Association of culture of mouse urogenital complexes in media containing rodent sera with the appearance of primordial germ cell-like cells

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Primordial germ cells differentiate into germ cells and have the ability to reacquire totipotency. Mouse primordial germ cells are identified by alkaline phosphatase staining of the extraembryonic mesoderm, and they proliferate and migrate to reach the genital ridges. Mouse primordial germ cells have never been maintained in culture exclusively for longer than a week without differentiation or dedifferentiation. Moreover, primordial germ cells have not been proliferated with urogenital complexes *in vitro*, because gonad culture has never been successful. It was thought that primordial germ cells could proliferate in a culture of urogenital complex under modified medium conditions resembling those *in vivo*; however, organ culture of mouse

gonad has been performed with fetal calf serum or equine serum, and those sera produce conditions different from those *in vivo*. Therefore, mouse urogenital complexes were cultured in media containing rodent sera. As a result, it was possible to proliferate primordial germ cell-like cells outside gonads, and these cells very closely resembled primordial germ cells. In addition, motile primordial germ cell-like cells could be obtained. The ability to maintain primordial germ cell-like cells in culture by this intra-species culture method is important in the study of gametogenesis. Furthermore, this method is useful as a source of stem cells such as embryonic germ cells.

Introduction

Primordial germ cells (PGCs) differentiate into germ cells and are able to reacquire totipotency in order to generate offspring. Mouse PGCs are first identified as the alkaline phosphatase-positive cells in an extraembryonic mesoderm. Subsequently, PGCs proliferate and migrate to reach the genital ridges at about day 13.5 after mating (Ginsberg *et al.*, 1990). The differentiation of the gonad starts after PGCs have migrated into the genital ridges (Minz and Russell, 1957; Tam and Snow, 1981; Ginsberg *et al.*, 1990). The developing mouse gonads first become distinguishable by sex at day 12.5 after mating (Byskov, 1986; Mackay and Smith, 1989). PGCs play an important role related to somatic cells in the organogenesis of the gonad. Therefore, the study of PGC proliferation and differentiation is important for understanding the mechanism of gonad formation.

Mouse PGCs have not been cultured exclusively and they have not been maintained in culture for a long period, because PGCs change their character very easily both *in vivo* and *in vitro*. PGCs are affected by some factors from somatic cells or the extracellular matrix.

For example, Steel factor (SCF) (Godin *et al.*, 1991) and transforming growth factor β (TGF- β) (Godin and Wylie, 1991) are regulatory factors of proliferation and migration. In previous studies, embryonic germ (EG) cells were obtained when mouse PGCs were cultured with SCF, leukaemia inhibitory factor (LIF) (Matsui *et al.*, 1991) and basic fibroblast growth factor (bFGF) (Resnick *et al.*, 1992). EG cells are known as totipotent stem cells and are different from PGCs.

Gonad culture has been used for the study of proliferation and differentiation of PGCs. Tissue culture is a useful technique for understanding the mechanisms of gametogenesis, for example, meiosis, genomic imprinting and signal transduction in germ cell and neighbouring somatic cells. However, PGCs could not be proliferated in urogenital complexes *in vitro*, because gonad culture has been difficult to perform.

Some researchers have reported culture of urogenital complexes at each stage of development (Taketo and Koide, 1981; Mackay *et al.*, 1986; Agelopoloul and Magre, 1987; Mackay and Smith, 1989; Tilmann and Capel, 1999). In a recent study, gametogenesis *in vitro* had limited success (Eppig and O'Brien, 1996) or required skilful manipulation techniques (Obata *et al.*, 2002). Some reports show that the serum generally improved the development, growth and stimulation of mitosis in cultures of gonads. The culture of urogenital

