Ultrastructural observations on the chorionic epithelium, parathyroid glands and bones from chick embryos developed in shell-less culture

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SUMMARY

Three-day-old chick embryos were cultured in Petri dishes until they reached developmental stages 37 or 39 (Hamburger & Hamilton, 1951). The electron microscopical study of the chorio-allantoic membranes showed that, even in the absence of the shell and the corresponding calcium supply, 'capillary-covering' and 'villus-cavity' cells differentiated well. The parathyroid glands from the cultured embryos showed ultrastructural signs indicative of active synthesis and secretion of the parathyroid hormone. This correlates well with the significant hypocalcemia (5.4 mg/100 ml) observed in these embryos.

In all cultured embryos mineralization of bones was greatly reduced as shown by alizarin bulk staining and confirmed by histological and electron microscopical analysis. The ultrastructural characteristics of osteoblasts and osteocytes, as well as of the bone matrix, appeared normal. The defect in mineralization appeared thus to be due to a deficiency in the availability of calcium and not to a delay in bone differentiation. This implies that the yolk sac appears to lack calcium regulatory capacity since it cannot compensate for the absence of shell by increasing its own contribution in order to assure an adequate mineralization of the embryonic bones.

INTRODUCTION

Although the concentration of calcium in the plasma of chick embryos increases during development (Taylor, 1963; Taylor, Shires & Baimbridge, 1975), it appears to be constant at any given age (Narbaitz, 1975; Taylor et al. 1975). Embryos thus appear to be capable of maintaining calcium homeostasis and it is possible that the regulatory factors involved are similar to those in the adult.

Thus, the parathyroid hormone (Narbaitz, 1972a; Narbaitz & Gartke, 1975; Narbaitz, 1975), calcitonin (Stoeckel & Porte, 1969; Taylor & Lewis, 1972) and vitamin D metabolites (Moriuchi & DeLuca, 1974; Narbaitz & Carrillo, 1976) appear to play a role in the regulation of calcium metabolism in the chick embryo.

Even if the factors involved in calcium regulation in the embryo are the same as those acting in the adult, the target organs on which these agents act are

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undoubtedly different. The embryo obtains 80% of the needed calcium from the egg-shell (Simkiss, 1961). The resorption of the shell mineral takes place through the chorionic epithelium and is especially active after the twelfth day of incubation (Johnston & Comar, 1955). Precisely at this time, two characteristic cell types appear in the epithelium. Both of them, the 'capillary-covering' cell (Coleman & Terepka, 1972) and the 'villus-cavity' cell (Skalinsky & Kondalenko, 1963; Coleman & Terepka, 1972), appear to be involved in the resorption of calcium from the shell (Owczarzak, 1971; Narbaitz, 1972b; Coleman & Terepka, 1972).

Experiments using ⁴⁵Ca (Johnston & Comar, 1955) appear to indicate that before the twelfth day of incubation, the contribution of the shell is small, and that the embryo obtains most of its calcium from the yolk. Nozaki, Horii & Takei (1954) have shown that part of the calcium resorbed from the shell goes to the yolk sac, which acts as an intermediate between the shell and the embryo. The relative importance of the roles of the chorion and the yolk sac in regulating the supply of calcium to the embryo is thus unclear.

Methods which allow the long-term culture of whole chick embryos have been developed (Dunn, 1974; Auerbach, Kubai, Knighton & Folkman, 1974). In such cultures, the embryos develop in the absence of shell and the system thus offers the possibility of analyzing separately the role of the yolk sac in embryonic calcium metabolism. In the present investigation, we have studied the fine structure of the chorionic epithelium, parathyroid glands and bones from cultured embryos as a first step in that direction.

