Studies on Development and Differentiation of Muscle. VI. Cytokinetic Analysis of Cell Proliferation by Using 3H-thymidine Autoradiography in Various Muscle Tissues of Chick Embryo

Tohoku Journal of Agricultural Research
Vol. 25, No. 1, June 1974
Printed in Japan

Studies on Development and Differentiation of Muscle. VI. Cytokinetic Analysis of Cell Proliferation by Using ^{3}H-thymidine Autoradiography in Various Muscle Tissues of Chick Embryo

Tateki KIKUCHI, Tamio NAGATANI* and Hideo TAMATE

Department of Animal Science, Faculty of Agriculture, Tohoku University, Sendai, Japan

(Received, June 30, 1974)

Summary

The main purpose of the present study is to analyze the cytokinetics of the presumptive myoblasts proliferating in various muscle tissues such as the M. complexus, the M. biceps femoris and the M. pectoralis at the different embryonic days by using ^{3}H-thymidine autoradiographic techniques. The labeling index (LI), the mitotic index (MI) and the proliferation index (PI) in these muscles began to decline after 9 days of incubation and showed a large difference in the pattern of decline between the M. complexus and the other muscles at 10, 12 and 13 days of incubation. During these developmental periods, the changes of these values in the M. complexus showed a more remarkable decline than the other muscles. The cytokinetic analysis of the presumptive myoblasts demonstrated that there was very little difference in the duration of the generation time and its component phases in various muscle species, but the length of the total mitotic cycle was prolonged progressively with the passing of the developmental stages. The lengthning of the generation time was mainly dependent upon the increase of the G_1-period of the mitotic cycle. Therefore, the reason why the LI, MI and PI values of the M. complexus at 10, 12 and 13 days of incubation indicated lower values primarily depends upon a rapid decline in the percentage of the actively dividing myoblasts. It seemed reasonable to assume that the growth and the proliferation of the successive generation myotubes affected the cell fusion of the myogenic cells which wait for a chance for cell contacts and cell fusion. The mode of increase in the number of myotubes within the primordia of the primary muscle fascicles were discussed.

Introduction

In our previous reports (1～4), the mode of increase in the number of skeletal muscle cells in chick embryos were studied with the electron and light microscope

* Present address: Technical Research Institute of National Federation of Daily Co-operating Association, Sayama, Saitama, Japan.

and the myogenic morphology of the M. complexus were compared with that of the M. pectoralis and the M. biceps femoris in chick embryos. The M. complexus, commonly called the "Hatching muscle", showed a remarkable growth during the later stage of development for the breaking of the egg shell at hatching time. The successive generation myotubes in the M. complexus, however, already began to enlarge more rapidly after 12 days of incubation than those in the other muscles. It was, therefore, strongly suspected that there are some characteristic differences in the mode of increase in the number and the size of myotubes between the M. complexus and the other muscles.

The muscle fibers in adult muscles are gathered in fascicles which consist of varying number of fibers and are surrounded by a layer of connective tissues. These fascicles, designated as primary muscle fascicles, may again be aggregated and gathered into larger fascicles. The primorida of such fascicles could already be distinguished in embyronic muscle tissues at 10 days of incubation. Two type of myotube were observed in the primordia of such fascicles; the primary myotubes (p-myotubes) and the successive generation myotubes (s-myotubes). The p-myotubes show a slight enlargement in cell size, but are nearly equal to various muscles during whole stages of development. However, the s-myotubes differentiate and enlarge more rapidly in the M. complexus after 12 days of incubation than in other muscles (2). Since the space in a given primary fascicle decreases when s-myotubes increase their number and cell size, the numbers of the myotubes in the M. complexus remain smaller than those in the other muscles (4). These mechanisms of the reciprocal relationship between the number and the size of s-myotubes in a given primary fascicle was also adapted for the total number of myotubes counted in the cross section of muscle tissues (4).

It has been pointed out that the formation of multinucleated myotubes is associated with a distinct change in the DNA metabolism. While mononucleated myoblasts synthesize DNA and divide, the incorporation of DNA precursors or mitotic figures have not been observed within multinucleated myotubes (5, 6). The "presumptive myoblast" which is mononucleate and synthesizes DNA and actively divides along the surface of the p-myotubes, contains no detectable myofibrils. Only "myoblasts which cease to synthesize DNA begin to produce muscle specific proteins. These myoblasts fuse with each other and with immature myotubes and form the multinucleated "myotubes" only at the G_1 phase of the mitotic cycles (7). It is interesting to note under what conditions the proliferating presumptive myoblasts stop cell division and fuse with the other myogenic cells and by what mechanisms the rate of increase of the multinucleated myotubes are controlled.

From the fact that there is a large difference in the mode of increase in the number and the growth of s-myotubes in various embyronic muscles, it is expected that there might be some differences in the absolute number and the

time of cell division cycle of the presumptive myoblasts. Since the mechanisms which control the proriferation of s-mytobues and presumptive myoblasts were not known, the main purpose of the present investigation was to analyze the cytokinetic of the presumptive myoblasts in three muscles such as the M. complexus, the M. biceps femoris and the M. pectoralis at the different incubation days by using ^{3}H-thymidine autoradiographic techniques.

