Fibroblasts Promote the Formation of a Continuous Basal Lamina during Myogenesis In Vitro

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Abstract. Analyses were made of the requirements for the formation of a continuous basal lamina during myogenesis of quail muscle in vitro. A culture system was developed in which mass cultures of differentiating muscle cells were embedded in a native gel of rat tail collagen. Fibroblastic cells, which were also present in the cultures, migrated into the gel and within a few days surrounded the newly formed myotubes. In this environment, a continuous basal lamina was formed at the surface of the myotubes as demonstrated by immunofluorescent staining with monoclonal antibodies against type IV collagen, laminin, and heparan sulfate, as well as by electron microscopic immunolocalization. To distinguish between the role of the fibroblasts and the collagen gel in promoting basal lamina formation, clones of quail muscle cells lacking fibroblasts were subsequently embedded in a native rat tail collagen gel. Under these conditions, only very limited fluorescent staining for basement membrane components was observed associated with the myotubes. However, the introduction of chick muscle or skin fibroblasts into the clonal cultures just before gel formation resulted in the formation of an extensive basal lamina on the surface of the myotubes. Conditioned medium from fibroblast cultures by itself was not effective in promoting basal lamina formation. These results clearly show that during myogenesis in vitro fibroblasts must be in close proximity to the myotubes for a continuous basal lamina to form. These results probably relate closely to the interactions that must occur during myogenesis in vivo between the muscle cells and the surrounding connective tissue including the developing tendons.

In skeletal muscle a continuous basal lamina surrounds each muscle fiber and acts as an attachment site for the collagen fibers of the endomysium (3) and tendon (22, 32). In previous studies, the muscle basal lamina was shown to contain type IV collagen, laminin, and heparan sulfate (1, 2, 29). However, the macromolecular organization of these components to form the lamina densa and lamina rara still remains poorly understood. In developing muscle, a basal lamina is formed only at the myotube surfaces that are in contact with surrounding interstitial connective tissue (7, 10, 14, 15, 26, 28), suggesting that an interaction with connective tissue may be required for basal lamina formation (23). Cell culture studies also indicate that an interaction between myogenic and fibroblastic cells may be required for successful basal lamina formation at the myotube surface (17, 18, 20). In the present series of experiments, our initial objective was to devise a culture system in which a continuous basal lamina was formed at the myotube surface during myogenesis in vitro. To achieve this, we found it was necessary to embed the myotubes within a native gel of rat tail type I collagen and also, more important, to have fibroblastic cells present within the gel.

Materials and Methods

Cell Cultures

Embryonic quail muscle was used to prepare myogenic clones since previous studies have shown that quail myogenic cells, but not fibroblastic cells, will grow successfully in clonal conditions (16). Quail eggs were obtained from GQF Manufacturing Co. (Savannah, GA), and myogenic clones were established from 9–10-d-old quail embryos (21). Breast muscle was removed, cleaned, minced, and mechanically dissociated by vortexing in complete medium (4). The cell suspension was filtered to remove undissociated clumps and plated at clonal density (8×10^3 cells/dish) in 100-mm Optilux tissue culture dishes (Falcon Plastics, Cockeysville, MD), which had been coated with type I collagen extracted from rat tail tendons. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2, 95% air. Growth medium consisted of Eagle's minimum essential medium, (Gibco, Grand Island, NY) supplemented with 15% horse serum (Gibco), 10% embryo extract, 1% antibiotic antimycotic solution, and 0.2 mM L-glutamine. Clones that developed under these conditions were predominantly myogenic, although an occasional fibroblastic clone was observed (<2% of total).

For mass cultures, cell suspensions were obtained (as described above) and plated on 35-mm collagen coated dishes at a density of 5×10^5 cells/dish. Growth medium (as above, except with 5% embryo extract) was replaced daily.

For fibroblast cultures, breast muscle from 11-d chick embryos was removed and mechanically dissociated in fresh balanced salt solution. After filtration, the cell suspension was diluted with fresh balanced salt solution and plated at 5×10^5 cells/ml on 100-mm tissue culture dishes for 30 min. Unattached cells

were removed and fresh growth medium was added. These cultures were enriched for fibroblasts that adhere to the culture dish more rapidly than myoblasts (33). After several days of growth, confluent cultures were passaged by trypsinization (0.25%) for 15 min in balanced salt solution. Generally, myotubes were not detected after the second passage.

Cell cultures were examined using a phase inverted microscope (Nikon model MS) and photographed using Kodak technical pan film 2415.

Preparation of Collagen Gels

Collagen used in the preparation of native gels was extracted from adult rat tail tendons (31). Tendons were removed, cleaned and placed in cold 0.5 M acetic acid (HAc) for 48 h at 4°C. The preparation was centrifuged at 5,000 *g* for 45 min at 4°C. Collagen was precipitated from the supernatant by the addition of an equal volume of 20% NaCl followed by centrifugation at 5,000 *g* for 45 min at 4°C. The precipitate was dissolved in cold 0.5 M HAc and dialyzed extensively against cold, sterile distilled H_2O and stored at 4°C. Stock solutions were prepared by dilution with sterile H_2O to a final collagen concentration of 2 mg/ml. Native collagen gels were prepared by rapid mixing (at 4°C) of solutions in the following proportions: 0.9 ml 10× concentrated minimal essential medium (Gibco), 0.2 ml sodium bicarbonate (7.5%), 9 ml collagen stock solution (31). Medium was removed from 35-mm dishes containing actively growing 3-d-old muscle cultures, and 2 ml of the above neutralized collagen solution was quickly pipetted into the dish. The collagen was allowed to gel at 37°C and after 30 min, 1 ml growth medium was added to each dish. The medium was replaced daily.

