MEMOIRS OF THE FACULTY OF SCIENCE, KYOTO UNIVERSITY, SERIES OF BIOLOGY
Vol. I, No. 2, pp. 167-175, March 1968

In vitro Formation of Multinucleated Fibers from the Dissociated Somite Cells of Chicken Embryos

By

Kazuo WATANABE

Laboratory of Developmental Biology, Department of Zoology, Kyoto University.

ABSTRACT The dissociated cells of the somites of 4-day chicken embryos (stage 24) were cultured *in vitro*, and the process of the formation of multinucleated fibers (MNF) was studied in comparison with that from the similarly cultured cells of the breast muscle of 12-day embryos (stage 38). The formation of MNF from the somite cells occurs much earlier period in culture than from the cells of the breast muscle. The pulse-chase experiments using ^{3}H-thymidine revealed that the myoblasts of the somite actively synthesize DNA since a very initial stage of culturing with a very short lag period. MNF formed from the cells of the breast muscle consists mostly of the labelled nuclei, whereas about 40% of the nuclei contained in MNF from the somite cells are left unlabelled, as long as ^{3}H-thymidine was given *in vitro* culture period.

Recently some important investigations have been made on the differentiation of multinucleated muscle fibers from the singly dispersed myoblasts in cell culture condition (reviews: Holtzer, 1961; Konigsberg, 1965). In these studies, which mostly concern chicken embryo as material, the cells from thigh or breast muscle of such older embryos as 11-12 days of incubation (stages 37-38, Hamburger and Hamilton, 1951) have been routinely employed to be cultured *in vitro*. The behavior of the pre-muscular tissue of the somites or limb buds of earlier embryos have so far been little studied in cell culture. In the condition of organ culture using plasma clot, however, Avery et al. (1956) reported the development of the early somite. Their results indicate that multinucleated myotubes are not formed from the somite taken out of the embryos earlier than stage 24, only mononucleated myoblasts being seen in such cultures. Necessity of the effect emanating from the neural tube was suggested for the formation of mature mus-

cles from the isolated somites.

Under such situations, it seemed to be of interest in regard to the problem of myogenesis, to cultivate early somite cells in the condition of cell culture and to compare their behavior with that of the cells from the 12 days muscle (stage 38).

Material and Methods

Chicken embryos were obtained by incubating the fertilized Rhoad Island Red eggs.

A) Preparation of the dissociated cells

Breast muscle of 12 day embryos (stage 38) was taken out after removing the overlying epidermis with fine forceps. This was minced with scissors and transferred to the dissociating medium (DM) consisting of 0.1 % trypsin (Difco; 1:250) and 0.014 % collagenase (Sigma chemical Co., U. S. A.) in calcium- and magnesium-free Earle's saline. After incubation in DM at 37°C for 30 min. and subsequent addition of the three volumes of Eagle's medium containing a few drops of calf serum, the soft clumps of the muscle were dissociated by gentle pipetting. The pellet obtained by centrifugation at ca. 1,200 rpm for 5 min. was resuspended with Eagle's medium and then filtrated through fine silk mesh to remove the undissociated aggregates.

The dorsal block composed of spinal cord, notochord, somite and covering epidermis was removed from the 10th to 20th somite level of 4 days embryos (stage 24). The blocks were briefly trypsinized and the somites were cleaned from the neighbouring tissues by microdissection with tangsten needles. The clean somites from several embryos were pooled and were transferred to DM for preparing the cell suspension with the same manner as in the case of the breast muscle.

B) The cell culture

The culture medium consisted of 10 parts of Ham's F-10 (amino acids: x 4 and vitamins: x 2; Coon, 1966), 3 parts of L-fraction of chick embryo extract (Coon, 1966), 2 parts of calf serum and 5 parts of Hanks's salt solution. 1 x 10^5 cells were inoculated in Falcon plastic dish (diameter: 60_{mm}) with 2 ml of the culture medium, and were incubated at 37°C in 5 % CO_2–95 % air.

C) Autoradiography

Labelling of DNA-synthesizing nuclei was made by adding 0.5μC of ^{3}H-thymidine (Daiichi Kagaku Co. Tokyo, specific activity: 5.0 curies/mM) to the culture medium. The cultures were fixed with 10 % neutral formalin in Earle's saline, washed with water, treated with 5 % trichloracetic acid at 5°C for 40 min. and repeatedly washed with distilled water. Autoradiography was made by coating method using Fuji stripping film. The bottoms of the culture dishes coated with film were exposed for about 3 weeks, developed by SDX-1 developer, washed and stained with Meyer's haematoxylin. In the chase experiments, the labelling medium with the isotope was thrown away at the desired stages of

culturing and the dishes were washed twice with Hanks's salt solution and another once with the same solution containing 5 γ/l of non-labelled(cold) thymidine. Finally the dishes were filled with fresh culture medium containing 0.2 γ/l of cold thymidine to continue further cultivation.

