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Possible Involvement of Folliculo-Stellate Cells in the Differentiation of Muscle Fibers during Monolayer Culture of Pituitary Cells

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ABSTRACT—A major objective of the present study was to examine the possibility that non-granular folliculo-stellate (FS) cells in the rat anterior pituitary are involved in the myogenesis that occurs during pituitary cell culture. Enzymatically dissociated anterior pituitary cells were fractionated by use of the Percoll gradient method. The proportion of FS cells was 5.8% on average before cell fractionation. After employing the Percoll gradient procedure, FS cells were enriched to a ratio of 12.2%. Three of five cell fractions were separately cultured, and the incidence of striated muscle fibers was quantitatively investigated. There was a good correlation between the numbers of muscle fibers and the proportions of FS cells in the fractions obtained from the Percoll gradient. These results suggest that FS cells are the cells that transform into striated muscles in pituitary monolayer cultures.

INTRODUCTION

Striated muscle fibers sometimes appear in monolayer cultures of non-muscular tissues (Watanabe *et al.*, 1981; Wekerle *et al.*, 1975). Of particular interest is the fact that differentiation of muscle fibers has been reported in monolayer cultures of rat anterior pituitary cells (Brunner and Tschank, 1982). After 5 to 6 days of culture of pituitary cells, spindle-shaped cells first appear and begin to fuse to form myotubes. Subsequently, these myotubes increase rapidly in size and number and differentiate into muscle (Brunner and Tschank, 1982; Spira *et al.*, 1988; Watanabe, 1989). To date, however, the cellular origin of such striated muscle fibers in pituitary monolayer cultures is still unknown. Inoue *et al.* (1987) reported the appearance of muscles in rat pituitary glands transplanted beneath the kidney capsule. Ultrastructural observations suggested close topological and cytological relationships between those striated muscles and folliculo-stellate (FS) cells. FS cells are a non-granular component of the adenohypophysis of a wide range of animal species (Dingemans and Feltkamp, 1972; Forbes, 1972; Gracia-Navarro *et al.*, 1983; Harrisson, 1978; Yamamoto *et al.*, 1982) and have some cytological characteristics in common with neuroglia, hence they are supposed to be derived from neuroectoderm (Cocchia and Miani, 1980; Velasco *et al.*, 1982). In view of the proposal that cells of neuroectodermal origin may have a myogenic potency (Lennon and Peterson, 1979; Nakamura *et al.*, 1984), it was considered worthwhile to investigate whether FS cells are the progenitor of striated muscle. At present, it is not technically possible to prepare a culture of separated individual FS cells. In this study, therefore, the Percoll gradient method was used in an attempt to obtain an FS cell-enriched fraction (Vankelecom *et al.*, 1989). The goal of the present experiment was to compare the incidence of striated muscle fibers in pituitary cell cultures that were rich or poor in FS cells.

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MATERIALS AND METHODS

Cell preparation

Pituitary glands were obtained from 3- to 4-month-old female rats of the Sprague-Dawley strain. After decapitation of the rats and complete removal of surrounding tissues, the pituitaries were placed in a Petri dish containing Dulbecco's phosphate buffered saline (DPBS, pH 7.4, without Ca^{2+}). During the subsequent surgical procedures, forceps and scissors were changed at each step to avoid contamination of pituitary cells with surrounding tissues. The neurointermediate lobes were removed with fine forceps under a dissecting microscope. Then the anterior pituitaries were transferred to a new dish containing DPBS and cut into small pieces approximately 1 mm in diameter with the use of iris scissors. These tissue pieces were transferred to a centrifuge tube. After the tissue fragments settled, the supernatant was discarded and the fragments were treated with a mixture of 0.25% trypsin (type IX), 0.0025% DNase I (type II), and 2 mM EDTA in DPBS-0.1% BSA (fraction V) at 37°C for 1 hr. Then the tissue blocks were dispersed into single cells by gentle pipetting. The dissociated cells were collected by centrifugation at 200 × g for 5 min at room temperature and subsequently treated with 1.25% collagenase (type V) and 1.25% hyaluronidase (type V) in DPBS containing 2 mM EDTA at 37°C for 30 min. All of these reagents were purchased from Sigma. Following gentle pipetting and centrifugation at 200 × g for 3 min, the cell pellet was resuspended in DPBS containing 2 mM EDTA. This procedure was repeated twice in order to remove the enzymes. The cell suspension was then filtered through gauze and centrifuged. Finally, the cell pellet was resuspended in α minimum essential

medium (αMEM, Gibco) supplemented with 300 μg/ml glutamine (Nissui), 10 μg/ml transferrin (Sigma), 5 μg/ml penicillin, 10 μg/ml streptomycin, 2 mg/ml $NaHCO_3$ (Wako), 5 μg/ml insulin, and 100 ng/ml cortisol (Sigma). Cell recovery was estimated with the aid of a hemocytometer, and cell viability was measured by the trypan blue exclusion test. With this method, the total cell yield was approximately 4.0×10^6 cells per anterior pituitary and viability was always better than 95%.

