Fibronectin Expression During Myogenesis

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ABSTRACT The biosynthesis and localization of fibronectin during chick muscle differentiation are described. This study employed two monoclonal antibodies, one that selectively killed mononucleated cells and one specific for avian fibronectin. These antibodies allowed precise analyses of fibronectin expression in well-defined cultures of myoblasts or myotubes and avoided the complications of exogenous fibronectin and contamination by fibroblasts or unfused myoblasts. Fibronectin synthesis, as a fraction of total protein synthesis, remains constant at 0.3-0.4% before and after myoblast fusion, suggesting that the absolute rate of fibronectin synthesis may increase somewhat when myotubes synthesize and accumulate myofibrillar proteins. The pattern of fibronectin arrangement does change during myogenesis. In myotube cultures, the appearance of pulse-labeled fibronectin at the cell surface and its secretion into the medium begin after a 2-3-h lag period, in contrast to the 30-min lag period observed in fibroblast cultures. This lag between polypeptide biosynthesis and the exteriorization of the new protein is thus a characteristic of each cell type rather than the protein. All of the major secretory proteins of myogenic cells, including fibronectin and collagenous components, share this 2-3-h intracellular transit time.

Fibronectin is a major extracellular connective tissue component of muscle (1-3). The function of fibronectin in muscle tissue is unknown, but in general, fibronectin has been implicated in embryonic tissue interactions and as substratum for cell migration and attachment (4, 5). Such a role for fibronectin has been demonstrated in vitro with the identification of serum fibronectin as the factor necessary for the attachment of myoblasts to collagen (6).

Several studies of the muscle cell surface in tissue culture have yielded data on the levels of fibronectin during myogenesis, but the data do not fit into an obvious pattern. Hynes et al. (7), using cell surface labeling techniques to examine pre- and post-fusion cultures of rat L8 cells, found an increase in fibronectin levels in postfusion myotube cultures. Similar data have been obtained with chick embryo myogenic cells (8) and cells of the mouse G8-1 clonal muscle cell line (9). In contrast with these reports, immunocytochemical observations on the rat L6 muscle cell line and on cloned human muscle cells indicate that as myoblasts fuse they lose their cell surface fibronectin (3, 10, 11). Furthermore, Chiquet et al. (6, 12) reported that primary chick myogenic cells depend upon serum fibronectin for in vitro attachment, and suggested that myogenic cells may not synthesize detectable levels of fibronectin. Given that myogenic cell lines may display abnormal biosynthetic capabilities and that primary cultures are usually composed of mixtures of cell types (including fibroblasts which synthesize fibronectin), it has remained questionable whether fibronectin is a normal biosynthetic product of myogenic cells.

In this paper we describe experiments, using monoclonal antibodies, which circumvent many of these problems. We have used primary cultures of muscle cells but can eliminate from the cultures all unfused cells. Therefore we can compare pure cultures of mononucleate cells and fused myotubes. We report that they do not differ in their rates of fibronectin synthesis relative to the overall rate of protein synthesis and that fibronectin is laid down by myoblasts and myotubes in a variety of arrangements that could play roles in cell substratum and/or cell-cell adhesion during myogenesis. With this system, the biosynthesis, transport, and exteriorization of fibronectin by myotubes was studied, and we conclude that the kinetics of these processes vary from one cell type to another.

MATERIALS AND METHODS

Fibronectin Isolation and Monoclonal Antibody Preparation: Cellular fibronectin was isolated from tertiary chick embryo fibroblast cultures grown in Falcon plastic roller bottles (3027; Falcon Labware, Oxnard, CA) as described by Yamada et al. (13) and the protein was fractionated by gelatin-Sepharose chromatography (14, 15). Fibronectin eluted from the gelatin columns with 8 M urea was essentially homogeneous by SDS PAGE analysis.
BALB/cf mice were injected intraperitoneally with 100 µg of cellular fibronectin in complete Freund's adjuvant. 4 wk later, mice were boosted with 100 µg of fibronectin without adjuvant. 3 d later the spleens were removed and lymphocytes were fractionated for hybridization with SP2/0 myeloma cells (16). The cell fusion was performed as described by Kennett et al. (17) with minor modifications. After HAT selection, the culture supernatants were screened for antibody production by an indirect radioimmunoassay and hybrids secreting antibodies were cloned in soft agar and grown as ascites in Balb/c mice for production of large amounts of antibody. The IgG fraction from ascitic fluid was purified by ammonium sulfate fractionation and chromatography on DEAE cellulose.

The monoclonal antibody #6 that bound to mononucleate myoblasts and fibroblasts was produced by essentially the same procedures except that the immunogen consisted of a crude plasma membrane fraction derived from homogenates of 14-d embryonic avian thigh muscle. This antibody was an IgM and was purified from ascitic fluid by ammonium sulfate fractionation and gel filtration on Ultratag Aca 22 (LKB).

Preparation of Myogenic Cultures: Primary cultures were prepared from the pectoral muscle of 1-d old chicken embryos. Cells were obtained by mechanical dissociation of minced tissue, filtered through gauze and silk to remove debris, and diluted with complete medium (Eagle's MEM with 2% embryo extract and 10% horse serum) to a concentration of 2 x 10^3 cells per ml. The embryo extract did not contain a detectable amount of fibronectin. The cells were then subjected to two sequential 30-min periods of preplating in Falcon 150-mm tissue culture dishes (not collagen-coated) at 37°C to remove fibroblast contamination. Nonadherent cells were collected and plated in collagen-coated tissue culture dishes cultures. Routinely, the cells from five embryos were plated into fifty 15-mm collagen-coated plates.

