Fibronectin Mediates Attachment of Chicken Myoblasts to a Gelatin-coated Substratum*  
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Myogenic cells derived from embryonic chicken muscle are conventionally cultured in a horse serum-containing medium. The serum is known to be the source of one or more factors that mediate the attachment of such cells to the gelatin-coated culture dish. In this paper, we demonstrate that fibronectin is the principal horse serum component responsible. In addition, we show that fibronectin synthesized and released into the medium by chicken fibroblasts also promotes the attachment of myogenic cells.

Antibodies against highly purified human plasma fibronectin (cold-insoluble globulin) precipitated a single component of horse serum. This horse serum fibronectin was purified by affinity chromatography on gelatin coupled to Sepharose (Engvall, E., and Ruoslahti, E. (1977) Int. J. Cancer 20, 1-5). When electrophoresed in sodium dodecyl sulfate-polyacrylamide gels under reducing conditions, human and horse fibronectins both ran as closely spaced doublet bands (M, = approximately 230,000).

Chicken embryo fibroblasts synthesized a protein precipitable by antibodies against human fibronectin. This chicken fibronectin was isolated by affinity chromatography from the medium of fibroblasts cultured in the absence of exogenous fibronectin. When electrophoresed in sodium dodecyl sulfate-polyacrylamide gels under reducing conditions, human and horse fibronectins both ran as a single band (not a doublet) of approximately the same mobility as the human and horse fibronectins.

Suspended myogenic cells were separated from attached fibroblasts in a serum-free medium and used to assay for attachment-promoting activity. Whether added directly to the medium or used to pretreat the (gelatinized) dishes, all three purified fibronectins promoted the attachment of myogenic cells and allowed their elongation. Horse serum depleted of fibronectin had low attachment-promoting activity. Purified antibodies against human fibronectin inhibited attachment by more than 85% when added to cells suspended in a medium that contained horse serum. Preincubation with these antibodies also removed almost all attachment-promoting activity from horse serum or from gelatinized dishes that had been pretreated with serum.

Attachment of cells to noncellular materials (extracellular matrices, basal laminae, collagen fibrils) is fundamental to normal metazoan development (1-4). Studies with cell cultures have shown that many growth- and differentiation-related processes depend on cell-substratum attachment (5-8).

Primary cultures of avian myogenic cells in serum-containing media have been widely used in studies of muscle differentiation (reviewed in Refs. 5, 9, and 10). Not only cell attachment and spreading, but also proliferation and differentiation of myogenic cells in such cultures, have been thought to require serum (5, 6). The formation of elongated myotubes in clonal cultures of chicken breast muscle cells depends on the presence of substrate-bound collagen (gelatin is as effective as native collagen) and of certain serum components (5, 6).

In high density cultures, presumably because the cells themselves provide sufficient collagen, serum alone is able to mediate cell attachment to uncoated culture dishes (5); with one exception (11), substratum attachment and elongation of myogenic cells as well as subsequent myotube formation have not been observed in the absence of serum (5). Hauschka and White (12) showed that the (unidentified) serum component(s) which mediate the attachment of myogenic cells do so by binding to certain segments of the collagen α1 (I) chain. Several investigators have isolated serum factors that mediate cell attachment or spreading, or both. These factors, called cell attachment protein (13), cell attachment factor (14), or spreading factor (15), have subsequently been shown to belong to a class of vertebrate serum proteins immunologically related to human cold-insoluble globulin, a dimeric plasma protein with a subunit molecular weight of about 220,000 (16-19). These plasma and serum proteins are thought to be the circulating forms of a cell surface protein that is the major ladinatable surface component of many normal cell types and that has been called LETS (large, external, transformation-sensitive) protein (20-22), CSP (cell-surface protein; Refs. 23-25), SFA (surface antigen; Refs. 26, 27), or fibronectin (the term we shall adopt; Ref. 27). Fibronectin binds to collagen and even more strongly to gelatin (28) and is believed to be important in cell-substratum attachment since it is present in reduced amounts on the surfaces of less adherent transformed cells (20, 21, 25, 26) and since its addition to transformed cells partially restores their normal morphology and adhesion (22, 24). Fibronectin-mediated cell attachment to collagen-coated substrata has been demonstrated for several kinds of cells (13, 14). It is thought that cells bind to an insoluble complex of fibronectin and collagen and that

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this makes possible the attachment of cells to the extracellular matrix (13, 14).