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complexes before the migrating stage of PGCs was attempted using equine serum (Taketo and Koide, 1981) and the urogenital complexes were capable of differentiation *in vitro*. Recently, urogenital complexes just at the start of the differentiation stage were cultured in a medium containing 10% fetal calf serum (FCS) for 30–44 h (Tilman and Capel, 1999). Furthermore, Obata *et al.* (2002) reported the successful *in vitro* maturation of mouse oocytes with the combination of organ culture of gonad and nuclear transplantation. These authors used Waymouth medium containing 10% fetal bovine serum. However, there are some reports that the serum has an inhibitory effect on organ culture (Agelopoloul and Magre, 1987; Mackay and Smith, 1989). A culture medium containing 15% FCS prevented the differentiation of testicular cords when the rat urogenital complexes were cultured for 4 days before sexual differentiation (Agelopoloul *et al.*, 1984). It was suggested by Agelopoloul and Magre (1987) that Sertoli cells secrete Mullerian inhibitor despite the absence of testicular cord formation in a culture with serum. In almost all cases, FCS or equine serum have been used as a supplement in the medium of organ culture. Namely, the addition of serum has been reported to produce an inhibitory effect but also some advantages in the differentiation of mouse gonad *in vitro*.

In the organ culture of mouse gonad *in vitro*, the cells emerge outside the male gonad on day 13 after mating (Byskov, 1978). In addition, characteristic round granular cells (RGCs) appeared outside the gonad cultured with equine serum and these cells were stained by alkaline phosphatase (MacLaren, 1983). However, there was no detailed description concerning the emergence of the round granular cells outside the gonad, the relation to PGC-like cells, and serum.

The present study examined whether PGCs could proliferate using urogenital complexes cultured with modified conditions using more appropriate sera. Previously, tissue culture of mouse gonad was carried out with FCS or horse serum. These cross-species conditions were different from the environmental conditions *in vivo*. In fact, some cross-species reactivities between mouse and cattle or horse were reported (Levery and Wainer, 1982; Diamond *et al.*, 1991), but these activities were low (Abreu *et al.*, 1981). Therefore, mouse urogenital complexes were cultured with rodent sera in this study.

Materials and Methods

Mice and embryos

ICR/Jcl mice (*Mus musculus*) obtained from Clea, Japan, Inc. (Tokyo) were used in this study. The adult female mice (about 2–3 months of age) were placed with males overnight; vaginal plugs were examined on the next morning and the mice were kept in a closed colony with 12 h light:12 h dark cycle at 23°C. The day on which

a vaginal plug was found was defined as day 1 after mating. Female mice at day 12.5 after mating were killed by dislocation of the cervical spine and embryos were collected in the tissue culture dish (Terumo Corporation, Tokyo) in PBS. Urogenital complexes (mesonephros and genital ridge) were obtained by microsurgery using the method of Cooke *et al.* (1993).

Organ culture of urogenital complexes

The organ culture was performed with the cell culture insert (Falcon, Becton Dickinson and Co., Franklin Lakes, NJ) of 0.4 µm pore size within a 24-well tissue culture plate (Falcon, Becton Dickinson and Co.). Dulbecco's modified eagle's medium (DMEM; Sigma, St Louis, MO) contained 1000 mg glucose l⁻¹, 0.054 g L-glutamine ml⁻¹, 3.7 g NaHCO₃ ml⁻¹ and 0.0004 g pyridoxine HCl ml⁻¹, supplemented with 25 mmol Hepes (Wako Pure Chemicals Industries, Osaka), 50 units penicillin G sodium ml⁻¹ and 50 µg streptomycin sulphate ml⁻¹ (Gibco, Grand Island, NY) as the control medium. The various animal sera were added to the control medium as 10% of the total volume of the experiment medium. Fetal calf serum (FCS; Gibco), horse, rat and mouse sera were used in this experiment. Sera of mice and rats were obtained from males, non-pregnant females, and pregnant females (day 12.5 after mating), respectively. The blood was obtained from horses, rats and mice, and the sera were separated. A set of urogenital complexes of 12.5 days after mating was placed on a cell culture insert membrane per well. These complexes were cultured at 37°C in air, 5% CO₂ and the medium was changed on alternate days during the culture period. These organs were cultured for 7 days.

