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FSH-initiated differentiation of newt spermatogonia to primary spermatocytes in germ-somatic cell reaggregates cultured within a collagen matrix

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ABSTRACT We previously cultured fragments of newt testes in chemically defined media and showed that mammalian follicle-stimulating hormone (FSH) stimulates proliferation of spermatogonia as well as their differentiation into primary spermatocytes (Ji *et al.,* 1992; Abe and Ji, 1994). Next, we indicated in cultures composed of spermatogonia and somatic cells (mainly Sertoli cells) that FSH stimulates germ cell proliferation via Sertoli cells (Maekawa *et al.,* 1995). However, the spermatogonia did not differentiate into primary spermatocytes, but instead died. In the present study, we embedded large reaggregates of spermatogonia and somatic cells (mainly Sertoli cells) within a collagen matrix and cultured the reaggregates on a filter that floated on chemically defined media containing FSH; in this revised culture system, spermatogonia proliferated and differentiated into primary spermatocytes. The viability and percentage of germ cells differentiating into primary spermatocytes were proportional to the percentage of somatic cells in the culture, indicating that differentiation of spermatogonia into primary spermatocytes is mediated by Sertoli cells.

KEY WORDS: *meiosis initiation, FSH, newt, reaggregated culture, spermatogonia, collagen matrix*

Introduction

The mechanism controlling the differentiation of proliferating spermatogonia to primary spermatocytes during vertebrate spermatogenesis requires elucidation. It is believed that Sertoli cells play a pivotal role during this process (Parvinen *et al.*, 1986; Griswold *et al.*, 1988; Skinner, 1991; Jegou, 1993). From mammals to reptiles, Sertoli cells maintain intimate contact with germ cells within seminiferous tubules throughout spermatogenesis and, in amphibians and fishes, do so within spermatocysts (Roosen-Runge, 1977; Parvinen *et al.*, 1986; Callard, 1991). Sertoli cells contain receptors for FSH and for androgens secreted by Leydig cells, and these receptors are considered to be their sole targets (Fritz, 1978). It is believed that FSH and androgens stimulate Sertoli cells to produce paracrine or autocrine factors, which in turn induce the proliferation and differentiation of germ cells (Skinner, 1991; Jegou, 1993); however, the actual mechanism controlling these processes is poorly understood, especially the factors inducing spermatogonial proliferation and their initiation of meiosis.

To examine this problem, we cultured fragments of newt testes in a chemically defined media, and found that mammalian follicle-stimulating hormone (FSH) alone stimulates spermatogonial proliferation and their differentiation into primary spermatocytes (Ji *et al.*, 1992; Abe and Ji, 1994). Next, we co-cultured spermatogonia with somatic cells (mainly Sertoli cells), and showed that the induced proliferation of spermatogonia by FSH is mediated by Sertoli cells, suggesting that paracrine factors produced by Sertoli cells stimulate spermatogonial proliferation (Maekawa *et al.*, 1995). However, the viability of the spermatogonia rapidly declined and no primary spermatocytes formed. In this report, we revised the culture system: larger reaggregates of cells were embedded within a collagen matrix and placed on a filter which floated on the medium. Under these conditions, spermatogonia differentiated into primary spermatocytes in the presence of FSH.

Results

Reconstituted culture of spermatogonia and somatic cells within a collagen matrix

To elucidate the roles of FSH and Sertoli cells in the initiation of meiosis, we established a culture system in which dissociated

Abbreviations used in this paper: FSH, follicle-stimulating hormone; OR-2, oocyte ringer-2; BSA, bovine serum albumin; BrdU, bromodeoxyuridine; PBS, phosphate-buffered saline; TPBS, Tween 20 in PBS; HRP, horse radish peroxidase; IgG, immunoglobulin G; ECM, extracellular matrix; RBM, reconstituted basement membrane.