For experiments in which chick fibroblasts were added to established quail clonal cultures, healthy clones containing extensive myotube formation were first sequestered by encircling them with 35-mm plastic rings. The rings were prepared by removing the bottom of 35-mm culture dishes and secured to the 100-mm dish with silicone grease. Fibroblasts were harvested from second passage cultures using 0.25% trypsin in balanced salt solution. After filtration, fibroblasts were diluted in growth medium (1×10^5 cells/ml) and added to myogenic clones. 6 h after the addition of fibroblasts a collagen gel was formed above the cells as described above.

For cultures grown on top of collagen gels, 1.5 ml of the neutralized collagen solution was first poured into a 35-mm dish and allowed to gel at 37°C. Muscle cells were then isolated as described above and grown as a mass culture (5×10^5 cells/dish) on the upper surface of the gel.

Preparation of Hybridoma and Production of Monoclonal Antibodies

The preparation and characterization of monoclonal antibody IA8 against type IV collagen was previously described in detail (11, 24). In brief, female SJL/J mice (Jackson Laboratories, Bar Harbor, ME) were immunized with type IV collagen isolated from chicken gizzards, and fusion of splenocytes was performed with NS-1 myeloma cells (19), from which hybridomas of the desired characteristics were subsequently selected.

Purification of Monoclonal Antibodies

After injection of hybridoma cells into the peritoneal cavity of athymic nude mice (nu/nu), ascites fluid was harvested, and immunoglobulin was purified as previously described (24). In brief, immunoglobulins were precipitated from ascites supernatant with ammonium sulfate (50% of saturation, 4°C) twice. Further purification was performed by affinity chromatography using a Sepharose-4B column to which unfractionated type IV collagen had been coupled. Localization of the epitope to the center of the $(F1)_2F2$ fragment of chick type IV collagen was previously demonstrated by rotary shadowing of a mixture of type IV collagen and antibody IA8 (24).

Monoclonal antibodies to laminin and heparan sulfate proteoglycan were a gift from Dr. Douglas M. Fambrough (Carnegie Institution of Washington, Baltimore, MD). The preparation and characterization of these antibodies was described previously (2).

All monoclonal antibodies used in this study were produced from immunogens isolated from chick tissues and showed extensive cross-reactivity with quail muscle by immunofluorescent staining.

Immunofluorescence Observations

Cell cultures were washed with 0.01 M phosphate-buffered saline (PBS), pH 7.2, and incubated for 30 min with antibody. After they were washed with PBS, culture dishes were incubated for 30 min with fluorescein-conjugated goat anti-mouse IgG diluted 1:40 (Cappel Laboratories, Cochranville, PA), washed in PBS, and mounted in 90% glycerol.

Cultures containing collagen gels were first washed extensively with PBS, and then each gel was loosened from the side of the dish with a scalpel blade. Cells were freed from the surface of the dish by gentle pipetting of PBS around the loosened edges of the gel. By carefully removing the gel from the dish, the myotubes remained associated with the gel. For cell surface immunofluorescent staining of unsectioned culture preparations, small squares (2 mm) of the gel were cut and incubated separately with primary and secondary antibody as described above. The squares were placed on glass slides and mounted in 90% glycerol. For frozen sections, pieces of the gel were embedded in Tissue-Tek II O.C.T. compound (Lab-Tek Products, Naperville, IL) and frozen with dry ice. Transverse sections (4 μm) were prepared in a cryostat (HR, Slee Ltd., London) at −18°C and transferred to glass slides. Fluorescence histochemistry was performed on the sections as described above for cell cultures.

All fluorescence examinations were with a Leitz Ortholux II microscope equipped with an epiilluminator and Ploemopak filter block I2. Photographs were taken with Kodak Tri-X film ASA 1,600 and processed with Diafine developer.

Electron Microscopic Observations

Segments of gels were rinsed in 0.1 M sodium cacodylate, pH 7.4, and fixed in paraformaldehyde (2%) and glutaraldehyde (2.5%) in 0.1 M sodium cacodylate, pH 7.4, at room temperature for 15 min. Gels were then transferred to fresh fixative containing 1% tannic acid for 1 h at room temperature. After gels were rinsed with distilled water, they were postfixed in 1% OsO_4 in 0.1 M sodium phosphate buffer, pH 6.2, for 30 min at 4°C. The gels were rinsed in 0.1 M sodium phosphate buffer and cut into strips for flat embedding. The strips were dehydrated in a graded series of alcohols and embedded in Durcupan ACM (Fluka AG, Buchs SG, Switzerland). Thin sections were cut with a diamond knife on an MT-2B Ultramicrotome (Dupont Instruments–Sorvall Biomedical Div., Newtown, CT), stained with aqueous uranyl acetate (1%) and lead citrate (0.2%), and examined at 80 kV on a JEOL-100 Cx electron microscope.

