CONCANAVALIN A INHIBITS FUSION OF MYOBLASTS AND APPEARANCE OF ACETYLCHOLINE RECEPTORS IN MUSCLE CULTURES

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1. Introduction

Mononucleated replicating cells derived from fetal skeletal muscle, when maintained in cell culture, withdraw from the cell cycle, align and subsequently fuse to form multinucleated muscle fibers [1]. These myogenic processes are associated with an elevated level of a number of muscle-specific proteins [2–5]. There is debate as to whether cell fusion and the appearance of characteristic muscle proteins are causally linked [6–18]. We have shown that inhibition of cell fusion by lowering Ca^{2+} concentration prevents the normal increase in the measured levels of acetylcholine receptors (AChR), creatine phosphokinase (CPK) and acetylcholinesterase (AChE) of rat muscle cultures, but that the levels of these proteins in chick muscle cells are hardly affected [19]. In order to exclude the possibility that Ca^{2+} is needed for the expression of these proteins in rat myoblasts (prior to onset of fusion), fusion was blocked by Concanavalin A (Con A). Con A is known to bind specifically to the cell membrane surface [20] and thus may play a role in preventing the fusion of myogenic cells. Using this method we show that while in the rat, fusion of myoblasts is a prerequisite for the appearance of the muscle proteins, in the chick this is not so.

2. Materials and methods

2.1. *Cell cultures*

Embryonic rat or chick skeletal thigh muscles were grown in cell culture essentially as in [2]. Cells were grown in 30 mm Nunc petri dishes, coated with collagen, in Dulbecco's modified Eagle medium supplemented with 10% horse serum and 2% chick embryo extract. After incubation for 20 h, 100 μg/ml of Con A were added to rat cultures, or 175 μg/ml to chick cultures.

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2.2. *Measurement of cell fusion*

Cell fusion was estimated by direct microscopic examination of methanol-fixed and Giemsa-stained cultures. Using an eyepiece grid and magnification of × 400, counts were made of the number of nuclei in single cells and in multinucleated myotubes in 5 randomly selected fields (370 × 1450 μm). Total counts/dish between 3000 and 6000 nuclei were obtained.

2.3. *Assay for AChR*

For the determination of AChR levels the intact culture dish was exposed to 6×10^{-8} M ^{125}I-labeled α-bungarotoxin of spec. act. 50–125 Ci/mmol [21]. After careful washing the labeled cells were counted directly on a 2 in diam. flat crystal γ-detector (Elscint, Haifa). Each dish of cells was then frozen in liquid nitrogen for enzyme assays or fixed in formalin for autoradiography [21].

2.4. *Determination of enzyme activity*

CPK activity was determined by recording ΔA_{340} at 30°C. The amount of ATP formed from ADP with creatine phosphate was determined by coupling the reaction with hexokinase and glucose 6-phosphate dehydrogenase, as in [2].

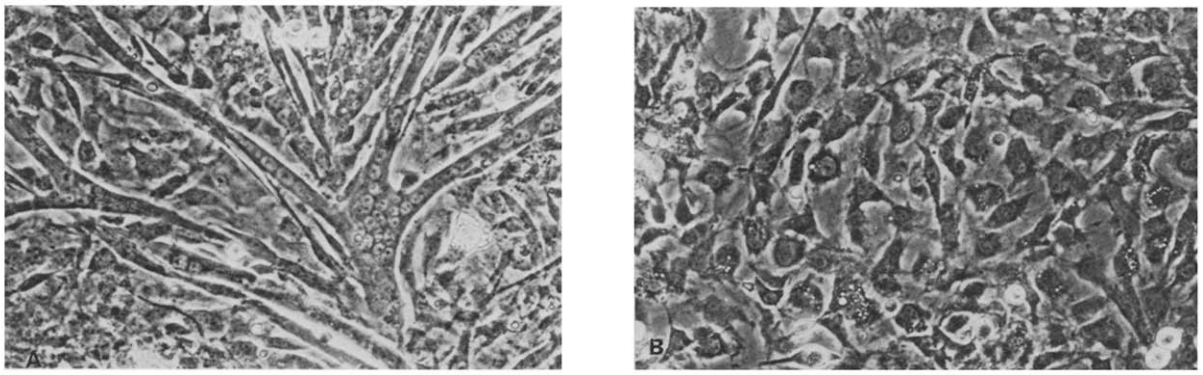

Fig.1. Phase-contrast photomicrographs of rat muscle after 4 days in culture. (A) Normal muscle fibers. (B) Con A-treated. Note the absence of myotubes (× 161.2).