Materials and Methods

Fertilized White Leghorn (Iwaya strain) eggs were incubated at 37.8°C for the desired length of time. The embyros were removed from the shell and sacrificed by decaptation, and immersed in Locke's solution at 37°C. The muscle tissues were removed carefully by a forcep and placed into Carnoy's fixing solution for 12 hours at room temperature.

1. *Preparation and Study of ^{3}H-thymidine Autoradigoraphy*

^{3}H-thymidines (5.0 Ci/mmole) were administrated to each embryo by injection into the chorioallantoic vein, following the procedure of Konigsberg (8). From the preliminary experiments, 5.0 μCi of ^{3}H-thymidine until 13 days of incubation and 2.0 μCi of ^{3}H-thymidine per gram weight embryo after 14 days of incubation were administered to each embryo. At the concentration of ^{3}H-thymidine used, a two week exposure was sufficient for detection of labeled nuclei. Its value agrees with that obtained by Marchok and Herrmann (9). After fixation, the muscle tissues were embedded in paraffin and sectioned at 5 μ. The paraffin was removed with xylene, the sections were passed through a graded series of alcohol to water, and dipped in Sakura Conidol-X at 20°C for 4 minutes, followed by

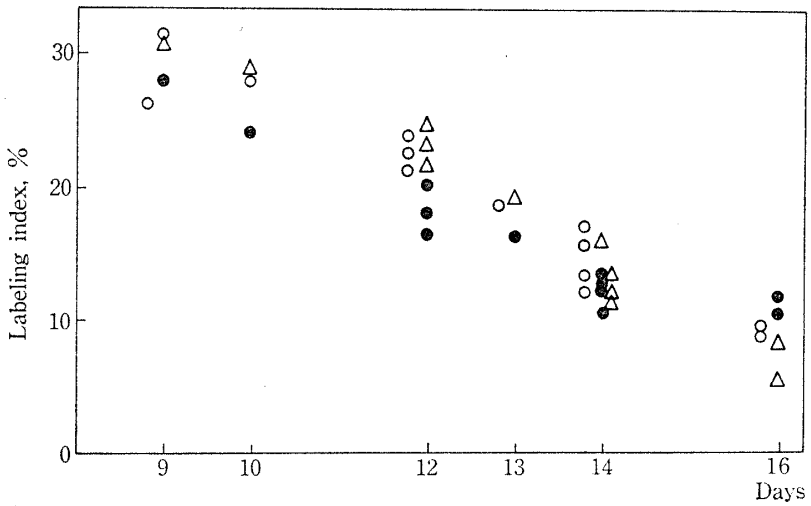

FIG. 1. The changes of Labeling index, 1 hour after injection of ^{3}H-thymidine (2–5 μCi/gm embryo weight), of development chick muscles (*M, complexus*: ●, *M, biceps femoris*: ○, *M, pectoralis*: △).

Sakura Conifix at 20°C. The sections which were not used for auotradiography were stained with PAS-hematoxylin staining for the purpose of calculating the mitotic index.

2. *Determination of the Mitotic Index (MI) and the Labeling Index (LI)*

The mitotic index was calculated by scoring the percentage of nuclei in mitosis. The labeling index was determined one hour after an injection with ^{3}H-thymidine and exposing the emulsion for 2–4 weeks (9, 10), and expressed as percentages of the total number of nuclei labeled with grains more than 4. Estimates were made with the M. complexus, the M. biceps femoris and the M. pectoralis at 9, 10, 12, 13, 14 and 16 days of incubation, respectively.

3. *Determination of Individual Periods of the Mitotic Cycle*

The mitotic cycle of embryonic muscle cells was analyzed by the method of "Labeled mitosis" of Quastler and Sherman (11). Since only the presumptive myoblasts could synthesize DNA and were proliferating frequently in muscle tissues, the mitotic cycle analyzed with labeled mitosis in these experiments was that of the presumptive myoblasts. The intervals between the time of labeling (time zero) and the time when 50 per cent of the metaphase figures appeared labeled represented therefore the average duration of G_2+M. The average generation time (G_1+S+G_2+M) was estimated as the interval between the plateau points on two successive ascending curves. The duration of the DNA synthetic period (S) was determined by measuring the interval between the 50 per cent points on the curve of frequency of labeled metaphase figures. The duration of mitosis (M) was estimated by mitotic index. The presynthetic time G_1 was determined by subtracting G_2, S, and M times from the generation time (7). The estimates were made with the M. complexus, the M. biceps femoris and the M. pectoralis at 10, 12 and 16 days of incubation, respectively.

4. *Determination of the Proliferation Index (PI)*

The durations of the generation time (T) and DNA synthetic phase (S) could be obtained by an analysis of the mitotic cycle. If all myoblasts did not cease to divide by fusion process and synchronized within the time interval of the duration of mitosis, the rate of S per T must be equal to the rate of the labeled cell number (Ns) per absolute cell number (N).

$$\frac{S}{T} \times 100 = \frac{Ns}{N} \times 100 \qquad ①$$

However, the labeling index practically obtained in our experiments showed lower values than the theoretical values obtained by the equation ①. These results were due to the fact that some portions of myoblasts were not synchronized

and entered into the nondividing phase.

$$LI = \frac{Ns'}{N} \times 100 \qquad ②$$

We expressed equations ① and ② by rearranging them as follows;

$$PI = \frac{Ns'}{Ns} \times 100 = \frac{LI \times S}{T}$$

This equation of the proliferation index (PI) represented the fraction of nuclei which were proliferating at several stages of muscle development.