Peroxidase Cytochemistry

Cultures were washed extensively in cold 0.1 M PBS, pH 7.3, and then fixed with 4% paraformaldehyde in 0.1 M PBS, pH 7.3, at 4°C for 2 h. After fixation, gels were removed from culture dishes and washed with three changes of cold 0.1 M PBS, pH 7.3, frozen in Tissue-Tek II O.C.T. compound (Lab-Tek Products), and 100-μm-thick sections were prepared on a sliding cryostat. Aldehydes generated from fixation were blocked by placing sections in 0.5 M ammonium chloride (in 0.01 M PBS, pH 7.3, 30 min at 4°C) and then washing with three changes of 0.1 M PBS, pH 7.3, 4°C. Sections were then incubated with monoclonal antibody IA8 for 1 h at 4°C with gentle agitation, washed extensively in cold PBS, and incubated with peroxidase-conjugated $F(ab')_2$ fragment of sheep anti-mouse IgG (Cappel Laboratories) for 1 h at 4°C. After they were washed in cold PBS, sections were fixed with 2% glutaraldehyde for 1 h and washed overnight in PBS containing 3.5% sucrose. Reaction product was generated with 0.05% diaminobenzidine and 0.01% H_2O_2 by the method of Graham and Karnovsky (12) at pH 6.0 (27), postfixed in 1% OsO_4 for 1.5 h, and dehydrated in alcohol, and the tissue was flat embedded. Ultrathin sections were stained for 1 min in 0.2% lead citrate.

Thick Sections

1-μm-thick sections were cut from the same block that had been embedded and prepared for electron microscopy (see above). Sections were placed on glass slides, stained with toluidine blue (1%), and mounted with a glass coverslip. Photographs were taken using Plus-X pan film.

Results

Electron microscopic observations by Lipton (20) first showed that clones of quail muscle cells grown in cell culture did not assemble a basal lamina on the surface of the myotubes. However, examination of a mass culture that also contained fibroblastic cells showed that a basal lamina was formed on the myotube surface. This observation suggests that muscle fibroblasts may be involved in basal lamina formation during myogenesis. We initially performed similar experiments in which quail muscle clones and mass cultures were examined for basal lamina formation by immunofluorescent staining with monoclonal antibodies to type IV collagen, laminin, and heparan sulfate (Fig. 1). With all three antibodies, patches of fluorescent staining were consistently observed in mass cul-