3. Results

Con A, at 100 μg/ml, was added to rat muscle cultures 24 h after plating, prior to onset of fiber formation. Normal fusion and formation of multinucleated myotubes took place only in cultures which had not received Con A. Those cultures which had received the lectin remained as mononucleated cells (fig.1).

Table 1 illustrates the inhibition of cell fusion by Con A. At 44 h all the cells were mononucleated. Between 44 h and 90 h, intense multinucleation took place in control cultures, reaching ~37% of nuclei in myotubes at 90 h, while the average fusion in Con A treated culture was <2%.

To examine the correlation between cell fusion and the appearance of muscle proteins the levels of AChR and CPK were measured. These proteins were found to be very low in mononucleated cells, as noted [2,19]. As soon as the fusion process started, it was accompanied by an increase in the activity of these proteins. In the presence of Con A from 1–7 days in rat or chick cultures, the appearance of these proteins was drastically inhibited. Although in chick cultures treated with Con A there was some increase in the levels of the proteins, nevertheless, no identifiable fusions were apparent. The inhibition of morphological and biochemical differentiation by Con A was found to be reversible. Addition of α-methyl-D-mannoside (which binds strongly to

Table 1
Effect of Con A on cell fusion

Age of culture	Treatment	Average no. of nuclei		% of fusion
		Within myotubes	Outside myotubes	
44 h	Control	9	4114	0.2
	Con A (2 h)	–	2835	0
68 h	Control	805	4352	15.6
	Con A (44 h)	27	4066	0.7
90 h	Control	2301	3831	37.5
	Con A (66 h)	93	4590	2.0

Rat muscle cultures were grown for 1 day in complete medium; then Con A at 100 μg/ml was added. At the indicated time nuclei within and outside the myotubes were counted in Giemsa-stained dishes. Each value represents the average number of nuclei in 5 randomly-selected fields

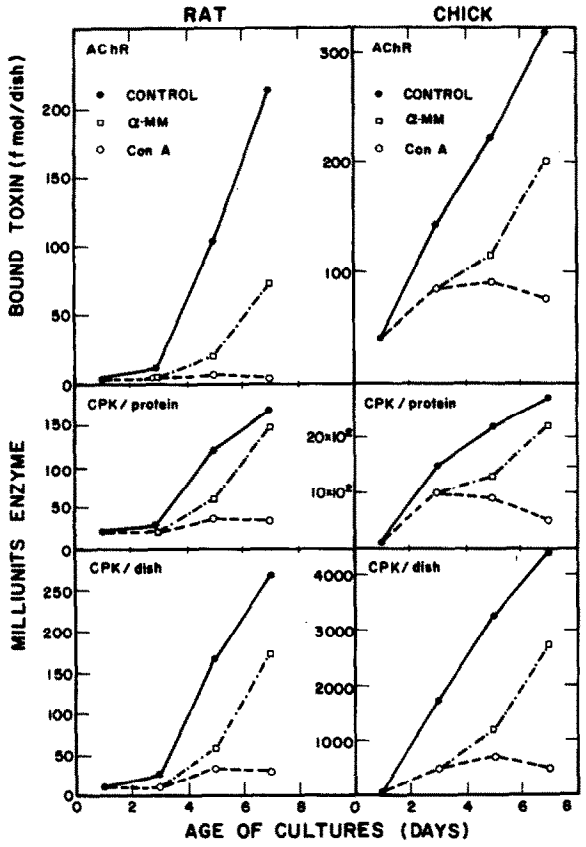

Fig.2. Appearance of acetylcholine receptors and creatine phosphokinase in muscle cultures. Rat or chick muscle cultures were grown in normal medium (●—●). Con A was added to 1 day old cultures (○- - -○). α-methyl-D-mannoside (10 mM) was added to 3 day old cultures treated with Con A (□ — · — · □).

Con A), to cultures grown in the presence of the lectin, resulted in initiation of cell fusion concomitant with elaboration of CPK and AChR (fig.2).

The possibility that Con A prevented cell fusion by interfering with the general synthetic machinery of the myoblasts, was tested. The rate of precursor incorporation into proteins or nucleic acids was measured. The results of such experiments show that 24 h after Con A treatment the rate of protein synthesis was almost the same as the control, although there is significant inhibition in nucleic acid synthesis (table 2).

