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Skeletal muscle cell populations:

Separation and partial characterization of fibroblast-like cells from embryonic tissue using density centrifugation

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Summary

Cell suspensions from the breast muscles of 10-day old chicken embryos were separated into non-myogenic, fibroblast-like cell fractions and a mononucleated, myogenic cell fraction by Percoll™ density centrifugation. Isolated populations were characterized by their morphology in both mass cultures and individual macroscopic clones and by the immunocytochemical detection of skeletal muscle- and smooth muscle-specific proteins in individual cells. Cell populations were also characterized by their protein patterns using sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The less dense, non-myogenic cells comprised 16% of the cells. In culture they were predominantly flattened, stellate cells and gave rise to clones lacking myotubes. These fibroblast-like cells were negative for skeletal muscle myosin or muscle type creatine phosphokinase. Less than 0.1 % of these cells demonstrated strong fluorescence when stained with anti-desmin or anti-smooth muscle specific actin. This observation suggested that the vast majority of these cells were not related to vascular smooth muscle cells. Also, over 99% of the non-myogenic cells did not display characteristic properties of endothelial cells. The denser myogenic cell fraction comprised over 80% of the cells and in clonal cultures gave rise to about 70% myogenic clones. An additional 30% of clones from this fraction were non-myogenic indicating heterogeneity in this population. We conclude that Percoll centrifugation can be employed for the isolation of myogenic and non-myogenic cell populations directly from the embryonic muscle. Moreover, this procedure allows the direct analysis of cell-specific proteins (e.g., by gel electrophoresis) without the need for cell culturing. The results thus obtained closely reflect the status of the cells in the intact muscle.

Introduction

Primary mass cultures derived from embryonic avian or mammalian skeletal muscle contain, in addition to myogenic cells, a population of so-called fibroblasts (Yaffe 1969; Abbott et al. 1974; Turner 1978). Clones of primary cells from muscle also consist of two types: myogenic (colonies that ultimately contain at least some terminally differentiated muscle cells) and non-muscle (colonies that consist of mononucleated flattened cells, presumably fibroblasts) (Konigsberg 1963; Hauschka and Konigsberg 1966; Hauschka 1974). However, the identification of the non-muscle cells has depended largely on their morphology; i.e.,

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because they are flattened and stellate, with no other distinguishing characteristics, they are often called fibroblasts. How heterogeneous this population may be, what types of molecules these cells may be capable of synthesizing, what cell lineages they may be part of or contribute to, are in large part unknown. Also, the tendency of various cell types to assume a flattened stellate morphology (under certain culture conditions) leads to such questions as whether or not there is a “true” fibroblast or whether many so-called fibroblasts represent phenotypic modulations or alternate states of other cell types (Garrett and Conrad 1979). It has been suggested that the non-muscle colonies represent both true fibroblasts and mesenchymal-like muscle precursors (Lipton 1977). It has also been suggested that the mesenchymal-like cells may express muscle differentiation under certain specific conditions, such as growth in the presence of conditioned medium (White and Hauschka 1971; White et al. 1975; Lipton 1977). However, large myogenic clones, which consist of hundreds of cells (Quinn et al. 1985), usually contain fibroblast-like cells in addition to myogenic and muscle cells (Abbott et al. 1974; Sasse et al. 1981). Based on this kind of observation, Abbott et al. (1974) suggested the existence of precursor cells which are ancestral to both myogenic and fibrogenic lineages. But, it is not clear whether any of the mononucleated cells in large myogenic clones are not myogenic and, if they are not, whether this is the result of a phenotypic modulation in some of the myogenic cells.

So-called muscle fibroblasts are most often produced by sequential passaging of primary cultures or by similar passaging of enriched non-myogenic cell populations obtained from muscle cell preparations by differential attachment (Yaffe 1969). Also, it has been shown that muscle-fibroblasts prepared this way contribute to the formation of basal laminae during myogenesis (Kühl et al. 1984; Sanderson et al. 1986). In order to begin to identify and study the presumed non-myogenic cells in vivo, in mass cultures, and in clones, and to avoid the possible changes that may occur in cells during long periods of growth in culture, we have developed a method for isolation of non-myogenic cells directly from the muscle tissue. Turner (1978) has described the use of a discontinuous gradient of Ficoll-400 to obtain enriched populations of muscle fibroblasts as well as several myogenic cell populations. The fibroblasts thus obtained were still contaminated with myoblasts to some degree. Also, except for the degree of myotube contamination, the fibroblasts were not characterized further. In this report we describe both the use of Percoll for the direct isolation of non-myogenic cells from muscle, and an initial characterization of some of the biochemical and immunocytochemical differences between the myogenic and non-myogenic cells.

Materials and methods

Source of cells

The cells for this study were from 10-day old chicken embryos (White Leghorn; Biological Supply, Bothell, Washington). Single cell suspensions were obtained by enzymatic digestion of the breast muscles (Robinson et al. 1984). Briefly, muscles were excised, finely minced, incubated in 0.1 % trypsin (GIBCO) for 30–45 min at 37° C, and centrifuged at approximately $300 \times g$ for 5 min. The trypsin solution was decanted and the pellet was resuspended in 5–10 ml of standard medium (see below). The cell suspension was recentrifuged as above and the final pellet was resuspended in 2.5–3 ml of standard medium.