We thought fibronectin the most likely candidate for the attachment-mediating serum component described by Haueckha (5). Accordingly, we purified fibronectin from horse serum and investigated its action on the attachment of chicken myogenic cells that had been cultured in suspension in serum-free medium (29).

MATERIALS AND METHODS

Isolation of Human Plasma Fibronectin for Immunization—Fifty milligrams (2500 units) of bovine thrombin (Hoffmann-La Roche) dissolved in 25 ml of 56% glycerol were added with stirring to 0.75 g of lyophylized human plasma Cohn Fraction I (Swiss Red Cross) dissolved in 750 ml of 7 mM sodium phosphate buffer, pH 6.4, containing 0.5 M NaCl and 2.5 mM CaCl2, and allowed to stand for 1 h at 20°C. After filtration to remove the fibrin clot (30), the defibrin Cohn Fraction I was loaded on a DEAE-Sephadex A-50 (Pharmacia) column (300 ml) equilibrated with 10 mM Tris-HCl, 10 mM sodium phosphate, pH 7.0. After washing with the same buffer, a linear gradient (2 liters) of sodium phosphate (from 10 to 300 mM) in 10 mM Tris-HCl, pH 7.0, was eluted with 0.5 M, = -55,090 was eluted with 0.5 M urea (ultrapure; Schwarz Mann) in BSS or with two steps of 0.5 M urea (gradient elution) or with 4 M urea; fibronectin was eluted with 4 M urea (gradient elution) or with 4 M urea; fibronectin was eluted (95,000), bovine serum albumin (68,000), rabbit muscle pyruvate kinase (45,000), and myoglobin (18,000) were clearly resolved. The column was further eluted with 4 M urea, about 0.5 mg of fibronectin was passed over a Sephadex G-25 (Pharmacia) column equilibrated with NaCl/P, containing 1 M sucrose and 2% Triton X-100, the immunoprecipitates were pelleted, dissolved in "sample buffer" (31), and subjected to SDS-acrylamide electrophoresis.

Isolation ofChicken Fibronectin from Fibroblast Cell Surfaces and from Fibroblast-conditioned Medium—Cell-surface fibronectin (CSF) was isolated from cultures of chicken embryo fibroblasts (32) subcultured in a medium containing fetal calf serum as described by Yamada et al. (23). Chicken cell-surface fibronectin was also extracted by the same procedure from primary fibroblast cultures grown for 2 days in serum-free LH-medium (29) and then for 5 days in LH-medium supplemented with 10% fibronectin-depleted horse serum. The conditioned medium from the latter fibroblast cultures was collected and a 50-ml sample passed over the gelatin-Sepharose column. By elution with 4 M urea, about 0.5 mg of fibronectin was recovered.

Immunoprecipitations—Samples (cell extracts, sera, fibronectin preparations) to be precipitated by anti-human fibronectin antiserum or IgG were diluted to 500 µl with NaCl/P, and centrifuged 30 min at 27,000 × g (33). The supernatants were incubated 30 min at 30°C and overnight at 4°C with 50 µl of anti-human fibronectin antiserum or with 57 µg of anti-human fibronectin IgG. Controls were performed with rabbit preimmune serum or rabbit preimmune IgG. Subsequent steps were as described by Periannon et al. (33): after underlaying with NaCl/P, containing 1 M sucrose and 2% Triton X-100, the immunoprecipitates were pelleted, dissolved in "sample buffer" (31), and subjected to SDS-acrylamide electrophoresis.

