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INNERVATION OF HUMAN DEVELOPING EXTRAOCULAR MUSCLES : IMMUNOHISTOCHEMICAL STUDY

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S-100 protein has two subunits, S-100α and S-100β. S-100α is predominant in neurons and slow-twitch muscle fibers, while S-100β exists in glial cells and Schwann cells. In the present study, the presence of S-100α and S-100β was investigated immunohistochemically in the extraocular muscles of 15 human embryos (Carnegie stages 13 to 23) and in one fetus (10 weeks of gestation). During stages 13-17, no immunoreactivities to S-100α and S-100β were found around the optic vesicle. At stage 18, immunoreactivity to S-100β appeared around the optic vesicles and increased as the embryonic stages advanced. In the fetus, the nerve fibers in the extraocular muscles were slightly immunoreactive to S-100α, but the muscle fibers were not. These findings suggested that extraocular muscles are innervated in the late embryonic period, around stage 18.

S-100 protein is a calcium-binding protein that has two subunits, S-100α and S-100β (1-3). S-100α is present in neurons (4, 5), and S-100β is mainly distributed in glial cells and Schwann cells (6, 7).

We previously showed that neuron-specific enolase (NSE) appears in extraocular muscles at stage 18 (8). Since the appearance of NSE is associated with the formation of synapses (9), it follows that the synapse formation of extraocular muscles starts in the late embryonic period.

In the present study, the presence of S-100 protein subunits, particularly NSE, were investigated.

MATERIALS AND METHODS

Fifteen externally normal human embryos in Carnegie stages 13 to 23 (4 to 8 weeks of gestation) and one fetus (10 weeks of gestation) were used in the present study (Table I). All tissues were obtained from surgical abortions of pregnancies, according to the Eugenic Protection Law of Japan (10). The embryos were observed under binocular microscope for external features, and their developmental stages were determined by the method of O'Rahilly (11). After removal, the whole embryos and fetus were placed in Schmechel's fixative (12), which is 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℃. One or two days later, specimens were transferred to Tris-buffered saline (TBS, 50 mM Tris-HCl buffer, pH 7.6 with 150 mM NaCl) and kept at 4℃. 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 the endogenous peroxidase activity and were embedded in paraffin. Serial sections, 5-μm thick, were prepared and immunostained with avidin-biotin-peroxidase complex (ABC) technique of Hsu *et al.* (13), using S-100α, S-100β, and NSE as primary antibodies. The preparation procedures and the specificity of the antibodies used in the present study have been described elsewhere (4, 6, 14). S-100α and S-100β antibodies are specific to the respective subunit of the S-100 protein and react not only with the respective homodimeric S-100 protein (αα or ββ) but also with the hybrid form (6). The cross-reactivity of the hybrid form to the S-100α and S-100β assay systems is about 25% of homodimeric S-100 protein (6). NSE antibody is specific to γ-subunits of human enolase (4). For controls, sections were incubated with normal rabbit serum, which gave no positive stainings, instead of the primary antibody.

Table I. Human embryos examined in the present study

Carnegie stage	Specimen 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±0.13	42
20	71025	19.2±0.26	46
21	52356	21.1±0.26	48
	52380	〃	〃
23	52377	28.0±1.02	52
Fetus	71021	35.5***	10 weeks

*Nishimura et al. (23)
**Nishimura (24)
***by actual measurement

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RESULTS

The summary of isoenzyme activity in the present study is given in Table II. During stages 13 to 17, no immunoreactivity to S-100 α, S-100 β, and NSE was observed around the optic vesicle. At stage 18, immunoreactivity to S-100 β and NSE appeared around the optic vesicle (Fig. 1b, 1c), but no immunoreactivity to S-100 α was observed (Fig. 1a). At stage 20, the muscle cells around the optic vesicle began to cluster, and immunoreactivities to S-100 β and NSE were observed between the muscle cells in the

Tabel II. Expression of isoenzyme in extraocular muscles

Carnegie stage	13	14	15	16	17	18	19	20	21	22	23	Fetus
S-100α												
nerve fiber	-	-	-	-	-	-		-	-		-	±
muscle fiber	-	-	-	-	-	-		-	-		-	-
S-100β												
glial cell	-	-	-	-	-	+		+	+		+	+
NSE*												
nerve fiber	-	-	-	-	-	+		+	+		+	+

