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Biochemical and morphological differences between fibroblasts and myoblasts from embryonic chicken skeletal muscle

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Summary. Non-myogenic cells were isolated from the breast muscle of 10-day-old chicken embryos employing Percoll density centrifugation. In culture, these cells exhibited the spread out, stellate morphology of fibroblast-like cells. They also exhibited receptor-mediated binding of platelet-derived growth factor (PDGF). Such binding was not detected in cultures of predominantly myogenic cells isolated by the Percoll density centrifugation from the same muscle. Percoll-isolated myogenic and fibrogenic cell populations were also analyzed by two-dimensional polyacrylamide gel electrophoresis immediately after removal from the muscle. This analysis revealed at least six polypeptides specific to the fibroblasts but not detected in the myogenic cell population. In addition, at least eight polypeptides found in the myogenic population were barely detectable, or lacking altogether from the fibroblast-like cells. Ultrastructural analysis of the freshly isolated cells demonstrated that the fibroblasts were larger than the myoblasts and that their cytoplasm contained many vesicles. We conclude that the fibrogenic and myogenic cells isolated by Percoll from embryonic muscle express cell type-specific characteristics. Moreover, based on the PDGF binding studies, the fibrogenic cells can be categorized as "true" fibroblasts.

Key words: Skeletal muscle – Fibroblasts – Myoblasts – Percoll – Platelet-derived growth factor receptor – Embryonic White Leghorn chick

Embryonic skeletal muscle consists of myogenic cells at various stages of differentiation. These cells occupy the developing muscle bundles (Fischman 1967, 1972; Yablonka-Reuveni and Nameroff 1987). Additional non-myogenic cells, predominantly fibroblasts, reside primarily in the connective tissue of the developing muscle. It has been reported that fibroblasts contribute to the formation of the muscle basal lamina during myogenesis (Kühl et al. 1984; Sanderson et al. 1986). Myogenic cultures prepared from embryos usually contain fibroblasts co-isolated with the myogenic cells. These fibroblasts contribute, depending on the species and the organismal age, up to 50% of the cultured cells (Konigsberg 1963; Yaffe 1969; Hauschka 1974; Abbott et al. 1974; Turner 1978; Yablonka-Reuveni and Nameroff 1987). It has been suggested that the cultured muscle-fibroblasts represent a mixture of both "true" fibroblasts and mesenchymal-like muscle precursors which may express muscle differentiation only under certain conditions (White et al. 1975; Lipton 1977). It has also been suggested that fibroblasts and myoblasts in embryonic muscle are descendants of precursor cells that are ancestral to both cell types (Abbott et al. 1974). The possibility that some fibroblasts in tissue culture may represent phenotypic modulations or alternate states of other cell types has also been raised (Garrett and Conrad 1979). In most studies, the identification of muscle fibroblasts in cultures has been based largely on their morphology; i.e., because they are flattened and stellate, do not fuse into myotubes, and have no other distinguishing characteristics, they have been called fibroblasts. However, the heterogeneity of these muscle fibroblasts, the types of molecules that they may be capable of synthesizing as well as their lineage origin are in large part unknown.

To characterize muscle fibroblasts we have developed a density centrifugation method that results in the separation of a fibroblast-like cell population and a mononucleated, myogenic cell population from embryonic as well as adult muscle (Yablonka-Reuveni and Nameroff 1987; Yablonka-Reuveni et al. 1987). Studying embryonic muscle, we have demonstrated that the Percoll-isolated fibroblasts are a virtually homogeneous cell population with a minimal number of contaminating smooth muscle and endothelial cells. This approach avoids the prolonged time in culture required when so-called "muscle fibroblasts" are produced by other methods; e.g., sequential passage of primary myogenic cultures or similar passage of enriched non-myogenic cell populations obtained from muscle cell preparations by differential attachment (Yaffe 1969). Studying the myogenic fraction isolated by Percoll from embryonic muscle, we have demonstrated that this population is comprised predominantly of myoblasts but contains some additional cells that resemble fibroblasts in both mass and clonal cultures. In this communication we further characterize the muscle fibroblasts. We demonstrate that these cells possess receptors for platelet-derived growth factor (PDGF), a characteristic property of fibroblasts from many other tissues (reviewed by Bowen-Pope et al. 1985). In addition, we detected several proteins in these cells that were not expressed by myogenic cells from the same embryonic muscle and demonstrated that the freshly isolated fibroblasts are large cells with a very distinct cytoplasm which contains many vesicles.