Cell Cultures—Tissue culture dishes (Falcon 3003; 10-cm diameter) were coated by rinsing with 1% gelatin (Gibco) and allowed to dry. Cell suspensions from 11-day-old chicken breast muscle were obtained as previously described (32); they normally contained some 80% myogenic and 20% fibrogenic cells. These two populations could be separated by plating 4 × 10⁶ cells/10-cm dish in 8 ml of LH-medium (29), which contains the "H fraction" of chicken embryo extract (34). Under these serum-free conditions, only fibroblasts reattached to the substrate, whereas viable myoblasts were found in suspension. Neither the H fraction nor the complete medium contained any material that reacted with anti-human fibronectin in double immunodiffusion tests (not shown). Details of this culture system are given elsewhere (29).

Attachment Assay—Sweated myoblasts were collected from primary cultures, described above, after 48 h and pooled (29). For the standard assay, 4-ml portions, containing between 8 × 10⁵ and 1 × 10⁶ cells, were plated on 6-cm tissue culture dishes (Lux) that had been gelatinized as described above. Fibronectin preparations were dialyzed against 200 volumes of BSS overnight; all dilutions of sera and fibronectin were made in BSS. The substance to be tested for attachment activity was added in the desired concentration directly to the cell suspension. Alternatively, as indicated under "Results," the gelatinized dishes were pretreated for 1 h at 37°C with different dilutions of the test preparation and washed three times with BSS prior to incubation with the myoblast suspension. At different times after beginning the incubation, the medium containing those cells that had not attached was decanted, the dishes were rinsed once with BSS, and the attached cells were fixed with 2.5% glutaraldehyde in BSS for at least 1 h.

After postfixation with 50% methanol for 1 h, cultures were stained with 1% Giemsa solution, washed, and dried. This fixation procedure gave minimal loss of attached cells at early time points. Attachment was quantitated by scoring about 0.18-mm² lines. The data are expressed as nuclei in attached cells/dish. At least 500 nuclei were scored for each estimate.

Other Procedures—Gel electrophoresis was performed according to Laemmli (31) on 1.5-mm slab gels containing 0.1% SDS. Except where indicated to the contrary, the concentrations of acrylamide (7.0% gel) or urea (37.5% for all gel) are given. The gel was 0.1% SDS. Except where indicated to the contrary, the concentrations of acrylamide (7.0% gel) or urea (37.5% for all gel) are given. The gel was subjected to SDS-acrylamide electrophoresis. Coomassie brilliant blue R (Sigma). Protein markers were (M): chicken myosin heavy chain (200,000), rabbit muscle phosphorylase b (95,000), bovine serum albumin (68,000), rabbit muscle pyruvate kinase (67,000), chicken pyruvate kinase (50,000), bovine chymotrypsinogen (24,000), and horse cytochrome c (12,000).

Protein was estimated by the method of Lowry (35) using crystal line bovine serum albumin (Fluka) as standard.

RESULTS

Isolation and Characterization of Horse Serum Fibronectin—Horse serum promotes attachment, to a gelatinized substratum, of myoblasts that have been cultured in suspension in serum-free LH-medium (29); see also Fig. 7). Horse serum contains a single component which reacts with anti-human...
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Fibronectin IgG in a double immunodiffusion test and which is immunologically related to human fibronectin (Fig. 1b). This component was isolated by affinity chromatography (cf. Fig. 1b). Electrophoresis of the preparation revealed two closely migrating protein bands which co-migrated with human fibronectin (cold-insoluble globulin; Mr = ~230,000) purified by the method of Chen and Mosesson (17) (Fig. 2). Affinity-purified antibodies against human fibronectin precipitated the same doublet, both from preparations of the affinity-purified material and from whole horse serum (Fig. 2). A doublet is seen after electrophoresis of whole horse serum (Fig. 3), and not only in immunoprecipitates of serum or in purified preparations. The doublet, therefore, does not arise in the course of the purification or immunoprecipitation procedures. As we do not know whether the two prominent bands in horse serum are also found in fresh plasma or in newly synthesized horse fibronectin, the origin of the doublet is unclear. A similar doublet of even more closely migrating bands can sometimes be detected in preparations of human plasma fibronectin (see Fig. 2 and Ref. 18); in this case, the subunit heterogeneity has been attributed to the action of proteases (18, 19).