In situ labelling was made by dropping 20μC of ^{3}H-thymidine in 0.5 ml Earle's saline directly onto the embryo through a small opening made in the shell. After incubation of the sealed eggs for desired period, the labelled embryos were drawn out and washed with 5 γ/l of cold thymidine. The dissection was made in Hanks's salt solution containing 1 γ/l of cold thymidine. The cell culture and the process of autoradiography were the same as mentioned above.

Results

A. Formation of multinucleated fibers (MNF) in vitro

1. The cells from the breast muscle (stage 38): Around 24 hrs. from inoculation, the cells are plated on the bottom of culture dishes. As soon as the cells flatten on the bottom, smaller and bipolar spindle-shaped myoblasts can be easily distinguishable from the fibroblasts which are larger and more extensively attached (Fig. 1-a). The multinucleated fibers (MNF) which probably arose

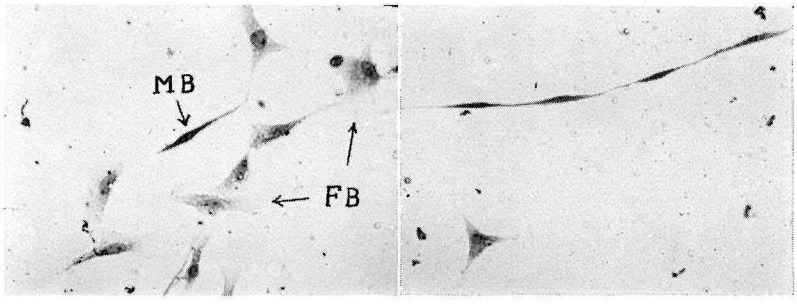

Fig. 1. a. Spindle-shaped myoblasts (MB) and flattened fibroblasts (FB) from the breast muscle cells (st. 38) in 72 hrs' culture; stained with Meyer's haematoxylin. (x 200)
b. MNF containing 4-nuclei from the breast muscle cells (st. 38) in 72 hrs' culture; stained with Meyer's haematoxylin. (x 200)

from the myoblasts, are abundant at 60 hrs. after inoculation (Fig. 1-b). They can be assumed to be primitive muscle fibers from their morphology.

2. The somite cells (stage 24): Plating of the inoculated cells are almost completed within about 6-10 hrs. after inoculation. Two cell types can be distinguished; the myoblast-like cells with spindle shape and the fibroblasts (Fig. 2-a). The latter cells are not large and spread as seen in the culture of the breast muscle material. The formation of MNF starts earlier than in the cultured breast muscle cells. At 24 hrs. after inoculation, a considerable number of MNF are seen (Fig. 2-b). In these cultures filopodia-like processes are abundant

at the surface of MNF and myoblasts (Fig. 3). It is probable that these filopodia might play some role in the rapid formation of MNF. We cultured MNF for 5-6 days *in vitro* without any sign of deterioration, but the sound indications to call them as muscle fibers, i. e. the presence of characteristic pattern of cross-striation and spontaneous contraction, are not fully available yet. So, we designate here the structure simply as "multinucleated fibers (MNF)".

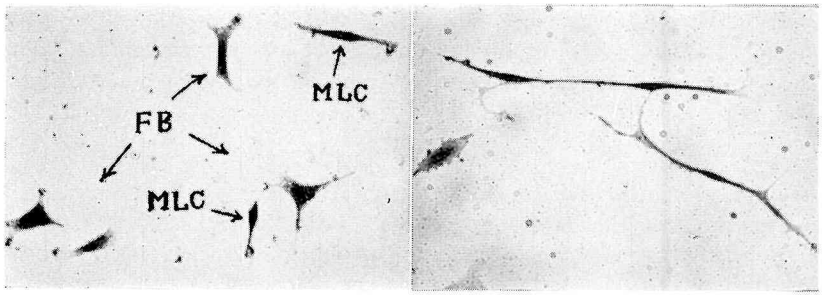

Fig. 2. a. Myoblast-like cells (MLC) and fibroblasts (FB) from the somite cells (st. 24) in 72 hrs' culture; stained with Meyer's haematoxylin. (x 200)
b. MNF containing 3-nuclei from the somite cells (st. 24) in 72 hrs' culture. The filopodia-like processes are seen. (x 200)

