11-15) have shown that myoblasts cultured in serum-containing media in nonadhesive dishes survive and fuse in suspension; in such cultures not only the fusion process, but also many other differentiative changes, proceed

Abbreviations:
LH, serum-free medium (16); EE, embryo extract; H-fraction, high-molecular weight fraction of EE according to (17); HS, horse serum; FN, fibronectin; a-FN, rabbit antibodies against human plasma FN; NaCl/Pi, phosphate-buffered saline (150 mM NaCl, 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.0).

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in a manner very similar to the changes observed in comparable monolayer cultures. Suspension cultures allow study of the effects of substances on myoblast-myoblast adhesion and fusion without the complications inherent in monolayer cultures (12,13). The presence of serum is, however, a drawback in analyzing the effects of substances that are themselves present in serum (e.g., fibronectin). Even if no serum is present in cell-cell adhesion and fusion assays (12), cells obtained from serum-containing cultures might retain serum proteins on their surfaces.

In a recent paper (16) we demonstrated that: (1) chicken myoblasts can be cultivated in suspension in serum-free LH medium; (2) the same culture conditions allow the efficient separation of myoblasts from contaminating fibroblasts, which remain attached to the culture dish; (3) myoblasts kept in LH medium fuse in suspension. The suspended myoblasts obtained with this culture system have not been treated with chelators and have not previously been exposed to serum. We now report a procedure for scoring fusion in our culture system. Using this procedure we have examined whether fibronectin or antibodies against fibronectin affect myoblast fusion in suspension.

MATERIALS AND METHODS

Primary cultures: Cells obtained by trypsinization of 11-day chick embryo breast muscle were plated in LH medium [82 % Leibovitz L-15, 16 % 150 mM $NaHCO_3$, 2 % H-Fraction of chick embryo extract (EE), supplemented with glutamine, antibiotics, and bovine insulin (200 ng/ml, added daily)] as described (16). The only modifications were the use of ungelatinized Lux tissue culture dishes and a higher plating density (1.2×10^6 cells in 4 ml).

Subcultures: The cells in suspension after 2 days were decanted and replated either in ungelatinized tissue culture dishes (Lux) or in bacteriological petri dishes (Nunc), and supplemented as described in the text. In ungelatinized Lux plates there was considerably more attachment with added horse serum (HS) or fibronectin (FN) than in unsupplemented cultures (data not shown). In Nunc plates, however, there was no cell-substrate attachment in the presence of either FN or HS (15). The Nunc plates could not be used for primary cultures: in LH medium, or in medium supplemented with HS and EE, myogenic as well as fibrogenic cells remain attached to the Nunc plates for at least 2 days, after which both detach, so that no separation of myogenic from fibrogenic cells is possible.

Fibronectin and anti-fibronectin: Horse serum FN was isolated by affinity chromatography on a gelatine-Sepharose column (18); FN-depleted HS was obtained by passing HS twice over the gelatine-Sepharose column (8). Rabbit antiserum against human plasma FN was obtained as previously described (8). A crude IgG fraction of antiserum and nonimmune serum was precipitated with 45 % saturated ammonium sulfate, redissolved in NaCl/Pi to the original volume and dialyzed against NaCl/Pi. The anti-fibronectin antibodies (a-FN) were tested, in our standard assay procedure (8), for their ability to block myoblast attachment to a gelatinized substrate mediated by 1 % HS; addition of 5 % a-FN (200 µl per 4 ml suspension) reduced attachment at 20 hr by more than 80 %.

Immunodiffusion: Double immunodiffusion tests were performed as previously described (8). The a-FN preparation precipitated a single component from HS (Fig.1). No reaction was seen with H-Fraction or with FN-depleted HS (Fig.1). Confluent chicken fibroblast cultures grown in the presence of FN-depleted HS release FN into the medium (8); with our a-FN preparation this chicken FN appeared serologically indistinguishable from horse FN (Fig.1).

Quantitation of cell fusion: For scoring nuclei in fused and unfused suspended cells, cells were centrifuged onto microscope slides, fixed with methanol, and stained with Giemsa's solution (18). Nuclei in multinucleated cells were expressed as a percentage of the total number of nuclei scored. Duplicate slides were scored for each time point (minimum of 500 nuclei/slide). Large aggregates found in most of the fixed preparations were not scored because of the difficulty in distinguishing fused from unfused cells. It was impossible to estimate fusion in cells treated with HS and/or EE for longer than 1 day because of the formation of large, rough-surfaced, opaque aggregates. Difficulties in quantitating the fusion of suspended cells have been noted before (12,16).

