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In vitro Separation of Embryonic Chick Skeletal Muscle Myoblasts and Fibroblasts: Comparison of Their Characteristics

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Summary

The aim of the present experiments was to test two methods of separating myoblasts and fibroblasts (selective plating, differential trypsinization) from chick embryonal skeletal muscle and to compare their characteristics. Ornithine decarboxylase (ODC) activity, the amount of incorporated [^{3}H]leucine into proteins and incorporation of [^{3}H]thymidine into DNA were significantly higher in myoblasts than in fibroblasts separated by selective plating. When comparing myoblasts and fibroblasts separated by differential trypsinization, significantly higher ODC activity and greater incorporation of [^{3}H]leucine into protein, but no incorporation of [^{3}H]thymidine into DNA, were found in myoblasts. Higher ODC activity and greater incorporation of labelled leucine were found in fibroblasts separated by the selective plating than in fibroblasts separated by differential trypsinization. The incorporation of labelled thymidine into DNA was higher in myoblasts separated by selective plating than in myoblasts obtained by differential trypsinization. The method of selective plating appears to be simpler and adequate for obtaining myoblastic and fibroblastic cell cultures with sufficiently low mutual contamination. The method of differential trypsinization involves a more drastic treatment of cells and is more time consuming.

Key words

Chicken – Fibroblast – Myoblast – Ornithine decarboxylase activity – Incorporation

Introduction

Embryonic skeletal muscles consist of myogenic cells at various stages of differentiation. Myogenic cultures prepared from embryos usually contain fibroblasts co-isolated with myogenic cells (Yablonka-Reuveni *et al.* 1988). The embryonic myoblasts are mononucleated cells, which fuse to form multinucleated myotubes, synthesize contractile proteins and ultimately develop into mature muscle fibres (McFarland *et al.* 1991). Fibroblasts that are nonmyogenic cells contribute, depending on the species and age, up to 50 % of the cultured cells (Königsberg 1963, Yaffe 1969, Yablonka-Reuveni and Nameroff 1987). Therefore, the contamination of myoblasts by fibroblasts in muscle cell culture depends on the stage of embryonic development at which the cells are isolated. Many authors have tried to separate myoblasts from fibroblasts using diverse methods based on different characteristics of myoblast and fibroblast cells (Richler and Yaffe 1970, Powers and Florini 1975, Yablonka-Reuveni *et al.* 1988, Autin and Ordahl 1991). Some methods were based on the different adherence of cells to the culture dish surface, i.e. the fact that bipolar myoblasts in mass culture are more loosely attached to the plastic surface than fibroblastic cells (Kaighn *et al.* 1966, Richler and Yaffe 1970). Richler and Yaffe (1970) separated myoblasts from fibroblasts shortly after seeding by aspiration of the medium containing floating myoblastic cells. On the other hand, Kaighn *et al.* (1966) separated the above mentioned types of cells from the primary culture before myoblast fusion by a similar method after previous trypsinization. Pure myoblast colonies were obtained by several passages of cell multiplication.

The aim of the present experiments was to test the different methods of myoblasts and fibroblasts separation from cultured chick embryonal skeletal muscles and to compare the activity of ornithine decarboxylase (ODC), incorporation of labelled thymidine into DNA and labelled leucine into protein in separated myoblast and fibroblast cultures.

Materials and Methods

Preparation of primary skeletal muscle cell cultures

Cells were isolated from thigh and breast muscles of 11-day-old embryos by trypsinization (Königsberg 1979) and seeded at 4 x 10^6 cells onto Petri dishes ϕ 100 mm in 7 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % TPB (tryptose phosphate broth), 10 % foetal calf serum and antibiotics (penicillin 10^5 U/l, streptomycin 25 mg/l, kanamycin 100 mg/l). The cultures were kept at 37 °C in a humidified incubator in an air atmosphere containing 5 % CO_2. Fibroblasts and myoblasts after separation (see below) were cultured in DMEM supplemented with 10 % calf serum. Myoblasts were incubated in gelatin-coated dishes and a growth medium containing 1 % chick serum.

Separation of myoblasts and fibroblasts by selective plating

A 40 min period was chosen as the optimal time, after which most fibroblastic and epithelial cells became attached to the surface, whereas most myoblastic cells still floated in the medium. This time was determined by observing the cultures under a microscope 20, 40, 60 and 180 min after seeding (4.0 x 10^6 cells onto ϕ 100 mm dishes). Forty min after seeding of isolated muscle cells the growth medium containing floating myoblastic cells, was collected by aspiration and pooled. The pooled medium was centrifuged and the myoblastic cells were counted and seeded at the appropriate density (1.5 x 10^6 cells onto ϕ 60 mm dishes coated with gelatin). The new growth medium was added to the original dishes containing fibroblastic cells (in density 2.5 x 10^6 cells onto ϕ 100 mm dish). The corresponding media with supplements were replaced in both cultures every 24 h.