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Materials and methods

Source of cells. Ten-day-old chicken embryos (White Leghorn) were used throughout this study. 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. Single cell suspensions were obtained by enzymatic digestion of the breast muscles (Yablonka-Reuveni and Nameroff 1987). Briefly, muscles from 6–12 embryos were excised, finely minced, incubated in trypsin (GIBCO, adjusted to 0.1% final concentration) for 30–45 min at 37° C, and centrifuged at approximately 300 g for 5 min. The trypsin solution was decanted and the pellet was resuspended in 5–10 ml of standard medium. The cell suspension was recentrifuged as above and 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 and immediately subjected to density centrifugation as described below.

Density centrifugation. Percoll density centrifugation was performed to separate a fibroblast-like cell population and a myogenic cell population as described (Yablonka-Reuveni and Nameroff 1987). Percoll (Pharmacia) was made isoosmotic by adding 1 part of 10× concentrated MEM to 9 parts of Percoll. Further dilution of the 90% Percoll was made with 1× MEM. Centrifugation was performed in 15 ml Corex tubes treated for 2 h with horse serum to minimize adherence of cells to the walls. About 11.5 ml of 20% Percoll in MEM were layered over a cushion of 60% Percoll (1.5 ml). The cell suspension (approximately 5×10^7 cells in 2 ml standard medium per tube) was then layered over the 20% Percoll and the sample was centrifuged for 5 min at 15000 g at 8° C with brakes off in a fixed angle rotor (Sorvall SS-34). Following centrifugation, fibroblast-like cells (containing less than 1% myogenic precursors) were recovered from the 20% Percoll region. A cell population that contained skeletal myogenic precursor cells was recovered from the 20/60% Percoll interface. Based on clonal assays, the latter myogenic fraction consisted of about 70% myogenic cells, whereas the remaining 30% did not express myogenic traits, e.g., expression of muscle specific proteins and myotube formation (Yablonka-Reuveni and Nameroff 1987). Cells were recovered from the Percoll solutions by centrifugation (300 g, 10 min) following dilution of the fractions with 5 volumes of complete medium. Cell pellets were resuspended in 2–5 ml of complete medium, counted in a hemocytometer and cultured as described below.

Cell culture. Cells were plated onto 35-mm tissue culture dishes coated with 2% gelatin and preincubated for 3 h with 25% horse serum in MEM to promote cellular adherence. Cells were plated at 6×10^4 cells in 1.5 ml of standard medium and the medium was changed 24 h after plating and every other day thereafter. Cultures were maintained at 37.5° C in a water-saturated atmosphere containing 5% CO_2 in air. For photographic purposes, cultures were fixed with 70% ethanol:formalin:acetic acid (20:2:1) as previously described (Yablonka-Reuveni and Nameroff 1986, 1987).

PDGF binding assay. Cells were plated onto 24-well trays in standard medium at 10^4–5×10^4 cells per well. After 1–3 days the cultures were rinsed with MEM and incubated for 16 h in medium lacking PDGF (2% bovine plasma-derived serum and antibiotics in MEM). Specific binding of ^{125}I-PDGF was determined as previously described (Bowen-Pope and Ross 1985). Briefly, cultures were exposed at 4° C for 3 h to increasing amounts of ^{125}I-PDGF in binding buffer (Ham's medium F-12 buffered at pH 7.4 with 25 mM HEPES and supplemented with 0.25% crude BSA). Cultures were then rinsed with cold binding buffer and cell-bound ^{125}I-PDGF was solubilized with 1% Triton X-100 in H_2O with 0.1% BSA and measured with a gamma counter. Non-specific binding of PDGF was determined by exposing cells to labeled PDGF with an excess of unlabeled PDGF and was substracted to determine the amount of specifically bound label. Labeled and unlabeled PGDF, prepared from human platelets as previously described (Raines and Ross 1982; Bowen-Pope and Ross 1982), were generous gifts of E. Raines and R. Ross.