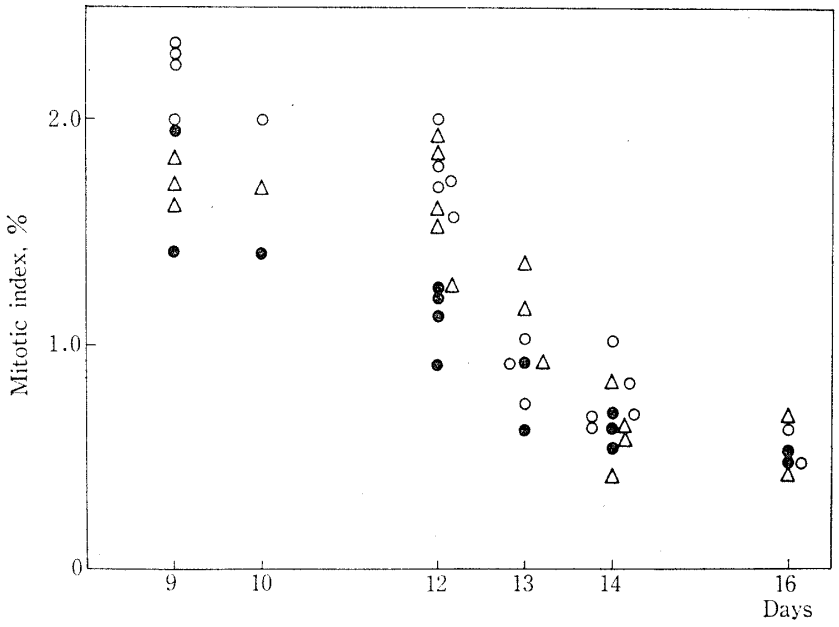

FIG. 2. The changes of Mitotic index (%) in developmental chick muscles (*M. complexus*: ●, *M. biceps femoris*: ○, *M. pectoralis*: △).

Results

The LI and MI values appeared approximately the same in three muscles examined at 9 days of incubation. The values began to decline after 10 days of incubation. A remarkable difference in the pattern of the decline was found between the M. complexus and the other muscles. The LI values in the M. pectoralis decreased from 30.09% at 9 days to 23.03% at 12 days and to 13.31% at 14 days of incubation. A similar decrease from 28.88% at 9 days to 22.5% at 12 days and to 14.78% at 14 days of incubation was observed in the M. biceps femoris. The changes in the LI value in the M. complexus showed a more remarkable decline than the other muscles, namely from 28.07% at 9 days to 18.24% at 12 days and to 16.40% at 13 days of incubation. It attained approximately the

same values as the other muscles at 14 days of incubation. At 16 days of incubation, the LI values appeared in contrast higher in the M. complexus than in the other two muscles, the values being 11.13%, 8.89% and 6.79% in the M. complexus, the M. biceps femoris and the M. pectoralis, respectively (Fig. 1).

The changes in the MI values were approximately parallel with that of the LI, and lower in the M. complexus than those of the other muscles from 10 to 13 days of incubation. The average values at 12 days of incubation were 1.10%, 1.77% and 1.64% in the M. complexus, the M. biceps femoris and the M. pectoralis, respectively (Fig. 2). The difference in the MI values became very small after 14 days of incubation in these muscles.

The cytokinetics of the presumptive myoblasts in the three muscles examined were analyzed by using changes in the percentage of the labeled mitosis at 10, 12 and 16 days of incubation (Fig. 3). At 10 days of incubation, the graphical analysis demonstrated that the durations of the generation time (T) were practically the same in these muscles; 10.25 hours in the M. complexus, 10.25 hours in the M. biceps femoris and 10.60 hours in the M. pectoralis. The durations of its component phases were also the same in these muscles. The average duration of S-, M-, G_2- and G_1- phase in these muscles were 6.79 hours, 0.25 hours, 1.62 hours, and 1.70 respectively. At 12 days of incubation, no differences in the generation time were observed among these muscles, although it was longer than that at 10 days of incubation. The time was 11.60 hours in the M. complexus, 11.50 hours in the M. biceps femoris and 11.60 hours in the M. pectoralis. The average durations of S-, M-, G_2-, and G_1- phases in these muscles were 6.69 hours, 0.24 hours, 1.04 hours and 3.58 hours, respectively. At 16 days of incubation, there were little differences in the generation time among these muscles, although it was still longer than that at 12 days of incubation. It was 13.88 hours in the M. complexus, 13.50 hours in the M. biceps femoris and 13.13 hours in the M. pectoralis. The average durations of S-, M-, G_2- and G_1-phase were 6.29 hours, 0.10 hours, 1.69 hours and 5.41 hours, respectively (Table. $1 \sim 3$).

From the above data, we may conclude that there were no differences in the duration of the generation time and its component phases of the proliferating presumptive myoblasts in various muscles, though the length of the total mitotic cycle was prolonged progressively with the passing of the developmental stages. The lengthening of the generation time was mainly dependent upon the increase of the G_1-period of the mitotic cycle.