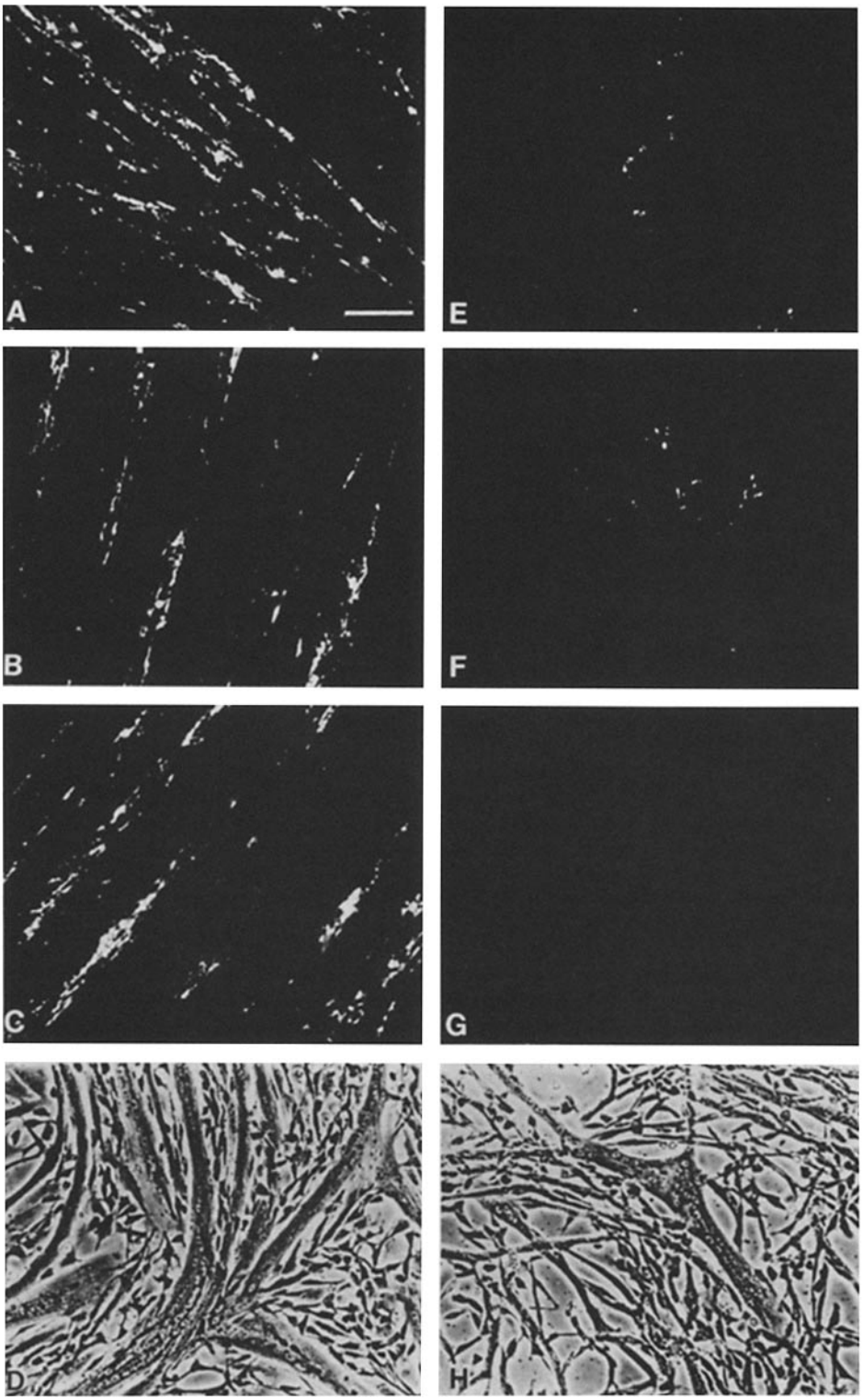

Figure 1. Fluorescence micrographs of 10-d-old quail muscle cultures which show the accumulation of basement membrane components on the surface of myotubes in mass cultures (*A–C*) and myogenic clones (*E–G*). Indirect immunofluorescent staining was performed using monoclonal antibodies to type IV collagen (*A* and *E*), heparan sulfate proteoglycan (*B* and *F*), and laminin (*C* and *G*). For all three basement membrane components, bright patchy staining was present on myotubes growing in the presence of fibroblasts, whereas only very limited staining was noted on myotubes growing in the absence of fibroblasts. Phase-contrast micrographs of a similar mass culture (*D*) and myogenic clone (*H*). Bar: 46.5 μm for *A–C* and *E–G*, respectively; 33.3 μm for *D* and *H*.

tures on the surface of the myotubes (Fig. 1, *A–C*). However, only an occasional patch of fluorescent staining was observed for muscle clones (Fig. 1, *E–G*). These results therefore confirm and extend the earlier results of Lipton (20) and suggest that fibroblasts may play a role in the assembly of the basal lamina on the myotube surface, as proposed previously (17, 18). However, the fluorescent staining pattern of the mass cultures suggested that a continuous basal lamina was not formed under these conditions, and subsequent electron microscopic observations of the cultures clearly showed that only patches of basal lamina were present on the surface of the myotubes (data not presented).

To further the analyses, a culture system was devised in which a continuous basal lamina was formed on the myotube surface. A mass culture of quail muscle cells, which included fibroblast-like cells, was grown for 3 d, until an extensive network of myotubes was present. The medium was then removed and a gel of native rat tail collagen was set above

the cells. After 1–2 h, fresh medium was added above the gel, and the culture was maintained for several days more. Fig. 2 shows a section of the culture after the gel was present above the cells for 7 d. During this time, extensive migration of fibroblastic-appearing cells into the gel occurred. Fluorescent staining of the cultures with a monoclonal antibody to type IV collagen was performed either by first freezing the gel and then preparing frozen cross-sections (Fig. 3, *A* and *B*) or by directly adding the antibody and secondary antibody to small segments of the gel (Fig. 3*C*). The results showed that a continuous, or almost continuous, basal lamina was formed around the myotubes when embedded within a collagen gel. With increasing time in culture, fluorescent staining of the basal lamina became more intense (compare the culture in Fig. 3*A*, which had been in a gel for 7 d, with that in Fig. 3*B*, which had been in a gel for 14 d). Similar staining patterns were also observed with monoclonal antibodies to laminin and heparan sulfate (data not presented).

Fig. 4*A* is an electron micrograph of a culture embedded within a gel for 7 d and fixed in the presence of tannic acid. An extensive basal lamina was present on the myotube surface. The electron micrograph at higher magnification (Fig. 4*B*) shows that both a lamina rara and a lamina densa were formed with fine fibrils passing across the lamina rara from the cell surface to the lamina densa. The fibroblastic cells were observed to migrate freely through the gel. They always showed an extensive rough endoplasmic reticulum and lacked a basal lamina. Cell fusion and the assembly of myofilaments were not observed with the cells migrating through the gel. Therefore, we regard these cells as authentic fibroblasts. This identification is further supported by the observations described below that show poor penetration of the myoblasts into the collagen gel.

Electron microscopic localization of type IV collagen was performed using a second antibody coupled to horseradish peroxidase. The reaction product was observed only in the developing basal lamina (Fig. 5). This experiment therefore localizes type IV collagen directly to the basal lamina, and thus extends the immunofluorescence observations described above.

From the experiments so far described, it was not possible to determine the potential roles of the collagen gel and the

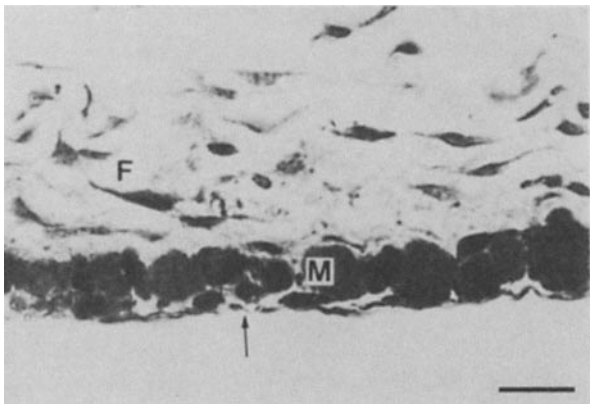

Figure 2. Toluidine blue-stained cross-section (1 μm) of a mass culture fixed and embedded for electron microscopy 7 d after the addition of the collagen gel. Note that fibroblasts (*F*) have penetrated the gel and proliferated, whereas myotubes (*M*) have remained on the surface of the culture dish. Fibroblastic cells also appear between and below the myotubes. Bar, 19.2 μm.

