Cytochemical and Immunocytochemical Demonstration of Acetylcholinesterase of the Prenatal Rat Lower Limb

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Summary. Acetylcholinesterase (AChE) activities in the prenatal rat lower limb were investigated by both cytochemistry and immunocytochemistry.

Results indicate that the epidermal cells show immunoreactions of AChE at a limited stage at prenatal day 15, and mesenchymal cells which are occasionally in contact with the basal lamina or with the adjacent myotubes begin to show AChE activities at prenatal day 17. Such AChE-positive mesenchymal cells, involved in the formation of the muscular tissues, have almost disappeared in the subepidermis by prenatal day 19.

This suggests that AChE independent of the neuromuscular system may be involved in the mesenchymal cell differentiation especially in the inductive process during myogenesis.

The progenitor cells of mammalian limb buds consist of at least two distinct subpopulations of mesenchymal cells: those of somatic mesodermal origin giving rise to the skeletal musculature, and those of somatopleurals mesodermal origin giving rise to the skeleton, although both kinds of cell populations are histologically indistinguishable from each other (CHEVALLIER et al., 1977; KOSHER and RODGERS, 1987). Little is known concerning the determinative and regulatory events of these mesenchymal cells involved in myogenesis and chondrogenesis of the lower limb.

The role of acetylcholinesterase (AChE) in the induction of cellular movement and interaction during morphogenesis was proposed by the light microscopic histochemistry of the developing chick lower limb (FALUGI and RAINERI, 1985). Although cytochemical investigations of AChE in the differentiating skeletal musculature have been done in a variety of mammals, such studies were mainly focused on the developing neuromuscular junction. Descriptions of the enzyme activity in mesenchymal progenitor cells involved in myogenesis have also been very limited (TENNYSON et al., 1971; KAENH et al., 1988).

AChE consists of globular monomers (G₁), dimers (G₂) and tetramers (G₄) of catalytic subunits (globular forms), and three asymmetric tetramers (A₄, A₈, A₁₂) covalently attached to noncatalytic collagen-like tail subunits (asymmetric forms). Biochemical analyses suggest that the asymmetric forms of AChE—which are highly concentrated in neuromuscular junctions—are assembled from previously assembled globular forms by addition of the collagen-like tail in a distal Golgi compartment (ROTUNDO, 1984), and that such a synthetic process first occurs between prenatal days 17 and 18 when the formation of the neuromuscular junction has already taken place in the cultured muscles from the rat lower limb (KÖNIG and VIGNY, 1978; KATO et al., 1980). However immunoelectron microscopy concerning the localization of globular and asymmetric forms of AChE in differentiating muscle cells of the rat lower limb has yet to be made available.

On these grounds, the present study was designed to investigate cytochemical and immunocytochemical AChE localizations of mesenchymal cells and myotubes of the prenatal rat lower limb. The purpose of this study was to throw some light on the role of AChE in the myogenesis by mesenchymal progenitor cells.

MATERIALS AND METHODS

Histochemistry

Lower limbs of Wistar rats at prenatal days 15, 17,
18 and 19 were used for the present study. Samples for cytochemical procedures were fixed either in 4% paraformaldehyde in 0.067 M cacodylate buffer (pH 7.4) or in a mixture of 2% paraformaldehyde and 1% glutaraldehyde (half Karnovsky) in the same buffer for 16 h at 4°C, rinsed in 0.1 M cacodylate buffer for 2 h at 4°C, and cut into approximately 100 μm-thick sections in a cryostat. Cytochemical stainings of AChE were done according to the copper thiocholine technique method by LEWIS and SHUTE (1966) for 30 min at room temperature with a slight modification. After incubation, sections were briefly rinsed in the buffer, fixed in 0.5% osmium tetroxide in the buffer, dehydrated in graded concentrations of acetone, and embedded in epoxy resin. A few sections approximately 60 μm in thickness were observed with a light microscope.

Ultrathin sections were made on a Portor-Blum ultramicrotome and observed in a JEM 1200 EX electron microscope after slight staining with uranyl acetate.

To check the specificity of the activities, sections were pre-incubated in 10^-6 M eserine (Wako Co.) for 30 min or incubated in the medium containing 2×10^-4 M iso-OMPA (Sigma Co.).

