Effects of heat treatment and concentration of fish serum on cell growth in adhesion culture of Chinese hamster ovary cells

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Running Title: Adhesive culture of CHO cells with fish serum

[Abstract]

The effects of heat treatment and concentration of fish serum (FS) on cell growth and human granulocyte-macrophage colony-stimulating factor (hGM-CSF) production in an adhesion culture of recombinant Chinese hamster ovary (CHO) cells, DR1000L4N, were investigated. The addition of heat treated FS instead of non-heat-treated FS improved cell growth in terms of cell density, which reached 60% that in 10% fetal calf serum (FCS) -containing medium (FCS medium). A decrease in FS concentration from 10% to 1.25% markedly increased cell density, which was 79% that in 10% FCS medium. The combination of heat treatment at 56℃ and the addition of FS at a low concentration (1.25%) showed an additive effect on cell growth and resulted in the same cell density as that in 10% FCS medium, whereas the hGM-CSF concentration in the culture using FS-containing medium (FS medium) was approximately 50% that in 10% FCS medium. The total lipid concentration in FS was more than three fold that in FCS. The effect of decreasing FS concentration on cell growth may be due to the low lipid concentration in FS medium, because addition of the lipids extracted from FS to 10% FCS and 1.25% FS media markedly decreased cell density. Consequently, the addition of heat-treated FS at low concentrations to medium may be useful for the growth of CHO cells without FCS.

[Keywords]

Fish serum, Chinese hamster ovary cells, lipid, heat treatment, low concentration

[Introduction]

Mammalian cell culture is an important and essential technology in pharmaceutical production and regenerative medicine. Cell adhesion and growth in culture generally require the addition of fetal calf serum (FCS) to a 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 effective substitutes for FCS is necessary. Such substitutes are required to have high medical safety, that is, no containing virulent factors for humans, and an effect on cell growth comparable to that of FCS.

To date, no DNA or RNA viruses infecting fish have been reported to infect humans (Yoshimizu and Kasai, 2005). Thus, employing fish serum (FS) in mammalian cell culture for medically related use should be safer than employing FCS. However, the stimulating activities of FS 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 (Norgarden 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 the North African catfish (*Clarias gariepinus*) (Rathore et al., 2001). Therefore, FS 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 our previous study, the stimulatory effects of FS from a red seabream (*P. major*) on the adhesion and proliferation of, and protein production by CHO cells were investigated (Fujiwara et al., 2007). It was concluded that the use of some FS lots resulted in a cell density of only 75% that in the FCS-containing medium (FCS medium), whereas the use of other FS lots resulted in very low cell density (data not shown). Thus, cell culture using FS-containing medium (FS medium) needs to be optimized to become as suitable as that with FCS medium.

Animal serum generally contains complement system proteins. A complement system cascade may be activated during cell culture and lead to the death of cultured cells. Thus, FCS is often heat treated at 56℃ for 30 min to inactivate the complement system before addition to the cell culture. Therefore, heat-treated FS was expected to be effective in improving cell growth.

FCS is generally added to a culture medium at a concentration of 10%. However, to date, the concentration of FS has not yet been optimized to obtain maximum cell growth. Therefore, optimization of the concentration of FS in a culture medium may also be effective in improving

cell growth.

In this study, the effects of heat treatment and concentration of FS on cell growth and hGM-CSF production in an adhesion culture of recombinant CHO cells were investigated and compared with those of FCS.

[Materials and Methods]

Cells

hGM-CSF-producing CHO DR1000L4N cells were used. CHO DR1000L4N was constructed by transfecting CHO DG44 with a plasmid vector carrying the dihydrofolate reductase (DHFR) and hGM-CSF genes under the control of an SV40 promoter (Yoshikawa et al., 2000).

Fish serum

FS from a Japanese amberjack (*Seriola quinqueradiata*, approximately 50-55 cm in length, approximately 2-2.5 kg in weight, farm-raised in Oita, Japan) provided from Nippon Suisan Kaisha, Ltd. (Tokyo, Japan) and a red seabream (*P. major*; approximately 30 cm in length, approximately 1 kg in weight, farm-raised in Ehime, Japan) was prepared as follows. Fish blood was collected from a vein using a syringe needle. The collected blood was left to stand for 2 h at room temperature and then refrigerated at 4℃ overnight. The fish blood was centrifuged (4℃,

1700 × g, 15 min) to remove blood clots and the supernatant was filtered through a 0.22-μm-membrane filter. The filtrate was stored at －20℃ until medium preparation.

