Contracting striated muscle fibres differentiated from primary rat pituitary cultures

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Summary. Whole pituitaries or adenohypophyses alone of adult female Wistar/Furth rats were dissociated into single cells by means of two different enzymic disintegration methods. The single-cell suspension was then seeded out and cultured for up to 8 months in tissue culture dishes with untreated and polylysine-coated surfaces. The cells were cultured in different sera (horse serum, newborn-calf serum, fetal-calf serum, mixtures of horse and newborn-calf serum, and isogenic rat serum) and also in a serum-free, hormone-supplemented medium. When the cells were cultured in medium containing horse serum (15 %) plus fetal-calf serum (3 %) on polylysine-treated surfaces, cell fusion and the development of myotubes could be observed between day 5 and 10 after seeding and, on about day twenty, the formation of multicellular microstructures could be seen. Myotubes in such microstructures differentiate into muscle fibres, and show spontaneous contraction. Striation is visible both light and electron microscopically. Such a differentiation into striated muscle cells depends on specific culture conditions: the serum used, the formation of microstructures, and the treatment of the culture dishes. There is apparently no previous report of striated muscle cells found in pituitary cultures.

Key words: Pituitary (Rat) – Culture, primary – Muscle, striated – Surface coat

Cells in culture have a different micro-environment than in the intact tissue. Moreover, enzymic dissociation results in a depolarization of the cell and a disturbance of the arrangement of the membrane components (Wanson et al. 1977). The supply of hormones and growth factors by serum supplementation, as commonly used, differs qualitatively and quantitatively from the *in vivo* state (Barnes and Sato 1980). Heterogeneous cell populations originating from tissue dispersions of single cells reorganize themselves according to the new microenvironmental conditions, forming multicellular microstructures. Under such conditions a cell

may express a phenotypic aspect not observed under the normal nonpathological situation.

In vitro experiments reported in this paper show that contracting striated muscle cells differentiate in primary cultures of dissociated rat pituitaries. This differentiation depends on i) the surface treatment of the culture dishes, ii) the type of serum used and iii) the formation of microstructures. Such a differentiation of striated muscle cells, which is not typical for the organ from which the original cell suspension had been obtained, has also been reported for long-term cultures of reticular cells from the thymus (Wekerle et al. 1975).

Materials and methods

Chemicals. All chemicals were obtained from SIGMA, Munich, FRG, unless otherwise stated.

Media. Phosphate-buffered saline (PBS), Ham's F12 and Dulbecco's modified Eagle's Medium (DMEM) (the 1:1 mixture of the latter two was used as the standard medium) were purchased from Boehringer (Mannheim, FRG). They were supplemented with 4 µg/ml Tylosin (Serva, Heidelberg, FRG), 2 µg/ml Amphotericin B (Sigma), 25 µg/ml Ampicillin (Serva), 50 µg/ml Streptomycin (Calbiochem, Giessen, FRG), and 40 U/ml Penicillin (Calbiochem). Versene was prepared according to Paul (1975).

Sera. Newborn calf serum (NCS) (Lot no. K900 115P), horse serum (HS) (Lot no. K900601) and fetal calf serum (FCS) were purchased from Gibco (Nunc, Wiesbaden, FRG). Syngenic serum from Wistar/Furth rats (males and females) was prepared as usual.

Dissociation solutions. Trypsin (E.C.3.4.21, Boehringer, 1 mg/ml PBS); Collagenase (E.C.3.4.24.3, Sigma) and Hyaluronidase (E.C.3.2.1.35, Sigma) – both 8 mg/ml PBS –; 1 mg/ml DNase (E.C.3.1.4.5, Serva, 1,000 Kunitz units/ml PBS); 1 mg/ml RNase (E.C.3.1.27.5, Boehringer, appr. 40 Kunitz units/ml PBS); Trypsin inhibitor (Boehringer, 1 mg/ml PBS).

Animals. Two month-old female Wistar/Furth rats were used for the cell-culture experiments.

