ORIGIN OF MYOTOME CELLS IN THE CHICK EMBRYO STUDIED BY ^3H -THYMIDINE AUTORADIOGRAPHY AND ACETYLCHOLINESTERASE HISTOCHEMISTRY

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^3H -thymidine autoradiography and acetylcholinesterase (AChE) histochemistry were performed on the same plastic sections to examine the origin of myotome cells in the chick embryo.

The myotome first forms in the dorso-medial angle of the dermomyotome and elongates ventro-laterally along the inner surface of the dermomyotome with development. The myotome consists of numerous AChE-positive cells and some AChE-negative cells.

Two hr after injection of ^3H -thymidine, a few AChE-negative cells but none of the AChE-positive myotome cells were labeled. Four hr after the injection, a few labeled AChE-positive and AChE-negative cells were observed throughout the myotome and thereafter, the labeling of the AChE-positive as well as AChE-negative cells increased with time. Twenty-four hr after the treatment, many labeled cells were distributed throughout the myotome, but unlabeled AChE-positive cells were also evident in the myotome.

In the present study, the proliferating ability and origin of myotome cells in the chick embryo are discussed.

Acetylcholinesterase (AChE) activity has been demonstrated histochemically in developing myogenic cells (1, 5, 6, 8, 14, 21, 24, 27, 29). It has also been reported that differentiated myogenic cells are no longer capable of proliferation (2, 22, 25, 30).

Our study, using the histochemical technique, has revealed that the myotome of the early chick embryo consists mainly of AChE-positive cells displaying enzymatic activity along the nuclear membrane and within the cytoplasm (18). Some AChE-negative cells were also noted throughout the myotome. Based on their morphological characteristics, the former were regarded as developing myogenic cells and the latter as undifferentiated cells.

In the present study, utilizing ^3H -thymidine autoradiography and AChE-histochemistry on the same sections (17), we examined the proliferating ability and AChE-activity of the myotome cells in the early chick embryo.

With regard to the origin of the myotome cells, considerable controversy exists. Some authors have stated that the myotome cells originate in the dorso-medial and ventro-lateral angles of the dermomyotome (3, 4, 28). Langman and Nelson (13) and Ikeda *et al.* (10) reported that the myotome cells originate throughout the length of the overlying dermomyotome, while others (15, 16) have emphasized that

cells of the sclerotome re-aggregate along the inner surface of the dermomyotome and form the myotome. In the present study, references are also made to the formation of the myotome and the origin of related cells.

MATERIALS AND METHODS

Fertilized eggs of White Leghorn fowls were artificially incubated in a humid atmosphere at 31°C, and 20 μ Ci of 3 H-thymidine (specific activity 21.5 Ci/mM, New England Nuclear, Boston, Mass) in a volume of 20 μ l sterile water was injected into the yolk sac of 2- and 3-day-old chick embryo. At intervals of 2, 4, 8, 12 and 24 hr after the injection, these embryos were removed and classified according to the stage series of Hamburger and Hamilton (8); they were then fixed in 4% paraformaldehyde dissolved in phosphate buffer (pH 7.3) according to Millonig (19) for 12 hr at 4°C and washed in the same buffer for 12 hr.

Non-frozen sections 100 μ m thick were cut with a Vibratome and incubated for 2 hr at 37°C in the medium of Karnovsky and Roots (10) containing acetylthiocholine iodide as substrate and 10^{-5} M iso-OMPA (tetraisopropyl pyrophosphoramidate) as an inhibitor of non-specific cholinesterase. After washing, without postfixation with osmium tetroxide, they were dehydrated and embedded in an epoxy resin mixture (Quetol 812; Nissin EM Co. Ltd. Tokyo).

Semithin sections 2 μ m thick were cut with glass knives, mounted serially on glass slides, dipped in a 1 : 1 diluted NR-M₂ emulsion (SAKURA, Tokyo) according to the method of Kopriwa and Leblond (11), exposed for 4 weeks at 4°C, and developed with SDX-1 (SAKURA, Tokyo).

Histochemical controls were performed as follows: (1) incubation in a substrate-free medium; (2) incubation in a medium containing 10^{-5} M BW284C51 (1,5-bis-(4-allyl-dimethyl-ammoniumphenyl)-pentan-3-one dibromide), an inhibitor for AChE.