Heat treatment of fish serum

One ml of FS in a 15-ml centrifuge tube (MS-56150; Sumilon, Tokyo, Japan) was incubated in a water bath for 30 min at various temperatures of 46, 51, 56, and 61℃.

Extraction of lipids from fish serum

FS (9 ml) from a Japanese amberjack (*S. quinqueradiata*) in a 50-ml test tube was mixed with 30 ml of a 3:1 mixture of diisopropyl ether and buthanol, and vortexed for 3 h. The mixture was centrifuged (750 × g, 2 min, room temperature) and the upper layer was concentrated using a rorary evaporator to obtain FS lipids. The FS lipids were then twice purified by ethanol addition and evaporation.

Media

Ham's F12-K medium (11.8 g/l; ICN, Aurora, OH, USA) containing $NaHCO_3$ (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 mixed with FCS (10%) (26140-079; Gibco, Grand Island, NY, USA) or FS (0, 1.25, 2.5, 5, 10, 20, and 30%). The medium containing FS

lipids was prepared by adding the extracted FS lipids to 10% FCS medium or 1.25% FS medium at various concentrations (0, 0.125, 0.25, 0.5, or 1.0 mg/ml).

Cell culture

Cells were inoculated onto a 24-well plate (MS-80240; Sumilon, Tokyo, Japan) at a density of 2×10^4 cells/cm^2 employing 10% FCS medium, and then incubated at 37℃ in an atmosphere containing 5% CO_2 for 24 h to attach cells onto culture plates. 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_2HPO_4, and 0.2 g/l KH_2PO_4, and further incubated for 96-144 h in a fresh medium.

General analysis

Cell concentration in the culture was determined by nuclei staining, in which adhering cells were incubated in a solution of 21 g/l citrate and 1 g/l crystal violet, and the stained nuclei were counted under a microscope (Sanford et al., 1951). Human GM-CSF concentration in the culture supernatant was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Pierce Endogen, Rockford, IL, USA). Lipid content in FS and FCS was determined using an automatic biochemical analyzer (AU-5421; Olympus Corporation, Tokyo, Japan).

[Results]

Effect of heat treatment of FS on cell growth

The effect of heat treatment of FS on cell growth was studied. After the adhesion of CHO cells in 10% FCS medium for 24 h, the cells were cultured for 120 h in several media containing heat-treated (46, 51, 56, and 61℃) 10% FS (*S. quinqueradiata*), non-heat-treated 10% FS (*S. quinqueradiata*), 10% FCS, and no serum (0%) (Fig. 1).

The cell densities in the heat-treated FS media in all tested temperatures (46-61℃) were higher than that in non-heat-treated FS medium. Among the tested temperatures, the medium with FS treated at 56℃ provided the highest cell density, which was 60% that in the 10% FCS medium.

Effect of concentration of FS on cell growth

To investigate the effect of FS concentration on cell growth, CHO cells were cultured for 120 h in several media containing FS (*S. quinqueradiata*) at different concentrations of 0, 1.25, 2.5, 5, 10, and 20% or 10% FCS after cell adhesion with 10% FCS medium for 24 h (Fig. 2). Cell density decreased during incubation at high FS concentrations (10 and 20%). However, cell density markedly increased as FS concentration decreased. The cell density in 1.25% FS medium was the highest among the tested concentrations and was 79% that in the 10% FCS medium.

Combined effects of heat treatment and low concentration of FS on cell growth

To investigate the combined effects of heat treatment and low concentration of FS on cell growth, CHO cells were cultured in several media containing FS (*S. quinqueradiata*) with or without heat treatment (56℃) at different concentrations of 0, 1.25, 5, 10, and 20% after cell adhesion with 10% FCS medium for 24 h (Fig. 3). FCS medium were similarly studied. The cell densities at higher concentrations of FS (5-20%) were markedly low and cell density was increased with a decrease in FS concentration. Heat-treated FS exhibited a combined stimulatory effect on cell growth in the FS concentration range from 1.25% to 20%. In contrast, cell density decreased with a decrease in FCS concentration. The effect of heat-treated FCS on cell growth was not observed in the culture with 10% FCS.