Cells were mechanically dissociated by 5 passages through a Pasteur pipet followed by 5 passages through an 18-gauge needle. The resulting cell suspension was filtered through a double layer of lens tissue to eliminate myotubes, counted in a hemocytometer and treated further or plated as described below.

Cell culture

Standard medium consisted of 85 parts Eagle's minimal essential medium (MEM, GIBCO), 10 parts horse serum (GIBCO), 5 parts embryo extract, penicillin and streptomycin at 10^5 units per liter each, Fungizone and Gentamicin at 2.5 and 5 mg per liter, respectively. Cells were plated onto tissue culture dishes which had been coated with 2% gelatin and preincubated for 3 h with 25% horse serum in MEM to promote cellular adherence (Quinn and Nameroff 1983). Cultures were maintained at 37.5°C in a water-saturated atmosphere containing 5% CO₂ in air. For mass cultures, cells were plated at 2×10^5 cells per 35 mm dish with 1.5 ml of standard medium and the medium was changed 24 h after plating and every other day thereafter. For clonal cultures 50–100 cells were plated per 60 mm dish and the medium was changed 24 h after plating and every third day thereafter. Clones were usually kept in culture for 10–12 days to allow development of macroscopic colonies. To enable effective detection of the clones, dishes were rinsed with MEM, fixed for 1–2 min with absolute methanol and reacted for 5 min with 1 % toluidine blue in 30% methanol. Cultures were then rinsed with phosphate buffered saline and individual clones were identified for microscopic examination.

Percoll density gradient centrifugation

Percoll (Pharmacia) was made iso-osmotic by adding 1 part of 10X concentrated MEM to 9 parts of Percoll. Further dilution of the 90% Percoll was made with 1X MEM. Centrifugation was performed using 15 ml Corex tubes which were pre-equilibrated for 2 h with horse serum to minimize adherence of cells to the glass walls. A suspension of single cells prepared as above (5×10^7 to 10^8 cells from 6 to 12 embryos in 2 ml standard medium) was layered onto 11.5 ml of 20% Percoll on a cushion (1.5 ml) of 60% Percoll. The sample was then centrifuged for 5 min at $15000 \times g$ at 8°C with brakes off in a fixed angle rotor (Sorvall SS-34). Following centrifugation, Percoll fractions were collected manually from the top of the tube (see Fig. 1) and were diluted with 5 volumes of MEM. Cells were recovered from these diluted fractions by centrifugation at $300 \times g$ for 10 min at room temperature. Cultures were prepared from the resulting cell pellets by resuspending the cells in standard medium. For protein or DNA analyses, cell pellets were resuspended in 2 ml of MEM, counted in a hemocytometer, recentrifuged, and processed as described below.

Extraction and analysis of cellular proteins

Cell pellets, prepared directly from the embryos as described above, were resuspended on ice in NP-40 buffer (250 mM NaCl, 10 mM Tris, 5 mM MgCl₂, 2 mM *N*-ethylmaleimide, 1% Nonidet P-40, pH 7.4 at 4°C). In preliminary experiments on extracts from well-fused muscle cultures, we found that the presence of *N*-ethylmaleimide in the extraction buffer completely inhibited degradation of desmin when examined by immunoblotting. Soybean trypsin inhibitor or phenylmethanesulfonyl fluoride (PMSF) did not eliminate this

degradation. Therefore, *N*-ethylmaleimide was routinely included in all cell extraction buffers. About 0.03 ml of NP-40 buffer were used for 10^6 cells. This extraction procedure breaks open the cell membrane but leaves nuclei and mitochondria intact. The cell extract was then centrifuged at $12000 \times g$ for 10 min at 4°C using a swinging bucket rotor. The supernatant fractions were removed and saved and the pellet fractions were suspended in NP-40 buffer which contained 8 *M* urea (about 0.02 ml buffer for each original 10^6 cells). Fractions were kept frozen at -70°C until further analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis SDS-PAGE according to Laemmli (1970) using 10%–20% acrylamide gradient gels.

DNA/cell cycle analysis

Cell pellets, prepared after Percoll centrifugation, were resuspended in nuclear isolation medium (NIM) containing 10 mg/ml 4,6-diamidino-2-phenyl-indole (DAPI) (Rabinovitch et al. 1982) (1.0 ml NIM-DAPI for 10^6 cells) and nuclear DNA content was analyzed using the ICP-22 epi-illumination flow-system.