In order to examine the presence of cells in mitosis in the myotome, 10 μ l of 10^{-5} M colchicine was injected into the yolk sac of 2- and 3-day-old chick embryo. One and 2 hr after the injection, these embryos were fixed for 1 hr in a mixture of 2.5% glutaraldehyde and 4% paraformaldehyde dissolved in the phosphate buffer, postfixed for 1 hr in 1% osmic acid solution, dehydrated and embedded in the epoxy resin mixture. Semithin sections were stained with toluidine blue and examined under an ordinary light microscope.

RESULTS

At the cervical level of the chick embryo, the myotome was first detected in the dorso-medial angle of the dermomyotome at stage 13 (2 days of incubation) (Fig. 1). As development progressed, it elongated ventro-laterally along the ventro-medial (inner) surface of the dermomyotome (Figs. 2, 3) and reached the ventro-lateral end of the dermomyotome at stage 17 to 18 (3 days of incubation). At these stages, the myotome was composed mainly of AChE-positive cells displaying enzymatic activity along the nuclear membrane and in the cytoplasm (Figs. 1-3). Some AChE-negative cells were also seen throughout the myotome (Fig. 2). Mitotic figures in the myotome were extremely rare and could be found only after an

FIG. 1. Transverse section of the somite at the cervical level. 2-day-old chick embryo, stage 13.

The myotome (*M*) is evident in the dorso-medial angle of the dermomyotome (*D*) and is mainly composed of AChE-positive cells. *IS*: interstitial cells, *S*: surface ectoderm. $\times 400$

FIG. 2. Transverse section of the somite at the cervical level. 2-day-old chick embryo, stage 14.

The myotome (*M*) extends ventro-laterally along the inner surface of the dermomyotome (*D*). Note some AChE-negative cells (*large arrowheads*) and a cell in mitosis (*small double arrowheads*) in the myotome. *IS*: interstitial cells, *S*: surface ectoderm. $\times 400$

FIG. 3. Transverse section of the somite at the cervical level. 2.5-day-old chick embryo, stage 16.

The myotome (*M*) is mainly composed of AChE-positive cells, but the cells in the most ventro-lateral portion of the myotome (*large arrowheads*) are AChE-negative. Almost all cells in the dermomyotome (*D*) are AChE-negative. Cells in mitosis (*small double arrowheads*) are seen along the inner surface of the dermomyotome. *IS*: interstitial cells, *S*: surface ectoderm. $\times 400$

FIG. 4. Transverse section of the somite at the cervical level. 2-day-old chick embryo, stage 13; treated with colchicine for 2 hr. Mitotic figures (*arrowheads*) are seen not only along the inner surface of the dermomyotome (*D*) but also in the myotome (*M*) and the dorso-medial angle (*A*) of the dermomyotome. *S*: surface ectoderm. *IS*: interstitial cells. $\times 400$

FIG. 5. Transverse section of the somite at the cervical level. 3-day-old chick embryo, stage 17; treated with colchicine for 1 hr. A cell in mitosis (*arrowhead*) is demonstrated in the myotome (*M*). *D*: dermomyotome, *S*: surface ectoderm, *IS*: interstitial cells. $\times 400$

extensive search (Fig. 2). On the other hand, almost all cells in the dermomyotome and the cells in the dorso-medial angle of the dermomyotome were AChE-negative (Figs. 1–3). Mitotically dividing cells were observed along the inner surface of the dermomyotome (Fig. 3). The interstitial cells located between the myotome and the neural tube were usually AChE-negative, but occasionally cells showed weak enzymatic activity along the nuclear membrane (Figs. 1–3). A few AChE-negative and AChE-weakly-positive cells were observed in the most ventro-lateral portion of the myotome (Fig. 3).

When 2- or 3-day-old chick embryos were treated for 1 or 2 hr with colchicine, mitotic figures were demonstrable not only along the inner surface of the dermomyotome but also in the myotome as well as in the dorso-medial angle of the dermomyotome (Figs. 4, 5).

Two hr after injection of ^3H -thymidine, AChE-positive myotome cells were not labeled (Figs. 6, 7), and the labeled cells distributed throughout the myotome were AChE-negative (Fig. 7). On the other hand, about 40% of the dermomyotome cells were labeled, and the labeled nuclei were observed mainly in the dorso-lateral (outer) half of the dermomyotome (Figs. 6, 7). The nuclei in the inner half of the dermomyotome and the cells in mitosis located along the inner surface of the dermomyotome were not labeled. About 40% of interstitial cells were also labeled.