NSE: neuron-specific enolase
*Oguni et al. (8)

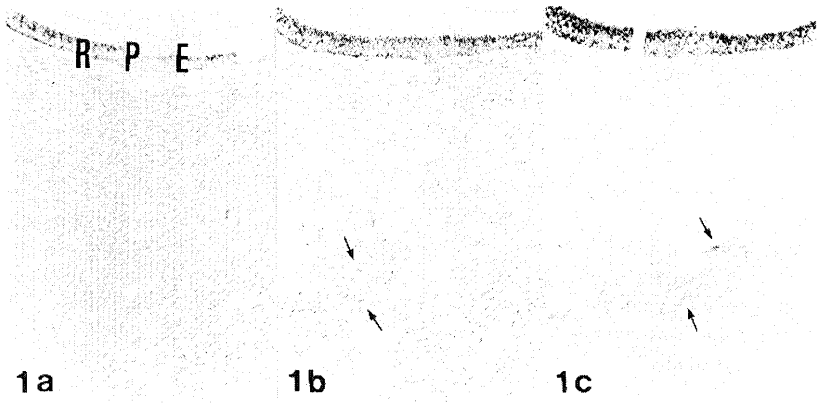

Fig. 1. Cross-sections of the extraocular muscles in a stage 18 embryo immunostained with S-100 α (a), S-100 β (b) and NSE antibodies (c). S-100 β and NSE immunoreactivities appear around the optic vesicle at stage 18 (b, c) (arrows), however, S-100 α is not observed at this stage. RPE: retinal pigment epithelium (a X 80, b X 80, c X 80)

Fig. 2. Cross-section of the extraocular muscles in a stage 21 embryo immunostained with S-100 β (a) and NSE (b) antibodies. Note that immunoreactivity to S-100 β and NSE is observed between the muscle cells of the extraocular muscles (arrows).
ON: optic nerve (a X 85, b X 85)

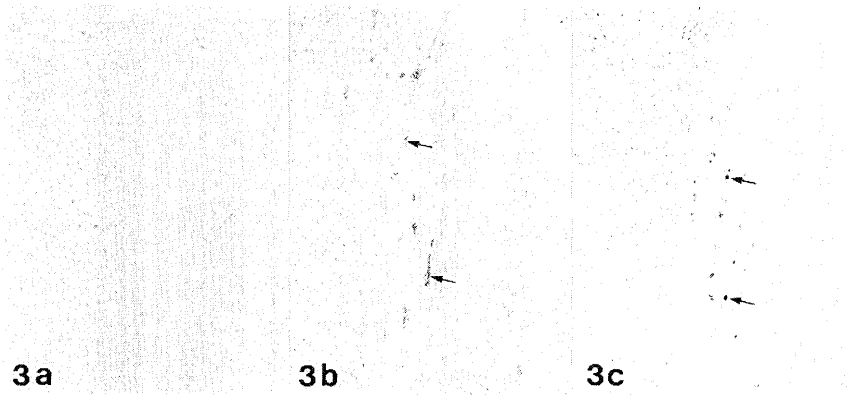

Fig. 3. Cross-section of the extraocular muscles in a stage 23 embryo immunostained with S-100 α (a), S-100 β (b) and NSE antibodies (c). Note that immunoreactivity to S-100 β and NSE is observed between the muscle cells of the extraocular muscles (arrows), while immunoreactivity to S-100 α is not seen in the extraocular muscles.
ON: optic nerve (a X 100, b X 100, c X 100)