Separation of myoblasts and fibroblasts by differential trypsinization of primary muscle cell cultures

Suspensions containing 4 x 10^6 muscle cells in 3 ml of the DMEM growth medium were seeded onto each ϕ 100 mm plastic dishes and incubated for 24 h. After this time the medium was removed and dishes were washed three times with saline to remove unattached cells and debris. Two ml of 0.025 % trypsin in saline were added, removed after a few minutes and the dishes with cells were incubated for 5–10 min. The exact duration of incubation after which the bipolar processes of myoblasts were retracted and the cells became rounded while the fibroblasts were unaffected was estimated under a microscope. Then the dishes were washed with 2 ml of the growth medium to collect the myoblasts. The suspension of myoblasts was transferred into a test tube, centrifuged, the cells were counted and seeded onto ϕ 60 mm plastic dishes coated with gelatine (at a density 1.5 x 10^6 cells). The fibroblasts were cultivated on original dishes after addition of the fresh growth medium. The proteosynthetic characteristics were determined in both cases after 48 h of cultivation.

The ODC activity was assayed as $^{14}CO_2$ generated from [^{14}C]ornithine using purified [^{14}C]ornithine hydrochloride (Hungarian Academy of Sciences, Budapest, Hungary, specific activity 520 MBq/mmol) and the method of Slotkin and Bartolome (1983).

The method of Robinson *et al.* (1976) was used for thymidine incorporation, estimation of DNA and incorporation of labelled leucine into the cell protein fraction. The cultures were incubated for 2 h at 37 °C in the presence of 37 kBq/ml of [methyl-^{3}H]thymidine (Adico, Prague, Czech Republic, specific activity 1500 GBq/mmol) or 37 kBq/ml L-[4,5-^{3}H]leucine (Adico, Prague, Czech Republic, specific activity 1620 GBq/mmol). An aliquot of the digest was taken for the estimation of proteins by the method of Lowry *et al.* (1951).

Experimental values are given as means ± S.E.M. The data were analyzed using Student's t-test.

Results

The differences in ODC activity, the incorporation of [^{3}H]leucine into proteins and of [^{3}H]thymidine into DNA between cultured myoblastic and fibroblastic cells separated by selective plating are presented in Table 1. The activity of ODC is significantly higher ($p<0.01$) in myoblasts. Myoblasts also incorporated higher amounts of labelled leucine into proteins ($p<0.05$) and labelled thymidine into DNA ($p<0.05$).

The differences in ODC activity, the amount of incorporated [^{3}H]leucine into proteins and the amount of incorporated [^{3}H]thymidine into DNA between cultured myoblastic and fibroblastic cells separated by differential trypsinization are summarized in Table 2. The ODC activity in myoblasts as well as the amount of incorporated [^{3}H]leucine into proteins were significantly higher in comparison with fibroblasts ($p<0.05$ and $p<0.001$, respectively). No differences between myoblasts and fibroblasts were found in the incorporation of labelled thymidine into DNA.

Table 1

Proteosynthetic characteristics of myoblasts and fibroblasts separated by selective plating

	Myoblasts	Fibroblasts
ODC activity (pmol $^{14}CO_2$/mg protein/h)	2057 ± 245**	1120 ± 113
Incorporation of [^{3}H]leucine (cpm/mg protein)	5971 ± 478*	4510 ± 270
Incorporation of [^{3}H]thymidine (cpm/μg DNA)	297 ± 43*	149 ± 26

* $p<0.05$, ** $p<0.01$ *in comparison with fibroblast culture; values represent the means from five Petri dishes ± S.E.M.*

Table 2

Proteosynthetic characteristics of myoblasts and fibroblasts separated by differential trypsinization

	Myoblasts	Fibroblasts
ODC activity (pmol $^{14}CO_2$/mg protein/h)	1543 ± 434*	415 ± 41
Incorporation of [^{3}H]leucine (cpm/mg protein)	6306 ± 297***	3289 ± 265
Incorporation of [^{3}H]thymidine (cpm/μg DNA)	130 ± 14	179 ± 38

* $p<0.05$, *** $p<0.001$ *in comparison with fibroblast culture; values represent the means from five Petri dishes ± S.E.M.*

A comparison of myoblasts obtained by different separation methods of myoblastic and fibroblastic cells has shown that a significantly higher amount of labelled thymidine is incorporated into DNA ($p<0.01$) of myoblasts isolated by selective plating than into myoblasts isolated by differential trypsinization. Fibroblasts isolated by selective plating exhibited significantly higher ODC activity ($p<0.01$) and significantly higher incorporation of labelled leucine into proteins ($p<0.05$) than fibroblasts obtained by differential trypsinization.