Effect of FS from red seabream on cell growth

To confirm whether the effects of heat treatment and low concentration of FS on cell growth are also observed for FS from a red seabream (*P. major*), CHO cells were cultured in several media containing heat-treated (56℃) FS from a red seabream (*P. major*) or non-heat-treated FS (*P. major*) at different concentrations of 2.5, 5, 10, 20, and 30% (Fig. 4). Cell density gradually increased with decreasing concentration of FS (*P. major*) and 2.5% provided the highest cell density among all tested concentrations (2.5-30%). Heat-treated FS appeared to be effective for enhancing cell growth at any concentrations of FS.

Effect of lipids in fish serum on cell growth

It was shown that FS (*S. quinqueradiata*) and FS (*P. major*) contained lipids at concentrations of 3.3-50-fold those in FCS (Table 1). Lipid content in several media containing 1.25% FS (*S. quinqueradiata*), 2.5% FS (*P. major*), and 10% FCS was calculated (Table 1).

In order to investigate the effect of high lipid concentration in FS, lipids extracted from FS (*S. quinqueradiata*) were added at different concentrations (0-1.0 mg/ml) to the media with 1.25% FS, 10% FCS, and 0% serum for cell culture. Yields of extracted lipids were 54% to 95% (Table 2). In the culture with 1.25% FS and 10% FCS media supplemented with various concentrations of FS lipids, cell density decreased as lipid concentration increased (Fig. 5). A similar correlation was observed between cell density and lipid concentration in the cultures with various FS concentrations from 1.25% to 10%.

Performance of cell growth and hGM-CSF production with heat-treated FS at low concentration

The time course of the culture with heat-treated FS at low concentration that was the optimum condition found was examined employing FS from *S. quinqueradiata*. CHO cells were cultured in media containing 1.25% FS with or without heat treatment (56℃) (Fig. 6a). Although the cell densities in 1.25% FS media with or without heat treatment were slightly lower than those in 10% FCS medium at 72 and 120 h, those densities were nearly the same as that in 10%

FCS medium at 168 h (3.2×10^5 cells/cm^2). The concentration of hGM-CSF increased throughout the entire period of culture with FS and FCS (Fig. 6b). The hGM-CSF concentration in the culture using 1.25% FS medium reached 2.0 ng/ml, whereas that in the culture using 10% FCS medium reached 3.9 ng/ml.

[Discussion]

CHO cell growth in FS medium was improved by the heat treatment of FS (*S. quinqueradiata*) for 30 min at various temperatures (Fig. 1). It was suggested that the inactivation of the complementary system in FS (*S. quinqueradiata*) by heat-treatment improved cell growth, because the optimum temperature was 56℃ and that is also optimum temperature for the inactivation of the complementary system in FCS. However, heat treatment could increase the cell density in FS medium only to 60% that in 10% FCS medium (Fig. 1).

Decreasing the FS (*S. quinqueradiata*) concentration to 1.25% markedly increased the cell density to approximately 79% that in 10% FCS medium, although no cell growth was found with 10% FS (Fig. 2). Therefore, decreasing FS (*S. quinqueradiata*) concentration was more effective for improving cell growth than heat treatment.

The combined effects of heat treatment (56℃) and decreasing concentration (1.25%) were observed not only for FS from *S. quinqueradiata* but also for FS from *P. major* (Fig. 4). Therefore, the effect of heat treatment and decreasing serum concentration might be commonly

observed for sera from several types of fish.

Lipid concentrations in both FS (*S. quinqueradiata* and *P. major*) were more than three fold those in FCS (Table 1). Addition of the lipids extracted from FS to 10% FCS and 1.25% FS media resulted in a marked decrease in cell density (Fig. 5). Consequently, the effect of decreasing FS concentration on cell growth may be due to decreasing lipid concentrations in FS media. Therefore, the difference in response to serum concentration between FS and FCS mentioned above may be due to the markedly higher concentration of lipids in FS than in FCS.