Identification of PGC-like cells and observation of tissue structure

The identification of PGC-like cells was accomplished by alkaline phosphatase (ALP) staining, carried out by the azo dye method of Brinster and Harstad (1977) that used naphthol AS-MX phosphate (Sigma) as the substrate of Fast blue RR salt (Sigma) as the dye. The cultured complexes were fixed with 10% formalin, 0.013 g CaCl₂ · H₂O ml⁻¹ solution (4°C, 15–20 min). After treatment, each well was washed five times with distilled water, and the reactive solution was added. The reactive solution containing 0.05 mol Tris buffer l⁻¹ solution was added with the substrate and azo dye. The cells outside the genital ridges were stained at days 3 and 7 of culture by ALP staining, and the number of ALP-positive cells was counted under a microscope. In the present study, the number of PGC-like cells was counted. Then, the tissue structure inside the organs was observed by paraffin wax section. The cultured organ was dehydrated in ethanol (4°C) for 24 h and embedded in paraffin wax and sectioned at 8 µm thickness. Each

Fig. 1. Mouse urogenital complex before culture at day 12.5 after mating and gonad cultured for 7 days with serum-free medium. (a) Male and (b) female urogenital complexes could be distinguished by the presence of primitive testicular cords (TC) in the male. (c) Male urogenital complex cultured for 7 days with serum-free medium. MN: mesonephros; GR: genital ridge. Scale bars represent 500 μm .

section was stained by eosin and observed under a light microscope.

Statistical analysis

Data for numbers of PGC-like cells were expressed as means $\pm$ SD. The statistical analysis was carried out using Student's *t* test. Significance was considered as $P < 0.05$. Experiments were examined at least in triplicate.

Results

Influence of various sera for structure of mouse urogenital complexes in vitro

The urogenital complexes of fetal mice on day 12.5 after mating began to show sexual differentiation. The sex of gonads could be determined before culture because

primitive testis cords were observed (Fig. 1a,b). In the control medium (serum-free), the tubules of the primitive testis cords extended and formed the testicular structures in male genital ridges after day 7 of culture (Fig. 1c). The extension of primitive testis cords was observed in male gonads at day 3 of culture. However, the female genital ridges cultured with the serum-free medium, FCS or horse serum did not change into any characteristic structure during these periods. These cultured organs changed into round structures. However, in the culture of organs with media containing mouse or rat serum, many round granular cells were observed outside the urogenital complex (Fig. 2a). These round granular cells appeared on the monolayer of fibroblast-like cells around the gonad, and these round granular cells were stained by ALP (Fig. 2b). Only fibroblast-like cells were observed in the culture containing FCS or equine serum (data not shown).

Fig. 2. Mouse primordial germ cell-like cells (PGCs) appeared around the urogenital complex. (a) Urogenital complex cultured for 7 days with male mouse serum was surrounded by PGCs (arrows). (b) High magnification of (a). UGC: urogenital complex, FC: fibroblast-like cells. PGCs were stained using alkaline phosphatase. Scale bars represent 100 μ m.

Fig. 3. Increase of primordial germ cell (PGC)-like cells outside urogenital complexes cultured with mouse and rat serum. ■: Mouse male serum†; □: mouse female serum; ▨: mouse pregnant serum; ▤: rat male serum; ▥: rat female serum†; □: rat pregnant serum†. *Number of PGC-like cells at day 7 of culture are significantly increased compared with day 3 culture ($P < 0.005$). †Significantly increased urogenital complex in males compared with females ($P < 0.005$). Bars represent mean $\pm$ SD.

Effects of rodent serum for PGC-like cells

PGC-like cells emerged outside the medium when rat or mouse serum were used. When gonads were cultured with rodent sera for 2 days, PGC-like cells appeared

around the urogenital complexes. The number of these cells increased with time of culture (Fig. 3).