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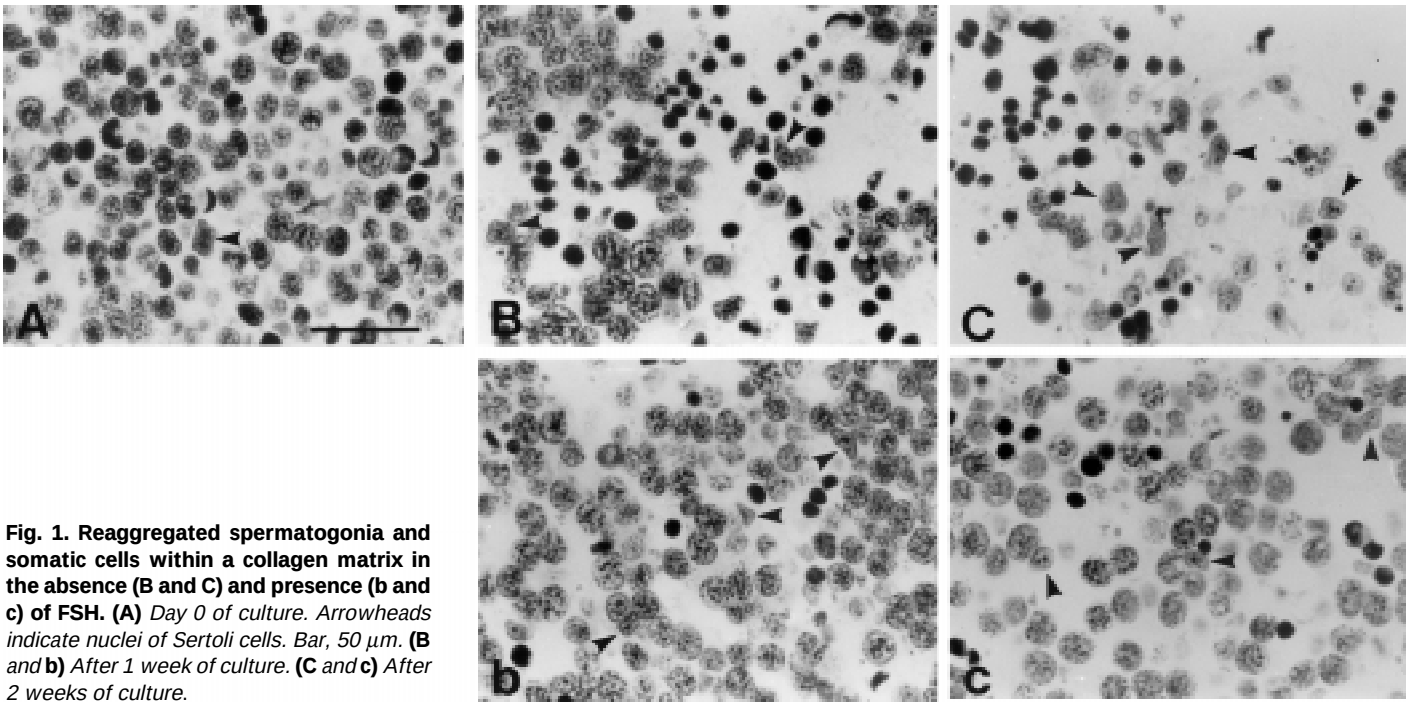

Fig. 1. Reaggregated spermatogonia and somatic cells within a collagen matrix in the absence (B and C) and presence (b and c) of FSH. (A) *Day 0 of culture. Arrowheads indicate nuclei of Sertoli cells. Bar, 50 μm.* **(B** *and* **b)** *After 1 week of culture.* **(C** *and* **c)** *After 2 weeks of culture.*

spermatogonia proliferate and then differentiate into primary spermatocytes in the presence of Sertoli cells and FSH. The steps and characteristics of these cultures are described below.

Dissociated testicular cells were reaggregated, centrifuged, and the pellet was cultured within a collagen gel on a filter. The pellet became slightly flat but retained a three dimensional structure. On day 0, the cells were closely adherent to each other with somatic cells distributed almost uniformly (Fig. 1A). Greater than 90% were live cells comprising spermatogonia and somatic cells in a ratio of 10:1. Among the somatic cells, more than 70% were Sertoli cells (Maekawa, *et al.*, 1995). Based on the live spermatogonia (100%) on day 0, the viability of cells in FSH medium was maintained at fairly high levels on day 7 (about 80%; Fig. 1b) and day 14 (about 65%; Fig. 1c) in contrast to those cells in basal medium in which viability decreased to about 60% on day 7 (Fig. 1B) and about 25% on day 14 (Fig. 1C). However, the percentage of live somatic cells remained fairly constant throughout the culture period irrespective of the presence or absence of FSH.