Removal of divalent cations: Medium was rendered free of Ca^{++} and Mg^{++} ions by treatment with Chelex 100 resin, 100-200 mesh (18). Stock solutions (100 mM) of $CaCl_2$ and $MgCl_2$ in 0.9 % NaCl were used to supplement the depleted medium to the desired concentration of divalent cations. Without Mg^{++} the cells were not viable; therefore the Chelex-treated medium was always supplemented with $MgCl_2$ to the Mg^{++} concentration (1.62 mM) of Leibovitz L-15 medium.

RESULTS AND DISCUSSION

Relation between Ca^{++} concentration and fusion of the suspended cells. Variation of the Ca^{++} concentration was chosen to test the adequacy of the scoring method because so much is known about the inhibitory effect of Ca^{++} depletion on myoblast fusion (12,19-21). Cultures contained a range of Ca^{++} concentrations from the time of plating. The separation of myogenic cells from fibroblasts, based on the failure of the former to remain attached, did not depend on the Ca^{++} concentration. Suspended cells were transferred to new plates after 2 days and scored for fusion on the fourth day. The values in Table 1 show, for myoblasts suspended in serum-free LH medium, a Ca^{++} concentration dependence of fusion comparable to that described for suspended (12) and attached (19-21) cells. We conclude that our method reliably measures fusion.

Neither a-FN nor FN affects the fusion of suspended cells. Myoblasts synthesize little or no FN, even in serum-containing medium (7). Fibroblasts produce FN in serum-containing medium (7,8; Fig.1), but little or none in LH medium: after 4 days, the medium from primary LH cultures (in which both fibroblasts and myoblasts are present) contained no detectable FN (Fig.1). However, if cell-derived FN were to affect myoblast fusion, the effective concentration might not be detectable. We therefore tested whether a-FN influences fusion in suspension.

TABLE 1 : Dependence of fusion in suspended cells on the concentration of Ca^{++} ions in the culture medium[a].

Ca^{++} concentration (mM)	Fusion[b] (%)
0	1.2
0.1	6.5
0.5	19.0
0.95	58.0

[a]After cells were plated in Chelex-treated medium the Mg^{++} concentration was brought to 1.62 mM and the indicated Ca^{++} concentrations established by addition of $CaCl_2$.

[b]The percentage of nuclei in multinucleated cells was estimated after 4 days of culture.

We had shown (8) that affinity-purified anti-FN-IgG at a concentration of 25 µg/ml inhibits, by more than 80 %, the myoblast attachment to gelatinized dishes mediated by 1 % HS. The crude IgG fraction used here (a-FN) had the same activity at a concentration of 5 %. As shown in Table 2 and Figure 2, 5 % a-NF did not affect cell fusion in suspension. The corresponding IgG fraction of nonimmune serum (5 %) also had no effect (not shown). The a-FN was not inactivated in the cultures. Suspended cells from cultures that had

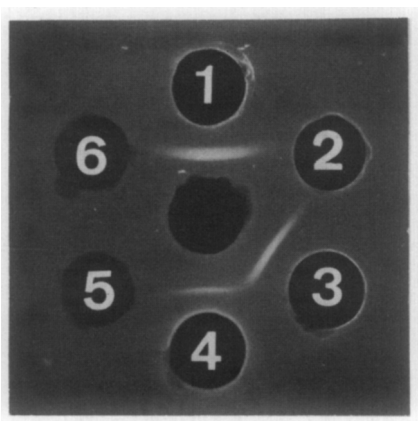

Fig. 1 : **Double immunodiffusion test. Center well: a-FN. Outer wells: 1) HS; 2) FN-depleted HS; 3) HS; 4) medium (5x concentrated by ultrafiltration) from confluent fibroblast cultures (medium contained 10 % FN-depleted HS); 5) 5x concentrated medium from 4-day primary cultures in LH medium; 6) H-fraction of EE.**

TABLE 2 : Comparison of percentage of fusion in control and a-FN-treated cultures.