Extraction and analysis of cellular proteins. Fibroblast-like and myogenic cell populations were recovered from the Percoll regions as described above except that MEM was used for Percoll dilutions and cell washes. This was done to eliminate possible contribution of proteins from the medium. Aliquots were removed from the final cell suspensions for cell counts and control cultures. The rest of the cells were subjected again to centrifugation and the cell pellets were resuspended on ice in NP-40 buffer (250 mM NaCl, 10 mM Tris, 5 mM $MgCl_2$, 2 mM N-ethylmaleimide, 1% Nonident P-40, pH 7.4); 0.03 ml of NP-40 buffer was used for 10^6 cells. This extraction procedure breaks open the cell membrane but leaves nuclei and mitochondria intact. The cell extracts were then centrifuged at 12000 *g* for 10 min at 4° C using a swinging bucket rotor. The supernatant (soluble) fractions were removed and urea was added to a final concentration of 8 M. The pellet (particulate) fractions, which included the nuclei, were suspended in NP-40 buffer containing 8 M urea (0.02 ml buffer for each original 10^6 cells). Fractions were kept frozen at −70° C until further analyzed. Two dimensional polyacrylamide gel electrophoresis was performed as described by O'Farrell (1975). For isoelectrofocusing, the procedure decribed by Bravo (1984) was used (10 ml of gel mixture including 0.6 ml of ampholyte, pH 5–7, and 0.12 ml of ampholyte, pH 7–9). The gels were 11 cm long. The second dimension was carried out in a Hoeffer SE 700 multigel unit according to Laemmli (1970) employing 10–20% acrylamide gradient gels and 0.1% SDS. Electrophoresis was conducted at 45 mA per slab gel until the bromophenol blue front emerged from the gel. Gels were fixed and stained with a solution containing 50% methanol, 7.5% acetic acid and 0.1% Coomassie Brilliant Blue. The gels were then extensively rinsed with a solution containing 5% methanol and 7.5% acetic acid followed by several rinses with 30% ethanol. They were then silver stained according to the procedure of Heukeshoven and Dernick (1985). The double staining enhances the detection level of minor proteins that were not easily visualized by each individual stain alone. Gels became extremely fragile during the double staining procedure. This often produced cracks as those seen in Fig. 3.

Electron microscopy. Fibrogenic and myogenic cell populations were collected from the Percoll regions with complete

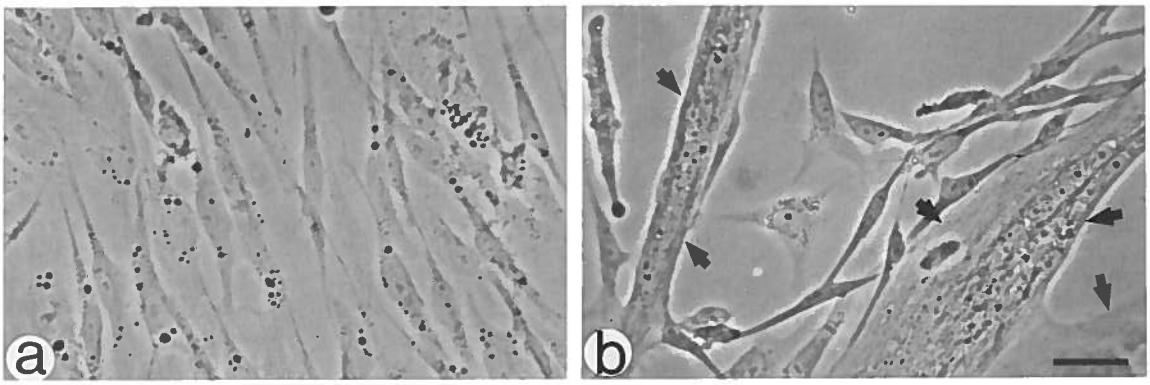


Fig. 1 a, b. Cultures of fibroblast and myogenic cell populations isolated from the breast muscle of 10-day-old chicken embryos using Percoll. **a** Muscle fibroblasts cultured for 3 days. **b** A myogenic precursor population cultured for 3 days. *Arrows* indicate myotubes. Bar = 30 μm; × 300

medium as described for cell cultures and then maintained for an additional 90 min in complete medium at room temperature to allow cell recovery. Cells were then washed twice with MEM, pelleted, and fixed overnight at 4° C in a mixed aldehyde fixative (2% paraformaldehyde, 2% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.4). After buffer washes, cells were post-fixed for 1 h in 1% aqueous OsO_4. To facilitate further handling cells were pelleted in Low Melting Point Agarose (Bethesda Research Laboratories, Rockville, MD), dehydrated in ethanol and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips 201 TEM operated at 60 kV.