**Immunocytochemistry**

For light microscopic immunocytochemistry, specimens...
from rat fetuses at prenatal days 15, 16, 17, 18 and 19 were fixed in a periodate-lysine-paraformaldehyde (PLP) fixative for 6 h at 4°C, embedded in OCT compound, sliced into approximately 6 μm in thickness in a cryostat at −20°C, treated with 0.3% H₂O₂ for 5 min to reduce the endogeneous peroxidase activity, and immunoreacted to mouse anti-AChE monoclonal antibody including both asymmetric and globular subunits or to that including a globular one only (Chemicon, Inc.), with a dilution of 1:100 for 1 h using the Biotin-Streptavidin (BSA) method. After rinsing in phosphate buffer saline (PBS), sections were developed in

Fig. 3. Cytochemical AChE activities in the rER of mesenchymal cells (MC) below the epidermis at prenatal day 17. ×7,200

Fig. 4. Myotubes (MT) show intense cytochemical AChE activities in the rER and nuclear membrane at prenatal day 18. The AChE-positive mesenchymal cells (MC) surround the myotubes. ×4,800
a mixture of 0.05% diaminobenzidine hydrogen peroxidase (DAB) conjugated with 0.01% H$_2$O$_2$.

For immunoelectron microscopy, specimens were fixed in a PLP fixative for 6 h at 4°C, cut into approximately 10 μm thick sections in a cryostat, incubated in the antibodies for 12–24 h at 4°C with a dilution of 1:100 in 0.1 M PBS containing 0.1% egg albumin, and treated with the BSA method. After washing twice in PBS, sections were developed with DAB for 10–15 min, then with 0.5% H$_2$O$_2$, and further with DAB-H$_2$O$_2$ for 15–30 min. They were washed twice in 0.1 M PBS, postfixed in 2% OsO$_4$ in 0.1 M PBS for 15 min, dehydrated by acetone, and embedded in epoxy resin. Ultrathin sections were examined in a JEM 1200 EX electron microscope without staining.

To confirm the specificity of the immunolabelings, normal mouse serum or PBS were substituted for the antisera.

RESULTS

Light and electron microscopic cytochemistry

At prenatal day 15, the epidermis of the lower limb consists of two or more layers of epithelial cells occasionally containing abundant glycogen particles. Mesenchymal cells aggregate below the epidermis and extend their cytoplasmic projections to the adjacent cells or to the basal lamina of the epidermis. Slight AChE activities are seen in the cytoplasm of both epidermal and mesenchymal cells. A few chondrocytes in the primordia of the lower limb bone show AChE activities in the Golgi cisterns at this stage (Fig. 1).

At prenatal day 17, mesenchymal cells below the epidermis reveal intense light microscopic AChE activities (Fig. 2), while the activities in the bone primordia and epidermis have almost completely disappeared. Electron microscopy shows the activities preferentially localized in the rough endoplasmic reticulum (rER) including the nuclear membrane, but not detectable on the plasma membrane (Fig. 3). At prenatal day 18, such AChE-positive mesenchymal cells aggregate to the adjacent myotubes which also show the activities in the rER, including the nuclear membrane (Fig. 4). In the present observations, most AChE-positive mesenchymal cells appear to lessen the intensity of the activities in rER at prenatal day 18 as shown in Figure 4. The incorporation of these AChE-positive mesenchymal cells to the myotubes is the most pronounced at this stage. At prenatal day 19, AChE activities have disappeared in almost all subepidermal mesenchymal cells.

Between prenatal days 17 and 19, the development of the sarcoplasmic reticulum (SR) which shows AChE activities is progressing as the myotomes mature (Fig. 5). On the other hand, AChE activities in the rER...
— including the nuclear membrane as revealed in the early myotubes — become much less intense. At these stages, AChE activities of the differentiating muscle cells become pronounced in the Golgi cisterns (Fig. 6A) and the smooth membranous system along the cell surface (Fig. 6B).

The invasion of nerve plexuses to the myotubes occurs around prenatal day 17, and the Schwann cells show AChE activities preferentially in the nuclear membrane (Fig. 7A). AChE activities at the neuromuscular junctions are detected at prenatal day 18 (Fig. 7B).

