F-3.2.1 Study on the mechanism of growth and differentiation of primordial germ cells

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Abstract Transplantation of primordial germ cells (PGCs) of endangered avian species into embryos of other birds is expected to be a feasible manner to preserve and multiply the endangered wild life. The final goal of this study was to examine the mechanism of in vitro growth and differentiation of quail PGCs. Toward this goal, a method for the purification of quail PGCs was developed. Then, monoclonal antibodies SSEA-1 and EMA-1 were shown to specifically stain quail PGCs. These monoclonal antibodies would be useful in the identification of quail PGCs cultured in vitro. Based on these achievements, quail and chicken PGCs were cultured in vitro on quail or chicken embryonic fibroblast feeder cells in the presence of various growth factors including chicken stem cell factor amd mouse leukemia inhibitory factor. Although a short-term proliferation of chicken PGCs under these conditions was seen, the growth of quail PGCs was not observed.

Key Words avian, primordial germ cells, endangered wildlife, in vitro culture, differentiation

1. Introduction

In chickens, the production of germline chimeras upon translpantation of primordial germ cells (PGCs) taken from the blood of embryos, and of viable progenies derived from the transplanted PGCs has been reported[1,2]. In addition, the cryopreservation of PGCs and subsequent production of germline chimeras by transfer of the cryopreserved PGCs have been performed[3,4]. Therefore, transplantation of PGCs of endangered avian species into other birds is expected to a feasible manner to preserve and multiply the wild life. However, as shown with chickens, the number of PGCs in the blood of an avian embryo is small [5] . Meanwhile, the in vitro proliferation of mouse PGCs on mouse fibroblast feeder cells with the aid of growth factors including mouse stem cell factor (SCF), and mouse leukemia inhibitory factor (LIF) has been reported[6,7,8]. Thus, if avian PGCs could proliferate in vitro as mouse PGCs do, the preservation of endangered birds by transplantation of their PGCs would be greatly facilitated. However, there is a possibility that the PGCs which have grown in vitro could lose the ability to differentiate into functional gametes. Therefore, the development of an experimental system for the evaluation of the PGCs

which are manipulated in vitro under various conditions for the ability to develop into functional gametes is required. In such a system, germline chimeras are made by transfer of the PGCs into embryos, and, after sexual maturation of the chimeras, the chimeras are examined by mating them with control birds. A series of techniques required for these processes have been established with chickens[1,2,3,4,5]. However, the sexual maturation period of chickens is long (5 months), making it inappropriate to use them for the evaluation of PGCs manipulated under various in vitro conditions for the ability to give rise to functional gametes. Quail is a more appropritate model species because sexual maturation period of quail (1.5 months) is much shorter than that of chickens. Thus, in 'Development of an experimental system for the evaluation of manipulated primordial germ cells for the ability to develop into functional gametes' (F-3.2.2), the techniques prerequisite for making quail germline chimeras have been developed.

In this study, a method for the purification of quail PGCs was developed. And, reactivity of quail PGCs to monoclonal antibodies (MAbs), SSEA-1[9] and EMA-1[10], was examind with a hope that these reagents could be used for the identification of quail PGCs. Then, in vitro culture of quail and chicken PGCs on quail or chicken fibroblast feeder cells was conducted.

2. Research objectives

(1) To develop a method for the purification of PGCs from the blood of quail embryos at 2.5 days of incubation.

(2) To examine whether SSEA-1 and/or EMA-1 stained quail PGCs in a cell-type specific manner.

(3) To examine whether quail and chicken PGCs are able to proliferate in vitro on avian fibroblast feeder cells in the presence of various growth factors.

3. Research methods

(1) Quail and chicken fertilized eggs used in this study

In this study, fertilized eggs of wild-type Japanese quail and of White Leghorn chickens were used. The eggs were incubated at 38° C and used in the experiments.