Proliferative activity of spermatogonia in reconstituted cultures

To examine the proliferative activity of spermatogonia, BrdU was added for 3 h on day 0,3 and 7 (Fig. 2). On day 0, only a few spermatogonia in the presence and absence of FSH incorporated BrdU and did so at similar percentages (Fig. 3). On day 3, the number of spermatogonia incorporating BrdU increased irrespective of the presence or absence of FSH (Fig. 2B,b); however, the proliferative activity was higher in the presence (about 24%) of FSH than in its absence (about 16%) (Fig. 3).

Some spermatogonia which incorporated BrdU on day 3 in the presence of FSH were grouped together (Fig. 2b), and on day 7 the size of the group increased (Fig. 2c), whereas no increase occurred in the absence of FSH (Fig. 2C). Finally, the proliferative activity of spermatogonia in FSH on day 7 (about 26%) was similar to that on day 3, whereas the activity of those in the basal medium dropped to about 5% (Fig. 3). These results indicate that FSH stimulates the proliferative activity of spermatogonia.

Differentiation of spermatogonia into primary spermatocytes in reconstituted culture

In basal medium very few spermatocytes (1.2±1.9%) developed even by day 14; however, when FSH was present, 45.9±8.6% of the germ cells were identified morphologically to be primary spermatocytes in zygotene or early pachytene stages (Fig. 1c). Our morphological observations were substantiated by detecting the presence of annexin V protein, a marker for newt primary spermatocytes (Yamamoto, *et al.*, 1996) (Fig. 4). On day 0, neither secondary spermatogonia nor somatic cells were stained by the monoclonal antibody against newt annexin V (Fig. 4A,B). However, after 2 weeks in the presence of FSH, a number of germ cells but not somatic cells stained with the antibody (Fig. 4a,b). In our negative control, preimmune serum failed to stain the cells in the presence of FSH on day 0 (Fig. 4C) and even after 2 weeks (Fig. 4c). These results demonstrate that spermatogonia differentiated into primary spermatocytes in the presence of FSH and somatic cells.

Culture of spermatogonia with variable numbers of somatic cells

To clarify the role of Sertoli cells in promoting differentiation from spermatogonia to primary spermatocytes, spermatogonia were co-cultured with variable numbers of somatic cells. To this end, spermatogonia were first separated from somatic cells by centrifugation through a continuous gradient of Metrizamide solution,

