F-3.1 Studies on the production of interspecific chimera using primordial germ cells.

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Abstract

Aims to proliferate a small number of primordial germ cells (PGCs) of threatened birds, the embryonic blood was analyzed as a environment of the PGCs, and KAv-1 medium having a pH 8.0 was established. Using this culture medium, the survival rate of PGCs *in vitro* is surprising improved, and they increased their number at over a hundred times for 7 days incubation on the embryonic feeder cells with growth factors.

Introduction

There are at least 49 species of threatened animals in Japan. In the present condition, we can not help to stock the cells include germ cells in liquid nitrogen for the future reproduce of extinct animals. However, it might become common knowledge that the freeze stock of the cells in liquid nitrogen is not so permanent, and so, we have to develop a new technique for the reproduce of extinct or threatened animals as soon as possible. Especially, it is impossible to cryopreserve the fertilized eggs in avian, because the eggs contains a great amount of yolks. For this reason, there is no technique to reproduce offspring after cryopreservation of sperms and oocytes in the case of avian species.

In spite of such faults in birds, we can already isolate PGCs, which are the

original cells of sperm and oocytes, from the embryonic blood stream, transplant the isolated PGCs into another embryonic blood stream, and get offspring derived from the transplanted PGCs in the chicken. This method might be a good because the donor embryo of PGCs is not damaged by the procedure of PGCs isolation. Furthermore, we already established the method of cryopreservation for the avian PGCs using liquid nitrogen.

Avian PGCs segregate from the hypoblast of the so-called germinal crescent region, and accumulate between the endoderm and ectoderm during stages 4 to 8 (staging of Hamburger & Hamilton, 1951). They begin to circulate via bloodstream along with the establishment of embryonic blood circulation till stage 10. After leaving the blood circulation from the capillaries near the GE, they migrate interstitially, as in mammalian PGCs and finally penetrate the developing gonad by 2.5 days of incubation (Swift, 1914; Fujimoto et al., 1976). In the circulating phase, PGCs could be easily isolated from embryonic blood samples. Moreover, by the transplantation of these isolated PGCs into blood vessels of other embryos, we could effectively produce PGC chimeras and gain offspring derived from donor PGCs (Kuwana, 1993). Avian PGCs may be the best candidate as a vehicle to increase threatened birds, and so it would be essential to culture them *in vitro* because it would be very difficult to collect a lot of PGCs from the fertilized eggs of threatened birds.

In cell culture of chick and quail, the primary culture is generally performed. In fact, it is not so difficult to obtain a cell population originating from adult tissues using a common culture medium at pH from 7.2 to 7.4 (Novero and Asem, 1993). Although cells proliferate rapidly during the initial period of subculture, their proliferation gradually decreases and stops within a few weeks. In the case of embryonic cells, however, such a long-term subculture has been difficult. The embryonic cells could be subcultured using the medium with chick embryo extracts at 7.2 to 7.4 (Watanabe et al., 1989). But, in chick or quail, there has been no report to our knowledge on subculture of cells originating from young embryo before 3 days incubation. Moreover, the development of subculture methods may succeed in establishing avian normal cell lines.

Though we have tried many culture mediums for chick or quail embryonic cells, the long-term subculture has not been performed at the standard pH range. The pH of the fluid around cells *in vivo* was finally realized to be one of the important factors in their culture. In the present study, the pH of embryonic blood, the yolk just behind the embryo and albumen as the environment of the embryonic cells, was measured in chick and quail embryos.

Research Objective

There are at least 49 species of threatened animals in Japan. In the present condition, we can not help to stock the cells include germ cells in liquid nitrogen for the future reproduce of extinct animals. However, it might become common knowledge that the freeze stock of the cells in liquid nitrogen is not so permanent, and so, we have to develop a new technique for the reproduce of extinct or threatened animals as soon as possible. Especially, it is impossible to cryopreserve the fertilized eggs in avian, because the eggs contains a great amount of yolks. For this reason, there is no technique to reproduce offspring after cryopreservation of sperms and oocytes in the case of avian species.

In spite of such faults in birds, we can already isolate PGCs, which are the original cells of sperm and oocytes, from the embryonic blood stream, transplant the isolated PGCs into another embryonic blood stream, and get offspring derived from the transplanted PGCs in the chicken. This method might be a good because the donor embryo of PGCs is not damaged by the procedure of PGCs isolation. Furthermore, we already established the method of cryopreservation for the avian PGCs using liquid nitrogen.