Antibody staining

Single- and double-immunolabeling of cells in cultures were performed by the indirect immunofluorescence technique as previously described (Robinson et al. 1984; Yablonka-Reuveni and Nameroff 1986). The guinea pig antiserum against chicken muscle myosin heavy chain and the rabbit antiserum against the muscle isozyme of creatine phosphokinase used in the present study have been described elsewhere (Quinn and Nameroff 1983; Robinson et al. 1984). A rabbit antiserum against chicken gizzard desmin was obtained from Dr H. Holtzer. The preparation and characterization of this antibody have been reported (Fellini et al. 1978; Bennett et al. 1978). The IgG fraction of this desmin antiserum was purified in our laboratory using protein A-sepharose (Yablonka-Reuveni and Nameroff 1986). A monoclonal antibody against chicken smooth muscle specific actin was provided by Drs. A.M. Gown and D. Gordon (Gown et al. 1985). Fluorescein- and tetramethylrhodamine-labeled secondary antibodies were from Cappel. Cultures were rinsed (3X) with 37°C MEM, fixed for 30s in ice-cold AFA (70% ethanol:formalin: acetic acid, 20:2:1), and rinsed (3X) with 4°C MEM. After fixation cultures were kept in sterile *Tris*-buffered saline containing normal goat serum (TBS-NGS; 0.05 *M Tris*, 1% NaCl, 1% normal goat serum, pH 7.6). Prior to adding the antibodies, cells were rinsed (3X) with *Tris* buffered saline containing Tween 20 (TBS-T20; 0.05 *M Tris*, 1% NaCl, 0.05% Tween 20, pH 7.6). Cultures were then exposed to various primary antibodies for 30 min at room temperature, rinsed with TBS-T20 (3X) and exposed to the appropriate secondary fluorescent antibody (as indicated) for an additional 30 min at room temperature. This was followed by another rinsing cycle with TBS-T20 and cultures were finally washed once with *Tris* buffer (TB; 0.05 *M Tris*, pH 7.6), and mounted in 90% glycerol containing p-phenylnediamine as an anti-fading agent (Johnson and Nogueira Araujo 1981). Observations were made with a Zeiss photo-microscope equipped for epifluorescence, and Kodak Ektachrome film (EL 135, 400 ASA) was used for photography.

Results

Density gradient separation and behavior of cells in mass and clonal cultures

Single cells were prepared from the breast muscle of 10-day embryonic chicks. About $0.5\text{--}1.0 \times 10^8$ cells were subjected to Percoll gradient centrifugation (Fig. 1). A very dense band of cells was observed at the 20%/60% Percoll interface. Some cell aggregates were found just above and below the dense band. Erythrocytes pelleted at the bottom of the tube. The remaining regions of the gradient showed some turbidity. The border region between the 20% Percoll and the original cell sample was the most turbid. In order to examine the cells in the different Percoll regions, fractions were withdrawn by hand from the top of the tube as described in Fig. 1. Cells were recovered from these fractions by centrifugation and were cultured on gelatin-coated dishes. Depending on the desired cell density, mass cultures were allowed to grow for 2 to 4 days. In Figs. 2 and 3 the morphology of the cells in cultures from the various Percoll regions is shown. The top 1 ml of the gradients was nearly devoid of cells and was discarded. Fractions 1–5 contained cells which, upon growth in culture, first exhibited a flattened, stellate morphology but, at dense concentrations, became packed and elongated. Very rarely, myoblasts or small myotubes were detected in cultures from these fractions. Their detection was aided by the use of an antibody against the heavy chain of skeletal muscle myosin. Such myosin-positive cells were observed in less than 5% of the examined microscopic fields in cultures from fractions 1–5. They usually appeared as a single cell or a small myotube, or in a cluster of a few cells or myotubes. No more myogenic cells were detected even after prolonged culturing of the cells for 8–10 days. A typical microscopic field with a differentiated myoblast is shown in Fig. 2j, n. These myosin positive cells constituted less than 1 % of the cells in Percoll fractions 1–5. Using an antibody against the muscle isozyme of creatine phosphokinase we obtained results identical to those described with anti-myosin (data not shown).

Fractions 6 and 7 contained cells that give rise to myotubes within 3 days of culture. These cultures were very similar to control cultures from muscle cell suspensions that were not subjected to Percoll fractionation. Fraction 7 also contained some erythrocytes. However, the vast majority of erythrocytes were in the pellet at the bottom of the centrifuge tube. Table 1 summarizes the numbers of cells recovered from the different Percoll fractions. About 16% of the cells recovered following the gradient centrifugation occupied the regions above the 20%/60% Percoll interface (using density marker beads we determined that the densities of these regions were in the range of 1.016–1.034 g/ml). Moreover, at least 99% of these cells were non-myogenic in their behavior in mass cultures and were “fibroblast-like” in their morphology. In addition, the cells recovered from the Percoll represented about 80–85% of those originally applied to the gradient. Hence, the Percoll isolation technique is highly efficient not only in separating fibro-blast-like cells but also in total cell recovery.

It was possible that the fibroblast-like cells in fractions 1–5 could actually give rise to myotubes (i.e., they were muscle precursor cells), but, under the mass culture conditions used, they did not proliferate sufficiently to produce terminally differentiated myoblasts or myotubes. In order to investigate this possibility, we cloned cells from the gradient fractions. Cells were seeded at a density of 50–100 per 60 mm dish and were grown for about 10–12

days. The cultures were then fixed, stained with toluidine blue, and the clones were scored for the presence of multinucleated myotubes. Photographs of clones from the different Percoll fractions are shown in Fig. 3. None of the clones from fractions 1–5 contained multinucleated myotubes. Cells in these clones were mostly flattened, stellate and mononucleated. Occasionally, a non-myogenic clone also contained an area with more packed cells presenting an elongated morphology as shown in Fig. 2 for fibroblast mass cultures. In fraction 6 (the cells at the 20%/60% Percoll interface), 70% of the clones developed myotubes and also contained both spindle-shaped and flat, stellate cells. The additional 30% of the clones from fraction 6 contained only flattened mononucleated cells and no myotubes. The non-myogenic clones in the various Percoll regions did not react with muscle specific antibodies such as anti-skeletal myosin or anti-muscle type creatine phosphokinase (data not shown). The distribution of myogenic and non-myogenic clones in fraction 7 was similar to that of fraction 6. We could identify size variations among the macroscopic non-myogenic clones from the various Percoll fractions, including fraction 6. However, in respect to the morphology of these macroscopic non-myogenic clones, we could not identify any obvious differences that allow sub-classification of clones.

