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学位論文

Immunohistochemical Study on the Development of
Extraocular Muscles of Human and Rat Embryos

1. M. Oguni
   Mode of expression of brain- and muscle-type glycogen phosphorylase in extraocular muscles of human embryos.
   Acta Histochem. Cytochem., (in press)

2. M. Oguni, O. Tanaka, T. Setogawa, H. Shinohara, K. Kato and R. Semba
   Immunohistochemical study on the development of extraocular muscles. I. Rat. Acta Histochem. Cytochem., 24; 39-45, 1991.

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   Immunohistochemical study on the development of extraocular muscles. II. Human embryos. Acta Histochem. Cytochem., 24; 47-53, 1991.

大國　昌美

MODE OF EXPRESSION OF BRAIN- AND MUSCLE-TYPE GLYCOGEN PHOSPHORYLASE IN EXTRAOCULAR MUSCLES OF HUMAN EMBRYOS

Masami OGUNI

Department of Ophthalmology, Shimane Medical University, Izumo 693, Japan

Running title : Glycogen Phosphorylase of Eye in Human embryos

Correspondence to : Masami Oguni, M. D. Department of Ophthalmology, Shimane Medical University, Izumo 693, Japan.

Abstract

The appearance of two molecular markers of muscle differentiation, brain (BGP) and muscle-brain (MB-GP) type of glycogen phosphorylase, was studied immunohistochemically in the extraocular muscles of human embryos (Carnegie stages 13 to 23). During stages 13-18, there was no immunoreactivity to BGP and MB-GP antibodies around the optic vesicle. At stage 20, myogenic cells around the optic vesicle became immunoreactive to both BGP and MB-GP antibodies, however, the number of MB-GP immnoreactive cells was larger than that of BGP immunoreactive cells. At stage 21, MB-GP immunoreactive cells increased in number, but BGP immunoreactive cells decreased. At stage 23, BGP immunoreactivity disappeared from the extraocular muscles. These findings suggest that BGP appears transiently in a restricted population of extraocular muscles in the embryonic period and the isoenzyme pattern becomes adult type (MGP) in the late embryonic period.

INTRODUCTION

Human extraocular muscles originate from the mesoderm around the prochordal space from Carnegie stage 13 to 16 (2). They are different from other muscles of human embryos in the appearance of the molecular markers of muscle differentiation, such as the muscle-type of creatine kinase (CK-M) and enolase (β-enolase) (13, 14, 20, 21).

Glycogen phosphorylase (GP)(E.C. 2. 4. 1. 1.), which catalyzes the release of glucose 1-phosphate from stored glycogen, plays an important role in the regulation of intracellular carbohydrate metabolism. This enzyme possesses three isoenzymes, i.e., muscle (MGP), liver (LGP) and brain type (BGP)(3, 9, 22, 24). BGP is the major isoenzyme of phosphorylase found in fetal tissues and tumor tissues (16, 17), but is also present in various peripheral tissues (1, 24, 25). MGP, which replaces BGP during muscle differentiation (17), and LGP are localized in mature skeletal muscles and liver cells, respectively (18).

Although GP is an excellent marker of skeletal muscle differentiation (7), there is no information available on the appearance of GP in the extraocular muscles of early human embryos. In the present study, the appearance of GP isoenzymes in the developing human extraocular muscles was studied immunohistochemically.

MATERIALS AND METHODS

Fifteen externally normal human embryos in Carnegie stage 13 to 23 (4 to 8 weeks of gestation) were used in the present study (Table. 1). All embryos were obtained from artificial abortion of pregnancies, according to the Eugenic Protection Law of Japan (10). These embryos were observed under a binocular microscope for external features and their developmental stages were determined according to O'Rahilly (15). After removal, the whole embryos were put into Schmechel's fixative (19) composed of 4% paraformaldehyde, 1% glutaraldehyde, 0.2% picric acid and 2% sucrose in 0.1M sodium acetate buffer, pH 6.0 and stored at 4 °C. One or two days later, they were transferred to Tris-buffered saline (TBS, 50mM Tris-HCl buffer, pH 7.6 with 150mM NaCl) and kept at 4 °C. After dehydration in a series of graded ethyl alcohols, the specimens were placed into 0.3% H_2O_2 in absolute methanol for 30 minutes to block endogenous peroxidase activity and embedded in paraffin. Serial sections of 5μ-thickness were prepared and were immunostained after avidin-biotin-peroxidase complex (ABC) method of Hsu (4), using BGP and MB-GP antibodies. The preparation procedures and the specificity of the antibodies used in the present study are described in other paper (5). BGP antibody is specific to brain-type phosphorylase and MB-GP antibody is common to brain and muscle phosphorylase (5). BGP and MB-GP showed no activity in liver cells in all embryos examined, suggesting that these

antibodies do not crossreact with LGP. For controls, sections were incubated with normal rabbit serum instead of the first antibody.

RESULTS

The results of the present study are shown in Table 2. Included in this table are the results of our previous study (14) for comparison and completion. During stages 13 to 18, the clusters of myogenic cells and BGP and MB-GP immunoreactive cells were not observed around the optic vesicle. At stage 20, the mesenchymal cells around the optic vesicle became condensed. Some myogenic cells in the mesenchymal condensation were BGP and MB-GP immunoreactive, however, the number of the former was smaller than that of the latter (Fig. 1a, 1b, 1c). At stage 21, MB-GP immunoreactive cells increased in number, but BGP immunoreactive cells decreased (Fig. 2a, 2b, 2c). At stage 23, the clusters of muscle cells in extraocular muscles elongated toward the optic vesicle along the optic nerve. BGP immunoreactivity was not observed in the extraocular muscles at this stage, while the number of MB-GP immunoreactive muscle cells increased (Fig. 3a, 3b).

DISCUSSION

Glycogen appears in the extraocular muscles at stage 19 (14). In the present study, BGP and MB-GP, which play an important role in the release of glucose from glycogen, appeared in the extraocular muscles at stage 20. Therefore, GP and glycogen appear almost at the same period, suggesting that the appearance of glycogen and GP is associated with the onset of glycogen metabolism in the extraocular muscles. Since BGP has a higher affinity to glycogen than MGP (18), the appearance of the former seems to play an important role in the initiation of glycogenolysis in the extraocular muscles. On the other hand, it was noted that BGP was expressed transiently in a restricted population during muscle differentiation. While the number of MB-GP immunoreactive cells increased with advancing development, that of BGP disappeared by stage 23. Thus, the isoenzyme pattern in the extraocular muscles become adult type (MGP) in the late embryonic period.

The enzyme activity of creatine kinase and glycogen phosphorylase increases after the fusion of myogenic cells (8). In a previous study, we showed that CK-M appeared at stage 18 in human extraocular muscles (14). Electron-microscopically, the fusion of myogenic cells in the extraocular muscles was observed at stage 20 (Oguni et al., submitted). In the present study, GP appeared in the myogenic cells of extraocular muscles at stage 20. Therefore, it

seems that the appearance of GP, which occurs later than CK-M in the course of muscle differentiation of human extraocular muscles, coincides with the fusion of myogenic cells.

We previously reported that neuron-specific enolase, which is present in the axon and in the synaptic plasma membrane of the neurons (6, 23), appears at stage 18 in the human extraocular muscles (14). This finding suggests that the innervation of human extraocular muscle occurs at this stage. The present study, in agreement with earlier reports (7), showed that GP appears after the innervation in the extraocular muscle.

Acknowledgements

Gratitude is extended to Professor Tomoichi Setogawa, Department of Ophthalmology and Professor Osamu Tanaka, Department of Anatomy, Shimane Medical Unversity, for support and valuable suggestion. The authors also thanks Dr. K. Kato, and Dr. H. Shinohara, Department of Biochemistry, Institute for Developmental Research, Aichi Prefectural Colony, for the preparation of the antibody and valuable suggestion, and Mr. F. Satow, Shimane Medical University, for technical assistance.

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Table 1. Human embryos examined in the present study

Carnegie stage	Specimen's number	Crown-rump Length(mm)*	Estimated postovulation days**
13	52368	5.1 ± 0.15	32
14	52383	6.8 ± 0.08	34-35
15	52379	8.0 ± 0.07	36
	52414	〃	〃
16	52382	9.2 ± 0.08	38
	52411	〃	〃
	52416	〃	〃
	71022	〃	〃
17	52417	11.5 ± 0.13	40
	71024	〃	〃
18	71020	13.5 ± 0.36	42
20	71025	19.2 ± 0.26	46
21	52356	21.1 ± 0.26	48
	52380	〃	〃
23	52377	28.0 ± 1.02	52

*Nishimura (11)
**Nishimura (12)

Table 2. Expression of Isoenzymes in the Extraocular Muscles

Carnegie stage	13	14	15	16	17	18	19	20	21	22	23
GP (muscle -brain type)	−	−	−	−	−	−		+	+		+
GP (brain type)	−	−	−	−	−	−		±	±		−
Glycogen*	−	−	−	−	−	−	+	+	+		
CK (muscle type)*	−	−	−	−	−	+	+	+	+		
CK (brain type)*	−	−	−	−	−	+	+	+	+		
β-enolase*	−	−	−	−	−	+	+	+	+		
NSE*	−	−	−	−	−	+	+	+	+		