Using the values obtained above for the durations of S-phase and of the total mitotic cycle and the average LI values, the fraction of nuclei which were proliferating at various stages of muscle development were calculated (Table 4). The values of 6.79 and 10.37 hours for the durations of the S phase and the generation time, respectively, of 10 days of incubation were used to calculate the PI from 9 and 10 days of incubation, the values of 6.69 and 11.57 hours at 12 days of incuba-

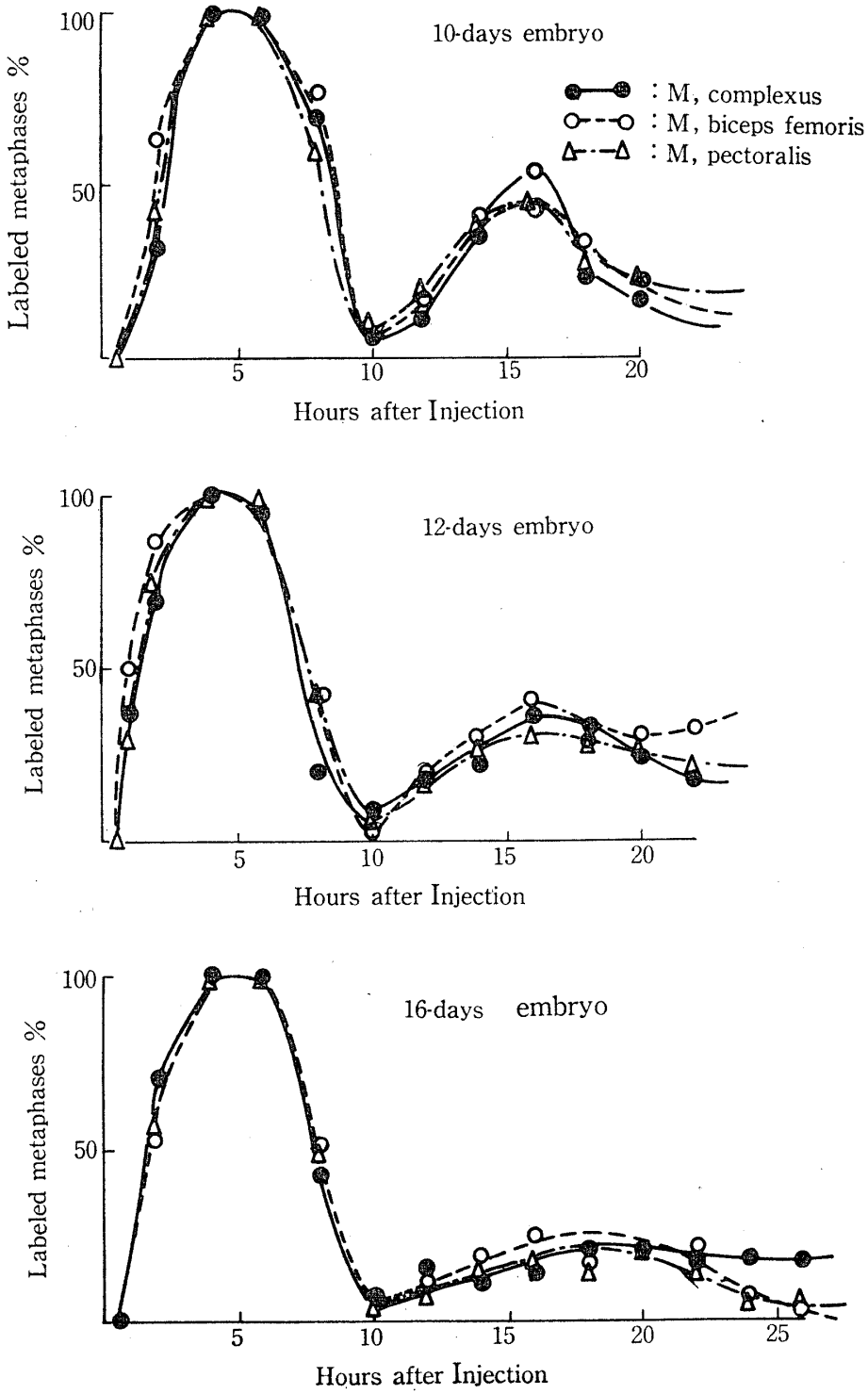

FIG. 3. The percentage of the labeled metaphases in 10, 12 and 16 days of incubation in various embryonic muscles at several time intervals after injection of ^{3}H-thymidine.

TABLE 1. *Duration (Hours) of the Cell cycle and its component phases at 10-days of incubation*

Muscle	G_2+1/2M	S	M	G_2	G_1	T
M, complexus	1.88	6.62	0.21	1.77	1.65	10.25
M, biceps femoris	1.75	6.88	0.30	1.60	1.47	10.25
M, pectoralis	1.63	6.87	0.25	1.50	1.98	10.60
Mean	1.75	6.79	0.25	1.62	1.70	10.37

TABLE 2. *Duration (Hours) of the Cell cycle and its component phases at 12-days of incubation*

Muscle	G_2+1/2M	S	M	G_2	G_1	T
M, complexus	1.25	6.63	0.18	1.16	3.63	11.60
M, biceps femoris	1.00	6.80	0.25	0.86	3.55	11.50
M, pectoralis	1.25	6.65	0.28	1.11	3.56	11.60
Mean	1.17	6.69	0.24	1.04	3.58	11.57