Four hr after the injection, most but not all of the AChE-positive myotome cells were free of labeling (Figs. 8, 9); they were observed not only in the dorso-medial but also in the more ventro-lateral portions of the myotome. Some of the AChE-negative myotome cells were also labeled (Figs. 8, 9). On the other hand, about

FIG. 6. 2-day-old chick embryo, stage 13; 2 hr after injection of ^3H -thymidine. No AChE-positive cells in the myotome (*M*) are labeled with ^3H -thymidine. Some of the cells in the dermomyotome (*D*) are radio-reactive, and the labeled nuclei are located mainly in the outer half of the dermomyotome. *NC*: migrating neural crest cells, *S*: surface ectoderm. $\times 400$

FIG. 7. 2-day-old chick embryo, stage 13; 2 hr after the injection. Note the labeled AChE-negative cells (arrowheads) in the myotome (*M*). No AChE-positive myotome cells are labeled. *D* dermomyotome, *S* surface ectoderm. $\times 400$

FIG. 8. 2-day-old chick embryo, stage 14; 4 hr after the injection. Some AChE-negative cells (arrowheads) in the myotome (*M*) are labeled with ^3H -thymidine, but no AChE-positive cells show labeling. Many cells in the dermomyotome (*D*) display autoradiographic grains, and labeled nuclei are seen in both the outer and the inner halves of the dermomyotome. *S*: surface ectoderm. $\times 400$

FIG. 9. 2-day-old chick embryo, stage 14; 4 hr after the injection. Note the labeled AChE-positive (large arrowhead) and AChE-negative cells (small double arrowheads) in the myotome (*M*). Many AChE-positive myotome cells are unlabeled with ^3H -thymidine. *D*: dermomyotome, *S*: surface ectoderm. $\times 400$

FIG. 10. 2-day-old chick embryo, stage 14; 8 hr after the injection. Almost all cells in the dermomyotome (*D*) are labeled with ^3H -thymidine. Note the labeled AChE-positive myotome cell (*arrowhead*), which is located in the ventro-lateral portion of the myotome (*M*). *S*: surface ectoderm. $\times 400$

FIG. 11. 2-day-old chick embryo, stage 14; 8 hr after the injection. Many of the AChE-positive cells in the myotome (*M*) are devoid of a ^3H -thymidine label. Note the labeled AChE-positive (*small double arrowheads*) and AChE-negative (*large arrowheads*) myotome cells. Almost all cells in the dermomyotome (*D*) and in the dorso-medial angle (*A*) of the dermomyotome are labeled. *S*: surface ectoderm. $\times 400$

70% of the cells in the dermomyotome were labeled, and labeled nuclei were observed in both the outer and the inner halves of the dermomyotome (Figs. 8, 9). Cells displaying mitotic figures belonged to the labeled elements.

Eight to 12 hr after the injection, some AChE-positive and AChE-negative myotome cells were clearly labeled and found distributed throughout the myotome; however, many unlabeled AChE-positive cells were also seen in the myotome (Figs. 10–12). Almost all cells in the dermomyotome as well as in the dorso-medial angle of the dermomyotome were labeled with ^3H -thymidine (Figs. 10–12). In addition, many of the interstitial cells were among the labeled elements.

Twenty-four hr after the treatment, numerous labeled AChE-positive and AChE-negative cells were distributed throughout the myotome, and many AChE-positive cells remained unlabeled (Fig. 13). In contrast, almost all cells in the dermomyotome and all interstitial cells located between the myotome and the neural tube were labeled with ^3H -thymidine.

DISCUSSION

Considerable controversy exists with regard to the origin of the myotome cells (3, 4, 10, 13, 15, 16, 28).

Using ^3H -thymidine autoradiography, Langman and Nelson (13) examined the origin of the myotome cells in the chick embryo and reported that 4–5 hr after injection of ^3H -thymidine, labeled cells appeared in the myotome, and after 10 hr the label was randomly distributed throughout the myotome. These authors concluded that since neither DNA synthesis nor cell division is ever observed in the cells of the myotome, and rarely in the dorso-medial angle of the dermomyotome, the myotome cells must have originated from the cells of the overlying dermomyotome.

