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**Fetal-calf-serum-free culture of Chinese hamster ovary cells employing fish serum**

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**Abstract**

The effects of fish serum on cell growth and human granulocyte-macrophage colony stimulating factor (hGM-CSF) production in an adhesion culture of Chinese hamster ovary (CHO) cells DR1000L4N were investigated and compared with those of fetal calf serum (FCS). Although fish serum did not stimulate the initial adhesion of CHO cells to culture dishes, it prompted cell growth after cell adhesion with FCS for 24 h. The cell density in the fish serum medium reached 75% that in the FCS medium. Fish serum promoted cell adhesion to and cell growth on collagen-coated

dishes. The cell specific production rate of hGM-CSF in the fish serum medium on collagen-coated dishes was almost the same as that in the FCS medium.

## **Introduction**

Mammalian cell culture is an important and essential technology for pharmaceutical production and regenerative medicine. Cell adhesion and growth in culture generally require the addition of fetal calf serum (FCS) to the medium, because FCS can supply trace amounts of essential elements, such as hormones, vitamins and growth factors. However, the use of FCS should be avoided owing to possible contamination by pathogens, such as prions derived from infected calves. Thus, the development of new useful substitutes in place of FCS is strongly desired. They are required to have high medical safety, that is, no-containing virulent factors for humans, and a comparable effect on cell growth with FCS.

No DNA or RNA viruses infecting fish have been reported to infect humans to date (Yoshimizu and Kasai 2005). Thus, employing fish serum in mammalian cell culture for medically related use is expected to be safe compared with employing FCS. However, the stimulating activities of fish serum for the adhesion and proliferation of mammalian cells are unknown.

Insulin-like growth factor-I (IGF-I), insulin, growth hormone (GH), and

thyroxine were detected in the plasma of fish such as gilthead seabream (*Sparus aurata*) (Funkenstein et al. 1989), coho salmon (*Oncorhynchus kisutch*) (Larsen et al. 2001), atlantic salmon (Nordgarden et al. 2005), and channel catfish (*Ictalurus punctatus*) (Small and Peterson 2005). A cDNA encoding fibroblast growth factor-2 (FGF-2) has been isolated from the cDNA library of rainbow trout (Hata et al. 1997). A probable fibroblast growth factor was obtained from the swim bladder of red seabream (*Pagrus major*) (Suzuki et al. 1994). Furthermore, primary cultures of cells from fish gills and kidneys were developed using serum from *Clarias gariepinus* (Rathore et al. 2001). Therefore, fish serum is expected to have some growth-stimulating effects on mammalian cells.

The Chinese hamster ovary (CHO) cell line is industrially important in the production of pharmaceutical proteins such as human granulocyte-macrophage colony stimulating factor (hGM-CSF). In this study, the stimulatory effects of fish serum on the adhesion and proliferation of, and protein production by CHO cells were investigated and compared with those of FCS. This study should be the first report of the application of fish serum for mammalian cell culture.

## Materials and methods

### Cells and Media

hGM-CSF-producing CHO DR1000L4N cells were used. CHO DR1000L4N was constructed by transfecting CHO DG44 with the plasmid vector carrying the dihydrofolate reductase (DHFR) and hGM-CSF genes under the control of the SV40 promoter (Yoshikawa et al. 2000). Ham's F12-K medium (11.8 g/l; ICN, Aurora, OH, USA) containing NaHCO<sub>3</sub> (2.25 g/l, Wako Pure Chemicals Industry, Ltd., Osaka, Japan), 0.1 mg/ml streptomycin (Sigma, St. Louis, MO, USA), and 100 U/ml penicillin (Sigma) was supplemented with 10% FCS (v/v) (26140-079; Gibco, Grand Island, NY, USA) or fish serum (0–30%).