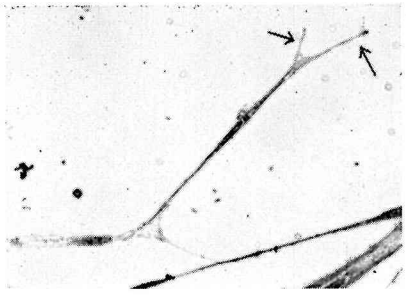

Fig. 3. Filopodia-like processes seen at the periphery of myoblasts from the somite cells (st. 24) in 48 hrs' culture; stained with Meyer's haematoxylin. (x 250)

B. Comparison of the process of MNF formation from the somite cells with that from the breast muscle cells

The formation of MNF from the somite cells starts at earlier stages of culturing than in the breast muscle cells. As shown in Table 1, a percentage of the nuclei incorporated into MNF to the whole nuclear number is 7.8 % in the somite cells after 24 hrs. in culture, while it is 2.8 % in the breast muscle culture. The data given in Table 1-b indicate that in the somite cells the formation of multinuclearity does not only start earlier but also it proceeds more rapidly than in the other cells. MNF with more than 4-5 nuclei appeared only in the somite cultures at 24 hrs' period. A gradual increase in the number of the nuclei incorporated within MNF during the first 24 hrs' period of somite cell culture

Table 1. Formation of MNF *in vitro* culture

a. Percentage of the "multinuclear" nuclei against the total nuclear number.

	24 hrs.	72 hrs.
Somite (st. 24)	7.8% (129/1630)*	7.3% (116/1601)
Breast muscle (st. 38)	2.8 (43/1516)	8.9 (138/1546)

* No. of nuclei in MNF / total nuclear number

b. Temporal changes in nuclear number contained in MNF.

	No. of nuclei in the individual MNF (N)	24 hrs.	72 hrs.
Somite (st. 24)	2	17/37*(46)**	7/34 (21)
	3	7/37 (19)	10/34 (29)
	4-5	5/37 (13)	4/34 (12)
	> 5	8/37 (22)	13/34 (38)
Breast muscle (st. 38)	2	5/7 (71)	17/29 (59)
	3	2/7 (29)	8/29 (28)
	4-5	0/7 (0)	4/29 (14)
	> 5	0/7 (0)	0/29 (0)

* No. of MNF containing (N) nuclei. / total number of MNF

** percentage

was recognized by counting at shorter intervals (Table 2). Therefore, the precocious appearance of multinuclearity in these cells is not simply due to a plating of undissociated or unfiltrated multinuclear cells, but it should be mostly due to a fusion of the mononucleated myoblasts.

Table 2. Increase of the multinuclearity counted at short intervals in the somite cells.

Time after inoculation (hrs.)	No. of nuclei found in MNF (M)	Total nuclear number (T)	% (M/T)
6	7	1502	0.5
10	24	1587	1.5
16	77	1599	4.8
20	73	1489	4.9
24*	104	1529	6.8

* The data at 24 hrs' period were derived from a different series of culture from that given in Table 1-a.

C. DNA synthesis and the formation of MNF

1. Comparison of the indices of ^{3}H-thymidine labelling between the somite cells and breast muscle cells.

When the cultures of breast material were labelled with ^{3}H-thymidine, almost of all the nuclei incorporated into MNF were labelled (96 % after 72 hrs; Fig. 4), whereas in the same structure derived from the somite cells labelling indices were much lower(52 % and 60 % after 24 and 72 hrs. respectively; see Table 3 and Fig. 5).

Table 3. Labelling indices of the somite cells and breast muscle cells cultured *in vitro*.

	time after inoculation* (hrs.)	MNF	myoblast	fibroblast
Somite (st. 24)	24	43/82 (52)**	16/27 (59)	212/354 (59)
	72	82/136 (60)	13/17 (76)	207/213 (97)
Breast muscle (st. 38)	24	———	9/38 (24)	26/117 (22)
	72	83/86 (96)	19/20 (95)	242/244 (99)

* The cells were in the medium containing ^{3}H-thymidine throughout the culture period before fixation for autoradiography.