Upon completion of fusion (72 h after plating) the cultures were fed with fresh medium and returned to incubation for 24 h. At this point the cultures were treated with the #6 antibody that binds to the remaining mononucleate, dividing cells, and in the presence of complement these cells were lysed. Lysis medium was prepared by adding #6 antibody to complete medium at a final concentration of 2 µg/ml. Guinea pig complement (Cappell Laboratories Inc., Cochraville, PA) was added at 20-fold dilution and the medium was filtered through a 2 µm filter (Nalge Co., Nalgene Labware Div, Rochester, NY). The normal medium was removed and replaced by lysis medium and the cultures were returned to incubation at 37°C for 60 min. The lysis medium was then removed and the cells were washed three times for 10 min each in sterile Hanks' Balanced Salt Solution containing 0.5% BSA and finally returned to normal medium.

The percentage of myotube formation or remaining mononucleate cells in cultures prepared as described above is expressed as the number of nuclei appearing in myotubes or mononucleated cells, multiplied by 100, divided by the total number of nuclei in all of the cells. Nuclei were visualized after fixation of cells in methanol, dehydration in 30%, 100% ethanol sequentially, and staining with 1% aqueous Giemsa (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY). Representative data are described in Table 1.

### Immunoautoradiography

- For some experiments monoclonal antifibronectin, control monoclonal immunoglobulin, or normal mouse immunoglobulin (IgG fraction) at 10 µg/ml was applied to either live or fixed cultures for 2 h at 22-23°C in HBSS with 10% horse serum. Fluorescein-labeled goat IgG directed against mouse IgG heavy and light chains (Cappell Laboratories) was used as second antibody in indirect immunofluorescent staining.
- For one experiments monoclonal antifibronectin MOPC 21 immunoglobulin, and normal mouse immunoglobulin were labeled directly with fluorescein or tetramethylrhodamine dye. In some cases, cells were permeabilized by acetone extraction after fixation. Covergips were immersed in 1:1 (vol/vol) acetone-water at 0°C for 2 min, acetone at -70°C for 2 min, 1:1 acetone-water at 0°C for 2 min, and then rinsed directly with PBS and preincubated for 30 min at room temperature with HBSS containing 1% horse serum before the addition of antibodies. All of the labeling procedures were adequate to saturate the exposed fibronectin sites. This was determined by testing subsequently for unoccupied sites with [35S]-labeled antifibronectin. In control experiments, the monoclonal antifibronectin antibody gave no detectable staining of murine fibroblasts grown in medium containing horse serum and embryo extract, confirming the antibody was not binding outside the possible interaction with fibronectin derived from the medium components.

### Pulse-Electron Flowscopy

- SDS polyacrylamide gels consisted of 5-15% linear gradients of polyacrylamide 1.5-mm thick, with a 3% stacking gel, and employed the buffer systems described by Laemmli (19). Immunoprecipitates, cell lysates, and conditioned medium were prepared for electrophoresis by the procedures reviewed by the conditioned sample buffer that yielded the final concentrations: 2% SDS, 10% glycerol, 0.125 M Tris- HCl, pH 6.8, 0.1 M dithiothreitol. Samples were boiled for 5 min before loading the equivalent of 106 cells in 10 µl of sample buffer.
- After completion of fusion (72 h after plating) the cultures were fed with fresh medium and returned to incubation for 24 h. At this point the cultures were treated with the #6 antibody that binds to the remaining mononucleate, dividing cells, and in the presence of complement these cells were lysed. Lysis medium was prepared by adding #6 antibody to complete medium at a final concentration of 2 µg/ml. Guinea pig complement (Cappell Laboratories Inc., Cochraville, PA) was added at 20-fold dilution and the medium was filtered through a 2 µm filter sterilizer (Naige Co., Nalgene Labware Div, Rochester, NY). The normal medium was removed and replaced by lysis medium and the cultures were returned to incubation at 37°C for 60 min. The lysis medium was then removed and the cells were washed three times for 10 min each in sterile Hanks' Balanced Salt Solution containing 0.5% BSA and finally returned to normal medium.

#### Immunoautoradiography

- Immunoautoradiography was performed by electrophoretic transfer of protein from 1-mm thick SDS polyacrylamide gel to nitrocellulose paper (0.45 µm pore size in roll from Millipore Corp., Bedford, MA). The transfer apparatus and blotting procedure were essentially as described by Towbin et al. (24).
Labeling of Cells: Metabolic labeling of cells was performed with \(^{35}\)S methionine (1,100 Ci/mmol; Amersham Corp., Arlington Heights, IL) at 100 µCi/ml or \(^{3}H\)leucine (50 Ci/mmol; New England Nuclear) at 100 µCi/ml. The medium was modified by omitting the appropriate unlabeled amino acid to improve incorporation, however, complete serum and embryo extract were used at levels in normal medium. The cells were rinsed with sterile Hank's Balanced Salt Solution at 37°C before the application of label. Similar procedures were used for labeling of cells with \(^{3}H\)proline (50 Ci/mmol; Amersham Corp.) and \(^{3}H\)glycine (30 Ci/mmol; New England Nuclear), which were included in complete medium at 100 µCi/ml each to preferentially label the collagenous polypeptides.