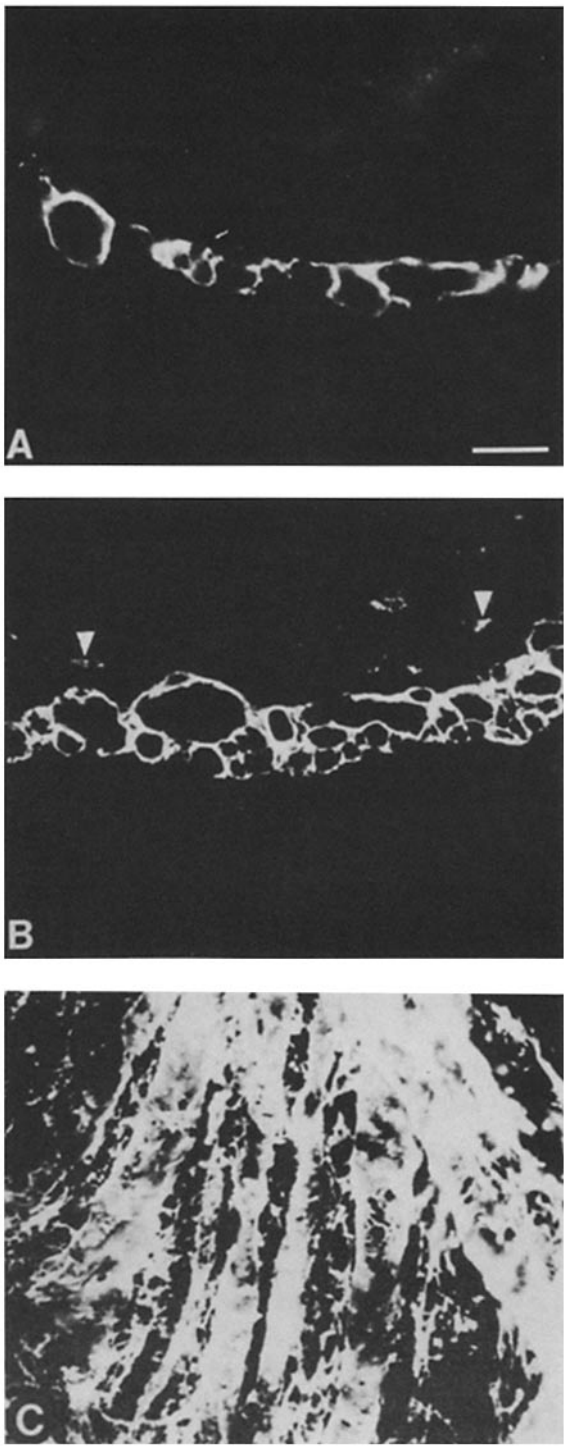

Figure 3. Assembly of a continuous basement membrane on myotube surfaces after the addition of a collagen gel to mass cultures. Fluorescence micrographs obtained using type IV–specific monoclonal antibodies on frozen cross-sections (4 μm) of cultures 7 d (*A*) and 14 d (*B*) after the addition of the gel above the developing muscle. Arrowheads in *B* indicate patches of fluorescent staining among fibroblasts growing within the gel. Fluorescent staining of the surface of the myotubes was also performed on unsectioned preparations (*C*). In this experiment a small area of the same culture as in *A* was cut from the gel before sectioning. For comparison, Fig. 1*A* represents a mass culture of the same age as that in *C* and in this figure was stained using the same type IV antibody, but the culture in Fig. 1*A* was without the addition of a gel. Bar: 24.1 μm for *A*; 38.5 μm for *B* and *C*.