### Fish serum

Fish blood was collected from a red seabream (*Pagrus major*, approximately 30 cm in body length, approximately 1 kg in body weight, farm-raised in Ehime, Japan) using a syringe needle (23 G). The collected blood was left to stand for 2 h at room temperature and refrigerated at 4°C overnight. The fish blood was centrifuged (4°C, 3000 rpm, 15 min) to remove blood clots and the supernatant was filtered through a 0.22- $\mu$  m membrane filter. The filtrate was stored at –20°C until medium preparation.

## Cell culture

Cells were inoculated onto a 12-well plate (MS-80120; Sumilon, Tokyo, Japan) or a type-I-collagen-coated 12-well plate (4815-010; Iwaki, Tokyo, Japan) in a density range of  $2-4 \times 10^4$  cells/cm<sup>2</sup> employing several media, and then incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub> for 24 h. Then, the cells were washed with phosphate-buffered saline (PBS), composed of 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na<sub>2</sub>HPO<sub>4</sub> and 0.2 g/l KH<sub>2</sub>PO<sub>4</sub>, and further incubated for 48–72 h using several fresh media.

## General analysis

The cell concentration in the culture was determined by nuclei staining, in which the adhering cells were incubated in a solution of 21 g/l citrate and 1 g/l crystal violet, and nuclei were counted under a microscope (Sanford et al. 1950). The hGM-CSF concentration in the culture supernatant was measured using an enzyme-linked immunosorbent assay kit (Pierce Endogen, Rockford, IL, USA). Specific production rate of hGM-CSF ( $q_{\text{hGM-CSF}}$ ) was calculated by the following equation:

$$q_{\text{hGM-CSF}} = \frac{1}{\bar{X}} \cdot \frac{dP}{dt} \quad (1)$$

Where  $\bar{X}$  is the mean of the cell density and P is the increase in product (hGM-CSF) concentration during culture period for  $dt$ .

## Results

### Effects of fish serum on cell adhesion and growth

The effects of fish serum on cell adhesion and growth were studied (Fig. 1). CHO cells were inoculated onto the culture dishes with three types of medium containing 10% FCS, 10% fish serum, and no serum. Although the 10% FCS medium and serum-free medium resulted in cell adhesion rates of 88% and 50% after 24 h, the 10% fish serum medium (25%) has a much lower cell adhesion rate. The medium was changed to 10% fish serum medium at 24 h for one of the cultures in which cell inoculation was performed in 10% FCS medium and cells grew even after the change. The cell density of the culture in which the medium was changed from 10% FCS medium to 10% fish serum medium reached  $6.6 \times 10^4$  cells/cm<sup>2</sup> at 93 h, which was 74% of the cell density in the 10% FCS medium throughout.

### Effects of fish serum concentration on cell growth and hGM-CSF production

To investigate the effects of fish serum concentration on cell growth and

hGM-CSF production, the medium was changed to fish serum (0-30%) medium at 24 h after the inoculation with 10% FCS medium (Fig. 2). Twenty percent fish serum gave the highest cell density at 72 h among the tested fish serum concentrations and the cell density was 75% that in the 10% FCS medium throughout. The specific production rate of hGM-CSF in the 20% fish serum medium was the highest among those in the fish serum media and was 64% that in the 10% FCS medium.

#### Effects of fish serum on cell adhesion to and growth on collagen-coated dishes

To study the effect of fish serum on cell adhesion to collagen-coated dishes, CHO cells were inoculated onto normal culture and collagen-coated dishes with several media containing 10% FCS or fish serum, and the density of adhering cells was determined after 24 h (Fig. 3). The cell densities in the 10% FCS medium were highest among those in several media on both dishes, and the adhesion rates were 138% and 112% on the normal culture and collagen-coated dishes, respectively. The cell densities in the fish serum media (0-30%) on the normal culture dishes were very low, and the adhesion rates in such media were less than 35%. The cell densities in the fish serum media on the collagen-coated dishes depended on the concentration of fish serum, and 20% fish serum gave the highest cell density of 1.5

$\times 10^4$  cells/cm<sup>2</sup>, for which the adhesion rate was 91%.