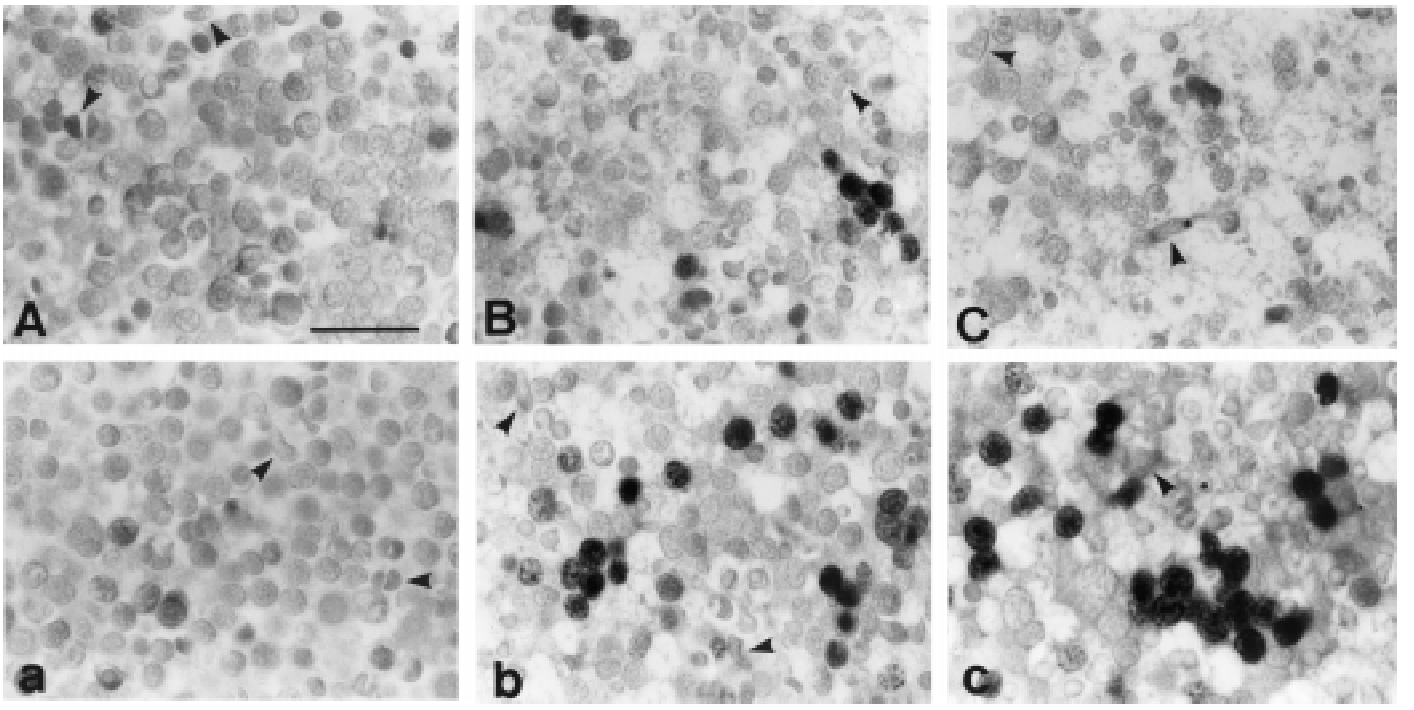

Fig. 2. Incorporation of BrdU into reaggregated spermatogonia and somatic cells within a collagen matrix in the absence (A,B and C) and presence (a,b and c) of FSH. (A *and* **a)** *Day 0 of culture. Arrowheads indicate nuclei of Sertoli cells. Bar, 50 μm.* **(B** *and* **b)** *Day 3 of culture.* **(C** *and* **c)** *Day 7 of culture.*

followed by sedimentation through a continuous gradient of BSA. The spermatogonia were organized into reaggregates, centrifuged and cultured within a collagen matrix. The purity of the spermatogonia was variable among experiments because complete fractionation of enriched spermatogonial fraction is difficult. Nevertheless, a role in meiosis could be established for Sertoli cells by comparing the percentage of primary spermatocytes formed in cultures with variable numbers of somatic cells.

When cultures enriched for spermatogonia contained as low as 1.3% somatic cells on day 0, the viability of germ cells declined rapidly to 15.0% and 12.8% after a week and to 4.9% and 4.0% after 2 weeks in the presence and absence of FSH, respectively. Differentiation into primary spermatocytes after 2 weeks was 8.0% in the presence of FSH and 2.9% in its absence. In the second experiment in which the percentage of somatic cells was higher, namely 2.2%, the viability of germ cells after 2 week was greater, 14.4% in the presence of FSH and 5.6% and in its absence. Also, the percentage of spermatocytes was higher in cultures: after 2 weeks 13.7% spermatocytes differentiated in the presence of FSH, whereas 3.3% did so in its absence. In the third experiment in which the percentage of somatic cells was much higher, namely 3.6%, the viability of germ cells after 2 week was much higher, 35.5% in the presence of FSH and 9.3% in its absence. Also, the percentage of spermatocytes was much higher in cultures containing a larger percentage of somatic cells: after 2 weeks 28.5% spermatocytes in the presence of FSH and 6.5% in its absence. Thus, the percentages of viable germ cells and spermatocytes was much higher when the percentage of somatic cells was greater and FSH was present than in those cultures containing less somatic cells and no FSH. These results combined with the cell reaggregates described above, demonstrate that both the viability of germ cells and their differentiation into primary spermatocytes in the presence of FSH is proportional to the percentage of somatic cells in the culture. This indicates that FSH promotes the differentiation of spermatogonia into primary spermatocytes via Sertoli cells.