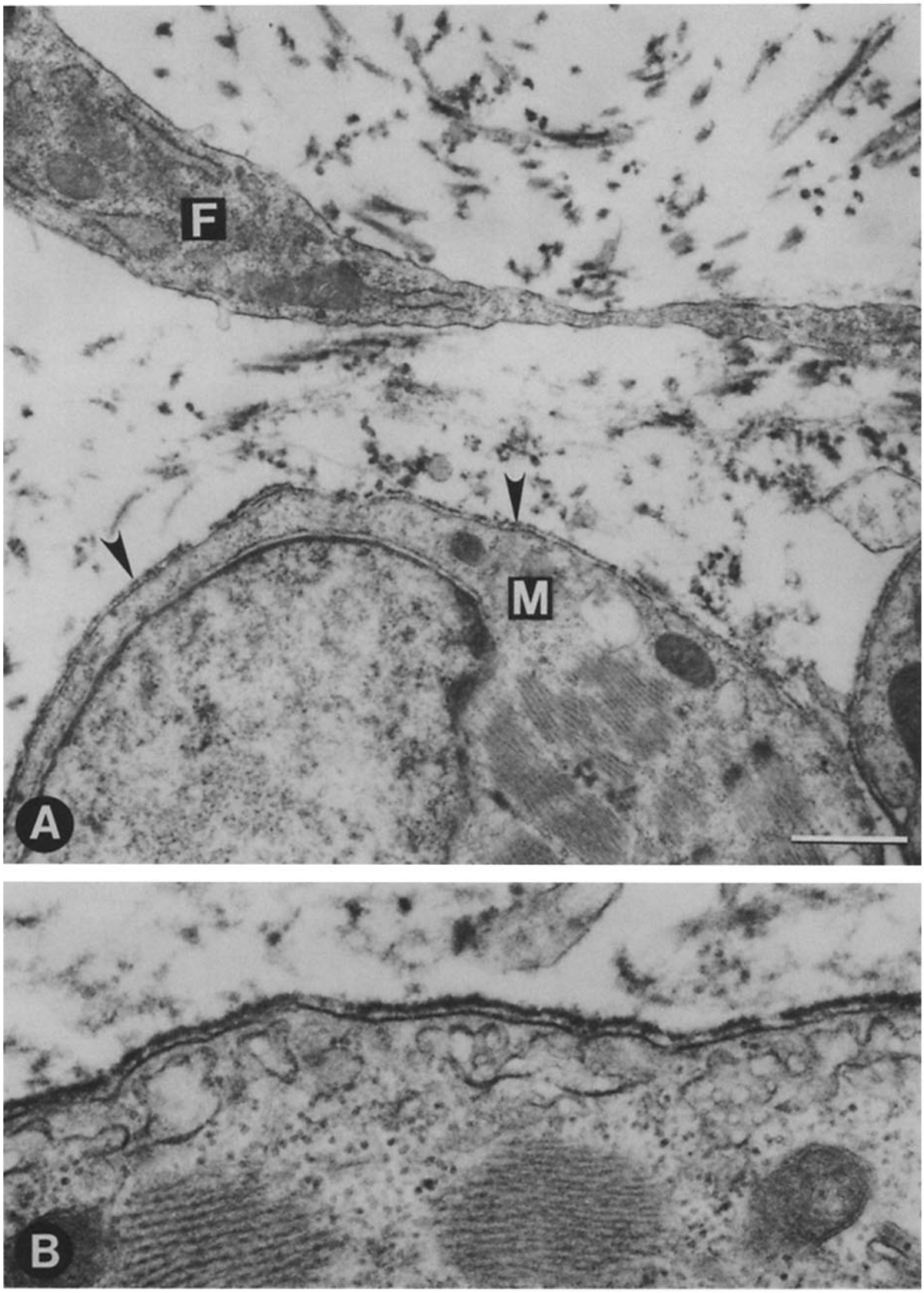

Figure 4. Electron micrographs of mass cultures 7 d after addition of a collagen gel previously fixed in the presence of 1% tannic acid. Note that in *A*, a basal lamina (arrowheads) is present on myotube (*M*) surfaces but is absent from the surface of fibroblasts (*F*). Collagen fibrils in cross and tangential section are present throughout the gel matrix. Higher magnification (*B*) shows a basal lamina consisting of both a lamina rara and lamina densa. Electron dense projections traverse the lamina rara and contact the myotube plasma membrane. Bar: 0.74 μm for *A*; 0.25 μm for *B*.

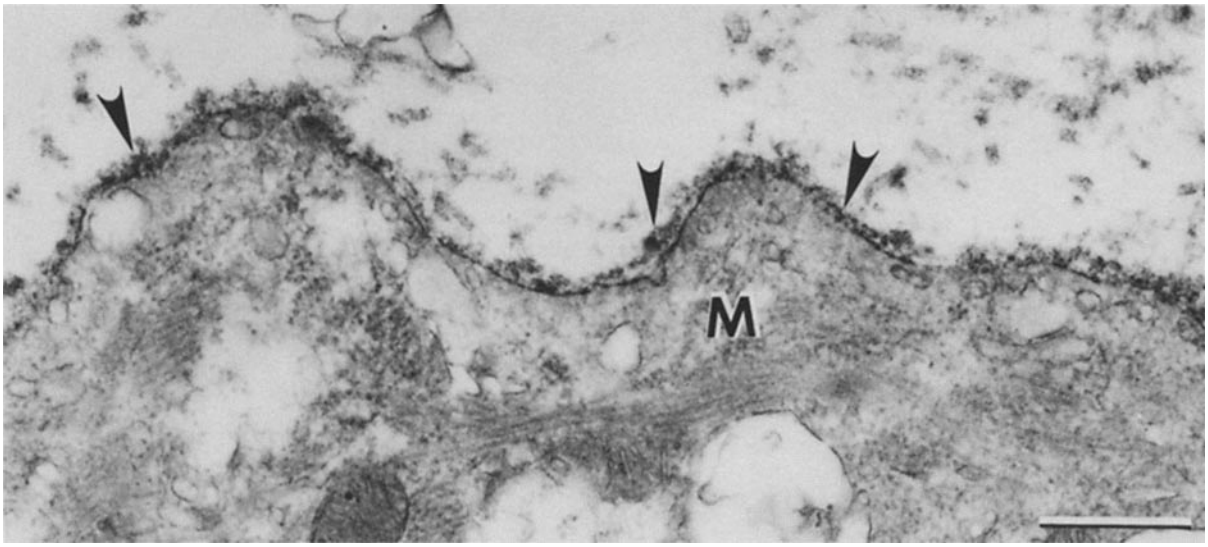

Figure 5. Electron microscopic localization of the monoclonal antibody specific for type IV collagen in mass cultures 7 days after addition of a collagen gel. Sections (100 μm) from the preparation were incubated with monoclonal antibody followed by peroxidase conjugated F(ab')$_2$ fragment of sheep anti-mouse IgG. Sections were then incubated with 0.01% H_2O_2 and 0.05% diaminobenzidine and prepared for ultrathin sectioning and electron microscopy. Reaction product is limited to the basal lamina (arrowheads) along the myotube (*M*) surface. Bar, 0.51 μm.

fibroblasts in promoting basal lamina formation. To distinguish among these possibilities, experiments were performed in which quail myoblasts were grown at clonal density, and, after myotube formation, the clones were covered with a native collagen gel. Fig. 6*A* shows the fluorescent staining pattern for type IV collagen in a typical clone after growth for an additional 7 d. Only very patchy staining for type IV collagen was observed, demonstrating that the collagen gel by itself is not sufficient to promote basal lamina formation. Further examination of these cultures by phase-contrast microscopy showed that quail myoblasts present in the clones failed to migrate to a significant extent into the collagen gel. This result therefore provides additional evidence that the cells observed migrating through the collagen gel in mass cultures are authentic fibroblasts. Experiments were also performed in which chick muscle fibroblasts were added to a culture of quail myoblast clones just prior to setting a gel above the cells. During the next 7 d the fibroblasts migrated extensively into the gel above the myotubes. Immunofluorescent staining of the cultures now revealed the accumulation of extensive amounts of type IV collagen on the surface of the myotubes (Fig. 6*B*). This correlated well with the formation of a basal lamina on the myotube surface as shown by electron microscopy (Fig. 7). These results provide direct evidence that, in culture, fibroblasts will promote basal lamina formation on developing myotube surfaces. The source of fibroblasts did not appear to be important, since skin fibroblasts were found to be equally effective as muscle fibroblasts (data not presented).