In the circulating phase, PGCs could be easily isolated from embryonic blood samples. Moreover, by the transplantation of these isolated PGCs into blood vessels of other embryos, we could effectively produce PGC chimeras and gain offspring derived from donor PGCs (Kuwana, 1993). Avian PGCs may be the best candidate as a vehicle to increase threatened birds, and so it would be essential to culture them *in vitro* because it would be very difficult to collect a lot of PGCs from the fertilized eggs of threatened birds.

This study aims to restore and reproduce the threatened birds by the biotechnological techniques using re-injection of primordial germ cells into the embryos of other host species similar to the technique used to obtain avian germ-line chimeras as in chick. Especially in the present study, the pH of embryonic blood, the yolk just behind the embryo and albumen as the environment of the embryonic cells, was measured in chick and quail embryos to establish the culture method for PGCs of threatened birds *in vitro*.

Research Methods

pH measurement

Fertilized eggs of White Leghorn and Japanese quail were incubated at 38.5°C

and 60% relative humidity in a forced air incubator (P-800, Showa Incubator Lab., Japan) to obtain embryos at various developmental stages (Hamburger and Hamilton, 1951). The pH levels of embryonic blood, yolk and albumen as the environment of the embryonic cells were measured at various developmental stages.

Embryonic blood samples of 2 μl were collected from the embryos at stages 12, 13, 14, 15, 16, 17, 18 and 25, and from 6- and 11-day embryos and at hatching. Yolks which presented just behind the embryo and thin albumen were collected from the eggs at stage 1 to hatching. All specimens were collected using glass micro-capillaries which were rinsed 3 times by double distilled water before use. After collecting from the eggs, approximately 2 μl of each specimen was immediately measured for pH by pH BOY-C1 (Shindengen, Japan).

Moreover, thin albumen and yolk just behind an embryonic disk from unincubated fertilized chick eggs were used for the pH measurement.

Source of cultured cells

Chick: Germinal crescent region at stage 3-4
Germinal crescent region at stage 3-5
Germinal crescent region at stage 4-8 (SPF embryo)
Whole embryo at stage 4-8 (SPF embryo)
Whole embryo at stage 13
Primordial germ cells (PGCs) from stage 15 embryo
Presumptive gonadal region and vascular area at stage 17
Whole embryo at stage 20
Developing gonads of stage 25 male embryo (SPF embryo)
Developing gonads of stage 25 female embryo (SPF embryo)
Whole male embryo at stage 25 (SPF embryo)
Whole female embryo at stage 25 (SPF embryo)
Developing gonads of day-4 embryo (stage 24)
Developing gonads of day-5 embryo (stage 27)
Developing gonads, heart and liver of day-7 embryo (stage 31)

Quail Germinal crescent region at stage 3-5 (stage 1-3 of Zacchei, 1961)
Whole embryonic body of stage 11 (L2 line)
Whole embryonic body of stage 11 (H2 line)
Presumptive gonadal region and Area vasculosa at stage 17 (stage 14 of Zacchei)

Developing gonads, heart and liver of day-7 embryo (stage 31; stage 22 of Zacchei)

Testis, heart and liver of hatching (male) (stage 46; stage 33 of Zacchei)

SPF eggs of White Leghorn were obtained from Nisseiken Co., Ltd. (Yamanashi, Japan). Fresh quail L2 and H2 inbred line eggs (the 47th generation) (Takahashi et al., 1984) were obtained from the animal center of the National Institute of Pollution.

Establishment of subculture conditions for embryonic cells in birds

Cells from chick and quail embryo were cultured with KAv-1 medium; α-MEM (GIBCO BRL, USA) added with 1 mM D-glucose, 5×10^{-5} M 2-ME, and 10 mM EPPS (Wako Chem., Japan), containing 5% of FBS (JRH Biosciences, USA) and CS (JRH Biosciences, USA). Medium pH was adjusted by NaCO3 to the designated pH (8.0), similar to that of the embryonic blood in air condition.

Five ml of the KAv-1 medium in the plastic culture flask (No. 25102S, CORNING Co., Ltd., USA) was changed every two days, and the cells were subcultured when the cells proliferated so as to cover 90% of the culture area.

Viability of PGCs

Viability of PGCs was compared at two different pHs using KAv-1 medium. Chick PGCs were collected from embryonic blood at stage 13-15 according to Kuwana & Fujimoto (1984) using KAv-1 medium. Each 100 PGCs were cultured with KAv-1 medium at pH 7.4 or 8.4. The PGCs were cultured in these conditions for 4 h or 24 h, and the viability of the PGCs was confirmed under phase contrast microscopy by morphological criteria and absorption test of trypan blue.