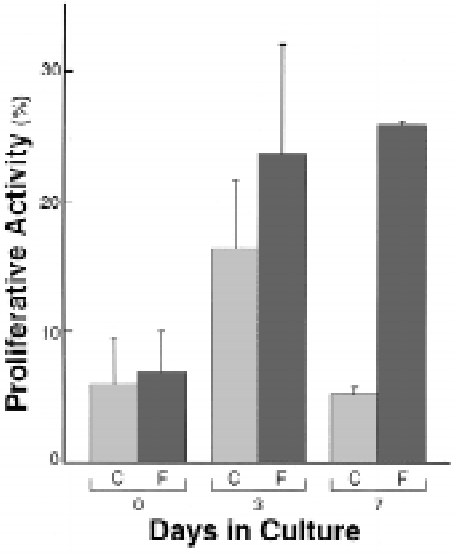

Fig. 3. Proliferative activity of spermatogonia assessed as the percentage of spermatogonia which incorporated BrdU among the total number of spermatogonia in reaggregated spermatogonia and somatic cells within a collagen matrix in the absence (C) and presence (F) of FSH. *Triplicate experiments were performed (Mean ± SE was shown).*

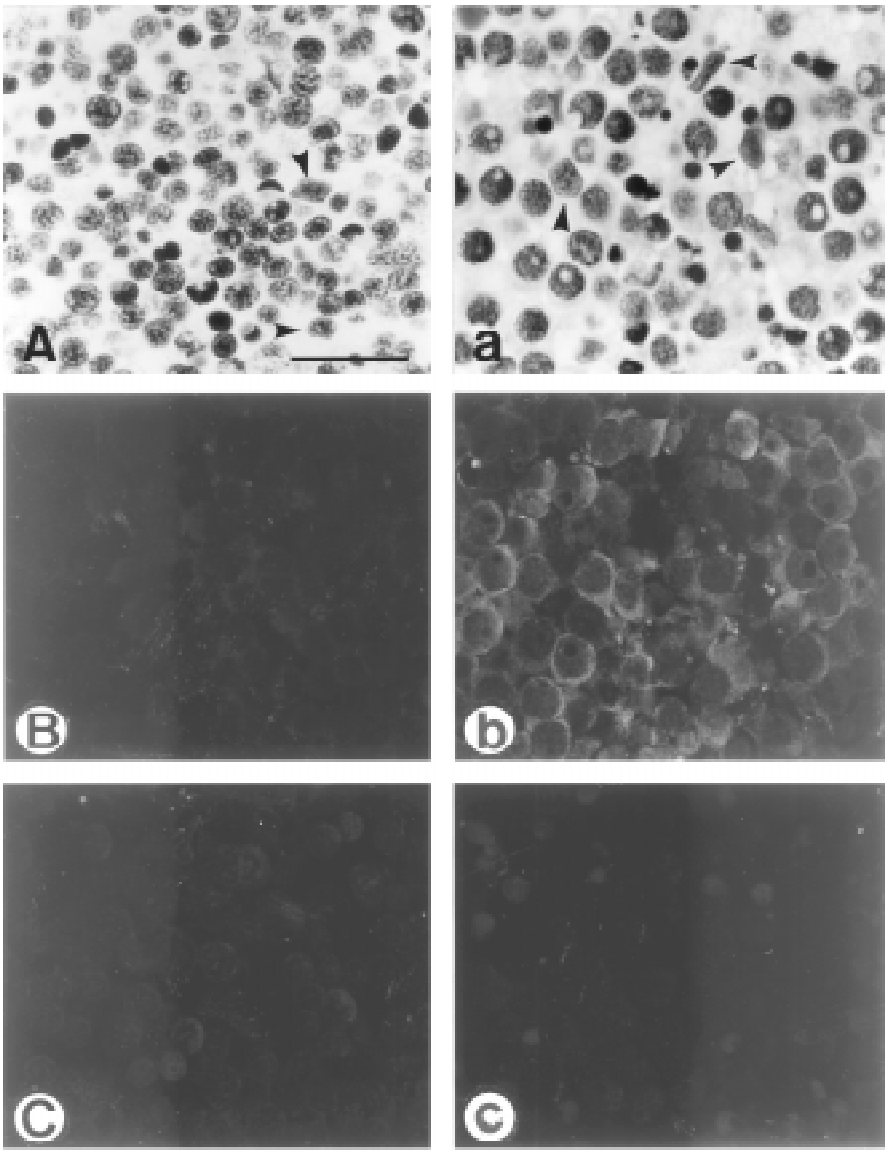

Fig. 4. Expression of newt annexin in reaggregated spermatogonia and somatic cells within a collagen matrix in the presence of FSH. (A,B *and* **C)** *Day 0 of culture.* **(a,b** *and* **c)** *After 2 weeks of culture.* **(A** *and* **a)** *Sections adjacent to those of (B and b), respectively, were stained with hematoxylin and eosin. Arrowheads indicate nuclei of Sertoli cells. Bar, 50 μm.* **(B** *and* **b)** *Sections were stained with monoclonal antibody against newt annexin V and with FITC-conjugated goat anti-mouse IgG antibody. (C and c) As controls, sections were stained with preimmune mouse serum and with FITC-conjugated goat anti-mouse IgG antibody.*

Discussion

One of our goals was to elucidate the mechanisms controlling the proliferation of spermatogonia and their differentiation into primary spermatocytes in the newt. To this end, we first established an organ culture system for testicular fragments in which spermatogonia differentiated into primary spermatocytes, and showed that FSH alone added to a chemically defined medium supported this differentiation (Ji *et al.*, 1992). In an attempt to simplify the culture system, we next combined dissociated spermatogonia and somatic (mainly Sertoli) cells, and indicated that FSH promotes the proliferation of spermatogonia via Sertoli cells (Maekawa *et al.*, 1995). However, the spermatogonia died and failed to differentiate into primary spermatocytes. We assumed that the death occurred because the reaggregates were too small and/or the cells were submerged in liquid medium. Accordingly, we prepared larger reaggregates by centrifuging the dissociated cells. Next, we embedded the pellet within a collagen matrix and placed this reaggregate on a nuclepore filter. Such composites were cultured in the same chemically defined medium that we used previously for organ cultures (Ji *et al.