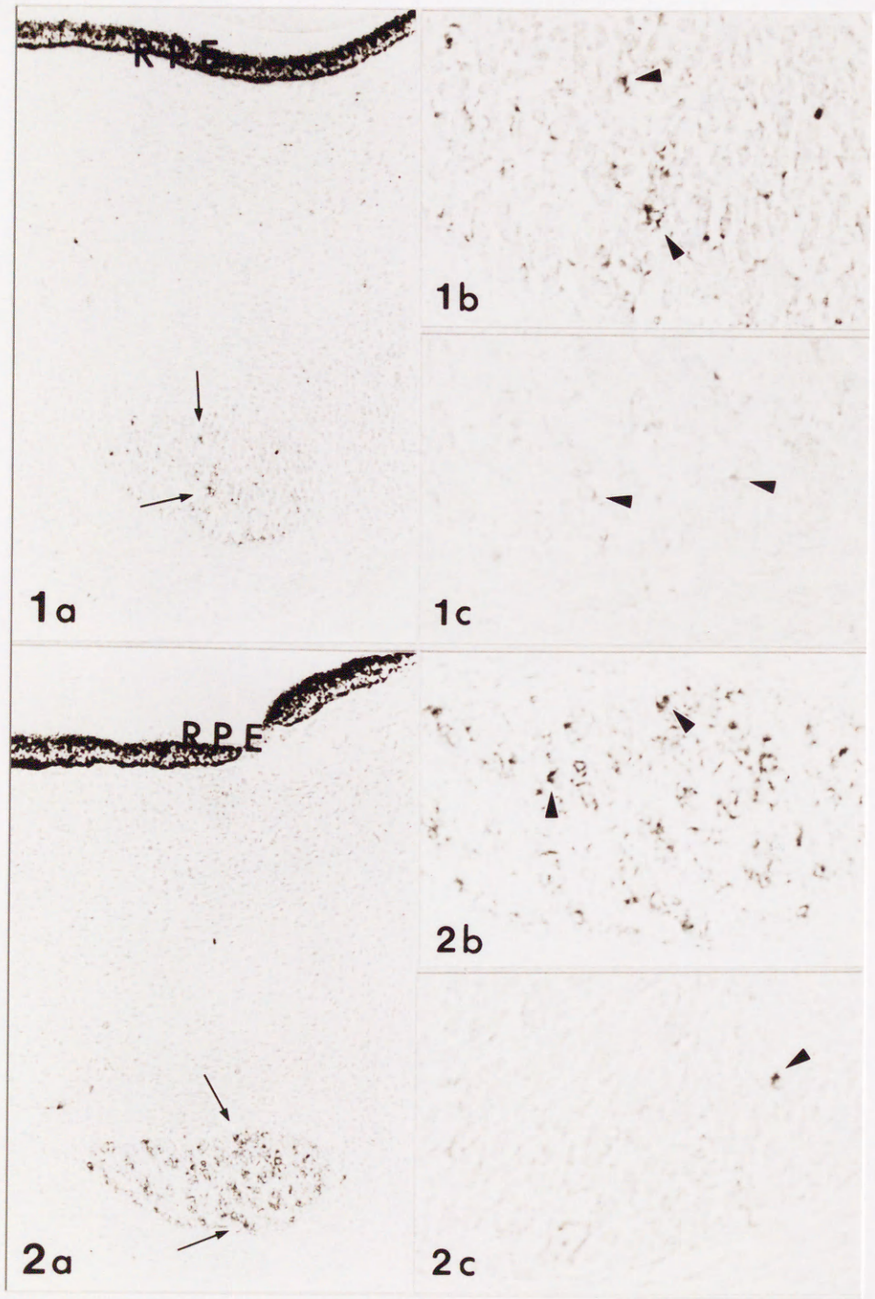
GP: glycogen phosphorylase, CK: creatine kinase,
NSE: neuron specific enolase
* Oguni et al. (14)

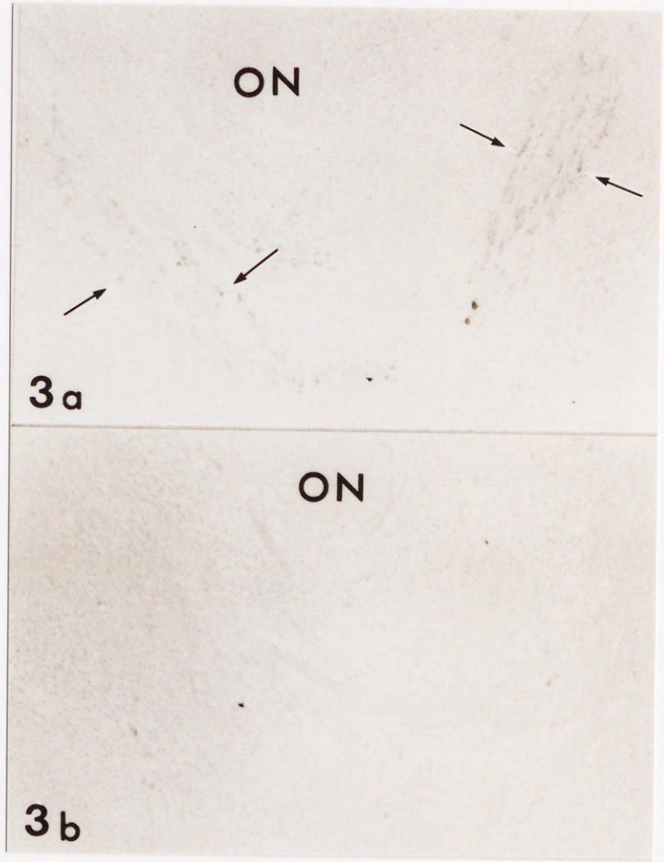
Figure Legends

Fig. 1 Cross sections of the extraocular muscles in a stage 20 embryo immunostained with MB-GP(a, b) and BGP (c) antibodies. 1a: At this stage, clusters of myogenic cells of extraocular muscles appear around the optic vesicles and some of them are immunoreactive to MB-GP antibody (arrows). 1b: Higher magnification of the clusters of myogenic cells in Fig. 1a. Note that MB-GP and BGP immunoreactive cells are observed in the clusters of myogenic cells (arrowheads). BGP immunoreactive cells are fewer than MB-GP immunoreactive cells. RPE: retinal pigment epithelium
(1a X 175, 1b X 370, 1c X 370)

Fig. 2 Cross sections of the extraocular muscles in a stage 21 embryo immunostained with MB-GP (a, b) and BGP (c) antibodies. 2a: At this stage, the number of MB-GP immunoreactive myogenic cells increased compared with that in stage 20 (Fig. 1a) (arrows). 2b: Higher magnification of the clusters of myogenic cells in Fig. 2a. 2c: The clusters of myogenic cells immunostained with BGP antibody. Note that MB-GP and BGP immunoreactive cells were observed in the clusters of myogenic cells (arrowheads), however, BGP immunoreactive cells decreased compared with the previous stage. RPE: retinal pigment epithlium
(2a, X 135, 2b, X 300, 2c X 300)

Fig. 3 Cross sections of the extraocular muscles in a stage 21 embryo immunostained with MB-GP (a) and BGP (b) antibodies. The clusters of muscle cells in the extraocular muscles elongate along the optic nerve (ON). Only MB-GP immunoreactive cells are observed (arrows) at this stage. (3a X 70, 3b X 70).


RPE
1a
1b
1c
RPE
2a
2b
2c


ON
3a
ON
3b

ACTA HISTOCHEM. CYTOCHEM. Vol. 24, No. 1, 1991

IMMUNOHISTOCHEMICAL STUDY ON THE DEVELOPMENT OF EXTRAOCULAR MUSCLES I. RAT

MASAMI OGUNI[1], OSASMU TANAKA[2], TOMOICHI SETOGAWA[1], HARUO SHINOHARA[3], KANEFUSA KATO[3], AND REIJI SEMBA[4]

Departments of Ophthalmology[1] and Anatomy[2], Shimane Medical University, Izumo 693 and Departments of Biochemistry[3] and Perinatology[4], Institute for Developmental Research, Aichi Prefectural Colony, Kasugai, Aichi 480-03

Received for publication June 13, 1990 and in revised form October 11, 1990

Development of extraocular muscles of rat was studied immunohistochemically using antibodies against three molecular markers of muscle differentiation: brain type (CK-B) and muscle type (CK-M) creatine kinase isoenzymes and muscle type enolase isoenzyme (β-enolase). The time of innervation of extraocular muscles was also studied immunohistochemically using antibody against neuron specific enolase (NSE). Periodic acid-Schiff (PAS) and PAS after diastase digestion stainings was used for the demonstration of glycogen. At embryonic day (E) 15, when muscle primordium of each extraocular muscle appears, β-enolase and glycogen were observed in all muscle primordia, while CK-B was immunoreactive only in lateral rectus (LR), superior rectus (SR), inferior rectus (IR) and inferior oblique (IO) muscle primordia. CK-B immunoreactivity appeared in superior oblique (SO) and medial rectus (MR) muscles at E17 and E18, respectively. By E18–19, CK-M immunoreactivity became positive in all muscles. NSE immunoreactive nerve fibers were first observed in LR, SR, IR and IO at E15, in SO at E16, and in MR at E17. Consequently, β-enolase immunoreactivity and glycogen appeared in all extraocular muscles at E15, while CK-M at E18. LR, SR, IR and IO muscles became immunoreactive to CK-B antibody at E15; however, SO and MR muscles became immunoreactive at E17 and E18, respectively. The sequence and time of appearance of CK-B in extraocular muscles were similar to those of NSE-immunoreactive nerve fibers. These findings suggest that in rats the expression of CK-B in each extraocular muscle coincides with the innervation of that muscle, while CK-M is expressed only after innervation.

Gilbert (8) reported that extraocular muscles originate from the mesenchymal cells near the prochordal plate. Subsequently, the cells form three pairs of mesenchymal condensations that are innervated by oculomotor, trochlear and abducens nerves. However, little is known about the time when extraocular muscles functionally differentiate.

Creatine kinase (CK) isoenzymes (EC. 2.7.3.2.) are involved in the reversible transfer of high-energy phosphate residue between adenosine triphosphate (ATP) and creatine. These isoenzymes are dimeric molecules composed of two immunologically distinct subunits, M and B, and they exist as MM, MB, and BB forms (5, 21). CK-MM and CK-MB are mainly present in skeletal muscle and heart, respectively (27). Biochemical studies have shown that CK-B is replaced by CK-M in the developing skeletal muscles of rats (6, 29) and early human fetuses (7). Therefore, the appearance of CK-M subunit seems to be a good event in the course of muscle differentiation (18).

Enolase isoenzymes (EC. 4.2.1.11) catalyze the interconversion of 2-phosphoglycerate and phosphoenolpyruvate in the glycolytic pathway. They are dimers composed of 3 immunologically distinct subunits, α, β and γ (22). α-Enolase is widely distributed in various tissues (12), while β-enolase appears in muscle tissues during differentiation (22). γ-Enolase is considered to be identical to the nervous-system-specific protein 14-3-2 (3, 17) and has been designated as neuron-specific enolase (NSE). NSE is one of the protein components of brain synaptic plasma membrane and axons (16). The onset of NSE immunoreactivity has been correlated with synapse formation (28).

CK-M and glycogen phosphorylase are considered to be excellent markers of differentiated

Correspondence to: Masami Oguni, M. D., Department of Ophthalmology, Shimane Medical University, Izumo 693, Japan.

skeletal muscles (18). Glycogen phosphorylase plays an important role in the release of glucose from glycogen. Since all differentiated muscles are glycogen-rich, the appearance of glycogen seems to be a good histochemical marker of muscle differentiation.