Enzymatic Digestions: Cell surface and substrate-bound fibronectin were removed by incubation of cultures with freshly solubilized trypsin (Koch Light Laboratory, Colnbrook Buck, U. K.) at 5 µg/ml in phosphate buffered saline for 5 min at room temperature. The cultures were observed by phase microscopy during and after the treatment and the myotubes remained adherent to the substrate yet the extracellular fibronectin was removed as detected by immunofluorescence. The cells were washed three times in PBS containing 2 mm PMSF and 1,500 µg/ml Soybean Trypsin Inhibitor (Sigma Chemical Co., St. Louis, MO). The cells were then lysed for immunoprecipitation or fixed for antibody staining.

Metabolically labeled myotube conditioned medium was digested with collagenase to further identify the secreted collagenous polypeptides. Purified bacterial collagenase (Advanced Biofactures) was added (20 units) and digestions carried out at 37°C for 1 h in the presence of 2 mm PMSF and 5 mm NEM. Aliquots were removed and the reaction was terminated by the addition of electrophoresis sample buffer and boiling.

Protein Synthesis: Protein synthesis was measured by the incorporation of labeled amino acid into trichloroacetic acid (TCA) insoluble cellular protein. Usually 50 µl aliquots of cell lysates or conditioned medium were spotted on Whatman 3-mm filter disks, Whatman Inc., Paper Div., Clifton, NJ), air dried and placed in 10% TCA containing 1 mg/ml unlabeled amino acid for 10 min, followed by hot 10% TCA (95°C) for 10 min and finally rinsed in ice cold 5% TCA. The filters were washed in 95% ethanol, ethanol:ether (3:1), and then ether and dried in a fume hood. The filters were placed in scintillation vials with 15 ml of Triton-toluene fluor and counted by liquid scintillation spectrometry.

Protein synthesis was inhibited by cyclohexamide (5 µg/ml) (Sigma Chemical Co.) or puromycin (10 µg/ml) (Calbiochem-Behring Corp., San Diego, CA).

RESULTS

A Novel Surface Antigen Expressed by Dividing Cells but Not by Multinucleated Myotubes

The hybrid myeloma technique has been used to generate a library of monoclonal antibodies for the analysis of muscle cell surface. One of these antibodies (#6), binds preferentially to mononucleated cells in primary myogenic cultures (E. K. Bayne, unpublished observations). Treatment of well-differentiated chick muscle cultures with hybridoma #6 IgM together with guinea pig complement results in the lysis of mononucleated cells (Fig. 1), while differentiated myotubes are left intact and viable. In younger cultures mononucleated cells possessing either the typical bipolarity of myoblasts or an irregular fibroblastlike morphology are susceptible to complement-mediated lysis by hybridoma #6 antibody. Young postfusion myotubes exhibit sufficient amounts of antigen to retain sensitivity to this treatment for ~24 h following fusion. Sub-

![Figure 1](https://example.com/figure1.png)

**Figure 1** Effects of cytotoxic monoclonal antibody on chick embryo myogenic cultures. (a) Live, differentiated myogenic culture (96 h after plating) viewed during treatment with cytotoxic antibody (2 µg/ml) and guinea pig complement (5% vol/vol). Note the lysis of mononucleated cells. (b) Prefusion myoblast culture (24 h after plating) prepared by serial preplating methods. (c) Postfusion myotube culture (5 d after plating), prepared by serial preplating methods and treatment with cytotoxic antibody 24 h earlier and returned to culture in normal medium. (d) Postfusion myotube culture (10 d after plating) prepared as described above, and after refeedings with fresh medium at 6 and 8 d of age in culture. Bar, 50 µm X 470.
sequent to this time myotubes are unaffected by exposure to antibody and complement as judged by dye exclusion, occurrence of vigorous, spontaneous contractions, healthy rates of amino acid incorporation, and unaltered morphology.

These findings make it apparent that the application of this technique at the appropriate time during myogenesis in vitro would result in the production of highly enriched cultures of postfusion myotubes. This strategy has been employed together with serial preplating techniques (25) to obtain the highest possible level of purity of myogenic cells (see Materials and Methods). This is important because dissociated embryonic muscle includes fibroblasts and unfused myoblasts that make it difficult to study separately myoblast or myotube-derived proteins. Examples of myogenic cultures obtained by applications of these selection procedures are shown in Fig. 1. All of the experiments described in this report were performed with such muscle cultures.

Characterization of a Monoclonal Antifibronectin Antibody

The monoclonal antibody to fibronectin was assayed for specificity of binding by antibody blotting methods (Fig. 2). The antibody stained chicken plasma and cellular fibronectin specifically (Fig. 2a–c). The antibody did not react with any components in horse serum (Fig. 2, track D) or with purified horse fibronectin (Fig. 2, track E), or with bovine serum, purified bovine fibronectin, rabbit serum, or murine serum. In summary, this monoclonal antibody displays a quite useful species selectivity and is capable of detecting both avian plasma fibronectin and cell surface fibronectin.