TABLE 3. *Duration (Hours) of the Cell cycle and its component phases at 16-days of incubation*

Muscle	G_2+1/2M	S	M	G_2	G_1	T
M, complexus	1.50	6.50	0.10	1.45	5.83	13.88
M, biceps femoris	2.00	6.13	0.10	1.95	5.32	13.50
M, pectoralis	1.75	6.25	0.11	1.69	5.08	13.13
Mean	1.75	6.29	0.10	1.69	5.41	13.50

tion were used for 12, 13, 14 days of incubation, and the values of 6.29 and 13.50 hours at 16 days of incubation were used. Although there were little differences in the proliferating fraction of nuclei at 9 days of incubation among these muscles, the percentage of the dividing nuclei in the M. complexus indicated more rapid decrease after 10 days of incubation than in the other muscles. Assays showed that at 12 days of incubation and in the M. complexus, 31.01% of all myogenic cells were in some phase of the mitotic cycle. At the same incubation days, PI values in the M. pectoralis and the M. biceps femoris showed higher values, 39.15% and 38.27%, respectively. This tendency continued from 10 to 13 days of incubation. At 16 days of incubation, the PI values of the M. complexus attained a higher level than that of the other muscles. These changes described above were parallel to the changes of the LI and MI values previously mentioned.

TABLE 4. *Percentage of Proliferating Nuclei (PI*) in Embryonic*

Age	T/S	M. complexus			
		% labeled nuclei	% dividing nuclei	% nondividing nuclei	% labeled nuclei
E-9	10.37/6.79	28.07	42.11	57.99	30.09
E-10	10.37/6.79	24.20	36.30	63.70	29.33
E-12	11.57/6.69	18.24	31.01	68.99	23.03
E-13	11.57/6.69	16.40	27.88	72.12	18.88
E-14	11.57/6.69	12.41	21.10	78.90	13.31
E-16	13.50/6.29	11.13	24.49	75.51	6.79

*PI=LI×T/S

Discussion

The myogenic cells are classified into the following three types; presumptive myoblast, myoblast, and myotube. The proliferating cells which become myosin synthesizing cells are the presumptive myoblasts. The mononucleated cells which actually engage in myosin synthesis are the myoblasts proper (5). The myoblasts continue to elongate and days later begin to fuse with other mononucleated cells and immature myotubes to form multinucleated myotubes. The degree of proliferation of the presumptive myoblasts along the wall of the p-myotube and their fusion by which the s-myotubes are formed has direct effects upon the number and the size of myotubes obtained at hatching time.

Comparing the development of the M. complexus with that of the other muscles, the p-myotubes show a slight enlargement in diameter, but are nearly equal among the differentiating muscle species. The s-myotubs which form along the wall of the p-myotubes by fusion with the proliferating presumptive myoblasts, however, undergo a more rapid hypertrophy in the M. complexus than those of the other muscle species after 10 days of incubation. Since the s-myotubes are deprived of space in a given primary fascicles and space on the surface of the p-myotube because of their successive forming, their number in the M. complexus remains smaller than those in the other muscles despite of remarkable enlargement of their myotube size (Fig. 4).

The schematic model in Figure 5 shows the stereographic structures of the relationships among the p-myotube, s-myotube and the presumptive myoblasts differentiating within the primordia of the primary muscle fascicle. The s-myotubes spring up from the cell fusion of myoblasts along the long axis of the p-myotube and gradually take the position of the second dimensional space on the surface of the p-myotube. As the developmental stages pass, they separate one by one from the surface membrane and occupy, as a next step, the third dimensional space within the primordia of the primary muscle fascicle. The cell number and the cell size obtained consequently at hatching time are prescribed by the degree of ocupancy of the second and the third dimensional space. If the s-

Muscles at the Different Stages of Development

M. pectoralis		M. biceps femoris		
% dividing nuclei	% nondividing nuclei	% labeled nuclei	% dividing nuclei	% nondividing nuclei
45.14	54.86	28.88	43.32	56.68
44.00	56.00	28.01	42.02	57.98
39.15	60.85	22.51	38.27	61.73
32.10	67.90	19.02	32.33	67.67
22.63	77.37	14.78	25.13	74.87
14.94	85.06	8.89	19.56	80.44