Cell density in the cultures with heat-treated or non-heat-treated 1.25% FS (*S. quinqueradiata*) was nearly the same as that in the culture with 10% FCS at 168 h, although it took a slightly longer culture time (Fig. 6a). Consequently, the medium with 1.25% FS may be applicable to practical production processes, although protein productivity should be improved. Although lower serum concentrations should offer the advantage of reducing process cost, they lead to low concentrations of growth factors in FS medium. This may be one of the reasons for the lower productivity of hGM-CSF in the culture using FS medium (Fig. 6b).

In conclusion, heat treatment and decreasing concentrations of FS might be effective in obtaining appropriate growth of CHO cells in adhesion culture without FCS.

[References]

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Figure Legends

Fig. 1 Effect of heat treatment of fish serum on adhesive growth of CHO cells. CHO DR1000L4N cells were cultivated in adhesion for 120 h in media containing 10% FCS, heat-treated 10% FS (*Seriola quinqueradiata*) (46, 51, 56, or 61℃), non-heat-treated 10% FS (NT), or no serum (0%) after initial adhesion for 24 h in 10% FCS medium. Mean ± SD (n=3)

Fig. 2 Effect of concentration of fish serum on adhesive growth of CHO cells. CHO DR1000L4N cells were cultivated in adhesion for 120 h in media containing 10% FCS, FS (*Seriola quinqueradiata*) (1.25, 2.5, 5, 10, or 20%) or no serum (0%) after initial adhesion for 24 h in 10% FCS medium. Mean ± SD (n=3)

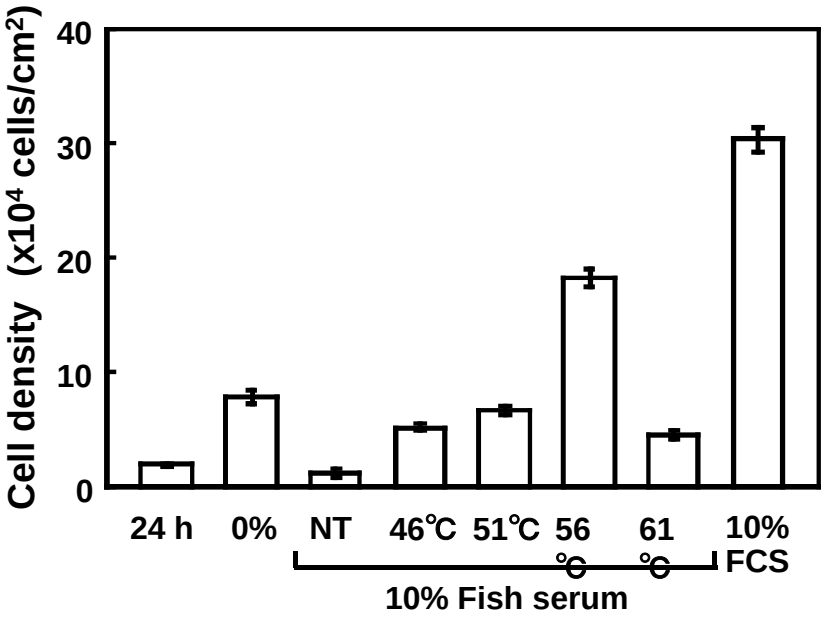
Fig. 3 Effects of heat treatment and concentration of FS and FCS on growth of CHO cells. CHO DR1000L4N cells were cultivated in adhesion for 120 h in medium containing FS (*Seriola quinqueradiata*) (circles) or FCS (squares) with (closed symbols) or without (open symbols) heat treatment (56℃) at various concentrations after initial adhesion for 24 h in 10% FCS medium. Mean ± SD (n=3)

Fig. 4 Effects of heat treatment and concentration of fish serum from red seabream (*Pagrus major*) on growth of CHO cells. CHO DR1000L4N cells were cultivated for 120 h in medium

containing FS (*Pagrus major*) with (closed bars) or without (open bars) heat treatment (56℃) at various concentrations after initial adhesion for 24 h in 10% FCS medium. Mean ± SD (n=3)