*, 1992). In the presence of FSH the spermatogonia proliferated and, in addition, differentiated into primary spermatocytes. As the number of spermatocytes was proportional to the percentage of somatic (mostly Sertoli) cells present, we conclude that FSH promotes the formation of primary spermatocytes via Sertoli cells.

Although the differentiation of spermatogonia into primary spermatocytes was achieved in several species, this success occurred in organ cultures of testes (rat, Steinberger and Steinberger, 1966; mouse, Aizawa and Nishimune, 1979; eel, Miura *et al.*, 1991a,b; rat, Boitani *et al.*, 1993). In fact, Miura *et al.* (1991a,b) obtained all stages of spermatogenesis from type A spermatogonia to spermatozoa in fragments of eel testes when human gonadotropin or 11-ketotestosterone was included in the chemically defined medium. In culture of immature rat testis, FSH was shown to be essential for the progression of type A spermatogonia up to the pachytene spermatocytes after 3 weeks (Boitani *et al.*, 1993). However, the progression of spermatogonia to primary spermatocytes in co-culture of spermatogonia with Sertoli cells was achieved previously only in the eel (Miura *et al.*, 1996).

At least three factors in our culture system probably account for our success, namely, the three dimensional structure of the cell reaggregates, availability of air (oxygen), and the inclusion of an extracellular matrix. First, when dissociated newt spermatogonia and Sertoli cells are cultured in conventional plastic dishes, the Sertoli cells gradually extend their cytoplasm radially, become flat, produce bubbles in the cytoplasm, and die after two weeks; in addition, the spermatogonia detach from the Sertoli cells as they flatten (data not shown). Mammalian germ cells, cultured under similar conditions, transform into squamous like cells and lose the highly polarized form observed *in vivo* (Steinberger *et al.*, 1975; Welsh and Weibe, 1975). Thus, germ cells co-cultured with Sertoli cells in conventional plastic dishes seem to require three dimensional histologic relations, otherwise the germ cells fail to differentiate (Steinberger, 1975).

