Establishment and some Biological Characteristics of a Cell Line Derived from Yamame Embryonal Liver

Tadaaki Moritomo,* Hiroyoshi Asakawa,* Masanori Nakano,*
and Tasuku Watanabe*

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We tried to cultivate fish liver cells using the embryonated eggs of Yamame, landlocked form of masu salmon, *Oncorhynchus masou*. A continuous cell line designated YEL-13 was established and has been subcultured more than 120 times over the period of 3 years. YEL-13 cells grow in the manner of contact inhibited monolayer and consisted of epithelial cells and a few fibroblastic cells. Electron microscopically, most of the cells had a large and round nucleus and well-developed rough surfaced endoplasmic reticulum in cytoplasm and smooth cell surface with poorly developed microvilli. Chromosome number of YEL-13 ranged widely and most of them were between 50 and 83. The cells with 56 chromosomes occupied 24% of those examined. YEL-13 cells have the permissible temperature range of 15–20°C for growth. The cell line has been producing and releasing a Yamame serum protein constantly which is one of the main functions of liver parenchymal cells.

In vitro cultivation of liver cells with functional characteristics common to *in vivo* liver cells is of potential value in many investigations. In recent years, there have been many attempts to cultivate liver cells using the liver tissues of mammals and lower vertebrates¹⁾ including fish.^{2,3)} Several continuous cell lines have been obtained from the liver of mammals,^{4,5)} amphibian⁶⁾ and fish.⁷⁾ Some of them retain several specific liver functions.^{8,9)} These cell lines are available in various studies such as cell differentiations, malignant transformation, or cell metabolisms.^{9–11)}

Recently, the importance of the functional cell culture, especially liver cell culture of fish have been recognized in the research field of fisheries sciences. Cultured fish liver cells will give us a good tool in studying physiology and biochemistry of fish liver. We tried to establish a cell line which had a differentiated liver function using embryonated eggs of Yamame, landlocked form of masu salmon *Oncorhynchus masou* indigenous to Japan.

dipped in a solutions containing 100 I.U./ml of penicillin G potassium for 24 h. After the surface of eggs was disinfected with 70% ethanol, egg membrane and yolk were removed under binocular microscope (Fig. 1). Liver tissues were transferred in sterilized petri dishes and minced into small fragments with scissors in small amount of TC199 medium supplemented with 10% fetal bovine serum (199-10), transferred into plastic tissue culture flask (Corning Glass Work, NY, USA) and then cultivated at 20°C. One half of the medium was replaced by fresh one twice a week throughout the period of primary culture. Each flask contained liver cells derived from 3–5 eggs for the primary culture and were designated YEL-. When cells grew to form confluent monolayer cell sheet, cells were trypsinized, and reseeded at the density of $1-3 \times 10^5$ cells per flask. The doubling time (D.T.) was calculated as described previously.¹²⁾ The cells after 20th passage were cultivated with Eagle's minimum essential medium supplemented with 10% fetal bovine serum (MEM-10).

Materials and Methods

Cell Culture

The embryonated eggs of Yamame obtained from Yamaguchi Fish Farm, Yamanashi. Upon the confirmation of few eggs hatched, eggs were

Chromosome Number

The cells between 85th and 90th passage of YEL-13 were used. Colchicine (Sigma, Mo., USA) was added in cultured medium to be a final concentration of 0.1 µg per ml. After in-

* Department of Veterinary Science, Nihon university, Kameino, Fujisawa 252, Japan (森友忠昭, 浅川洋美, 中野雅功, 渡辺 翼: 日本大学農獣医学部).

Fig. 1. Yamame embryo after removing the egg membrane and yolk. Arrow indicates liver.

Fig. 2. Phase contrast micrograph of the primary culture of Yamame embryonal liver 2 days after incubation. The cells migrating from tissue fragment.

Fig. 3. Phase contrast micrograph of the primary culture of Yamame embryonal liver 4 day after incubation. Small colony of epithelial cells.

Fig. 5. Monolayer cell sheet of YEL-13 cells, consisting of large epithelial cells and a few fibroblastic spindle-shaped cells.

Fig. 6. Electron micrograph of one of the typical YEL-13 cells. The cells had smooth surface with poorly developed microvilli and well-developed rough surfaced endoplasmic reticulum. $\times 17000$

cubated with the colchicine medium for 24 h at 20°C, the cells were trypsinized and treated with 0.075 M KCl hypotonic solution for 40 min. After swelling the cells were fixed with acetic acid-methanol (1:3) and stained with Giemsa solution.