Expression of Fibronectin During Myogenesis In Vitro

Immunofluorescence localizations of fibronectin during several stages of myogenesis are shown in Fig. 3. Intensely staining tufts of extracellular fibronectin fibrils were present on solitary myoblasts as early as 24 h after plating (Fig. 3A, b). These cells possess a characteristic bipolar shape and a small ruffled membrane, usually at one end of the cell. In addition to these fibrillar tufts that extend beyond each end of the cell onto the substrate, fibronectin was also stained in punctate structures and small fibrils predominantly on the upper surface of the myoblast cell body.

Just before cell fusion, the motile nature of myoblasts is readily apparent as the cells migrate into aligned positions, adhere and aggregate. As myogenic cells begin to exhibit this social activity (especially during the second day in vitro), the deposition of fibronectin becomes more extensive (Fig. 3c and d). Fibronectin fibrils primarily under and between cells extend substantially beyond the cellular outlines in arrays approximately parallel to the major axis of the cell. Often extensive extracellular fibrillar meshworks and the "footprints" of solitary cells remain on the substratum after the cells have retracted or moved away, attesting to the high degree of motility exhibited by the prefusion myoblast and relatively static nature of the fibronectin deposits.

A major alteration in the distribution of fibronectin becomes visible between 2 and 3 d in vitro. The clumps and aggregates of cells continue to be associated with fibrillar staining on the substratum, but careful focussing showed that fibronectin is present in discrete punctate blocks of stain higher up on the cell surface (Fig. 3e and f). These focal accumulations of fibronectin are present at the abutting surfaces of closely apposed cells within the aggregates and on the free surfaces of the cells as well. The appearance of this staining pattern coincides with a period of intense fusion of myoblasts.

Newly formed myotubes display very little fibronectin on their cell surfaces, although the amount of fibronectin in the culture dish continues to accumulate during this period, as measured by radioimmunoassay (data not shown). The majority (>90%) of the myotubes during this stage of myogenesis are devoid of cell-surface-associated fibronectin and the areas underneath the cells appear as dark shadows in the midst of the uniform mat of fibronectin which continues to amass on the substratum. A smaller percentage of the myotubes (5–10%) retain some fibronectin both uniformly distributed on their cell surfaces and in the form of short fibrils or stitches located predominately on the under surfaces of the cells. Treatment of 4-d old cultures with hybridoma antibody #6, together with complement to kill the remaining mononucleated cells did not result in any changes in the patterns of immunofluorescence observed on myotubes.

In these pure myotube cultures two distinct populations of myotubes were observed. By 5–6 d in culture ~50% of the myotubes had some form of well-defined fibronectin organization associated with their cell surfaces. This percentage increased to 75% after 10 d in culture (the oldest cultures observed having ~95% of the viable nuclei in myotubes). A second population of mature myotubes was totally devoid of detectable fibronectin staining at 5 and 10 d in culture (50% and 25% of the cells respectively). Myotubes that were scored positive for...
fibronectin showed several staining patterns (Fig. 4). The cell margins of myotubes were often associated with borders of fibronectin staining, more intense than either adjacent areas of the collagenous substratum or the tops or bottoms of the cells. The majority of the myotubes regained fibronectin fibrils that remain closely associated with the cell surface. These fibrils extended from the cell margins and the upper or lower cell surface to points of substratum contact away from the myotubes. These fibrils were invariably associated with the ends of myotube branches and extended from the pseudopods of well spread myotubes (Fig. 4).

The accumulation of fibronectin on the substratum continued to increase steadily with time in culture as judged by the fluorescence intensity. The deposition of fibronectin beneath the cells appeared to be variable and in general lagged behind the accumulation of cell derived fibronectin on cell free areas.
FIGURE 4 Localization of fibronectin on 5-d old differentiated myotubes. 5-d old myotubes were fixed and immunolabeled for fibronectin followed by fluorescein-conjugated goat anti mouse antibody. (a-f) Phase and fluorescent photomicrographs of myotube associated fibronectin. Note the appreciable build-up of cellular fibronectin on the collagenous substrate. Dark shadows under myotubes were not due to immunoglobulin in accessibility since permeabilization revealed no further substrate staining, suggesting the cells remove fibronectin from substrate or accumulate less there. Bar, 20 μm x 500.

of the substratum. Permeabilization of the cells did not reveal any additional fibronectin accumulations at the level of the cell-substratum interface (described below, Fig. 9), indicating that the variable staining under cells was not due to incomplete penetration of immunoglobulin into areas beneath the cells. The staining patterns on the underside of the myotubes ranged from dark shadows under younger myotubes (Fig. 4) to streaks, short fibrils of fibronectin and uniform mats of fibronectin beneath 10-d old myotubes (Fig. 5). Occasionally extensive extracellular fibrillar meshworks were present under cells. Fibronectin clusters of the type observed on rat myotube surfaces (10) were never observed.

Biosynthesis of Fibronectin During Myogenesis

Estimates of the rate of fibronectin synthesis relative to total protein synthesis of myoblasts and myotubes were obtained by labeling for 30 min with [35S]methionine, lysing the cells with 2% deoxycholate, then immunoprecipitating with monoclonal antifibronectin, electrophoresing in SDS gels and counting.
radioactivity in the fibronectin band. Table II shows that the synthesis of fibronectin represents ~0.4% of the total protein synthesis in myoblasts after 24 h in culture and similarly, ~0.35% of the total protein synthesized in 5-d old post-fusion myotubes. Synthesis of fibronectin remains detectable in 10-d old cultures of fibroblast-free myotubes at a rate close to that observed for younger cells.

