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Expression of Chicken Troponin T Isoforms in Cultured Muscle Cells

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ABSTRACT—Cells prepared from chicken skeletal muscles of different developmental stages were cultured to study their troponin T isoform expression, using antisera specific to the fast- and slow-muscle-type isoforms. We found that the cultured myogenic cells from chickens and chick embryos were classified into two types, fast type and fast/slow type in which fast- and slow-muscle-type isoforms were coexpressed. Cells expressing only slow-muscle-type troponin T isoforms could not be found. Most cells prepared from *pectoralis major* (fast muscle) and *gastrocnemius* (mixed muscle) of 11-day old embryos belonged to the latter, with only a small fraction belonging to the former. The percentage of fast type cells in those cells prepared from *pectoralis major* increased along development to over 90% by the 17th day of incubation, while, in the cells prepared from *gastrocnemius*, it reached a plateau of 30-40% by the 13th day of incubation. All the cells from *anterior latissimus dorsi* (slow muscle) belonged to the fast/slow type. Ratios of these two types of muscle cells varied depending on their origins and stages. The *in vitro* expression of troponin T isoforms was different from the *in vivo* expression, and each muscle seems to be determined differently in the composition of cell types during the developmental course.

INTRODUCTION

In skeletal muscles, proteins specific to myofibril and sarcoplasmic reticulum and enzymes essential for metabolism are uniquely expressed. Among them, contractile proteins have families consisting of very similar isoforms, and different sets of those families are often coordinately regulated within functionally distinct muscle fiber types with respect to the speed of contraction (Bandman, 1992). In some cases, the heterogeneity of muscle fibers is caused by nerve control and hormonal signals. The sequential generations of muscle fibers and the expression of unique proteins depend on the developmental programs of myogenic cells and are diversified by extracellular cues (Cossu and Molinaro, 1987; Miller, 1992; Stockdale, 1992).

In many studies, myosin heavy chain was frequently used as a marker of muscle fiber types. Since different isoforms of myosin heavy chain are expressed in different muscle fibers and at different stages of development, myosin heavy chain is considered useful for studying myogenic cell lineage (Cho *et al.*, 1993; DiMario *et al.*, 1993; Hartley *et al.*, 1992; Schafer *et al.*, 1987). On the other hand, using troponin T (TnT) as a marker, we showed by tissue transplantation experiments that expression patterns of fast-muscle-type (F-type) and slow-muscle-type (S-type) TnT isoforms were seemed to be fixed in cell lineage (Yao *et al.*, 1992, 1994).

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TnT is one of the three subunits of troponin complex. It has been shown that there are three classes of TnT isoforms specific to different fiber types of striated muscle: F-type TnT, S-type TnT, and cardiac-muscle-type (C-type) TnT (Perry, 1985; Tsukui and Ebashi, 1973). The three classes of isoforms are encoded by three different genes (Cooper and Ordahl, 1984; Gahlmann *et al.*, 1987; Smille *et al.*, 1988).

In this study, the expression of TnT isoforms has been investigated by immunohistochemical techniques in cultured cells prepared from chicken skeletal muscles at various developmental stages in order to investigate the stability of TnT expression pattern *in vitro*. We have shown that the *in vitro* expression of TnT isoforms was apparently different from the *in vivo* expression.

MATERIALS AND METHODS

Animals

White leghorn chickens (*Gallus domesticus* (L)) and their fertilized eggs were obtained from commercial sources.

Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out essentially according to the method of Laemmli (1970) with 12% acrylamide gels.

Preparation of antisera and immunoblotting

To prepare an antiserum against F-type TnT, the immunogen was prepared by the method of Ebashi *et al.* (1971), further purified by SDS-PAGE, and cut out from the gels. The gels were dialyzed at

4°C against phosphate buffered saline (PBS) for one day, mixed with Freund's complete adjuvant, and injected into the back of a guinea pig. The anti-F-type TnT (guinea pig serum) reacted with F-type isoforms of TnT (Fig. 1). The anti-S-type TnT (rabbit serum) has been described by Yao *et al.* (1992). The anti-C-type TnT (rabbit serum) is a personal gift from Dr. M. Oishi, Kitasato University.