(2) Preparation of quail PGCs

For the preparation of PGCs from the blood of quail embryos at 2.5 days of incubation, the blood was collected with a fine glass pipette, and suspended in M199 medium containing 10% fetal calf serum (FCS) (M199/10% FCS). After centrifugation, the cell pellet was suspended in 100 μl M199/10% FCS, and then mixed with 900 μl of M199/10% FCS containing various concentrations of Ficoll (Type 400DL, Sigma). This suspension was placed in a 1.5 ml eppendorf tube, and 200 μl of PBS was gently placed onto the suspension. After centrifugation of the tube at 1200 g for 30 min, a portion around the interphase (400 μl) was saved. After washing, the number and purity of PGCs in the interphase was examined under a phase-contrast microscope.

(3) Staining of quail PGCs with MAbs SSEA-1 and EMA-1

SSEA-1 and EMA-1 were purchased from the Developmental Studies Hybridoma Bank. These MAbs were labeled with fluorescein isothiocyanate (FITC) using a standard protocol. Ten μl of the blood of quail embryos at 2.5 days of incubation was washed,

and incubated with the MAbs labeled with FITC (250 μg/ml) in M199 containing 1% FCS (M199/1% FCS) at 4° C for 1h. After washing with M199/1% FCS, the cells were observed under a fluorescence microscope.

To examine the reactivity of the MAbs to quail PGCs in the gonads of embryos at 5 days of incubation, the gonads were cut out from the embryos and treated with 0.05% trypsin/ 0.53% EDTA. After washing, the resulting cell suspensions were stained with the MAbs as described above, and then observed under a fluorescence microscope .

(4) Culture of quail and chicken embryonic fibroblast cells

Quail and chicken embryos at stages 14 - 16[11] were taken, and the heads and internal organs of the embryos were removed. The remaining parts were cut into tiny pieces and digested with 0.05% trypsin/ 0.53% EDTA. The resulting cells were cultured in a 75 ml plastic culture bottle with 10 ml of KAv-1 medium[12] containing 5% FCS and 5% chick serum (complete KAv-1 medium). Subculture of the cells was done every 3 - 4 days.

(5) In vitro culture of quail and chicken PGCs

The confluent culture of quail and chicken fibroblast cells were digested with 0.0 5% trypsin/ 0.53% EDTA, and cell suspensions at a concentration of 2 x 10^6/ml were made. To each well of a 96-well plastic culture plate, 200 μl of the cell suspension was seeded, and incubated overnight at 37° C. After removing the medium, 400 to 1000 quail or chicken PGCs purified as described in Research methods (2) were placed on the layer of the fibroblast cells. Then, PGCs were cultured in 200 μl of the complete KAv-1 medium or M199/20% FCS both of which contained growth factors in several combinations. Half of the medium was changed with fresh one every 2 days.

Growth factors used in the culture included recombinant chicken SCF (100 ng/ml), recombinant mouse LIF (104 units/ml), recombinant human interleukin-6 (IL-6) (50 ng/ml), bovine basic fibroblast growth factor (bFGF)(10 ng/ml), rat ciliary neurotrophic factor (CNTF)(20 ng/ml), and human transforming growth factor-α (TGF-α)(10 ng/ml). Recombinant chicken SCF was prepared by us[13], and other factors were commercially pur chased.

4. Results

(1) Purification of quail PGCs

It was found that the recovery rate and purity of PGCs were high when the Ficoll density gradient centrifugation was performed after the blood cell suspension was mixed with M199/20% FCS containing Ficoll at a concentration of 16.0%. Under these conditions, 15 - 30 PGCs per embryo with a purity of 55% on average were obtained.

