Development and use of shell-less quail chorio-allantoic-membrane cultures to study developing skeletal tissues; a qualitative study

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Summary. A technique is described for in vitro culture of the quail embryo from the 1st to the 18th day of development. The embryos are cultured in Teflon hammocks, suspended in glass supports and kept in a humidified atmosphere at 36.5 °C. The quail CAM is used as support and cell source for developing non-quail cartilage and bone. The quail cells can be identified histologically and easily recognized by Feulgen-staining which is demonstrated in the presence of quail chondro- or osteoclasts in a mouse long bone rudiment cultured on the CAM.

Key words. Quail chorio-allantoic-membrane; shell-less cultures; chimera-technique.

For the in vitro study of developing cartilage and bone the use of chicken chorio-allantoic-membrane (CAM) is an established procedure[1–4]. The avian embryo is considered immuno-incompetent and explants of chicken, quail or mouse are well accepted by the membrane without a host-immune response. There is a good survival of the grafted tissues because of excellent vascularization of the CAM. In developmental biology, quail tissues are often used in combination with other tissues to study the origin of certain cells. Quail tissues are unique because their cells can be identified using Feulgen-staining, which reacts with the large amount of heterochromatic DNA associated with the nucleus. In many studies the chimera technique has been used to study the invasion of non-quail cells into quail tissues (e.g. in studies on the origin of the osteoclast in developing long bones)[5]. However, since it is much easier to recognize a Feulgen-marked quail cell in non-quail host tissues than vice versa, it was decided to develop a quail-CAM system as support and cell source for developing non-quail cartilage and bone grafts. In order to have a relatively large area to graft tissues onto, and to be able to inspect the viability of the embryo, it was decided to use a shell-less culture technique.

In this paper a technique is described for successful shell-less quail CAM cultures. The usefulness of this technique is evaluated using various non-quail embryonic tissue grafts.

Materials and methods. The Japanese quails used for egg production were 5–6 months old, one male matched with 1 or 2 females. The birds were kept under a 12/12 light/dark regimen and fed ad libitum. Eggs were collected daily and eggs used for the experiment were not older than 8 days.

To facilitate the culture of shell-less quail CAMs the following method was developed. In the lower end of 4 × 4 cm glass cylinders, 3 equidistant squares (1 × 1 cm) were cut (fig. 1). Found-cut gauzes (Teflon T.E.P.-fils 10–15 µm, Janssens MBL, St. Niklaas, Belgium) were suspended like hammocks on top of the cylinders. The eggs were cleaned with alcohol (70%) and with a 1% antimycine solution. With a pair of scissors a circular incision was made at the blunt end of the eggs corresponding with the position of the air chamber. This part of the shell was removed (fig. 2a). Subsequently the egg was turned over slowly and with the help of a second cut in the shell (fig. 2b), the contents were carefully allowed to flow into the hammock. This was done in such a way that the blastoderm, recognizable as a whitish disc on the yolk[6], was always facing upwards. The identification of the blastoderm is highly facilitated by placing a piece of dark blue cardboard under the unit. No media or antibiotics were added to the egg contents. After the units were filled with the egg contents they were covered with the glass lid of a petri dish and were placed in a sterilized glass box. This box was filled up to approximately 0.3 cm with sterile water, covered with Teflon and placed in a 36.5 °C incubator, to guarantee a sterile and stabilized, humid environment (fig. 3).

The usefulness of the shell-less CAM technique was investigated using various non-quail tissue explants. High density cultures of aggregated chondrocytes served as explants. These aggregates consisted of chondrocytes which were isolated from the epiphyses of tibiae of 15-day-old chicken embryos[7]. Furthermore, whole epiphyses of tibiae of 15-day-old chicken embryos were used as explants. The epiphyses were inserted into a shallow slit made in the allantoic membrane close to a blood vessel, as described by Krukowski et al.[4]. Finally, metatarsal rudiments (without a perichondrium) from the hind limbs of 17-day-old mouse embryos[8] were grafted onto the CAMs, and covered with a piece of Teflon to prevent desiccation.

After about one week, 30 mg sterilized $CaCO_3$ powder was sprinkled on to the CAM to supply calcium[9]. After various periods of culture on the CAM, the explants, together with the adjacent part of the CAM, were excised and fixed in Bouin-Hollande for 10 days and embedded in paraffin. Histological 5-µm serial sections were made and stained with Hematoxylin, with Alcian Blue (pH 2.5) or with Feulgen-staining to enable nuclear recognition.

Results. 1. Culture technique. Owing to the absence of any selection based on the fertility of the eggs, a substantial quantity (40–50%) did not develop into healthy embryos. A similar fertility was found in eggs kept in a standard egg-incubator. It is hard to explain why some of these fertile ones still failed to develop into a healthy CAM. The eggs should be used between 4 and 8 days after they have been laid. After 5 days of development the CAM can be used as a tissue culture substratum. Embryos could be kept alive for at least 1 week and 50% of them for 2 weeks. In some cases embryos even survived for 18 days. Since the CAM

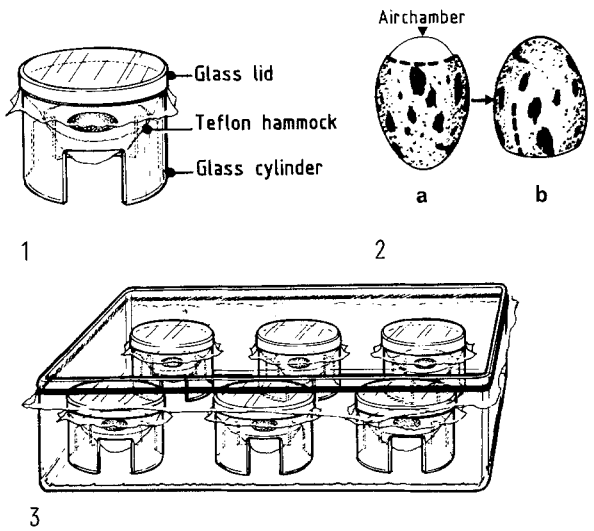

Figure 1. A culture unit consisting of a glass cylinder, with a Teflon hammock and a Petri dish lid.