After electrophoresis, proteins in gels were transferred to a nitrocellulose membrane by a combination of the methods of Towbin *et al.* (1979) and Franke *et al.* (1981) at 300 mA for 3 hr at 0°C.

Immunoblotting was performed using anti-F-type TnT, anti-S-type TnT, and anti-C-type TnT as the first antibodies. The second antibodies were rhodamine-conjugated anti-rabbit IgG goat serum (Jackson Immunoresearch Laboratories) and rhodamine-conjugated anti-guinea pig IgG goat serum (Cappel).

Cell isolation

Cells were prepared from *pectoralis major*, *gastrocnemius*, and *anterior latissimus dorsi* (ALD) of 11-, 13- and 17-day old embryos and 1- and 60-day old chickens. Muscle tissue (2-5 g) was minced with sharp scissors into 1-2 mm fragments. The fragments were washed twice with Ca^{++}- and Mg^{++}-free Tyrode's solution (CMF) and treated with trypsin at a final concentration of 0.1% in CMF for 30 min at 37°C with constant shaking. The ratio of the trypsin solution to muscle tissue was kept at 4-5 ml solution/g tissue. After the treatment, the suspension was centrifuged at approximately 100×g for 5 min to collect liberated single cells. The pellet was resuspended in 2 ml of a growth medium (37.5% Dulbecco's modified Eagle's minimum essential medium (DMEM), 37.5% Ham's F12, 20% fetal bovine serum, 5% chick embryo extract, Gentamicin at 4.0 mg/l of medium). Cells were then counted with a hemocytometer. The chick embryo extract was prepared essentially according to the method of Hauschka and Konigsberg (1966).

Differential trypsinization

To obtain purer populations of myoblasts, we used the method by Kaighn *et al.* (1966) with some modifications as follows.

Two ml suspension of the cells (10^2-10^3 cells) was incubated in a 60 mm collagen-coated plastic dish for 3 days. Before the myoblasts fused, the medium was removed and the plate washed twice with CMF to remove unattached cells and debris. After 2 ml of 0.01% trypsin in CMF was added, the plate was incubated at 37°C in 5% CO_2. After about 3-5 min bipolar processes of myoblasts retracted and the cells rounded up, while flattened fibroblasts remained unaffected. Then, the plate was swirled to suspend cells and the suspension was transferred to a centrifuge tube. The cells in the solution were collected by centrifugation at approximately 100×g for 5 min and resuspended in 3 ml of the growth medium.

Differential cell adhesion

We performed this preparation procedure essentially according to the method of Yaffe (1968) to obtain purer populations of myoblasts.

The cell suspension was plated on non-collagen-coated plastic dishes and incubated at 37°C in 5% CO_2. After 30 min of incubation, the medium was collected. The plating was repeated once more, and the floating cells were collected.

Cell culture

The collected cells were counted and plated at a concentration of 1×10^3 cells/well with 0.3 ml of the growth medium on a collagen-coated Celltight C-1 Celldesk (Sumilon) in one well of a 24-well plate to culture at 37°C in 5% CO_2. When myoblasts began to fuse in culture, after about 2 days, the growth medium was exchanged with a differentiation medium (47.5% DMEM, 47.5% Ham's F12, 4% fetal bovine serum, 1% chick embryo extract, Gentamicin at 4.0 mg/l of medium). Thereafter, the differentiation medium was changed every other day. The cells adhering to Celldesks were examined on the 6th day after plating.

Indirect immunofluorescence microscopy

Immunohistochemistry was carried out with the anti-F-type TnT, anti-S-type TnT, and anti-C-type TnT according to the method used by Yao *et al.* (1994).

Immunocytochemistry was carried out by the method of Hirai and Hirabayashi (1986) with some modifications as follows. The cultured cells adhering to Celldesk were fixed with 3% paraformaldehyde in PBS for 20 min at 0°C, permeabilized with 0.1% Triton X-100 in PBS for 3 min at 0°C, and treated with 0.1 M glycine in PBS for 20 min at room temperature. The cells were firstly incubated with the anti-S-type TnT for 1 hr at room temperature, and washed 5 times with PBS for 2 min and secondly incubated with the fluorescein-conjugate anti-rabbit IgG goat serum for 1 hr. The same process as this was repeated with the anti-F-type TnT and the rhodamine-conjugated anti-guinea pig IgG goat serum.