Cell separation

A 9 ml discontinuous Percoll gradient was prepared in a 10 ml centrifuge tube, as described by Vankelecom *et al.* (1989) with a modification. Nine volumes of Percoll (Pharmacia) were diluted with 1 volume of 10-fold Hanks solution to produce an iso-osmotic solution. Then this solution was diluted with DPBS to make 20, 30, 40, 50, 60, and 70% Percoll solutions. The volume of each concentration of Percoll was 1.5 ml. One ml of αMEM containing $2.5\text{-}3.0 \times 10^7$ cells was placed on the top of the gradient. After centrifugation at 650 × g for 20 min in a swing-out rotor at room temperature, cell layers at interfaces were carefully harvested and washed twice with DPBS to remove the Percoll. These cells were finally resuspended in αMEM for use in the procedures described below. The proportions of each cell type in each fraction were estimated by immunocytochemistry. For this technique a cell suspension was dropped onto a slide and allowed to dry at 35°C for 1 hr. The attached cells were fixed in Bouin's solution for 10 min and washed with 70% ethanol overnight at room temperature. After rinsing in 0.02 M phosphate buffered saline (PBS, pH 7.4) for 30 min, the cells were stained by the immunocytochemical method, as will be described below.

Cell culture

In this experiment, cell cultures were confined to the central parts of dishes since myogenesis was seen most frequently at areas where cells were very sparse. Dissociated cells (1.2 to 1.5×10^5) were first seeded in a drop (100 μl) of αMEM that was applied to the center of 35 mm tissue culture dishes (Nunc). About 2 ml of the culture medium containing 10% fetal bovine serum (Gibco) was gently added the following day. Dishes were maintained at 37°C in a humidified atmosphere of 5% CO_2. The culture medium was changed every 4 days and cultures were maintained for 4 weeks. The resulting monolayers were then washed twice with DPBS, fixed in Bouin's solution for 10 min and washed with 70% ethanol overnight. After rinsing with distilled water, they were stained for 10 sec with Halmi's trichrome solution (Halmi, 1952), washed with 100% ethanol and mounted in glycerin. Finally, the number of differentiating striated muscles was counted for each fraction and expressed as a mean value per dish. In this study, a myotube is defined as a multinucleated syncytium without any cross striations, whereas a muscle is defined as one with clear striations.

Immunocytochemistry

To detect growth hormone (GH), prolactin (PRL), luteinizing hormone β subunit (LHβ), and adrenocorticotropic hormone (ACTH), the peroxidase-antiperoxidase complex (PAP) method was employed. Briefly, cells were treated at room temperature in the following sequences: 1) the primary antisera (1,000 × in PBS); anti-rat GH and PRL (HAC-RT25-01-RBP85 and HAC-RT26-01-RBP85, respectively, gifts from Dr. K. Wakabayashi, Gunma University), anti-ovine LHβ (1,000 ×, this laboratory), anti-human ACTH (1,000 ×, NIAMDD) for 2 hr; 2) guinea pig anti-rabbit IgG (100 ×) for 1 hr; 3) rabbit PAP complex (100 ×, Dakopatts) for 30 min. For staining of thyroid stimulating hormone (TSH) β subunit and S-100 protein, the strepto-avidin-biotin method (SAB, Nichirei) was used. The SAB method was performed by treating sections with the following reagents: 1) 0.3% H_2O_2 in distilled water for 10 min; 2) goat serum for 10 min; 3) the primary antisera, anti-human TSHβ (1,000 ×, NIAMDD) and anti-bovine S-100 protein (200 ×, Dakopatts) for 1 hr; 4) biotinylated goat anti-rabbit IgG for 10 min; 5) peroxidase conjugated streptavidin for 5 min. Finally, the reaction product was visualized with 3,3'-diaminobenzidine tetrahydrochloride in 0.05 M Tris buffer (pH 7.6) containing H_2O_2. The immunospecificity of these antisera have been already described (Carbajo-Perez *et al.*, 1989; Watanabe and Carbajo-Perez, 1990; Watanabe,1991). For each fraction 200 to 400 cells per slide were counted. The number of immunostained cells was expressed as a percentage of the total number of cells.