**MATERIAL AND METHODS**

White Leghorn eggs were incubated in a forced-draught incubator for 3 days. Their surface was then cleaned with a solution of benzalconium chloride (Zephiran, Winthrop, 1:750) and their contents were cultured (Auerbach et al., 1974) by placing the egg contents in the bottom of a 20 × 100 mm plastic Petri dish (Falcon, no. 3003) after cracking the underside of the egg against the edge of the dish. It is then placed inside a larger 25 × 100 mm plastic dish (Falcon, no. 1013) containing some water for humidification; neither tissue culture medium nor antibiotics were added to the cultures. The dishes were then incubated at 37 °C in a humidified air atmosphere, for either 10 or 12 days. At that time, small portions of chorio-allantoic membrane and parathyroid glands and one complete tibia were dissected from each embryo. The length of tibiae was measured and their diaphyses were cut into small pieces. All tissues were fixed in half-strength Karnovsky’s (1965) fixative for 6 h. They were then washed in cacodylate buffer at pH 7.4 with 0.2 M sucrose, post-fixed in 1% osmium tetroxide for 2 h, dehydrated with ethyl alcohol, and embedded in Araldite. Sections (1 μm) were stained with toluidine blue for observation with the optical microscope; for electron microscopy, thin sections were stained with
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Amnion v > Chorio-allantoic membrane

Albumen

Yolk

Fig. 1. Schematic representation of the accessory membranes in a shell-less cultured embryo.

uranyl acetate and lead citrate according to Reynolds (1963). During sectioning, thin sections of bone were floated on distilled water alkalinized up to pH 9 with 0.1 N NaOH in order to reduce loss of calcium. Sections were observed with a Philips E.M. 300.

After dissecting the above-mentioned organs the remaining portions of the embryos were fixed in neutral 10% formalin; their developmental stage was established following Hamburger & Hamilton's (1951) description. After this, the embryos were eviscerated and stained in bulk with alizarin according to Karnofsky (1965). Since the cultured embryos used in this study reached stage 37 or 39, normally incubated embryos of the corresponding stages were processed similarly and used as controls.

Blood was obtained from six cultured embryos (stage 39) and six controls of similar stage and the concentration of calcium in their sera was determined with an automatic calcium analyzer (Corning, Scientific Instruments, Medfield, Mass.).

RESULTS

Only 40 of the 98 embryos explanted reached the age required for our experiments; most of the embryos which failed to survive died during the first 3 days after explantation. The degree of growth and differentiation of the surviving embryos was fairly uniform; the 25 embryos cultured up to a total age of 13 days (3 d in ovo + 10 d in vitro) reached stage 37 (ca. 11 d in ovo) and the 15 embryos cultured up to a total age of 15 days (3 d in ovo + 12 d in vitro) reached stage 39 (ca. 13 d in ovo).

Figure 1 illustrates the organization of the accessory membranes in the cultured embryos. Similar to controls, the embryos were surrounded by the amniotic sac and the yolk by a well developed yolk sac connected to the embryo by a narrow yolk stalk. The chorio-allantoic membrane, which in the embryos developed in ovo faces the shell membrane, faced the air phase in the cultures. The albumen in the cultures was not encircled by an albumen sac as happens in ovo.
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Chorionic epithelium. The chorionic epithelium differentiated adequately in culture and no signs of keratinization were observed. In the embryos at both stage 37 and stage 39 the chorionic organization was comparable to that found in the corresponding controls. In experimental as well as control embryos the rich superficial vascular network and the chorionic cell types had differentiated by stage 39 but not by stage 37. However, in cultured embryos, the differentiation of the vascular spaces was not uniform throughout the membrane; in some zones their wall was separated from the air phase only by a thin cytoplasmic process (Fig. 2), but in others they were still deeply located (Fig. 3).

The 'villus-cavity' cells in the stage-39 cultured embryos were similar to those found in controls; their cytoplasm was rich in mitochondria; numerous apical vacuolae and microvilli were present (Fig. 3). Microvilli, however, were shorter than in controls and the large apical cavity, after which the cells were named, was absent.

Typical ‘capillary-covering’ cells were also present (Fig. 2). They were poor in organelles, contained numerous microfilaments and possessed thin cytoplasmic processes overlying the vascular spaces (Fig. 2). The degenerating cells with very electron-dense cytoplasm and swollen mitochondria which are usually present in the chorion (Skalinsky & Kondalenko, 1963; Narbaitz, 1972b) were never observed in the chorion of the cultured embryos, although they were always present in the controls.

