Ultrastructural study of developing rabbit diaphragm

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INTRODUCTION

Numerous studies have been conducted on the development, ultrastructure, histochemistry and cytochemistry of skeletal muscle (Allen & Pepe, 1965; Davies & Gunn, 1972; Rowe, 1973; Gauthier, Lowey & Hobbs, 1978). Skeletal muscle fibres are classified into three basic types, namely, white, intermediate and red fibres according to their morphological and functional characteristics (Rowe, 1973; Gauthier et al. 1978).

The diaphragm is a skeletal muscle which develops early in fetal life (Hewer, 1934; Gamble, Fenton & Allsopp, 1978). The functional differentiation of the diaphragm in the neonatal animal is important because it is required for respiratory function and hence survival; any abnormality in the diaphragm can result in death (Muller et al. 1979). Histochemical and cytochemical characteristics of the diaphragm of various animals have been reported (George & Susheela, 1961; Davies & Gunn, 1972; Gauthier & Lowey, 1977) as well as the lipid, protein and glycogen concentrations of the diaphragm during the last third of gestation in rabbits (Metzger & Brachet, 1978). However, there has been no detailed ultrastructural study of developing diaphragm.

In the light of the enormous amount of information currently available on myogenesis (Holtzer et al. 1974; Ontell, 1977; Gamble et al. 1978) as well as on types of muscle fibre (Gauthier & Padykula, 1966; Davies & Gunn, 1972; Rowe, 1973), the ultrastructural investigation of the morphological differentiation of developing rabbit diaphragm was related to the changes reported in its lipid, protein and glycogen concentrations (Metzger & Brachet, 1978).

MATERIALS AND METHODS

Ten gravid New Zealand White rabbits (Oryctolagus cuniculus) were obtained from a commercial breeder. The gestational ages were calculated by counting the day of breeding as day zero. Fetuses from four gestational ages (20, 22, 25 and 30 days) were studied during the last third of gestation, as well as neonates at one week of age. The diaphragms of fetuses of 20 days gestation were collected by the use of a dissecting microscope. Litters from two gravid does in each age group were delivered by Caesarean section after intravenous injection of sodium pentobarbital (25–35 mg/kg of body weight). Ten fetuses obtained from two does constituted each age group. They were matched for developmental stages as described by Edwards (1968). The diaphragms from five one week old rabbits were collected for the study of postnatal development. Adipose tissues from the same animals were used for other investigations (Stopps, Yamashiro & Harris, 1981).
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All fetuses were decapitated immediately after removal from the uterus and small pieces of diaphragm were fixed by immersion in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 6 hours. The samples were then washed in the buffer and post-fixed in 2.0% osmium tetroxide for 1 hour. The tissue pieces were processed for embedding in Epon 812 (Shell Chemical Co.). The procedure for transmission electron microscopy has been reported elsewhere (Yamashiro & Clandinin, 1980). One micrometre sections of the tissue were stained with toluidine blue and selected areas were sectioned for electron microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate, examined and photographed in a Jeol JEM 100S electron microscope. Serial sections were made from some of the blocks when the position of certain cells required clarifying.

RESULTS

Fetuses at 20 days of gestation

The diaphragms of these fetuses showed early signs of developing myotubes. The myotubes were of variable sizes and contained numerous lipid droplets and moderate amounts of glycogen particles. They also contained a few mitochondria and some sarcoplasmic reticulum but no transverse tubules or triad formations were observed. Their nuclei were central and were arranged in a row (Fig. 1). Myofibrils were sparse and showed a loose organisation with dark bodies (Z line precursors). Many myoblasts in the vicinity of the myotubes appeared to be in the process of fusion (Fig. 2). Some of them were in mitosis: myoblasts already partially fused with a myotube were still undergoing mitosis (Fig. 3). Myoblasts were readily identified not only by their location but also by their characteristic features. They contained an abundance of free polyriboosomes and some glycogen particles in the cytoplasm but, in contrast to fibroblasts, granular endoplasmic reticulum was not prominent. Each myoblast contained a nucleus with fine chromatin (Fig. 2). In addition, a few strands of collagen fibrils were seen near the myotubes.

No motor end plate was observed in these developing myotubes. The mesothelial cells lining the serous membrane (either pleura or peritoneum) exhibited distended

Fig. 1. Electron micrograph of the diaphragm from a fetal rabbit at 20 days gestation. Portions of myotubes show centrally located nuclei, a few strands of myofibrils, lipid droplets of various sizes and glycogen particles. Myoblasts (M.B.) are also seen near the myotubes. × 6300.