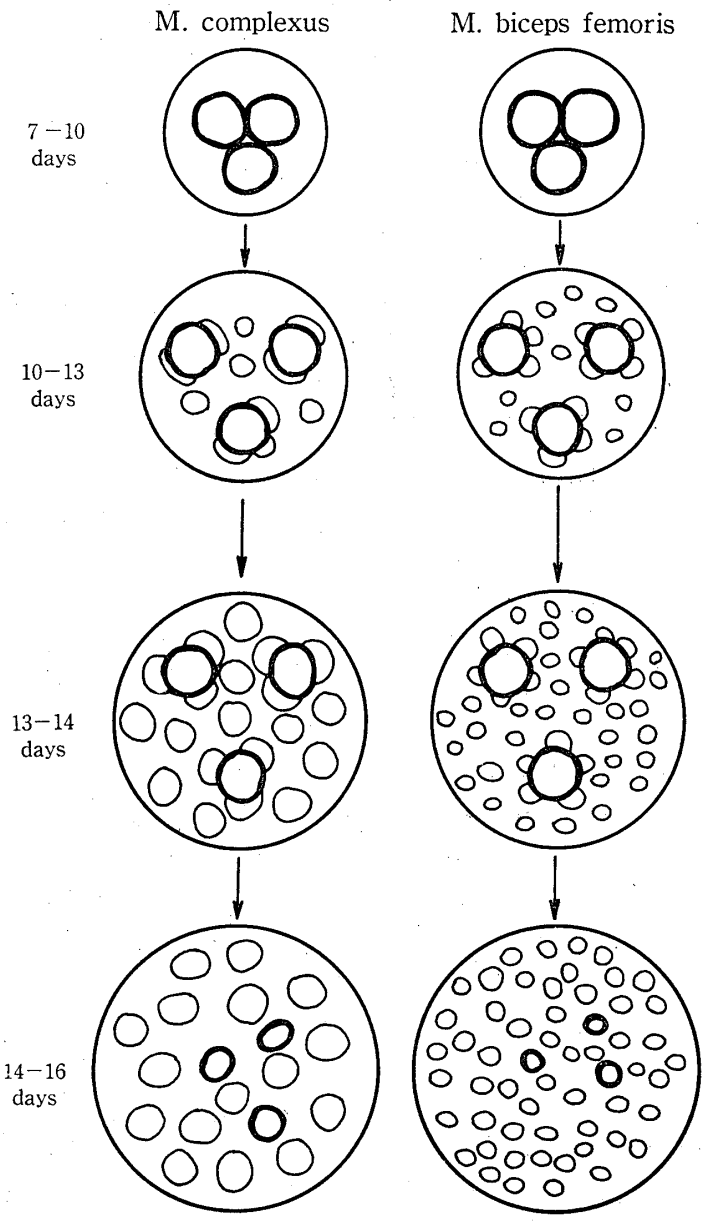

FIG. 4. A schematic drawing of a cross section passing through the primordia of the primary muscle fascicle in which the s-myotubes spring up along the surface membrane of the p-myotube and separate one after another. Note that in the M. complexus the s-myotubes grow rapidly and lose their proliferation space within a primary muscle fascicle.

myotubes are small in size and develop slowly, they will have a wide second dimensional space on the surface of the p-myotube and a large third dimensional space between each p-myotubes. On the contrary, the s-myotubes which indicate a remarkable growth and are large in their cell size will lose both of dimensional spaces. As mentioned previously, the degree of proliferation in the presumptive myoblasts and their fusion has direct effects upon the growth of s-myotubes. It was observed that the M. complexus is composed of s-myotubes which grow remarkably and has a small number of presumptive myoblasts surrounding the p-myotube, while the other muscles are composed of the s-myotubes developing slowly and have a large amount of presumptive myoblasts.

In order to verify these results, the changes of the MI and LI at certain stages of development were investigated. The changes of both values were approximately parallel with each other and decreased rapidly after 9 days of incubation. On the other hand, the changes of the values in the M. complexus showed a more remarkable decline than the other two muscles during the period from 10 days to 13 days of incubation, and attained approximately the same values with the other muscles at 14 days of incubation.

Several papers have been published on the cytokinetic analysis of embryonic muscles at the different developmental stages. Most of them were, however, concerned with a special embryonic muscle and not with the difference between various muscle species (9, 12, 13, 14). It has been suggested that the results obtained from LI and MI values at various incubation days bring up the following problems: (1) why the gradual decreases in the both values were seen during the development of the chick muscles and (2) why the both values of the M. complexus at 10, 12 and 13 days of incubation were lower than the other muscles. Both values are an indicator of the rate of proliferation of a cellular population. This is because this parameter is primarily dependent on the length of the average cell cycle relative to the length of the DNA synthetic phase and mitotic phase, and on the percentage of the population that is actively dividing. Consequently, a gradually decline of the LI and the MI values during development might be due primarily to the shift of phase from actively dividing muscles to nondividing ones, or to the respective reduction of the mitotic phase and DNA synthetic phase.

Marchok and Herrmann (9) analyzed the mitotic cycle and the duration of its components of chicken leg muscle following the procedure of Quastler and Sherman (11). They showed that the decline of the LI and the MI values after 7 days of incubation was the result of an increase in the number of cells which remain in the G_1 phase and did not enter into another mitotic cycle and to increased duration of the G_1 phase of the presumptive myoblasts (9, 15). A complete mitotic cycle took 10.5 hours in 9 day embryonic leg muscle, but took 16.5 hours in 16 day one. A value of 3.9 hours was obtained for G_1 in 9 day leg muscle and 8.9 hours for 16 day one (9). We obtained a generation time of 10.25 hours, G_1 of 1.65