Extracts and coating procedure

1. *Pituitary deoxycholate extracts.* Only pituitaries from female Wistar/Furth rats, two months old, were used. The organs of 10–20 rats were immersed in ice-cold PBS and washed twice with the same reagent to remove the erythrocytes. They were then minced and the tissue pieces suspended in 10 ml PBS containing 1% w/v 7-deoxy-cholic acid (sodium salt) and homogenized with an Ultraturrax for 1 min at maximal power. Homogenisation was carried out in an ice bath. Debris was removed by centrifuging the homogenate at 1,200 rpm, 10 min ($800 g_{av} \cdot \text{min}$) at 4° C. The supernatant was stirred for 1 h in the cold. Then the suspension was centrifuged at 50,000 rpm, 35 min ($6 \times 10^6 g_{av} \cdot \text{min}$) at 4° C. The supernatant was the rat pituitary deoxycholate extract used for coating the culture dishes.

2. *Aqueous pituitary extracts.* The procedure was the same as described above for the deoxycholate extracts, only the deoxycholate step was omitted.

3. *Rat muscle deoxycholate extracts.* Femoral muscles from female Wistar/Furth rats were used for preparing the extracts. For procedure, see 1 (pituitary extracts).

4. *Aqueous muscle extracts* were prepared in the same way as the aqueous pituitary extracts.

Coating procedure

1. *Polylysine coat.* 2 ml PBS (room temperature) containing 0.1 mg poly-L-lysine hydrobromide (Sigma, mw 150,000) were serially transferred from one culture dish to another (2 ml solution for about 20

dishes). The dishes were then washed twice with double distilled water at room temperature. They were stored dry at room temperature until used.

In some experiments the dishes, prepared as described above, were modified with the four different types of extracts as follows: 2 ml PBS (at room temperature) were pipetted per 35 mm Costar dish (Costar, Tecnomara, Hüttenberg, FRG). Aliquots of the extracts (200 µg protein/dish) were added and the dishes were agitated for 1 h on a rocker plate at room temperature. Then the liquid was aspirated and the dishes were washed twice with PBS. Sterilization was carried out by UV irradiation at 254 nm for 60 min. No liposome formation was observed after the addition of cells.

2. *Collagen coat.* Collagen (Sigma, Type II, from bovine achilles tendon) was brought into suspension by stirring for 24 h in the cold (500 mg Collagen/100 ml 1 M acetic acid in distilled water). Aggregates were spun down by centrifuging the suspension at 900 rpm, 10 min ($500 g_{av} \cdot \text{min}$) at 4° C. Aliquots of 340 µl were spread on 35 mm Costar dishes. The dishes were then air-dried overnight on the flow bench and sterilized with UV-irradiation (254 nm, 1 h). The quality of the coating was examined by phase contrast microscopy.

Dissociation of pituitaries

1. The standard method developed in our laboratory: Two month-old female Wistar/Furth rats were killed with chloroform and decapitated. The pituitaries were removed and suspended in PBS (37° C, 10 ml/100 mm Costar dish). During the entire procedure extreme care was taken to change the surgical instruments between the different steps of the preparation in order to exclude contamination with surrounding muscle tissue. The whole organs or adenohypophyses alone were minced, and the culture dish was placed on a warming plate (37° C). Ten ml of trypsin solution were added immediately and 4 ml DNase/RNase solution were added 10 min later. Five min later 5 ml of hyaluronidase/collagenase solution were added and, 10 min later, the tissue pieces were agitated by repeatedly drawing them into a Pasteur pipette at intervals of 5 min during the following 30 min. Then 10 ml of trypsin inhibitor were added and, 5 min later, 5 ml DNase/RNase. After 5 more min the suspension was filtered through a nylon filter with a 30 µm aperture (G. Maienthaler, Köln-Lindenthal, FRG). The cells were collected by centrifuging at room temperature at 900 rpm, 10 min ($500 g_{av} \cdot \text{min}$). The supernatant was discarded and the pellet resuspended in prewarmed F12/DMEM. The cells were counted with a hemocytometer, and the vitality of the cells was found to be between 90 and 95 %, the yield being approximately 1×10^6 cells per pituitary. The cells were then diluted in the different serum/medium combinations and plated on 35-mm polystyrene Costar dishes.

2. The method proposed by Hopkins and Farquhar (1973) was slightly modified by filtering the cell suspension through a 30-µm nylon filter. This method was used to test the influence of enzymatic disintegration on muscle differentiation. To test the influence of aggregates, an experiment employing both disintegration methods was carried out without filtering the final suspension.

