Oocyte growth and ovarian tissue differentiation in organ culture of mouse fetal gonads

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Abstract

Mammalian oocytes undergo growth and development through prenatal and postnatal periods. This is an investigation of development in vitro of oocytes and ovarian tissues from fetal gonads of the 12.5 days post coitus. Cultured tissues survived and the oocytes entered the growth phase, subsequently achieving long-term (14-30 days) culture. Growing oocytes were observed in their general morphology and were enclosed within 1-2 layers of granulosa cells, which resembled primary or secondary follicles. However, no follicular structures were also found in the cultures despite the existence of growing oocytes. The oocytes enclosed in very flattened cells were detected as in histological structure on day 18 of culture. The oocyte diameter became approximately 50 μm under culture conditions supplemented with 15% fetal bovine serum or Ehrlich ascites tumor fluid. These results indicate that the maximum culture period for oocyte growth in this organ culture is approximately 3 weeks and that the oocytes can grow in vitro to at least a medium size even if normal follicular structure is not formed.

Keywords: organ culture, primordial germ cells, fetal gonads, oocyte growth and development, folliculogenesis

Introduction

Mouse primordial germ cells (PGCs) are first recognizable in the posterior region of the extraembryonic mesoderm at 7.25 days post coitum (dpc) [1, 2, 3]. In the female embryo, the germ cells enter the prophase of the first meiotic division at about 13.5 dpc; they pass through leptotene, zygotene and pachytene, and arrest after birth in diplotene. The newborn mouse ovary contains only primordial follicles in which the primary oocyte is surrounded by a single layer of flattened somatic cells. In primordial follicle activation, a first wave begins at 2–5 days old. During that time oocyte

growth and differentiation is coordinated with multiplication and differentiation of the follicular cells [4]. This activated follicle is a primary preantral follicle; subsequently, it becomes a secondary preantral and antral follicle. During follicle development, the oocyte volume increases greatly through protein synthesis [5].

In the fetal germ cells, the most suitable best culture system for growth and development is probably organ culture of fetal gonads [6, 7, 8]. We examined about development in vitro of fetal germ cells [9, 10]. However, the limits of oocyte growth and gonadal differentiation in organ culture were unclear. Thus, We investigated the limits of oocyte

growth and tissue differentiation in long-term organ culture of female fetal gonads.

Materials and Methods

Organ culture

F₁ female mice (C57BL/6J × CBA/JNCrj) were mated with males and inspected for vaginal plugs the next day (0.5 dpc). Pregnant females were sacrificed at 12.5 dpc and fetuses were extracted. The stage of fetal development was defined according to the shape of the hind limb bud [8]. Female gonads were torn into half fragments using a 27-gauge needle in Leibovitz's L-15 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals Inc., Norcross, GA). We cultured 10–15 fragments on Millicell-PC membrane (Millipore) in six-well cluster dishes (Corning), each well contained approximately 1.5–1.7 ml of medium at 37°C in an atmosphere of 5% CO₂ in air. The culture medium was Waymouth's MB752/1 medium (Invitrogen) supplemented with 15% FBS and 1 mM sodium pyruvate (Sigma). Every 2 days, the culture was refreshed with 1 ml of new medium.

In some experiments, Ehrlich ascites tumor fluid (EATF) was added to the culture medium instead of FBS at the same concentration. The ascites fluid was produced from CD-1 (ICR) female mice injected intraperitoneally with approximately 10⁷ Ehrlich ascites tumor cells that had been passed once (12–13 days) in vivo. After centrifugation for 20 min at 3000 rpm, the cell-free fluid was sterilized by filtration through 0.45 µm filters, then stored at -20°C until use.

Histology and Staining

Isolation of the PGCs from the gonads was carried out using a Nycodenz method [9]. The alkaline phosphatase (ALP) detection of the PGCs was performed using an ALP staining kit (Sigma).

At the end of organ culture, the gonads were fixed in 10% formalin in PBS for 24 h, embedded in paraffin, sectioned at 2 µm and stained with hematoxylin and eosin.