Electron microscopy

Monolayers were washed twice with DPBS, fixed in 2.5% glutaraldehyde in 0.07 M DPBS at room temperature for 1.5 hr and then postfixed in 1% OsO_4 in the same buffer for 1 hr. After dehydration in an ascending ethanol series, cells were embedded in a mixture of Epon and Araldite. Ultrathin sections were stained with lead citrate and observed with a Hitachi H-300 electron microscope.

RESULTS

By centrifuging the Percoll gradient that contained dissociated pituitary cells several cell fractions were obtained. Those fractions that contained only a small number of pituitary cells were discarded. The majority of the anterior pituitary cells were found in three fractions, which were designated fractions 3, 4 and 5. Cell viability of these fractions exceeded 93% as measured by the trypan blue exclusion test. The cellular composition of each fraction was examined by immunocytochemistry (Table 1). The percentage of FS cells was 5.5 ± 0.6% before fractionation. After fractionation, it increased to 12.2 ± 1.8% in Fraction 3. This value was approximately 2.2 times as high as that of the unfractionated cells. In fractions 4 and 5, FS cells scored 4.8 ± 1.0% and 2.7 ± 1.4 %, respectively. The majority of the cells (70 to 80%) in these fractions consisted of GH and PRL cells.

As stated above, cells from these fractions were cultured

Table 1. Proportion of anterior pituitary cells before and after discontinuous Percoll gradient

	Interface (% of Percoll)	S-100	PRL	GH	ACTH	TSHβ	LHβ	Unstained
Unfractionated		5.5 ± 0.6	47.8 ± 2.0	30.9 ± 2.1	2.9 ± 0.5	2.2 ± 0.3	3.7 ± 0.4	6.9 ± 1.7
Fraction 3	30/40	12.2 ± 1.8	66.9 ± 1.1	8.6 ± 0.8	3.1 ± 1.3	0.5 ± 0.1	2.1 ± 0.4	5.1 ± 4.2
4	40/50	4.8 ± 1.0	62.3 ± 6.7	13.2 ± 5.4	3.7 ± 0.4	3.0 ± 0.9	8.0 ± 0.4	4.7 ± 1.8
5	50/60	2.7 ± 1.4	10.5 ± 0.3	73.2 ± 2.9	1.3 ± 0.4	1.1 ± 0.5	2.1 ± 0.3	8.7 ± 1.6

Fractions were numbered from top to bottom. Fractions 1, 2 and 6 are not shown because they contained only small number of cells. Red blood cells sedimented at the bottom. Values are represented as mean ± SE of 3-5 experiments.

as monolayers for 4 weeks. Striated muscles differentiated in all fractions (Fig. 1a, b). Electron microscopic observation confirmed the formation of myofibrils in their cytoplasm (Fig. 1c). When the incidence of striated muscles was determined, there was a good correlation between the incidence of FS cells and that of striated muscles. Representative results are shown in Fig. 2. Monolayer cultures from fraction 3 were found to contain the greatest number of striated muscles. On the other hand, cultures from fractions 4 and 5 which contained a low percentage of FS cells had a small number of muscles fibers. To determine whether Percoll fractionation itself might affect the differentiation ability of pituitary cells, the incidence of muscles in cultures of simply dispersed cells was compared with that in cultures of fractionated cells that were recombined. In both cases myogenic cells appeared after 1 week of culture. The incidence of striated muscles was very similar in the two groups of cultures (data not shown).

DISCUSSION

Because the incidence of cell proliferation is very low in the normal pituitary both *in vivo* (Carbajo-Perez *et al.*, 1989) and *in vitro* (Watanabe and Carbajo-Perez, 1990), the routine methods for cloning culture of a given type of cell are unsuccessful. In most of the techniques, enzymatically dispersed pituitary cells are separated into several fractions by the use of a Percoll or BSA gradient. Of these two methods, the Percoll gradient is simpler and consequently is widely used by many investigators (Burris and Freeman, 1993; Geffroy-Roisne *et al.*, 1992; Gonzalez de Aguilar *et al.*, 1994; Heyward *et al.*, 1993; Hu and Lawson, 1994; Velkeniers *et al.*, 1994). It must be stressed, however, that these gradient procedures only permit us to obtain enrichment of the respective types of cells. In the present study, FS cells were invariably collected from the lightest fraction as has been found to be true by other investigators (Baes *et al.*, 1987; Tatsuno *et al.*, 1991; Vankelecom *et al.*, 1989). This fraction also contained a considerable population of PRL cells which differs from the results of others. The wide variety in size of secretory granules among subpopulations of PRL cells (Nogami and Yoshimura, 1982; Nogami, 1984) may explain why these cells were recovered from several fractions. As far as FS cells are concerned, especially by use of the discontinuous density gradient, we could obtain fractions with a rich and poor concentration of this type of cell. Our results have shown that there is a good correlation between the incidence of FS cells and that of muscle fibers. Our assumption that in monolayer culture FS cells may transform into muscle fibers came from the observation made by Inoue *et al.* (1987) who found that striated muscles appeared in close topological association with