Further experiments were performed in which myogenic clones were grown in fibroblast-conditioned medium in order to investigate if this would be sufficient to promote basal lamina formation. In these experiments, a continuous basal lamina was not formed on the surface of the myotubes. This was true even when co-cultures were prepared in which myogenic clones and fibroblasts were present in separate com-

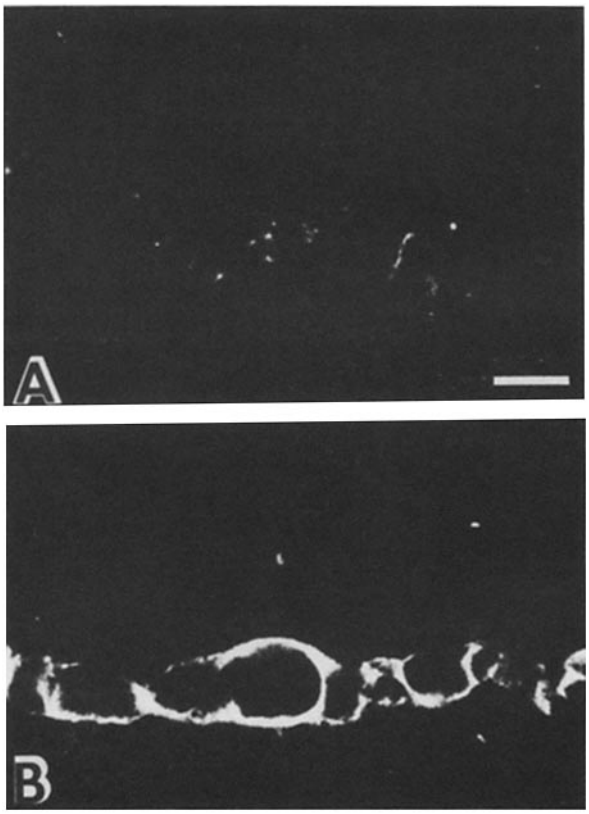

Figure 6. Addition of chick fibroblasts to quail myogenic clones results in basal lamina formation. Myogenic clones were grown for 7 d followed by the addition of a collagen gel to the surface of the clone. 7 d after addition of the gel only very sparse immunofluorescent staining was noted using an antibody specific for type IV collagen (*A*). Introduction of chick fibroblasts to quail clonal cultures, followed by addition of a collagen gel, resulted in the assembly of a basal lamina (*B*). Bar: 31.7 μm for *A*; 16 μm for *B*.

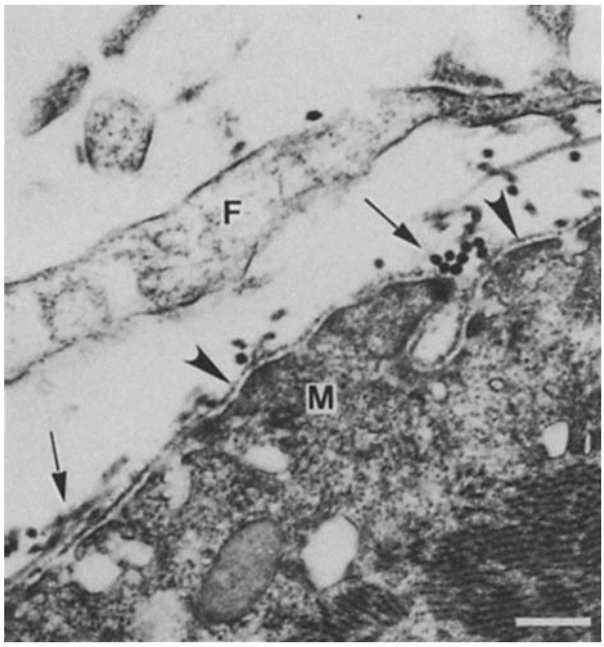

Figure 7. Electron micrograph showing the formation of a continuous basal lamina after the addition of fibroblasts and a collagen gel to quail muscle clones. Basal lamina (arrowheads) was present on myotubes (*M*) but not on fibroblasts (*F*). Collagen fibrils (arrows) often appear closely associated with the basal lamina. Bar, 0.5 μm.