Results

PDGF binding. The appearance of fibroblast-like cells and myogenic cells in culture following their isolation by Percoll is shown in Fig. 1. We have previously demonstrated that the fibroblast-like cells first exhibit a flattened, stellate morphology and contain punctate material, but at more dense concentrations become packed and elongated (Yablonka-Reuveni and Nameroff 1987). By measuring the concentration dependent binding of ^{125}I-PDGF to cell cultures we determined that the fibroblast cultures but not the myogenic cultures express saturable, high-affinity binding sites for ^{125}I-PDGF (Fig. 2). The fibroblast-like cells exhibited such binding regardless of culture age and cell density (data not shown). Myogenic cultures, on the other hand, did not exhibit binding regardless of the differentiative stage of the cells or the cell density. As shown in Fig. 2, neither myoblasts (2-day-old culture) nor myotubes (4-day-old cultures) possessed detectable PDGF binding sites.

Two-dimensional gel analysis. Our earlier studies, employing one-dimensional SDS-polyacrylamide gel electrophoresis, suggested that the two Percoll-isolated cell populations may express several different polypeptides (Yablonka-Reuveni and Nameroff 1987). In the present study we extended these analyses employing two-dimensional gel electrophoresis. As in the previous study, the cell extracts were fractionated into soluble (cytoplasmic) and particulate (nuclear) compartments. We performed this fractionation to obtain some indication of the cellular localization of potential cell-specific proteins. To ensure that the results were reproducible,

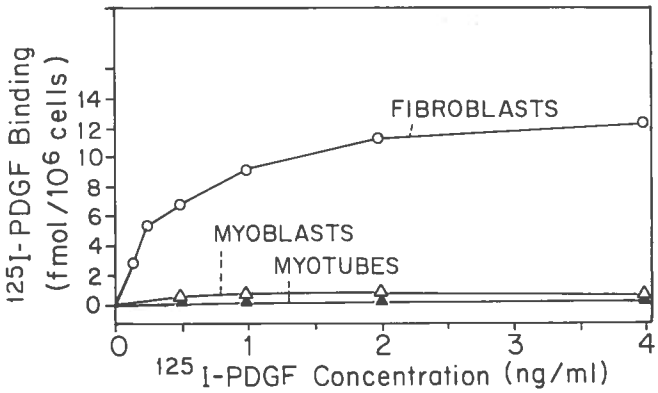


Fig. 2. Specific binding of ^{125}I-PDGF to fibrogenic and myogenic cultures. Cells were cultured in complete medium and about 16 h prior to the PDGF binding assay the medium was replaced with MEM supplemented with 2% bovine plasma-derived serum to eliminate exogenous PDGF. At the time of the assay fibroblast and myoblast cultures were 40-h old; myotube-containing cultures were 88-h old. The plotted values represent the average binding to triplicate cultures. Cell numbers were determined on parallel cultures; cell numbers for myogenic cultures were calculated based on the number of mononucleated cells and the number of nuclei within myotubes in sampled microscopic fields

we performed at least six independent analyses of this kind using different preparations of cells. Figure 3 demonstrates a typical two-dimensional gel analysis of the freshly isolated fibroblast population and the predominantly myogenic cell population following the fractionation of the populations to particulate and soluble cell compartments. By comparing the soluble and particulate compartments of the two cell populations we could identify at least six polypeptides in the fibroblasts, all but one in the soluble compartment (marked by arrows in Fig. 3b and d), which were not detected in the myogenic cell fraction. This is a conservative estimate of the number of different polypeptides since only polypeptides in regions with comparable staining intensity in both the fibroblast and the myoblast gels are indicated by arrows in the figure. We routinely applied to the gels samples representing equal numbers of cells and observed that some regions in the gels of the two different populations stained with different intensity. This suggested that some proteins, shared by the different populations, do not exist in the same concentration in the two cell types. Differ-