Preparation of meningeal cells: Meninges were carefully removed from the skull, subjected to the same disintegration method as described under 1) and kept in culture for forty days.

Cell culture

Cells were plated at a density of 4×10^5 cells/35 mm Costar dish. The medium was changed at 24 h and 72 h after plating and then every 5th day after plating. Nine such culture experiments were carried out for periods of up to more than 6 months. In two experiments some dishes were used for subculturing. The cells were harvested after treatment with Versene. Cells from one dish were transferred to two dishes. Subculture was performed three to four days after plating, depending on the differentiation status of the cells (prior to myoblast fusion). Serum-free, hormone-supplemented cell culture was carried out according to Florini and Roberts (1978). Phase contrast microscopy and microphotography were performed using a Zeiss microscope IM 35, an Olympus camera OM 2 and Ilford Pan F film.

Electron microscopy

Cells were scraped from the culture dish and fixed in cacodylate buffer (0.1 M Na cacodylate; 0.05 % CaCl_2 , pH 7.3) containing 0.8 % glutaraldehyde and 0.3 % osmium tetroxide. The cells were kept in this

solution for 1 h at 4°C. After three washes with cacodylate buffer (4°C) the cells were postfixed in 1% osmium tetroxide, supplemented with 6.8% sucrose, and washed three times as above.

The samples were dehydrated in graded ethanol solutions (50%, 60%, 70%) and stained with 1% phosphotungstic acid and 0.5% uranyl acetate.

Ultrathin sections of material embedded in Epon 812 were stained with uranyl acetate and lead citrate and viewed with a Zeiss EM 9 transmission electron microscope.

Results

Depending on the culture conditions, contracting striated muscle fibres were found in cultures of primary pituitary cells from female Wistar/Furth rats.

Five days after cultures had been raised from pituitaries dispersed into single cells (Fig. 1) cell fusions and myotube formations were observed with the phase contrast microscope (Fig. 2), and from day 10 onward striation and spontaneous contractions were found. Branched striated muscle fibres were observed in clusters; as many as five per culture dish. The fibres (150–200 µm) showed spontaneous contractions of variable frequency (from two to thirty contractions/min). The frequency increased when 1 µmol acetylcholine was added or when the culture dishes were left outside the incubator over longer periods (10 min). As shown in Fig. 3 the striation could be recognized with the phase contrast microscope without staining. However, the detection of the muscle fibres was somewhat difficult since they were mainly found in association with “multicellular microstructures” (Fig. 4), the myotubes being partially surrounded by other cells.

The nature of the striated elements could be further clarified by transmission electron microscopy (Fig. 5), based on the classical criteria (myofibrils, M, Z bands) of striated muscle fibres.

The formation of these muscle fibres depends on the serum conditions as well as on the surface treatment of the culture dishes. From all the sera used (see Materials and methods) a combination of 15% horse serum and 3% newborn-calf serum was most effective in inducing muscle cell differentiation when the cells were plated on polylysine-treated culture dishes. In untreated dishes muscle differentiation was not observed. Whereas fetal-calf serum and syngenic rat serum did not induce muscle differentiation in polylysine-treated dishes, newborn-calf serum and horse serum alone in some cases induced muscle formation on polylysine coats. However, the

Fig. 1. Single cell suspension obtained by enzymic dissociation of the pituitary (see dissociation method 1). Bar represents 20 µm

Fig. 2. Myotubes formed between days 5 and 10 in polylysine-treated culture dishes (medium supplemented with 15% horse serum and 3% newborn-calf serum). Some nuclei are marked by *arrows*. Phase contrast micrograph. Bar represents 20 µm

Fig. 3. Phase contrast micrograph of a striated (*arrows*) muscle fibre. Bar represents 15 µm

Fig. 4. Microstructures of primary pituitary cells in which the striated muscle fibres predominantly occur. These structures develop from original cultures of single dispersed cells in polylysine-treated culture dishes (medium supplemented with 15% horse serum and 3% newborn-calf serum). Bar represents 40 µm

Fig. 5. Electron micrograph of cell containing myofibrillar Z- and M-bands. Three month-old cultures. (Bar represents 0.5 μm)

occurrence of fusions and striated muscle fibres was delayed under these serum conditions and single clusters per dish were rarely observed. When polylysine was combined with aqueous or deoxycholate extracts of rat pituitary or muscle tissue for coating, occasional muscle fibre formation was also found, using horse serum and newborn-calf serum in the medium. Collagen coats did not induce any differentiation into muscle cells.