Cell cycle analysis

The migration of cells to different regions of the Percoll gradient could have resulted from differences in DNA content among cells at different points in the cell cycle. That this was not a very significant cause of differential migration is shown in Table 2 which summarizes the results of flow-cytometric DNA analyses of cells from the different Percoll fractions. The cell layer at the 20%/60% Percoll interface (fraction 6, which produced myotubes in culture) was composed of 71.4% cells in G1 and 18.2% cells in S. The cell fractions which did not produce myotubes in tissue culture (fractions 1–5) demonstrated a somewhat higher percentage of cells in G1 (83.4–79.9) and a correspondingly lower percent of cells in S (9.8–12.1).

Analysis of proteins in gradient-separated cells

One of the reasons for developing a method for the separation of myogenic and presumed fibrogenic cells was to identify and characterize proteins in cells prepared directly from the animal. By means of the cell culture assays described above, we could not observe any obvious morphological differences between cells in the mass cultures or clones of the apparently non-myogenic gradient fractions (fractions 1–5). We therefore decided to pool these fractions for further analysis, although we recognized the possibility that the non-myogenic cells could also be heterogeneous. The pooled non-myogenic cell population was then compared with the major myogenic cell population (fraction 6) by means of SDS-PAGE. The protease inhibitor N-ethylmaleimide was included in the extraction buffer to prevent possible protein degradation (see Materials and methods). To increase the detection level of minor proteins, extracts of the cells were split into soluble and particulate compartments. The particulate compartment included the nuclei since the extraction buffer did not break open the nuclear membranes. This was demonstrated by the detection of histones only in the particulate compartment. Mitochondria, cell membranes and insoluble proteins were also found in this fraction. As shown in Fig. 5, SDS-PAGE demonstrated several differences in proteins between the two cell populations. Some of these differences

are indicated by dots in the figure. In the particulate samples, the myogenic cell fraction contained a polypeptide with a molecular weight of 25000 which was either absent or present at very low levels in the non-myogenic cell fraction. A polypeptide with a molecular weight of 38000 was detected only in the non-myogenic cell population. This latter polypeptide is positioned just below another polypeptide which can be detected in both cell populations. When the soluble samples were compared, a polypeptide with a molecular weight of 29000 was predominant in extracts from the myogenic cell fraction. This polypeptide is positioned just above another polypeptide common to both cell populations. Obviously, characterization of these proteins as absolutely population-specific is not possible from results obtained solely by one-dimensional electrophoresis, since several polypeptides could migrate to the same position on the gel. However, two-dimensional gel electrophoretic analysis of the different extracts has also revealed differences between the two cell populations (Yablonka-Reuveni et al. to be published). Two additional polypeptides which migrated on the gel to the same position as globins from 10-day old chicken embryos, were detected in the soluble samples from the myogenic cell fraction. Repetitive centrifugations of this fraction, in 20% over 60% Percoll, reduced the intensity of these two bands. Fractionation of the myogenic cell population with 35% Percoll (which separated the cells into several myogenic populations) completely eliminated these bands (data not shown). These findings indicated that the two (presumably globin) polypeptides originated from some contaminating erythrocytes which were trapped at the 20%/60% Percoll interface.

In summary, the protein content of cells prepared directly from the embryo could be easily analyzed by SDS-PAGE. Furthermore, the myogenic and non-myogenic populations contained polypeptides which are unique for each population or at least present in higher quantities in one population compared to the other.

Immunofluorescent staining with anti-desmin

Antibodies against desmin have been used to identify terminally differentiated myoblasts and myotubes (Bennett et al. 1978; Lazarides 1982). We have also employed an anti-desmin antibody in order to detect terminally differentiated myoblasts and small myotubes in the fibroblast-like cell cultures. We observed that isolated presumed fibroblasts stained faintly with fluorescent anti-desmin. The pattern of staining resembled that of cardiac myocytes (Wallace et al. 1983), and appeared as a fibrous network with more concentrated staining at the perinuclear region. We also could detect staining at the cell borders and in very narrow and extended pseudopodia. However, the level of fluorescence was usually too low to obtain a good fluorescence photograph. In Fig. 6b, e, we attempted to show some of the staining that was captured by the photographic film. This low-level staining with anti-desmin was detected in fibroblast-like cells which were well separated from each other but was barely detectable in parallel cells in confluent cultures where the morphology of the cells changed from flattened and spread out to elongated and bipolar. It is very unlikely that this low-level staining is due to cross-reactivity of the desmin antibody with other proteins since the anti-desmin did not react with partially purified vimentin or with other proteins from well-fused muscle cultures (Yablonka-Reuveni and Nameroff 1986). Also, low levels of desmin in presumed fibroblasts have been reported by others (Gard et al. 1979; Wallace et al. 1983). In addition to the low-level staining, we could detect among the isolated fibroblasts, although