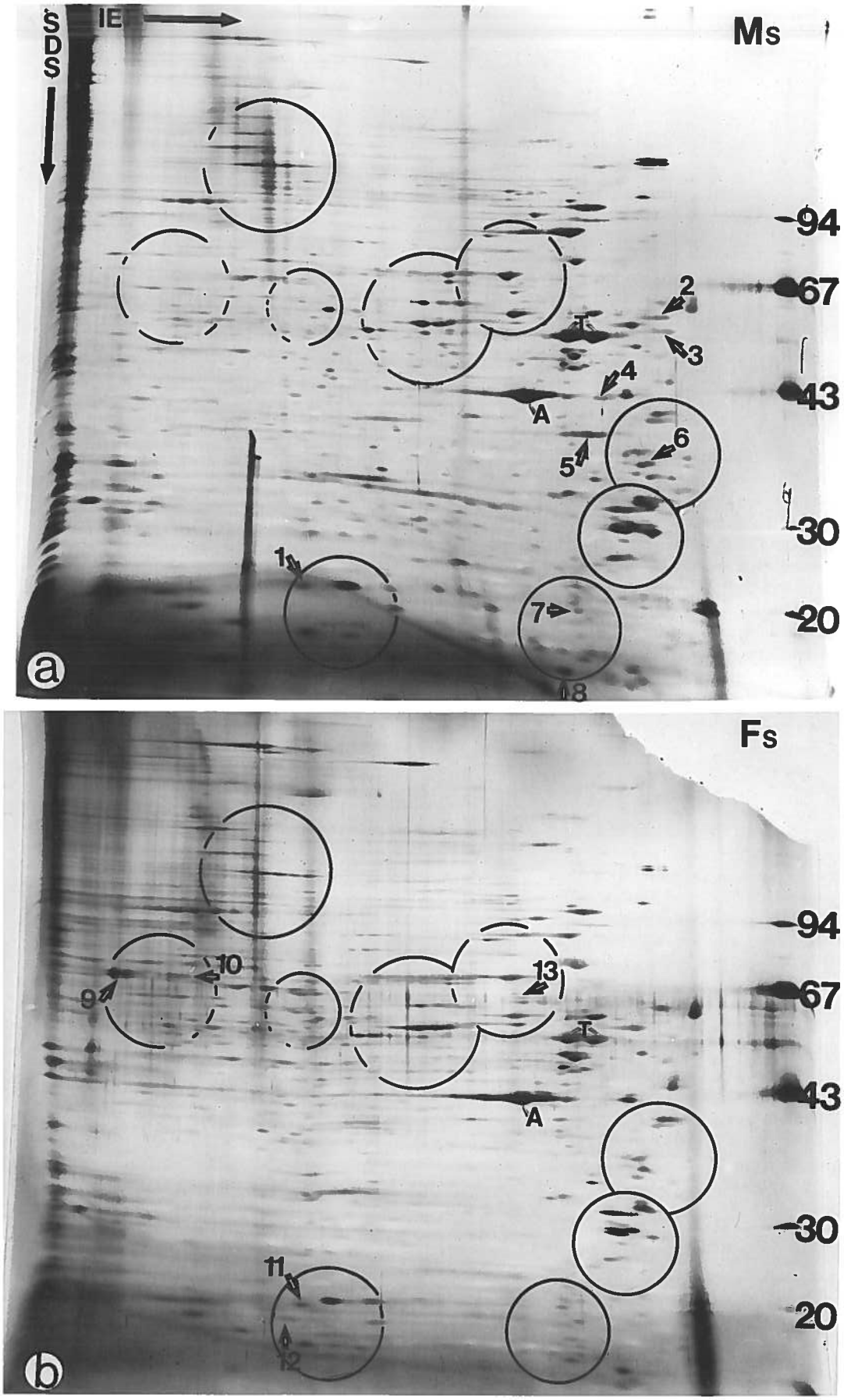
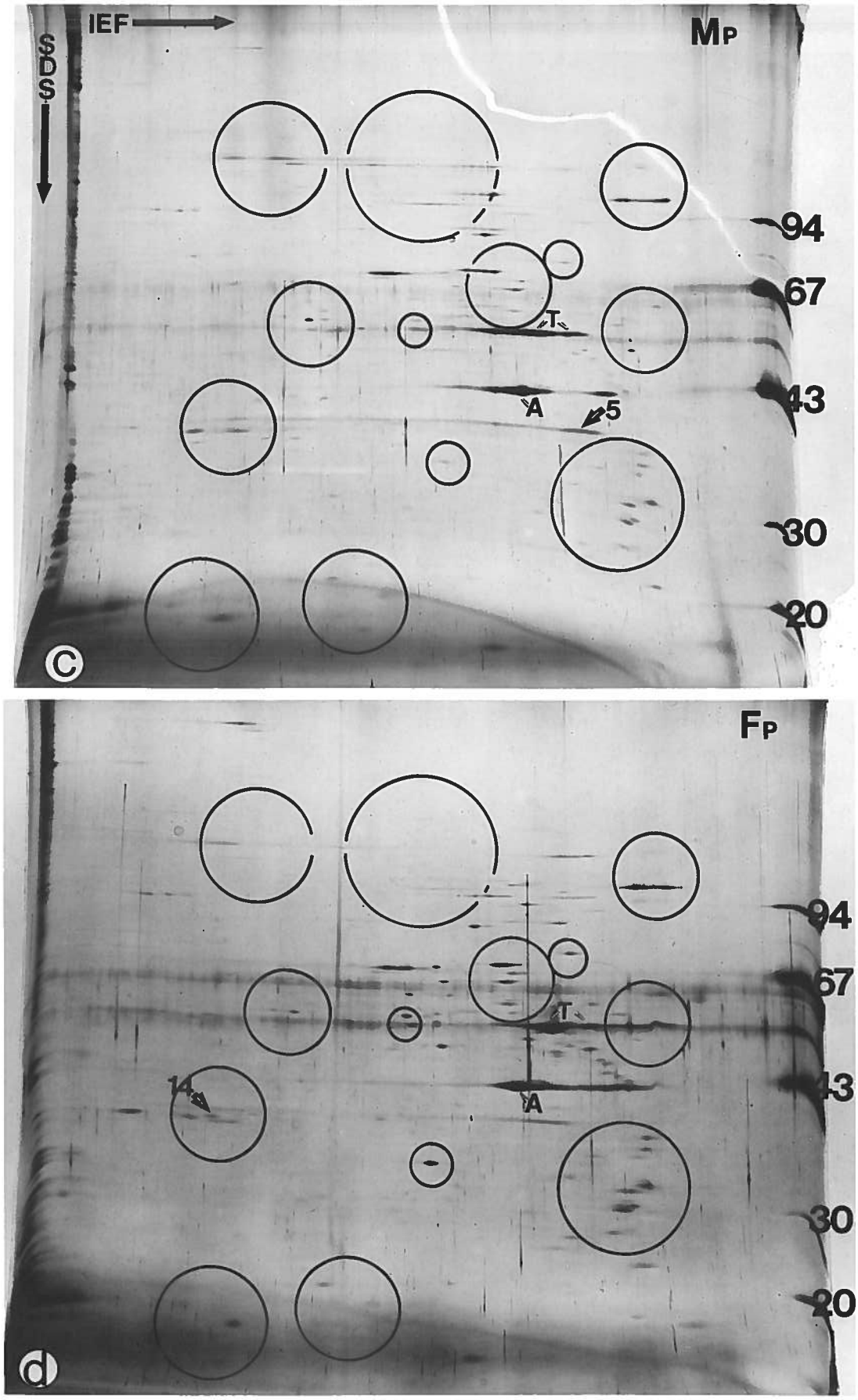