The present study was undertaken to determine immunohistochemically the stage of functional differentiation of extraocular muscles in rats using molecular markers of muscle differentiation and innervation.

MATERIALS AND METHODS

Embryos of Jcl: Wistar rats between embryonic day (E) 14 end 19 were used in the present study. The day on which a vaginal plug was present was designated as E0. After the pregnant animals were anesthetized with pentobarbital solution, the embryos were obtained by Cesarean section.

Histochemistry

Embryos were immersed in Lillie's fluid (15), a mixture composed of 10 ml of 40% formaldehyde, 5 ml of glacial acetic acid, and 85 ml of absolute alcohol, and kept at 4 °C. After dehydration in a graded series of ethyl alcohols, the head was embedded in paraffin. Horizontal and sagittal sections of 5μm thickness were prepared and periodic acid-Schiff (PAS) or PAS after 1% diastase digestion staining was performed.

Immunohistochemistry

Embryos for immunohistochemistry were briefly perfused with physiological saline by inserting a fine glass capillary into the umbilical vein, followed by perfusion with Schmechel's fixative (23), which was composed of 4% paraformaldehyde, 1% glutalaldehyde, 0.2% picric acid and 2% sucrose in 0.1M sodium acetate buffer, pH 6.0 and stored at 4°C. One or two days later, they were transferred to Tris-buffered saline

TABLE 1. *Immunohistochemical study of rat extraocular muscles*

muscle primordium						glycogen					
	E14	E15	E16	E17	E18		E14	E15	E16	E17	E18
LR	−	+	+	+	+	LR	−	+	+	+	+
SO	−	+	+	+	+	SO	−	+	+	+	+
IR	−	+	+	+	+	SR	−	+	+	+	+
MR	−	+	+	+	+	IR	−	+	+	+	+
IO	−	+	+	+	+	MR	−	+	+	+	+
	−	+	+	+	+	IO	−	+	+	+	+

CK-M							CK-B					
	E14	E15	E16	E17	E18	E19		E14	E15	E16	E17	E18
LR	−	−	−	−	±	+	LR	−	+	+	+	+
SO	−	−	−	−	±	+	SO	−	−	−	±	+
SR	−	−	−	−	±	+	SR	−	±	+	+	+
IR	−	−	−	−	±	+	IR	−	±	±	±	+
MR	−	−	−	−	±	+	MR	−	−	−	−	+
IO	−	−	−	−	±	+	IO	−	±	+	+	+

β-enolase						NSE-R					
E18	E14	E15	E16	E17			E18	E14	E15	E16	E17
LR	−	+	+	+	+	LR	−	+	+	+	+
SO	−	+	+	+	+	SO	−	−	±	±	+
SR	−	+	+	+	+	SR	−	±	±	±	+
IR	−	+	+	+	+	IR	−	±	±	±	+
MR	−	+	+	+	+	MR	−	−	−	±	+
IO	−	+	+	+	+	IO	−	+	+	+	+

+: positive in all rat embryos −: negative in all rat embryos
±: some rat embryos are immunopositive, and others are immunonegative

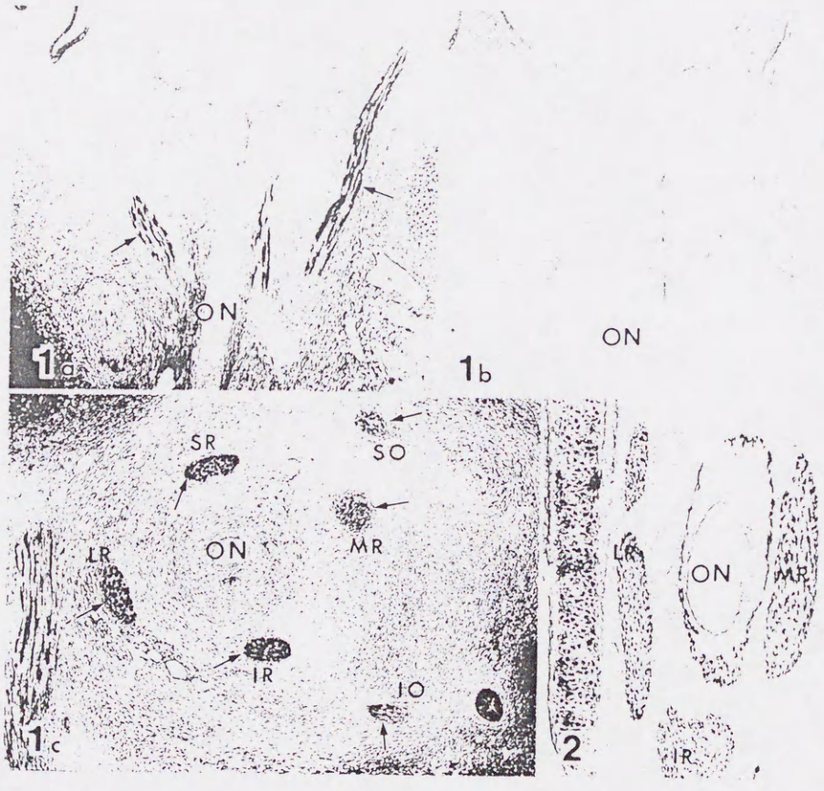
(TBS, 50mM Tris-HCL buffer, pH 7.6 with 150mM NaCl) and kept at 4°C. After dehydration in a series of graded ethyl alcohols, the specimens were placed in 0.3% H_2O_2 in absolute methanol for 30 min to block the endogenous peroxidase activity and then embedded in paraffin. Horizontal and sagittal sections of 5μm thickness of the head were prepared and were immunostained after avidin-biotin-peroxidase complex (ABC) method of Hsu (9) using CK-B, CK-M, β-enolase and NSE antibodies. The preparation procedures of the antibodies used in the present study are described in other papers (11, 13, 14). For controls, sections were incubated with normal rabbit serum.

RESULTS

Muscle primordium, glycogen staining and immunoreactivity to CK-B, CK-M, β-enolase and NSE antibodies in each extraocular muscle are summarized in Table 1.

Histochemistry

At E14, muscle primordium and PAS-positive substances were not observed around the optic vesicle. At E15, muscle primordia of each extraocular muscle appeared around the optic vesicle, containing PAS-positive substances (Figs. 1a, c). These PAS- positive substances disappeared after treatment with 1% diastase digestion (Figs. 1b). With the advancement of the embryonic day, clusters of muscle cells and

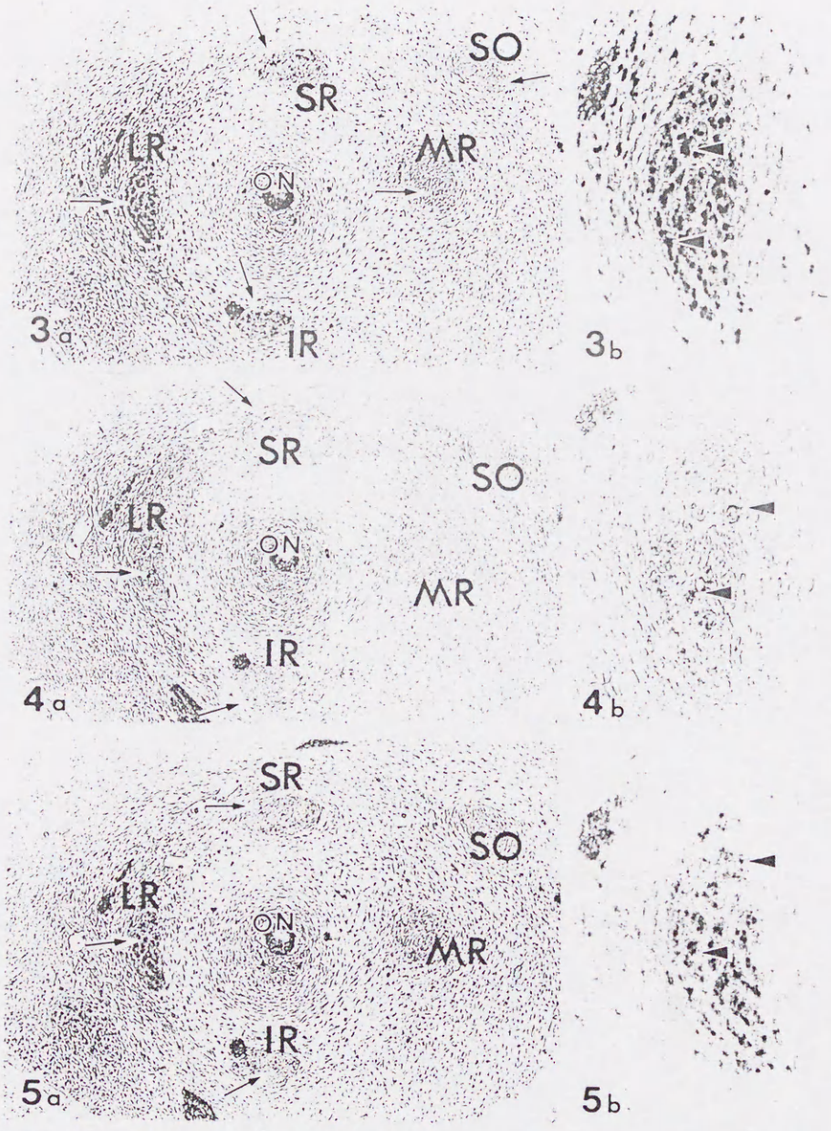


Abbreviations used in the Figures

SR: superior rectus muscle	MR: medial rectus muscle	IR: inferior rectus muscle
LR: lateral rectus muscle	SO: superior oblique muscle	IO: inferior oblique muscle
ON: optic nerve		

FIGS. 1a–b. Horizontal section (a), and sagittal section (c) of the head of E15 rat stained with periodic acid-Schiff (PAS), and horizontal section of PAS after 1% diastase digestion (b). Note that the muscle primordia and PAS-positive substances appear in all extraocular muscle primordia(arrows). PAS-positive sustances in extraocular muscle primordia disappear after treatment with diastase digestion staining (b). a. ×70, b. ×70, c. ×70