The relative rate of fibronectin synthesis by myogenic cells is four- to five-fold lower than that measured in parallel cultures of subconfluent, tertiary chick embryo fibroblasts at equal cell density. Similar results were obtained when [3H]-leucine was used in the pulse labeling. Thus it was important for our study to minimize the contamination of the cultures by fibroblasts. Calculations of the fibroblast contribution to fibronectin synthesis in our myogenic cultures (even assuming that all the mononucleated cells were fibroblasts synthesizing fibronectin as 3% of their total [35S]methionine incorporation into protein) indicate that fibronectin synthesis represents >0.25% of the total synthetic activity of the myogenic cells at all stages of development.

**Fibronectin Export, Secretion, and Incorporation into the Extracellular Matrix of Myotubes**

Fig. 6 shows SDS polyacrylamide gel analysis of the proteins synthesized and secreted by 5- to 6-d old pure myotube cultures. After [35S]methionine labeling, the most prominent electrophoretic bands had apparent molecular weights of approximately 230,000, 180,000, 140,000 and 45,000 daltons (lane 1). The 230,000-dalton polypeptide was specifically isolated by indirect immunoprecipitation with the monoclonal antifibronectin antibody (lane 3), indicating that fibronectin is one of the major proteins secreted by chicken myotubes.Two collagenous proteins of apparent molecular weight 180,000 and 160,000 daltons were also present in the profile of myotube secreted proteins. These two showed enhanced labeling with glycine and proline and were easily digested by purified bacterial collagenase (lanes 2, 5, and 6).

The rate of appearance of fibronectin in the medium of myotube cultures was measured directly by quantitative immunoprecipitation of aliquots of culture medium taken from cells labeled for various times with [35S]methionine and analysis of precipitated fibronectin on SDS polyacrylamide gels. Fibronectin accumulation in the medium was linear after a lag of between 2–3 h (Fig. 7a and b) that presumably reflects the intracellular transit time before secretion. Similar results were obtained by observing the appearance of fibronectin and other secreted proteins in the culture medium directly (Fig. 7c). Quantitation of [3H]glycine-/[3H]proline-labeled proteins secreted into the medium showed that fibronectin and the two major collagenous species were accumulated linearly after a lag of 2–3 h. The other major secreted proteins show very
similar kinetics of secretion. This time period is markedly different from that observed with parallel cultures of subconfluent tertiary fibroblast cultures (Fig. 7 b). The intracellular transit time for fibronectin in fibroblasts was ~30 min after which the appearance of fibronectin in the medium was linear with time.

An analysis of the rate of appearance of fibronectin on the cell surface or substratum of muscle cultures also confirmed the duration of the 2-3 h intracellular transit time for newly synthesized, labeled fibronectin (Fig. 8). The amount of trypsin-sensitive fibronectin in live muscle cultures represents the quantity of fibronectin on the surface and accessible to trypsin, while the intracellular pool of fibronectin is trypsin-resistant. As shown in Fig. 8, labeled fibronectin does not acquire detectable trypsin sensitivity until the muscle cells have been in labeled amino acid for ~2-3 h which is in close agreement with the delayed appearance of labeled fibronectin in the medium of myotube cultures. After the 2-3 h lag, labeled fibronectin accumulated linearly for 6 h in the cultures not treated with trypsin. The observation that the rate of fibronectin accumulation remains approximately constant throughout the course of the experiment for the control cultures (Fig. 8) indicates that only a small proportion of the exteriorized fibronectin goes rapidly into solution. The majority of fibronectin molecules appear to become associated with the collagenous substrate or the fibrillar matrix surrounding the cells.

The time course of appearance of newly synthesized fibronectin in the external compartments (surface and medium) suggests that precursor fibronectin molecules reside at intracellular locations, presumably in structures involved in processing and secretion of glycoproteins. To confirm the existence of such intracellular pools of fibronectin and to examine their locations within the cell, muscle cultures were briefly fixed with paraformaldehyde and then treated with 10 μg/ml of antifibronectin monoclonal antibody that was directly conjugated to the fluorescent dye tetramethylrhodamine. This treatment was effective in saturating the antigenic sites of the extracellular fibronectin in the myotube cultures, since specific binding of 125I-labeled antifibronectin antibody applied subsequently was inhibited by 99% (data not shown). The unbound rhodamine conjugate was removed, cultures fixed again with paraformaldehyde, extracted with acetone, and then incubated with directly labeled fluorescein antifibronectin. The rhodamine-labeled antifibronectin was localized with fibronectin present in a uniform mat on acellular areas of the substrate and to lesser and variable degrees beneath the myotubes (Fig. 9a'). In addition, fibronectin fibrils were seen extending from the cell margins, and the upper and lower surfaces of the myotubes to points away from the myotube cell surface. Essentially identical results were obtained with indirect immunofluorescence techniques as described above. After permeabilization of cells with acetone and treatment with fluorescein-labeled antifibronectin, the staining was localized to granular, perinuclear structures (Fig. 9a''). This staining pattern was not in the substrate focal plane or the upper cell surface, but was almost totally in the focal plane which included the nucleus. The fluorescent staining extended into a network of vesicular structures but did not usually extend into cell processes. In some cases the fibronectin in permeabilized cells was located close to the plasma mem-