Pictures were taken with a combination of a Nikon Optiphot microscope and a Nikon FX-35WA camera.

RESULTS

Specificity of antisera

SDS-PAGE and immunoblotting patterns of tissue extracts prepared from adult muscles of chicken were presented in Fig. 1. The immunoblotting patterns were obtained with three kinds of antisera: anti-F-type TnT, anti-S-type TnT, and anti-C-type TnT.

Reacting with the anti-F-type TnT, extracts of *pectoralis major*, *triceps brachii*, and *gastrocnemius* gave 2 bands (Fig. 1b, lane 1), 7 bands (Fig. 1b, lane 2), and 4 bands (Fig. 1b, lane 3), respectively. *Triceps brachii* was used here only to monitor the variety of F-type TnT isoforms. These bands corresponded to the F-type isoform compositions of TnT as described in our previous papers (Imai *et al.*, 1986; Yao *et al.*, 1992).

In the reaction with the anti-S-type TnT, extracts of *gastrocnemius* and ALD gave one band (Fig. 1c, lanes 3 and 4) corresponding to the S-type isoform composition as reported previously (Yao *et al.*, 1992).

Reacting with the anti-C-type TnT only the extract of *ventricle* gave a single band (Fig. 1d, lane 5).

Expression of TnT isoforms in developing skeletal muscles

Immunohistochemical studies of serial sections of *pectoralis major*, *gastrocnemius*, and ALD of different stages were performed (Table 1). In some reports, tissue specific expression pattern of TnT isoforms has been established in chicken and other vertebrate species (Dhoot and Perry, 1979; Obinata, 1985; Reiser *et al.*, 1992; Swynghedauw, 1986). But so far, no information was reported about the distribution of F-type and S-type TnT isoforms in the developing muscles of chicken. Longitudinal serial sections of *pectoralis major*, *gastrocnemius*, ALD , and *ventricle* muscles from chickens were stained with the three kinds of antisera. All fibers in the adult *pectoralis major* were stained with anti-F-type TnT. Fibers in adult *gastrocnemius* were of three types; many fibers were stained only with anti-F-type TnT, some stained with both the antisera and occasional fibers stained only with anti-S-type TnT. All fibers in adult ALD were stained only with anti-S-type TnT and all fibers in ventricle were stained with only anti-C-