Parathyroid glands. The parathyroid glands from both the cultured and the control embryos appeared well differentiated. Cord cells had oval-shaped nuclei and their cytoplasm contained numerous mitochondria, a fair amount of profiles of rough endoplasmic reticulum and an extensive Golgi complex with numerous small pro-secretory granules and coated vesicles (Fig. 4); large secretory granules were sometimes present (Fig. 5) in the cultured embryos but never in the controls. Although the quantitative distribution of organelles varied from cell to cell and from gland to gland, making exact comparisons very difficult, we obtained the impression that the amount of rough endoplasmic reticulum found in the glands from cultured embryos was somewhat greater than in controls of the same age.

Bones. The study of embryos stained in block with alizarin gave an approximate idea of the degree of calcification of their bones. Extensive calcification of most bones could be observed in control embryos of both stages 37 and 39. In long bones, the diaphysis of the cartilaginous primordia was surrounded by a

Figures 2 and 3

Fig. 2. Electron micrograph of the chorion from a cultured embryo at stage 39. A.P., Air phase; B.S., blood sinus; C.C., ‘capillary-covering’ cell.

Fig. 3. A ‘villus-cavity’ cell (V.C.) in the same chorion. A.P., Air phase; B.S., blood sinus.
collar of calcified bone (Fig. 6a). Eight out of 25 cultured embryos in stage 37 did not stain at all with alizarin, indicating that no bone had undergone calcification. Some staining was present in the other 17 embryos: however, the zone of mineralized bone was restricted to a much shorter extension of the diaphysis (Fig. 6b). In many cases, the tibiae were soft and laterally bent (Fig. 7). All embryos cultured up to stage 39 had bones which had calcified to a larger extent than those in stage 37; however, their calcification was significantly retarded with regards to the corresponding controls, this difference being more evident in long bones. In all cases, the length of tibiae in cultured embryos was similar to that in controls: in stage 37, 11 ± 0.2 mm in cultured embryos and 10.9 ± 0.1 mm in controls; in stage 39, 14.1 ± 0.5 mm in cultured embryos and 14.2 ± 0.3 mm in controls.

The examination of 1 μm sections with the optical microscope showed that in all bones from embryos in stage 37 the cartilaginous primordium was surrounded by two to three rows of anastomosing trabeculae of bone. There was no detectable difference between cultured and control embryos with respect to number and pattern of arrangement of trabeculae. Figure 8 shows a section of the tibia of one of the embryos which with alizarin staining had shown no signs of calcification. Apparently normal trabeculae are surrounding the cartilage.

The defective calcification of the bones from cultured embryos became evident at the time of thin-sectioning for the electron microscopical study; these bones were sectioned with great ease as compared with the controls. Figure 9 is an electron micrograph of a bone trabecula of the tibia from a control embryo in stage 37. The matrix is extensively calcified, and most of the mineral has been preserved during the processing of the tissue. Figure 10 shows a bone trabecula from one of the cultured embryos in stage 37 which did not bind alizarin. No mineral is present in the bone matrix which contains numerous collagen fibers. As judged by the presence of large amounts of rough ER, the osteoblasts were active (Fig. 10). Figure 11 corresponds to a bone trabecula of a tibia from a similar embryo. The higher magnification allows us to observe the typical collagen fibers in the matrix; very few crystals are present. The osteocyte in the same figure appears also to be active and normal.

**Calcium concentration in serum.** The concentration of calcium in cultured stage-39 embryos was very low (5.4 mg/100 ml ± 0.80) with respect to the controls of similar stage (9.18 mg/100 ml ± 0.6). This difference was statistically significant (P < 0.001; Student’s test).

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**Figures 4 and 5**

Fig. 4. Electron micrograph of a parathyroid gland from a cultured embryo at stage 37. G, Golgi complex.

Fig. 5. Same parathyroid gland. S, Secretory granules; L, lipid droplet.
DISCUSSION

In the present investigation we have used the culture method described by Auerbach et al. (1974), but omitted the CO₂-enriched atmosphere; the respiratory vascular network differentiated adequately and no signs of keratinization of the chorion were observed. The very high mortality rate during the first days of culture was attributed by Auerbach et al. (1974) to lesions in the yolk sac produced during explantation.