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**TABLE II**

| Cell type         | (A) Pulse labelings with [35S]methionine | (B) Comparison of biosynthetic labeling with: |
|-------------------|----------------------------------------|--------------------------------------------|
|                   | (N = 3)                                 | [35S]methionine | [3H]leucine |
| CEF*              | 1.61 ± 0.03§                           | 1.65          | 2.4        |
| 24-h myoblasts*   | 0.39 ± 0.04                            | 0.042         | 0.060      |
| 5-d myotubes      | 0.35 ± 0.05                            | 0.44          | 0.55       |
| 10-d myotubes     | 0.32§                                  |               |            |

* CEF and 24-h myoblasts were of equal cell density of ~5 x 10⁴ cells/35-mm dish at the time of labeling. 
§ Indicates the standard error of the mean for each determination.

Tertiary chick embryo fibroblasts (CEF) or primary myogenic cells at different stages of development were washed three times with Hank's Balanced Salt Solution (37°C) and were pulse-labeled with 100 μCi/ml [35S]methionine or [3H]leucine for 30 min at 37°C. The cells were washed three times with PBS, collected by scraping the dishes into 0.5 ml of lysis buffer containing 2% deoxycholate, 20 mM Tris, pH 8.0, 2 mM PMSF, 2 mM EDTA and 5 mM N-ethylmaleimide. Lysates were centrifuged at 10,000 g for 15 min to remove insoluble residue. These pellets contained no detectable pulse labeled fibronectin as judged by electrophoresis and autoradiography of gels. Aliquots of cell lysates were used to determine incorporation into protein by precipitation with 10% TCA, or by immunoprecipitation and SDS PAGE analysis as described in Materials and Methods. The results of a typical [35S]methionine labeling were: immunoprecipitated fibronectin radioactivity was 2,959 cpm, CEF; 745 cpm, 24-h myoblasts; 1,808 cpm, 5-d myotubes; 1,877 cpm, 10-d myotubes; 37 cpm, 5-d myotubes with nonspecific immunoprecipitation by MOPC 21 immunoglobulin substituted for antifibronectin as a nonspecific antibody. The total acid-insoluble cpm were 182,660 cpm, CEF; 177,440 cpm, 24-h myoblasts; 515,762 cpm, 5-d myotubes; 430,312 cpm, 10-d old myotubes.

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**FIGURE 6** SDS PAGE of the proteins synthesized and secreted by differentiated myotubes. 5-d old myotube cultures were labeled for 24 h with [35S]methionine or [3H]proline/[3H]glycine and the conditioned medium harvested and centrifuged to remove cellular debris. Aliquots were either analyzed directly, used for immunoprecipitation or subjected to collagenase digestion. (Tracks 1-4) [35S]methionine-labeled secreted proteins: track 7, control, analyzed directly; track 2, collagenase-digested; track 3, double antibody immunoprecipitation of fibronectin with monoclonal antifibronectin; track 4, double antibody immunoprecipitation with MOPC 21 immunoglobulin substituted for antifibronectin as a nonspecific precipitation control. (Tracks 5-8) [3H]glycine/[3H]proline-labeled secreted proteins: track 5, control; track 6, collagenase-digested; track 7, fibronectin immunoprecipitation; track 8, nonspecific immunoprecipitation control. This fluorograph of a 5-15 gradient SDS polyacrylamide gel was exposed to X-ray film for 48 h. Molecular weight markers included fibronectin (F) (230 kdaotton), β galactosidase (116 kdaotton), serum albumin (68 kdaotton) and ovalbumin (43 kdaotton). The apparent molecular weights of the collagenous peptides (C) were 160 and 170 kdaotton, respectively.
Figure 7  Kinetics of appearance of fibronectin and other secreted proteins in the medium of cultured chick myotubes. (a) Fluorogram of SDS-polyacrylamide gradient gel (5-15%) which represents the analysis of fibronectin immunoprecipitations from aliquots of culture medium of myotubes labeled for various times with [35S]methionine. Increasing length of time in labeled amino acid measured in minutes is from left to right as depicted above the fluorogram. (b) Scanning densitometer quantitation of the radioactivity represented in fluorogram (a) of myotube fibronectin secretion kinetics is represented as filled circles (O). Notice the approximate 2-h lag before the assumption of linear fibronectin accumulation in the culture medium. The results of the analysis of a similar experiment performed with tertiary chick embryo fibroblasts is also shown (O). The input TCA-insoluble counts in the immunoprecipitations reactions were adjusted so that the identical normalization factor was used for both myotube and fibroblast peak areas. Note the appearance of fibroblast fibronectin in the medium begins after a much shorter lag period—30 min. (c) Fluorogram of SDS gel of [3H]glycine/[3H]proline-labeled proteins appearing in the medium of myotube cultures. Incubation times (min) in labeled amino acids are indicated across the top of the gel. Again a 2-h lag period was observed before the appearance of labeled protein in the medium. Densitometer scans indicated that the collagenous peptides (see Fig. 6) and fibronectin were accumulated with identical kinetics. Molecular weight markers were identical to those described in Fig. 6.