very rarely (less than 0.1% of the cells), cells which exhibit very strong staining with anti-desmin. These monocucleated, flattened, strongly positive cells did not react with anti-skeletal muscle myosin. One possibility is that such cells were smooth muscle, since desmin has been reported to be detectable not only in terminally differentiated myoblasts, myotubes, and cardiac myocytes, but also in smooth muscle cells (Fellini et al. 1978; Bennett et al. 1978; Lazarides 1982; Wallace et al. 1983). These desmin-positive, myosin-negative cells are shown in Fig. 6 along with the positive staining of the myogenic cells from the interface fraction. When immunoblots were performed on both the fibroblast-like cells and the myogenic cells (fraction 6) immediately following the Percoll separation, very small but similar amounts of desmin were detected in the two cell populations (data not shown). It is probable that some desmin detected on the immunoblots originated from smooth muscle cells and a few terminally differentiated myoblasts, and that additional desmin came from the small amounts present in the other cells. It should be emphasized that the anti-desmin did not react with partially purified vimentin or with other proteins from well-fused muscle cultures (Yablonka-Reuveni and Nameroff 1986).

Immunofluorescent staining with anti-smooth muscle actin

In view of the weak but positive staining of the fibroblast-like cells with anti-desmin, the possibility was raised that some of the isolated non-myogenic cells might be smooth muscle. Such cells could come from the blood vessel walls in the skeletal muscle tissue. In order to study this possibility, the Percoll isolated non-myogenic cells were cultured for 3 days and reacted with anti-smooth muscle actin. Cultures from the myogenic cell fraction were treated similarly. The results of this study are shown in Fig. 7. Cells positive for smooth muscle actin were very rare (less than 0.1%) in cultures from both fibroblast-like cells and myogenic cells (fraction 6). The positive cells were usually very spread out and displayed a typical network of "stress fibers" when reacted with anti-actin. It is noteworthy that in experiments where the cultures were doubly-labeled with antibodies against smooth muscle actin and desmin, the spread out cells exhibited the presence of either desmin or actin but not both (data not shown; see also discussion by Yablonka-Reuveni and Nameroff 1986). At any rate, all these presumed smooth muscle cells accounted for less than 0.1% of the fibroblast-like cells recovered from the 20% Percoll regions.

In summary, the immunocytochemical studies suggest that cells which resemble smooth muscle cells in respect to the expression of desmin and actin comprised less than 0.1% of the isolated fibroblast-like cells.

Analysis of cell heterogeneity in the intact muscle

To examine the heterogeneity among muscle cells in ovo the histology of breast muscles from 10-day old embryo was studied (Fig. 8). Muscle bundles (MB), surrounded by connective tissue (CN) were prominent. The isolated non-myogenic cells probably originated from the connective tissue. Occasionally, a blood vessel (BV) with a wall of at least two cell layers and erythrocytes in the lumen could be detected. Cell such as presumptive or differentiated smooth muscle cells as well as endothelial cells originating from these blood vessel walls could contribute to the Per-coll-isolated fibroblasts. The contribution of smooth muscle cells was studied by immunofluorescence, as described

above. In addition, we tried to assess the possibility that some of the fibroblasts were related to endothelial cells. Morphologically, the isolated muscle-fibroblasts did not resemble vascular endothelial cells prepared from the aortic arches of a 10-day old chicken embryo. Moreover, endothelial cells from these aortic arches could be identified both in the intact embryo and in cultures by their uptake of a fluorescent probe, acetylated-low density lipoprotein (Yablonka-Reuveni, submitted; Voyta et al. 1984). The vast majority (99%-100%) of the Percoll-isolated fibroblasts, however, did not incorporate the fluorescent lipoprotein probe.

High power examination of the muscle sections (Fig. 8 b), clearly showed that the muscle bundles (indicated as MB) contained different cells. Cells marked with M generally contained a peripheral ring of myofibrils, and were in fact myotubes (Fischman 1972). Additional cells which were darkly stained (indicated as *) have usually been described as myoblasts (Fischman 1972). Whether some of the mononucleated cells in the muscle bundles are actually non-myogenic (e.g., presumptive endomysium cells) remains an open question.

Discussion

We have described a method of density gradient centrifugation for the direct isolation of fibroblast-like cells from skeletal muscle of embryonic chicken. The method yields a population of which over 99% are non-myogenic cells as assayed by clonal analysis. The density gradient medium was Percoll, a preparation of colloidal silica particles coated with polyvinylpyrrolidone. It is non-toxic to cells, does not penetrate biological membranes, and has been successfully used for the isolation of a variety of cells (Pertoft et al. 1977; Gmelig-Meyling and Waldmann 1980; Schweizer et al. 1984). This approach does not require the culturing (pre-plating) or sequential subculturing which have been commonly used for the preparation of muscle fibroblasts, and thus allows direct studies on the isolated cells immediately after separation. Turner (1978), has also reported that non-myogenic cells (presumably fibroblasts) can be separated from myogenic cells when cell suspensions from embryonic chicken muscle are subjected to discontinuous Ficoll-400 density gradient centrifugation. However, some myotubes were still observed in cultures derived from presumptive fibroblasts and no further studies were reported on the non-myogenic cells. In the present study, myogenic cells or myotubes were rarely observed when cells from fractions 1–5 were tested by the indirect immunofluorescence technique using skeletal muscle specific antibodies. The increased ability to separate fibroblast-like cells in the current study was probably due to the use of a large volume of 20% Percoll.