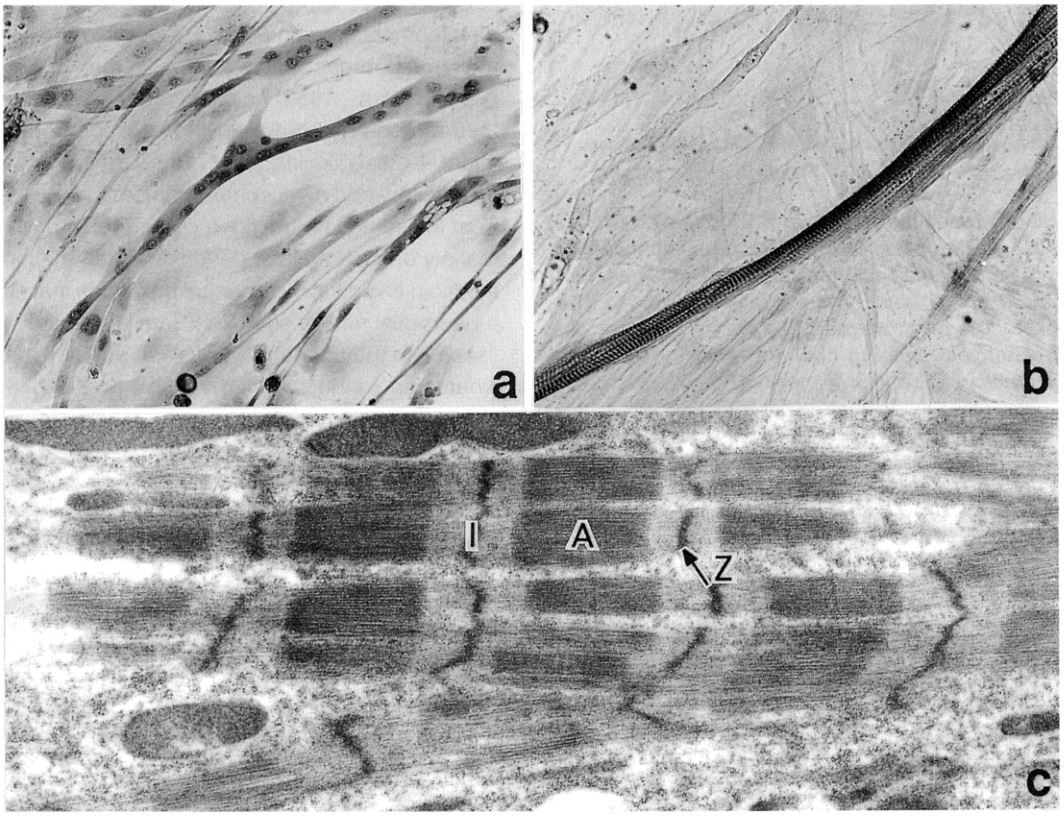

Fig.1. Micrographs of the striated muscles after 4 weeks of culture of pituitary cells. (**a**) Multinucleated myotubes stained with hematoxylin. × 350. (**b**) A striated muscle fiber stained with Halmi's trichrome solution. × 500. (**c**) Electron micrograph of a striated muscle fiber, showing the A-, I-, and Z-bands. × 16,000.