In our materials, 2 hr after injection of ^3H -thymidine, about 40% of the cells in the dermomyotome were labeled and the labeled nuclei were located mainly in the outer half of the dermomyotome. Mitotic figures seen along the inner surface of the dermomyotome were not labeled at this time, but first at 4 hr after the injection. These results favor the concept that the somite walls consist of a pseudo-stratified epithelium and the nuclei undergo interkinetic migration (13). However, as described by these authors, if the products of cell division throughout the length of the overlying dermomyotome are the primary source of the myotome, the myotome cells would not be labeled with ^3H -thymidine before the cells undergoing mitosis in the dermomyotome are labeled. Our radiographic results, however, showed that a few AChE-negative myotome cells gave an autoradiographic reaction before the

FIG. 12. 2.5-day-old chick embryo, stage 16; 12 hr after the injection of ^3H -thymidine. Many AChE-positive cells in the myotome (*M*) are not labeled. Note some labeled cells (*arrowheads*) distributed throughout the myotome. Almost all cells in the dermomyotome (*D*) and almost all interstitial cells (*IS*) show distinct labeling. *S*: surface ectoderm. $\times 400$

FIG. 13. 3-day-old chick embryo, stage 18; 24 hr after the injection of ^3H -thymidine. Many labeled cells are distributed throughout the myotome (*M*); note many unlabeled AChE-positive myotome cells (*arrowheads*). Almost all cells in the dermomyotome (*D*) and all interstitial cells (*IS*) are labeled with ^3H -thymidine. *S*: surface ectoderm. $\times 400$

mitotic figures in the dermomyotome were labeled. As described by others (13, 23), mitotic figures were rare in the myotome, but the present study showed also that when the embryos were treated with colchicine for 1 or 2 hr, mitotic figures were demonstrable in the dorso-medial angle of the dermomyotome and the myotome. These findings indicate that the AChE-negative cells in the myotome incorporate ^3H -thymidine for DNA-synthesis. In addition, the dermomyotome cells are attached by terminal bars at their inner ends (18, 26), and cells migrating from the dermomyotome into the myotome were rarely seen in our material or in that of Christ *et al.* (4). These findings do not support the opinion of Langman and Nelson (13).

In the present study, a few AChE-positive myotome cells labeled with ^3H -thymidine were first noted at 4 hr after the injection, and thereafter the labeled cells increased in number with time. Several authors emphasized that the differentiated myogenic cells no longer possess a proliferating ability (2, 22, 25, 30). Fluck and Strohmman (7) stated that AChE appears in mononucleated myogenic cells after DNA-synthesis has ceased and before fusion of the mononucleated cells has begun. Previously, we have reported that the AChE-positive cells in the myotome can be regarded as developing myogenic cells and AChE-negative cells, as undifferentiated cells, based on their morphological characteristics (18). All these previous findings taken together suggest that the AChE-positive myotome cells are no longer capable of proliferation, and also that some of the AChE-positive myotome cells may be derived from the AChE-negative myotome cells.

On the basis of the autoradiographic study using ^{125}I - α -bungarotoxin (α -BGT), Meiniel and Bourgeois (15) described that since some mesenchyme cells bordering the medial (inner) surface of the myotome were observed to bind α -BGT, these cells might belong to the myogenic line, and thus be destined to contribute to the development of the myotome. Previously, using ^3H - α -BGT autoradiography and AChE histochemistry on the same sections, we found that the specific labeling of α -BGT was restricted to the AChE-positive myotome cells, but no sclerotome (mesenchyme) cells were specifically labeled with α -BGT (19). We also reported that almost all cells in the sclerotome including the cells bordering the inner surface of the myotome were AChE-negative and morphologically undifferentiated (18). These findings do not support the opinion of Meiniel and Bourgeois (15).

In the present study as well as in the previous study (18), the AChE-positive cells first appeared in the dorso-medial angle of the dermomyotome. The myotome, which consisted of many AChE-positive and some AChE-negative cells, elongated ventro-laterally along the inner surface of the dermomyotome, as the development progressed. In addition, there was a distinct ventro-dorsal progression of the morphological differentiation (18) as well as of the density of ACh-receptors of the myotome cells (19). These findings suggest that the myotome cells originate mainly in the dorso-medial angle of the dermomyotome and migrate ventro-laterally along the inner surface of the dermomyotome. Some authors have described that the myotome cells originate in the dorso-medial and ventro-lateral angles of the dermomyotome (3, 4, 28). Unfortunately we cannot at present determine whether the ventro-lateral angle of the dermomyotome contributes to the myotome or not.

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