Many studies on fibroblast-like cells have used cell populations prepared by sequential passaging. This approach is necessary when studying cell aging but in many other instances is potentially subject to a number of problems. The cells may change with time in culture. Also, if the cells do not actually represent a single population then, following prolonged culturing, the frequency of some sub-populations may no longer represent the original composition of the cells. When studying muscle cells in culture, the myogenic precursors may give rise to cells which morphologically resemble fibroblasts but are not necessarily identical to those isolated directly from the embryo (i.e., it is possible that the flattened, non-

fusing cells in individual myogenic clones do not represent “true” fibroblasts). The separation procedure reported here reduces some of the potential difficulties as primary cultures of fibroblasts can be obtained without prolonged culturing.

In addition, the separation procedure has made it possible to compare the behavior of the myogenic and non-myogenic populations and the proteins contained in these cell populations. Since populations were analyzed directly following their isolation from the embryo, the protein patterns obtained by SDS-PAGE presumably reflect the *in vivo* status of these populations. One-dimensional gel electrophoresis demonstrated several differences in polypeptides between the two cell populations in both the particulate and soluble compartments. These findings are supported by two dimensional gel electrophoresis analyses which have revealed additional differences between the two populations (Yablonka-Reuveni et al., in preparation). A population-specific polypeptide such as the one with a molecular weight of 38000, described in Results for the fibroblast cells, may be a useful marker for part or all of the isolated muscle fibroblasts. Obviously, in view of the many reports on fibroblast heterogeneity we have no way of knowing, currently, whether this polypeptide is present in part or all of the isolated fibroblasts. Moreover, the specific polypeptides which were detected for the myogenic population (fraction 6) may not necessarily be produced by the myogenic precursor cells. They might be all made by the non-myogenic cells detected in fraction 6 by clonal analysis. Furthermore, based on the SDS-PAGE analysis we cannot rule out the possibility that the non-myogenic cells in fraction 6 differ from those in fractions 1–5. The availability of antibodies against such specific protein markers will facilitate future study on the question of fibroblast as well as myoblast heterogeneity (see Quinn et al. 1985). Recently, a comparison of proteins made by human skin fibroblasts in tissue culture with those made by human muscle cultures was described (Graham et al. 1984). This study suggested the existence of several fibroblast-specific polypeptides but did not rule out the possibility that the fibroblast-like cells from skin differed from those originating in muscle. Obviously, the lineage origin of fibroblasts from different tissues and organs is not clear, and it is possible that different fibroblasts reflect differentiative stages of cells from different lineages. Indeed, several reports have suggested that there are differences between fibroblasts from different tissues of the same species (Garrett and Conrad 1979; Ndumbe and Levinsky 1985).

The lineage origin of the fibroblasts isolated in the current study is obviously not clear. They could have risen from myogenic precursor cells in the embryo in a similar fashion to the appearance of fibroblast-like cells in myogenic clones. Connective tissue which appears as an integral part of the breast muscle and is apparent at the studied developmental age (Fig. 8) could also have given rise to some of these fibroblasts. In addition, the vascular system can be detected in cross sections from the breast muscle of a 10-day old embryo (Fig. 8). Thus, “differentiated” or prospective smooth muscle and endothelial cells from the blood vessel walls of the skeletal muscle tissue could have been isolated as well. Staining with anti-desmin or anti-smooth muscle actin revealed little reaction of blood vessels at this developmental stage (data not shown). Immunoblotting of extracts from the non-myogenic cell fraction immediately after the Percoll isolation showed only trace amounts of desmin and only a very low number of isolated, non-myogenic cells could be characterized as “differentiated” smooth muscle cells by immunofluorescence microscopy (Figs. 6 and 7).

Thus it is unlikely that the majority of the isolated non-myogenic cells arose from smooth muscle cells. It is interesting to note however, that in avian aortas the larger elastic arterial medias have well differentiated smooth muscle cells and “interlaminar” cells which more closely resemble fibroblasts and do not react with anti-desmin (Moss and Benditt 1970; Lauper et al. 1975; Schmid et al. 1982). Also, some reports suggest that proliferating smooth muscle cells lack desmin (Gabbiani et al. 1982). Identifiable endothelial cells also contribute very small numbers to the isolated fibroblasts. Despite the findings that skeletal myogenic precursor cells, “differentiated” smooth muscle cells, and endothelial cells contribute very low numbers of cells to the isolated muscle fibroblasts, we cannot exclude the possibility that the isolated fibroblasts represent more than one cell population.