Discussion

Muscle cells in the whole organism interact with other tissues and are affected by their presence. Cell culture allows us to separate these interactions (McFarland 1992). Primary cultures of embryonic muscle cells contain a mixture of at least two cell types – myoblasts and fibroblasts – which influence each other. In cultures, the fibroblasts accompany myogenic cells in greater or smaller numbers depending on the age of the organism from which they had been isolated. Eleven-day-old chick embryos were chosen for the

present experiments because this is the period of intense skeletal muscle growth as well as the period of rapid protein synthesis in the chicken.

Fibroblasts produce the fibroblast growth factor (FGF), which is necessary for cell growth. FGF has been shown to stimulate cellular proliferation in rat myoblasts (Johnson and Allen 1990) and rat satellite cells (Allen *et al.* 1984). Myotubes grown in culture with the possible assistance of fibroblasts, assemble the extracellular matrix which contains collagens (Muntz 1990). Hauschka and Königsberg (1966) have shown that myotubes could develop from myoblasts in culture if the condition medium was replaced by collagen. As was shown later, myoblast adhesion to collagen was mediated by fibronectin (Chiquet *et al.* 1979). Because of the above mentioned function of collagen, the dishes used for cultivation of myoblasts are usually coated with collagen or gelatine.

The morphological differences between myoblasts and fibroblasts are also accompanied by biochemical differences. ODC is a rate-limiting enzyme in the conversion of ornithine into polyamines and polyamines are intimately involved in the DNA, RNA and protein synthesis machinery of the cell (Heby 1981, Pegg and McCann 1982). High activity of ODC in whole embryo from the 3rd to the 16th day of chick embryonal development was also found by Russel and Snyder (1968). The observed higher ODC activity in myoblasts compared with fibroblasts (separated by both methods under defined conditions) corresponds to the higher protein synthesis indicated by higher incorporation of [^{3}H]leucine into proteins and [^{3}H]thymidine into DNA.

As has already been mentioned, the amount of [^{3}H]leucine incorporated into cell proteins was higher in myoblasts than in fibroblasts regardless of the separation method. The higher incorporation of labelled leucine reflects the higher content of protein in myoblasts. The increased protein content is probably related to a higher density of cytoplasm and a smaller number of vesicles in myoblast cells.

The initial stage of the terminal differentiation in muscle cells is marked by the end of DNA synthesis in the mononucleate myoblasts. Myoblast fusion and the synthesis of muscle-specific proteins are two separate events that usually occur at the same time (Muntz 1990). Whether myoblasts fuse before or after the synthesis of muscle-specific proteins (α-actin, myosin) depends on the age of the embryo (Okazaki and Holtzer 1966). The amount of labelled thymidine incorporated into DNA was higher in myoblasts isolated by selective plating of cells than in fibroblasts. On the other hand, no significant differences in the incorporation of labelled thymidine were found between fibroblasts and myoblasts isolated by differential trypsinization. The difference in the results obtained by the two separation methods could be caused by differences in the stage of differentiation of myoblastic cells. Though the incubation time from separation was the same in both experiments (48 h), the time from plating to separation of myoblastic and fibroblastic cells differed between the two methods (40 min and 24 h respectively; see Materials and Methods). The higher incorporation of labelled thymidine into DNA of myoblastic cells separated by selective plating therefore reflects a higher proliferation of myoblasts before fusion. However, no differences were found in the incorporation of labelled thymidine into DNA between myoblasts and fibroblasts isolated by differential trypsinization which reflects the fact that myoblasts had fused into myotubes (65–75 % of the culture) and that synthesis of DNA had terminated. These results are in agreement with the results of Powers and Florini (1975), showing that multinucleated skeletal muscle fibres fail to incorporate thymidine into DNA after fusion and that fused myoblasts are withdrawn from the mitotic cycle.

A comparison of cultures obtained by two methods of separation has shown that the activity of ODC in fibroblasts after selective plating is higher than after differential trypsinization. It probably reflects a higher protein synthesis, since a comparison of the two fibroblast cultures has shown that the incorporation of labelled leucine into proteins of fibroblasts obtained by the first method is higher. The higher incorporation of labelled thymidine into myoblasts after selective plating in comparison with differential trypsinization could be due to differences in the stage of cell proliferation resulting from different time from plating to cell separation in each method as was discussed above.

The efficiency of both separation methods was practically the same, the final mutual contamination being about 8 %. The method of selective plating appears to be simpler and sufficient for obtaining myoblastic and fibroblastic cell cultures. The method of differential trypsinization involves more drastic treatment of cells and therefore the yield of myoblasts and fibroblasts from the original culture was lower in comparison with selective plating and is more time consuming.

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