Second, cell reaggregates require oxygen via air. Testicular fragments of newts submerged in medium undergo cell death in the central part, but this area survives when the fragments are cultured

on a filter floating on the medium (data not shown). Similarly, murine fetal thymuses grow better on membranes floating on the medium than those submerged in the medium; only when oxygen concentration in the medium is increased to 60-80% does the submerged tissue equal the growth of the floating tissue (Watanabe and Katsura, 1993).

Third, collagen matrix seems to provide an appropriate extracellular matrix (ECM) for Sertoli cells to support the differentiation of germ cells. Reaggregates of newt spermatogonia and Sertoli cells on a filter lacking a collagen matrix flatten and no spermatocytes form (data not shown), indicating that a three dimensional structure of the cell aggregates is required for further differentiation in culture. In the present study, proliferating spermatogonia probably underwent synchronous divisions and formed cyst-like structures. However, a further study is needed to reveal ultrastructure of the reconstituted culture in order to see whether cyst-like structures are formed or not.

We should also consider the possibility that the collagen matrix retains growth factors that are secreted by Sertoli cells and required for spermatogonial proliferation. The exact mechanism whereby ECMs support spermatogenesis *in vitro* remains to be explained. Nevertheless, many studies in mammals reveal that ECMs support cell viability and cellular architecture *in vitro*. For example, Sertoli cells resemble in morphology their *in vivo* counterpart when cultured on a substratum of seminiferous tubule biomatrix (Tung and Fritz, 1984), on matrix deposited by Sertoli-myoid cell co-cultures (Hadley *et al.*, 1985), or on type IV collagen and laminin (constituents of native basal laminae of rat testes) (Suarez-Quian *et al.*, 1985). However, Sertoli cells eventually spread out in long-term culture on any substratum. Grown on top of the reconstituted basement membrane (RBM), Sertoli cells formed polarized monolayers virtually identical to those *in vivo* and maintained germ cells for long time, but the germ cells did not differentiate. Finally, Sertoli cells and spermatogonia cultured within reconstituted basement membrane reorganized into cords resembling those present *in vivo* and eventually primary spermatocytes formed (Hadley *et al.*, 1985).

In the future we plan to identify the factors produced by newt Sertoli cells that stimulate spermatogonial proliferation and meiotic initiation by using our current culture system to assay the effect of various candidates on these processes.

Materials and Methods

Dissociation of testes and separation of testicular cells

Dissociation of testes and separation of testicular cells were performed as described previously (Maekawa, *et al.*, 1995). Briefly, the testes were sterilized in 70% ethanol for a few seconds, and then the immature part of each testis, containing spermatogonia, was removed and cut into small fragments. This and other operations were performed at room temperature (22°C) unless noted otherwise. The cells of the fragments were dissociated by 0.1% collagenase (type N-2, Nitta Zaratin Co., Tokyo, Japan), containing 0.05% trypsin inhibitor (type I-S from soybean, Sigma) in L-15 medium (1.5 h) on a rotary shaker (double shaker R-30, Taitek Co., Kosigaya, Japan) at 70 rpm, followed by treatment with 0.005% DNase I (type IV, Sigma) for 20 min. Next, the cell suspension was pipetted about 50 times and further incubated on a rotary shaker for an additional 1.5 h. The cell suspension was again pipetted about 50 times and then filtered through a 100 μm nylon mesh filter to remove undissociated cells. The filtrate was centrifuged (5 min, 250g) and washed in L-15 medium. The pellet was suspended in 5 ml L-15 medium. To remove dead cells, the cell suspension was layered on 15% Metrizamide (Centrifugation grade, NYCOMED, Oslo, Norway) solution in OR-2 (Wallace *et al.*, 1973) and centrifuged (20 min, 1,800g).