Bailey et al. (1979), studying the chick muscle, have demonstrated a spatial distribution of the isomorphic forms of collagen. Type I was mainly present in epi- and perimysium, type III in the perimysium and type V in the endomysium. This raised the possibility that fibroblasts isolated from the muscle as described in the present study could be further classified according to the collagen which they synthesized. Analysis of isotopically labeled procollagens from cultures of the various Percoll isolated fibroblast fractions (Fig. 1, 1–5) did not reveal any differences in the synthesis of procollagen type I and procollagen type III (Yablonka-Reuveni, unpublished). Obviously, the Percoll centrifugation might not offer the separation needed to differentiate between the different fibroblasts. Furthermore, differences in the production of collagens may not exist at the studied embryonic age or may be lost in cell culture.

The degree of homogeneity of the cells at the 20%/60% Percoll interface is not clear. In tissue culture, these cells give rise to both myoblasts capable of fusion and to cells which morphologically resemble fibroblasts and do not fuse to form myotubes. These latter fibroblast-like cells could represent additional non-myogenic cells which are identical to or different from those isolated by the 20% Percoll. Alternatively, the fibroblast-like cells in tissue cultures from the myogenic fraction could have arisen, at least in part, from myogenic cells which, through divisions in tissues culture, underwent phenotypic modulation. Or, they could have arisen from precursor cells which are genetically programmed to produce both fibroblasts and myoblasts. We have attempted to fractionate further the major cell population containing the myogenic precursor cells (the cells at the 20%/60% Percoll interface). Employing a cell sorter (using 90° light scattering as a parameter for sorting, Yablonka-Reuveni, unpublished), we could obtain a myogenic sub-population which gave rise only to myogenic clones. Subjecting the Percoll isolated-myogenic cell population to additional density centrifugations (i.e., 35% Percoll instead of 20% Percoll or step gradient of 20–60% Percoll for the separation), we obtained several myogenic cell populations with different sedimentation characteristics. However, judging by clonal analysis or by protein separations on SDS-PAGE we could not detect any obvious difference between these different populations. In addition, using the Percoll centrifugation technique we have been able to obtain from older animals a nearly pure population of myogenic precursor cells (presumably satellite cells) which give rise to large myogenic clones (Yablonka-Reuveni et al. 1987).

In conclusion, the data presented in this report clearly demonstrate the usefulness of employing Percoll density centrifugation for the isolation and analysis of myogenic and non-myogenic cells from chicken embryonic muscle. This approach eliminates the need for sequential culturing of muscle cells to obtain pure muscle fibroblasts. Moreover, it allows the analysis of various cellular proteins. Such an analysis should closely reflect the *in vivo* status as the cells are prepared directly from the animal.

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Fig. 1.

Schematic representation of the Percoll fractionation of a single cell suspension from chicken embryonic breast muscle. Mononucleated cells were prepared from the trypsinized muscle tissue and resuspended in complete medium. About 5×10^7 – 10^8 cells in 2 ml complete medium were then applied onto 20% Percoll as described to the left of the diagram. Following centrifugation a sharp band was generated at the 20%/60% Percoll interphase and turbidity was detected all along the gradient but more enhanced in fraction 1. Most of the erythrocytes (*RBC*) pelleted at the bottom of the tube. Numbers at the right indicate the fraction numbers as referred to in the text; fractions 1–4 had a volume of 2.5 ml each, fractions 5, 6, 7 had a volume of 2.1, 0.5 and 1.4 ml, respectively

Fig. 2.

a-p. Phase and fluorescence micrographs of cell cultures from the various Percoll regions following centrifugation. Cells were cultured at a concentration of 2×10^5 per 35 mm dish and fixed after 3–4 days in culture. Cultures were then reacted with antiserum against skeletal muscle heavy chain (1:60) and rhodamine-conjugated goat anti-guinea pig IgG (1:60). **a, b, c, d, i** phase and **e, f, g, h, m** fluorescent staining of cultures from fractions 1, 2, 3, 4 and 5, respectively, **j** phase and **n** fluorescent staining showing one myogenic cell in

fraction 5. **k, l** phase and **o, p** fluorescent staining of cultures from fractions 6 and 7, respectively, demonstrating myotubes. *Bar* = 30 μ m

Fig. 3.
a-e. The stellate morphology of the cells isolated from Percoll gradient fractions 1–5 following 2 days in culture. Cells were prepared, cultured and fixed as described in the legend to Fig. 2 except that cultures were carried only for about 2 days, **a-e** cultures from Percoll regions 1 through 5, respectively. This spread out, stellate morphology changes to that shown in Fig. 2 after more time in culture. *Bar* = 30 μ m

Fig. 4.

a-d. Representative fields of macroscopic clones from the different Percoll fractions. Cells were recovered from the gradient following centrifugation and cultured at a concentration of 50–100 cells per 60 mm dish. After 10–12 days the clonal cultures were fixed and stained with toluidine blue. Macroscopic clones were then identified and examined by phase microscopy. **a** phase micrograph of a representative clone from cells in Percoll fraction 3; clones from fractions 1, 2, 4 and 5 had similar morphology, **b-d** phase micrographs of clones from cells in Percoll fraction 6; **b** a non-myogenic clone, **c, d** two different myogenic clones exhibiting different morphologies. Arrows indicate myotubes and/or cells that reacted positively with skeletal myosin antiserum. *Bar* = 48 μ m

Fig. 5.