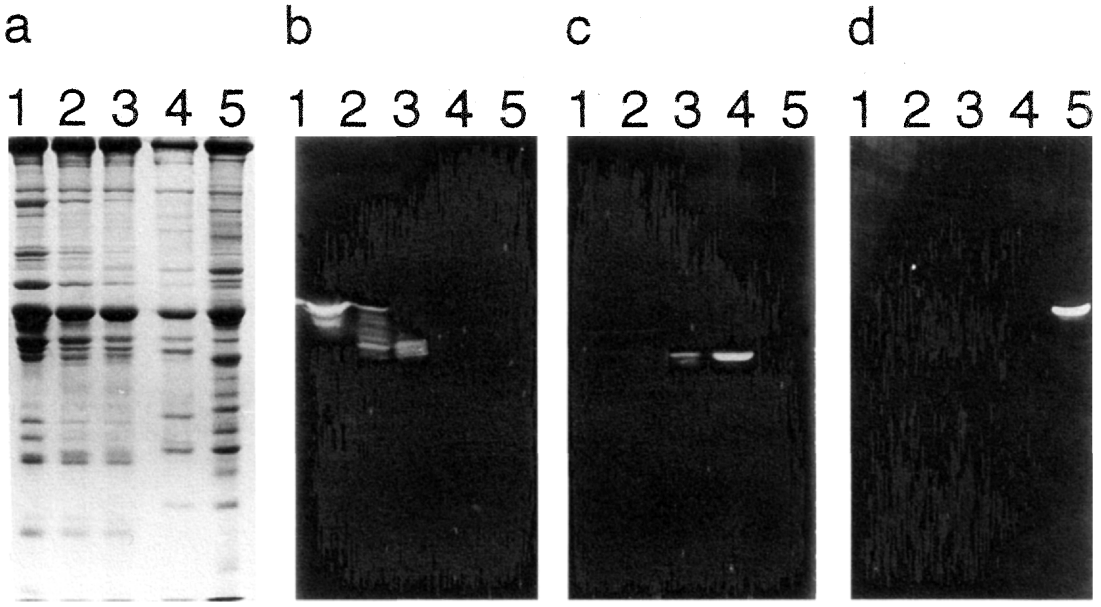

Fig. 1. Specificity of antisera. The specificity of anti-fast-muscle-type troponin T (b), anti-slow-muscle-type troponin T (c), and anti-cardiac-muscle-type troponin T (d) was investigated by immunoblotting of the extracts from *pectoralis major* (lane 1), *triceps brachii* (lane 2), *gastrocnemius* (lane 3), *anterior latissimus dorsi* (lane 4), and *ventricle* (lane 5). a, SDS-PAGE pattern stained with Coomassie brilliant blue R.

Table 1. Reactivity of muscle tissues with antisera

Stage	*Pectoralis major*	*Gastrocnemius*	*Anterior latissimus dorsi*
E11	F/S/C	F/S	F/S/C
E13	F/S/C	F/S	F/S
E15	F	F, F/S, S	F/S
E17	F	F, F/S, S	S
E19	F	F, F/S, S	S
H1	F	F, F/S, S	S
H60	F	F, F/S, S	S
Adult	F	F, F/S, S	S

The results from immunofluorescence analysis of serial sections of *pectoralis major*, *gastrocnemius*, and *anterior latissimus dorsi* muscles at various stages were summarized. The serial sections were stained separately with anti-fast-muscle-type troponin T, anti-slow-muscle-type troponin T or anti-cardiac-muscle-type troponin T. F, anti-fast-muscle-type troponin T positive; S, anti-slow-muscle-type troponin T positive; C, anti-cardiac-muscle-type troponin T positive; Slashes mean that single fibers are positive with 2 or 3 antisera; E11~19, 11~19-day old embryos; H1 and H60, 1- and 60-day old chickens.

type TnT. These immunohistochemical observations were compatible with the results of immunoblotting of these adult muscle extracts (Fig. 1). All fibers of *pectoralis major* from 15-day old embryos (E15) and older ones were stained only with anti-F-type TnT, but those from 11- and 13-day old embryos (E11 and E13) were stained with all of the three kinds of antisera. In contrast to these, all fibers of ALD from 17-day old embryos and older ones were stained only with anti-S-type TnT, but those from 13- and 15-day old embryos were stained with both anti-F-type TnT and anti-S-type TnT, and all fibers of ALD from 11-day old embryos were stained with the three kinds of antisera. While, in *gastrocnemius* from 15-day old embryos and older ones, fibers were stained with either anti-F-type TnT or anti-S-type TnT, or with both the antisera, and all fibers from 11- and 13-day old embryos were stained with both anti-F-type TnT and anti-S-type TnT.

Expression of TnT isoforms in cultured muscle cells

The expression pattern of TnT isoforms in chicken skeletal muscles seemed to be changed on around the 15th day of incubation (Table 1). In addition, the previous studies (Yao *et al.*, 1992, 1994) suggested that the expression pattern of TnT isoforms was fixed in cell lineage. Therefore, we thought that characteristics of TnT isoform expression in myogenic cells might have been fixed on around the 13th day of incubation, and changes in the isoform expression might be detected in cultured cells prepared from the embryos of around the 13th day of incubation.

Myogenic cells were isolated from *pectoralis major*, *gastrocnemius*, and ALD of chickens of different stages, using differential trypsinization and cell adhesion techniques, cultured for 9 days, and double-immunostained with the anti-F-type TnT and anti-S-type TnT to examine which type of TnT isoforms was expressed in each cell.

Representative results were presented in Fig. 2. Cells from *pectoralis major* of 11-day old embryos (Fig. 2a-d), *gastrocnemius* of 13-day old embryos (Fig. 2e-h), and ALD of 1-day old chicks (Fig. 2i and j) were stained. Cells stained with both anti-F-type TnT and anti-S-type TnT were in the majority at these stages (Fig. 2c-f, i, and j) There were, however, some cells stained only with the anti-F-type TnT among those from *pectoralis major* and *gastrocnemius* (Fig. 2a, b, g, and h).