To investigate the effect of fish serum on the growth of CHO cells on collagen-coated dishes, the cells were inoculated into 10% FCS or 20% fish serum medium and the medium was changed to 20% fish serum or serum-free medium after 24 h (Fig. 4). The cell density at 72 h ( $4.2 \times 10^4$  cells/cm<sup>2</sup>) in the 20% fish serum medium was markedly higher than that in the culture in which the medium was changed from 20% fish serum medium to serum-free medium. The growth rate (24-72 h) in the 20% fish serum medium was almost the same as that in the culture in which the medium was changed from 10% FCS medium to 20% fish serum medium, whereas the growth rate in the 10% FCS medium was higher than that in the culture in which the medium was changed from 10% FCS medium to 20% fish serum medium.

#### hGM-CSF production on collagen-coated dishes

The specific production rate of hGM-CSF during culture for 72 h on collagen-coated dishes using 10% FCS or 20% fish serum was determined (Table 1). There was no marked difference between the specific production rates of hGM-CSF in the media with 20% fish serum and 10% FCS.

## Discussion

Since there should be no report that fish serum is employed for mammalian cell culture, the effects of fish serum on the adhesion and growth of CHO cells were compared with those of FCS in this study. The density of adhering cells after 24-h incubation in the serum-free medium was only 57% that in the 10% FCS medium (Fig. 1). The density of adhering cells after 24-h incubation in the 10% fish serum medium was even lower than that in the serum-free medium. Thus, fish serum may inhibit cell adhesion on culture dishes. However, fish serum stimulated cell adhesion on the collagen-coated dishes (Fig. 3). The reason for the inhibitory effect of fish serum on cell adhesion is still unclear and whether this characteristic is common to other fish sera than *Pagrus major* serum should be studied in the future.

After cell adhesion to the culture dishes with the 10% FCS medium, the cell density in the 10% fish serum medium increased until 72 h and thereafter remained constant (Fig. 1). This time course of cell density was analogous to that of cell density in the 10% FCS medium. However, the concentration of 10% for fish serum should not be enough to stimulate cell growth as shown in Fig. 2. On the other hand, the cell density in the 20% fish serum medium after initial adhesion in the 10% FCS medium on the culture dishes was markedly higher than those for 0% and 10% fish

serum media and reached 75% that in the 10% FCS medium (Fig. 2). So, the promotion of cell growth needed the concentration of 20% for fish serum. The average cell doubling time in the 20% fish serum medium on the collagen-coated dishes (33 h) was comparable to that in the 10% FCS medium (19 h) (Fig. 4). Consequently, the growth-stimulating activity of serum from *Pagrus major* might not be markedly high but is sufficient for CHO cells.

The specific hGM-CSF production rate of cells grown in the 20% fish serum medium after initial adhesion in the 10% FCS medium was 64% that of cells grown in the 10% FCS medium (Fig. 2). Moreover, the cells harvested on the collagen-coated dishes with the 20% fish serum medium throughout entire culture period showed almost the same specific production rate of hGM-CSF as the cells cultivated in the 10% FCS medium (Table 1). Also, there was no apparent difference in the morphology of the CHO cells observed under a phase contrast microscope between the cells cultivated in the fish serum medium and those cultivated in the FCS medium (data not shown). Thus, there may be no difference in cell quality in practical hGM-CSF production between the cells grown in the medium containing fish serum and those grown in the medium containing FCS.

The cell growth and hGM-CSF production of cells after initial adhesion in the 10% FCS medium on the culture dishes (Fig. 2) and cell adhesion to the

collagen-coated dishes (Fig. 3) were maximum at the same concentration of fish serum (20%). Thus, this concentration (20%) may be sub-optimum for the effect of serum from *Pagrus major*. The sub-optimum concentration of 20% for fish serum was indeed higher than the common concentration of 10% for FCS. The cost of 20% fish serum medium should be compared with that of 10% FCS medium when the price of fish serum is decided in the future.