The fact that 'capillary-covering' and 'villus-cavity' cells differentiated in the chorion of the cultured embryos indicates that the contact with the shell membranes and the massive transport of calcium from the shell are not essential for this to occur. The 'villus-cavity' cells did not present the typical apical cavity which is always present in the controls but the presence of apical vacuoles, microvilli and large numbers of mitochondria suggests that they were functionally active.

In normal chick embryos the chorion contains degenerating cells with very electron-dense cytoplasm and swollen mitochondria. Skalinsky & Kondalenko (1963) suggested that this process of degeneration is produced by the high concentration of calcium in the cytoplasm. Such cells were never found in our cultures, perhaps because their presence is connected in some way with the process of calcium absorption from the shell.

The parathyroid glands from all cultured embryos appeared to be active since they presented all the ultrastructural characteristics which have been shown to be associated with the synthesis and secretion of the hormone (Nevalainen, 1969; Youshak & Capen, 1970). The rough endoplasmic reticulum seemed to be more abundant in the glands from the cultured embryos than in the controls, suggesting that more hormone is being synthesized. A correspondingly intense hypocalcemia was found in these embryos.

The mineralization of the bones from the cultured embryos was defective. The length of the tibiae and the number of bone trabeculae surrounding their diaphyses were similar in cultured and in control embryos. Since the osteoblasts and osteocytes in the bones from cultured embryos appeared to be active as judged by their fine structure and the presence of numerous collagen fibers

FIGURES 6–9

Fig. 6. (a) Tibia from a stage-37 normal embryo. Alizarin stain. (b) Tibia from a stage-37 cultured embryo. Alizarin stain.
Fig. 7. Tibia from a stage-37 cultured embryo. Alizarin stain.
Fig. 8. Photomicrograph of a cross-section of the tibial diaphysis from a stage-37 cultured embryo. Cart, Cartilage primordium.
Fig. 9. Electron micrograph of tibia from a stage-37 normal embryo. Note the extensive mineralization of the bone matrix.
forming the bone matrix, the defect in the mineralization can probably be attributed to a deficit in the availability of the ions required to produce the mineral and not to an intrinsic defect in the differentiation of bone.

Phosphate originates from the phosphoproteins in the yolk (Williams, 1967) and since this appeared to be actively utilized by the cultured embryos these probably had a sufficient supply of phosphate. The calcium in the embryo has a double origin: experiments by Johnston & Comar (1955) indicate that before the tenth day of incubation, most of the embryonic calcium originates in the yolk; there is some shell resorption after the ninth day but it is only after the twelfth day that the shell becomes the main source. Our results, showing that even in the absence of shell most of the cultured embryos had partially mineralized bones, agree with Johnston & Comar's (1955) conclusion that the embryo utilizes at this time the calcium present in the yolk.

However, in the embryos cultured up to stage 37 (corresponding to 11-day controls), there was a notable defect in bone mineralization. If the results obtained in in vitro experiments are representative of what happens in ovo, this would suggest that even before the eleventh day, the relative proportion of calcium coming from the shell is significant, and that in the absence of shell, the yolk sac cannot compensate for the deficit of calcium by increasing its own contribution. The fact that embryos cultured up to stage 39 had a significant hypocalcemia suggests also the inability of the yolk sac to regulate the supply of calcium and stresses the importance of the shell supply for the maintenance of calcium homeostasis.

Additional studies on the pH and the concentration of bicarbonate and phosphate ions in blood from cultured embryos are needed in order to establish if this experimental model is comparable to in ovo development.

REFERENCES


Figures 10 and 11

Fig. 10. Bone trabeculae from a stage-37 cultured embryo. The bone matrix (Mat.) is not mineralized. Ob, Osteoblast; Oc, osteocyte.

Fig. 11. Trabeculae from the same bone seen with greater magnification. Few mineral crystals can be seen in the bone matrix (Mat.). Oc, Osteocyte.


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