Fig. 5 Influences of lipids extracted from fish serum on growth of CHO cells. CHO DR1000L4N cells were cultivated in adhesion for 120 h in media containing 10% FCS supplemented with lipids extracted from FS (*Seriola quinqueradiata*) (open circles), heat-treated 1.25% FS (*Seriola quinqueradiata*) supplemented with lipids (closed squares), and no serum supplemented with lipids (open triangles), after initial adhesion for 24 h in 10% FCS medium. For comparison, the same cultivations were performed in media with various concentrations of lipids originally containted in heat-treated FS (*Seriola quinqueradiata*) (open squares). Data represent the means of triplicate cultures.

Fig. 6 Time course of cell density and hGM-CSF concentration in adhesion culture of CHO cells using fish serum. (A) Cell density, (B) hGM-CSF concentration. CHO DR1000L4N cells were cultivated in adhesion for 168 h in media containing 10% FCS (open circles), 1.25% fish serum (*Seriola quinqueradiata*) with (closed squares) or without (open squares) heat treatment, and no serum (0%) (open triangles) after initial adhesion for 24 h in 10% FCS medium. Data represent the means of triplicate cultures.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24 h
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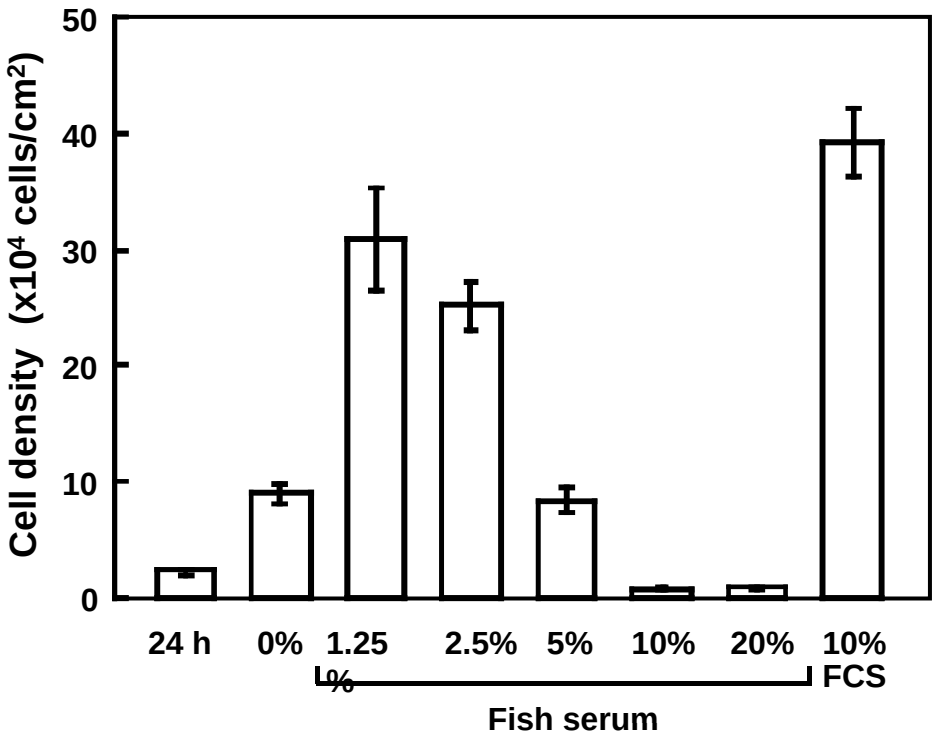
NT
46℃
51℃
56 ℃
61 ℃
10% FCS
10% Fish serum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30
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0
24 h
0%
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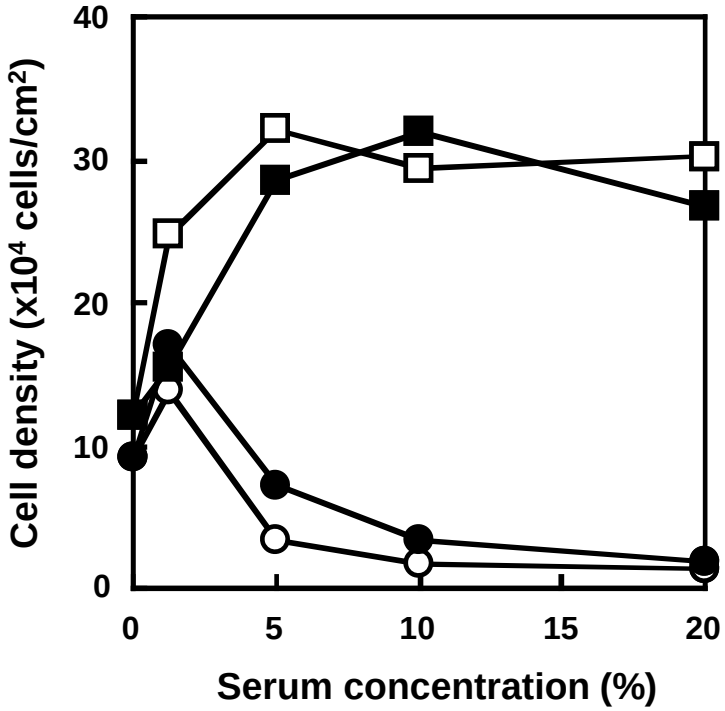
2.5%
5%
10%
20%
10%
%
FCS
Fish serum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5
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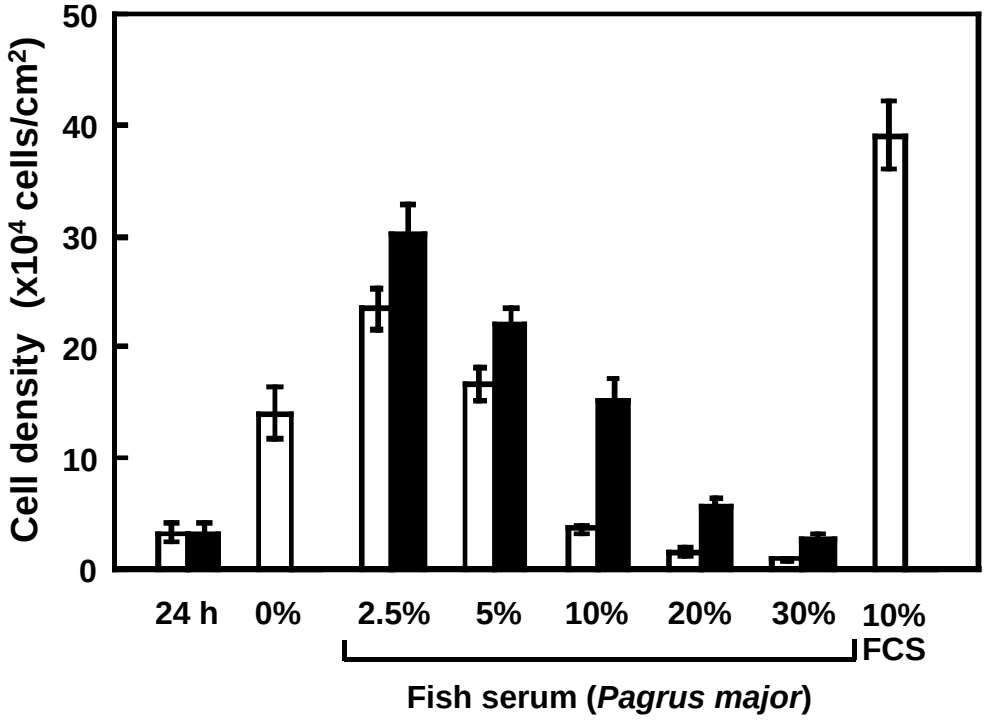
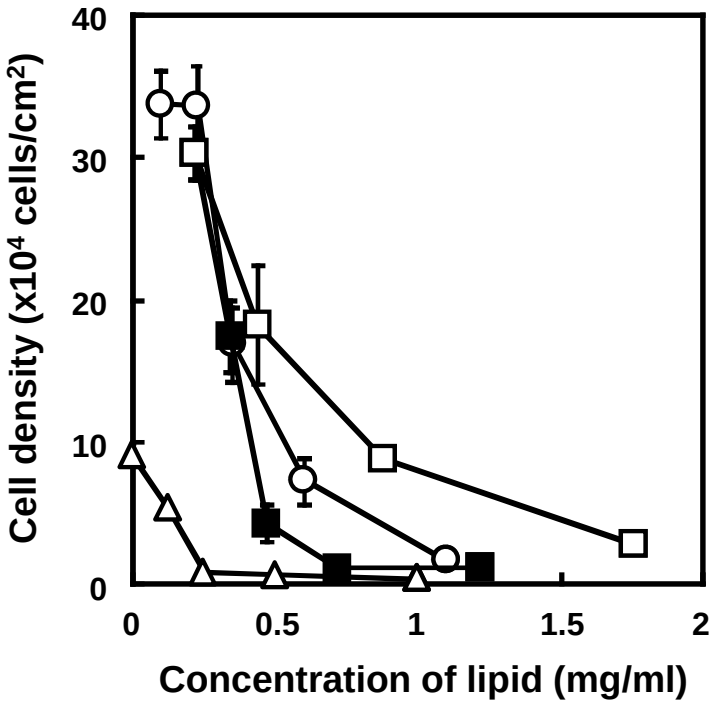
Cell density (x10⁴ cells/cm²)
Serum concentration (%)