The yield of horse serum fibronectin was about 0.6 to 0.7 mg of protein/ml of serum (or 0.7 to 0.8% of the total horse serum protein), compared to about 0.2 mg of human fibronectin/ml of serum (or 0.22% of the total serum protein) isolated by the same method. The latter value agrees well with esti-

\[ \text{V. Bieri, personal communication.} \]
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FIG. 4. Precipitation by anti-human fibronectin of fibronectin synthesized by chicken fibroblasts. After 2 days in LH-medium, primary chicken fibroblast cultures were supplemented with 10% horse serum and with 10 μg/ml of [35S]methionine. Eighteen hours later, the cultures were washed three times with BSS and then extracted according to the method of Yamada et al. (23). Samples containing 5×10⁴ dpm were incubated with antibody, after which the immunoprecipitates were collected and electrophoresed. The gels were impregnated with 2, 5-diphenyloxazole (PPO), dried, and exposed to x-ray film (36). Precipitation was with: a, 50 μl of preimmune serum; b, 50 μl of anti-human fibronectin antiserum; c, 39 μg of preimmune IgG; d, 57 μg of anti-human fibronectin IgG.

mates of the fibronectin content of human serum based on immunological methods (17). Since fibronectin binding to and recovery from the affinity column is essentially 100% efficient (Ref. 28; cf. also Fig. 2), the fibronectin concentration in horse serum must be at least 3 times that in human serum.

Chicken Fibronectin from Fibroblast Cell Surface and from Conditioned Medium—Cell surface fibronectin (CSP) extracted from chicken fibroblasts that had been cultured in a serum-containing medium contained a major protein band that co-migrated with human plasma fibronectin (Fig. 3). If the cells were first labeled with [35S]methionine and then extracted, radioactive chicken fibronectin could be specifically precipitated from the extracts with anti-human fibronectin (Fig. 4). This confirmed that the extracted fibronectin was (at least partially) of cellular origin. In contrast to the fibronectins isolated from human plasma or horse serum, the immunoprecipitated, newly synthesized, chicken fibronectin ran not as a doublet, but as a single protein band (Fig. 4). Even at shorter exposure times, no evidence of a doublet was detected on the x-ray films.

Fibroblasts proliferated rapidly in LH-medium supplemented with fibronectin-depleted horse serum; after 4 days, the originally fibronectin-free medium contained a component (which ran as a single protein band, not a doublet) with the same electrophoretic mobility as human and horse fibronectin (Fig. 3). This chicken fibronectin is released into the medium by the fibroblasts. It is precipitable with anti-human fibronectin. It was isolated by affinity chromatography (Fig. 3, Lane c).

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Serum- and Fibronectin-mediated Attachment to a Gelatinized Substratum and Subsequent Elongation of Myogenic Cells—Fig. 5 shows the relationship between plated and attached myoblasts under different assay conditions. For a given horse serum concentration and incubation time, the number

FIG. 5. Dependence of number of attached cells on number of plated cells for different horse serum concentrations. Suspended myoblasts cultured for 2 days in LH-medium were pooled, centrifuged for 8 min at 300×g, and resuspended in 1/3 the original volume of LH-medium. This suspension containing about 4.5×10⁶ cells/ml (estimated by counting in a hemocytometer) was diluted 1:1, 2:3, 1:3, 1:6, and 1:12 with LH-medium. Four milliliters of diluted cell suspension were plated/gelatinized 6-cm dish and horse serum was added to the indicated concentrations: [ ], 3%; [ ], 0.3%; or [ ], 0.03%. Cultures were fixed after 3 h (---) and 20 h (— — ).
of attached cells was roughly proportional to the number of plated cells over a wide range (between $10^5$ and $1.2 \times 10^6$ plated cells/6-cm dish).