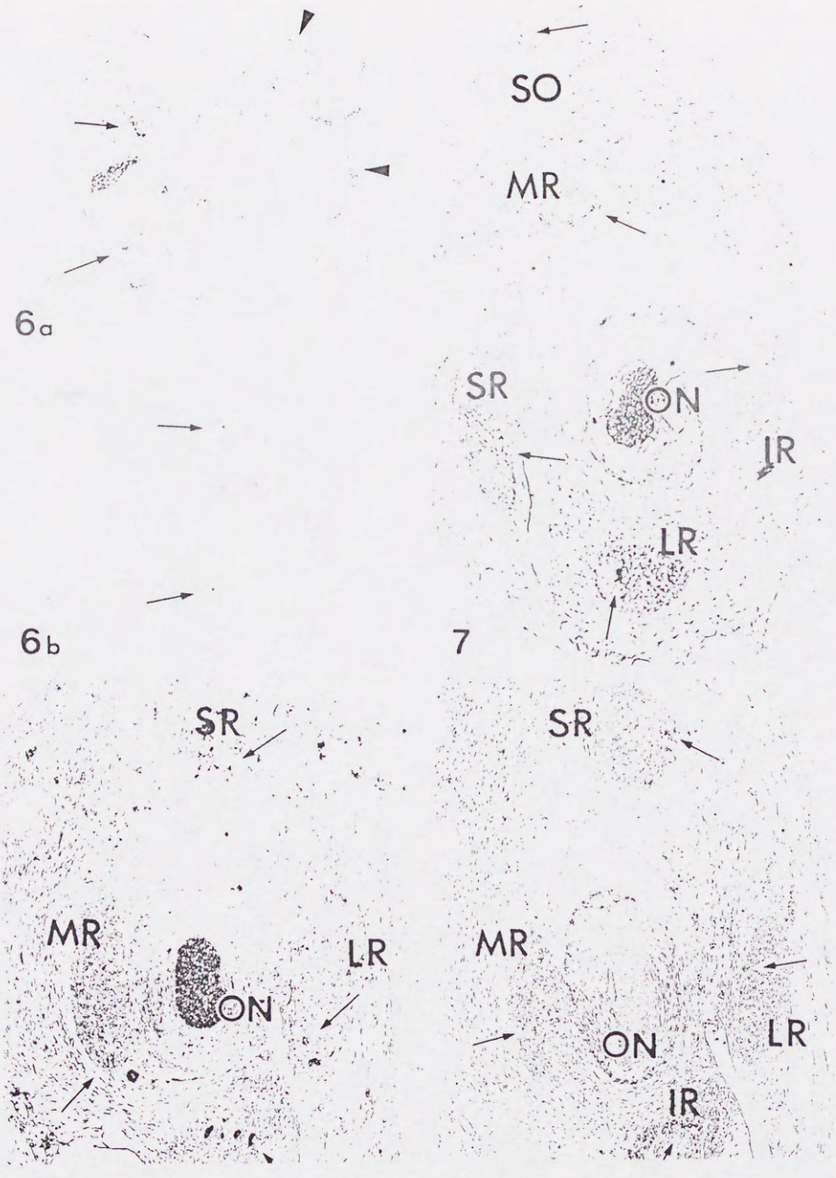
FIG. 2. Sagittal section of the head of E18 rat stained with PAS. Note that PAS-positive substances have increased in each extraocular muscle. ×100



FIGS. 3a–b. Sagittal sections of the head of E15 rat immunostained with β-enolase antibody. 3a. Note that all extraocular muscle primordia are immunoreactive against β-enolase antibody (arrows). 3b. Magnified view of LR muscle. Some muscular cells in the muscle primordium of LR muscle are immunoreactive with β-enolase antibody (arrowheads). a. ×100, b. ×350

FIGS. 4a–b. Sagittal sections of the head of E15 rat immunostained with CK-B antibody. 4a. Note that muscle primordia of SR, IR and LR muscles (arrows) are immunoreactive with CK-B antibody. 4b. Magnified view of primordium of LR muscle. Some muscular cells or nerve fibers in the muscle primordium of LR muscle are immunoreactive with CK-B antibody (arrowheads). a. ×100, b. ×350

FIGS. 5a–b. Sagittal sections of the head of E15 rat immunostained with NSE antibody. 5a: Note that nerve fibers in MR, IR and LR muscle primordia are immunoreactive with NSE antibody (arrows). 5b: Magnified view of primordium of LR muscle. Some nerve fibers in the muscle primordium of LR muscle are immunoreactive with NSE antibody (arrowheads). a. ×100, b. ×350



FIGS. 6a–b Sagittal sections of the head of E17 rat immunostained with CK-B antibody. 6a. Note that not only muscular cells (arrows) but also nerve fibers (arrowheads), CK-B immunoreactivity in muscular cells of SO muscle appears at this embryonic day. a. ×300, b ×300 of LR muscle are immunoreactive with CK-B antibodies, 6b:

FIG. 7. Sagittal section of the head of E17 rat immunostained with NSE antibody. Note that immunoreactivity with NSE antibody is observed in MR, SR, LR, IR and SO muscles (arrows). Optic nerve (ON) and nerve fibers are immunoreactive to NSE antibody ×80

FIG. 8. Sagittal section of the head of E18 rat immunostained with CK-B antibody. Note that immunoreactivity with CK-B antibody is present not only in muscle cells of IR, SR, LR and MR muscles are also immunoreactive to CK-B antibody (arrows). ×80

FIG. 9. Sagittal section of the head of E18 rat immunostained with CK-M antibody. Note that immunoreactivity with CK-M antibody appears in MR, IR, SR and LR muscles (arrows). ×80

PAS-positive substances increased in each muscle (Fig. 2).

Immunohistochemistry

At E14, there was no immunoreactivity against CK-B, CK-M, β-enolase or NSE antibodies around the optic vesicle. At E15, immunoreactivity to β-enolase antibody appeared in muscle cells of all extraocular muscles (Figs. 3a, b). Immunoreactivity to CK-B antibody appeared in lateral rectus (LR), superior rectus (SR), inferior rectus (IR) and inferior oblique (IO) muscles (Figs. 4a, b). On the other hand, NSE immunoreactive nerve fibers appeared in LR, SR, IR and IO muscles and in SO muscle, at E15 (Figs. 5a, b) and at E16, respectively. At E17, muscle cells of SO muscle and nerve fibers of LR muscle became immunoreactive (Figs. 6a, b), while the nerve fibers of MR muscle became immunoreactive to NSE (Fig. 7). CK-B immunoreactivity in muscle cells and nerve fibers of MR muscle was noted at E18 (Fig. 8). By that time CK-M antibody appeared in all muscle cells (Fig. 9). As the embryonic day advanced from E15 to E18, β-enolase immunoreactivity in muscle cells increased, while immunoreactivity to CK-B antibody in muscle cells of all extraocular muscles remained constant. NSE and CK-B immunoreactivity of nerve fibers in each extraocular muscle increased with advancing development.

DISCUSSION

CK-B is reported to be present mainly in the myoblast, while CK-M has been closely linked to the increase in number of the myotubes (10). In the rat skeletal muscles of the upper and lower extremities, CK-M activity appears at E17 and increases thereafter, while CK-B activity rapidly decreases between E10 and E20 (29). We have shown that in rat extraocular muscles CK-B first appears between E15 and E17, while CK-M appears between E18 and E19. Therefore, the appearance of CK-M in extraocular muscles almost coincides with that of muscles in the extremities, although no decrease of CK-B in extraocular muscles was observed before E19.

The concentration of β-enolase is believed to be closely correlated with the functional state of the muscular tissue(13, 19). Formation of the myotome starts at E9 or E10 in rats (4), while β-enlase and CK-M immunoreactive cells appear at E12 and E16, respectively (25). In the present study, the time course and sequence of appearance of β-enolase and CK-M immunoreactivities and glycogen in the extraocular muscles of rat embryos are similar to those of the upper limb muscles of human embryos (26). On the other hand, it is controversial whether extraocular muscles develop synchronously (24) or independently at different times (8). In this study, the expression of CK-M, β-enolase and glycogen in each extraocular muscle was synchronous, however, it was independent of that of CK-B and NSE.

Nag and Cheng (20) reported that neuromuscular junctions of the rat superior rectus muscle are formed by the end of the first week of the postnatal period. We showed that the immunoreactivity to NSE in extraocular muscles appeared in LR, SR, IR and IO at E15, in SO at E16, and in MR at E17. NSE is one of the protein components of rat brain synaptic plasma membrane and is also present in the axons (16). In addition, NSE appears with the formation of synapses (28). Therefore, it is noteworthy that while the morphological differentiation of the neuromuscular junction takes place in the postnatal period, the nerve fibers containing NSE appear in extraocular muscles in the embryonic period. In addition, the sequential expression of CK-B in the extraocular muscles was coincident with the appearance of NSE in the corresponding muscles, suggesting that the appearance of CK-B is closely associated with the innervation of extraocular muscles.

In the present study, we showed that the immunoreactivities to NSE and CK-B antibodies in SR, IO and IR muscles, which are innervated by the oculomotor nerve, appear earlier than those of the SO muscle, which is innervated by the trochlear nerve. These results are contrary to the sequence of neurogenesis in the brain stem (1, 2). A study on the expressions of NSE and CK-B in oculomotor and trochlear nerve nuclei of the brain stem in rat embryos is in progress.

ACKNOWLEDGEMENT

The authors are indebted to Dr. Antoine Abu Musa for critical comments.

