Mitochondrial ultrastructure in embryos after implantation

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Information on the morphology of mitochondria during embryogenesis is scattered in the literature, but there appears to be a consistent pattern. During early organogenesis, the embryo is in a state of relative hypoxia associated with a major decrease in terminal electron transport system activity and a marked increase in anaerobic glycolysis. Ultrastructural studies of a 14-somite monkey embryo and day 10 and 12 rat embryos, together with a review of the literature, led us to determine that this hypoxic stage is characterized by vesiculation of the mitochondrial inner membranes, or cristae. Starting in the late morula stage and continuing during early postimplantation embryogenesis, the cristae increase but appear tubular or vesicular. After the end of neurulation, and with the onset of vascular perfusion of embryonic tissues, the cristae gradually become lamellated; by the limb bud stage they appear more mature. We suggest that new cristae derive from blebs of the inner mitochondrial membrane and that with maturation these blebs collapse, giving them a lamelliform appearance. The delamellated state of the cristae might inactivate oxidative phosphorylation to protect the embryo from toxic respiratory end-products that could accumulate in an embryo before there is vascular perfusion. Consistent with this hypothesis, mitochondrial diameters in the developing heart of monkey and rat embryos were approximately twice those found in skin and neural tube.

Key words: embryo genesis/mitochondria/mitochondrial cristae/oxidative phosphorylation/ultrastructure

Introduction

Changes in the shape of mitochondria during embryogenesis could help our understanding of their growth and development in early post-implantational embryonic development. Little systematic attention has been directed to this subject (Luft, 1971; Fawcett, 1981). To our knowledge, the morphogenesis of the mitochondrial inner membranes (or cristae, the site of the respiratory chain enzymes) during this period of embryogenesis in mammals had not been specifically addressed prior to our published studies (Shepard et al., 1998).

Our studies of a 14-somite monkey embryo and rat embryos from days 10 and 12 and a review of the literature led us to propose a mechanism by which the mitochondrial cristae form. An extensive search of the literature on
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the ultrastructure of embryonic mitochondria preceded our general conclusions relating to morphogenetic changes. In several papers, the exact state of the cristae was not mentioned and our interpretations were drawn from examination of the published figures. Additionally, several authors helped by replying to our letters requesting further descriptions.

In the early stages of the preimplantation embryo, the cristae are sparse but lamellated. Starting in the late morula stage and continuing during gastrulation and the early somite stage, the cristae begin increasing in number and length; during this period; however, they appear tubular or vesicular, with separation of the inner mitochondrial membranes. After the end of neurulation they become increasingly closely lamellated, eventually appearing mature by the limb bud stage. The delamellated state of the cristae is coincident with anaerobic glycolysis, during which terminal electron transport phosphorylation activity is very low (Mackler et al., 1970; 1973). We suggest that the new cristae first appear as blebs from the inner mitochondrial membrane and subsequently, with maturation, these blebs collapse, giving them the lamelliform appearance (Shepard et al., 1998).

Electron microscopic analysis of embryonic mitochondria

Details of the breeding, housing, and timing of the rat embryos has been previously published (Shepard et al., 1998). The monkey embryo (Macaca nemestrina) was obtained from the Regional Primate Research Center, University of Washington. The timed pregnancy was observed by ultrasound and when the diameter of the chorionic sac measured 14 mm, a hysterotomy was performed to obtain the specimen.

Embryos from both species were removed from the amniotic cavity and were rinsed briefly in Hanks’ solution and then fixed in cold fixative of 2% glutaraldehyde in 0.1 mol/l phosphate buffer pH 7.4. The time interval from removal to fixation was 35 min for the monkey and 5–10 min for the rats. After 12–24 h fixation, the embryos were dehydrated in graded alcohol and critical point dried in CO₂ (PVT-3B SAM DRI, Tousimis Company, Rockville, MD, USA). The embryos were mounted on stubs with silver paint (Ernest Fullam, Latham, NY, USA). They were then sputter-coated for two 4-min periods with gold palladium (E5100; Polaron Instruments, Cambridge, MA, USA). Scanning electron microscopy was carried at 20 kV, using an ETEC (Autoscan SEMTEC, Stockton, CA, USA) or, for higher magnification (×2000–90 000), a field emission scanning electron microscope (JSM 6400F JEOL, Tokyo, Japan) at 1–15 kV.