Fig. 2. A myoblast is shown in the process of fusing with a myotube. The myoblast has a nucleus containing fine chromatin and a distinct nucleolus. The outer membrane of the nuclear envelope is continuous with the granular endoplasmic reticulum. A portion of the myotube shows developing myofibrils, mitochondria, glycogen particles and vacuoles. 20 days gestation. × 8800.

Fig. 3. A partially fused myoblast in the centre of the micrograph is in mitosis. Portions of three myotubes are shown in the area; these are surrounded by myoblasts. 20 days gestation. × 5500.

Fig. 4. Oblique section of a myotube, exhibiting the mitotic activity of a newly fused cell. A centriole and mitotic spindles are seen in the centre and there is a distinct basal lamina (arrows). Developing myofibrils are noted on one side of the cell. 22 days gestation. × 11100.

Fig. 5. Myotubes at 22 days gestation, showing nuclei with fine chromatin (arrows) and a moderate number of myofibrils. Some of the myoblast-like cells contain a condensed nucleus (open arrowheads). These uninucleate cells with coarse chromatin were identified as satellite cells. × 3500.

Fig. 6. Portions of two or more myotubes are seen. In one, a nucleus is located at the periphery while another is centrally placed. Mitochondria are distributed throughout the myotube and numerous glycogen particles are seen in both myotubes and myoblasts. 22 days gestation. × 5250.
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cisternae of granular endoplasmic reticulum and the nuclei contained fine chromatin with prominent nucleoli. A tendon was not observed at the margins of the developing muscle.

_Fetuses at 22 days of gestation_

Occasionally, recently fused myogenic cells that were undergoing mitosis lay within the basal laminae of the myotubes (Fig. 4). Other dividing myoblasts were partially used with a myotube. The myoblasts showed an abundance of free polyribosomes and two types of nuclei were observed in them. Myoblasts with nuclei containing fine chromatin appeared to be newly recruited cells destined to fuse with the myotubes. These nuclei resembled those of the myotubes. The other type of nuclei exhibited clumped chromatin and the cells resembled satellite cells (Fig. 5). The myotubes contained a few lipid droplets and large amounts of glycogen particles. Some of them exhibited peripherally located nuclei with prominent nucleoli, a relatively small number of mitochondria and a relatively large number of myofibrils (Fig. 6). An undulation of the sarcolemma was observed in the myotubes wherever unmyelinated nerve fibres were in close proximity. The sarcolemma in this region was electron-dense (Fig. 7) and was covered by a thin basal lamina. The region of the sarcolemmal undulations, which were not as deep as the secondary synaptic clefts of fully developed motor end plates, was identified as a developing motor end plate. Many unmyelinated axons and Schwann cells were observed between the myotubes and numerous small vesicles were found in the axon terminals. Some Schwann cells exhibited centrioles. Also in the vicinity of the myotubes were fibroblasts with well developed granular endoplasmic reticulum and small bundles of collagen fibrils. Tendon had not yet differentiated in these diaphragms.

_Fetuses at 25 days of gestation_

Mitoses were frequently observed in the myoblasts. Myoblasts and satellite cells were observed at the periphery of the myotube. Myotubes contained large amounts of glycogen and a few lipid droplets, and basal laminae were clearly recognisable.

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Fig. 7. The Schwann cells surrounding small axonal processes which are forming a neuromuscular junction with a developing muscle fibre. Axon terminals (A.T.) contain many small vesicles and a few mitochondria. The sarcolemma of the myotube shows undulations and is electron-opaque (arrows) at the junction. × 12840.

Fig. 8. Muscle fibres seen in cross section are filled with myofibrils. A myoneural junction is well differentiated. The sarcolemma at the junction shows deep indentations, forming secondary clefts (arrows). 25 days gestation. × 6300.

Fig. 9. A fibroblast (F.B.) and a cytoplasmic process of another fibroblast (arrows) in the interstitium exhibit well developed granular endoplasmic reticulum. Portions of muscle fibres show large numbers of myofibrils and glycogen particles in the subsarcolemmal region. 25 days gestation. × 8750.