** Percentage of the number of the labelled nuclei to the total nuclear number of the given cell type.

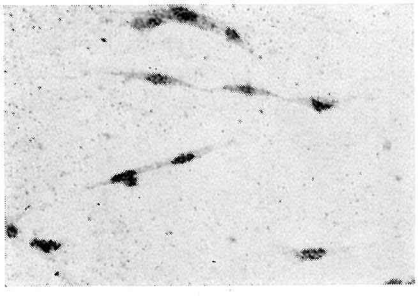

Fig. 4. Labelling for 72 hrs. *in vitro* culture of the breast muscle from the embryos at stage 38. All the nuclei within MNF are labelled. (x 200)

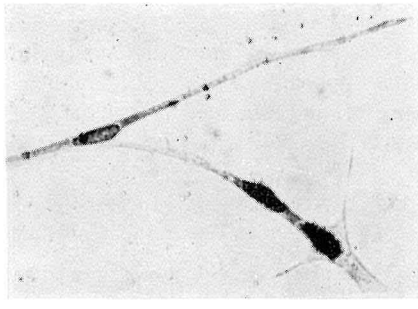

Fig. 5. Labelling for 72 hrs. *in vitro* culture of the somite cells from the embryos at stage 24, showing MNF with one unlabelled nuclei which is in the process of fusion with other labelled cells. (x 300)

2. The process of the formation of MNF in the somite cells revealed by autoradiography.

To examine the precocious formation of MNF in the somite cells more precisely, the behavior of the labelled nuclei was traced by means of autoradiography.

Pulse-chase experiments

The pulse-chase experiments with 6-10 hrs' labelling period were conducted within initial 24 hrs. of culturing (Table 4). The labelling indices of 10-20 % were obtained in MNF at whatever period the pulse was given. The labelled nuclei by the pulse at the terminal stage (from 18 hrs. to 24 hrs.) appear to be

Table 4. Pulse-chase experiment at various times during 24 hrs' period of the somite cultures.

pulse time after inoculation (hrs.)*	MNF	mononuclear cell
0-10	21/183 (16)**	10/102 (10)**
6-12	27/149 (18)	24/294 (8)
12-18	11/89 (12)	23/360 (6)
18-24	18/76 (24)	29/199 (15)

* In each experiment, the cultures were fixed at 24 hrs.
** Percentage of the number of the labelled nuclei to the total nuclear number.

generally located in the outermost part of MNF. These nuclei may be newly incorporated into this structure. In the all series of the pulse-chase experiments the labelling indices of nuclei of MNF are always higher than those of the mononucleated cells. The finding suggests that the myoblasts to fuse into MNF *in vitro* may be more actively proliferating population than other population to remain as mononuclear.

In situ labelling experiments

To know the proliferating activity of the cells of the somite at pre-culturing period, labelling was done *in situ* and subsequently the cells of the dissected somites of the labelling embryos were cultured (Fig. 6). As seen in Table 5, after 10 hrs' *in situ* and subsequent 24 hrs' *in vitro* labelling, 14 % of the nuclei in MNF remains unlabelled. The result indicates that the nuclei which did not proliferate at least during these *in situ* and *in vitro* labelling periods can be

Table 5. *In situ* labelling and subsequent culture *in vitro* with or without ^{3}H-thymidine.

24 hrs culture	after 5 hrs' *in situ* labelling		after 10 hrs' *in situ* labelling	
	MNF	mononuclear cell	MNF	mononuclear cell
with cold thymidine	121/239 (51)*	236/483 (49)	162/223 (73)	361/452 (80)
with ^{3}H-thymidine	140/217 (65)	334/498 (67)	212/246 (86)	425/455 (93)

* Percentage of the number of the labelled nuclei to the total nuclear number of the given cell type (labelling index).

incorporated into the formation of MNF. On the other hand the fact that labelling indices of MNF formed in the cultures with the isotope *in vitro* period are higher than those in the cultures with the cold medium shows an involvement of the freshly divided nuclei during *in vitro* period into MNF formation.