Specimens for transmission electron microscopy were fixed in glutaraldehyde and post-fixed in 1% osmium tetroxide, followed by 1% aqueous uranyl acetate. After scanning electron microscopy the embryos were freed from the stubs and immersed in propylene oxide. Decreasing concentrations of propylene oxide with increasing Polybed 812 were employed over 4 days, ending with pure Polybed (Polyscience, Warrington, PA). Sections (1 μm) were stained with Richardson’s blue (Richardson et al., 1960) and the adjacent ultrathin sections were stained sequentially with saturated uranyl acetate and Reynolds’s lead citrate (Reynolds, 1962). A Phillips 420 transmission electron microscope or JEM 1200 EX II was used. The entire monkey embryo was sectioned with orienting thick sections taken about every 13 μm.

For measurement of the widest diameter across the mitochondria and for counting the number of lamellated cristae from the heart, skin and neural tube of the monkey, sections adjacent to four of the 42 thick sections through the heart were chosen by a random number method. After the heart, skin or neural
Figure 1. Day 24 monkey embryo. Scanning electron micrograph. Crown–rump length = 2.5 mm, 14 somites. Y = yolk sac; A = amnion; cranial arrow to otic placode; caudal arrow to open caudal neural tube. Bar = 200 µm. (Reproduced from Shepard et al., 1998, with permission).

Mitochondria ultrastructure in a monkey embryo

The 24-day monkey embryo was 2.5 mm in crown–rump length, with 14 somites, and corresponded to early Streeter’s stage 11 (O’Rahilly and Müller, 1987). The cephalic neuropore was partially closed and the otic placodes, primitive circulation, and pronephros were present (Figure 1). Numerous blood islands were present in the yolk sac, which measured ~3.2 mm, but only rare red blood cells were seen in sections of the embryonic blood vessels. The heart was beating slowly. The embryo was slightly retroflexed and the caudal neural tube was open.

There were many mitotic figures in all tissues examined. Cells in the heart contained empty areas interpreted as glycogen; myofibrillar bundles with z-bands were present. The neural tube lumen contained numerous mitotic figures and was lined by microvilli. There were many cell processes abutting its cavity. The skin consisted of one to two layers of cells but no continuous basement membrane. There were many microvilli on its surface.

The mitochondria of all monkey embryo tissues contained many ovoid vesicles, which varied from 0.03–0.2 µm in diameter. They were located mostly in the periphery of the mitochondria and many could be seen to be connected to the inner membrane (Figures 2, 3). A few lamellated cristae were present, especially in the myocardium (see Shepard et al., 1998 for details). The mitochondria of the skin were dense, with a mean greatest diameter of 0.19 µm (Figure 4). There was marked variation in the widths of skin mitochondria, depending on the location examined. The neural tube mitochondria were less dense, with an average diameter of 0.23 µm (Figure 5). The heart mitochondria, often in association with the empty spaces (glycogen), were the least dense and were about twice the diameter of the mitochondria in other tissues, with an average diameter of 0.37 µm (significantly greater than the diameters of mitochondria of the skin and neural tube, see Shepard et al., 1998 for details). A larger proportion of mitochondrial cristae in the heart were lamellated compared with skin and neural tissue.

Mitochondrial ultrastructure in rat embryos

The rat embryos removed at 10:00–11:00 on day 10 and 12 had crown–rump lengths of 2.0

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Figure 2. Day 24 monkey embryo. (A) Thick cross-section at the level of the myocardium (M) and thyroid diverticulum (T). Arrow shows neural tube; O = otic placode. Bar = 100 μm. (B) Heart mitochondria showing vesicular inner membranes, or cristae. Bar = 0.2 μm. (Reproduced from Shepard et al., 1998, with permission).

and 5.5 mm, and average somite counts of 11 and 35 respectively (see Figures 6 and 7). The mitochondria of day 10 heart, neural tube, and skin were very similar in morphology to the corresponding mitochondria of the 14-somite monkey embryo. At this stage, the average diameter of the heart mitochondria was 0.49 μm (Figure 6), significantly larger than that of the neural tube mitochondria. On day 12, the mitochondria of the rat embryo tissue contained numerous cristae, the majority of which were lamellated (Figure 7). Only a few vesicular cristae were found. On day 12 the average diameters of the mitochondria of skin, neural tube and heart were 0.31, 0.27 and 0.39 μm respectively.