Fig. 10. At a myotendinous junction, the terminal portion of the muscle fibre exhibits protrusions and indentations. Myofilaments of the fibrils are anchored at dense bodies of the sarcolemma. Collagen fibrils are closely associated with indented portions of the muscle fibre. 30 days gestation. × 11 700.

Fig. 11. A portion of a fully differentiated muscle fibre exhibits chains of mitochondria between the myofibrils. Lipid droplets are observed in contact with the mitochondria. The myofibrils show ultrastructural features of the intermediate fibre. One week post partum. × 17600.

Fig. 12. Differentiated muscle fibre from a one week old rabbit showing the characteristic features of the red fibre. × 17 500.
around them. Well organised myofibrils were abundant in the myotubes and mitochondria were also frequently seen. In addition, further differentiation of the neuromuscular junction was observed (Fig. 8).

The interstitium of the diaphragm contained typical fibroblasts in an active secretory stage and a few bundles of collagen fibrils (Fig. 9). A few small fascicles containing unmyelinated and large fascicles with myelinated nerves were also observed. Tendon formation was noted at the periphery of the diaphragm.

**Fetuses at 30 days of gestation**

There was a moderate number of myoblasts around well differentiated myotubes. The myotubes were now so well developed that their appearance was almost that of mature muscle fibres. They contained glycogen particles between the myofibrils and in the sarcoplasm beneath the sarcolemma. At similar locations, mitochondria were observed in rows or in clusters. There were also well developed sarcotubules, motor end plates and transverse tubules. These developing muscle fibres showed protrusions and invaginations at myotendinous junctions (Fig. 10). Near the tips of the fibres, dark bodies were noted on the inside of the sarcolemma where the myofilaments were anchored. Fibroblasts with distended cisternae or granular endoplasmic reticulum and some lipid droplets were seen at the junction. In addition, collagen fibrils were seen in bundles. The mesothelium adjacent to the diaphragm was squamous in type and its nuclei were condensed. There were many large nerve fascicles containing myelinated fibres of various sizes.

**One week post partum**

The diaphragm showed complete differentiation with mature skeletal muscle fibres (Figs. 11, 12). Uninucleated myoblasts were not observed. The muscle fibres possessed abundant mitochondria between the myofibrils and within the subsarcolemmal zone. Lipid droplets were also present near the mitochondria. The ultrastructure of the diaphragm was similar to that reported by Gauthier & Padykula (1966) in adults.

**DISCUSSION**

The diaphragm of a fetal rabbit develops very rapidly in the last third of gestation (Metzger & Brachet, 1978), increasing from a weight of 8·8 mg at 21 days to 116 mg at 30 days gestation. At 20 days of gestation, the diaphragm was so small that samples could be collected only with the aid of a dissecting microscope. No reports have been published on the morphology of the rabbit diaphragm as early as 20 days of gestation. Despite its size, myogenic activity is already in progress. Myoblasts are numerous and many of them are in mitosis (Fig. 3); this may be the reason for the differences in gross appearance of the organ between 20 and 22 days of gestation (Nag & Foster, 1981).

Myogenesis as observed in this investigation is similar in nature and sequence to that reported by others (Kelly & Zacks, 1969a; Holtzer et al. 1974; Gamble et al. 1978) in other species. However, development of certain structures occurs at different stages of gestation compared with skeletal muscles in other anatomical locations and in other species. From 22 days to 30 days gestation, two types of uninucleated cells are noted in the vicinity of the myotubes. Cells with nuclei containing fine chromatin are identified as myoblasts and those with condensed nuclei as satellite cells (Gamble et al. 1978; Lipton & Schultz, 1979). The paucity of cells with the morphological
characteristics of satellite cells at 20 days of gestation may suggest that either the precursors of the myotubes (namely, myoblasts) are formed prior to the formation of satellite cells, or the satellite cells do not exhibit their characteristic form at this stage of development. The satellite cells, which are enclosed in a basal lamina, have been reported in fetal muscles (Ontell, 1977; Gamble et al. 1978) as well as in regenerating muscles (Lipton & Schultz, 1979; Summers & Parsons, 1981). Satellite cells have been shown to differentiate into myoblasts during the regeneration of skeletal muscle both in vivo (Lipton & Schultz, 1979) and under in vitro (Konigsberg, Lipton & Konigsberg, 1975; Nag & Foster, 1981) conditions. Their role in muscular dystrophy is considered to be significant (Wakayama, 1974; Summers & Parsons, 1981). The mechanism of myotube formation by fusion of myoblasts has been studied in detail (Delain & Wahrman, 1975; Fulton, Prives, Farmer & Penman, 1981; Fumagali, Brigonzi, Tachikawa & Clementi, 1981).