The results of double staining of cultured muscle with the

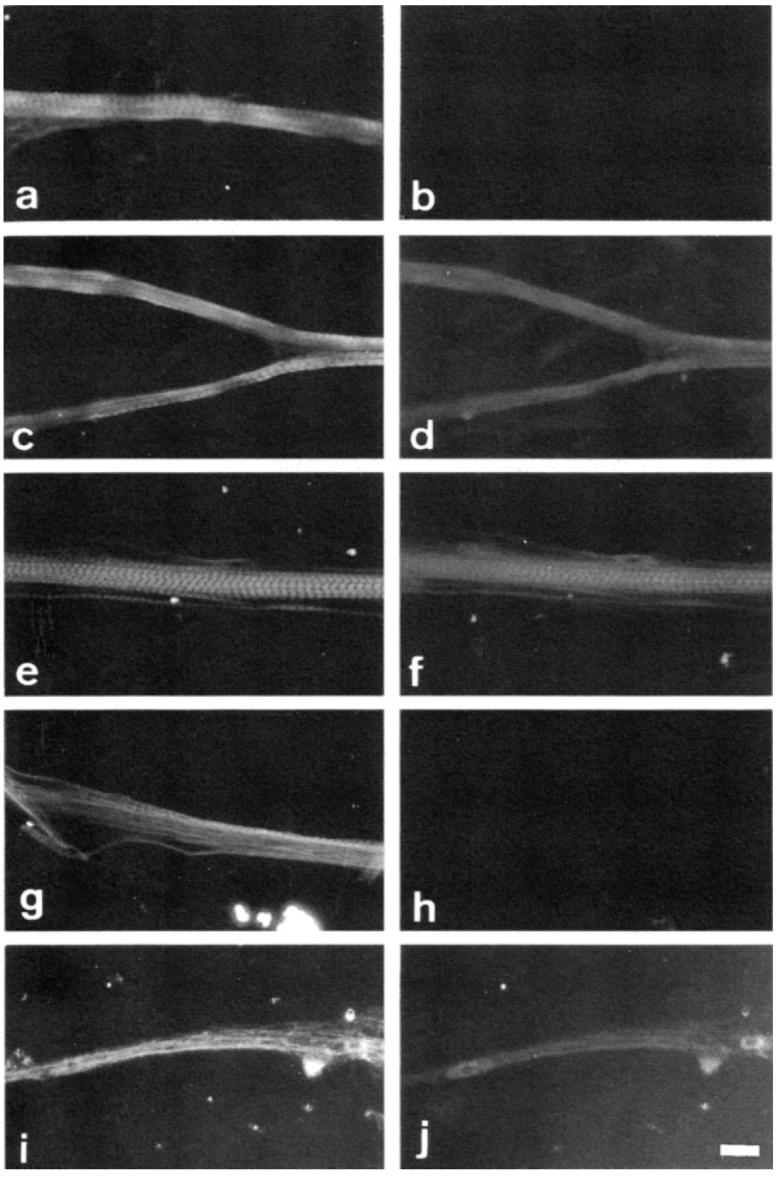

Fig. 2. Double immunofluorescence analysis of troponin T in cultured muscle cells. Myoblasts were prepared from *pectoralis major* of 11-day old embryos (a-d), *gastrocnemius* of 13-day old embryos (e-h) and *anterior latissimus dorsi* of 1-day old chicks (i and j) and cultured for 9 days. Cells were fixed and processed for double immunofluorescence as described in Materials and Methods. Rhodamine (a, c, e, g, and i) and fluorescein (b, d, f, h, and j) fluorescence represents expression of fast-muscle-type troponin T and slow-muscle-type troponin T, respectively, in the same myotubes. ×400. Bar=10 μm.

anti-F-type TnT and S-type TnT were summarized in Table 2. Most cells from *pectoralis major* and *gastrocnemius* from the youngest embryos (E11) were stained with both anti-F-type TnT and anti-S-type TnT ("Fast/Slow" in Table 2), leaving only small fractions (7.2% and 1.0%, respectively) stained only with the anti-F-type TnT ("Fast" in Table 2). Differences in the percentages of stained cells along development were found between *pectoralis major* and *gastrocnemius*: In the former the percentages of cells stained only with anti-F-type TnT increased to over 90% by the 17th day of incubation, while those in the latter reached a plateau of 30-40% by the 13th day of incubation.