In all culture media containing mouse sera, the number of PGC-like cells outside of the male gonads increased to about 1000 at day 7 of culture. In contrast,

Fig. 4. Changes in (a,c) male or (b,d) female mouse urogenital complexes cultured for 7 days in medium supplemented with (a,b) male mouse serum or (c,d) male rat serum. These sections were stained by alkaline phosphatase and eosin. Arrows show primordial germ cell-like cells stained by alkaline phosphatase. Scale bars represent 200 μm .

the increase in the number of PGC-like cells outside of female gonads was only almost half that observed outside male gonads.

In the rat serum, PGC-like cells outside of the male gonad increased in number to approximately 1300 compared with 800 for the female gonad at day 7 of culture. This effect also occurred with mouse serum (Fig. 3). Particularly, in the pregnant rat serum, the number of PGC-like cells outside of the male gonads increased to about 2000 (1912 ± 143) at day 7 of culture. There were some differences for each rat serum; serum from non-pregnant rats had little effect (563 ± 83 from female gonad) and serum from pregnant rats had a large effect (1912 ± 143 from male gonad) in increasing the number of PGC-like cells. However, only a few PGC-like cells and small spherical cells outside the gonads were observed during the culture periods in FCS (< 50), equine serum (< 20) and the serum-free medium (none or a few) (data not shown).

Changes of the internal structure of genital ridges in culture

Increased numbers of PGC-like cells appeared outside the urogenital complexes cultured in medium containing the rodent sera. It is possible that the number of PGC-like cells inside the gonad increased or decreased after the emergence of PGC-like cells outside the gonad during

organ culture. Changes in the organ structures and the distribution of ALP-positive PGC-like cells were observed in tissue sections (Fig. 4).

Almost all organs cultured with media containing rodent sera had cleft spaces in the urogenital complex (Fig. 4). Before culture, many PGC-like cells were observed inside the genital ridges (Fig. 5a,b). However, few PGC-like cells were observed in the cultured urogenital complexes. These PGC-like cells were located near the surface of the tissue (Fig. 4). In the urogenital complexes cultured with FCS, the morphology of the gonads did not change, and few ALP-positive cells were found inside the organs (Fig. 5e,f). These results were similar to those in the serum-free medium: cells positive for ALP did not appear outside the tissues in the serum-free medium (Fig. 5c,d). Cells positive for ALP were observed in a primitive testicular cord-like structure (Fig. 5c). Urogenital complexes cultured with horse serum were similar to those cultured with FCS (data not shown).

Discussion

The objective of this study was to achieve the proliferation of PGCs in culture. In this study, PGC-like cells emerged outside gonads cultured with rodent sera and proliferated in this culture medium.

The successful maturation of mouse germ cells *in vitro* was reported using serum (Eppig and O'Brien 1996;

Fig. 5. Mouse urogenital complexes at day 12.5 after mating (a,b) before culture and changes in urogenital complexes with (c,d) serum-free medium or (e,f) fetal calf serum. (a,c,e) Male gonad; (b,d,f) female gonad. Sections were stained by alkaline phosphatase and eosin. MN: mesonephros, GR: genital ridge, TC: primitive testicular cord-like structure, PGCs: primordial germ cell-like cells stained by alkaline phosphatase. Scale bars represent 200 μm .

Obata *et al.*, 2002). It required changes in the medium conditions during culture periods and skilful techniques (Obata *et al.*, 2002). In a previous study, germ cells have been able to progress up to the diplotene stage of the first meiosis without nuclear transplantation. Therefore, it was thought that serum in the medium has an important role in the initial condition of gonad culture *in vitro*. However, there have been no reports using sera other than FCS or horse serum for the culture of the urogenital complex. These conditions are different from the *in vivo* environment of mice. Cross-species reactivity was reported but rat fibroblast-derived interferon had low reactivity in cattle and high reactivity in mice (Aberu *et al.*, 1981). Therefore, unidentified factors in sera were thought to act effectively in the intra-species