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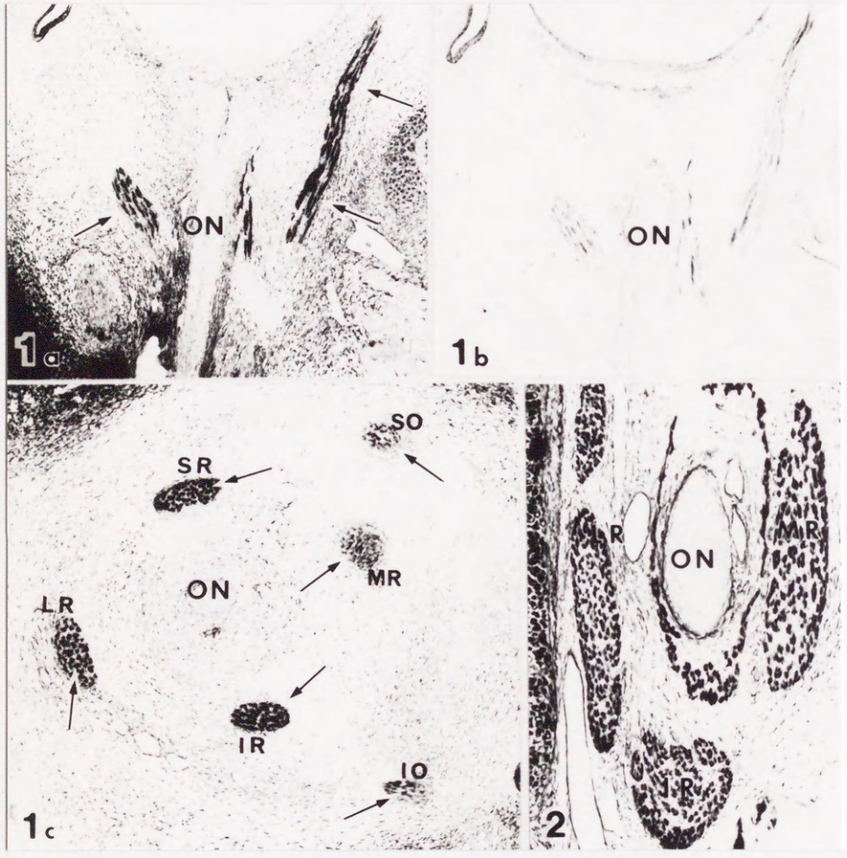
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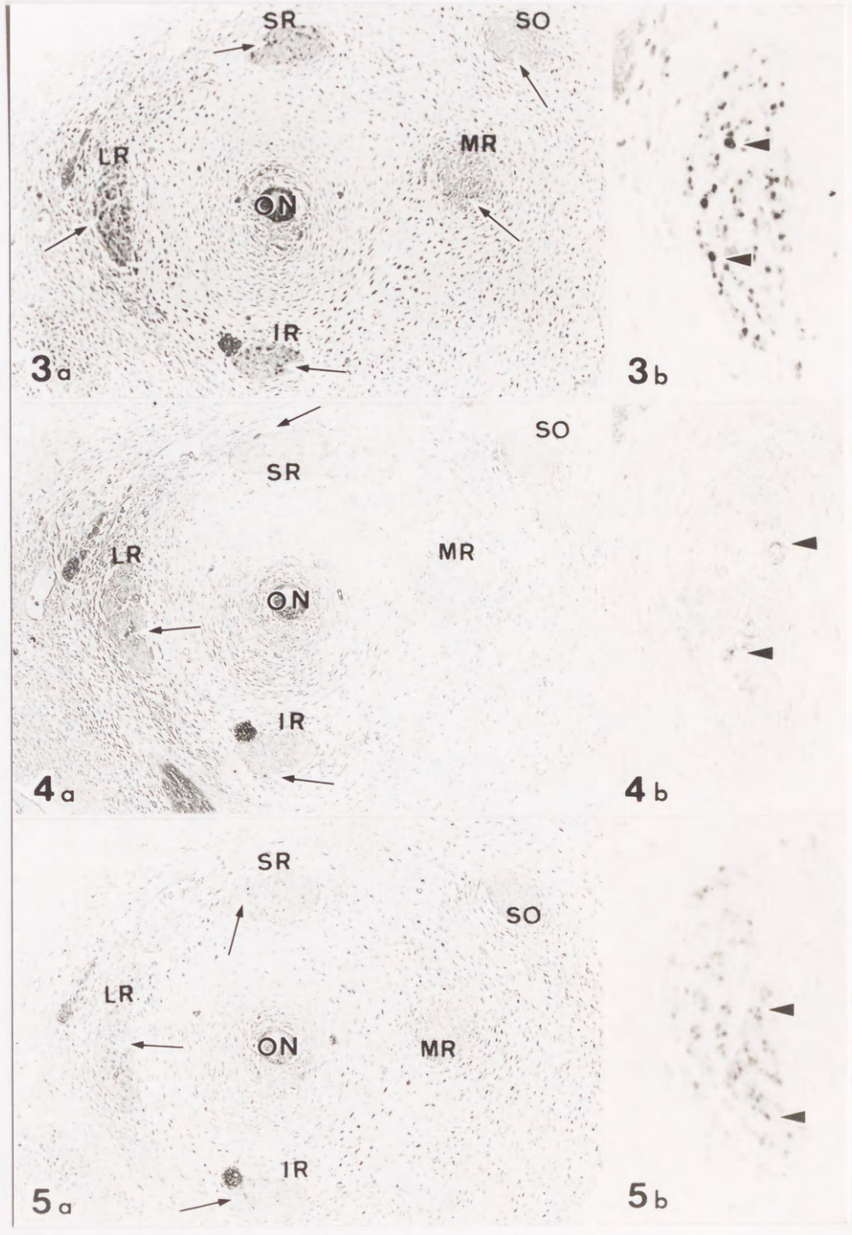
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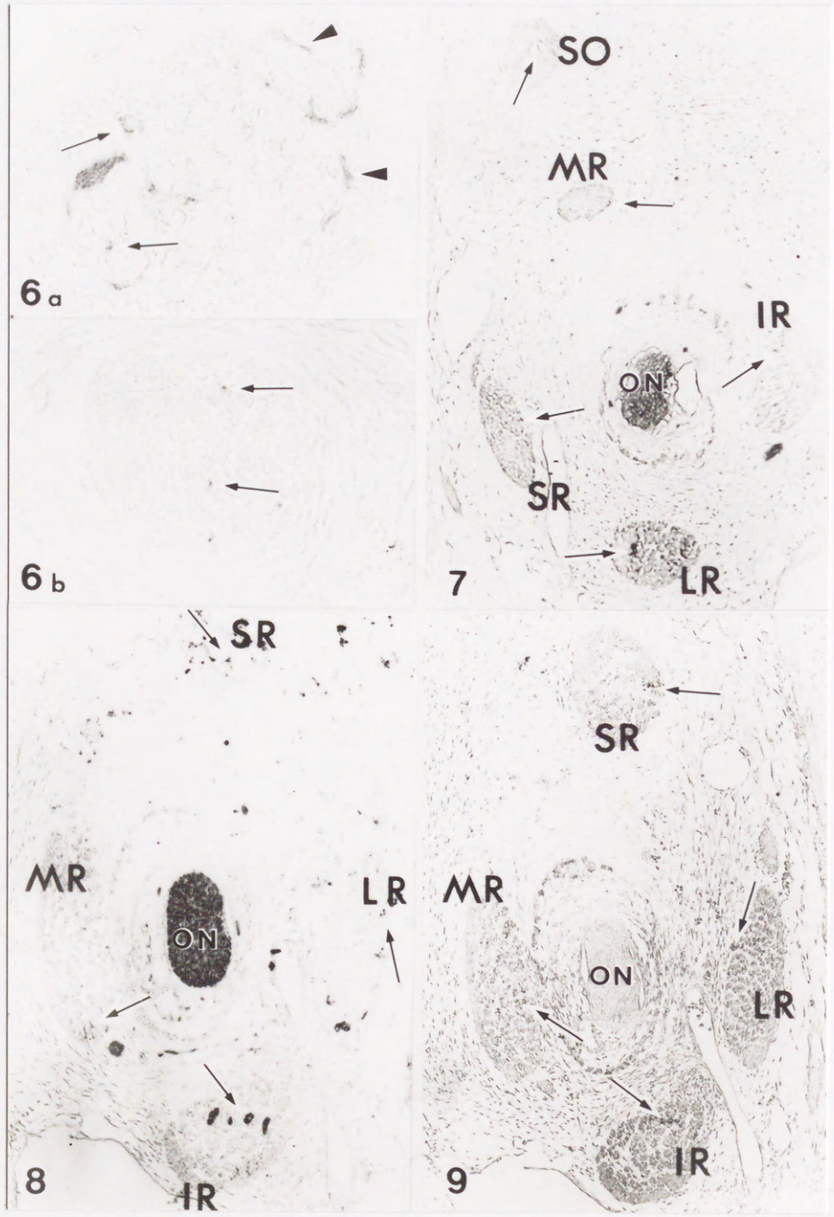
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ACTA HISTOCHEM. CYTOCHEM. Vol. 24, No. 1, 1991

IMMUNOHISTOCHEMICAL STUDY ON THE DEVELOPMENT OF EXTRAOCULAR MUSCLES II. HUMAN EMBRYOS

MASAMI OGUNI[1], OSAMU TANAKA[2], TOMOICHI SETOGAWA[1], HARUO SHINOHARA[3], KANEFUSA KATO[3] AND REIJI SEMBA[4]

Departments of Ophthalmology[1] and Anatomy[2], Shimane Medical University, Izumo 693 and Departments of Biochemistry[3] and Perinatology[4], Institute for Developmental Research, Aichi Prefectural Colony, Kasugai, Aichi 480-03

Received for publication June 13, 1990 and in revised form October 11, 1990

Development of extraocular muscles was studied in externally normal human embryos (Carnegie stages 13 to 21), using three antibodies as molecular markers of muscle differentiation: brain type (CK-B) and muscle type (CK-M) creatine kinase isoenzymes, and muscle type enolase isoenzyme (β-enolase). The innervation of extraocular muscles was also studied immunohistochemically, using antibody against neuron specific enolase (NSE). Periodic acid-Schiff (PAS) and modified PAS stainings were used to demonstrate glycogen. During stages 13–17, neither immunoreactivity to CK-B, CK-M, β-enolase and NSE nor glycogen could be detected around the optic vesicle. At stage 18, myogenic cells around the optic vesicle became immunoreactive to CK-B, CK-M, and β-enolase antibodies, and the nerve fibers in each extraocular muscle were immunoreactive to NSE antibody; however, glycogen was still undetectable. Glycogen began to appear at stage 19 in the clusters of the myogenic cells. These findings suggest that some of the muscle-type isoenzymes for glycolytic pathway and ATP production appear synchronously in human extraocular muscles, and they are in close association with the storage of glycogen and innervation of extraocular muscles.

Human extraocular muscles originate from the mesoderm around the prochordal space from stage 13 to 16 (8). They are differrent from other muscles in structural and physiological properties, such as the morphology of myofibrils and the distribution of cholinesterase activity in neuromuscular junctions (4, 18). In the rat, such structural and physiological differences of extraocular muscles are already present during the embryonic perod (25), however, immunohistochemical study on the development of extraocular muscles in early human emnbryos is not availiable. In the present study, we used CK-B, CK-M and β-enolase antibodies as molecular markers of muscle differentiation and NSE antibody as a marker of innervation, to assess the development of extraocular muscles in human embryos.

Creatine kinase (CK) isoenzyme (EC. 2.7.3.2) is involved in the reversible transfer of high-energy phosphate residue between adenosine triphosphate (ATP) and creatine. This enzyme is a dimeric molecule composed of two immunologically distinct subunits, M and B, and exists as MM, MB, and BB forms (5, 31). CK-MM and CK-MB are mainly present in skeletal muscle and heart, respectively (39). Biochemical studies have shown that CK-B is replaced by CK-M in the developing skeletal muscles of rat (6,42) and early human fetus (7).