In this study, although serum from *Pagrus major* inhibited the initial adhesion of CHO cells to the culture dishes, its stimulatory effect on cell growth after cell adhesion with FCS was comparable with that of FCS. And it stimulated cell adhesion to and cell growth on the collagen-coated dishes. Thus, fish serum should be utilized instead of FCS in large-scale microcarrier culture with collagen microcarriers such as Cytodex 3. The application of fish serum to suspension culture should be investigated in the future.

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Fig. 1 Effects of fish serum on cell adhesion and growth of CHO cells. CHO DR1000L4N cells were inoculated onto media containing 10% FCS (○, ▲), no serum (△) and 10% fish serum (□). The medium was changed from 10% FCS medium to 10% fish serum medium at 24 h (▲). A dashed line represents the level of inoculum cell density ( $3.3 \times 10^4$  cells/cm<sup>2</sup>). Data represent the mean of triplicate

cultures.

Fig. 2 Effects of concentration of fish serum on cell growth and hGM-CSF production. CHO DR1000L4N cells were cultivated for 72 h on medium containing 0, 10, 20, or 30% fish serum or 10% FCS after cultivation for 24 h on 10% FCS medium. Data represent mean  $\pm$  SD (n=3). (\*:  $P < 0.05$ )

Fig. 3 Effect of fish serum on adhesion of CHO cells on collagen-coated dishes. CHO DR1000L4N cells were inoculated onto several media containing 10% FCS or fish serum (0 – 30%) on normal culture (open bars) and type-I-collagen-coated (closed bars) dishes. Density of adhering cells was determined after 24 h. Data represent mean  $\pm$  SD (n=3). (\*:  $P < 0.05$ )

Fig. 4 Effect of fish serum on cell growth on collagen-coated dishes. CHO DR1000L4N cells were inoculated onto collagen-coated dishes with media containing 10% FCS ( $\square$ ,  $\circ$ ) or 20% fish serum ( $\bullet$ ,  $\blacktriangle$ ). The medium was changed to 20% fish serum medium ( $\circ$ ) or serum-free medium ( $\blacktriangle$ ) at 24 h. A dashed line represents the level of inoculum cell density ( $2.0 \times 10^4$  cells/cm<sup>2</sup>). Data represent the mean of triplicate cultures.

Table 1 Specific production rates of hGM-CSF for CHO cells cultivated on collagen-coated dishes

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Serum <sup>a</sup>	Specific production rate of hGM-CSF <sup>b</sup> (10 <sup>-13</sup> mg/cell/h)
Fish serum (20%)	1.26 ± 0.31
FCS (10%)	1.21 ± 0.32

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<sup>a</sup>CHO DR1000L4N cells were inoculated onto collagen-coated dishes containing 10% FCS medium and 20% fish serum medium, and cultivated for 72 h

<sup>b</sup>Data represent mean ± SD (n=3)