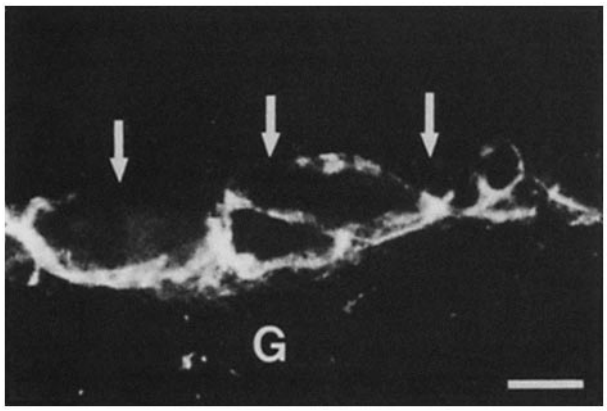

Figure 8. Growth of a mass culture on the upper surface of a collagen gel (*G*). After 8 d a basal lamina was detected on myotube surfaces in contact with the collagen gel which, during the culture period, was invaded by proliferating fibroblasts. The upper surface of the myotubes (arrows), which were in contact with the culture medium, showed only sparse patches of basal lamina formation. Immunofluorescence staining was obtained using type IV collagen specific monoclonal antibodies. Bar, 16.1 μm.

partments of the same culture dish and shared a common culture medium. Experiments were next performed in which a mass culture was grown on the upper surface of a collagen gel into which fibroblasts migrated and grew below the myotubes. Immunofluorescent staining of a section of the gel for type IV collagen now revealed the formation of a basal lamina on the underside of the myotubes that were in contact with the gel (Fig. 8). However, only occasional patchy fluorescent staining was present on the upper surface of the myotubes that were in contact with the culture medium. This result suggests that the fibroblasts may have to be near the myotube surface for successful basal lamina formation and that the presence of the collagen gel may have a permissive effect on this interaction.

Discussion

The present series of experiments clearly demonstrates that, in culture, fibroblasts promote the formation of a continuous basal lamina on the surface of myotubes. These experiments therefore agree with earlier observations (17, 18, 20). However, the present experiments differ from previous experiments in several important respects. A culture system has now been developed in which a continuous basal lamina is formed on the myotube surface, allowing for analysis both by immunofluorescent staining and electron microscopy. This differs from previous studies in which only patches of fluorescent staining for basement membrane components were observed on the surface of the myotubes (17, 18, 30). Furthermore, we have clearly shown that the addition of fibroblasts to clonal myotube cultures promotes basal lamina formation on the surface of myotubes. Such an experiment has not been previously performed. Kühl et al. (17) showed, using species-specific polyclonal antibodies to type IV collagen and mixed cultures of mouse and chick or quail cells, that mouse fibroblasts could contribute type IV collagen to the surface of avian myotubes and vice versa. We have recently prepared a monoclonal antibody to rat type IV collagen that shows no cross-reactivity to quail type IV collagen as shown by enzyme-linked immunosorbent assay or immunofluorescence (Sanderson, R. D., and R. Mayne, unpublished experiments). Experiments with this antibody will be used to investigate by electron microscopic immunolocalization if rat fibroblasts can synthesize type IV collagen that will be incorporated into the basal lamina of a quail myotube.

We cannot currently evaluate the relative contributions by myoblasts and fibroblasts of components present in the muscle basal lamina. Previously, Kühl et al. (18) reported that both cell types synthesize laminin and type IV collagen by immunoprecipitation with specific antibodies. In our experiments, clonal cultures showed very limited immunofluorescent staining for basement membrane components (Fig. 1), but if such myogenic clones were covered with a collagen gel and kept for several weeks, then the fluorescent staining eventually became more extensive, although a continuous basal lamina was never formed. Fibroblastic cells within a gel also sometimes showed limited extracellular fluorescent staining for type IV collagen (Fig. 3 *B*), but this was not a consistent observation. With monoclonal antibodies to type IV collagen and laminin, we could not detect the extensive intracellular staining for these components as observed previously with polyclonal antibodies (17, 18). The reason for this discrepancy between our results obtained using monoclonal antibodies and previous results using polyclonal antibodies is unclear.

For several cell types in culture, growth on a collagen gel is sufficient to promote basal lamina formation (8, 9, 13, 25). However, for myotubes, a collagen gel alone is not enough for basal lamina assembly and the presence of fibroblasts also is required. This finding may relate to the necessity for the myofibers to be joined to the collagen fibers of the tendon (22, 32) and endomysium (3). In the tendon the collagen fibers appear to insert directly into the muscle basal lamina by an unknown mechanism (22, 32).

During development the muscle cells of a limb are derived by migration from the somites, whereas the fibroblastic cells have a somatopleural origin (5, 6). Interactions between the muscle cells and the tendon cells or fibroblasts of the intercellular matrix must therefore be carefully co-ordinated as the muscles develop. This organizing function appears to reside largely with the mesenchymal cells and not with the myogenic precursor cells (5). The present observation of an interaction between fibroblastic cells and myoblasts during myogenesis in vitro may be a reflection of the kinds of interactions that must occur between these two cell types during myogenesis in vivo.

During myogenesis of limb muscles, a basal lamina initially separates a "cluster" of myoblasts and myotubes from the extracellular matrix that contains the fibroblastic cells (7, 10, 14, 15, 26, 28). Basal lamina components were not detected by immunofluorescent staining or by electron microscopic immunolocalization between the individual cells of a cluster (Irwin, M. H., R. D. Sanderson, and R. Mayne, unpublished observations). Only later in development, when the individual cells of a cluster separate (14), does the basal lamina begin to surround each myofiber (7, 15, 28). At this time, the connective tissue of the endomysium begins to form, and fibroblastic cells are observed between the myofibers. Thus, during myogenesis in vivo, the myogenic cells are always sequestered by a basal lamina that forms only where the muscle cells are in contact with the extracellular matrix containing fibroblastic cells. These results from in vivo analyses of basal lamina formation during myogenesis are therefore consistent with the results in this paper that show a requirement for fibroblasts in muscle basal lamina formation.

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