Fig. 3a–d. Two-dimensional polyacrylamide gel electrophoresis analyses of the fibroblast and the myogenic cell populations. Freshly-isolated cell populations were obtained by Percoll centrifugation. The cells were fractionated to soluble and particulate compartments using an extraction buffer containing Nonidet P-40. For each sample the equivalent of about 5×10^5 cells was applied. Gels were sequentially stained with Coomassie Brilliant Blue and silver. **a** and **c** Soluble and particulate compartments of the myogenic cell population. **b** and **d** Soluble and particulate compartments of the fibroblast cell population. *Ms* and *Fs* soluble compartments of the



myogenic and fibrogenic cell populations, respectively; *Mp* and *Fp* particulate compartments of the myogenic and fibrogenic cell populations, respectively. *Circled areas* indicate identical regions in *Ms* and *Fs* samples or in *Mp* and *Fp* samples and can be used as landmarks for protein pattern comparison. *Numbered arrows* indicate population-specific polypeptides; *A* and *T* indicate the migration position of actin and tubulin, respectively. The migration positions of known molecular weight markers ($\times 10^{-3}$) are indicated to the right

ences in staining intensity were also observed when equal amounts of proteins rather than equal numbers of cells were anlyzed by gel electrophoresis. In addition, by limiting the analysis to regions with comparable staining intensity, we could identify at least eight polypeptides in the myogenic cell population (marked by arrows in Fig. 3a and c) that were not detected in the fibroblasts or appeared in very reduced amounts. The latter were all detected in the soluble compartment of the myogenic fraction. One of these polypeptides (number 5) seems to appear in both the soluble and particulate compartments.