ALD presented a clearcut result: All cells from ALD were stained with both anti-F-type TnT and anti-S-type TnT irrespective of the stage of source materials. Preparation of the muscle cells from the embryos was so difficult that we could not determine the types of the cells from ALD of 17-day old embryos or younger one (Table 2).

Another clearcut result in Table 2 was that no cell was stained only with the anti-S-type TnT as far as this experiment was concerned ("Slow" in Table 2).

DISCUSSION

In vivo expression of TnT isoforms

Muscle fiber types could be distinguished on the basis of the expression of TnT isoforms (Dhoot and Perry, 1979; Perry, 1985). In the chicken, it was functionally defined by Swynghedauw (1986) that *pectoralis major* was a fast muscle, ALD a slow one, and *gastrocnemius* a mixed one, and these were in good agreement with results of this study. Therefore, TnT isoforms are reasonably considered to be good markers of chicken muscle fiber types.

All fibers of *pectoralis major* from 15-day old embryos (E15) and older ones were stained only with the anti-F-type TnT, but those from 11- and 13-day old embryos (E11 and

Table 2. Reactivity of cultured muscle cells with antisera

Source of cells		Percentage (numbers) of cells reacted			
Muscle	Stage	Fast	Fast/Slow	Slow	Numbers of fields
Pectoralis major	E11	7.2 (15)	92.8 (194)	0 (0)	3
	E13	34.0 (223)	66.0 (432)	0 (0)	2
	E17	93.8 (436)	6.2 (29)	0 (0)	3
	H1	93.4 (99)	6.6 (7)	0 (0)	3
	H60	95.0 (165)	5.0 (9)	0 (0)	3
Gastrocnemius					
	E11	1.0 (4)	99.0 (380)	0 (0)	2
	E13	33.0 (148)	67.0 (301)	0 (0)	3
	E17	43.1 (260)	56.9 (343)	0 (0)	3
	H1	37.4 (43)	62.6 (72)	0 (0)	3
	H60	37.6 (74)	62.4 (123)	0 (0)	3
Anterior latissimus dorsi	H1	0 (0)	100 (82)	0 (0)	3
	H60	0 (0)	100 (75)	0 (0)	2

The results from double immunofluorescence analysis of cultured cells prepared from *pectoralis major*, *gastrocnemius* and *anterior latissimus dorsi* were summarized. The cells stained with the antisera were counted, changing the microscope field several times (numbers of fields). Fast, only anti-fast-muscle-type troponin T positive; Fast/Slow, both anti-fast- and anti-slow-muscle-type troponin T positive; Slow, only anti-slow-muscle-type troponin T positive; E11~17, 11~17-day old embryos; H1 and H60, 1- and 60-day old chickens.

E13) were stained with all the three kinds of antisera. Therefore, differential expression of TnT isoforms occurred during myogenesis, supporting Obinata's results that showed transition of TnT isoforms from cardiac-muscle-type to fast-muscle-type in myogenesis of chicken breast muscle (Obinata, 1985). However no information was so far reported about the expression of S-type TnT isoforms. In *gastrocnemius* and ALD from 17-day old embryos and older ones, fibers expressed the same TnT isoforms as adult fibers do, while, fibers of 13- or 15-day old embryos and younger ones did not (Table 1).

To make sure of the results of the tissue immunostaining of *pectoralis major* from 11- and 13-day old embryos, we performed immunoblotting and confirmed that the fibers of these muscles expressed all three kinds of TnT (data not shown).

Our results also showed changes in expression of TnT isoforms in *gastrocnemius* and ALD. Taking these results together, it can be concluded that the expression pattern of TnT isoforms in chicken skeletal muscles transits in the embryonic muscle tissues, and that muscle fiber type is probably determined on around the 15th day of incubation.