Growth Rate

Five $\times 10^5$ cells of YEL-13 were seeded in tissue culture glass tubes and incubated at 10, 15, 20, and 25°C. Cell numbers were counted triplicate tubes on the 2nd, 4th, 7th, 11th, 15th, 19th and 23rd culture days. The media were renewed every second day. The growth curve of the cultured YEL-13 cells were made and compared at each temperature.

Immunological Methods

Rabbit antiserum against Yamame serum was prepared by intravenous injections at intervals of 7 days for 4 times and collected at the 7th day after the last injection. Antiserum was heated at 63°C for 20 min before use.

For indirect fluorescent antibody methods, YEL-13 cells were grown on glass coverslips for two days, fixed in cold acetone and stored at -20°C until use. In the staining procedures, diluted antiserum was applied to the cells for 1 h. After washing with phosphate buffered saline (pH 7.4, PBS), the cells were overlaid with a solutions of FITC-conjugated goat IgG against rabbit IgG (Cappel Labo. Inc., USA) for another 1 h. Then,

the cells were washed with PBS again and examined with a fluorescence microscope.

Cultured media, which had been in contact with the cells of YEL-8, 12 or 13 for 7 days, were harvested, pooled, concentrated 10 times and stored at -20°C until use. These concentrated cultured media were examined by immunodiffusion and immunoelectrophoresis using the methods described by Ouchterlony and Nillson with anti-Yamame whole serum.¹³⁾ Fetal bovine serum (FBS) and Yamame whole serum were used in both examinations as control.

Results

Primary Cell Culture

Flasks seeded with Yamame embryonated liver cells were observed daily with a phase contrast tissue culture microscope. Two day after the primary culture, epithelial and fibroblastic cells were observed migrating from tissue fragments in every flask (Fig. 2). After 3-4 days, several colonies consisted of epithelial cells were observed in various parts of the flasks (Fig. 3). These epithelial cells were multiplied to form colonies and terminated the growth in the manner of contact-inhibited monolayer in 28-38th day of primary cell culture. These colonies were trypsinized and reseeded in new flasks. Cell growth was not observed in 8 flasks, and fibroblastic cells grew and were predominant in other 2 flasks (Table 1).

Fig. 4. Doubling times and passages of YEL- cells from 2nd to 20th culture.

Fig. 7. Electron micrograph of a YEL-13 cell. Note the expanded rough surfaced endoplasmic reticulum (arrows). $\times 9300$

Fig. 8. Electron micrograph of a YEL-13 cell. Note the filaments in the cytoplasm (arrows). $\times 7500$

Fig. 9. Electron micrograph of a YEL-13 cell. Extensive electron-densed granules were observed in the leaf border of cytoplasm. $\times 6200$

Fig. 12. Immunofluorescent micrograph of YEL-13 cells. The cytoplasm were stained intensely by immunofluorescence with antiserum to Yamame serum.

Fig. 13. Immunoelectrophoresis of the mediums of YEL-cells (A) and diagram (B).

Table 1. Culture of Yamame embryonated liver cells

Flask number	Period of primary culture (days)	Passage number in 10th May, 1988
YEL- 1	—	
YEL- 2	—	
YEL- 3	37	
YEL- 4	33(F)	
YEL- 5	—	
YEL- 6	38	
YEL- 7	38	14
YEL- 8	38	40
YEL- 9	29	
YEL-10	—	
YEL-11	32	
YEL-12	29	21
YEL-13	28	120
YEL-14	30	
YEL-15	27	16
YEL-16	—	
YEL-17	28	20
YEL-18	—	
YEL-19	21(F)	
YEL-20	—	
YEL-21	26	
YEL-22	—	

—: No growth (F): Fibroblastic cells Others: Epithelial cells.