The generation time was calculated from the growth curves of cells treated with Con A. It was

Table 2
Effect of Con A on DNA, RNA and protein synthesis in cultured muscle cells

Precursor	Treatment	cpm/mg protein	%
[^{3}H]Thymidine	Control	247 000	100
	Con A	171 000	69
[^{3}H]Uridine	Control	198 000	100
	Con A	144 000	73
[^{3}H]Alanine	Control	167 000	100
	Con A	161 000	97

Primary rat muscle cultures, 1 day old, received Con A 100 μg/ml for 24 h. Then 3 replicates of cultures were exposed to the indicated precursors for 1 h, after which the cells were washed, harvested and homogenized. Trichloroacetic acid precipitates were dissolved in 1 N NaOH and counted in a liquid scintillation counter. Protein was measured by the Lowry method

found that the cells do multiply under these conditions, though at a slightly lower rate (17 h compared with 13 h for cells grown in normal medium).

4. Discussion

The main event in muscle differentiation is the fusion of mononucleated myoblasts to form myotubes and a concomitant elaboration of various muscle-specific proteins. The question arises whether the morphological and biochemical differentiation of the muscle are causally, or only temporarily related. Apparently inconsistent conclusions have been reached by various groups:

(i) That appearance of muscle-specific proteins is tightly coupled to fusion [2–4,22];
(ii) that cytoplasmic and structural proteins, unlike the membrane proteins, are linked to fusion [7];
(iii) that fusion is not causally linked to biochemical differentiation of the muscle [6,8–18].

We have attempted to settle the argument by showing that a fundamental difference exists between chick and rat myoblasts grown in vitro. Both cytoplasmic (CPK) and membrane-specific proteins (AChE and AChR) in muscle cultures, treated with low Ca^{2+} to inhibit cell fusion, are indeed coupled to the process of fusion in rat-originated myoblasts. However, this coupling does not exist in chick muscle cultures [19].

Here we confirm our studies that fusion of myoblasts is coupled to the appearance of muscle-specific proteins in rat cultures, but only partially in chick cultures. We have developed a new method to inhibit cell fusion, replacing low Ca^{2+} (because it is evident that AChR has high affinity for Ca^{2+} and a large amount of Ca^{2+} is tightly bound to AChR molecule [23]). Furthermore, the activity of cytoplasmic enzymes and CPK were shown to be dependent on Ca^{2+} concentration, although the number of nuclei in fibers were the same [13,24]. To exclude the possibility that Ca^{2+} is required for detection of these proteins in rat myoblasts, grown in low Ca^{2+}, we used Con A to inhibit cell fusion. This method is based on the property of Con A to bind carbohydrate residues which are abundant on the surface of the myoblasts. Using this method it was shown that in rat muscle, inhibition of cell fusion prevents the appearance of membrane and cytoplasmic proteins (fig.2). Furthermore, even in chick muscle cultures inhibition of cell fusion considerably reduced elaboration of AChR and CPK. Thus, our results show a coupling between biochemical and morphological differentiation. Fusion of rat myoblasts is probably a prerequisite for the expression of AChR and CPK.

AChR is known to have a carbohydrate moiety, and to bind Con A. The possibility that Con A competes with α-bungarotoxin for AChR was excluded by washing the cells before AChR was determined. We have shown that Con A does not inhibit significantly the binding of the toxin to the receptor (unpublished results).

The mechanism by which Con A inhibits fusion of the myoblast is not known. We have shown that removal of the lectin, or addition of α-methyl-D-mannoside, resulted in the initiation of cell fusion. Furthermore, uninterrupted protein synthesis was shown to be essential for the process of fusion [2]. Con A was found in this study to inhibit protein synthesis only slightly, which cannot explain its effect on arresting cell fusion. It order to obtain further insight into the mechanism of Con A action, we grew myoblasts in low Ca^{2+} until they were crowded in a monolayer; then the cells received simultaneously Ca^{2+} and Con A. If Con A, a big molecule compared to Ca^{2+}, could not penetrate into the intercellular space, it would not prevent cell fusion. Our results showed that Con A indeed prevented cell fusion (data not published). These experiments suggest that Con A and low Ca^{2+} suppress cell fusion probably through a similar mechanism. The ability of lectin to inhibit cell fusion is not restricted to Con A; similar results were obtained with phytohemagglutinin. Whether the lectins prevent fusion by inhibiting cell movement, by preventing membrane–membrane interaction or by binding to proteins necessary for the fusion, remains to be explored.

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