Attachment of myoblasts after addition of horse serum or horse serum fibronectin occurred rapidly, reaching characteristic values, which depended on the serum or fibronectin concentration, by 2 h (Fig. 6). The attached cells were still rounded at this time, although two short processes were frequently seen, particularly in the presence of purified horse fibronectin (Fig. 7). The elongation of the bipolar myoblasts was complete only after about 24 h (Fig. 7); elongation in cultures supplemented with isolated fibronectin was indistinguishable from that observed with whole horse serum.

Horse serum from which fibronectin was selectively removed by gelatin affinity chromatography lost most of its attachment-promoting activity. In one experiment, $6.1 \times 10^4$ and $1.0 \times 10^5$ cells/dish, respectively, were attached at 3 h in 1 and 3% depleted serum, while attached cells numbered $6.3 \times 10^5$ and $7.6 \times 10^5$ in 1 and 3% untreated serum. At 20 h, the values for 1 and 3% depleted serum were $7.2 \times 10^5$ and $1.1 \times 10^5$, respectively, compared to $7.0 \times 10^5$ and $5.4 \times 10^5$ in full serum; Table I shows a similar result obtained in a separate experiment. The activity of fibronectin-depleted serum was fully restored by addition of purified fibronectin.

The number of attached cells continued to increase between 2 and 20 h after addition of horse serum, although at a lower rate than in the first 2 h; this was seen even at low serum concentrations in which attachment during the first 2 h was well below the maximum (Fig. 6). In contrast, there was a net loss of attached cells during the same period in cultures supplemented with purified fibronectin even at high concentrations (Fig. 6). This difference in behavior between cultures supplemented with whole serum and fibronectin, respectively, could be overcome by adding fibronectin-depleted horse serum together with fibronectin (see below and Table I).

Attachment of chicken myoblasts was also mediated by serum or fibronectin previously adsorbed to the gelatinized substratum. The time course of attachment was similar to that after addition of horse serum or horse fibronectin to the culture medium (not shown); to reach similar attachment levels, however, higher concentrations were necessary. Over the entire range tested (up to 100% horse serum and 400 µg/ml of fibronectin), attachment increased with increasing concentration of horse serum or fibronectin in the pretreatment solution.

### Table I

| Fibronectin concentration | No additional supplement | Plus 1% fibronectin-depleted horse serum |
|---------------------------|--------------------------|----------------------------------------|
| µg/ml                     | Attached cells/dish x 10^5 at 20 h | % of control* | Attached cells/dish x 10^5 at 20 h | % of control* |
| 0                         | 0.38                     | 5           | 1.93                     | 26          |
| 2                         | 0.66                     | 9           | 4.58                     | 61          |
| 5                         | 2.77                     | 37          | 6.18                     | 83          |
| 10                        | 3.61                     | 48          | 6.68                     | 90          |
| 20                        | 5.11                     | 68          | 6.88                     | 92          |

*Attachment in parallel cultures supplemented with 1% horse serum (7.46 x 10^5 cells/dish) is taken to be 100%.

Fig. 7. Attachment and elongation of chicken myoblasts on a gelatinized substratum is mediated by purified fibronectins. a and b, 10% horse serum; a, 2 h and b, 20 h after addition. c and d, purified horse serum fibronectin (135 µg/ml); c, 2 h and d, 20 h after addition. e, chicken fibronectin (20 µg/ml) isolated from conditioned medium, 20 h after addition. f, no supplement, 20 h after additions to other cultures. At the times indicated, the medium with nonattached cells was decanted from the dishes, the cultures were washed with BSS, fixed with 2.5% glutaraldehyde in BSS and photographed with phase contrast optics. Magnification, x 200.
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Table II
Attachment-promoting activity of fibronectins prepared from chicken fibroblast cultures compared to horse serum fibronectin

| Supplement to standard assay | Protein concentration µg/ml | Attached cells/plate at 90 h (x10^5) |
|-----------------------------|----------------------------|-------------------------------------|
| None                        | 190                        | 0.17                                |
| Horse serum (3%)            | 2290                       | 4.04                                |
| Horse serum fibronectin     | 4                          | 0.12                                |
|                             | 14                         | 0.17                                |
|                             | 42                         | 3.05                                |
|                             | 136                        | 2.74                                |
| CNP from fibroblasts grown in the presence of serum | /                          | 0.36                                |
|                             | 14                         | 0.75                                |
|                             | 24                         | 2.41                                |
| Chicken fibronectin from serum-free medium conditioned by fibroblasts | 2                          | 0.11                                |
|                             | 6                          | 0.17                                |
|                             | 20                         | 3.05                                |
|                             | 60                         | 5.28                                |
| Serum-free medium conditioned by fibroblasts (3%) | N.D.*                      | 6.29                                |