Enolase isoenzymes (EC. 4.2.1.11) catalyze the interconversion of 2-phosphoglycerate and phosphoenolpyruvate in the glycolytic pathway. They are dimers composed of 3 immunologically distinct subunits, α, β and γ (32). α-Enolase is widely distributed in various tissues (12), while β-Enolase appears in muscle tissues during differentiation (32). γ-Enolase is considered to be identical to the nervous-system-specific protein 14-3-2 (3, 21) and has been designated as neuron-specific enolase (NSE). NSE is one of the protein components of brain synaptic plasma membrane and axons (20), and appears with the formation of synapses (41).

In addition to creatine kinase isoenzymes, glycogen phosphorylase is an excellent marker of skeletal muscle differentiaiton (23). Glycogen

Corresponcence to: Masami Oguni, M. D., Department of Ophthalmology, Shimane Medical University, Izumo 693, Japan.

phosphorylase plays an important role in the release of glucose from glycogn. Subsequently, the process of glycolysis, in which enolase isoenzymes are involved, proceeds and ATP which is needed for muscle contraction is synthesized. Therefore, the presence of glycogen in muscle cells indicates their role as an energy source of muscle contraction.

MATERIALS AND METHODS

Thirty-four externally normal human embryos used in the present study, ranging from Carnegie stage 13 to 21 (6.3 mm to 23.9 mm in crown rump length) are listed in Table 1. All were obtained through collaboration with obstetricians in Japan and were the products of interrupted pregnancies: this procedure is in accord with the Japanese Eugenic Protection law (26). These embryos were observed under a binocular microscope for external features and their developmental stages were determined according to O'Rahilly (30).

Histochemistry

A total of 22 embryos, within 2 hr after acquisition, were fixed in Lillie's fluid (19) (formaldehyde 40%, 10 ml; glacial acetic acid, 5 ml; absolute alcohol, 85 ml) and kept at 4°C. After dehydration in a graded series of ethyl alcohols, the specimens were embedded in paraffin and 7 μ-thick sections of the head were prepared. Sections were stained with periodic acid-Schiff (PAS) and modified PAS (PARS), an improved staining method for the demonstration of glycogen (10).

Immunohistochemistry

12 externally normal human embryos were used. After abortion, the whole embyos were put into Schmechel's fixative (33) composed of 4% paraformaldehyde, 1% glutalaldehyde, 0.2% picric acid and 2% sucrose in 0.1 M sodium acetate buffer, pH 6.0 and stored at 4°C. One or two days later, they were transferred to Tris-buffered saline (TBS. 50 M Tris-HCL buffer, pH 7.6 with 150 mM NaCl) and kept at 4°C. After dehydration in a series of graded ethyl alcohols, the specimens were placed in 0.3% H_2O_2 in absolute methanol for 30 min for the blocking of endogenous peroxidase activity and then embedded in paraffin. Serial sections of 5 μ-thickness were prepared and were immunostained after abidin-biotin-peroxidase complex (ABC) method of Hsu (11), using CK-B, CK-M, β-enolase and NSE antibodies. The preparation procedures and the specificity of the antibodies used in the present study are described in other papers (13-15, 17). CK-M antibody was immunoreactive to CK-MM and CK-MB, but not to CK-BB, while CK-B antibody was immunoreactive to CK-BB and CK-MB, without cross-reactivity to CK-MM (15, 17). β-enolase antibody was specific to the β-subunits with no cross-reaction with the γ-subunits (NSE) of human enolase (13). For controls, sections were incubated with normal rabbit serum.

RESULTS

Histochemistry

During stages 13 to 18, neither clusters of myogenic cells of extraocular muscles nor PAS-positive substances could be detected around the optic vesicle (Fig. 1). At stage 19, myogenic cells, some of which were PAS-positive, began to cluster around the optic vesicle (Figs. 2a, b). As the embryonic stages advanced through stages 20 and 21, myogenic cells and

TABLE 1. *Human embryos examined*

Carnegie stage	Estimated postovulation days*	Numbers of Embryos	
		Histochemical	Immunohistochemical
13	32	1	1
14	34–35	2	1
15	36	1	2
16	38	2	3
17	40	3	1
18	42	4	2
19	44	5	
20	46	2	1
21	48	2	1
total		22	12

* Nishimura (27)

PAS-positive substances increased in each extraocular muscle (Figs. 3a, b).

Immunohistochemistry

During stages 13 to 17, there was no immunoreactivity to CK-B, CK-M, β-enolase and NSE antibodies around the optic vesicle. At stage 18, not only immunoreactive muscle cells to CK-B, CK-M and β-enolase antibodies (Figs. 4a–c), but also immunoreactive nerve fibers to NSE antibody appeared in extraocular muscles (Figs. 4d). At stage 21, the clusters of muscle cells elongated toward the optic vesicle along the optic nerve, and the immunoreactivity of muscle

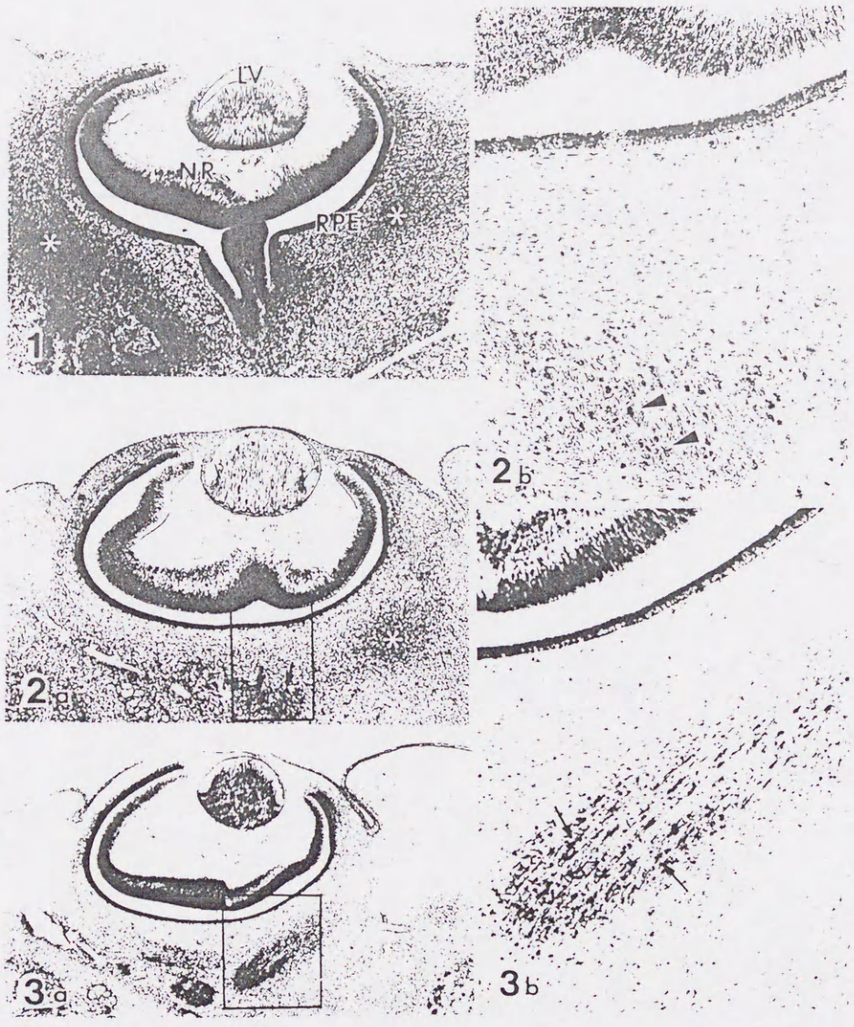
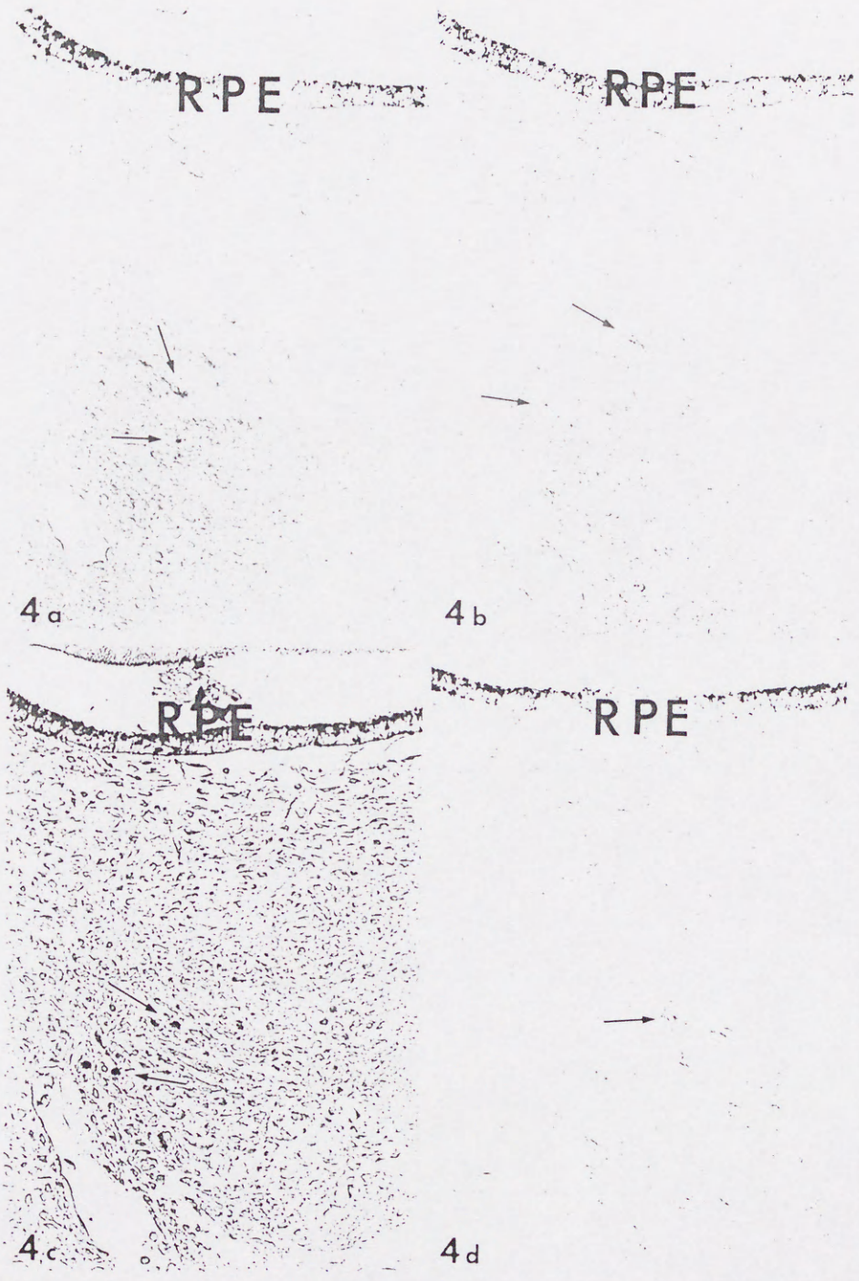