Figure 3. Day 24 monkey heart mitochondrion to show 'blebbing' of inner membrane (arrows). Bar = 0.1 μm. (Reproduced from Shepard et al., 1998, with permission).

Mitochondrial development during embryogenesis

Before the late morula stage, mitochondrial cristae are sparse but lamelliform. This has
been reported for the sea urchin (Berg and Long, 1964), wasp (Amy, 1975), pig (Hytell and Niemann, 1990), sheep (Calarco and McLaren, 1976; Ferrer et al., 1995); monkey (Enders and Schlafke, 1981) and human (Sundstrom et al., 1981; Trounson and Sathananthan, 1984). From the late morula period until neural tube closure (during implantation, gastrulation, and neurulation: a time of relative tissue hypoxia) embryonic mitochondrial cristae are generally characterized by delamellation, which gives the cristae a vesicular or tubular appearance. These findings are almost universal among 26 previous publications dealing with a wide number of embryonic species, from the sea urchin to humans (see Table I) and have been recorded in the chick (Bancroft and Bellairs, 1975; de Paz et al.,
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Figure 5. Day 24 monkey neural tube. (A) Low power. L = lumen; M = mitotic figure. Bar = 2 μm. (B) High power, showing mitochondria (arrows). Bar = 0.5 μm. (Reproduced from Shepard et al., 1998, with permission).

1986; Kowazaki and Hiruma, 1994), the rat (Mackler et al., 1970; Merker and Villegas, 1970; Takeuchi, 1980; Yang et al., 1995) and the rabbit (Andersen et al., 1970; Sordhal, 1972).

The work that specifically studied changes in mitochondrial morphology and correlated them with functional changes was that of Mackler et al. (1970, 1973), who reported a gradual increase in lamellar forms of heart mitochondrial cristae after day 10 in rat embryogenesis, becoming mature on day 14 and paralleling the maturation of the electron transport chain. Our morphological studies in the rat on day 10 and day 12 closely approximate to theirs and our studies in the monkey and rat extend the findings of cristal delamellation to tissues other than the heart. Published studies of embryos after neural tube closure show an increasing number of lamellar cristae, as in our day 12 rat study. These findings have been reported in the chick (Pollak and Shoreg, 1967; Holman, 1969), the rat (Lambson, 1966; Mackler et al., 1970; Jollie, 1984), the guinea pig (Dempsey, 1953), the monkey (Jerome and Hendrickx, 1988) and the human (Sako, 1975) (see Table I).

Based on these findings, we postulate that
the vesicular forms of mitochondrial cristae represent stages in their normal formation, with initial blebbing from the inner membrane followed by the persistence of dilated structures during hypoxic conditions, and then the eventual condensation of these structures to the more mature, lamellated form with establishment of the fetal circulation and relief from hypoxia. The changes in inner membranal configuration are probably associated with new mitochondria being formed during these stages. Our findings that heart mitochondria in both the monkey and day 10 rat are approximately twice the diameter of mitochondria in other tissues could be the result of vesicle formation and expansion of the outer and inner mitochondrial membrane as part of a more rapid growth and maturation in this organ in preparation for a switch to aerobic oxidation, as shown by histochemical studies of developing rat embryos (Shepard et al., 1968).

The oxidative electron transport system located in the mitochondrial inner membrane has been shown to be relatively inactive during the early stages after implantation of rat embryo, whereas it gradually matures after establishment of vascular circulation (Mackler et al., 1970, 1973). The inactivity of this important energy source coincides with the vesicular stage of the cristae. Although it is difficult to prove functional changes from morphological observation, the high rate of glucose metabolism during this early stage of embryogenesis is further evidence that the terminal electron transport system is inactive at this time and that anaerobic glycolysis is being relied on for ATP production (Shepard et al., 1970; Tanimura and Shepard, 1970; Neubert, 1973; Clough and Whittingham, 1983, Ellington, 1987; Shepard et al., 1997). Therefore the vesiculated or delamellated state of the cristae might prevent inadvertent production of reactive oxygen species.