Passage of YEL

Secondarily passaged seven flasks, YEL-6, -7, -8, -12, -13, -15 and -17 showed epithelial morphology and grew rapidly to form a monolayer cell sheet until contact-inhibition restricted the growth. Fig. 4 shows the relationships between the doubling time and passage numbers of YEL-

8, -12, and -13. From the 3rd to 9th passage, cell growth of YEL-13 was quite slow. But the cells had shortened their doubling time gradually, and were able to be transferred at the intervals of 7–10 days after 10 passages. Cell growth of YEL-8 and -12 became slowly from 8th to 9th passage. But after then, growth of the cells were getting fast like YEL-13. As shown in Table 1, these cells were subcultured for 14 to 125 until April, 1988. YEL-13 has been subcultured for 125 times from its initiation in October, 1984.

Morphology of YEL-13

YEL-13 cells grew on the manner of contact inhibited monolayer until the formations of confluent cell sheets. The cells consisted of epithelial cells with round nucleus and abundant granular intracytoplasmic organelle suspected to be mitochondria (Fig. 5). A few fibroblastic spindle-shaped cells with small oval nucleus were observed.

Electron microscopically, most of the YEL-13 had a large and round nucleus and cell surface with poorly developed microvilli and well-developed rough surfaced endoplasmic reticulum (rER) in cytoplasm (Fig. 6). Some of these cells had expanded rER (Fig. 7). There were a few cells with an elongated nucleus often deeply indented and small filaments in cytoplasm as shown in Fig. 8. In the cytoplasm of those cells, numerous ribosomes were observed. And some of them had extensive electron-densed granules in the border of cytoplasm as shown in Fig. 9.

Chromosome Number

Fig. 10 represents the chromosomal distribu-

Fig. 10. Chromosome numbers of YEL-13 cells in colchicine metaphase.

Fig. 11. Growth curve of YEL-13 at various temperature.

tion patterns of YEL-13. Chromosome number of YEL-13 ranged widely and most of them were between 50 to 83. Count were made on 118 cells and 64% of them had chromosomes between 50 and 60. The cells with 56 chromosomes were 24% counted cell. Occasionally, tetraploid-like cells with more than 100 chromosomes were observed.

Protein Synthesis by YEL Cells

YEL-13 cells were examined under a fluorescent microscope, after reacting with rabbit serum against Yamame serum and with FITC-conjugated goat IgG against rabbit IgG. As shown in Fig. 12, the cytoplasm of YEL-13 was stained intensely by immunofluorescence with antiserum against Yamame serum.

A Yamame serum protein was detected in the concentrated culture medium, which were harvested from YEL-8, -12, and YEL-13 by immunoelectrophoretic study. As shown in Fig. 13, only one precipitating line was observed between these medium and antiserum against Yamame serum. No precipitating line was observed between FBS and antiserum. The double diffusion tests also showed that one of the serum protein were produced and released by YEL-8, -12, and -13.

Growth Rate of YEL-13 at Various Temperature

Fig. 11 represents the growth curve of YEL-13 cells at various temperature. YEL-13 cells grew

between 15°C and 20°C and grew most rapidly at 20°C. But at 25°C, all of the cells came off from the surface of the culture tubes within 10 days. Low temperature less than 10°C inhibited the cell growth of YEL-13.

Discussion

We succeeded in culturing liver cells from embryonated egg of Yamame for long period. Cells of YEL-7, -8, -13, -15, and -17 grew rapidly and were transferable. Of them, YEL-13 has been subcultured more than 120 times. Recently many efforts have been made to establish cell lines with properties of their original cells of tissues. Production of serum protein is one of the most important properties of liver parenchymal cells.^{14,15} In the culture media of YEL-8, -12, and -13, we detected a protein which reacted with antiserum against Yamame serum. These facts indicate that YEL- cells have the properties of producing and releasing one of Yamame serum proteins into culture medium. Continuously growing cell lines derived from mammalian liver tissues often have stable function, some keeping their functions even after long cultivation,^{5,8} while others losing their functions along with passage.^{4,16} Even after subcultured more than 120 times, it was confirmed that YEL-13 has been retained the property of production of the serum protein.

It is known that healthy Yamame (or masu salmon) has 66 chromosomes.^{17,18} The chromosome number of YEL-13 cells ranged widely and most of them were between 50 and 83. Such heteroploidy has not been reported in the tissues of Yamame. YEL-13 cell line suspected to be transformed and adapted to *in vitro* condition. But it is also well known that fish cell lines, which have been cultured for long period have varied chromosome numbers, if not cloned.^{19,20}

This cell line is supposed to consist of two or three cells morphologically. Cloning of the cells was not succeeded yet. An other liver functional study remain for further investigation.

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