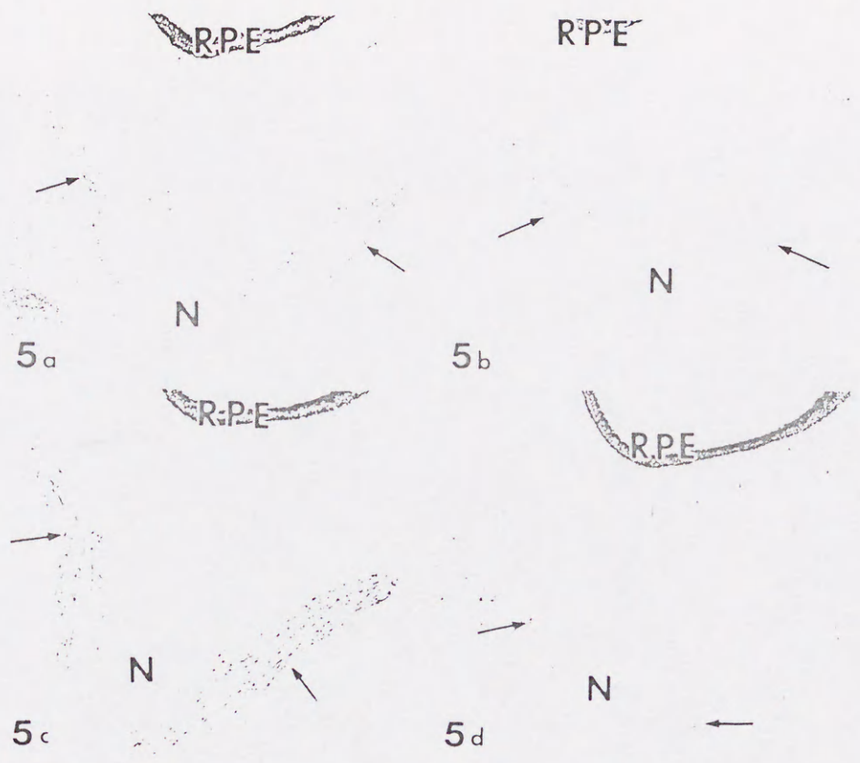
Fig. 1. Cross section of stage 18 embryo stained with the modified method (PARS) of periodic acid-Schiff staining. Note that the clusters of myogenic cells of extraocular muscle are not evident, while, scleral condensations (indicated by asterisks) are clearly identified around the optic vesicle at this stage. PARS positive substances are not present around the optic vesicle. LV: lens vesicle, NR: Neural retina, RPE: retinal pigment epithelium. ×40

Figs. 2a–b. Cross section of stage 19 embryo stained with PARS. 2a. The myogenic cells (arrows) begin to cluster around the optic vesicle at this stage. 2b. Magnified view of the area indicated with a rectangle in Fig. 2a. PARS positive substances are present in the clusters of myogenic cells (arrowheads). Scleral condensation is indicated by an asterisk. a. ×40, b. ×180

Figs. 3a–b. Cross section of stage 21 embryo stained with PARS. 3a. Note that the clusters of myogenic cells elongate toward the optic vesicle and they become identifiable as extraocular muscles. 3b. Magnified view of the area indicated with a rectangle in Fig. 3a. PARS-positive substances have increased in extraocular muscle (indicated by arrows). a. ×30, b. ×160



FIGS. 4a–d. Cross section of stage 18 embryo immunostained with CK-M (a), CK-B (b), β-enolase (c) and NSE (d) antibodies. At this stage, myogenic cells are immunoreactive to CK-M, CK-B, β-enolase (a, b, c), and immunoreactivity to NSE is noted (arrows). RPE: retinal pigment epithelium. a. ×270, b. ×270, c ×270, d. ×270



FIGS. 5a–d. Cross section of stage 21 embryo immunostained with CK-M (a), CK-B (b), β-enolase (c) and NSE (d) antibodies. Note that the immunoreactive cells to CK-M, β-enolase antibodies have increased in number (arrows). The decrease of immunoreactivity to CK-B antibody is not apparent at this stage. Note that the number of nerve fibers, that are immunoreactive to NSE antibody, have increased compared with the stage 18 embryo (Fig. 4d). RPE: retinal pigment epithelium. N: optic nerve a. ×50, b. ×50, c×50, d×50.

cells to CK-M, CK-B and β-enolase and of nerve fibers to NSE increased (Figs. 5a–d).

DISCUSSION

Skeletal muscle differentiation and the functional state of muscle tissue have been correlated with CK-M (23) and β-enolase (16, 24) isoenzymes, respectively. However, differences in the expression of CK-M during muscle differentiation between chicks and rats (6), and between humans and rats (36) were noted. While it has been shown that the immunoreactivity to β-enolase and CK-M antibodies in myogenic cells of rat extraocular muscles appears at embryonic day 15 and 18, respectively (29), the present study shows that such immunoreactivity in myogenic cells of human extraocular muscles appears at the same embryonic period of stage 18. Moreover, it has been reported that CK-B appears earlier than CK-M in extraocular muscles of rat embryos (29), however, our results show that CK-M and CK-B appear in myogenic cells of extraocular muscles also at the same embryonic period of stage 18. Therefore, the muscle development of human extraocular muscles is different from that of rats, so far as the expression of CK-M, CK-B and β-enolase genes is concerned.

Studies on glycogen, glycogen phosphorylase and myofibrillar ATPase, have shown that extraocular and limb muscles of human adult are different in oxidative function and glycolytic pathway (33). On the other hand, the appearance of β-enolase and glycogen is earlier than CK-M in myogenic cells of human upper limb bud (37). In this study, β-enolase, CK-M and glycogen appeared at the same embryonic stage in human extraocular muscles. These findings suggest not only that human extraocular muscles are different from human upper limb muscles in the appearance of CK isoenzymes, β-enolase and glycogen, but also such difference is already present in the embryonic period.

A biochemical study revealed that the isoenzyme

pattern changes from CK-B to CK-M in the human quadriceps muscle between late embryonic and early fetal periods (7). It has been reported that CK-B appears earlier than CK-M in upper limb muscles of human embryos (9, 38). Our previous study showed that CK-B is replaced by CK-M in the trunk muscles of human embryos during the late embryonic period (28). In the present study, the staining intensity to CK-M and CK-B antibodies increased in the extraocular muscle as the embryonic stages advanced up to stage 21. Therefore, it seems that the CK subunit of extraocular muscles either changes from CK-B to CK-M after stage 21, or remains as a hybrid CK-MB, as in the case of heart muscle (6).

Thymidine-radiographic studies have shown that the neurons innervating extraocular muscles are generated on the 11th or 12th embryonic day in the developint rat brain stem (1, 2). We have previouly shown that NSE immunoreactivity appears in the extraocular muscles of rat between embryonic day 15-17 (29). On the other hand, NSE immunoreactive neurons in the mesencephalon, which innervate extraocular muscles, appear in the stage 14 human embryo (35). In the present study, NSE immunoreactive nerve fibers appeared in extraocular muscles in stage 18, the time CK isoenzymes and glycogen were first noted in extraocular muscles. Therefore, these findings confirm the earlier reports that CK isoenzymes are directly correlated with innervation (23).

ACKNOWLEDGEMENT

The authors are indebted to Mr. F. Satow, Shimane Medical University, for technical assistance and to Dr. Antoine Abu Musa for critical comments.