Figre 2. The partial removal of the eggshell (a) and the place of the second cut (b) to facilitate the removal of the egg content.

Figure 3. The glass box with 6 units, ready for incubation.

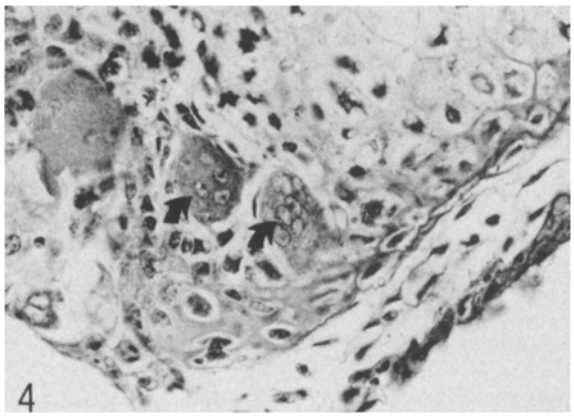

Figure 4. A mouse-quail chimera section, showing quail chondroclasts (indicated by the arrow) lying against the calcified cartilage of a mouse long bone rudiment.

can be used after 5 days of development, this means that on an average these quail-CAMs can be used during 7–10 days to culture tissue grafts. It turned out that slightly decreasing the temperature from 37 °C to 36.4 °C improved the survival rate of the embryos. It was observed that the muscle activity increased when $CaCO_3$ was supplied.

2. Explants. The usefulness of the described technique was studied by placing various tissues on the CAM. Aggregates consisting of isolated chondrocytes which are devoid of matrix grew poorly in contrast to the growth in a standard liquid culture medium. Judged from the Alcian Blue-stained histological sections, synthesis and deposit of matrix only occurred during the first days of CAM-culture. Thereafter the occurrence of pycnotic nuclei indicated unfavorable conditions. On the other hand in pieces of intact epiphyseal cartilage, which were inserted in a shallow slit in the CAM, no cell deterioration could be observed. Small capillaries, branching off from larger vessels in the CAM, could be seen around the cartilage graft. No invasion of capillaries or of quail cells were found inside the graft. Most successful was the grafting of mice metatarsal bone rudiments. After 7 days of culture on the CAM, histological sections showed healthy tissues and no sign of degeneration. During this culture period calcification of the hypertrophic cartilage continued and a marrow cavity was formed in the mineralized center of the rudiment. Blood vessels containing quail erythrocytes had invaded the calcified rudiments and multinucleated chondroclasts were found containing nuclei with the chromatin pattern typical of Feulgen-stained quail nuclei (fig. 4).

Discussion. This study describes the integration of two different techniques for cultures in biology: The successful CAM technique with chicken is well established[1,2]. The replacement of chicken by the quail[5] was introduced for better identification of migrating cells. Previously, however, the quail CAM within the shell was used. The advantages of working with a shell-less embryo in the culture are obvious. There is a widely-spread surface of the CAM that permits a good view, surgical manipulations, and grafting of a number of explants.

This study demonstrates that it is possible to culture embryos even from the first day on without incubating them for the first 3 days within the shell[1,2]. This fact could be useful for physiological studies of development. Moreover, the units are inexpensive and easy to assemble and to sterilize, whilst the gas-permeability of the Teflon gauze hammocks remains unchanged.

A disadvantage of the removal of the shell is the lack of a Ca supply after the first week of development. For normal differentiation of the CAM the presence and active transport of large amounts of Ca from the eggshell is not necessary[10]. Chick embryos maintained in shell-less cultures are retarded in their gross development[11]. The structural characteristics of osteoblasts and osteocytes, however, appear normal[12]. To overcome the lack of Ca in shell-less cultures Tuan[13] added pieces of the shells to the system. In the present study, encouraging preliminary results were obtained by supplying inorganic $CaCO_3$ to the CAM. Although the vitality of the embryo increased in combination with enhanced muscle-activity, it could not be shown that absence of the shell affects the long bone explant during the investigated periods.

The usefulness of the CAM as a support and cell source was investigated using various explants. However, the aggregates, consisting of isolated chondrocytes which had been enzymatically liberated from the matrix, showed limited growth and vitality. Probably the presence of matrix around the cells means protection in terms of a favorable environment and is also a prerequisite for CAM-cultures in contrast to cultures in liquid media. This is also suggested by the fact that pieces of epiphyseal cartilage with an intact matrix did not show any degeneration. The long bone rudiments of 17-day-old mouse embryos had been stripped clean of adhering perichondrium and periosteum in order to remove the source of osteoclast-progenitor cells in the rudiment[13]. In the marrow cavity, which developed during the 7 days' culture period, nucleated quail erythrocytes and chondroclasts of quail origin were found. Our results show that the use of glass supports with Teflon hammocks leads to successful culture of quail CAMs. The problem of lack of calcium can be overcome by supplying inorganic $CaCO_3$. This culture technique can be useful as a support and cell source for studies of the origin of specific cell types, such as the chondro- or osteoclast. Besides that, it makes observation of physiological and morphological development possible.

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0014-4754/87/020199-02$1.50 + 0.20/0
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