Cell density (x10⁴ cells/cm²)
50
40
30
20
10
0
24 h
0%
2.5%
5%
10%
20%
30%
10% FCS
Fish serum (Pagrus major)


40
30
20
10
0
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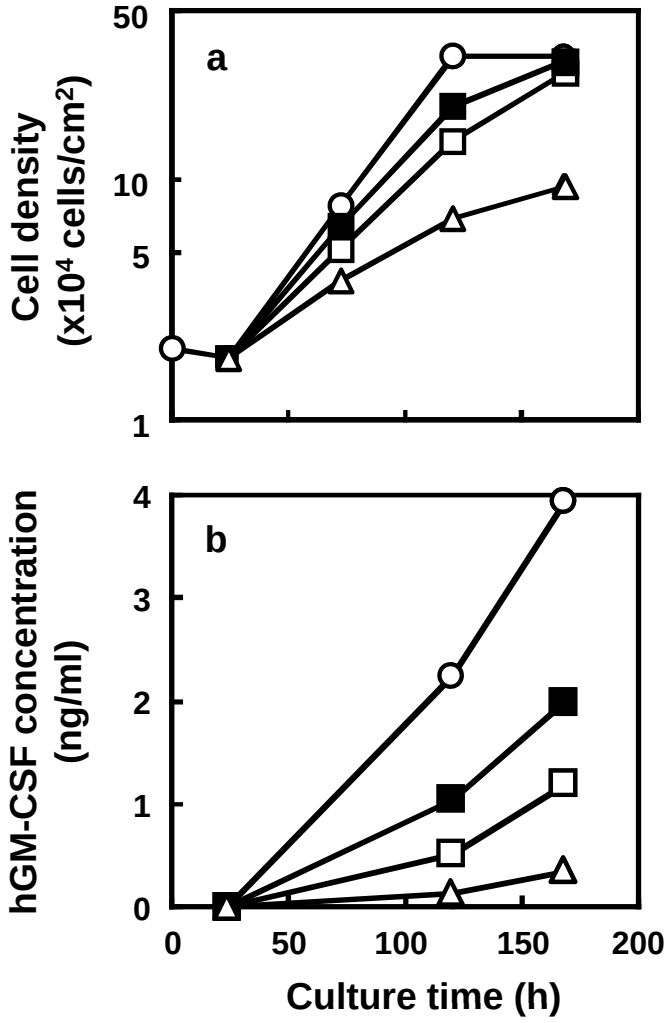
1
1.5
2
Concentration of lipid (mg/ml)


a
Cell density
(x10⁴ cells/cm²)
50
10
5
1
b
hGM-CSF concentration
(ng/ml)
4
3
2
1
0
0
50
100
150
200
Culture time (h)