Further support for the normality of this relatively hypoxic environment of the early postimplantional stages of embryogenesis comes from the observation that oxygen is toxic to day 10 rat embryos, a finding known for some time (Shepard et al., 1969; New and Coppola, 1970), and it is standard laboratory practice at this early stage to culture explanted rat and mouse embryos in $<10\%$ oxygen.

Figure 6. Day 10 rat embryo. (A) Scanning electron micrograph. Arrow to optic vesicle; $A =$ amnion. Bar = 200 $\mu$m. (B) Heart mitochondria show blebbing of inner membrane (arrows). Bar = 0.2 $\mu$m. (Reproduced from Shepard et al., 1998, with permission).
Figure 7. Day 12 rat embryo. (A) Scanning electron micrograph. L = limb; arrow to nasal placode. Bar = 2 mm. (B) Heart mitochondria showing mostly lamellated cristae (arrows). Bar = 0.5 μm. (Reproduced from Shepard et al., 1998, with permission).

Figure 8. Embryonic maturation of mitochondrial inner membranes, illustrating the proposed formation of mitochondrial cristae from blebbing of inner mitochondrial membrane in early embryogenesis, commencing from the late morula through implantation, gastrulation (pre-somite), and the end of neural tube formation. With maturation, these blebs collapse to form lamellated mature cristae. (Reproduced from Shepard et al., 1998, with permission).

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(New, 1973). Furthermore, exposure to carbon monoxide, which inhibits the terminal electron transport system, is known to have no adverse effects on the day 10 rat embryo in vitro (Robkin, 1997). On the other hand, following neurulation, embryonic tissues require 95% oxygen to continue to survive in vitro, and carbon monoxide is toxic to oxidative phosphorylation.

The delamellated state of the cristae could be a mechanism by which the embryo partially inactivates its mitochondrial electron transport system in order to protect non-perfused tissues from the toxic end-products of inefficient respiration, i.e. hydrogen peroxide, superoxide, and hydroxyl radicals (the reactive oxygen species). A lack of vascular perfusion might otherwise cause these toxic respiratory end-products to accumulate in relatively hypoxic conditions. (An alternative possibility is that delamellation of the cristae might constitute a pathological reaction to hypoxia, although this seems unlikely, because there is an overall growth in the number of mitochondria and the amount of mitochondrial inner membrane during early embryogenesis. Yet this and other aspects should be amenable to experimental study in tissue culture systems.)

Our hypothesis that the vesicular forms of the cristae are a normal stage in their morphogenic development implies that despite their relative hypoxic state, the embryonic tissues are nevertheless steadfastly developing the apparatus needed to switch to the more efficient ATP production seen with oxidative phosphorylation (and associated with mature, lamellated cristae) once the hypoxia is relieved by the establishment of the embryonic cardiovascular system. See Figure 8 for a summary of our hypothesis.

There are other descriptions of delamellated mitochondrial cristae in the literature. In the rhizopod Dictyostelium discoideum (a primitive amoeba-like organism) and the plasmodial slime mould Physarum polycephalum, the
Table I. Studies of embryonic mitochondria from different species