**Light and electron microscopic immunocytochemistry**

Light microscopic immunocytochemistry shows intense AChE immunoreactions of the epidermis between prenatal days 15 and 16 (Fig. 8). Intense immunoreactions are seen in the subepidermal mesenchymal cells at prenatal day 17 (Fig. 9), but have almost disappeared by prenatal day 19. Chondrocytes in the cartilaginous tissue do not show immunoreactions after prenatal day 17 as did the data from the cytochemistry.

In immuno-electron microscopy using either antibodies including the asymmetric and globular forms or those including the globular one only, the cytoplasmic vesicles of the muscle cells (Fig. 10) and the intercellular space of the adjacent cells (Fig. 11) are immunoreacted exclusively in the former sample between prenatal days 17 and 18.
DISCUSSION

At prenatal day 15, the epidermal cells of the lower limb reveal intense AChE immunoreactions, although the AChE activities were hardly demonstrable by the present cytochemistry. This discrepancy is under investigation. At prenatal day 17, the subepidermal mesenchymal cells, which are occasionally in contact with the epidermal basal lamina or with the adjacent myotubes by cytoplasmic projections, show intense cytochemical and immunocytochemical AChE activities. FALUGI and RAINERI (1985) proposed AChE-dependent ectodermal-mesenchymal interactions. Since our AChE-positive mesenchymal cells appear to be incorporated into the myotubes, it seems likely that AChE independent of the neuromuscular system may be involved in the inductive process of the cellular differentiation and aggregation in the process of the myogenesis.

In the present study, precartilaginous mesenchymal cells appear to produce AChE by prenatal day 15 prior to the onset of production of this enzyme in myogenic mesenchymal cells. This may signify the spatiotemporal difference in expression of AChE synthesis between the two cell populations as previously cited (DREWS, 1975; CHEVALLIER et al., 1977; CHRIST et al., 1977; KOSHER and RODGERS, 1987).

Immunoelectron microscopy using the antibodies containing the asymmetric forms exclusively shows intense immunoreactions of AChE on the plasma membrane in contact areas of the adjacent muscle cells. As the immunocytochemical AChE activities in the Golgi cisterns and Golgi-derived vesicles of the

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**Fig. 7.** Cytochemical AChE activities in the nuclear membrane of a Schwann cell (SW) (A) and the intercellular space between nerve (NV) and muscle cells (B) at prenatal day 18. A: ×16,000, B: ×84,000
Fig. 8. Light microscopic AChE immunoreactions of the epidermis (EP) at prenatal day 15. Asymmetric and globular forms. ×500

Fig. 9. Light microscopic AChE immunoreactions of the subepidermal mesenchymal cells (MC) at prenatal day 17. The immunoreactions of the epidermis (EP) have almost disappeared. Asymmetric and globular forms. ×500

Fig. 10. AChE immunoreactions of the plasma membrane (arrows) and vesicles (arrowheads) in a muscle cell at prenatal day 18. Asymmetric and globular forms. ×20,000

Fig. 11. AChE immunoreactions of the intercellular space (arrows) between the adjacent muscle cells of the myotube at prenatal day 18. Asymmetric and globular forms. ×18,000
myotubes become more pronounced, those of the cell membrane come to be more readily detected after prenatal day 17. Thus, it is conceivable that AChE on the plasma membrane manifested by the asymmetric forms is delivered from the Golgi complexes and plays an essential role in the formation of myotubes by myogenic mesenchymal cells.

Nerve profiles are in contact with the muscle cells and exhibit intense AChE activities in the intercellular space after prenatal day 17. Since the immunoreactions at the neuromuscular junctions can not be obtained when we use the antibodies containing globular forms only, it seems likely that the formation of the primitive neuromuscular junction is mainly induced by the asymmetric forms derived from Golgi complexes of the myotubes, although we do not rule out the possibility that AChE in such regions is delivered from Schwann cells (DoNoso and Fernandez, 1985).

Our data indicate that the primitive neuromuscular junction is established after prenatal day 17. This is fundamentally consistent with the ultrastructural data of the rat intercostal muscles by Teräväinen (1968), but argues against the histochemical data by Kupfer and Koelle (1951) that the neuromuscular junction does not establish until prenatal day 21 in the rat forelimb. Furthermore, Dennis et al. (1981) recorded the endplate potentials at prenatal day 13. Such discrepancies are under investigation.

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REFERENCES