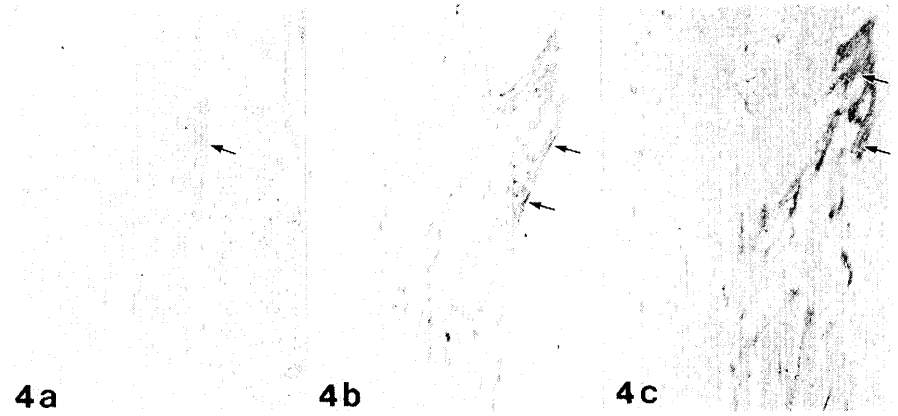

Fig. 4. Cross-sections of the extraocular muscles in a 10-week-old fetus immunostained with S-100α (a), S-100β (b) and NSE antibodies (c). 4a: The nerve fibers in the extraocular muscles are slightly immunoreactive to S-100α (arrow). 4b, 4c: The nerve fibers in the extraocular muscles are more intensely immunoreactive to NSE than S-100α, while glial cells around the neurons are immunoreactive to S-100β (arrows).
(a X 100, b X 100, c X 100)

clusters (Fig. 2a, 2b). At stage 23, immunoreactivity to S-100β and NSE was observed in the elongating extraocular muscles (Fig. 3b, 3c), while no immunoreactivity to S-100α was found (Fig. 3a). In the 10-week-old fetus, some nerve fibers were slightly immunoreactive to S-100α, however, muscle fibers were not immunoreactive to this antibody (Fig. 4a). The nerve fibers in the extraocular muscles were more intensely immunoreactive to NSE than S-100β (Fig. 4a, 4c), while the Schwann cells around the neurons were immunoreactive to S-100β (Fig. 4b).

DISCUSSION

It has been reported that S-100 protein is primarily localized in glial cells in the nervous system (15, 16). Since S-100β is present in Schwann cells and glial cells (6, 7), while S-100α occurs in neurons (5, 7, 17), it is noteworthy that S-100β appears earlier than S-100α in the neuromuscular junctions of extraocular muscles.

In the present study, S-100β appeared in the extraocular muscles at stage 18, the period when NSE appears (8). Since NSE is one of the protein components of brain synaptic plasma membrane and axons (18), and appears with the formation of synapses (9), we suggested that the synaptic formation in the extraocular muscles begins around stage 18 in extraocular muscles (8). The present study revealed that S-100β appeared at the same stage in almost the same portion around the optic vesicle as NSE (8). Since S-100β is present in Schwann cells but not in neurons and muscle fibers (5, 6, 7), it appears that S-100β-immunoreactive cells are Schwann cells around the peripheral nerve fibers. These findings strengthen the notion that nerve fibers, together with Schwann cells, which are connected with extraocular muscles, extend and have synaptic connections in some parts with myogenic cells in extraocular muscles at stage 18.

In the present study, S-100α, which is localized in the slow-twitch muscle fibers and nerve fibers (19), appeared in the early fetal period. Since the immunoreactive portions to S-100α were also immunoreactive to NSE, it is thought that S-100α is localized in the nerve fibers in extraocular muscles. It has been reported that fast muscle fibers are more numerous than slow-twitch muscle fibers in early development (20). In our previous study, carbonic anhydrase-III (CA-III), which is present in the slow-twitch muscle fibers (21), appears at stage 20 in the extraocular muscles (22). These findings suggest that the expression of S-100α immunoreactive muscle fibers takes place at a considerably late stage of myogenesis in the extraocular muscles, as compared with the other marker of slow-twitch muscle fibers (22).

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REFERENCES

1) Isobe, T., and Okuyama, T. (1978) The amino-acid sequence of S-100 protein (PAP I-b protein) and its relation to the calcium binding proteins. Eur. J. Biochem.,89, 379-388
2) Isobe, T., and Okuyama, T. (1981) The amino-acid sequence of the subunit in bovine brain S-100β protein. Eur. J. Biochem., 116, 79-86
3) Isobe, T., Ishioka, N., Masuda, T., Takahashi, Y., Ganno, S., and Okuyama, T. (1983) A rapid separation of S100 subunits by high performance liquid chromatography: the subunit composition of S100 protein. Biochem. Int., 6, 419-426
4) Kato, K., Arai, R., Shimizu, A., Suzuki, F., and Ariyoshi, Y. (1983) Immunoassay of three enolase isozymes in human serum and in blood cells. Clin. Chim. Acta 127, 353-363
5) Takahashi, K., Isobe,T., Ohtsuki, Y., Akagi, T., Sonobe, H., and Okuyama, T. (1984) Immunohistochemical study on the distribution of a and b subunits of S-100 protein in human neoplasm and normal tissues. Virchows Arch. (Cell Pathol.), 45, 385-396
6) Kimura, S., Kato, K., Semba, R., and Isobe, T.