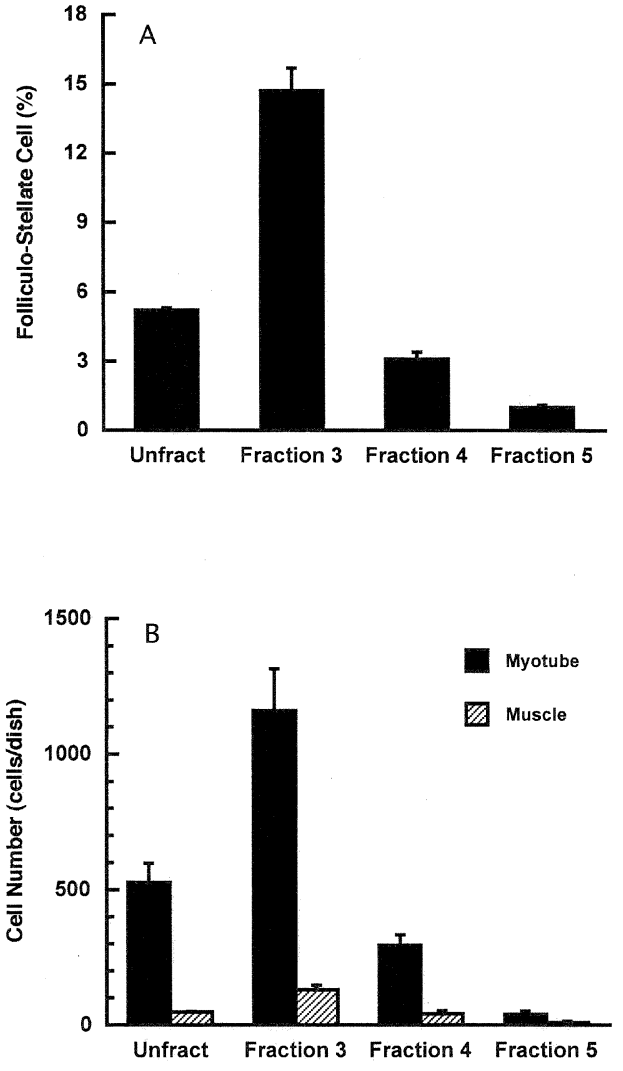

Fig. 2. Correlation between the proportion of folliculo-stellate (FS) cell and that of striated muscle cells in fractions separated by discontinuous Percoll gradient. These data were obtained from a single experiment. Unfract : unfractionated cells. (**A**) The proportion of FS cells in each fraction. FS cells were identified by immunocytochemical staining of S-100 protein. Data are expressed as mean ± SE of 4 specimens. (**B**) The incidence of myotubes and striated muscle fibers in each fraction. Values are represented as mean ± SE of 3-4 samples.

FS cells in rat pituitary glands transplanted under the kidney capsule. Apart from the pituitary gland, other tissues containing S-100 immunoreactive stellate-shaped cells have also been shown to yield striated muscle fibers *in vitro*. Thus in primary cultures of the thymus (Wekerle *et al.*, 1975) and pineal gland (Watanabe *et al.*, 1981), unexpected differentiation of muscle fibers was observed. *In vivo*, too, striated muscle fibers were detectable in the thymus (Henry, 1968) and pineal gland (Krstić, 1972) at the electron microscopic level. In view of the fact that S-100 protein is also detected in skeletal muscles (Haimoto and Kato, 1987; Haimoto *et al.*, 1987; Zimmer, 1991), it is tempting to generalize that tissues containing S-100 positive stellate cells have a myogenic potency.

The adenohypophysis was thought to be formed from an evagination of the oral ectoderm. Chimera experiments between the chick and quail (Couly and Le Douarin, 1987; Le Douarin *et al.*, 1986), however, have demonstrated that the anterior neural ridge is involved in organogenesis of the adenohypophysis. Such a contribution of neural tissue has also been reported in the developing hypophysis of the toad (Kawamura and Kikuyama, 1992). Although no evidence is at present available for mammals, it is highly possible that cells of neural origin are also contained in the adenohypophysis. If this is the case, the appearance of muscle fibers in pituitary cultures is not surprising because cells of nervous tissue are known to have myogenic potency. Lennon *et al.* (1979) observed the appearance of striated muscles in a neuroglial cell line that was derived from a nitrosoethylurea-induced brain tumor of rats (Schubert *et al.*, 1974). There are also several reports that describe the presence of muscles in tumors of the nervous system (Auer and Becker, 1983; Goldman, 1969). Furthermore, striated muscle fibers were found in non-neoplastic tissue of the human central nervous system (Ambler, 1977; Hoffman and Rorke, 1971; Nakamura *et al.*, 1984). According to Nakamura *et al.* (1984), these muscle fibers were found in the proximity of glial cells that are immunoreactive to glial fibrillary acidic protein (GFAP) and S-100 protein.

In view of these findings, FS cells are the most likely candidate for the progenitor of striated muscles. Although the developmental origin of the FS cells is not known at present, their immunoreactivity to neuroglial marker S-100 and GFAP (Velasco *et al.*, 1982) has led some investigators to think that they may be derived from the neurectoderm (Cocchia and Miani, 1980; Nakajima *et al.*, 1980). In the chick embryo, both the pituitary and diencephalic primordia arise as a single entity from the ventral neural ridge (Takor Takor and Pearse, 1975). In view of such a close developmental relationship between the brain and pituitary, it cannot be denied that cells possessing a neuroglial nature such as FS cells migrate from the neural tissue into the adenohypophysis. It remains to be determined what factors induce their myogenic potency.

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