* N.D., not determined.

Specific Attachment Activity of Different Fibronectins Compared to Serum—Fig. 8 shows the dependence of number of attached cells on the concentration of horse serum and horse fibronectin at 3 and 20 h after addition. At 3 h, half-maximal attachment activity was reached with about 700 µg/ml of horse serum protein (0.9% horse serum) or 25 µg/ml of purified horse serum fibronectin. For purified fibronectin, the concentration giving half-maximal activity at 20 h was the same as at 3 h, while with horse serum, attachment had reached a higher absolute level by 20 h and half-maximal activity was seen at about 300 µg/ml of protein (0.4% horse serum). This finding, taken together with the loss of attached cells between 3 and 20 h after addition of fibronectin in the absence of other serum factors (Fig. 6), suggests that horse serum factors other than fibronectin enhance cell attachment, perhaps indirectly by affecting cell metabolism or viability. This synergistic effect is demonstrated in Table I: fibronectin-depleted serum enhances, by about 10-fold, the apparent specific activity of purified horse fibronectin measured at 20 h.

Since affinity-purified fibronectin represents about 0.8% of total horse serum protein, one would expect a 125-fold purification if the activity yield were 100%. The reason for the low recovery (about 25%, based on 30-fold increase in specific activity assayed at 3 h; Fig. 8) in our affinity purification of horse serum fibronectin is not known. Horse serum fibronectin isolated (37) by the method of Klebe (13) had about the same specific activity as the affinity-purified material (not shown). The specific activity of purified human fibronectin was about 2 to 3 times higher than that of horse fibronectin (Table II). Human serum is at least 2 times less active than horse serum (as expected from its approximately 4-fold lower content of fibronectin) and toxic at concentrations over 1%.

The chicken fibronectin used in this experiment was extracted from fibroblast cultures grown in medium containing fibronectin-depleted horse serum. This was necessary to ensure that the extracted fibronectin was derived from endogenous serum fibronectin that had adsorbed to the cells. Chicken cell-surface fibronectin isolated in this way promoted attachment of chicken myoblasts; half-maximal activity appeared to be similar to that found for human and horse fibronectin (Table II).

After conditioning by fibroblasts, LH-medium supplemented with fibronectin-depleted horse serum became a very potent promotor of myoblast attachment: more myoblasts attached within 20 h with only 3% conditioned medium in the assay medium than with 2% horse serum (Table II). The greatly enhanced activity was at least partially due to release of fibronectin by the fibroblasts: chicken fibronectin isolated from conditioned medium by affinity chromatography was at least as active in promoting attachment of chicken myoblasts as chicken cell-surface fibronectin (Table II).

It is noteworthy that, despite the differences seen in the electrophoretic patterns, cell surface fibronectin (chicken), fibronectin from conditioned medium (chicken), and plasma or serum fibronectin (human and horse) had similar specific activities in our assay. (The only exception was human plasma fibronectin isolated by thrombin treatment of plasma Cohn Fraction I, which was less active (37).)

Anti-human Fibronectin Inhibits Serum- and Fibronectin-mediated Attachment and Spreading of Chicken Myoblasts—The model experiments show two control experiments. 1) Preincubation of the antibody with purified horse fibronectin eliminated its action on serum-mediated attachment (Table III). 2) As part of the experiment shown in Table III, other aliquots of the cell suspension were supplemented with 0.3% horse serum and with anti-human fibronectin antisera (1.3%) or antiserum that had first been passed over a fibronectin-Sepharose column (1.3%); attachment after 20 h was, respectively, 2.1 x 10^5 and 6.8 x 10^5 cells/dish.