This coat and serum dependency occurred in all experiments performed. During all nine experiments, lasting up to half a year, myotube formations were observed; eight experiments resulted in differentiation of contracting striated muscle fibres.

The two different methods of enzymatic dissociation yielded identical results. There was no difference whether the cultures were started with single cells (Fig. 1), or with aggregates, which partially retained the histological structure of the pituitary. Cultures raised from whole pituitaries or from adenohypophyses alone showed the same differentiation pattern.

Subculturing before the appearance of fusions seems to have a stimulatory effect on the differentiation of muscle fibres and results in a higher yield. A serum-free, hormone-supplemented medium, developed for rat myoblasts (Florini and Roberts 1978), was also tested for its ability to induce muscle differentiation, however, the results were negative. Since two reports exist (Lipow et al. 1978; Reine and Horoupian 1980) that striated muscle fibres have been observed within

meninges, a control experiment under conditions for optimal muscle fibre differentiation was performed with cultures of dura mater cells. After 50 days in culture no muscular structures were found.

Discussion

As can be seen from the results reported here, striated muscle cells can differentiate in pituitary primary cultures. This process is dependent on (i) the serum used, and also (ii) on the surface coat. The striated muscle cells mainly differentiate (iii) within specific multicellular microstructures that are formed by the heterogeneous cell population initially present at the onset of the culture period. The possibility that the muscle cells originated from contaminating tissue was not very likely, since new surgical instruments were always used for the different stages of removal. The anatomical position of the pituitary is itself a strong argument against contamination. However, there is most probably no method of removing the hypophysis ensuring that it is entirely free of pia mater or of connective tissue cells derived from the walls of small blood vessels. These extraneous pieces of connective tissue may carry with them primitive mesenchymal cells, so that fibroblasts, smooth muscle cells or even striated muscle cells could conceivably develop from these elements. Control experiments with cultures of isolated meningeal tissue showed that these cells do not differentiate into muscle cells under identical culture conditions.

For some brain compartments the heterotopic appearance of striated muscle fibres is reported: in the cornu ammonis (Zipper 1968), within leptomeninges (Lipow et al. 1978; Reine and Horoupian 1980) and within the pineal body (Diehl 1978). However, to date there exists no report that striated muscle cells occur in the pituitary, either under normal or pathological conditions. The three case reports (Luse and Kernohan 1955; Satyamurti and Huntington 1972; Symon et al. 1971) on the occurrence of myoblastomas concern the human neurohypophysis. A control experiment using only the isolated adenohypophysis was therefore performed. In this experiment also, contracting striated muscle fibres developed in the culture dish. Therefore, we conclude that the striated muscle cells do not occur normally in the pituitary, and that those found in the culture dishes are the products of a differentiation process, whatever their progenitor cell type may have been.

The large number of different tumors in the pituitary (Horvath and Kovacs 1980) may be considered as evidence for numerous distinct differentiation processes occurring in this organ (Furth 1969).

The interesting fact emerging from these experiments is, from our point of view (Brunner 1977), that the environmentally dependent induction of muscle differentiation is normally not found *in vivo*.

Muscle differentiation from pituitary cultures requires special serum conditions (mixture of 15% horse serum and 3% newborn-calf serum) and a special surface coat (polylysine). Other sera (syngenic rat serum, fetal-calf serum, horse serum or newborn-calf serum alone) were not capable of inducing myogenesis, suggesting a specific requirement for growth factors and hormones. Similar results have been reported by Yaffe (1972) where fetal-calf serum was used for propagation of myoblasts without induction of fusion, and horse serum was used for differentiation. In accordance, Wekerle et al. (1975) found horse serum to be more efficient

than fetal-calf serum, inducing muscle differentiation in epithelial cultures of thymus cells. The dependency on a certain microenvironment is shown by the inability of coats other than polylysine to induce myogenesis.

A recent report (Wahrmann et al. 1981) on the influence of positively charged surfaces on myogenesis is consistent with the reported findings concerning polylysine coats.

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