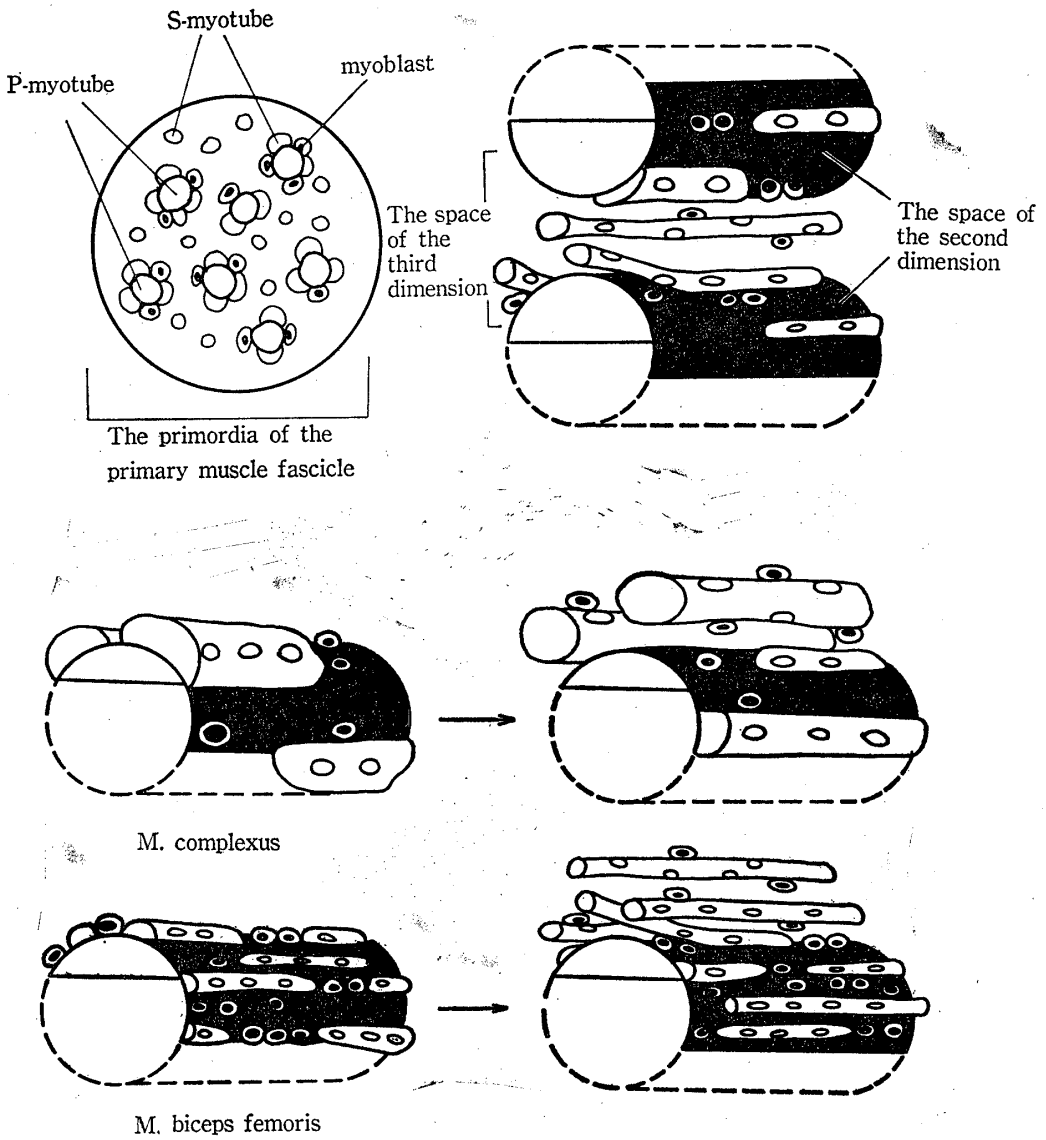

FIG. 5. Schematic model indicating the stereographic structures of the relationship among the p-myotube, the s-myotube and the presumptive myoblasts proliferating within the primordia of the primary muscle fascicle. The s-myotubes spring up from the cell fusion of myoblasts along the long axis of the p-myotube and gradually take the position of second dimensional space on surface membrane of the p-myotube. They occupy, as a next step, third dimensional space within the primordia of the primary muscle fascicle. Lower diagram shows the comparison of the mode of the proliferating myoblasts and their myotube formation between the M. complexus and the M. biceps femoris.

hours, S of 6.62 hours in the M. complexus at 10 days of incubation and a generation time of 13.88, G_1 of 5.83 hours, S of 6.50 hours in the M. complexus at 16 days of incubation. Therefore, an increase in the duration of the mitotic cycle during development from 10 to 16 days of incubation depend upon that of the G_1-period. This value for the length of S between 10 and 16 days of incubation agrees with most of the values for S that have been obtained previously in chick embryos

during from 2 days of embryonic life to 35 days after hatching (Fujita, 1962: 4–6 hours; Cameron, 1964: 5–6 hours; Okazaki and Holtzer, 1966: 5 hours; Marchok and Herrmann, 1967: 5.85 hours; Janners and Searls, 1970: 5.6 hours) (9, 16, 17, 18). On the other hand, there is very little change in the length of the cell cycle or its component phase during this period among the M. complexus, the M. biceps femoris and M. pectoralis. Therefore, the reason that the LI and MI values of the M. complexus were lower at 10, 12 and 13 days of incubation may be a rapid decline of the percentage of the actively dividing myoblasts occured in this muscle.

Janners and Searls (18) have described the changes in the rate of cell division in various regions of embryonic chick wing-bud during the first 48 hours of development. At stage 19, the PI in all regions of wing is 100%. At stage 24, the PI in the dorsal-proximal, ventral-proximal, and subridge region was 75% and the PI in the cartilage forming region was 25%. Graphical analysis of the continuous labeling data showed that there is very little change in the length of the cell cycle or its component phases and that the decrease in LI was almost entirely due to a cessation of cell division. Our results obtained in this study showed that the mitotic cycle and its component phases of the myogenic cells in various muscle species differed little, even though in the M. complexus undergoing a rapid hypertrophy of myotubes.