(1984) Regional distribution of S-100a_0 ($\alpha\ \alpha$), S-100a ($\alpha\ \beta$) and S-100b ($\beta\ \beta$) in the bovine central nervous tissue determined with a sensitive enzyme immunoassay system. Neurochem. Int., 6, 513-518

7) Kato, K., and Kimura, S. (1985) S100a_0 ($\alpha\ \alpha$) protein is mainly located in the heart and striated muscles. Biochim. Biophys. Acta., 842, 146-150

8) Oguni, M., Tanaka, O., Setogawa, T., Shinohara, H., Kato, K., and Semba, R. (1991) Immunohistochemical study on the development of extraocular muscles. II. Human embryos. Acta Histochem. Cytochem., 24, 47-53

9) Whitehead, M. C., Marangos, P. J., Connolly, S. M., and Morest, D. K. (1982) Synapse formation is related to the onset of neuron-specific enolase immunoreactivity in the avian auditory and vestibular system. Dev. Neurosci., 5, 298-307

10) Nishimura, H. (1975) Prenatal versus postnatal malformations based on the Japanese experience on induced abortions in the human being. In: Aging Gametes, (Blandau, R. J.) pp. 349-368, S. Karger, Basel

11) O'Rahilly, R. (1972) Guide to the staging of human embryos. Anat. Anz., 130, 556-559

12) Schmechel, D. E., Brightman, M. and Barker, J. L. (1980) Localization of neuron-specific enolase in mouse spinal neurons grown in tissue culture. Brain Res., 181,391-400

13) Hsu, S. M., Raine,L., and Franger, H. (1981) Use of avidin-biotin- peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedure. J. Histochem. Cytochem., 29, 577-580

14) Kato, K., Suzuki, F., Kurobe, N., Okajima, K., Ogasawara, N., Nagaya, M., and Yamanaka, T. (1990) Enhancement of S-100β protein in blood of patients with Down's syndrome. J. Mol. Neurosci., 2, 109-113

15) Hyden, H., and McEwen, B. (1966) A glial protein specific for the nervous system. Proc. Natl. Acad. Sci. USA, 55, 354-358

16) Sviridov, S. M., Korochkin, L. I., Ivanov, V. N., Maletskaya, E. I., and Bakhtina, T. K. (1972) Immunohistochemical studies of S-100 protein during postnatal ontogenesis of the brain of two strains of rats. J. Neurochem., 19, 713-718

17) Schmechel, D. E., Marangos, P. J., Brightman, M., and Goodwin, F. K. (1978) Brain enolases as specific markers of neuronal and glial cells. Science, 199, 313-315

18) Lim, L., Hall, C., Leung, T., Mahadevan, L., and Whatley, S. (1983) Neuron-specific enolase and creatine phosphokinase are protein components of rat brain synaptic plasma membranes. J. Neurochem., 41, 1177-1182

19) Haimoto, H., and Kato, K. (1987) S100a_0($\alpha\ \alpha$) protein, a calcium-binding protein, is localized in the slow-twitch muscle fiber. J. Neurochem., 48, 917-923

20) Fenichel, G. M. (1966) A histochemical study of developing human skeletal muscle. Neurology, 16, 741-745

21) Shima, K., Tashiro, K., Hibi, N., Tsukada, Y., and Hirai, H. (1983) Carbonic anhydrase-III immunohistochemical localization in human skeletal muscle. Acta Neuropathol. (Berl), 59, 237-239

22) Oguni, M., Setogawa, T., Tanaka, O., Shinohara, H., and Kato, K. (1991) Immunohistochemical study of carbonic anhydrase III in the extraocular muscles of human embryos. Acta Anat., 144, 316-319

23) Nishimura, H., Takano, K., Tanimura, T. and Yasuda, M. (1968) Normal and abnormal development of human embryos: first report of the analysis of 1,213 intact embryos. Teratology 1, 281-290

24) Nishimura, H. (1983) Introduction. In: Atlas of Human Prenatal Histology, (Nishimura, H.) pp. 1-4, Igaku-shoin, Tokyo