In vitro expression of TnT isoforms

On the basis of the expression of TnT isoforms, cultured muscle cells were classified into two types: One is fast type cells stained only with anti-F-type TnT and the other is fast/slow type cells stained with both anti-F-type TnT and anti-S-type TnT. No cells could be found which were stained only with anti-S-type TnT even in the muscle cells prepared from *gastrocnemius* and ALD (Table 2 and Fig. 2). However, the fibers stained only with anti-S-type TnT were present in the tissues of these muscles (Table 1). Why were slow type cells (the cells expressing only S-type TnT) absent in culture?

We can not remove completely the possibility that the discrepancy in S-type TnT isoform expression between cultured cells and muscle tissues was caused by cell selection during cell preparation. We do not think, however, that cell selection was a major reason for this discrepancy, because we could not find slow type cells among the cells prepared by a method in which processes of differential trypsinization and differential cell adhesion were omitted (data not shown). Then, how do the cells in muscle tissue express only S-type TnT isoform? We think there are two possibilities: One is that the intrinsic potential of slow type cells to express F-type TnT isoforms is suppressed *in vivo*. This possibility was supported by similar experiments with S-type myosin heavy chain isoforms in cultured cells from human fast muscle (Cho *et al.*, 1993) and with C-type TnT isoforms in cultured cells from chicken fast muscle (Toyota and Shimada, 1983). The other possibility is that muscle cell differentiation was delayed in cultured cells. Cells cultured in the differentiation medium for 6 days might have been still immature to express specific isoforms, and expressed both F-type and S-type isoforms like *in vivo* muscle cells at earlier developmental stages. In the previous study, Yao *et al.* (1994) transplanted ALD into *pectoralis major*, and detected the fibers expressing only S-type TnT on the 58th day after the operation. Therefore, a long-time culture as in their experiments might have made it possible for the cells to express only S-type TnT.

Since we think the two cases are equally possible and have no further experimental bases to remove either one, we cannot determine which is more likely, or rather we would like to say that both are the cases.

Ratios of fast/slow type to fast type cells varied depending on the stage and origin of the muscle examined. In the cultured cells from 11-day old embryos, most cells belonged to the fast/slow type, while, in the cells from 13-day old embryos, the ratio of fast/slow type to fast type was 3:1. The ratios in the cultured cells prepared from embryos older than the 17th day of incubation were seemed to be fixed depending on the cell sources. The developmental change in the ratio seemed to reflect the appearance of satellite cells (Feldman and Stockdale, 1992; Hartley *et al.*, 1992) rather than the phase of myogenesis, such as primary or secondary myogenesis (Miller and Stockdale, 1986).

Myosin heavy chain isoforms have been used as good markers of muscle fiber types, and their expressions have been described in detail (Feldman and Stockdale, 1991; Miller *et al.*, 1985; Schafer *et al.*, 1987; Stockdale and Miller, 1987). However, the results reported here are not in good agreement with those works based on the expression of myosin heavy chain isoform. With respect to myosin heavy chain, most cultured cells prepared from *pectoralis major* of 11-day old embryos were fast type (Miller *et al.*, 1985), all cultured cells from adult *pectoralis major* were fast type, and those from

adult ALD were a mixture of fast type and fast/slow type (Feldman and Stockdale, 1991).

The reason for the discrepancy between the results with TnT and myosin heavy chain is not clear, but probably it would be due to the fiber type markers used in the two experiments. On the basis of TnT expression, there were only slow type fibers in adult ALD , but on the basis of myosin heavy chain expression, there were fast type and slow type fibers (Feldman and Stockdale, 1991). It is conceivable that regulatory mechanisms of expressions are different between TnT and myosin heavy chain.

As far as we know, this is the first report in which the preparation of muscle cells from ALD of 1-day old chicks was succeeded. In fact, preparation of muscle cells from the young ALD was very difficult, so that no one uses these young tissues to prepare cells for culture.

In conclusion, the expression of TnT in the cells from chicken muscles of these stages reflects their origins and stages, although it apparently deviates *in vitro* from that *in vivo*. The expression of TnT isoforms is stable and reproducible, so that this protein can be a good marker of myogenic cell lineage, together with myosin heavy chain.

In the next study, we will perform clonal analysis to investigate further the stability or otherwise of TnT isoform expression in muscle cells prepared from various stages.

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