In a separate experiment, anti-human fibronectin likewise inhibited the attachment activity of purified fibronectin. Half-maximal attachment at 20 h (2.5 x 10^5 cells/dish) was obtained with 35 µg/ml of purified horse fibronectin (cf. Fig. 8); simultaneous addition of anti-human fibronectin IgG (35 µg/ml) reduced attachment by about 60% (to 1.0 x 10^5 cells/dish). Inspection of Fig. 8 shows that 0.3% horse serum (~290 µg/ml, containing about 1.7 µg/ml of fibronectin) gives attachment comparable to that with 35 µg/ml of purified horse fibronectin. It is, therefore, not surprising that the inhibition

Table III
Horse serum-dependent myoblast attachment is blocked by antibodies against fibronectin

| Horse serum | Supplements to standard assay | Attached cells/dish at 20 h (x10^5) |
|-------------|-------------------------------|-------------------------------------|
| %           | Purified IgG µg/ml | Anti-fibronectin IgG |
| 0           | 0.48                         | 7.33                                |
| 1           | 28.8                         | 9.98*                               |
| 1 Anti-fibronectin IgG | 14.4                         | 2.87                                |
| 1 Control IgG | 19.5                         | 7.40                                |

* This loss of activity could be prevented by preincubating the IgG (28.8 µg/ml) with purified horse fibronectin (see method for immunoprecipitation in "Materials and Methods"); at concentrations of 0.43, 0.96, and 1.74 µg/µg of IgG, attached cells (~x10^5) were 4.90, 5.06, and 6.75, respectively.
was not more marked in this experiment. Attachment mediated by purified horse fibronectin, however, could be inhibited by over 90% with an anti-human fibronectin antiserum which had a higher titer than the preparation of purified antibody used (not shown).

To exclude the possibility that the immunoreaction occurring in the culture medium blocked cell attachment by indirect mechanisms, such as cell agglutination, horse serum was preincubated with anti-human fibronectin IgG and, after removal of the immunoprecipitate, added to a suspension of myoblasts. In this experiment, 35 μg of anti-human fibronectin IgG reduced the attachment activity of 10 μl of horse serum by 80% (Table III). This demonstrates that the inhibitory effect of anti-fibronectin is indeed due to specific removal of fibronectin from horse serum.

In a further experiment, gelatinized dishes were pretreated with 30% horse serum; in the absence of a second pretreatment, 1.8 x 10^4 cells/dish attached by 20 h. A second pretreatment with anti-human fibronectin IgG (0.14 and 0.29 mg/ml) reduced attachment of chicken myoblasts to the serum-treated dishes by up to 90% (2.6 x 10^3 and 8 x 10^3 cells/dish at 20 h, respectively). Control IgG (0.39 mg/ml) in the second pretreatment was ineffective in blocking adhesion (1.9 x 10^3 cells attached/dish at 20 h). This confirmed that the attachment-promoting serum component which binds to the gelatinized substrate is fibronectin; the antibody evidently inhibits attachment of myoblasts by blocking their substratum binding sites. Taken together with the results on the specific removal of fibronectin from serum by affinity chromatography (see above), the experiments reported in this section establish that fibronectin is the principal serum factor responsible for promoting myoblast attachment.

**DISCUSSION**

**Elucidation of the Functions of Fibronectin is Facilitated by the Use of Culture Conditions Free of Exogenous Fibronectin**

