IMMUNOHISTOCHEMICAL STUDY ON THE DEVELOPMENT OF EXTRAOCULAR MUSCLES I. RAT

MASAMI OGUNI¹, OSAMU TANAKA², TOMOICHI SETOGAWA¹, HARUO SHINOHARA³,
KANEFUSA KATO³ AND REIJI SEMBA⁴

*Departments of Ophthalmology¹ and Anatomy², Shimane Medical University, Izumo 693 and
Departments of Biochemistry³ and Perinatology⁴, Institute for Developmental Research,
Aichi Prefectural Colony, Kasugai, Aichi 480-03*

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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 and 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 Caesarean 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 5 μ m 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% 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

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% H₂O₂ 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 substances 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. $\times 100$, b. $\times 350$

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. $\times 100$, b. $\times 350$

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. $\times 100$, b. $\times 350$

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) of LR muscle are immunoreactive with CK-B antibodies. 6b. CK-B immunoreactivity in muscular cells of SO muscle appears at this embryonic day. a. $\times 300$, b $\times 300$

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 $\times 80$

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). $\times 80$

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). $\times 80$

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 -enolase 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.

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