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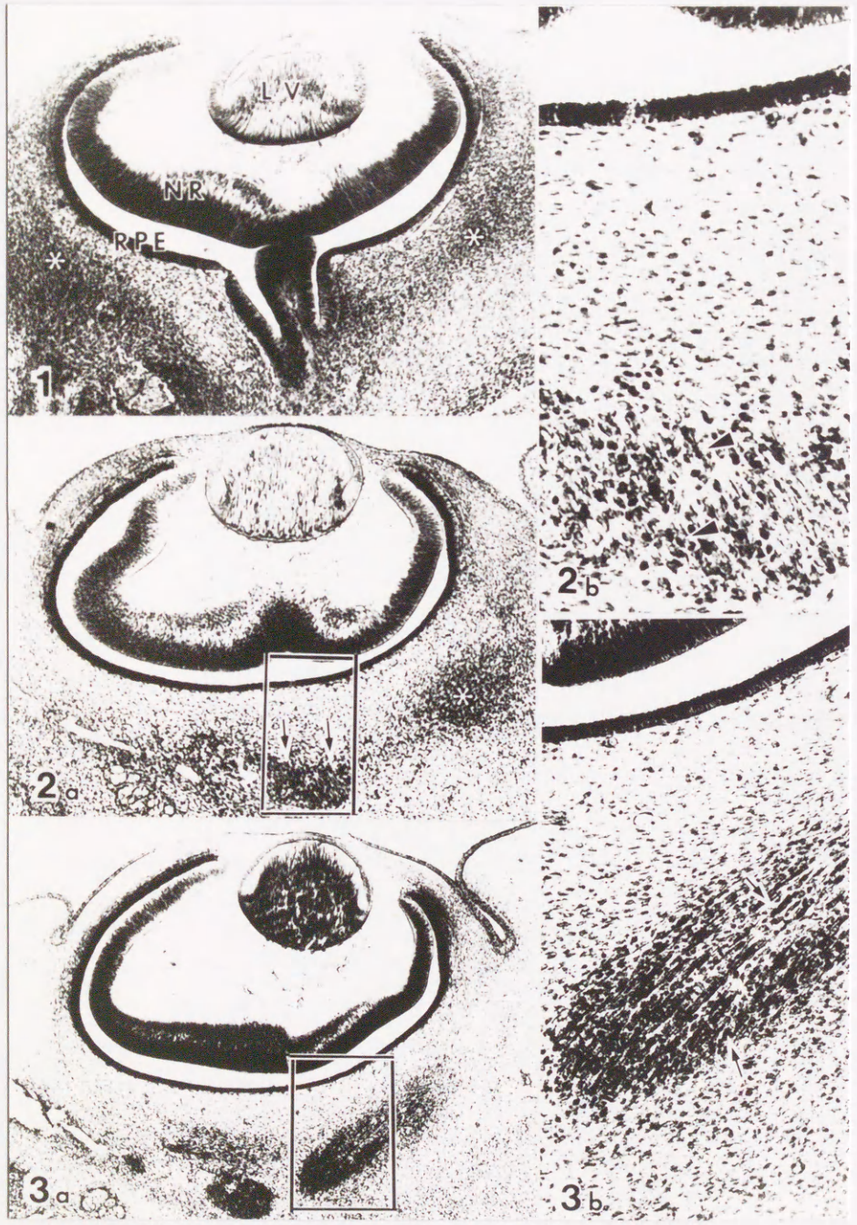
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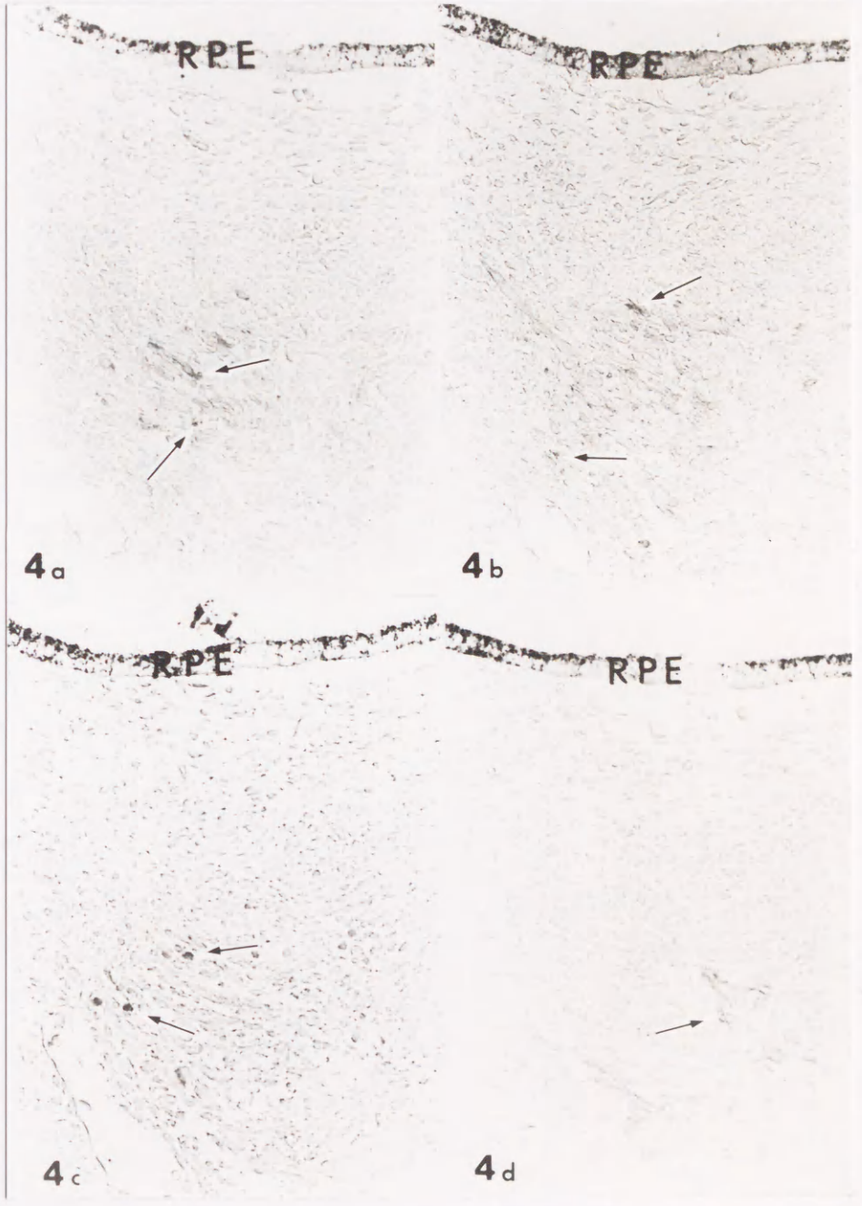
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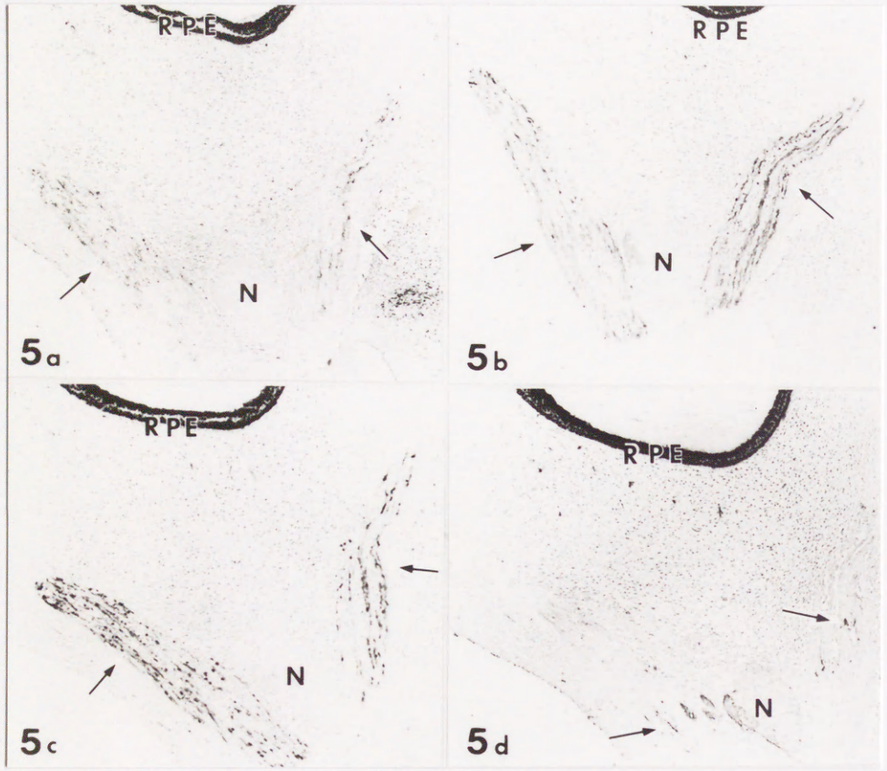
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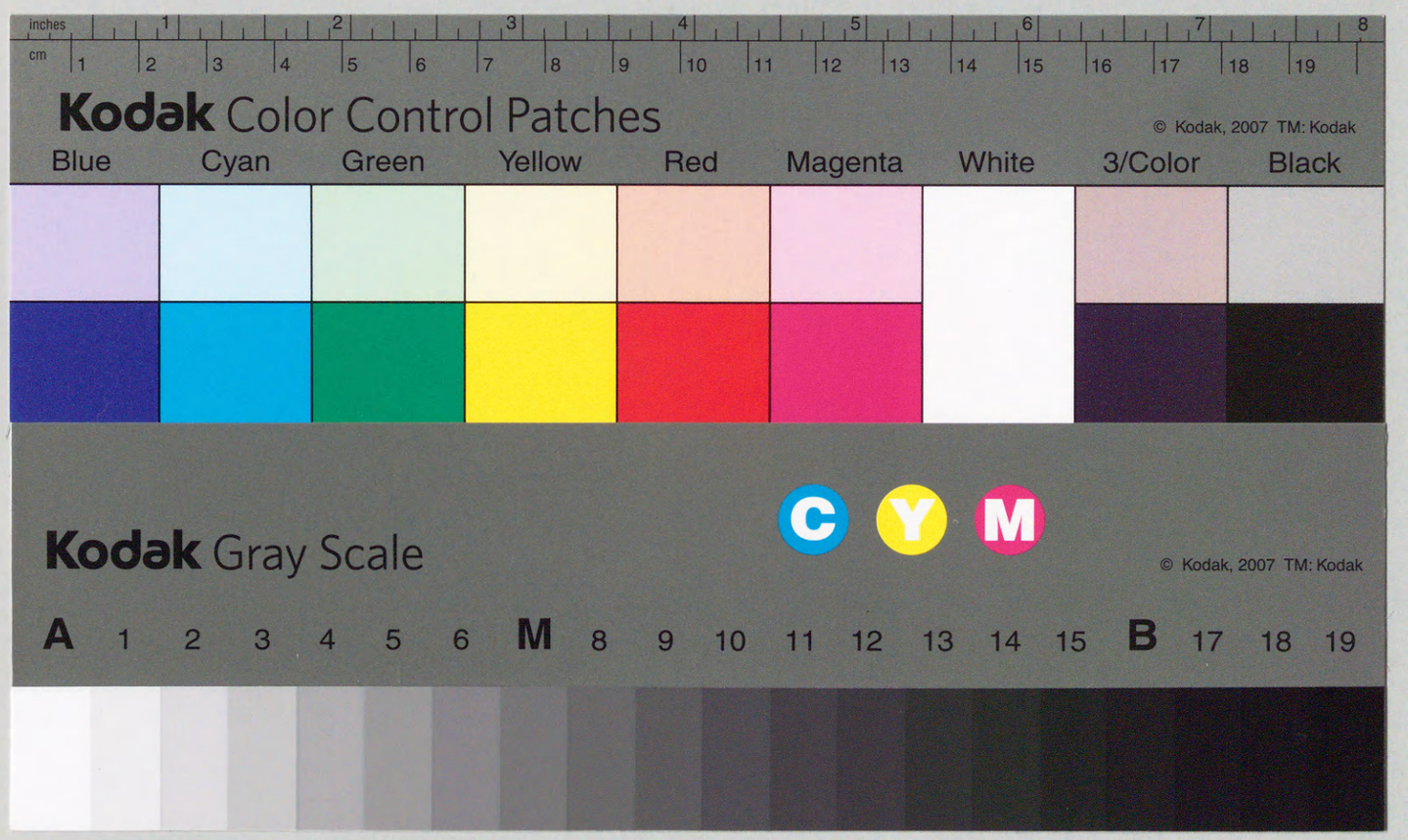
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