SDS-PAGE patterns of proteins of cells isolated by Percoll centrifugation. Cells in Percoll regions 1–5, pooled to one fraction designated *F*, and cells in Percoll fraction 6 designated *M* (cells at the 20%/60% Percoll interphase), as well as the erythrocyte pellet were fractionated to particulate (*p*) and soluble (*s*) sub-cellular compartments. These compartments were electrophoresed on a 10–20% SDS polyacrylamide gel and stained with Coomassie brilliant blue. 1 particulate fraction from Percoll regions 1–5, about 4×10^5 cells. 2 particulate fraction from Percoll regions 1–5, about 10^6 cells. 3 particulate fraction from Percoll region 6, about 10^6 cells. 4 soluble fraction from Percoll regions 1–5, about 4×10^5 cells. 5 soluble fraction from Percoll regions 1–5, about 10^6 cells. 6 soluble fraction from Percoll region 6, about 10^6 cells. 7 soluble fraction from the erythrocyte pellet, about 3×10^3 cell; this sample demonstrates the migration position of globins from chicken embryos. 8, 9 as in lanes 5 and 6, respectively, but prepared from a different batch of cells. Lane 10 demonstrates a blank sample prepared from all Percoll regions combined together following the fractionation of standard medium alone (no cells) by the Percoll centrifugation; the Percoll regions were diluted and subjected to centrifugation as described for regions with cells, the “alleged” pellet thus obtained was then subjected to gel electrophoresis without further separation to particulate and soluble fractions; this control sample was analyzed to ensure that the contribution of proteins from the standard medium

itself and/or from the horse serum used to preincubate the centrifuge tube is minimal; a polypeptide with a molecular weight of about 66000 was detected. Dots indicate some polypeptides discussed in the text. Migration position and molecular weights ($\times 10^{-3}$) of molecular weight markers (*MWM*) are indicated

Fig. 6.

a-f. Immunofluorescent staining with anti-desmin of fibroblast-like cells and myogenic cells prepared by Percoll centrifugation and grown in culture. Cells were prepared and fixed as described in the legend to Fig. 2. Cultures were then reacted with anti-desmin (IgG fraction, 0.1 mg/ml) and fluorescein-conjugated goat anti-rabbit IgG (1:60). **a, d** phase and fluorescence micrographs of cultured cells prepared from Percoll fraction 2 and demonstrating a single cell which is desmin positive. **b, e** phase and fluorescence micrographs of cultured cells prepared from Percoll fraction 2 and demonstrating cells which react positively with the anti-desmin but exhibit only faint fluorescence. Cells as in **d** and **e** were detected in other non-myogenic Percoll fractions as well; cells such as in **d** represent much less than 0.1% of cells, **c, f** phase and fluorescence micrographs of cultured

cells prepared from Percoll fraction 6 and demonstrating the positive reaction of myotubes with anti-desmin. *Bar* = 30 μ m

Fig. 7.

a-d. Immunofluorescent staining with anti-smooth muscle-specific actin of fibroblast-like cells and myogenic cells prepared by Percoll centrifugation and grown in culture. Cells were prepared and fixed as described in the legend to Fig. 2. This was followed by sequential incubations with anti-smooth actin (1:40) and fluorescein-conjugated goat anti-mouse IgM + IgG (1:40). **a, c** phase and fluorescence micrographs of cultured cells prepared from the pooled fibroblast-like fractions (Percoll fractions 1–5). **b, d** phase and fluorescence micrographs of cultured cells prepared from the 20%/60% Percoll interphase fraction. Note that the actin-positive cells represent much less than 0.1% of cells in both cell populations. *Bar* = 30 μ m

Fig. 8.

a and b. Cross section of breast muscle from 10-day-old chicken embryo. The breast tissue was immersion fixed with 2% paraformaldehyde – 2% glutaraldehyde in 0.1 *M* cacodylate, pH 7.4, post-fixed in 1% OsO₄, dehydrated in a graded series of ethanol and embedded in EPON. 1 μ m thick sections were then stained with Richardson's stain, **a** bar = 50 μ m; **b** higher resolution of blood vessel and surrounding area in **a**. Bar = 20 μ m. BV blood vessel;

CN connective tissue; *MB* muscle bundle; *M* myotube with a ring of myofibrils; * mononucleated cell, possibly a myoblast; → fibroblast in CN

Table 1

Distribution of cells from embryonic breast muscle in the various Percoll regions following centrifugation

Fraction ^a	Number of cells $\times 10^5$	% of total cells ^b
1	12.0	2.5
2	11.4	2.3
3	13.6	2.8
4	19.0	3.9
5	24.8	5.1
6	396.0	81.4
7	9.8	2.0
Total	486.6	100.0

^aFraction numbers correspond with those indicated in Fig. 1^bCalculated from total recovered cells; 80% of applied cells were recovered

Table 2

Cell cycle analysis of the populations in the different Percoll fractions

Fraction ^a	% cells in G1	% cells in S	% cells in G2 + M
1	83.8	9.4	6.8
2	82.9	10.2	6.9
3	81.9	11.0	7.1
4	81.5	11.6	6.9
5	80.1	11.9	8.0
6	71.4	18.2	10.4
7 ^b	77.2	14.8	8.0
UF ^c	76.3	15.1	8.6

^aFraction numbers correspond with those indicated in Fig. 1^bAlso contain erythrocytes^cUF, unfractionated cells or cells that were not subjected to Percoll fractionation