Mitosis is most frequently seen at 20 days of gestation but is less frequent in the diaphragms of older fetuses, although the rate of increase in weight is reported to be higher during the later stages of gestation (Metzger & Brachet, 1978). It is of interest that myoblasts continue to divide after fusing with the myotubes (Fig. 4); this has not been reported in earlier in vivo studies. According to Metzger & Brachet (1978), glycogen concentration increases during development of the diaphragm and is at a peak at 27 days of gestation. In contrast, lipid concentration decreases from 21 days of gestation. Although no quantitative analysis has been done in the present study, similar trends are observed histologically.

Neuromuscular junctions developed early in the differentiating myotubes (see Fig. 7). This may be a characteristic feature of the diaphragm (Hewer, 1934) or specific to the rabbit. Gamble et al. (1978) discuss the species differences in the development of the motor end plate with respect to rat (Kelly & Zacks, 1969b) and man (Gamble et al. 1978). Rabbit fetuses may be similar to those of the rat in terms of the development of the diaphragm during gestation because both show an almost explosive succession of events in the development of this muscle (Kelly & Zacks, 1969b). The secondary synaptic clefts characterised by an undulating sarcolemma are shallow, but the axon terminals are filled with synaptic vesicles. These characteristics suggest the functional differentiation of the motor end plate (see Fig. 7). The morphological features of the developing neuromuscular junctions are similar to those of rat intercostal muscle reported by Kelly & Zacks (1969b). The basal lamina of the myotubes also becomes identifiable at 22 days of gestation and its first appearance coincides with a possible functional differentiation of the motor end plates.

At 25 days gestation, there are marked increases in the number of myofibrils in the developing muscle fibres (Fig. 8), and a large number of fibroblasts are noted in the interstitium (Fig. 9). The presence of many fibroblasts containing well developed granular endoplasmic reticulum, with distended cisternae appears to indicate the formation of endo- and perimysium. The distribution and arrangement of mitochondria in the muscle of the diaphragm have been reported to be of special functional significance (Bakeeva, Chentsov & Skulachev, 1978). The distribution and appearance of mitochondria in the rabbit diaphragm are similar to those in cardiac muscle of the rat (Yamashiro & Clandinin, 1980). The differentiation of muscle fibres at 30 days gestation, as exhibited by the growth in length at myotendinous junctions (Fig. 10), suggests that the diaphragm of the fetal rabbit is structurally almost complete by 30 days of gestation.

At one week post partum, the presence of three types of mature muscle fibres, as
identified by the appearance of the Z line (Rowe, 1973) and a fully developed interstitial tissue, suggests the complete development of the organ. The ultrastructure of the diaphragm of one week old animals is similar to that of the mature rabbit diaphragm, as reported previously (Gauthier & Padykula, 1966).

These observations suggest that morphological development of the rabbit diaphragm is very rapid in comparison with the myogenesis of skeletal muscles of different anatomical locations and species (Kelly & Zacks, 1969a; Gamble et al. 1978). Further quantitative study is in progress.

SUMMARY

Diaphragms obtained from forty rabbit fetuses during the last third of gestation and from five rabbits one week old were studied ultrastructurally. Four gestational ages were chosen for the investigation. The diaphragm at 20 days gestation revealed early myogenesis which was characterised by many myogenic cells in the process of mitosis and by fusion of myoblasts to form myotubes. A large number of lipid droplets and a moderate quantity of glycogen were observed in the myotubes. These myotubes contained a few myofibrils peripherally. At 22 days of gestation, the myotubes exhibited a relatively large number of myofibrils and large amounts of glycogen. They also showed sarcolemmal modifications which appeared to be developing motor end plates. Mitosis of the myogenic cells persisted even after fusion. At 25 days of gestation, connective tissue sheaths appeared around the muscle fibres as well as further differentiation of the muscle fibres. At 30 days of gestation, the muscle fibres exhibited nearly complete differentiation, with the formation of myotendinous junctions. At one week post partum, the diaphragm showed full development of its muscle fibres. These morphological observations suggest a very rapid functional differentiation of the rabbit diaphragm during the last third of gestation.

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