Ultrastructure. The ability to separate fibroblasts by density centrifugation indicated that these cells are less dense than those recovered from the 20/60% interface. Also, our preliminary studies using light microscopy suggested that the Percoll-isolated fibroblasts were larger than the cells in the myogenic population. To detect other possible differences between the fibroblasts and myoblasts and to analyze the heterogeneity of the myogenic population, we examined cell suspensions of the Percoll-isolated populations by electron microscopy (Fig. 4). The results indicated that the fibroblast population was homogeneous in regard to the morphology of the cells. The average diameter of the vast majority of the cells in this population (Fig. 4a) was about 1.7–2 times larger than that of the cells in the myogenic fraction (Fig. 4b). The fibroblast-like cells contained large numbers of vesicles in their cytoplasm. In addition, these cells contained multivesicular bodies, numerous Golgi apparatuses, and cytoplasmic filaments (Fig. 4c). Cells with morphologies other than the above were rare in the fibroblast preparation (less than 5% of the total cells). Cells in the myogenic fraction were smaller than the fibroblasts and the majority of these cells were lacking vesicles altogether or contained only a few small vesicles. In addition, the majority of the cells in the myogenic population had dense cytoplasm with numerous polyribosomes, one or two Golgi apparatuses, and rare if any filaments in the cytoplasm. Some cells in the myogenic fraction contained a higher number of larger vesicles (Fig. 4b) and had ultrastructural features like those described above for the fibroblast population. These cells, which amount to about 10–15% of the total, probably represent the fibroblast-like cells co-purified with the myoblasts in the Percoll centrifugation. It is notworthy that when cultures were prepared from the Percoll-isolated populations we routinely observed plating efficiency of 80–90%. Therefore, the different morphologies of the cells can not be attributed to differing viability of the cells in the two populations.

Discussion

The present study is a continuation of our attempt to characterize the fibroblasts in embryonic skeletal muscle. We have demonstrated in the current study that the muscle fibroblasts but not the myoblasts possess binding sites for PDGF. We have also identified several proteins that are expressed by fibroblasts but not by the myoblasts. Moreover, we have shown that the cytoplasm of the fibroblasts is less dense than that of the myoblasts and is highly enriched with vesicles. The above characteristics offer new tools for the identification of muscle fibroblasts which, in the past, has been most often based on cell morphology. Moreover, the analysis was performed with freshly isolated cells or cells that were cultured for a short time only. Therefore the characteristic properties detected in the current study should closely reflect the in vivo status.

Receptor-mediated binding of PDGF is a characteristic property of fibroblasts from different tissues (Bowen-Pope et al. 1985). Hence, the findings that the Percoll-isolated muscle fibroblasts express PDGF receptors suggest that these cells are likely to represent "true" fibroblasts. Endothelial cells and smooth muscle cells, can be detected in the muscle vascular system (Yablonka-Reuveni and Nameroff 1986, 1987). Similar to fibroblasts, the latter cells also express PDGF receptors (Bowen-Pope et al. 1985). However, we have shown that the contribution of the endothelial and smooth muscle cells to the fibroblast cell population is minimal and amounts to less than 0.1% of the cells (Yablonka-Reuveni and Nameroff 1987). Our findings that the Percoll-isolated myogenic cell population does not possess detectable binding sites for PDGF confirms the findings of Hauschka, Bowen-Pope and coworkers who could not detect PDGF binding by myogenic cultures from embryonic human and from a mouse cell line (Linkhart et al. 1982; Bowen-Pope et al. 1985).

As previously reported, the Percoll-isolated myogenic fraction also contains some non-myogenic cells (Yablonka-Reuveni and Nameroff 1987). These cells resemble, in their spread-out morphology and punctate cytoplasm, the muscle fibroblasts isolated by Percoll. If these non-myogenic cells are indeed identical to the isolated fibroblasts then the Percoll-isolated myogenic cell population should express some PDGF binding activity. However, it is possible that the number of non-myogenic cells in the myogenic cultures is insufficient to allow detection of significant binding. This is supported by our ability to demonstrate saturable, high-affinity PDGF binding to cultures prepared by sequential passage from the primary Percoll-isolated myogenic culture (Yablonka-Reuveni and Bowen-Pope, unpublished). The latter cultures are devoid of myogenic cells and the "contaminating" fibroblasts become dominant.