To separate spermatogonia from somatic cells, the cells were layered on a 4-10% continuous Metrizamide solution (15 ml), itself underlayered by 15% Metrizamide (5 ml). Centrifugation (1,800g, 20 min) resulted in two bands, one containing somatic cells at the interface between L-15 and 4% Metrizamide and the other containing primarily spermatogonia between 8 and 9% Metrizamide. To separate aggregates containing spermatogonia and somatic cells from the enriched spermatogonial fraction, the cell suspension (5 ml) was layered on a 2-4% continuous bovine serum albumin (BSA) in L-15 (15 ml), which itself was underlayered by 15% Metrizamide (5 ml). After 20 min the upper part of the BSA solution comprised single spermatogonial cells whereas the lower part contained cell aggregates.

Reconstituted culture within a collagen matrix

Spermatogonia or a population of spermatogonia mixed with somatic cells was aggregated on a rotary shaker (60 rpm) in a 35 mm plastic dish (Falcon #1008), previously coated with 4% BSA in L-15 medium (>30 min) and then rinsed. The reaggregates were centrifuged (250g, 5 min) and the resultant pellet was suspended in L-15 medium (about 200 μl). The cell suspension was placed in a 0.5 ml tube (3-5x10^5 cells/tube) and centrifuged (1,000g, 5 min). The medium was discarded and a cold collagen solution (150 μl) consisting of 120 μl of 0.3% collagen (KOKEN CELLGEN, I-PC; KOKEN Inc.) and 30 μl of 5xL-15 (pH 7.4), was added to the tube. The pellet was detached from the tube by using a needle. The collagen solution containing the pellet was aspirated with a broad mouth pipette and placed on a nuclepore filter (25 mm, pore size 0.2 μm; Costar) for 1-1.5 h until the collagen hardened. The filter was floated on medium (2 ml) with or without FSH in a 35 mm plastic dish; the medium was replaced weekly. Triplicate experiments were performed.

Histology - measurement of viability and extent of differentiation

Pellets on filters from each culture were fixed at 0,1 and 2 weeks in Bouin solution. The samples were dehydrated in an ethanol series, removed from the filters and embedded in paraffin blocks (paraplast; Oxford Labware). The blocks were sectioned serially (5 μm) and stained according to the Delafield hematoxylin-eosin method. To estimate the viability of cells, two sections about 50 μm apart were randomly chosen among 30 sections. The central area (about 450 μmx600 μm) of each section was photographed and the number of viable and dead cells was counted. The viable cells were classified as spermatogonia, primary spermatocytes or somatic cells, and the percentage of primary spermatocytes among the germ cells indicated the extent of differentiation.

Measurement of proliferative activity of spermatogonia

On culture day 0,3 and 7, 4 μl of bromodeoxyuridine (BrdU) labeling reagent (Amersham) was added to 2 ml of medium and incubated for 3 h. Then the samples were fixed in Bouin solution and prepared for histological sections. From among 30 sections derived from the central part of the fragment, every 6th section was stained with anti-BrdU mouse monoclonal antibody according to the following procedure. After dehydration by an ethanol series, the sections were washed in PBS, blocked with 1% BSA in PBS (30 min), and then washed in PBS (5 min). Anti-BrdU mouse monoclonal antibody + nuclease (Amersham; 10 μl) was added to a section and the sample was incubated (1 h). Next, the section was washed in PBS (3 times), and horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG (Bio-Rad; 10 μl) was added to the section for 1 h. Then, the section was washed in PBS (3 times) and 50 μl of DAB substrate (Vectastain DAB substrate kit, Funakoshi, Japan) was added for 2-10 min. Finally, the sections were washed in tap water (>5 min) and counterstained by eosin.

Two sections were randomly selected and the central areas (450 μmx600 μm) of each section were photographed. The percentage of spermatogonia which incorporated BrdU indicated their proliferative activity. Triplicate experiments were performed.

Immunocytochemistry with anti-newt annexin antibody

Following dehydration by an ethanol series, sections were washed in 0.1% Tween 20 in PBS (TPBS) for 24 h and blocked by 3% BSA in TPBS for 1 h. After the section was washed in PBS (20 min), 10 μl of anti-newt annexin monoclonal antibody (Yamamoto *et al.*, 1996) was added and the sample was incubated (16 h, 4°C). Finally, the section was washed in TPBS (3 times, 20 min each) and 10 μl of fluorescein-conjugated goat anti-mouse IgG (CAPPEL) diluted 50 times by TPBS was added for 1.5 h.

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