culture. In the present study, we examined the culture of urogenital complexes containing rodent sera in order to approximate the conditions *in vivo*; as a result, round granular cells appeared outside the gonads. These round granular cells resembled PGCs, being stained by ALP and being morphologically similar (Chiquoine, 1954). In previous reports, the fibroblast-like cells and round cells were described outside the gonads in serum-free medium or horse serum (Byskov, 1978; McLaren, 1983). In the present study, the fibroblast-like cells observed were similar to those in previous reports, but the round cells emerged from the urogenital complexes cultured with rodent serum and did not emerge in the serum-free medium. It is thought that the appearance of PGCs reported by Byskov (1978) and McLaren (1983) was via

the use of the genital ridges without mesonephros, and it was thought that the genital ridges had been damaged by microsurgery. The c-kit and SCF were reported as being the factors related to migration of PGCs (Matsui *et al.*, 1991, 1992; Pesce *et al.*, 1997). SCF is expressed on the path toward the genital ridges and fibroblast cells, and c-kit is expressed on PGCs. It was also reported that PGCs emerged from epiblasts at day 6.0–6.5 after mating on STO feeder cells or on fibronectin (Yoshimizu *et al.*, 2001). Fibronectin is an extracellular matrix that is related to adhesion and migration of cells (Berrios *et al.*, 2001; Park *et al.*, 2001). Some motile PGC-like cells were observed in the present study. It was suggested that PGC-like cells appearing outside the gonads might involve these factors, because PGC-like cells adhered to fibroblast-like cells, and many migrating PGC-like cells were observed in this study.

In addition, PGC-like cells increased in numbers, maintaining ALP activity in cultures with rodent sera *in vitro*. This result showed that the increase in number of PGC-like cells was greater than that of PGCs derived from embryos at day 11.5 after mating (Koshimizu *et al.*, 1996). Three reasons are proposed to account for the appearance and proliferation of PGC-like cells outside the gonads: (1) The medium supplemented with rodent serum was similar to that inside the gonads, (2) PGCs emerged outside the gonads because of the increase in number of PGCs, which filled the gonads, and (3) PGCs moved to the outside of the gonads, because the internal structures of the cultured organ were disorganized and changed the internal environment. Generally, the number of germ cells decreases sharply when meiosis is initiated; Allan *et al.* (1987) reported that this decrease occurs by apoptosis. Apoptosis factors are expressed in gonads (De Felici *et al.*, 1999; Reynaud and Driancourt, 2000; Riccioli *et al.*, 2000; Xu *et al.*, 2000). Fas, Fas-ligand, and Bax are expressed in adult and fetal testis (Francavilla *et al.*, 2000; Rucker *et al.*, 2000). The ovary expresses Fas-ligand (Guo *et al.*, 1997), Bax (De Felici *et al.*, 1999) and TGF- β (Kolaja and Klaassen, 1998). In addition, Fas and Fas-ligand are present in the serum (Iwama *et al.*, 2000). Saito *et al.* (1999) suggested that the Fas-ligand in the serum is able to cause damage to the tissues. If cells inside of the gonads expressed apoptosis-inducing factors, it was thought that the supplemented sera disorganized the structures of fetal gonads, and PGCs lost the locations where they normally differentiate and they emerged outside the gonads via the SCF and c-kit system, or the motility affected the extracellular matrix between fibroblast cells. The marked increase in PGC-like cells and the disorganization might therefore be caused by factors derived from rodent sera in the intra-species culture compared with the other sera. More studies on the emergence of PGC-like cells may elucidate the mechanism of migration of PGCs.

PGCs at day 12.5 after mating have the capability to differentiate gametes and dedifferentiate stem cells. The

disappearance of totipotency and differentiation of PGCs might be related to the fact that the majority of genomic imprinting is erased during 11–12 days after mating (Tada *et al.*, 1998; Durcova-Hills *et al.*, 2001; Obata *et al.*, 2002). However, the entire process of genomic imprinting and maturation factors has never been clarified. PGCs are an interesting source for the study of meiosis, genomic imprinting, mobility and totipotency. Therefore, this method of culturing PGC-like cells is important in the study of gametogenesis, because this method is useful in maintaining PGCs *in vitro*. Furthermore, by using this method, it is thought that EG cells may be easily obtained, and thus this method may be useful in studying stem cells and germ cells.

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