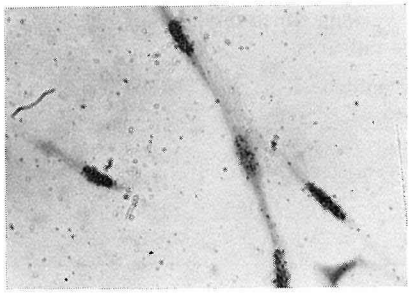

Fig. 6. Labelling for 10 hrs. *in situ* and subsequent 24 hrs' culturing *in vitro* without the isotope; showing the labelled nuclei within MNF. (x 200)

Discussion

The present results demonstrate the *in vitro* formation of MNF from the somite cells of stage 24 embryos. Since the nuclei once incorporated into MNF do not usually synthesize DNA (Konigsberg et al., 1960; Stockdale and Holtzer, 1961) and the nuclei within MNF are always diploid (Strehler et al., 1963), the ^{3}H-thymidine labelled nuclei observed in MNF in the *in vitro* labelling culture should be introduced by the process of cell fusion, after DNA synthesis and cell division of the progenitor mononucleated myoblasts.

According to the present observations, the somite cells can form MNF in culture more rapidly than the breast muscle myoblasts do. Though quantitative data have so far not yet been available, the dissociated somite cells attach on the bottom of the petri dish more rapidly than the cells from the breast muscle do. Can this difference simply be the cause for precocious formation of multinuclearity in the somite material ? A partial answer to this question is obtained from autoradiographic studies. Their results reveal that all the breast muscle cells proliferate *in vitro* prior to fusion, whereas in the somite cells only about a half of the nuclei of the precociously formed MNF within 24 hrs' *in vitro* had passed through DNA synthesis. An occurrence of DNA synthesis in very initial period of culturing is shown by the pulse-chase experiments, in which as many as 10 % of the nuclei in MNF did synthesize DNA within 10 hrs' period (Table 4). Some of the myoblasts of the somite may proliferate with unusually short lag-period after inoculation *in vitro*. The cells with such a high proliferating activity can be almost immediately introduced into the fusion-phase of myogenesis in the *in vitro* culture.

There is another point to arise from the comparison of the behavior of the somite cells and of the breast muscle cells. In MNF from the somite cells nearly half of the nuclei remained unlabelled (48 % after 24 hrs. and 40 % after 72

hrs.), while almost of all the nuclei of MNF from the breast muscle were labelled (only 4 % being left unlabelled; see Table 3). A presence of unlabelled nuclei within MNF formed from the somite cells can be explained by assuming either (1) precocious fusion *in vitro* of the myoblasts which did synthesize DNA just before operation, but not *in vitro* culture period or (2) an occurrence of cell fusion without prior DNA synthesis.

The results of *in situ* labelling experiments may be relevant to this point. If the first assumption were true, all the nuclei in MNF would be labelled in the through-labelling experiments both *in situ* and *in vitro*. But the results do not support this assumption. Actually 14 % of the nuclei in MNF of this experiment remained unlabelled. Therefore, *in vitro* fusion of some myoblasts which did not pass a replicating phase may be presumed. At the same time, it is also probable that some of the unlabelled nuclei could be derived from the MNF which had been preformed *in situ* far before operation.

Acknowledgement

The author is very grateful to Prof. T. S. Okada for his kind advice and reading the manuscript.

References

Avery, G., M. Chow and H. Holtzer (1956). An experimental analysis of the development of the spinal column. V) Reactivity of chick somites. J. exp. Zool., *132*, 409-423.

Coon. H. G. (1966). Clonal stability and phenotypic expression of chick cartilage cells *in vitro*. Proc. Natl. Acad. Sci., *55*, 66-73.

Hamburger, V. and H. L. Hamilton (1951). A series of normal stages in the development of the chick embryo. J. Morph., *88*, 49-92.

Holtzer, H. (1961). Aspects of chondrogenesis and myogenesis. *In* "Synthesis of molecular and cellular structure" ed. D. Rudnick, Ronald Press, New York, pp. 35-87.

Konigsberg, I. R., N. McElvain, M. Tootle and H. Herrmann (1960). The dissociability of deoxyribonucleic acid synthesis from the development of multinuclearity of muscle cells in culture. J. Biophys. Biochem. Cytol., *8*, 333-343.

Konigsberg, I. R. (1965). Aspects of cytodifferentiation of skeletal muscle. *In* "Organogenesis" ed. R. L. DeHaan & H. Ursprung, Holt, Rinehart and Winston, Inc., pp. 337-358.

Stockdale, F. E. and H. Holtzer (1961). DNA synthesis and myogenesis. Exptl. Cell Res., *24*, 508-520.

Strehler, B. L., I. R. Konigsberg and J. E. T. Kelley (1963). Ploidy of myotube nuclei developing *in vitro* as determined with a recording double beam microspectrophotometer. Exptl. Cell Res., *32*, 232-241.