brane and groups of nuclei (usually in narrow myotubes) in granular structures (Fig. 9b'). These staining patterns were not altered by pretreatment of myotube cultures with trypsin (5 µg/ml, 5 min, room temperature), which removed extracellular fibronectin as detected with rhodamine antifibronectin (Fig. 9b' and c'). Under these experimental conditions (10 µg/ml of directly labeled antifibronectin applied to acetone-extracted myotubes for 2 h at room temperature) it was possible to detect intracellular staining in ~30-40% of the myotubes. Pretreatment of the living cells for 4 h with cyclohexamide (5 µg/ml) or puromycin (10 µg/ml) at 37°C completely abolished this intracellular staining (Fig. 9c'). In addition, no staining was observed with fluorescein-labeled antifibronectin in cultures that had been pretreated with the rhodamine-labeled conjugate unless the cultures were acetone extracted before application of fluorescein labeled antibody. Substitution of fluorescein-labeled MOPC-21 IgG or normal mouse immunoglobulin for the directly labeled antifibronectin resulted in the complete absence of stain. These results confirm the existence of an intracellular population of fibronectin molecules whose form, structure, location, and disappearance in the presence of protein synthesis inhibitors are consistent with its role as precursor to cell surface and secreted fibronectin. In addition, these observations stand as further direct evidence of the synthesis of fibronectin by differentiated muscle cells.

Discussion

The major findings of this study are: (a) the synthesis of fibronectin occurs throughout myogenesis in vitro, at similar rates before and after myoblast fusion; (b) the rate of fibronectin biosynthesis is approximately four- to five-fold lower in muscle cells than in fibroblasts; (c) the kinetics of biosynthetic processing and intracellular transport of fibronectin in differentiated myotubes are distinctly slower than those in fibroblasts; (d) alterations in the deposition of fibronectin by muscle

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FIGURE 8 Kinetics of labeling of intracellular and total bound fibronectin in myotube cultures. Myotube cultures were labeled with [35S]methionine for various times. Cells were removed from incubation at intervals, trypsin-treated (t) or not (c), washed with protease inhibitors, lysed, and immunoprecipitated with monoclonal antibody. (a) Fluorograph and SDS gel to analyze the precipitations. (t or c) represents trypsin-treated cells or control cells and time in labeled amino acid is in hours from left to right. (b) Densitometer analysis of fluorograph in a. • represents accumulation of both intracellular and extracellular bound fibronectin in control cultures; O represents labeling of intracellular fibronectin in trypsin-treated cells. Notice the trypsin-sensitive (i.e. extracellular) fibronectin does not become detectable until between 2-3 h of labeling.

FIGURE 9 Localization of the intracellular pool of fibronectin in myotubes. (a, b, and c) Phase images of differentiated myotubes which were treated in various ways to reveal the presumptive intracellular pool of newly synthesized fibronectin. (a', b', and c') nonpermeabilized cells were incubated with directly conjugated, rhodamine-antifibronectin monoclonal antibody after trypsin treatment (b' or c') or not (a') and formaldehyde fixation. Note the localization of rhodamine-antifibronectin to extracellular sites in myotube free areas, under cells and in cell-associated fibrils as described earlier and the absence of staining in the trypsin-treated cultures b' and c'. (a'', b'', and c'') were the same cultures incubated with directly conjugated fluorescein-antifibronectin antibody after rhodamine antibody staining, repeated formaldehyde fixation and acetone permeabilization. Note the revelation of new fibronectin containing structures in a'' and b'', which were in the same plane of focus as the nuclei, and in the form of perinuclear vesicular structures which were not available for trypsin digestion but were affected by incubation of the cells in cyclohexamide for 4 h (c'') before initial fixation. Bar, 20 μm X 500.
cells occur during differentiation and may be correlated with cellular behavior.

**Fibronectin Biosynthesis in Myogenic Cultures**

In situ, muscle fibers are encircled by a continuous basal lamina which consists of an electron-lucent zone close to the plasma membrane and the lamina densa, 10-15-nm thick, located farther away from the cell membrane. Recently, immunoelectronmicroscopic investigations of muscle basal lamina have revealed that fibronectin, laminin, and type IV collagen are present in both zones and in the synaptic as well as the extra-synaptic regions of muscle basal lamina (26). These molecules are likely to play important roles in the formation and maintenance of muscle structure although their functions in muscle are unknown at present. Also of importance in understanding the function of these molecules in muscle tissue is the identification of their cellular sources and the time of their expression during development.

Prior investigations of the muscle cell surface have yielded contrasting observations on the deployment of fibronectin by muscle cells during myogenesis in vitro. Surface labeling procedures have detected increases in fibronectin levels when mononucleated myoblasts form myotubes in culture (7-9). However, morphological studies of myogenesis suggested that cultured myoblasts possess fibronectin matrices that subsequently disappear upon myotube formation (3, 10, 11). Interpretation of these results has been difficult since it was not possible to identify precisely the source or time of appearance of fibronectin in these studies.