The two-dimensional gel analyses revealed at least six polypeptides that were specific to the fibroblasts and not shared by the myoblasts. The ability to detect fibroblast-specific polypeptides clearly demonstrates biochemical differences between the muscle fibroblasts and myoblasts. A comparison of protein secreted by cultured human skin fibroblasts with those secreted by human muscle cultures has been described (Graham et al. 1984). This latter study also detected several fibroblast-specific polypeptides but did not rule out the possibility that the fibroblast-like cells from skin differed from muscle fibroblasts. In contrast to the latter study, we compared myoblasts with fibroblast-like cells from the same tissue and organismal age. Moreover, the fibroblasts in the current study were obtained directly from the tissue and not by sequential passages and prolonged maintenance in cultures. The fact that we could not identify the fibroblast-specific polypeptides in the myogenic fraction suggests that the contribution of the non-myogenic cells to the myogenic fraction is too small to be detected by gel electrophoresis. Also, the electron-microscopic studies support the idea that the contribution of fibroblast-like cells to the myogenic fraction is small. Therefore, despite the contribution of the non-myogenic cells, we suggest that the specific polypeptides in the myogenic fraction represent polypeptides of myoblasts. Surprisingly, despite differences in species and methodology, a polypeptide similar in its

migration position to polypeptide number 5 (Fig. 3a) was detected in myoblast cultures from the calf (Whalen et al. 1976).

Ultrastructural studies have established a morphological difference between the Percoll-isolated fibroblasts and myoblasts. Also, these studies suggested that the differences in size and cytoplasmic structure (presence of vesicles) between muscle fibroblasts and myoblasts contributed to our ability to separate these two cell populations by Percoll density centrifugation. The cells in the fibroblast fraction were large and contained many vesicles, whereas over 85% of the cells in the myogenic fraction exhibited dense cytoplasm and contained either one or two small vesicles or none at all. The nature of the vesicles in the fibroblasts is not clear. We observed such vesicles even when the cells were exposed to the aldehyde fixative for short period of times (up to one hour). Furthermore, the appearance of numerous regions of rough endoplasmic reticulum and Golgi apparatuses in the fibroblasts (Fig. 4c) do not support the posssibility that the vesicles represent such structures which might have been damaged during fixation. Also, we observed enlarged vesicles in the fibroblasts using high-magnification light microscopy avoiding any fixation. Nevertheless, it is possible that the cell isolation procedure (e.g., trypsination and/or exposure to Percoll) gave rise to these vesicles. However, the fact that we could culture these cells at high plating efficiency (80–90%) clearly demonstrates that they were viable after isolation. Even if the vesicles are a result of the isolation procedures these procedures affect the different cell types in the muscle and the morphologies reflect a true difference beetween the myogenic and fibrogenic cells. Also, Lipton (1977) studied the fine structure of different cell types in avian myogenic cultures and suggested that cultured muscle fibroblasts but not myoblasts are highly enriched with vesicles. Furthermore, the additional ultrastructural characteristics described in this study for fibroblasts and myoblast are in agreement with those reported by Lipton (1977) for cultured cells.

Our cell culture studies have previously indicated that, of the clonable cells in the Percoll-isolated myogenic fraction, about 70% could give rise to large myogenic clones (Yablonka-Reuveni and Nameroff 1987). The current electron-microscopic studies suggested that 10–15% of the cells in the Percoll-isolated myogenic fraction had a fibroblast-like morphology. Hence, both cell culture and structural analysis suggest that the Percoll-isolated myogenic fraction is heterogeneous but that myoblasts are predominant. Cell culture gives a lower estimate of myogenic cells because some of the cells may give rise to myogenic clones only in the presence of additional factors such as conditioned medium (Hauschka 1974; White et al. 1975). In addition, the estimate based on the cell culture is less accurate because it relied on clonable cells only.

We conclude, that the Percoll-isolated fibroblasts can be categorized as "true" fibroblasts based on the findings that these cells possess receptors for PDGF. The latter property is not expressed by myoblasts. Moreover, the isolated fibroblasts contain several proteins that are not expressed by myogenic cells and exhibit a different cytoplasmic structure then the myoblasts. Some of the fibroblast characteristics can now be used as markers to identify embryonic muscle fibroblasts and may help to shed some light on the lineage relationships of the fibrogenic and myogenic cells.

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