<table>
<thead>
<tr>
<th>Species</th>
<th>Embryonic stage and mitochondrial morphology</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Sea urchin (<em>L.anamesus</em>)</td>
<td>Mitochondria enlarge and cristae increase in number at gastrulation</td>
<td>Berg and Long, 1964</td>
</tr>
<tr>
<td>Wasp (<em>Habrobracon juglandis</em>)</td>
<td>Poorly developed cristae during first 29 h</td>
<td>Amy, 1975</td>
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<tr>
<td>Chick</td>
<td>Presomite. Cristae occasionally dilated. Volume of mitochondria measured</td>
<td>de Paz et al., 1986</td>
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<tr>
<td>Chick</td>
<td>Neural plate. Some dilated cristae</td>
<td>Bancroft and Bellairs, 1975</td>
</tr>
<tr>
<td>Chick</td>
<td>Day 2–7, heart. Dense and vacuolated mitochondria, occasional dilated cristae</td>
<td>Kowazaki and Hiruma, 1994</td>
</tr>
<tr>
<td>Chick</td>
<td>Day 11–14, intestine. Mature</td>
<td>Holman, 1969</td>
</tr>
<tr>
<td>Chick</td>
<td>Day 5–6, genital ridge. Mature</td>
<td>Pollak and Shoreg, 1967</td>
</tr>
<tr>
<td>Rat</td>
<td>Day 10, vacuolated cristae</td>
<td>Mackler et al., 1970</td>
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<tr>
<td>Rat</td>
<td>Day 14, mature</td>
<td></td>
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<tr>
<td>Rat</td>
<td>Day 9.5, yolk sac. Immature</td>
<td>Takeuchi, 1980</td>
</tr>
<tr>
<td>Rat</td>
<td>Day 9, few swollen cristae</td>
<td>Yang et al., 1995</td>
</tr>
<tr>
<td>Rat</td>
<td>Day 7–10, visceral yolk sac. Dilated cristae</td>
<td>Merker and Villegas, 1970</td>
</tr>
<tr>
<td>Rat</td>
<td>Day 10, yolk sac. Sparse, short cristae</td>
<td>Lambson, 1966</td>
</tr>
<tr>
<td>Rat</td>
<td>Day 11.5, yolk sac. Mature mitochondria</td>
<td>Jollie, 1984</td>
</tr>
<tr>
<td>Hamster</td>
<td>Day 7.5, oral membrane. Dilated cristae</td>
<td>Waterman, 1977</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>Crown–rump length ≥50 mm, visceral yolk sac. Lamellar cristae</td>
<td>Dempsey, 1953</td>
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<tr>
<td>Rabbit</td>
<td>Blastocyst and inner cell mass. Cristae delaminated</td>
<td>Andersen et al., 1970</td>
</tr>
<tr>
<td>Pig</td>
<td>Day 19, heart. Smaller mitochondria and dilated cristae</td>
<td>Sordhal, 1972</td>
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<tr>
<td>Sheep</td>
<td>Morula. Tubular cristae</td>
<td>Hyttel and Niemann, 1990</td>
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<tr>
<td>Sheep</td>
<td>Morula to blastocyst. Decreased number of cristae</td>
<td>Ferrer et al., 1995</td>
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<tr>
<td>Prosimian (<em>Galago crassicandus</em>)</td>
<td>Day 27, germ cells. Sparse dilated cristae</td>
<td>Calarco and McLaren, 1976</td>
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<td>Monkey (<em>M.mulatta</em>)</td>
<td>Day 35, notochord. Lamellar cristae with some focal swelling</td>
<td>Jerome and Hendrickx, 1988</td>
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<tr>
<td>Monkey (<em>M.rhesus</em>)</td>
<td>Preimplantation. Reduced numbers of lamellar cristae</td>
<td>Enders and Schlafke, 1981</td>
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<tr>
<td>Human</td>
<td>Day 20 to 31, genital ridge. Vesicular cristae</td>
<td>Trounson and Sathananthan, 1984</td>
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<tr>
<td>Human</td>
<td>2–4 cell. Sparse ‘arch like’ cristae</td>
<td>Sundstrom et al., 1981</td>
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<td>Human</td>
<td>Day 54, heart. Lamellar cristae</td>
<td>Fukuda, 1976</td>
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<tr>
<td>Human</td>
<td>2–4 cell. Sparse ‘arch like’ cristae</td>
<td>Sako, 1975</td>
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Mitochondrial cristae are tubular throughout development (Luft, 1971; Loomis, 1975). Delaminated tubular cristae are also seen in mammalian steroid-producing cells of the adrenal cortex and testes (Idelman, 1970; Fawcett, 1981; Kerr, 1991, Lea et al., 1994). The cross-sections of the cristae in these examples differ from those of the embryos in that they are much smaller and are less variable in size. The delamination of cristae and inactivation of the terminal electron transport system during steroid production, however, may protect steroid hydroxylation by limiting the production of reactive oxygen species.

Based on our studies in rat and monkey embryos and our review of the literature, we propose that, after the onset of mitochondrial proliferation following implantation, but during relatively hypoxic conditions, the mitochondria produce new cristae by blebbing from the inner membrane; these form vesicles that, after neurulation and with a return to relatively oxygen-rich conditions as the cardiovascular system develops, gradually collapse into the mature lamellated forms that conduct efficient oxidative phosphorylation. These changes are first seen in the developing myocardium.
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