Figure 1. Development in vitro of the female gonads at 12.5 dpc in organ culture. (a) Primordial germ cells (PGCs) exhibited alkaline phosphatase activity (arrow). (b) Half fragment of gonads (arrow) with the mesonephric region. (c) Gonadal ridges developed in vitro for 18 d. Growing oocytes and preantral follicle-like structures were visible. Scale bar = a: 50 µm, b, c: 200 µm.

Results

Tissue differentiation

We observed that ovarian structures and oocytes developed in long-term cultures of 12.5 dpc female gonads (Fig. 1, 2). Almost all cultures

Figure 2. Histological sections in tissues developed in vitro for 18 days from 12.5 dpc gonads. (a) The oocytes were generally enclosed within 1–2 layers of granulosa-like cells and a theca-like cell layer. (b) No follicular structures were found in the cultures despite the existence of growing oocytes. The oocytes enclosed in flattened cells were detected (arrows). Scale bar = 50 μ m.

survived and germ cells entered meiosis and the growth phase. Growing oocytes and follicular structures were observed in their general morphology and histological structure on day 18 of culture (Fig. 1C, Fig. 2). These oocytes were generally enclosed within 1–2 layers of granulosa cell-like somatic cells, which resembled primary or secondary follicles of in vivo ovaries (Fig. 2). In some cases, however, growing oocytes enclosed in very flattened cells without follicular structures detected in histological observation (Fig. 2b).

Oocyte growth

Oocytes in cultures of 12.5 dpc with mesonephric region were $33.2 \pm 0.9 \mu$ m in diameter on day 14; their mean diameter increased rapidly to

Figure 3. Oocyte growth in long-term culture of 12.5 dpc gonads. Means with different letters are significantly different ($P < 0.01$).

$48.0 \pm 0.6 \mu$ m on day 18 (Fig. 3). Further extension of the culture period, however, did not show a remarkable size increase (Fig. 3).

Oocytes in the gonads cultured in the FBS supplemented medium did not continue growth beyond a maximum size of 50–55 μ m. For that reason, we tested the culture medium supplemented with EATF instead of FBS in culture of 12.5 dpc without a mesonephric region. However, EATF showed no effect of oocyte growth beyond that of FBS (Table 1).

Discussion

The 12.5 dpc female fetal gonads formed a preantral follicle-like structure during long-term culture. This result agrees with those of previous reports [10, 11]. Oocytes grown in vitro were generally enclosed within 1–2 layers of granulosa cells. However, histological examination in this study detected growing oocytes without normal follicular structure in vitro. These oocytes were enclosed in mono-layered flat cells. Thus, appropriate differentiation of follicular cells may not be always necessary for a primary stage of oocyte growth.

The developmental limit of the fetal gonads in vitro is probably our understanding of the nutritional

Table 1. Effect of fetal bovine serum (FBS) and Ehrlich's ascites tumor fluid (EATF) on oocyte growth in vitro from 12.5 dpc gonads.

Supplements	Days of culture	No. of oocytes	Oocyte diameter (μm , mean $\pm$ SEM)
FBS	18	71	49.0 $\pm$ 0.6
	24	30	49.8 $\pm$ 1.1
EATF	18	71	48.6 $\pm$ 0.6
	24	39	49.7 $\pm$ 1.1

/ developmental requirements of the tissue. In organ culture, the oocyte growth dulled after about 3 week of culture; the limit of growth was approximately 50 μm mean diameter (Fig. 3), which is almost equivalent to the diameter of oocytes in preantral follicles of prepuberal mice [12]. Thus, it might be sufficient that a female gonad of 12.5 dpc is made to develop in vitro to preantral follicles – secondary follicles – with midsize oocytes. However, the oocyte development following the preantral stage requires physical space around the follicles, which would eliminate growth inhibition. In fact, the antrum – a physical space – is formed following the preantral follicle stage. Its formation is essential for full oocyte growth in vivo. Even if somatic cells surrounding oocytes have sufficient competence to allow oocyte growth, it is difficult for the oocyte to grow further under an environment with high density of growing oocytes. This study showed the limits of oocyte growth and tissue differentiation in long-term organ culture of female fetal gonads.

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