PGCs proliferation in vitro

Chick PGCs were collected from the embryonic blood of stage 13 chick embryos, and purified under phase contrast microscopy. Isolated pure PGCs were collected and put on the chick feeder cells originated from stage 16 SPF chick embryonic fibroblast which had been treated by mitomycin C (MMC; 4 μg/ml for 2 hrs) just before the experiment. After 7 days incubation, the number of the PGCs were calculated under phase contrast microscopy in the cases with growth and without factors.

Result

a) Transition in pH as environment of embryonic cells in chick

1) Embryonic blood

Chick embryonic blood circulation begins at stage 10, and one can suck out 2 μl of blood only after stage 12. The transition in the blood pH was shown in Table 1 and Figure 1. The pH at stage 12 indicated 8.4 and then ranged 7.7 to 8.4 throughout the embryonic period. The blood pH at stages 12 to 18 ranged from 8.1 to 8.5 (average 8.3).

2) Yolk

The pH of yolk just behind the embryo was measured after stage 1 till embryos were 6 days old (stage 25). The pH value of the yolk just behind the embryo indicated an acidic value till 35 h after incubation and then increased gradually till stage 25 (108 h of incubation time).

3) Albumen

Unincubated fertilized egg albumen had a pH of approximately 9.3, then decreased gradually.

Throughout stage 12 to 25, the pH of embryonic blood consistently ranged between that of the yolk and the albumen.

The transition in pH in quail also showed a result similar to that in chick (data not shown).

b) Establishment of subculture conditions for embryonic cells in birds

In the present study, the culture medium pH was adjusted to 8.0 in air. All the cells proliferated well in this culture condition, and 6 kinds of cells, which were tried to subculture for a long-term, were subcultured for over 3 months (20 passages) except liver and primordial germ cells. Finally, all the subcultured cells was stocked in liquid nitrogen at the respective number of passages.

In preliminary experiments, we attempted to culture the chick cells of germinal crescent region at stage 3-5 with KAv-1 adjusted to pH 7.2 and KAv-1 adjusted to pH 8.0. At pH 8.0, the cells had been subcultured over 3 months as described above. In contrast, when the culture medium pH was adjusted to 7.2, all the same cells had died at the 10th and 14th passage.

d) Viability of PGCs

When 100 PGCs were cultured at pH 7.4, their viability was 5.75% after 4 h

and 3.75 % after 24 h respectively. When 100 PGCs were cultured at pH 8.0, their viability was 95% after 4 h and 18.25 % after 24 h, respectively (Table 3). In the present experiments, the PGCs were considered dead cells when their periphery was not demarcated, bubbling occurred on their surfaces or they were taken to be completely destroyed. Additionally, the viability of the PGCs was reconfirmed by the absorption test of trypan blue.

e) PGCs proliferation in vitro

After 7 days, the PGCs still alive in this situation but the number of the cells does not increased meaningfully. In contrast, when the PGCs was cultured in this situation with additional growth factor, the number of the cells increased approximately a hundred times after 7 days incubation compared to that of the controls.

Discussion

There are no established conditions to culture avian cells for a long time. The present study indicates that the pH of embryonic blood as the environment of the embryonic cells was higher than that of the former medium conventionally used for cell culture (7.2 to 7.4). Additionally, the cells from all tested tissues except liver and PGCs could be subcultured at pH 8.0. In the case of PGCs and liver, cells did not proliferate in our culture condition.

In the present study, the high pH (8.0) of culture medium as an environment was demonstrated to be essential for long term culture of avian embryonic cells. Though it was not be clear why such a high pH is essential for such long-term cultivation, embryonic blood pH seemed to be controlled by a balance between the pH of the albumen (pH 9.4-9.65) and that of the yolk (pH 6.1-7.9), as shown in Table 1 and Figure 1. As shown in Table 1, the pH of blood was over 8.0 till 108 hr incubation (the end of early embryogenesis). This result suggests that the high pH may play some role in the proliferation of undifferentiated cells in the early development of the avian embryo and that the decrease of pH may trigger the next developmental steps.

This pH condition for avian cell culture may make it possible to establish new cell lines from not only embryonic cells but also adult cells. Moreover, the viability of the PGCs *in vitro* greatly improved by using KAv-1 medium at pH 8.0. This suggests that a culture medium of pH 8.0 might have wide application in the field of avian embryonic manipulations. Furthermore, the PGCs proliferated at over hundred times on the chick embryonic feeder cells using KAv-1 medium with cell growth factors for

7days suggesting the high possibility of the recovery of threatened birds by our biotechnological method.