In the present work a protocol was developed that employed conventional prelabeling techniques followed by application of a cytotoxic monoclonal antibody to obtain cultures that were not contaminated with fibroblasts or unfused myoblasts to any significant extent. These cultures provided for a clean division between the stages of culture development: a proliferative stage composed almost entirely of mononucleated cells and a period after fusion in which the cell population was composed almost entirely of multinuclear muscle fibers. Immunofluorescence studies of fibronectin appearance in these avian muscle cell cultures, showed that both myoblasts and multinuclear muscle fibers were capable of fibronectin production. Metabolic labeling and identification of the fibronectin polypeptide subunits by immunoprecipitation at various stages during myogenic differentiation, clearly demonstrated that muscle cells were capable of fibronectin synthesis at significant rates relative to total protein synthesis and at a constant rate before and after fusion. Direct visualization of intracellular precursor pools of fibronectin furnished further evidence of fibronectin production by myogenic cells.

The patterns of fibronectin synthesis observed in this study indicate that synthesis of fibronectin is not regulated coordinately with the synthesis of contractile proteins during myogenesis. The time of first appearance and rates of synthesis of contractile proteins are coordinated with muscle differentiation (27). The observation that fibronectin synthesis remains constant relative to total protein synthesis before and after fusion suggests that the absolute rate of fibronectin synthesis may increase about twofold following fusion, in concert with a twofold increase in overall rate of protein synthesis in postfusion myotubes (27).

The time course of intracellular transit of newly synthesized fibronectin in differentiated myotubes is in agreement with the previous determinations of the time course of intracellular transport and externalization of an integral membrane protein, the acetylcholine receptor (23), and acetylcholinesterase in muscle. The latter protein is both secreted into the medium and resides on the cell surface of muscle cells (28). These results suggest, but do not prove, that membrane and secretory glycoproteins share a common intracellular pathway in muscle cells. Our results clearly show that this pathway differs kinetically from the maturation pathway for glycoproteins in culture is transported over a 2-3-h period to the cell surface where it is either released into the medium or incorporated into the myotube extracellular matrix. Fibroblastic production of fibronectin, on the other hand, is characterized by a transit period of ~30 min (29, 30, and this study). It remains to be seen whether the pronounced delay in the externalization of muscle cell glycoproteins is related to a slow time course of posttranslational modifications. The maturation of fibronectin's asparagine-linked oligosaccharides occurs rapidly in fibroblasts (30, 31).

**Fibronectin Distribution during Myogenesis In Vitro**

The typical bipolar, spindle-shaped myoblast that invariably gives rise to muscle colonies as demonstrated by clonal analysis (32, 33), exhibits extracellular fibronectin soon after plating as fibrillar tufts at the tips of cells, which extend beyond the cell processes onto the substratum. Within the cytoplasm of the distal extremities of these cells there is an abundance of 5-nm filaments that appear to merge with the plasma membrane (33, 34). These filaments, termed cortical filaments, bind heavy meromyosin, are aligned parallel to the longitudinal axis of the myoblast, and are presumably composed of actin. Given the known ultrastructural localization of these microfilaments and the notable presence of fibronectin at the ends of myoblasts seen in the present work, it is reasonable to propose that an adhesion plaque or a fibronexus (35) exists at the tips of the bipolar myoblast, which may be characterized by a transmembrane relationship between fibronectin and actin. Several studies have presented data suggesting such associations between fibronectin and actin in fibroblasts (35-37). Double label immunofluorescent and immunoelectron microscopic studies should confirm whether or not such associations and structures exist in mononucleated myogenic cells.

The prefusion alignment of myoblasts into linear aggregates is accompanied by the appearance of a more extensive extracellular fibrillar matrix of fibronectin. These structures remain approximately parallel to the linear arrays of myoblasts and are often seen on the substratum, unattended by cells. There is an accumulation of focal patches containing fibronectin on clumps and aggregates of myoblasts about to begin or in the process of fusion. It is possible that the fibronectin foci observed on the myoblast cell surface in this study are related to the clearance of cell surface fibronectin which results in the attainment of a permissive state for fusion (see reference 38). This process would result in the virtual absence of fibronectin from the surfaces of newly formed myotubes, as we have observed. The loss of fibronectin from the myotube cell surface is probably not the result of a change in the rate of synthesis of fibronectin since the synthetic rates are similar before and after fusion and the amount of celluarily derived fibronectin present in the culture dish continues to increase during the period before and after fusion. It is likely that the fusing myoblasts
and young postfusion myotubes are producing fibronectin that can attach to the collagenous substrate but is not retained by the cells. Our data indicate that as postfusion myotubes mature in culture, there is an increasing return of fibrillar fibronectin organization in the regions of cell to substratum contact. Perhaps this phenomenon is related to a return of a cell surface receptor for fibronectin or the appearance of a new molecule that can coordinate or stabilize the binding of fibronectin to the muscle cell surface.

Evidence is mounting that components of extracellular matrices and basement membranes can be synthesized by non-connec-tive tissue cells and are thus not solely the products of fibroblasts, chondrocytes, or osteocytes. In addition to fibronectin, myogenic cell lines have been shown to synthesize several forms of collagen (39, 40). Recently it has been shown that several basement membrane glycoproteins are produced by neuroblastoma cells (41) and by a Schwann cell line (42). Our results show that normal myoblasts and differentiating muscle cells synthesize fibronectin and a variety of collagenous polypeptides. The purified primary muscle cell cultures developed for this study provide an in vitro system for the study of the synthesis and assembly of other muscle extracellular matrix and basal lamina components.

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