Fig. 1

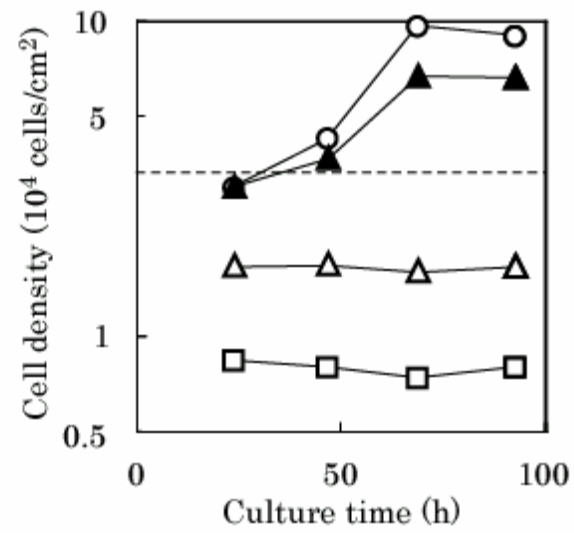


Fig. 2

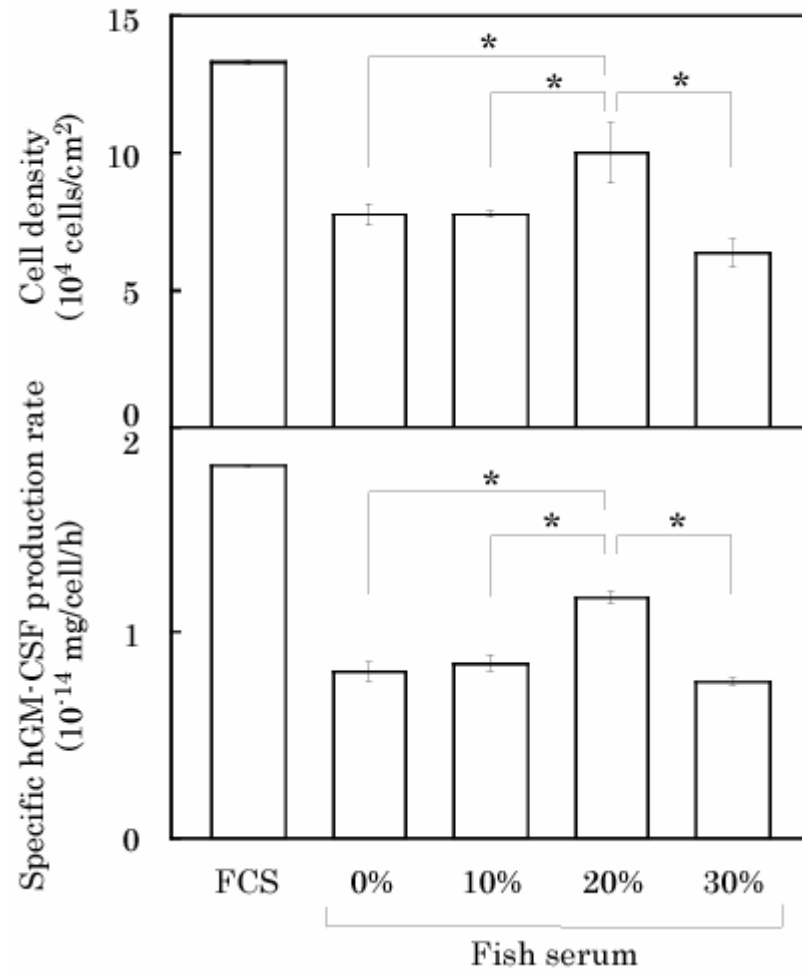


Fig. 3

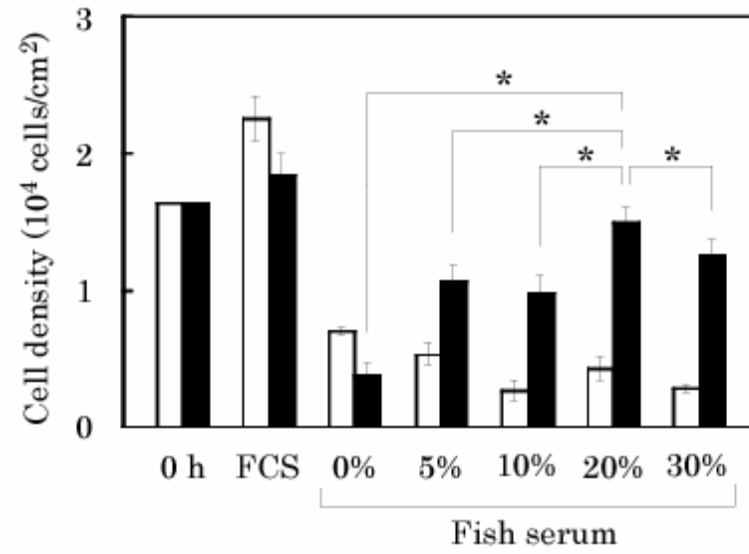


Fig. 4