Medium	days in culture				
	1	2	3	4	5
LH (control)	14.8	43.3	62.0	60.7	70.7
LH + a-FN[a]	16.5	42.9	62.3	61.5	65.6

[a] a-FN (200 µl/plate; 5 %) was added at zero time and on the second and third days of culture.

or had not been supplemented with a-FN were decanted into gelatinized Lux plates after 2 and 3 days with or without the addition of 1 % HS. There was no appreciable attachment to the substrate in cultures supplemented with a-FN. Cultures not treated with a-FN showed normal attachment in 1 % HS. As part of the experiment shown in Table 2, FN (21 µg/ml) was added to primary cultures at the time of plating. The FN did not reduce the yield of suspended cells on day 2. (With continued culture in Lux plates there was myoblast reattachment, however.) Aliquots of the suspended cells were decanted after 2 days into

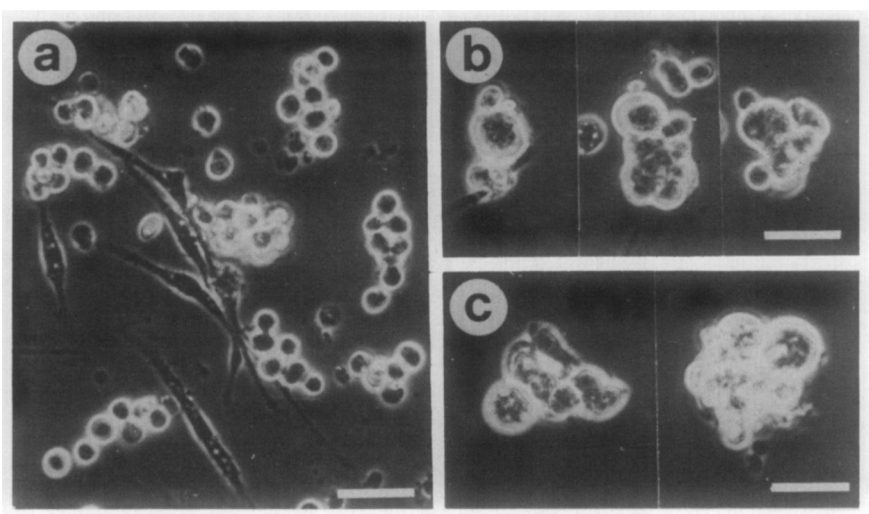

Fig. 2 : Fused and unfused cells in suspension. Cells were examined with phase contrast three days after plating. a) LH medium pretreated to remove Ca^{++}; note that aggregation occurs but fusion does not. b) LH medium; the composite photograph shows many multinucleate myoballs. c) LH medium supplemented at the time of plating and again on day 2 with 5 % a-FN; fusion as in untreated LH. Bar 40 µm.

TABLE 3 : Serum and embryo extract stimulate fusion in suspension.

Supplement (final concentration)	% Fusion after 3 days
none	41.1
5 % a-FN	41.8
21 µg/ml FN[a]	43.1
70 µg/ml FN[a]	44.0
3 % HS	53.1
10 % HS	62.0
14 % FN-free HS[b]	63.4
10 % HS + 3,5 % EE	80.2

[a]21 and 70 µg/ml FN are roughly equivalent to the amount of FN in 3 and 10 % HS, respectively.

[b]The protein concentration of 14 % FN-depleted HS is equivalent to that of 10 % untreated HS.

Nunc plates and cultured further. Fusion on days 1-6 was: 14.5, 41.8, 56.5, 59.8, 62.9, and 60.6 %, respectively. Exogenous FN thus has no significant effect on fusion.

Enhancement of the fusion of suspended cells. After 2 days the suspended cells in primary cultures were collected, pooled, subcultured in Nunc plates, and supplemented with the substances to be tested at the desired concentrations. Fusion was scored one day later (Table 3). The percentage of fusion at 3 days in control cultures was lower than in the experiment described in Table 2. Reduced fusion has been observed whenever cells have been collected, pooled and subcultured in new plates; the reason for this is not clear. Addition of a-FN (5 %; 200 µl/plate) or exogenous FN again did not appreciably influence cell fusion. Addition of HS significantly enhanced the fusion of suspended cells. With addition of both EE and HS a very high level of fusion (80 %) was attained. The fact that HS free of FN stimulated fusion to the same extent as whole HS, whereas purified FN did not, further supports our contention that FN does not influence the fusion of chick myoblasts.

Conclusion. We report a procedure for quantitating the fusion of suspended myoblasts not previously exposed to serum. With this assay, we demonstrated an inhibition of fusion in low-calcium medium as well as enhancement of fusion in the presence of embryo extract, serum, or FN-free serum. Thus, if exogenous

FN had any significant influence on the fusion process, we should have been able to detect it. Added FN known to be active in promoting cell-substrate attachment had no effect. Since excess a-FN (shown to react with chicken FN) also had no effect, it seems very unlikely that cell-derived FN (produced by myogenic cells themselves or released by fibroblasts) is involved in myoblast fusion. Our experiments suggest that the myoblast-myoblast interactions prior to fusion also do not depend on FN, but do not rule out the possibility that FN influences some process, such as cell migration, required for cell fusion in monolayer cultures.

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