Fibronectin has been reported to promote cell-substratum attachment (13, 14), morphological changes associated with spreading (22, 24, 38), cell aggregation (23), and cell motility (39). One difficulty that has become apparent in studies of fibronectin is that this glycoprotein does not have a unique location on the cell surface or in the immediate surroundings of the cell. Aside from that (presumably largely newly synthesized) fibronectin found intracellularly, fibronectin may occur either intimately associated with the cell surface (20, 23) or as a protein released from the cell (25, 40). Released fibronectin, in turn, is found both as soluble protein (40) and as part of an insoluble matrix (26). Even if one knows that fibronectin is involved in a given activity, it is difficult to ascertain which class or classes of the variously located fibronectin molecules is responsible. Compounding this problem is the possibility that fibronectin in different locations may be structurally distinct. The readily soluble fibronectin released by cells into the medium and the fibronectin extractable from cell surfaces appear to have identical primary structures (40). It is known, however, that some fibronectin molecules occur as polymers linked by disulfide bridges (41) and that fibronectin can be cross-linked by transglutaminase (42, 43). The degree of cross-linking might influence the biological activity.

An additional complication in studies with cell cultures, which we believe has received too little attention heretofore, is the problem of distinguishing between effects of exogenously supplied fibronectin and that produced by the cells being studied. Serum, generally added at some stage as a supplement to the culture medium, is one obvious source of exogenous fibronectin. A second potential source are cells others than

those under study: those present as contaminants in primary cultures (e. g. fibroblasts in primary myogenic cultures) or those at other stages of differentiation in cultures of established cell lines.

The myogenic cells we use are homogeneous with respect to developmental stage (virtually all are postmitotic myoblasts), are essentially free of fibroblast contamination, have never been exposed to serum in culture, and do not need to be treated with protease or other dissociating agents in order to obtain a suspension for the attachment test (29). Myoblast attachment depends upon the addition of exogenous fibronectin. This suggests that, if myoblasts themselves produce fibronectin, the amount is insufficient to mediate attachment (cf. Ref. 37).

**The Attachment Assay**—To investigate attachment and spreading activity of serum or serum factors, several different attachment assays with cells from established lines (13-15, 38) and with primary cells (44) have been developed. The cells are obtained either from monolayer cultures or tissues by dissociation with proteases (13, 14) or from suspension cultures grown in medium free of divalent cations (15, 38). The cell suspensions are plated on untreated (15, 38, 44) or collagen-coated (13, 14) dishes, and serum or serum factors are either added to the culture medium (15, 38) or used to pretreat the culture dishes (13-15, 38, 44, 45). Appreciable attachment in the absence of added serum or fibronectin complicates the analysis of cell substratum attachment; in two instances with trypsin-treated cells, this was overcome by washing collagenized dishes with urea before use (13, 14). Moreover, certain cells attach to untreated dishes even faster in the absence than in the presence of serum (38) or "spreading factor" (15), which in this case promotes cell spreading only (15). It is an advantage of our culture system that both substratum attachment and elongation of the suspended primary chicken myoblasts strongly depend on the presence of serum or isolated fibronectin. Because of differences among the various assay systems, comparison of our data with those of others is difficult. Nevertheless, the active concentrations of serum and fibronectin as well as the time course of myoblast attachment appear to be similar to those observed for other cell types and assay systems (13-15, 38, 44).

Although fibronectin alone is able to mediate both attachment and elongation of chicken myoblasts, there is some cell loss over longer times. Our studies reveal that other serum factors synergistically enhance fibronectin-mediated myoblast attachment and elongation between 3 and 20 h; with the aid of our assay system, we hope to elucidate their nature.

**Fibronectin and Collagen in Muscle Development**—Hauschka (5) demonstrated that at least one factor present in horse serum was required for the attachment of myogenic cells to collagen-coated culture plates. From our work, it appears that Hauschka's active horse serum component is the horse serum form of fibronectin. Strongly supporting this notion is the finding (45) that a "cell attachment protein" from fetal calf serum (13), (which, like the horse serum fibronectin described here, is immunologically closely related to human fibronectin (19)) binds to the very same fragments of the α1(1) chain of collagen that had earlier been shown to promote myogenesis in clonal cultures of chicken myogenic cells (12). Homologous (chicken) fibronectin is also active in promoting myoblast attachment to gelatinized plates. This is an important piece of information if it is to be proposed (37) that fibronectin-mediated anchorage of cells to an extracellular matrix or basal lamina may be part of normal myogenesis in the chicken.

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