(2) Staining of quail PGCs with MAbs SSEA-1 and EMA-1

In the blood of quail embryos at 2.5 days of incubation, the proportion of PGCs is approximately 0.01%. Upon the staining of the blood with the MAbs, it was found that both of SSEA-1 and EMA-1 positively stained PGCs, and that either MAbs did not stain any other kind of cells. However, the intensity of staining with either MAb was low, and it appeared that only a part of PGCs could be recognized as positively stained. Meanwhile, a small proportion of PGCs prepared from the gonads of 5-day embryos was found to be positive for staining with EMA-1 and this MAbs did not stain any other cells. However, the intensity of staining with EMA-1 was much lower than that

observed with PGCs from the blood. By contrast, specific staining of PGCs from the gonads with SSEA-1 was not detected.

(3) In vitro culture of quail and chicken PGCs

Cultures with all combinations of feeder cells and PGCs (quail PGCs on quail feeder cells, quail PGCs on chicken feeder cells, and so on) were performed. The growth of chicken PGCs were observed when they were cultured on quail feeder cells in complete KAv-1 medium containing all of the six growth factors. The PGCs appeared to grow within 6 days from the beginning of the culture, and the number of PGCs increased by approximately 5 fold. However, the number of the PGCs decreased thereafter. By contrast, the growth of quail PGCs were not observed under the same conditions. Under other conditions tested, no growth of quail or chicken PGCs was seen.

5. Discussion

(1) Purification of quail PGCs

The number and purity of quail PGCs obtained by the procedure described in Results (2) are comparable to those reported on the purification of chicken PGCs[5,14], indicat ing that the method for purification of quail PGCs has been established.

(2) Staining of quail PGCs with MAbs

It has been reported that mouse PGCs are positive for staining with SSEA-1 and EMA -1[10]. In this study, at least a part of quail PGCs in the blood of embryos were shown to be positive for staining with SSEA-1 and EMA-1. This staining is specific in a sense that any other kind of cells in the blood were not positively stained. The chicken PGCs in the blood were positive for SSEA-1 and EMA-1 (unpublished data by us). However, the intensity of the staining of quail PGCs with these MAbs was much lower th an that of chicken PGCs, suggesting that the antigens recognized by these MAbs are expressed on quail PGCs at much lower levels than those on chicken PGCs. As with PGCs prepared from the gonads, only a small part of them were recognized as positive for staining with EMA-1. It appears that the expression level of the antigen recognized by EMA-1 decreases as the development of embryos proceeds. However, also in this case, the staining with EMA-1 was specific in a sense that other kinds of cells in the gonadal cell preparation were not stained positively. Therefore, it is conceivable th at quail PGCs could be identified with these MAbs. However, for this purpose, it would be required to increase the sensitivity of the staining, for example, by using MAbs labeled with phycoerythrin.

Yoshinaga et al. have reported the specfic binding of lectins, WFA and STA, to quail PGCs[15]. Meanwile, Pardanaud et al. have reported that a MAb QH-1 binds quail PGCs and endothelial cells in a cell-type specific manner[16]. In this study, the reactivity of these reagents to quail PGCs was also examined. Specific staining of PGCs with WFA labeled with FITC was observed when the blood of embryos were stained with this lectin. However, when the cells of the gonads were stained with WFA, cells other than PGCs were also positively stained. The QH-1 was found to stain the cells prepared from the gonads in a non-specific manner. On the other hand, no positive staining of cells including PGCs with STA was obserbed.

(3) In vitro culture of quail and chicken PGCs

It has been reported that mouse PGCs grow in vitro on mouse fibroblast feeder

cells in the presence of growth factors including SCF and LIF[6,7,8]. Based on these results, and according to the suggestion by Dr. T. Kuwana at National Institute for Minamata Disease (personal communication), in vitro culture of quail and chicken on avian embryonic feeder cells was conducted. As a result, in vitro growth of only chicken PGCs was observed, while the growth of quail PGCs was not seen. However, because the cytological characteristics of quail PGCs are similar to those of chicken PGCs, it is conceivable that quail PGCs would proliferate in vitro under the conditions similar to those described in this study. In this sense, the result in this study would provide a basis for in vitro culture of quail PGCs.

6. References

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