By what mechanism did the proliferating myoblasts in the M. complexus shift rapidly from the dividing phase to the nondividing phase? The mode of growth of the s-myotubes might give the key to answer this question. Although there were little differences in the PI values between various muscle tissues at 9 days of incubation, the percentage of the dividing nuclei in the M. complexus indicated more rapid decrease after 10 days of incubation than that in the other muscles. Since the rapid forming and elongation of s-myotubes elevated the probability of the myogenic cell fusion, the proliferating cells might cease to divide and not enter the next cell division. Recently Konigsberg (19) suggested that the population density might affect the initiation of cell fusion and at the higher cell densities, cell-to-cell contacts might occur more frequently, thus increasing the probability of encounters between two cell's components to fuse. The difference in the cellular environments in the M. complexus at 10, 12 and 13 days of incubation might be higher cell densities because of the remarkable growth of s-myotubes around the wall of the p-myotubes. It has been demonstrated that the fusion of muscle cells occur only at G_1, and that the last cell division places these cells in a distinct, postmitotic state preparatory to fusion (5, 14, 15, 20, 21). Since the duration of G_1-phase did not change among various muscle species at certain incubation days, it seemed reasonable to assume that the s-myotubes actively affected the fusion of the presumptive myoblasts which wait for a chance for cell contacts and cell fusion.

Ashmore et al. (22, 23) identified histochemically the myotubes in various

animal species as α or β dependent upon their myosin ATP-ase activity. They identified the p-myotubes as those destined to become β-fibers and the s-myotubes as α-fibers and suggested that the proportion of α-fibers might be related to the mechanism in which muscle fascicles were formed. In our earlier report (4), it was suggested that there is no significant difference in the number of the p-myotubes in a given primary muscle fascicle between the M. complexus and the M. biceps femoris from 11 to 13 days of incubation. Since there were little differences in the volume of the primary muscle fascicles during this period, the s/p myotube ratio might be relatively restricted by the growth rate of the s-myotubes. Where the formation and the separation of the s-myotubes were restrained by the loss of the second and the third dimensional space, the subsequent proliferation of s-myotube might be restricted.

Acknowledgement

The authores wish to thank Miss. Y. Kamioka for her assistance in preparing the manuscript. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education.

References

1) Kikuchi, T., Hoshino, T., and Ichikawa, O., *Jap. J. Zootch. Sci.*, **39**, 306 (1968)
2) Kikuchi, T., *Tohoku J. Agr. Res.*, **22**, 1 (1971)
3) Kikuchi, T., *Tohoku J. Agr. Res.*, **23**, 92 (1972)
4) Kikuchi, T., Nagatani, T., and Tamate, H., *Tohoku J. Agr. Res.*, **23**, 149 (1972)
5) Okazaki, K., and Holtzer, H., *Proc. Natl. Acad. Sci. U.S.*, **56**, 1484 (1966)
6) Yaffe, D., In "*current topics in developmental biology*" (A.A. Moscona, ed.), p. 37. Academic press, New York (1969)
7) Okazaki, K., and Holtzer, H., *J. Histochem. Cytochem.*, **13**, 726 (1965)
8) Konigsberg, I., *J. Cellular Comp Physiol.*, **52**, 13 (1958)
9) Marchok, A.C., and Herrmann, H., *Develop. Biol.*, **15**, 129 (1967)
10) Matsuzawa, T., and Hatakeyama, T., *Nipp. Act. Radiol.*, **27**, 13 (1967)
11) Quastler, H., and Sherman, F.G., *Exptl. Cell Research.*, **17**, 420 (1959)
12) Zhinkin, L.N., and Andreeva, F., *J. Embryol. Exptl. Morph.*, **11**, 353 (1963)
13) Mitoriu, P., Lang, W., and Maurer, W., *Z. Zellforsch. Mikrosk. Anat.*, **90**, 68 (1968)
14) Bischoff, R., and Holtzer, H., *J. Cell Biol.*, **41**, 188 (1969)
15) Hermann, H., Heywood, S.M., and Marchok, A.C., In "*current topics in developmental biology*" (A.A. Moscona, ed.), p. 181 Academic press, New York (1969)
16) Fujita, S., *Exp. Cell Res.*, **28**, 52 (1962)
17) Cameron, I.L., *J. Cell Biol.*, **20**, 185 (1962)
18) Janners, M.Y., and Searls, R.L., *Develop. Biol.*, **23**, 136 (1970)
19) Konigsberg, I.R., *Develop. Biol.*, **26**, 133 (1971)
20) O'Neil, M.C., and Stockdale, F.E., *Develop. Biol.*, **29**, 410 (1972)
21) Bischoff, R., and Holtzer, H., *J. Cell Biol.*, **36**, 111 (1968)

22) Ashmore, C.R., Robinson, D.W., Rattray, P., and Doerr, L., *Exp Neurol.*, **37**, 241 (1972)
23) Ashmore, C.R., Addis, P.B., Doerr, L., and Stokes, H., *J. Histochem. Cytochem.*, **21**, 266 (1973)